Molecular Cardiology

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

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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. On various types of stimuli, macrophages respond with either classic (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 opportunity 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 (miR-223) and its role in controlling macrophage functions in adipose tissue inflammation and systemic insulin resistance. miR-223−/− mice on a high-fat diet exhibited an increased severity of systemic insulin resistance compared with wild-type mice that 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, we demonstrated that miR-223 is a novel regulator of macrophage polarization, which suppresses classic proinflammatory pathways and enhances the alternative antiinflammatory responses. In addition, we identified Pknox1 as a genuine miR-223 target gene and an essential regulator for macrophage polarization.

Conclusion—For the first time, this study demonstrates that miR-223 acts to inhibit Pknox1, suppressing proinflammatory 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. (Circulation. 2012;125:2892-2903.)

Key Words: adipose tissue • insulin resistance • macrophages • microRNAs

A adipose tissue inflammation is a hallmark of obesity and a causal factor of metabolic disorders such as insulin resistance1–5 and a wide variety of metabolic diseases, including atherosclerosis and type 2 diabetes mellitus.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 the production of proinflammatory cytokines.1,2 Consequently, adipocytes produce a number of inflammatory mediators that contribute to atherosclerotic cardiovascular disease.7,8 Importantly, elevated adipose tissue inflammation is a significant factor contributing to systemic insulin resistance,9–14 which is an additional risk factor for cardiovascular disease 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.

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Several reports demonstrate that macrophages are key regulators of adipose tissue inflammatory responses.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.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, the receptor for the C-C motif chemo-

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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 or nuclear factor-κB in myeloid cells protects mice from diet-induced insulin resistance. Conversely, phenotypic switching of adipose tissue macrophages involving alternative activation (M2) provides antiinflammatory modulation of adipose tissue function and systemic insulin resistance. Within this context, peroxisome proliferator–activated receptor (PPAR)-α or PPARγ activation leads to M2 polarization in adipose tissue and in turn improves adipose tissue functions and systemic insulin sensitivity. In contrast, mice with macrophage-specific PPARγ deletion exhibited blunted macrophage M2 response and increased classic proinflammatory (M1) activation, thereby enhancing systemic insulin resistance. Thus, regulators that are crucial for macrophage polarization also exert pivotal functions in modulating adipose tissue inflammatory responses and systemic insulin sensitivity.

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 noncoding RNAs (~22 nucleotides). By base pairing with complementary sites within target mRNAs, miRNAs trigger either a block in translation and/or mRNA degradation. Numerous studies in multiple model organisms have provided compelling evidence that miRNAs are key regulators of cell fate determination and significant contributors to the pathogenesis of complex diseases, including obesity-associated metabolic diseases. Among the known miRNAs, miRNA-223 (miR-223) is a potent regulator of some inflammatory responses. When challenged by endotoxin, miR-223–deficient mice exhibited increased inflammatory lung lesions, and altered expression of miR-223 has been linked to several immune disorders, including rheumatoid arthritis and type 2 diabetes mellitus. During monocyctic differentiation into macrophages, miR-223 is downregulated; 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.39 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 5 to 6 weeks of age were used for both feeding and bone marrow isolation and macrophage activation analyses. For dietary feeding studies, mice were fed an HFD (60% fat calories, 20% protein calories, and 20% carbohydrate calories) or a low-fat diet (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, and tissue histological and immunohistochemical analyses.4,5 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 (BMDMs) were generated using protocols as previously described.21 In brief, bone marrow cells from miR-223−/− mice and WT mice were isolated, followed by erythrocyte lysis with ammonium chloride (Stem Cell Technologies), and seeded in 12-well plates at a concentration of 2×10⁶ cells per 1 mL. Cells were induced for differentiation to monocytes with RPMI 1640 medium containing 10% FBS and 15% L929 culture supernatant for 7 days. The formation of mature monocytes was evaluated on day 7 through the use of flow cytometry with fluorescence-conjugated antibodies against CD11b and F4/80.

Macrophage Polarization Analysis

To analyze macrophage polarization, BMDMs were stimulated by lipopolysaccharide (LPS; 100 ng/mL) or interleukin (IL)-4 (10 ng/mL). Surface antigens, CD69, CD80, and CD86, were examined with flow cytometry at 2, 5, 24, 48, and 72 hours after 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 with Western blot and quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) analysis. For each experiment, BMDMs from at least 3 mice were tested individually, and results were analyzed for statistical differences.

BMDM and Adipocyte Co-Culture Assay

BMDMs derived from miR-223−/− or WT bone marrow were co-cultured with differentiated 3T3-L1 adipocytes as previously described.4 After differentiation for 8 days, adipocytes were cultured with BMDMs at a ratio of 10:1.23 To determine changes in insulin signaling, the cells were treated with or without insulin (100 nmol/L) for 30 minutes before 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 with TargetScan Mouse 5.1 (www.targetscan.org) and PicTar (pictar.mdc-berlin.de).41,42 To validate miR-223–predicted targets, the luciferase reporter assay was
carried out with 3' untranslated regions of candidate genes containing potential WT or mutated miR-223 binding sites inserted downstream from the Renilla luciferase gene. The reporter constructs were cotransfected with miR-223 mimic oligonucleotides or negative control oligonucleotides into HEK293 cells. Forty-eight hours after cotransfection, the activities of Renilla luciferase were measured with 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 3 biological repeats (each biological repeat contains 3 technical repeats).

Bone Marrow Transplantation

Bone marrow transplantation analyses were performed as previously described.36 Six-week-old miR-223−/− mice (C57BL/6J background, CD45.1) or age-matched WT (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 2 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 5×10^6 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 an HFD for 8 weeks before the insulin resistance test and tissue collections.

Data and Statistical Analyses

For overall group-effect significance, data were analyzed with 2-way ANOVA and Bonferroni post test for each factor at individual times. Each data point derived from quantitative RT-PCR assays represents an average of 3 technical replicates, and data were averaged over independently replicated experiments (n=5–8 independently collected samples) and analyzed with the Student t test, presented as the mean±SEM. Data analysis was performed with the Graphpad Prism version 5.01 software. A value of P<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 WT C57BL/6J mice using quantitative miRNA RT-PCR analysis (ABI). Consistent with previous studies,39 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 nondetectable. 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 WT mice an HFD for 12 weeks. Mice maintained on a low-fat diet served as experimental controls. miR-223 ablation was confirmed with quantitative RT-PCR assays (Figure I in the online-only Data Supplement). In the WT control mice, the expression pattern in adipose tissues and bone marrow cells was not affected by an HFD (Figure 1C). miR-223−/− mice maintained on a low-fat diet did not differ from WT control mice with respect to fasting plasma levels of glucose and insulin (Figure 1D), but they exhibited a slight increase in insulin resistance and glucose intolerance (Figure 1E). On an HFD, miR-223−/− mice gained a slight but insignificant increase in body weight and showed no difference in food intake (Figure II in the online-only Data Supplement). Surprisingly, miR-223−/− mice on an HFD exhibited dramatically increased insulin response to glucose (Fed in Figure 1D) despite similar insulin and glucose levels after fasting for 16 hours (Fasted in Figure 1D). We did not observe significant effects of miR-223 deficiency on lipid metabolism or mitochondria functions of adipose tissue of mice on an HFD (Figure III in the online-only Data Supplement). It is well documented that HFD-induced adipose tissue inflammation and systemic insulin resistance, we conducted glucose tolerance and insulin resistance analyses. HFD-fed miR-223−/− mice showed a greater increase in the severity of insulin resistance and glucose intolerance than WT mice after 12 weeks on this feeding regimen (Figure 1E and 1F).

miR-223 Deficiency Enhanced M1 Macrophage Infiltration in HFD-Fed Mice

We observed a slight increase in visceral fat and adiposity in HFD-fed miR-223−/− mice compared with control mice (Figure 2A and Figure IV in the online-only Data Supplement). This was accompanied by enhanced activation of inflammatory pathways as evidenced by both increased nuclear factor-κB p65 phosphorylation in adipose tissues compared with controls (Figure II in the online-only Data Supplement). Surprisingly, miR-223−/− mice on an HFD exhibited dramatically increased insulin response to glucose (Fed in Figure 1D) despite similar insulin and glucose levels after fasting for 16 hours (Fasted in Figure 1D). We did not observe significant effects of miR-223 deficiency on lipid metabolism or mitochondria functions of adipose tissue of mice on an HFD (Figure III in the online-only Data Supplement). It is well documented that HFD-induced adipose tissue inflammation is a major contributor to systemic insulin resistance. To determine whether miR-223 is a novel regulator for HFD-induced adipose tissue inflammation and systemic insulin resistance, we conducted glucose tolerance and insulin resistance analyses. HFD-fed miR-223−/− mice showed a greater increase in the severity of insulin resistance and glucose intolerance than WT mice after 12 weeks on this feeding regimen (Figure 1E and 1F).

Immunohistochemical analysis of adipose tissue sections from HFD-fed mice showed that the size of adipocytes in HFD-fed miR-223−/− mice did not differ from those in

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**Figure 4.** Bone marrow transplantation analysis. A and B, The engraftment was examined through the use of flow cytometry analysis with antibodies against CD45.1 (donor) and CD45.2 (recipient). WT indicates wild type. C, After 3 months, microRNA-223 (miR-223) expression in the bone marrow of transplanted mice was examined with quantitative reverse transcriptase–polymerase chain reaction to confirm the reconstitution. D, Glucose and (E) insulin tolerance test in transplanted mice (BMT) on a high-fat diet (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). IL indicates interleukin; MCP1, monocyte chemotactic protein-1. G, Nuclear factor-κB activation in adipose tissues collected from HFD-fed recipient mice. Western blots were performed with antibodies against p65 and phosphorylated p65. Data are presented as mean±SEM. *P<0.05; **P<0.001.
MicroRNA-223 (miR-223) regulates macrophage polarization. 

A. Differentially expressed miR-223 in bone marrow-derived macrophages (BMDMs) on lipopolysaccharide (LPS; M1) or interleukin (IL)-4 (M2) was measured at various time points after stimulation. Data are presented as mean±SE; n=4.

B. The purity of mature BMDMs (CD11b+/F4/80+) derived from bone marrow cells isolated from wild-type (WT) or miR-223−/− mice (n=3). 

C. Cytokine, peroxisome proliferator-activated receptor-γ (PPARγ), and arginase 1 expression was determined by quantitative reverse transcriptase-polymerase chain reaction in BMDMs at 24 hours after either LPS (100 ng/mL) or IL-4 (10 ng/mL) stimulation (n=3, normalized to β-actin). TNFα indicates tumor necrosis factor-α.

D. The activation-related surface markers CD69, CD80, and CD86 were analyzed by flow cytometry after stimulation. Data are presented as mean±SEM; n=4. *P<0.05; **P<0.001.
controls (Figure 3A). Both WT and miR-223−/− mice on HFD developed fatty livers, but the severity of lipid accumulation in hepatocytes and liver weights were similar (Figure VA and VB in the online-only Data Supplement), and liver triglyceride levels were comparable in both groups (Figure VC in the online-only Data Supplement). In addition, there were no differences between miR-223−/− and WT control mice with the respect to plasma triglyceride levels on either fed or fasted (16 hours) status (Figure VI in the online-only Data Supplement). However, HFD-fed miR-223−/− mice exhibited a higher macrophage infiltration in adipose tissues compared with WT mice (Figure 3A). This was confirmed by the increased percentage of adipose tissue macrophages (CD11b+F4/80+) in visceral fat stromal cells from HFD-fed miR-223−/− mice compared with control mice (Figure 3B). Among these macrophages (CD11b+F4/80+), the proportion of M1 (CD11c+CD206−) was significantly increased in visceral stromal cells of miR-223−/− mice; in contrast, the percentage of M2 (CD11c−CD206+) in miR-223−/− visceral stromal cells was slightly less (P=0.057) than in the WT mice. Additionally, flow cytometry results indicated a higher proportion of proinflammatory macrophages (CD11b+F4/80+CD11c−CD206+) in stromal cells of HFD-fed miR-223−/− mice compared with control mice (Figure 3C and 3D), and this has been closely correlated with insulin resistance. These results demonstrate that miR-223 plays a critical role in macrophage activation and that ablation of miR-223 exacerbates M1 macrophage–mediated adipose tissue inflammation and insulin resistance.

Transplanted Mice With Myeloid Cell–Specific miR-223 Deficiency Recapitulated Phenotypes in miR-223 Mice on HFD

To confirm that the adipose tissue inflammation and insulin resistance in miR-223−/− mice are due primarily to miR-223 ablation in myeloid cells, we conducted bone marrow transplantation assays. To introduce myeloid cell–specific miR-223 ablation, we transplanted syngeneic WT mice with bone marrow cells isolated from miR-223−/− mice (BMT–miR-223−/−), and age-matched WT donor mice (BMT-WT) were used as control in the study. The engraftment of donor cells (CD45.1) in lethally irradiated recipient mice (CD45.2) was confirmed by the presence of donor-derived cells (Figure 4A and 4B). Once confirmed, the recipient mice were fed an HFD for 8 weeks and subjected to insulin sensitivity and glucose tolerance tests. There were no differences in the body weight gain and food intake between the 2 groups (Figure VII in the online-only Data Supplement). Various tissues were then collected, and the engraftment was further confirmed with 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 (Figure VIII in the online-only Data Supplement). In addition, BMT–miR-223−/− mice exhibited increased severity of glucose intolerance (Figure 4D) and insulin resistance (Figure 4E) compared with BMT-WT mice. As expected, elevated proinflammatory cytokines (Figure 4F) accompanied by enhanced nuclear factor-κB activation (Figure 4G) were observed in adipose tissues collected from BMT–miR-223−/− mice compared with those from control mice. We did not observe differences in plasma insulin, glucose, or triglyceride levels or visceral adiposity between the 2 groups (Figure IX in the online-only Data Supplement). Taken together, our results suggest that exacerbated adipose tissue inflammation and insulin resistance in miR-223–deficient mice are due mainly to enhanced proinflammatory response of myeloid cells with miR-223 ablation.
miR-223 Is a Novel Regulator for Macrophage Polarization

To determine whether the 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 low-fat diet–fed mice. Consistent with a previous report, the neutrophil portion was slightly increased in miR-223−/− compared with control mice.39 No significant differences were detected in the macrophage population (CD11b+ Gr-1+; Figure X in the online-only Data Supplement). 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/monocyte or colony forming unit–granulocyte/erythrocyte/megakaryocyte/monocyte (Figure XI in the online-only Data Supplement), 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 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 on M1 or M2 activation. Dramatically elevated miR-223 levels were observed in BMDMs 5 hours after treatment with IL-4, and levels remained high for up to 72 hours (Figure 5A), whereas LPS stimulation slightly decreased miR-223 levels in BMDMs (Figure 5A). During the 7-day course of macrophage differentiation, there were no differences in the mature macrophage purity in BMDMs from either miR-223−/− or WT mice, as evidenced by fluorescence-activated cell sorter analysis with antibodies against CD11b and F4/80 (Figure 5B). Quantitative RT-PCR analysis showed that proinflammatory cytokine IL-1α, IL-6, and tumor necrosis factor-α were significantly elevated in miR-223−/− macrophages compared with WT macrophages on LPS stimulation. Expression of M2-associated genes PPARγ and arginase 1 was increased in miR-223−/− macrophages compared with control cells after IL-4 stimulation (Figure 5C). miR-223−/− macrophages exhibited enhanced M1 but decreased M2 responses, as judged by fluorescence-activated cell sorter analysis with antibodies against activation surface markers CD69, CD80, and CD86 at various time points after stimulation (Figure 5D).

To examine the direct impact of isolated BMDM on adipocytes, we used an in vitro co-culture assay. miR-223−/− BMDM-treated WT adipocytes exhibited a slight but significant increase in nuclear factor-κB p65 phosphorylation compared with control adipocytes (Figure 6A). Additionally, in miR-223−/− BMDM-treated WT adipocytes, there was a decrease in insulin-stimulated Akt (Ser473) phosphorylation (Figure 6B) and an increase in proinflammatory cytokines on LPS stimulation (Figure 6C). These results recapitulated adipose tissue inflammatory and metabolic responses of HFD-fed miR-223−/− mice (Figure 2B and 2D) and BMT–miR-223−/− mice (Figure 4F and 4G) and clearly demonstrate that miR-223 is indeed an important regulator of macrophage polarization.

Figure 7. Pknox1 is a microRNA-223 (miR-223) target. A, Predicted miR-223 binding site in the 3′ untranslated region (UTR) of Pknox1 and a mutated version of the seed match region (red). Reporter constructs containing a 3′ UTR region with wild-type (WT; B) or mutated miR-223 binding site of Pknox1 (mut) (C) were cotransfected with an 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 (lipopolysaccharide [LPS] or interleukin [IL]-4) bone marrow–derived macrophages from WT or miR-223−/− mice was examined with quantitative reverse transcriptase–polymerase chain reaction (normalized to β-actin). Data are presented as mean ± SEM; n = 4. E, Levels of Pknox1 protein in adipose tissues collected from high-fat diet–fed WT or miR-223−/− mice were determined by Western blots. *P < 0.05; **P < 0.001. Mmu indicates Mus musculus.
Figure 8. Role of Pknox1 in macrophage polarization. A, Pknox1 was targeted with siRNA (siPknox1) in bone marrow–derived macrophages (BMDMs) with microRNA-223 (miR-223) deletion. Scrambled siRNA was used as control (Ctrl). The knockdown was confirmed with quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR; n=8). Cells were then stimulated with either lipopolysaccharide (LPS; 100 ng/mL) or interleukin (IL)-4 (10 ng/mL) for 24 hours. B, IL-1β production and arginase 1 expression were measured.
Pkn01 Is a Bona Fide miR-223 Target Gene 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 with luciferase reporter assays. Among 8 tested potential targets, Pkn01 was identified as a genuine target of miR-223 (Figure 7A). Luciferase activity was repressed in cells transfected with constructs containing 3′ untranslated 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). Pkn01 expression was higher in miR-223−/− BMDMs stimulated with LPS (Figure 7D and Figure XII in the online-only Data Supplement) compared with BMDMs from control mice. Consistent with in vitro observations, Pkn01 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 Pkn01 in macrophage polarization was further investigated with the use of gene-specific short interfering RNAs to knock down elevated levels of Pkn01 (Figure 8A). Knockdown of Pkn01 (siPkn01) in miR-223−/− BMDMs decreased pro-inflammatory cytokine production (IL-1β; Figure 8B) and partially blocked M1 response as indicated by fluorescence-activated cell sorter analysis (Figure 8C). M2 activation was also partially restored in miR-223−/− BMDMs with siPkn01 knockdown as judged by elevated arginase 1 levels (Figure 8B). To further confirm the function of Pkn01 in macrophage polarization, we introduced ectopic expression of this protein in BMDMs by lentiviral infection (Figure XIII in the online-only Data Supplement). Pkn01 overexpression partially recapitulated the miR-223−/− macrophage response to LPS with a significantly enhanced shift in surface markers (Figure 8D) and elevated inflammatory cytokine production (Figure 8E). These results demonstrated that Pkn01 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.1,2,15,16 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 with 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 proinflammatory 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 by inappropriate adipokine expression, which are indicators for adipose tissue 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 nuclear factor-κB and insulin signaling pathways in adipocytes treated with miR-223−/− macrophages recapitulated the defects observed in adipose tissue of miR-223−/− mice on HFD. Thus, miR-223 expression in macrophages is an important component of adipocyte inflammatory and metabolic responses.

Macrophage accumulation was significantly higher in adipose tissue from HFD-fed miR-223−/− mice than in WT 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 (Figure V in the online-only Data Supplement). 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 monocyte chemotactic protein-1,44 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.1,2,14 This is further validated by our enhanced adipose tissue inflammatory responses in miR-223−/− mice. Moreover, HFD-fed miR-223−/− deficient mice exhibited adipose tissue macrophage infiltration, proinflammatory cytokine expression, and nuclear factor-κB p65 phosphorylation. Genes that are crucial for metabolism were not directly affected by the loss of miR-223 in both adipose tissue and liver (Figure III in the online-only Data Supplement). 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 a combination of computational analysis and luciferase reporter assays, we identified Pkn01 as a genuine target of miR-223. The expression of Pkn01 is inversely correlated with miR-223 levels in either activated BMDMs or adipose cell sorter; n=3. MFI indicates median fluorescence intensity. BMDMs from wild-type mice were lentivirally infected to introduce ectopic Pkn01 expression. At 24 hours after infection, cells were stimulated with LPS or IL-4, (D) activation-related surface markers (CD69, CD80, and CD86) were examined with flow cytometry, and (E) cytokine production was measured with qRT-PCR (normalized to β-actin). TNFα indicates tumor necrosis factor-α. *P<0.05; **P<0.001.
tissues. The function of Pknox1 as a target of miR-223 in regulating macrophage polarization was validated in our gain-of-function and loss-of-function analyses 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 be involved in miR-223–regulated macrophage function. Indeed, we identified several genes besides Pknox1 that may play important roles in modulating macrophage activation; their function will be validated further.

Conclusions

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 and the crucial target gene Pknox1 in modulating macrophage function provided novel insights into the network governing macrophage-mediated adipose tissue inflammatory response and metabolic regulation. These unique observations indicate that 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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Disclosures

None.

References


**CLINICAL PERSPECTIVE**

Macrophage-mediated adipose tissue inflammation is a major contributor to the pathogenesis of obesity-associated chronic diseases, including the 2 types of most prevailing diseases, namely type 2 diabetes mellitus and cardiovascular diseases. The activation status of macrophages is a key determinant for the onset of these diseases, and despite extensive research in this area, major questions about macrophages function and their interactions with host tissues remain unanswered. The discovery of microRNAs (miRNAs) has opened a new window for understanding the regulatory networks related to homeostasis and disease. MiRNAs are a family of small noncoding RNAs that have been demonstrated to be crucial regulators in multiple physiological processes and disease pathogenesis. Altered expression patterns of miRNAs are tightly associated with chronic inflammatory diseases. However, miRNA-mediated regulatory networks in modulating macrophage polarization in adipose tissue inflammation have not been previously investigated. In this study, we demonstrated that miR-223 is a novel and crucial regulator of macrophage polarization and is indicated for suppressing proinflammatory and enhancing antiinflammatory responses. Our results identify a new miRNA-based paradigm for the regulation of insulin sensitivity and provide the basis for using miRNA analogs to treat insulin resistance–related diseases.
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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.
Genes that are key regulators for lipogenesis, mitochondrial function, lipolysis were measured in the adipose tissues (A) and liver (B) collected from miR223\(^{-/-}\) 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.
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).