A Novel Regulator of Macrophage Activation: miR-223 in Obesity Associated Adipose Tissue Inflammation

Running title: Zhuang et al.; miR-223 regulation of macrophage activation

Guoqing Zhuang, PhD1*; Cong Meng, BS1,2*; Xin Guo, PhD2; Patali S. Cheruku3; Lei Shi, MD, PhD1,4; Hang Xu, BS2; Honggui Li, MS2; Gang Wang, BS1; Ashley R. Evans5; Stephen Safe, PhD1; Chaodong Wu, MD, PhD2; Beiyan Zhou, PhD1

1Dept of Veterinary Physiology & Pharmacology, College of Veterinary Med & Biomed Sciences, 2Intercollegiate Faculty of Nutrition Program, 3Dept of Biology, College of Science, 4Biomed Sciences Program, College of Veterinary Med & Biomed Sciences, Texas A&M University, College Station, TX; 5Dept of Oncology, Renmin Hospital of Wuhan University, Wuhan, China; *contributed equally

Correspondence:
Chaodong Wu, MD, PhD
Dept of Nutrition & Food Science
Texas A&M University
Cater Mattil 214D
College Station, TX, 77843
Tel: 979-458-1521
Fax: 979-458-1521
E-mail: cdwu@tamu.edu

Beiyan Zhou, PhD
Physiology & Pharmacology, MS 4466
Texas A&M University
VMR 1197, Room 422b
College Station, TX, 77843
Tel: 979-845-7175
Fax: 979-845-7175
E-mail: bzhou@cvm.tamu.edu

Abstract:

**Background** - Macrophage activation plays a crucial role in regulating adipose tissue inflammation and is a major contributor to the pathogenesis of obesity-associated cardiovascular diseases. Upon various types of stimuli, macrophages respond with either classical (M1) or alternative (M2) activation. M1 and M2-mediated signaling pathways and corresponding cytokine production profiles are not completely understood. The discovery of microRNAs provides a new window to understand this complicated but crucial network for macrophage activation and adipose tissue function.

**Methods and Results** - We have examined the activity of microRNA-223 and its role in controlling macrophage functions in adipose tissue inflammation and systemic insulin resistance. miR-223−/− mice on HFD exhibited an increased severity of systemic insulin resistance compared to wild-type mice, and this was accompanied by a marked increase in adipose tissue inflammation. The specific regulatory effects of miR-223 in myeloid cell-mediated regulation of adipose tissue inflammation and insulin resistance were then confirmed by transplantation analysis. Moreover, using bone marrow derived macrophages (BMDM) we demonstrated that miR-223 is a novel regulator of macrophage polarization, which suppresses classic pro-inflammatory pathways and enhances the alternative anti-inflammatory responses. In addition, we identified Pknox1 is a genuine miR-223 target gene and an essential regulators for macrophage polarization.

**Conclusions** - For the first time, this study demonstrates that miR-223 acts to inhibit Pknox1, suppressing pro-inflammatory activation of macrophages, thus it is a crucial regulator of macrophage polarization and protects against diet-induced adipose tissue inflammatory response and systemic insulin resistance.

**Key words:** microRNA, macrophage polarization, adipose tissue inflammation; insulin resistance
Adipose tissue inflammation is a hallmark of obesity and a causal factor of metabolic disorders such as insulin resistance\textsuperscript{1-5} and a wide variety of metabolic diseases including atherosclerosis and type 2 diabetes\textsuperscript{4,6}. Mice fed a high-fat diet (HFD) frequently develop chronic low-grade inflammation within adipose tissues, characterized by increased infiltration of immune cells and production of pro-inflammatory cytokines\textsuperscript{1,2}. Consequently, adipocytes produce a number of inflammatory mediators that contribute to atherosclerotic cardiovascular disease (CVD)\textsuperscript{7,8}. Importantly, elevated adipose tissue inflammation is a significant contributing factor to systemic insulin resistance\textsuperscript{9-14}, which is an additional risk factor for CVD through both inflammation-dependent and independent mechanisms. Given the importance of adipose tissue inflammation in metabolic diseases, there is a critical need to better understand the mechanisms underlying these inflammatory processes.

Several reports demonstrate that macrophages are key regulators of adipose tissue inflammatory responses\textsuperscript{1,2,15-17}. For example, in mice lacking osteopontin, a secreted matrix glycoprotein and proinflammatory cytokine, inhibition of macrophage recruitment suppresses adipose tissue inflammatory response\textsuperscript{18}. As a consequence, osteopontin-deficient mice are protected from HFD-induced insulin resistance. A similar result has been observed in mice lacking C-C motif chemokine receptor 2 (CCR2), the receptor for the C-C motif chemokine ligand 2 (CCL2, also known as monocyte chemotactic protein-1 (MCP-1))\textsuperscript{16}. In addition, altering inflammatory signaling in myeloid cells including macrophages is sufficient to modulate adipose tissue inflammatory responses and systemic insulin sensitivity. In support of this assertion, disruption of inflammatory signaling through Toll-like receptor 4 (TLR4) or nuclear factor κB (NF-κB) in myeloid cells protects mice from diet-induced insulin resistance\textsuperscript{17,19}. Conversely, phenotypic switching of adipose tissue macrophages involving alternative activation (M2)
provides anti-inflammatory modulation of adipose tissue function and systemic insulin resistance
20. Within this context, peroxisome proliferator-activated receptor γ (PPARγ) and PPARδ are
two best known intracellular regulators of the alternative macrophage activation pathways.
PPARγ or PPARδ activation leads to M2 polarization in adipose tissue and in turn improves
adipose tissue functions and systemic insulin sensitivity.3,21-24 In contrast, mice with
macrophage-specific PPARγ deletion exhibited blunted macrophage M2 response and increased
classical proinflammatory (M1) activation, thereby enhancing systemic insulin resistance.21.
Thus, regulators that are crucial for macrophage polarization also exert pivotal functions in
modulating adipose tissue inflammatory responses and systemic insulin sensitivity.3,19,21,23.
However, despite the importance of this process to metabolic diseases, the mechanisms
underlying macrophage polarization remain poorly understood.

MicroRNAs (miRNAs) are a group of highly conserved, small non-coding RNAs (~22
nucleotides). By base pairing with complementary sites within target mRNAs, miRNAs trigger
either a block in translation and/or mRNA degradation.25-29 Numerous studies in multiple model
organisms have provided compelling evidence indicating that miRNAs are key regulators of cell
fate determination and significant contributors to the pathogenesis of complex diseases including
obesity-associated metabolic diseases.30-37 Among the known miRNAs, microRNA-223 (miR-
223) is a potent regulator of some inflammatory responses.38,39 When challenged by endotoxin,
miR-223-deficient mice exhibited increased inflammatory lung lesions,40 and altered expression
of miR-223 has been linked with several immune disorders, including rheumatoid arthritis and
type 2 diabetes.34,41 During monocytic differentiation into macrophages, miR-223 is
downregulated,38 however, the role of miR-223 in regulating downstream processes such as
macrophage activation and subsequent adipose tissue inflammation and systemic insulin
resistance is unknown. The present study provides evidence to support a novel role of miR-223 in modulating macrophage polarization in a pattern that protects mice from diet-induced adipose tissue inflammation and systemic insulin resistance.

Methods

Animal experiments

Generation of miR-223-deficient mice has been described. Wild-type (WT) C57BL/6J mice were used as controls. All mice were maintained on a 12:12-hour light-dark cycle. All mice were fed ad libitum except those that were used for dietary feeding study. Male mice of 5-6 weeks of age were used for both feeding and bone marrow isolation and macrophage activation analysis. For dietary feeding studies, mice were fed with an HFD (60% fat calories, 20% protein calories, and 20% carbohydrate calories) or low-fat diet (LFD) (10% fat calories, 20% protein calories, and 70% carbohydrate calories) (Research Diets, Inc.) for 12 weeks. After the feeding regimen, mice were subjected to phenotype characterization and metabolic assays including measurements of plasma metabolic parameters, insulin and glucose tolerance tests, tissue histological and immunohistochemical analyses. All study protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Texas A&M University.

Bone marrow isolation and macrophage differentiation

Bone marrow derived macrophages were generated using protocols as previous described. In brief, bone marrow cells from miR-223−/− mice and wild-type mice were isolated followed by erythrocytes lysis using ammonium chloride (Stem Cell Technologies), and seeded in 12-well plates at a concentration of 2 × 10^6 cells/ml. Cells were induced for differentiation to monocytes with RPMI 1640 medium containing 10% fetal bovine serum and 15% L929 culture supernatant.
for 7 days. The formation of mature monocytes was evaluated on day 7 using flow cytometry with fluorescence conjugated antibodies against CD11b and F4/80.

**Macrophage polarization analysis**

To analyze macrophage polarization, BMDMs were stimulated by LPS (100 ng/ml) or IL-4 (10 ng/ml). Surface antigens, CD69, CD80 and CD86, were examined using flow cytometry at 2, 5, 24, 48 and 72 hours post stimulation. Total RNAs were extracted from activated BMDMs at these same time points and subjected to gene expression analysis. Activation of the signaling pathway was determined using western blot and quantitative RT-PCR analysis. For each experiment, BMDMs from at least three mice were tested individually and results were analyzed for statistical differences.

**BMDM and adipocyte co-culture assay**

BMDMs derived from miR-223<sup>−/−</sup> or wild type bone marrow were co-cultured with differentiated 3T3-L1 adipocytes as previously described<sup>4</sup>. After differentiated for 8 days, adipocytes were cultured with BMDM at a ratio of 10:1.<sup>23</sup> To determine changes in insulin signaling, the cells were treated with or without insulin (100 nM) for 30 min prior to harvest. Cell lysates were prepared and used to examine inflammatory and insulin signaling by Western blots.

**miR-223 target gene prediction and validation**

miR-223 target gene prediction was conducted using TargetScanMouse 5.1<sup>(www.targetscan.com)</sup> and PicTar<sup>(pictar.mdc-berlin.de)</sup><sup>42,43</sup>. To validate miR-223 predicted targets, the luciferase reporter assay was carried out using 3' untranslated regions of candidate genes containing potential wild type or mutated miR-223 binding sites inserted downstream from the Renilla luciferase gene. The reporter constructs were co-transfected with miR-223 mimic oligo nucleotides or negative control oligo nucleotides into HEK293 cells. Forty eight hours after
co-transfection, the activities of Renilla luciferase were measured using the Dual-Glo luciferase reporter system (Promega) and normalized to the internal control firefly luciferase activity. Repressive effects of miR-223 on gene targets were plotted as the percentage of repression of three biological repeats (each biological repeat contains three technical replicates).

**Bone marrow transplantation**

Bone marrow transplantation analyses are performed as previously described. Six week old miR-223−/− mice (C57BL/6J background, CD45.1) or age-matched wild type (CD45.1) mice were used as donor mice. Six-week old syngeneic male mice (CD45.2, C57BL/6J) were purchased from the Jackson Laboratory and used as recipients. A total of 10 mice received bone marrow transplantation in each group in two independent tests. Primary bone marrow cells from donor mice are isolated as described above. Recipient mice were subjected to 10 Gy lethal dose irradiation and 4 hours later received 5X10^6 bone marrow cells (red blood cell depleted) from donor mice. The engraftment was monitored by flow cytometry analysis with peripheral blood samples obtained from each mouse 4 weeks after transplantation. Recipient mice were then fed on a high-fat diet for 8 weeks before insulin resistance test and tissue collections.

**Data analysis and Statistics**

For overall group-effect significance, data were analyzed using two-way analysis of variance (ANOVA) analysis and Bonferroni post-test for each factor at individual times. For each data point derived from qRT-PCR assays represents an average of three technical replicates and data were averaged over independently replicated experiments (n=5 to 8 independently collected samples) and analyzed using Student t test, presented as the Mean fold-change over the mean of the control group ± SEM (Standard Error of the Mean). Data analysis was performed using software Graphpad Prism V5.01. A P value < 0.05 was considered statistically significant.
Results

miR-223 deficiency exacerbates HFD-induced adipose tissue inflammation and systemic insulin resistance

To profile miR-223 expression patterns we first sought to examine miR-223 levels in key metabolic and hematopoietic tissues of wild type C57BL/6J (WT) mice using quantitative microRNA RT-PCR analysis (ABI). Consistent with previous studies, miR-223 was preferentially expressed in the bone marrow which consists of the major population of myeloid cells (Figure 1A). The expression of miR-223 in other tissues, including muscle, spleen, heart and liver, was low or non-detectable. miR-223 was detected at low levels in various adipose tissues, which may be due to the presence of blood cells, especially myeloid cells, in the adipose tissues. The expression of miR-223 in visceral fat stromal cells is slightly higher than in adipocytes, but lower than in macrophages (Figure 1B).

To address the potential role of miR-223 in regulating adipose tissue function in relation to systemic insulin resistance, we fed both miR-223–/– mice and wild-type mice a HFD for 12 weeks. Mice maintained on a LFD served as experimental controls. miR-223 ablation was confirmed using quantitative RT-PCR assays (Supplemental Figure 1). In the WT control mice, the expression pattern in adipose tissues and bone marrow cells was not affected by HFD (Figure 1C). miR-223–/– mice maintained on a LFD did not differ from WT control mice with respect to fasting plasma levels of glucose and insulin (Figure 1D), but did exhibit a slight increase in insulin resistance and glucose intolerance (Figure 1E). On a HFD, miR-223–/– mice gained a slight but insignificant increase in body weight and showed no difference in food intake (Supplemental Figure 2). Surprisingly, miR-223–/– mice on a HFD exhibited dramatically increased insulin response to glucose and hyperglycemia (Fed, Figure 1D), despite similar
insulin and glucose levels after fasting for 16 hours (Fasted, Figure 1D). We did not observe significant effects of miR-223 deficiency on lipid metabolism or mitochondria functions adipose tissue of mice on a HFD (Supplemental Figure 3). It is well documented that HFD-induced adipose tissue inflammation is a major contributor to systemic insulin resistance. To determine if miR-223 is a novel regulator for HFD induced adipose tissue inflammation and systemic insulin resistance, we conducted glucose tolerance and insulin resistance analysis. HFD-fed miR-223<sup>−/−</sup> mice showed a greater increase in the severity of insulin resistance and glucose intolerance than wild-type mice after 12 weeks on this feeding regimen (Figure 1E and F).

miR-223 deficiency enhanced M1 macrophage infiltration in HFD-fed mice. We observed a slight increase of visceral fat and adiposity in HFD-fed miR-223<sup>−/−</sup> mice compared with control mice (Figure 2A and Supplemental Figure 4). This was accompanied by enhanced activation of inflammatory pathways as evidenced by both increased NF-κB p65 phosphorylation in adipose tissues compared to controls (Figure 2B) as well as increased adipose tissue expression of inflammatory mediators including MCP-1, TNFα, IL-1b, and IL-6 (Figure 2C). Moreover, the severity of HFD-induced adipose tissue dysfunction in miR-223<sup>−/−</sup> mice was also higher than in controls as indicated by a decrease in insulin-induced adipose tissue Akt (Ser473) phosphorylation (Figure 2D) and increased resistin mRNA levels (Figure 2E).

Immunohistochemical analysis of adipose tissue sections from HFD-fed mice showed that the size of adipocytes in HFD-fed miR-223<sup>−/−</sup> mice did not differ from those in controls (Figure 3A). Both wild type and miR-223<sup>−/−</sup> mice on HFD developed fatty livers but the severity of lipid accumulation in hepatocytes and liver weights were similar (Supplemental Figures 5A and B), and liver triglyceride levels were comparable in both groups (Supplemental Figure 5C).
In addition, there were no differences between miR-223+/− and wild type control mice with the respect to plasma triglyceride levels on either fed or fasted (16 hours) status (Supplemental Figure 6). Whereas HFD-fed miR-223+/− mice exhibited a higher macrophage infiltration in adipose tissues compared to wild type mice (Figure 3A). This was confirmed by the increased percentage of adipose tissue macrophages (ATM) (CD11b+F4/80+) in visceral fat stromal cells from HFD-fed miR223+/− mice compared to control mice (Figure 3B). Among these macrophages (CD11b+F4/80+), the proportion of M1 (CD11c+CD206−) was significantly increased in VSCs of miR-223+/− mice; in contrast, the percentage of M2 (CD11c-CD206+) in miR-223+/− VSCs was slightly less (P=0.057) than in the wild type mice. Additionally, flow cytometry results also indicated a higher proportion of proinflammatory macrophages (CD11b+F4/80+CD11c+CD206+) in stromal cells of HFD-fed miR-223+/− mice compared to control mice (Figure 3C and 3D), and this has been closely correlated with insulin resistance 44.

These results demonstrate that miR-223 plays a critical role in macrophages activation and ablation of miR-223 exacerbates M1 macrophage mediated adipose tissue inflammation and insulin resistance.

Transplanted mice with myeloid cell specific miR-223 deficiency re-capitulated phenotypes in miR-223 mice on HFD

To confirm the adipose tissue inflammation and insulin resistance in miR-223+/− mice is primarily due to miR-223 ablation in myeloid cells, we conducted bone marrow transplantation assays. To introduce myeloid cell-specific miR-223 ablation, we transplanted syngeneic wild type mice with bone marrow cells isolated from miR-223+/− mice (BMT-miR-223+/−) and age matched wild type donor mice were used as control in the study (BMT-WT). The engraftment of donor cells (CD45.1) in lethally irradiated recipient mice (CD45.2) were confirmed by the
presence of donor-derived cells (Figure 4A and 4B). Once confirmed, the recipient mice were fed with a HFD for eight weeks and subjected to insulin sensitivity and glucose tolerance tests. There were no differences in the body weight gain and food intake between two groups (Supplemental Figure 7). Various tissues were then collected and the engraftment was further confirmed using flow cytometry and quantitative PCR analysis. More than 90% of bone marrow and circulating cells were CD45.1+ (donor derived) and the expression of miR-223 was depleted in the bone marrow in BMT-miR-223−/− mice (Figure 4C), suggesting a successful long-term stem cell repopulation in the recipients (Supplemental Figure 8). In addition, BMT-miR223−/− mice exhibited increased severity of glucose intolerance (Figure 4D) and insulin resistance (Figure 4E) compared to BMT-WT mice. As expected, elevated proinflammatory cytokines (Figure 4F) accompanied by enhanced NFkB activation (Figure 4G) were observed in adipose tissues collected from BMT-miR-223−/− mice compared to those from control mice. We did not observe differences in plasma insulin, glucose or triglyceride levels or visceral adiposity between the two groups (Supplemental Figure 9). Taken together, our results suggest that exacerbated adipose tissue inflammation and insulin resistance in miR-223 deficient mice are mainly due to enhanced pro-inflammatory response of myeloid cells with miR-223 ablation.

**miR-223 is a novel regulator for macrophage polarization**

To determine if ablation of miR-223 in mice results in altered macrophage production, we initially examined the proportion of monocytes in the peripheral blood samples from either HFD or LFD–fed mice. Consistent with a previous report, the neutrophil portion was slightly increased in miR-223−/− compared to control mice 39. No significant differences were detected in macrophage population (CD11b+Gr-1+) (Supplemental Figure 10). We next examined the differentiation capacity of bone marrow progenitors within the context of miR-223 deletion using
colony forming assays. Interestingly, no significant differences were observed in either colony forming unit-granulocyte/erythocyte/megakaryocyte/monocyte (CFU-GEMM) or colony forming unit-granulocyte/monocyte (CFU-GM) (Supplemental Figure 11), indicating that increased adipose tissue inflammation is likely due to the alternation of macrophage activation instead of production.

To further investigate the effects of miR-223 on macrophage activation, we generated bone marrow derived macrophages (BMDMs) and treated them with either LPS (100 ng/ml) or IL-4 (10 ng/ml) to induce M1 or M2 activation respectively. Surprisingly, miR-223 levels in BMDMs significantly altered upon M1 or M2 activation. Dramatically elevated miR-223 levels were observed in BMDMs 5 hours after treatment with IL-4 and levels remained high levels for up to 72 hours (Figure 5A), whereas LPS stimulation slightly decreased miR-223 level in BMDMs (Figure 5A). During the 7 day course of macrophage differentiation, there were no differences in the mature macrophage purity in BMDM from either miR-223−/− or wild type mice, evidenced by FACS analysis using antibodies against CD11b and F4/80 (Figure 5B).

Quantitative RT-PCR analysis showed that proinflammatory cytokine IL-1β, IL-6 and TNFα were significantly elevated in miR-223−/− macrophages compared to wild type macrophages upon LPS stimulation. Expression of M2 associated genes PPARγ and Arginase 1 were decreased in miR-223−/− macrophages compared to control cells following IL-4 stimulation (Figure 5C). miR-223−/− macrophages exhibited enhanced M1 but decreased M2 responses, as judged by FACS analysis using antibodies against activation surface markers CD69, CD80 and CD86 at various time point post stimulation (Figure 5D).

To examine the direct impact of isolated BMDM on adipocytes, we used an in vitro coculture assay. miR-223−/− BMDM-treated wild type adipocytes exhibited a slight but significant
increase in NF-κB p65 phosphorylation compared to control adipocytes (Figure 6A).

Additionally, in miR-223+/− BMDM-treated wild type adipocytes there was a decrease in insulin-stimulated Akt (Ser473) phosphorylation (Figure 6B), and an increase in proinflammatory cytokines upon LPS stimulation (Figure 6C). These results re-capitulated adipose tissue inflammatory and metabolic responses of HFD-fed miR-223+/− mice (Figure 2B and 2D) and BMT-miR-223+/− mice (Figure 4F and G), and clearly demonstrate that miR-223 is indeed an important regulator of macrophage polarization.

**Pknox1 is a bona fide miR-223 target genes that partially mediates its function during macrophage polarization**

To better understand the role of miR-223 in regulating macrophage polarization, we used multiple target gene prediction algorithms, including TargetScan Mouse 5.1 and PicTar to screen for miR-223 target genes followed by confirmation using luciferase reporter assays. Among 8 tested potential targets, Pknox1 was identified as a genuine target of miR-223 (Figure 7A). Luciferase activity was repressed in cells transfected with constructs containing 3'UTR regions with miR-223 binding sites in the presence of miR-223, whereas these inhibitory effects were not observed using constructs with miR-223 binding site mutations (Figure 7B and 7C). Pknox1 expression was higher in miR-223+/− BMDMs stimulated with LPS (Figure 7D and Supplemental Figure 12) compared to BMDMs from control mice. In consistent with in vitro observation, Pknox1 protein levels in the adipose tissues collected from HFD fed mice were inversely correlated with miR-223 expression levels (Figure 7E).

The importance of miR-223-mediated suppression of Pknox1 in macrophage polarization was further investigated using gene specific short interfering RNAs (siRNAs) to knockdown elevated levels of Pknox1 (Figure 8A). Knockdown of Pknox1 (siPknox1) in miR-223+/−
BMDMs decreased proinflammatory cytokine production (IL-1β, Figure 8B) and partially blocked M1 response as indicated by FACS analysis (Figure 8C). M2 activation was also partially restored in miR-223+/− BMDM with siPknox1 knockdown as judged by elevated Arginase 1 levels (Figure 8B). To further confirm the function of Pknox1 in macrophage polarization, we introduced ectopic expression of this protein in BMDMs by lentiviral infection (Supplemental Figure 13). Pknox1 overexpression partially recapitulated the miR-223–/– macrophage response to LPS with significantly enhanced surface markers’ shift (Figure 8D) and elevated inflammatory cytokine production (Figure 8E). These results demonstrated that Pknox1 is a bona fide target of miR-223 and plays a role in regulating macrophage polarization.

Discussion

Macrophage polarization is a critical component of the inflammatory response in metabolic tissues, and is of particular importance in adipose tissue. The present study provides evidence for the first time to support an essential role for miRNAs in regulating macrophage polarization. Notably, miR-223 is differentially expressed during macrophage polarization and miR-223-deficient macrophages were hypersensitive to LPS stimulation and exhibited delayed responses to IL-4 compared to controls (Figure 5). These results, together with increases in M1 and decreases in M2 polarization biomarkers in miR-223–/– macrophages, demonstrate a suppressive effect of miR-223 on macrophage pro-inflammatory activation and a stimulatory effect on anti-inflammatory activation. miR-223 regulated macrophage polarization is important for adipose tissue function. In the present study, miR-223-deficient mice exhibited an increase in adipose tissue inflammatory responses and decreased adipose tissue insulin signaling, accompanied with inappropriate adipokines expression which are indicators for adipose tissue function.
dysfunction. Using bone marrow transplantation analysis, we demonstrated that myeloid cell-specific deficiency of miR-223 is sufficient to exacerbate adipose tissue inflammation and systemic insulin resistance. The impacts of macrophages with miR-223 ablation on adipocytes were further confirmed in our co-culture study. Notably, changes in NF-κB and insulin signaling pathways in adipocytes treated with miR-223-deficient macrophages recapitulated the defects observed in adipose tissue of miR-223-deficient mice on HFD. Thus, miR-223 expression in macrophages is an important component of adipocyte inflammatory and metabolic responses.

Macrophage accumulation in adipose tissue from HFD-fed miR-223−/− mice was significantly higher than in wild-type mice, suggesting that miR-223-deficient macrophages exhibit an increased ability for infiltration. However, we did not observe an increased presence of macrophages/Kupffer cells in the liver (Supplemental Figure 5). Thus, it is likely that miR-223 deficiency has a limited role in increasing the infiltration ability of macrophages, whereas loss of miR-223 in adipocytes contributes, in large part, to increased macrophage infiltration. This is consistent with increased expression of MCP-1, a chemokine marker of macrophage infiltration into adipose tissue in both adipose tissue and primary adipocytes isolated from miR-223−/− mice (Figure 2C). Adipose tissue inflammation is well documented as an important contributor to systemic insulin resistance. This is further validated by our enhanced adipose tissue inflammatory responses in miR223−/− mice. Moreover, HFD-fed miR-223-deficient mice exhibited adipose tissue macrophage infiltration, proinflammatory cytokine expression, and NF-κB p65 phosphorylation. Genes that are crucial for metabolism were not directly affected by loss of miR-223 in both adipose tissue and liver (Supplemental Figure 3). Thus, increased adipose tissue inflammation resulting from miR-223 deficiency contributed, in large part, to systemic insulin resistance in miR-223-deficient mice.
miRNAs are critical regulators for multiple physiological processes by negatively regulating target genes expression. Using combination of computational analysis and luciferase reporter assays, we identified Pknox1 as a genuine target of miR-223. The expression of Pknox1 is reversely correlated with miR-223 levels in either activated BMDMs or adipose tissues. The function of Pknox1 as a target of miR-223 in regulating macrophage polarization was validated in our gain- and loss- of function analysis in BMDMs. Of note, altered expression of Pknox1 in BMDMs only partially recapitulated the phenotypes in miR-223−/− BMDMs, suggesting that other genes may also involved in miR-223 regulated macrophage function. Indeed, we identified several genes additional to Pknox1 that are may play important roles in modulating macrophage activation, their function will be further validated.

In summary, the present study provides new evidence to support a critical role for miR-223 in regulating macrophage polarization, which directly contributes to the protective effect of miR-223 against obesity-associated insulin resistance. Mechanistically, identification of miR-223, as well as a crucial target gene Pknox1, in modulating macrophage function provided novel insights to understand the network governing macrophage-mediated adipose tissue inflammatory response and metabolic regulation. Based on these unique observations, it is possible that miR-223 mimics would serve as a novel approach to prevent and/or treat insulin resistance associated diseases.

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Conflict of Interest Disclosures: None

References:


Figure Legends:

**Figure 1.** miR-223 deficiency exacerbates HFD induced insulin resistance. (A) The expression levels of miR-223 in various tissues were examined using quantitative RT-PCR. White adipose tissue (WAT) samples included mesenteric (Mes), perinephric (Peri), and epididymal (Epi) fat pads. BAT, brown adipose tissue; BM, bone marrow. (B) The expression of miR-223 was examined in macrophages, mature adipocytes and visceral stromal cells (VSC) isolated from epididymal fat tissues. For (A) and (B), tissue samples were collected from chow diet-fed male wild-type mice. Data are presented as means ± SEM, n = 3. (C) The dietary effects on miR-223 expression were examined in the metabolic related tissues using qRT-PCR from mice (n=3) on either HFD or LFD for 12 weeks. (D) Plasma glucose and insulin levels in miR-223−/− or wild type (WT) mice fed on HFD (Fed) or fasted for 16 hours (Fasted). (E) Glucose and (F) insulin tolerance tests. For (E and F), data are presented as mean ± SEM, n = 5. †, P < 0.05, HFD-miR-223−/− vs. HFD-wild-type; *, p < 0.05 LFD-miR-223−/− vs. LFD-wild-type.

**Figure 2.** miR-223 regulates adipose tissue inflammation and insulin signaling. (A) Visceral fat content and adiposity of male wild type (WT) or miR-223−/− mice on HFD. (B) NF-kB activation. Western blots were performed using antibodies against p65 and phos-p65. (C) Cytokines and MCP1 production in adipose tissues were examined using qRT-PCR (n=3, normalized to β-actin). (D) Adipose tissue insulin signaling. After fasting, mice were injected with insulin (1 U/kg) and adipose tissues were collected after 5 minutes. Western blots were performed using antibodies against Akt1/2 and phos-Akt (ser473). (E) Adipokine expression levels were examined by qRT-PCR (n=3, normalized to β-actin). *, p < 0.05; **, p < 0.001.
**Figure 3.** miR-223 deficiency enhances macrophage infiltration. (A) Adipose tissue sections of HFD fed mice were stained with H&E (top panels) or F4/80 for macrophage infiltration (bottom panels). (B) The adipose tissue macrophages (ATM) (CD11b+F4/80+) were examined by flow cytometry analysis from visceral stromal cells. (C) and (D), Macrophage subtypes were further analyzed by FACS using antibodies against CD11c and CD206. Data in (B) and (D) are presented as mean ± SEM, n = 5.

**Figure 4.** Bone marrow transplantation analysis. (A) and (B) The engraftment was examined using flow cytometry analysis with antibodies against CD45.1 (donor) and CD45.2 (recipient). (C) After three months, miR-223 expression in the bone marrow of transplanted mice was examined using qRT-PCR to confirm the reconstitution. (D) Glucose and (E) insulin tolerance test in transplanted mice (BMT) on HFD for 8 weeks. Data are presented as mean± SE, n = 8. (F) Cytokine expression in the adipose tissues collected from HFD fed mice with transplantation (n=4, normalized to β-actin). (G) NF-kB activation in adipose tissues collected from HFD fed recipient mice. Western blots were performed using antibodies against p65 and phos-p65. Data are presented as mean± SEM; *, p < 0.05; **, p < 0.001.

**Figure 5.** miR-223 regulates macrophage polarization. (A) Differentially expressed miR-223 in BMDMs upon LPS (M1) or IL-4(M2) were measured at various time point after stimulation. Data are presented as ± SE, n = 4. (B) The purity of mature BMDM (CD11b+F4/80+) derived from bone marrow cells isolated from wild type (WT) or miR-223−/− mice (n=5). (C) Cytokines, PPARγ and Arginase 1 expression were determined by qRT-PCR in BMDMs at 24 hours after either LPS (100ng/ml) or IL-4 (10ng/ml) stimulation (normalized to β-actin, n=3). (D)
Activation related surface markers, CD69, CD80 and CD86 were analyzed by flow cytometry after stimulation. MFI, median fluorescence intensity. Data are presented as mean ± SEM, n = 4; *, p < 0.05; **, p < 0.001.

**Figure 6.** Macrophage effects on adipocyte insulin signaling. BMDMs derived from wild type or miR-223−/− mice were co-cultured with differentiated 3T3-L1 cells at a ratio of 10:1. (A) 48 hours after co-culture, cells were collected and examined for activation of NFκB. (B) Cells were treated with insulin (100 nM) for 30 min before harvest. The activation of Akt were examined using antibodies against Akt and phos-Akt (ser473). (C) The cytokine expression levels in co-cultured cells were examined using qRT-PCR assays (normalized to β-actin, n=3). Data are presented as mean± SEM; *, p < 0.05; **, p < 0.001.

**Figure 7.** Pknox1 is a miR-223 target. (A) Predicted miR-223 binding site in the 3′ UTR of Pknox1 and a mutated version of the seed match region (red). (B) Reporter constructs containing a 3′UTR region with wild-type (B) or mutated miR-223 binding site of Pknox1 (C) were co-transfected with a miR-223 mimic oligo or control oligo (mimic ctrl) into HEK293 cells. Luciferase activity was analyzed 48 hours after transfection to evaluate the inhibitory effects of miR-223. Data are presented as mean ± SEM, n = 9. (D) Pknox1 expression in activated (LPS or IL-4) BMDM from wild type (WT) or miR223−/− mice were examined using qRT-PCR (normalized to β-actin). Data are presented as mean± SEM; n=4. (E) Levels of Pknox1 protein in adipose tissues collected from HFD fed wild type or miR-223−/− mice were determined by Western blots. *, p < 0.05; **, p < 0.001.
Figure 8. Role of Pknox1 in macrophage polarization. (A) Pknox1 was targeted with siRNA (siPknox1) in BMDMs with miR-223 deletion. Scrambled siRNA was used as control (Ctrl). The knockdown was confirmed using qRT-PCR (n=8). Cells were then stimulated with either LPS (100 ng/ml) or IL-4 (10 ng/ml) for 24 hours. (B) IL-1β production and Arginase 1 expression was measured using qRT-PCR (normalized to β-actin). (C) Surface markers (CD86 and CD69) were examined by FACS (MFI, median florescence intensity, n=3). BMDMs from wild type mice were lentiviral infected to introduce ectopic Pknox1 expression. 24 hours after infection, cells were stimulated with LPS or IL-4, (D) activation related surface markers (CD69, CD80 and CD86) were examined using flow cytometry and (E) cytokine production was measured using qRT-PCR (normalized to β-actin). *, P < 0.05; **, P < 0.001
Relative Abundance

Glucose (mg/dl)

Heart Lung Spleen Liver Kidney Muscle Pancreas Mes-fat Peri-fat Epi-fat BAT Colon BM

0 20 40 60 80 100

Relative Abundance

Adipocytes VSC Macrophage

0 2 4 6

Relative Abundance

HFD LFD HFD LFD HFD LFD HFD LFD


HFD LFD HFD LFD HFD LFD HFD LFD

Glucose Level (mg/dl)

Glucose Level (mg/dl)

Insulin (ng/ml)

Peri-fat Epi-fat BAT Muscle BM

Glucose (mg/dl)

HFD-WT HFD-miR223−/− LFD-WT LFD-miR223−/−


HFD-WT HFD-miR223−/− LFD-WT LFD-miR223−/−

% of initial glucose levels

0 30 60 90 120

% of initial glucose levels

0 15 30 45 60

†††
**Relative Abundance**

**BMT BMT**

**WT         miR-223-/−**

**miR-223 Expression**

(Bone Marrow)

<table>
<thead>
<tr>
<th></th>
<th>BMT</th>
<th>BMT-miR-223–/–</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of CD45.1+ Cells in Total Cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMT-WT</td>
<td>23.9%</td>
<td>25.5%</td>
</tr>
<tr>
<td>BMT-miR-223–/–</td>
<td>65.3%</td>
<td>67.4%</td>
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</table>

**Glucose Level (mg/dl)**

<table>
<thead>
<tr>
<th></th>
<th>BMT-WT</th>
<th>BMT-miR-223–/–</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of Initial Glucose Levels</td>
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<td></td>
</tr>
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<td>400</td>
</tr>
<tr>
<td>30</td>
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<td>300</td>
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<tr>
<td>60</td>
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**BMT Engraftment**

<table>
<thead>
<tr>
<th></th>
<th>BMT</th>
<th>BMT-miR-223–/–</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of CD45.1+ Cells in Total Cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMT-WT</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>BMT-miR-223–/–</td>
<td>60</td>
<td>60</td>
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</tbody>
</table>

**miR-223 Expression (Bone Marrow)**

- **BMT-WT**: 2
- **BMT-miR-223–/–**: 4

**Glucose Level (mg/dl) % of Initial Glucose Levels**

<table>
<thead>
<tr>
<th></th>
<th>BMT-WT</th>
<th>BMT-miR-223–/–</th>
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</thead>
<tbody>
<tr>
<td>Glucose Level (mg/dl)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>30</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>60</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

**A**

- **BMT-WT**: 23.9% (CD45.1), 65.3% (CD45.2)
- **BMT-miR-223–/–**: 25.5% (CD45.1), 67.4% (CD45.2)

**D**

- **Glucose Level (mg/dl)**
  - **BMT-WT**: Decreasing
  - **BMT-miR-223–/–**: Decreasing

**E**

- **% of Initial Glucose Levels**
  - **BMT-WT**: Decreasing
  - **BMT-miR-223–/–**: Decreasing

**F**

- **Relative Abundance**
  - **IL-1β**: 2
  - **IL-6**: 4
  - **MCP1**: 6

**G**

- **Pp65/p65 (Fold)**
  - **BMT-WT**: 2
  - **BMT-miR-223–/–**: 4

- **Tubulin**
  - **BMT-WT**: Expression
  - **BMT-miR-223–/–**: Expression

**Donor (CD45.1)**

**Recipient (CD45.2)**

**Glucose Level (mg/dl)**

<table>
<thead>
<tr>
<th></th>
<th>BMT-WT</th>
<th>BMT-miR-223–/–</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of Initial Glucose Levels</td>
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<tr>
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<tr>
<td>60</td>
<td>90</td>
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**Pp65/p65 (Fold)**

- **BMT-WT**: 2
- **BMT-miR-223–/–**: 4

**Tubulin**

- **BMT-WT**: Expression
- **BMT-miR-223–/–**: Expression
A. **Adipocytes**

- Pp65
- p65
- Tubulin

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>miR-223–/–</th>
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<tbody>
<tr>
<td>Pp65/p65</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
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<td>–</td>
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<tr>
<td>Pp65/p65</td>
<td><strong>1.5</strong></td>
<td><strong>1.5</strong></td>
</tr>
<tr>
<td>p65</td>
<td><strong>1.0</strong></td>
<td><strong>1.0</strong></td>
</tr>
<tr>
<td>Tubulin</td>
<td>0.5</td>
<td>0.5</td>
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</table>

B. **Adipocytes**

- BMDM
- WT
- miR-223–/–

<table>
<thead>
<tr>
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<th>BMDM</th>
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<td></td>
<td>Basal</td>
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<tr>
<td></td>
<td>Insulin</td>
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C. **Relative Abundance**

<table>
<thead>
<tr>
<th></th>
<th>IL-1β</th>
<th>IL-6</th>
<th>TNFα</th>
<th>MCP1</th>
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<tbody>
<tr>
<td>WT</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>miR-223–/–</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

- *: P < 0.05
- **: P < 0.01
miR-223

A

Pknox1

UTR WT: 5’ ...AAGUUCGGUCUUUGAAACUGACA...

MiMu-223 3’ ACCCCAUAAACUGU UUGACUGU

UTR Mutated: 5’ ...AAGUUCGGUCUUUGAAGATATCA...

MiMu-223 3’ ACCCCAUAAACUGU UUGACUGU

B

Relative Inhibition

PKnox1 Igf1r Fbox8 Dusp2 Lmo2 Tgfbr3 Sp3

C

D

Pknox1

Relative Abundance

24 Hours Post Activation

LPS IL-4

E

WT miR-223–/–

Relative Abundance

Tubulin

24 Hours Post Activation

WT miR-223–/–
**D**

- **Ctrl** vs. **Pknox1-oe**

- **MFI**

  - **CD69**
  - **CD80**
  - **CD86**

**E**

- **LPS** vs. **IL4**

- **Relative Abundance**

  - **IL-1 β**
  - **TNF α**
A Novel Regulator of Macrophage Activation: miR-223 in Obesity Associated Adipose Tissue Inflammation
Guoqing Zhuang, Cong Meng, Xin Guo, Patali S. Cheruku, Lei Shi, Hang Xu, Honggui Li, Gang Wang, Ashley R. Evans, Stephen Safe, Chaodong Wu and Beiyan Zhou

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Supplemental Material
Total RNA was extracted from various tissues of wild type (WT) or miR-223 knockout (miR-223−/−) mice. The expression of miR-223 was measured using Taqman miRNA Assay kits (ABI). Relative abundance of miR-223 in each sample was normalized to Sno202 and statistics was calculated based on results from results from three biological samples (tissues from three mice in each group) using student t test. **, p<0.001; ND, non detectable.
Age matched wild type (CD57/BL6, WT) or miR-223⁻/⁻ mice were fed on high fat diet (60% fatty acid) at the age of 5 week. The body weight and food intake were measured every week for 12 weeks. The results of body weight represent 2 individual experiments with total of 9 mice in each group. Data are mean ± SE, n=9.
Figure S3

Genes that are key regulators for lipogenesis, mitochondrial function, lipolysis were measured in the adipose tissues (A) and liver (B) collected from miR223<sup>−/−</sup> or wild type (WT) mice on HFD using qRT-PCR (normalized to β-actin). Data are means ± SE, n=4. ACC, acetyl-CoA carboxylase alpha; FAS, fatty acid synthetase; SCD1, stearoyl-CoA desaturase-1; PGC1b, peroxisome proliferator-activated receptor gamma, coactivator 1 beta; CPT1, carnitine palmitoyltransferase 1; HSL, hormone-sensitive lipase; G6pase, glucose 6-phosphatase; GK, glucokinase; PEPCK, phosphoenolpyruvate carboxykinase 2.
Adipose tissues were collected from wild type (WT) or miR-223−/− mice after 12 weeks of HFD feeding. Tissue weight was measured and analyzed between two groups.
Livers were collected from wild type (WT) or miR-223–/– mice after 12 weeks of HFD feeding regimen. Tissues were fixed and used for H&E staining and immunohistochemical staining with an antibody against F4/80 (A). The weight (B) and the triglyceride content (C) in liver did not differ between wild type and miR-223 deficient groups.
At 5 – 6 weeks of age, male miR-223–/– mice and wild-type (WT) control mice were fed an HFD for 12 weeks. After the feeding regimen, blood samples were collected from mice after feeding (Fed) or after 16 hours of fasting (Fasted). Triglyceride content from each sample was measured using metabolic kit. Data are mean ± SE, n = 5 – 8.
After engraftment was confirmed, mice received either bone marrow cells from either wild type donor (BMT-WT) or miR-223\(^{-/-}\) mice (BMT-miR-223\(^{-/-}\)) were fed on a HFD (60% fatty acid). The body weight and food intake were measured every week for eight weeks. The results of body weight represent 2 individual transplantation experiments with total of 10 mice in each group.
Three months after transplantation, peripheral blood (PB) samples (n=4) were collected from recipient mice to evaluate the donor cell engraftment. After mice were sacrificed for the tissue collection, the engraftment was further examined in the bone marrow samples using flow cytometry (n=4).
Metabolites in the plasma of transplanted mice were measured after mice were fed or fasted for 16 hours after 8 week of HFD feeding: glucose (A), insulin (B) and triglyceride (C). The weight of visceral fat (D) and liver (E) were measured after mice were sacrificed. Visceral fat adiposity were calculated with respect to the body weight (F)
The percentage of monocytes (CD11b+Gr-1-) were analyzed from peripheral blood samples (PB) or spleen of wild type (WT) and miR-223−/− mice using flow cytometry. No significant differences were observed between two groups in the proportion of monocytes.
Bone marrow cells were extracted from wild type (WT) or miR223−/− mice after 12 weeks of HFD feeding. Red blood cells were lysed with NH4Cl (StemCell Technologies) and plated in the methocult® GF 3434 (StemCell Technologies). CFU-GEMM and CFU-GM were counted after 10 days culture and analyzed to compare the effects of miR-223 on myeloid progenitors. Each group contained cell preparations from five mice.
Figure S12

BMDMs derived from HFD fed wild type or miR-223\(^{-/-}\) mice were stimulated with LPS (100 ng/ml). The protein levels of Pknox1 were examined using Western blots. The intensity of Pknox1 bands in each sample were normalized to the tubulin loading Controls and calculated for statistic analysis using student t test. *, p<0.05.
Ectopic expression of Pknox1 was introduced by lentiviral infection (pLenti6.2-Pknox1) followed by confirmation with Western blots. An empty lentiviral was used as a control. The intensities of each Pknox1 band were normalized to the loading control (Tubulin).
Supplementary Methods

Flow cytometry analysis
Unless specified, antibodies were purchased from eBiosciences (San Diego, CA). For cell lineage detection, fluorescence-tagged antibodies: anti-F4/80, anti-CD11b and anti-Gr-1 for myeloid cells; anti-CD80, anti-CD69, anti-CD86, anti-CD11c and anti-CD206 (Biolegend) for myeloid cell activation; anti-CD19, anti-B220 and anti-CD3e for lymphocytes were utilized. Cells were then analyzed using an Accuri C6 Flow Cytometer System (BD Biosciences, San Diego, CA). Data were analysed using Flowjo software or Accuri C6 software.

Bone marrow progenitor colony forming assays
The frequency of bone marrow myeloid progenitors was determined using colony forming assays. Total isolated bone marrow cells were seeded at $2 \times 10^5$ in a 12-well plate with methylcellulose medium supplemented with SCF (20 ng/ml), IL3 (10 ng/ml), IL-6 (10 ng/ml) and EPO (5 U/ml). Colonies were scored after 10 days culture to assess the effect of miR-223 on colony initiation capacity of hematopoietic progenitor cells, including CFU-GEMM and CFU-GM.
Western blots

Lysates were prepared from frozen tissue samples and cultured cells. The levels of NFκB activation were determined using antibodies against p65 and phospho-p65 (Cell Signaling Technology); insulin signaling was determined using antibodies against Akt1/2, and phospho-Akt (Ser473).