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

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-
ligand 2 (also known as monocyte chemotactic protein-1). 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 and PPARγ are the 2 best-known intracellular regulators of the alternative macrophage activation pathways. PPARγ or PPARα activation leads to M2 polarization in adipose tissue and in turn improves adipose tissue functions and systemic insulin sensitivity. 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)-γ and PPARδ are the 2 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. 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 monocytic 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 insulin resistance.
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 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. 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. 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 2×10^6 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. After differentiation for 8 days, adipocytes were cultured with BMDMs at a ratio of 10:1. 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). 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 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 5×10⁶ 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 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 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.24-20 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).
Figure 5. 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 = 5). **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+ transcript) in visceral fat stromal cells from HFD-fed miR-223−/− mice compared with control mice (Figure 3B). Among these macrophages (CD11b+ transcript), 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+ transcript/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.43 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. 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/erythrocyte/megakaryocyte/monocyte or colony forming unit–granulocyte/monocyte (Figure XI in the online-only Data Supplement), indicating that increased adipose tissue inflammation is likely due to the alteration of macrophage production 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-1β, 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 decreased 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 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.
Pkn0x1 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, Pkn0x1 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). Pkn0x1 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, Pkn0x1 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 Pkn0x1 in macrophage polarization was further investigated with the use of gene-specific short interfering RNAs to knock down elevated levels of Pkn0x1 (Figure 8A). Knockdown of Pkn0x1 (siPkn0x1) 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 siPkn0x1 knockdown as judged by elevated arginase 1 levels (Figure 8B). To further confirm the function of Pkn0x1 in macrophage polarization, we introduced ectopic expression of this protein in BMDMs by lentiviral infection (Figure XIII in the online-only Data Supplement). Pkn0x1 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 Pkn0x1 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.1,2,21–24 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–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 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 infiltrating 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.1,2,4 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 Pkn0x1 as a genuine target of miR-223. The expression of Pkn0x1 is inversely 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-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

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.