MICRORNA-223 REGULATES MACROPHAGE POLARIZATION AND DIET-INDUCED INSULIN RESISTANCE

A Thesis

by

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ABSTRACT

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.

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 pro-inflammatory pathways and enhances the alternative anti-inflammatory responses. In addition, we identified Pknox1 as a genuine miR-223 target gene and an essential regulator for macrophage polarization.

For the first time, this study demonstrates that miR-223 acts to inhibit Pknox 1, 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.

DEDICATION

To my favorite younger brother-

Yanbo

God bless you

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NOMENCLATURE

ATM	Adipose Tissue Macrophage
BAT	Brown Adipose Tissue
BM	Bone Marrow
CCL-2	Chemokine (C-C motif) ligand 2
Ctrl	Control
DUSP2	Dual specificity protein phosphatase 2
Epi-fat	Epididymal Fat
FBOX-8	F-box Protein 8
GTT	Glucose Tolerance Test
H&E	Hematoxylin Eosin
HFD	High fat Diet
IL-1β	Interleukin-1β
IL-4	Interleukin-4
IL-6	Interleukin-6
IRS	Insulin Receptor Substrate
ITT	Insulin Tolerance Test
LFD	Low Fat Diet
IGF1R	Insulin-like Growth Factor 1 Receptor
LMO2	LIM domain only 2
LPS	Lipopolysaccharides

MCP-1	Monocyte Chemotactic Protein-1
Mes-fat	Mesenteric Fat
miR-223	microRNA-223
miRs	microRNAs
NFκB	Nuclear Factor κ B
PBS	Phosphate-Buffered Saline
Peri-fat	Perinephric Fat
PKNOX1	PBX/Knotted 1 Homeobox
PPARγ	Peroxisome Proliferator-activated Receptor-γ
PPARδ	Peroxisome Proliferator-activated Receptor-δ
qRT-PCR	Quantitative RT-PCR
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SiRNA	Small interfering RNA
SP3	Sp3 transcription factor
T2DM	Type 2 Diabetes Mellitus
TGFBR3	Transforming Growth Factor, Beta Receptor III
UTR	Untranslated Region
WT	Wild-type

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW*

Introduction

Type 2 diabetes is a major pandemic problem in the United States and worldwide and is characterized by the inability to utilize insulin resulting in high blood sugar levels. This phenomenon is known as insulin resistance and is the hallmark of type 2 diabetes. Insulin resistance can be induced by adipose tissue inflammation, a characteristic feature of obesity resulting from nutrient excess (HFD).[1]

Recent in research has linked obesity with inflammation and insulin resistance; for example, Hotamisiligil and coworkers have reported a relationship between obesity-induced inflammation and insulin resistance.[1,2] Inflammation associated with obesity is characterized by: 1) elevated levels of inflammatory molecules in the blood plasma and adipose tissue;[3,4] 2) infiltration and activation of macrophages in the adipose tissue.[1,5] Adipose tissue inflammation ultimately results in insulin resistance by inhibiting insulin receptor substrate (IRS) 1 or 2 (IRS-1/2), which interferes with the insulin signaling pathways.[6] Additionally, inflammation may also interfere with the insulin functioning by increasing concentrations, of circulating free fatty acid, TNF-α or IL-1β and decreasing

* Portion of this thesis are reprinted with the permission from Circulation Journal articles: A Novel Regulator of Macrophage Activation: miR-223 in Obesity-Associated Adipose Tissue Inflammation. Zhuang G*, Meng C* (equal contribution), Wang G, Guo X, Li H, Shi L, Stephen Safe, Wu C, Zhou B. (Epub: May 11, 2012)

adiponectin.[1,3,7] Both inflammatory cytokines as well as free fatty acids can target insulin receptor leading to the development of insulin resistance.[7,8] In addition, macrophage infiltration and activation in adipose tissues can trigger multiple signaling pathways resulting in systemic insulin resistance. Activation of macrophages can occur through two processes namely classic activation (M1) and alternative activation (M2). Classical activation results in production of pro-inflammatory cytokines whereas activation through alternative pathways leads to production of anti-inflammatory cytokines. Although the role of macrophages in obesity-induced inflammation is well established, the regulation of macrophage polarization is not well understood.

Recently, some studies have reported the roles of epigenetic regulators including microRNAs in the pathogenesis of diabetes and this opened a new window for investigating mechanisms governing obesity induced inflammation and diabetes.[9] MicroRNAs (miRs) are small noncoding RNAs of approximately 22-24 nucleotides in length. They are highly conserved in species and can modulate the expression of their target mRNAs by base pairing at complimentary sites leading to either blockage of protein translation or mRNA degradation.[10] Many studies have revealed the roles of miRs in cell fate determination and pathogenesis of many diseases including obesity associated metabolic disorders.[11] However, regulation in adipose tissue inflammation by miRs is not well understood. In addition to the known miRs regulators of adipogenesis, we have recently identified miR-223 as a microRNA regulator, which is highly expressed in myeloid cells including macrophages. Our studies revealed that: 1) compared to wild-type, miR-223 deficient (miR-223$^{-/-}$) HFD-fed mice exhibited a higher

percentage of infiltrated macrophages in the adipose tissue; 2) mice lacking miR-223 displayed increased insulin resistance and glucose intolerance; 3) macrophages in the miR-223$^{-/-}$ adipose tissue were activated through M1 pathways; 4) miR-223 regulates macrophage polarization associated insulin resistance through its target gene PKnox1 and others, as shown in Figure 1.[12] All these observations suggest that miR-223 plays a crucial role on regulating macrophage activation and associated insulin resistance.

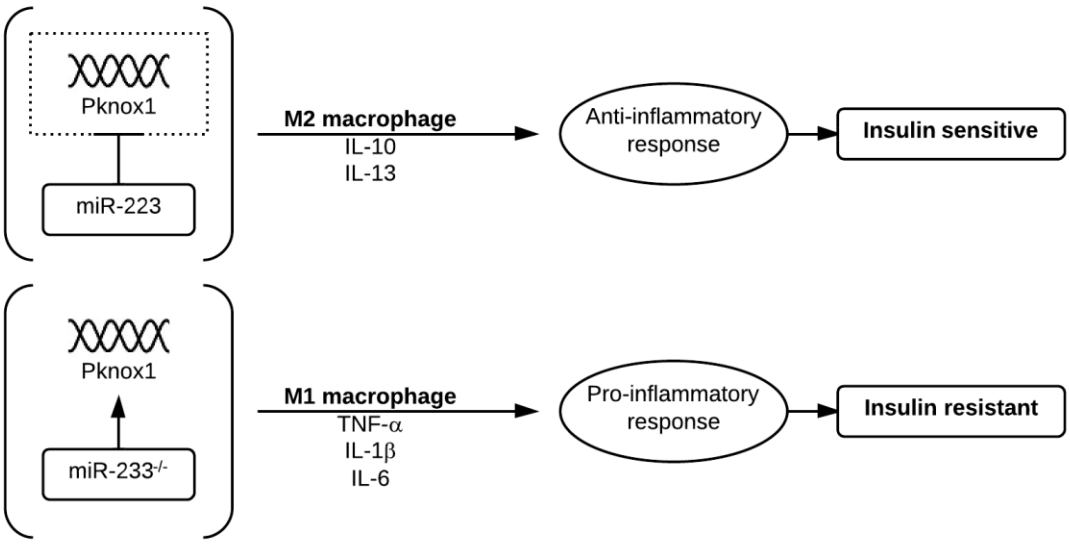


Figure 1. Schematic mechanism of the miR-223 regulatory role in the macrophage polarization associated insulin resistance. Adapted and modified based on Jiandie Lin, et al.[12]

Literature review

Molecular Mechanisms that Affect Obesity Related Comorbidities

Obesity is one of the leading health issues in developed nations, affecting nearly 1/3 of the population and 17% of children in US. The public health crisis associated with obesity is due to comorbidities such as diabetes which greatly affect the quality of life leading to more serious health outcomes such as heart disease.[4,13] While diet and exercise can decrease one's body mass index (BMI) and be an effective way for dealing with obesity related metabolic disorders. Other essential strategies can also be beneficial including modulation of inflammatory responses to attenuate metabolic disorders. Many of the comorbidities associated with obesity such as type 2 diabetes and other metabolic syndromes are due to low-grade chronic inflammation.[14] Understanding the molecular links between obesity, inflammation, the immune response and finally the comorbidities associated with obesity may provide insights on pharmaceutical interventions for treating these diseases.

Adipose Tissue Inflammation and Insulin Resistance

One of the possible links between obesity and metabolic disorders is due to macrophage polarization which can be stimulated by the production of inflammatory cytokines by adipocytes.[14-16] After activation of adipose tissue macrophages (ATMs), there is an inflammatory cascade that leads to an increased inflammation which is an etiologic function in diseases such as diabetes, atherosclerosis, or metabolic

syndromes.[3,17,18] Adipocytes as well as ATMs are both sources of either inflammatory or anti-inflammatory cytokines and other mediators. Therefore, to better understand the comorbidities associated with obesity, we have investigated pathways in which adipose cells promote polarization of ATMs, and the mechanism of polarized activation of ATMs since the inflammatory cascade that results from the polarization of ATM[19] leads to the other diseases.

Under lean conditions most macrophages are found in an anti-inflammatory state,[20] which may be due to the secretion of cytokines such as adiponection by adipocytes,[21] that can polarizes ATM into an M2 phenotype which exhibits an anti-inflammatory profile.[17,22] However, in obese individuals, adiponectin is down-regulated, and there is an increase probability that macrophages will assume a pro-inflammatory M1 phenotype, as shown in Figure 2 and Figure 3.[23] It has been shown both *in vivo* and *in vitro* that adiponectin can prevent the polarization of ATM's into the M1 phenotype,[24] and down-regulation of adiponectin under obese conditions may be a viable method for blunting the metabolic comorbidities associated with obesity.

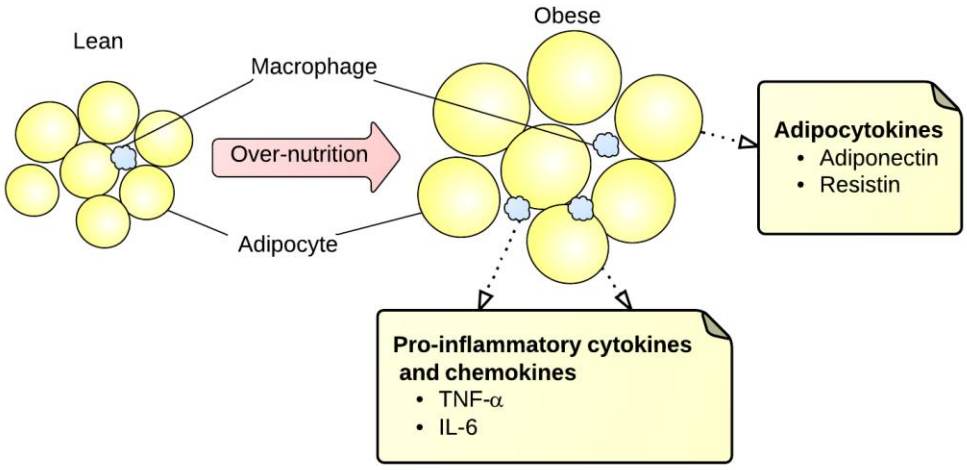


Figure 2. Production of pro-inflammation responses in over-nutrition induced obesity. Adapted and modified based on Herbert Tilg, et al.[25]

Mechanisms Controlling Macrophage Polarization

One possible factor that may control the levels of adiponectin may be the activation of the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) in ATM's.[1] PPARγ is activated in the presence of fatty acids, and in conjunction with RXR and is responsible for regulating levels of adiponectin and controlling polarization of macrophages into the M2 phenotype. This suggests that PPARγ plays an anti-inflammatory role on two levels, namely, preventing the differentiation of monocytes which induces the inflammatory M1 phenotype, and controlling expression of anti-inflammatory cytokines in adipocytes.[26] This strongly suggests that PPARγ may play a role in the body's response to the short term effects of inflammation due to the level of fatty acids in the organism.

PPARδ also responds to TH2 cytokines, and under the influence of TH2, PPARδ controls polarization of the macrophages to the M2 phenotype.[27] However, it should be

noted that PPARγ and PPARδ are similar in terms of both their ligands and their DNA binding sequences.[28] Therefore the fact that both of these nuclear receptors have been implicated in the control of macrophage differentiation to the M2 phenotype suggests that there may be either significant off-target effects or significant crosstalk between their respective signaling pathways. It may be possible to utilize some of the new generation highly specific PPARδ agonists to probe the differentiation of monocytes *in vitro*, or via the use of knockout models to determine whether the γ and δ subtypes of PPAR compensate for the loss of the other receptors. However, PPARγ and PPARδ seem to play a critical role in the control of monocyte differentiation, and it is likely that both receptors control the anti-inflammatory effects, seen in response to fatty acids. It has been shown that up-regulation or activation of different PPARs via compounds such as rosiglitazone can have positive effects on inhibiting inflammation, enhancing insulin production, and insulin resistance. [23] However, it is unclear whether this response is solely due to decreasing the overall level of inflammation or suppressing monocyte differentiation, or due to other metabolic effects given the important role of PPARγ in regulating glucose metabolism and adipogenesis.[23]

It has been well-established in the literature that adipocytes and ATM's play important roles in the development of chronic inflammation associated with obesity. However, few have investigated the mechanisms of the anti-inflammatory responses, and this will be important for understanding an increase in the overall level of inflammation.[22] While fatty acids can lead to the activation of PPARγ, over the long term, involvement of PPARγ is not sufficient to prevent the pro-inflammatory responses, associated with

obesity. To date, it is unclear whether long term exposure to fatty acids can mediate a secondary tolerance mechanism in which the PPARγ is down-regulated leading to the loss of sensitivity to fatty acids, and therefore dysregulation of adiponectin levels.[26] It has been observed that in addition to the anti-inflammatory signals/mechanisms, adipocytes that are stimulated with fatty acids adipocytes can also secrete chemokines, including an important component is CCL-2[29] which is responsible for the recruitment of macrophages. The presence of the CCL-2 chemokine exacerbates the inflammatory conditions and may serve as a counterbalance factor to the anti-inflammatory activity of PPARγ. Another issue that has not previously been adequately addressed are the different magnitudes, of these different effects and whether one serves as a compensatory mechanism for the other, or whether these mechanisms function independently.

The accumulation of macrophages with classic M1 phenotypes increases the amount of TNF-α that is secreted, and TNF-α can then cause the subsequent polarization of monocytes through the classical NFκB/JNK pathway. Activated NFκB then up-regulates the expression of genes containing the AP1, ISRE, or kB response elements,[33] which in turn enhance the polarization of macrophages. Furthermore, activation of JNK is also associated with insulin resistance, as shown in Figure 4.[15]

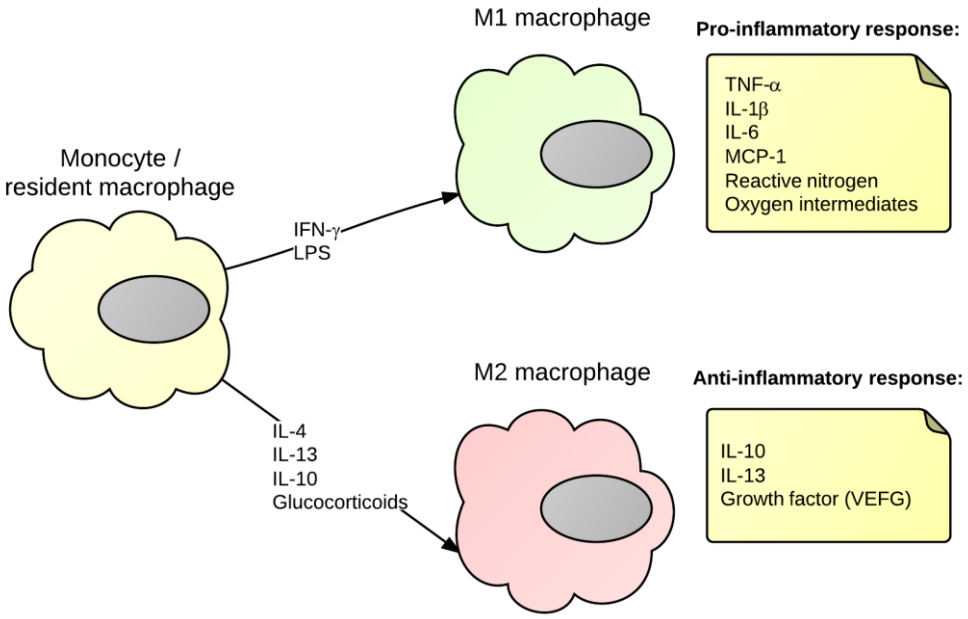


Figure 3. Macrophage polarization and its activation by cytokines.[17-29]

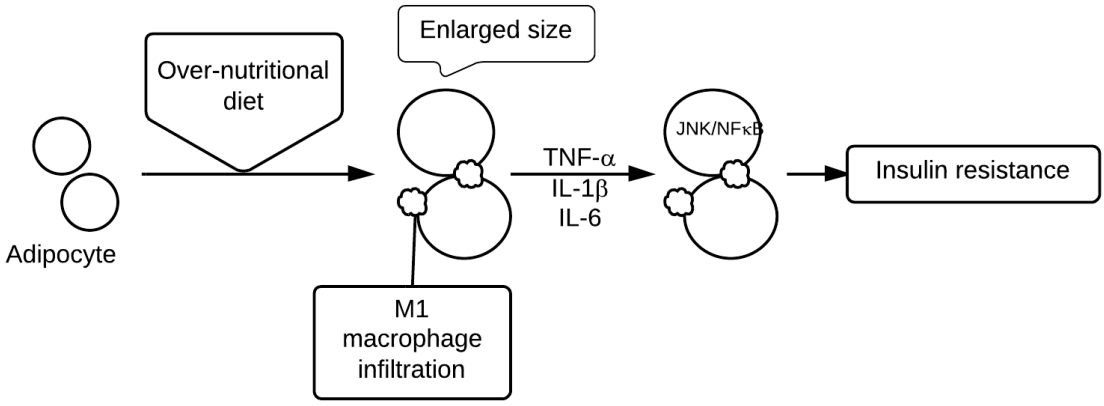


Figure 4. M1 macrophage activation is involved in the JNK/NFκB pathway. Adapted and modified based on Kathryn E. Wellen, et al.[15]

microRNAs and the Importance of miR-223

Obesity leads to a chronic state of low-grade inflammation and various pathways such as chemotaxis and the compensatory anti-inflammatory pathways via PPARγ and adiponectin are counter-balances to inflammation and it is important to understand how inflammation can lead to obesity related comorbidities such as diabetes or atherosclerosis. Identification the overall mechanisms, of inflammation may allow us to develop macrophage polarization associated treatments.

One mechanism by which inflammation can trigger insulin resistance is through the induction of miRs transcription.[30] The presence of specific miRs' such as miR-375 which has been identified in pancreatic islets and beta cells can change the ability of cells to secrete insulin through binding to one of its target genes, Mtpn.[31] Additionally, miRs have been shown to play a role in controlling cell proliferation and differentiation,[32] and the expression of certain miRs can also have profound effects upon the development of cell lineages.[33] MiRs are small noncoding RNAs of approximately 22-24 nucleotides in length. They normally reside either in the intronic region of genes or in the noncoding region of the genome. From the ENCODE project, it was found that over 90% of the genome was actively being transcribed, which indicates the importance of miRs and other non-coding RNAs in maintaining cellular homeostasis. They play regulatory roles in post-transcriptional gene expression primarily by binding to the 3' UTR of the target genes resulting in RNA degradation, or by translational repression, illustrated in Figure 5.

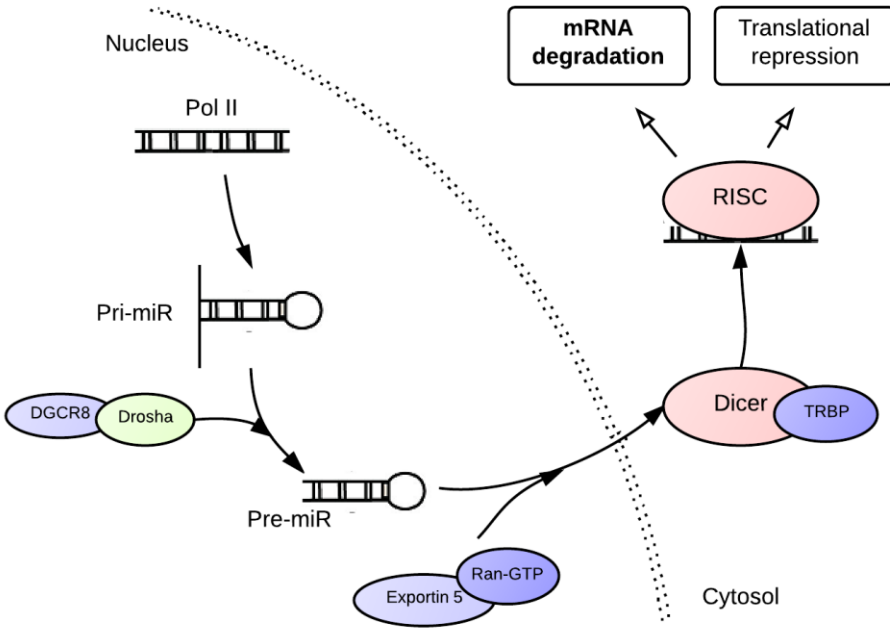


Figure 5. Biogenesis and molecular function of miRs. Adapted and modified based on Stefanie Dimmeler, et al.[35]

MiRs may play significant roles in low-grade inflammation as well as the co-morbidities associated with obesity. In the process of low-grade inflammation and the control of monocyte differentiation, it was reported that miR-154, miR-221, and miR-222 control the differentiation of monocytes into either the M2 anti-inflammatory fate or the M1 pro-inflammatory orientation,[31] and that the control of these miRs was dependent on whether a pro-inflammatory cytokine was the primary activator or whether an anti-inflammatory cytokine was the activator.

It was also found that miR-375 and miR-9 are important for regulating insulin secretion. MiR-375 was found to be regulated by NFκB that is one of the transcription factors that is activated via TNF-α, which is one of the pro-inflammatory cytokines present in an

inflammatory state. This therefore can be one of mechanism which leads to the lack of insulin production in obese individuals, or those with significant levels of inflammation.[34]

However, in addition to the lack of insulin production, obese subjects also experience significant insulin resistance i.e. their tissues do not properly take up glucose when stimulated by insulin. It was found that miR-143 can control the expression of GLUT4,[24] an important glucose transporter. Furthermore, it was reported that PPARγ is also regulated by the expression of miR-143. However it should be noted that miR-143 works indirectly in this pathway, and decreases GLUT4 transporter or PPARγ expression through ERK activation, which in turn down regulates these proteins. [23]

Other obesity-mediated phenomena such as atherosclerosis, are also linked to the expression and induction of miRs. MiR-126 was found to control the TNF-α mediated expression of VCAM-1 and thus affects the adhesion of endothelial cells,[34] and this in turn affects the integrity of the overall vasculature. Coupled with the expression of miR-155, miR-17-92 increases, the presence of granulation as well as the further differentiation of monocytes. These two components later make up a substantial portion of the atherosclerosis lesions.

While many different miRs may play roles in the comorbidities associated with obesity, there is one specific miR, miR-223, which plays a role in atherosclerosis because of its effect controlling the differentiation of immune cells and the process of granulation which leads to the formation of scar tissue.[35] Furthermore, miR-223 also function in the down-regulation of various glucose transporters, which lead to insulin resistance.

MiR-223 was first discovered as a crucial regulator of neutrophil differentiation. [36,37] Mice with miR-223 ablation were viable but had significantly higher levels of immature neutrophils. MiR-223 expression is controlled by the C/EBP-α transcription factor that is expressed in neutrophil cell lines. What is notable about the C/EBP-α transcription factor is that it is activated during the latter stages of adipocyte maturation.[38] Therefore, an increase in the number and size of adipocytes can lead to the activation of the C/EBP-α, which then induces expression of miR-223. In addition, tt was found that the activation of PPARγ leads to the up-regulation of C/EBP-α, and thus one may function as a negative feedback mechanism for the other.[46] This suggests that while PPARγ may play a role in limiting inflammation, one of its targets, C/EBP-α may play the opposite role.[38] This may be one of the mechanism that leads to the paradoxical effects of fatty acids, namely the anti-inflammatory effects mediated by fatty acid activation of PPARγ and PPARδ, and the long term inflammatory responses mediated by C/EBP-α.[39] Furthermore, it appears that if the pathway is mediated by C/EBP-α, glucose intolerance that is observed is more a function of activation via C/EBP-α and its effect upon the glucose transporter, and that inflammation while an important component in obesity does not play a direct role.

In general, chronic low-grade inflammation associated with obesity has many components. Based upon the involvement of different signaling pathways via production of various cytokines, adipose tissue inflammation can be regulated by governing the macrophage status and switching from a pro-inflammatory state to anti-inflammatory state. In an effort to investigate the overall mechanisms leading to specific co-

morbidities, it is important to take a more overall holistic approach in which the systems as a whole is investigated rather than individual pathways. While it may not be possible to isolate multiple components within a signal experiment, the incorporation of systems biology and sophisticated modeling approaches may represent a way to organize the existing knowledge base and to synthesize new hypotheses that are not apparent from results of individual experiments.

CHAPTER II

SUMMARY AND CONCLUSION

Overview

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.

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 pro-inflammatory pathways and enhances the alternative anti-inflammatory responses. In addition, we identified Pknox1

as a genuine miR-223 target gene and an essential regulator for macrophage polarization.

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.

Introduction

Adipose tissue inflammation is a hallmark of obesity and a causal factor of metabolic disorders such as insulin resistance[1,40-43] and a wide variety of metabolic diseases, including atherosclerosis and type 2 diabetes mellitus.[42-44] 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 pro-inflammatory cytokines.[1,40] Consequently, adipocytes produce a number of inflammatory mediators that contribute to atherosclerotic cardiovascular disease.[17,45] Importantly, elevated adipose tissue inflammation is a significant factor contributing to systemic insulin resistance,[46-51] 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.

Several reports demonstrate that macrophages are key regulators of adipose tissue inflammatory responses.[1,5,40,52,53] For example, in mice lacking osteopontin, a secreted

matrix glycoprotein and pro-inflammatory cytokine, inhibition of macrophage recruitment suppresses adipose tissue inflammatory response.[54] 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 chemokine ligand 2 (also known as monocyte chemotactic protein-1.[52] 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.[53,55] Conversely, phenotypic switching of adipose tissue macrophages involving alternative activation (M2) provides anti-inflammatory modulation of adipose tissue function and systemic insulin resistance.[26] 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.[23,27,41,56,57] In contrast, mice with macrophage specific PPARγ deletion exhibited blunted macrophage M2 response and increased classic pro-inflammatory (M1) activation, thereby enhancing systemic insulin resistance.[23] Thus, regulators that are crucial for macrophage polarization also exert pivotal functions in modulating adipose tissue inflammatory responses and systemic insulin sensitivity.[23,41,55,56] However, despite the importance of this process to metabolic diseases, the mechanisms underlying macrophage polarization

remain poorly understood.

MicroRNAs (miRs) are a group of highly conserved, small noncoding RNAs (≈22 nucleotides). By base pairing with complementary sites within target mRNAs, miRs trigger either a block in translation and/or mRNA degradation.[10,30,58-60] Numerous studies in multiple model organisms have provided compelling evidence that miRs are key regulators of cell fate determination and significant contributors to the pathogenesis of complex diseases, including obesity-associated metabolic diseases.[11,32,61-66] Among the known miRs, miR-223 (miR-223) is a potent regulator of some inflammatory responses.[39,67] When challenged by endotoxin, miR-223-deficient mice exhibited increased inflammatory lung lesions,[39] and altered expression of miR-223 has been linked to several immune disorders, including rheumatoid arthritis and type 2 diabetes mellitus.[64,68] During monocytic differentiation into macrophages, miR-223 is downregulated[67]; 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.[42,43] 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 previous described.[23] 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.[42] 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).[69,70] 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 down stream 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.[51] 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 miR 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 6A). 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 6B). 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 7).In the WT control mice, the expression pattern in adipose tissues and bone marrow cells was not affected by an HFD (Figure 6C). 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 6D), but they exhibited a slight increase in insulin resistance and glucose intolerance (Figure 6E). On an HFD, miR-223$^{-/-}$ mice gained a slight but insignificant increase in body weight and showed no difference in food intake (Figure 8). Surprisingly, miR-223$^{-/-}$ mice on an HFD exhibited dramatically increased insulin response to glucose (Fed in Figure 6D) despite similar insulin and glucose levels after fasting for 16 hours (Fasted in Figure 6D). 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 9). It is well documented that HFD- induced adipose tissue inflammation is a major contributor to systemic insulin resistance.[26,40,42] 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 6E and 7F).

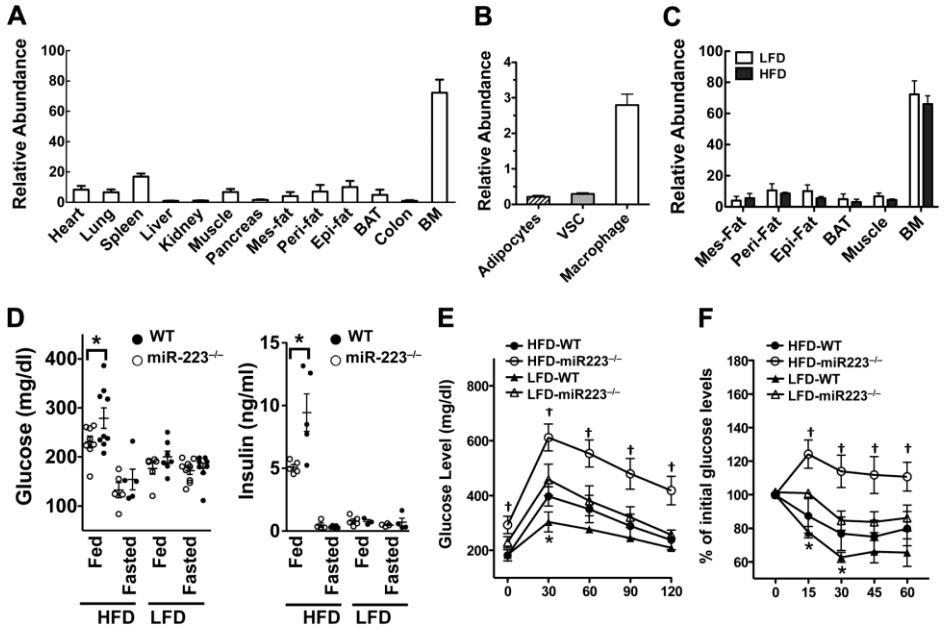


Figure 6. MicroRNA-223 (miR-223) deficiency exacerbates high-fat diet (HFD)-induced insulin resistance. A, The expression levels of miR-223 in various tissues were examined with quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). White adipose tissue (WAT) samples included mesenteric (Mes), perinephric (Peri), and epididymal (Epi) fat pads. BAT indicates brown adipose tissue; BM, bone marrow. B, The expression of miR-223 was examined in macrophages, mature adipocytes, and visceral stromal cells (VSCs) isolated from epididymal fat tissues. For A and B, tissue samples were collected from chow diet-fed male wild-type (WT) mice. Data are presented as mean±SEM; n=3. C, The dietary effects on miR-223 expression were examined in the metabolic-related tissues with quantitative RT-PCR from mice (n=3) on either an HFD or a low-fat diet (LFD) for 12 weeks. D, Plasma glucose and insulin levels in miR-223$^{-/-}$ or WT mice fed an 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-WT; *P<0.05, LFD-miR-223$^{-/-}$ vs LFD-WT.(A and B are contributed by Guoqing Zhuang, D,E and F are contributed by Guoqing Zhuang, Gang Wang and Xin Guo)

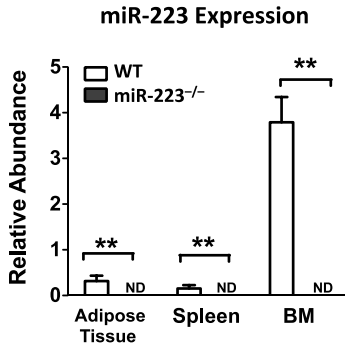
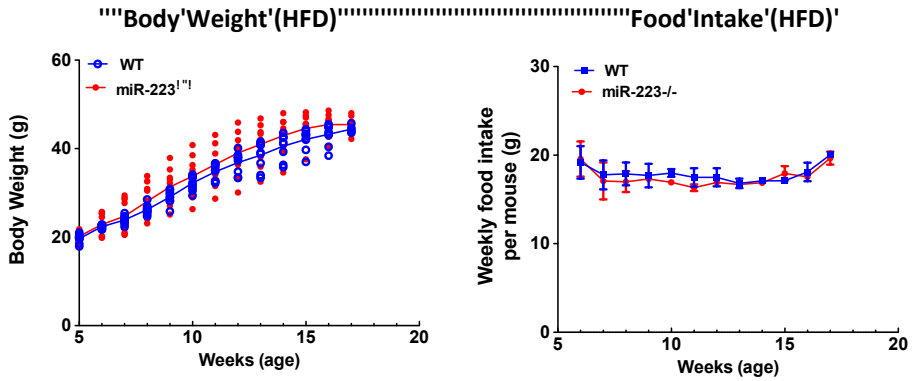


Figure 7. miR-223 expression level in adipose tissue, spleen and bone marrow. Total RNA was extracted from various 6ssues of wild type (WT) or miR-223 knockout (miR-223$^{-/-}$) mice. The expression of miR-223 was measured using Taqman miR Assay kits (ABI). Relative abundance of miR-223 in each sample 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

Figure 8. Body weight and food intake in WT and miR-223$^{-/-}$ mice. 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. (Contributed by Guoqing Zhuang and Gang Wang)

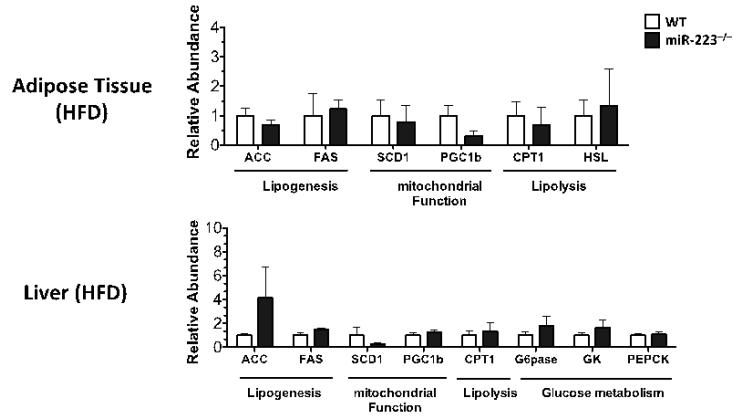


Figure 9. Expression level of genes that are key regulators for lipogenesis, mitochondrial function, lipolysis. Genes that are key regulators for lipogenesis, mitochondrial function, lipolysis were measured in the adipose tissues (A) and liver (B) collected from miR223$^{-/-}$ or wild type (WT) mice 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-ac6vated receptor gamma, coactivator 1 beta; CPT1, carnitine palmitoyltransferase 1; HSL, hormone-sensi6ve lipase; G6pase, glucose 6- phosphatase; GK, glucokinase; PEPCK, phosphoenolpyruvate carboxykinase 2.

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 10 and Figure 11). This was accompanied by enhanced activation of inflammatory pathways as evidenced by both increased nuclear factor κ B p65 phosphorylation in adipose tissues com- pared with controls (Figure 10B) and increased adipose tissue expression of inflammatory mediators, including monocyte chemotactic protein-1, tumor necrosis factor-α, IL-1β, and IL-6 (Figure 10C). Moreover, the severity of HFD-induced adipose tissue dysfunction in miR-223$^{-/-}$ mice was also higher than in controls, as indicated by a decrease in insulin- induced adipose tissue Akt

(Ser473) phosphorylation (Figure 10D) and increased resist in mRNA levels (Figure 10E).

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 controls (Figure 13A). 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 12A and 12B), and liver triglyceride levels were comparable in both groups (Figure 12C). 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 12D). However, HFD-fed miR-223$^{-/-}$ mice exhibited a higher macrophage infiltration in adipose tissues compared with WT mice (Figure 13A). 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 13B). 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 pro-inflammatory macrophages (CD11b^{+}F4/ 80^{+}CD11c^{+}CD206^{+}) in stromal cells of HFD-fed miR-223$^{-/-}$ mice compared with control mice (Figure 13C and 13D), and this has been closely correlated with insulin resistance.[71] 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.

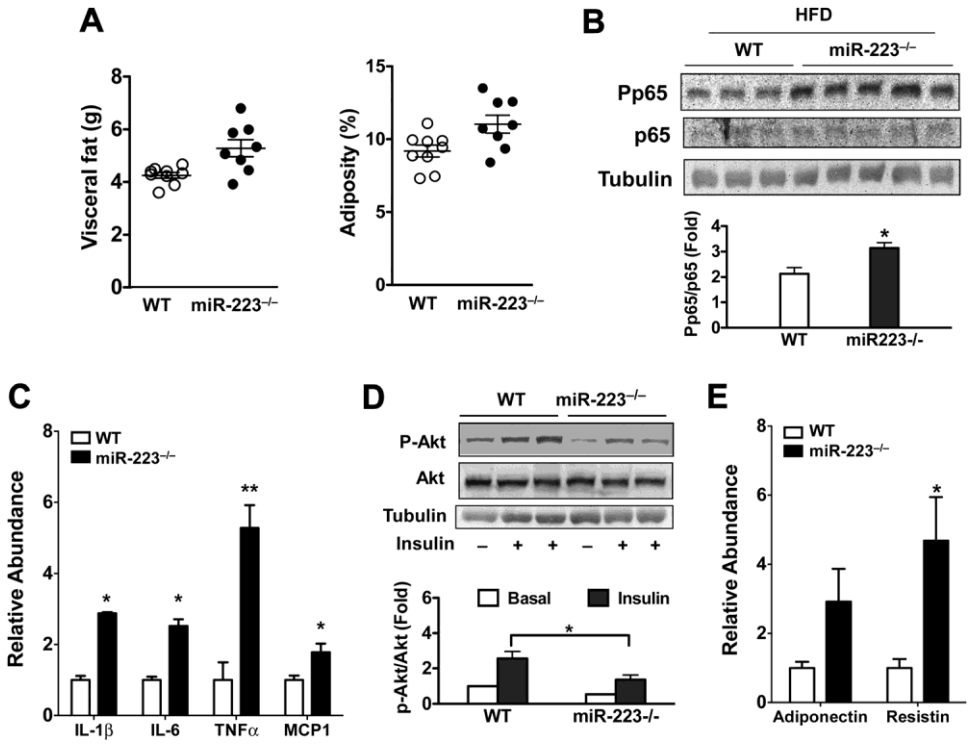


Figure 10. MicroRNA-223 (miR-223) regulates adipose tissue inflammation and insulin signaling. A, Visceral fat content and adiposity of male wild-type (WT) or miR-223$^{-/-}$ mice on a high-fat diet (HFD). B, Nuclear factor κ B activation. Western blots were performed with antibodies against p65 and phosphorylated p65. C, Cytokine and monocyte chemotactic protein-1 production in adipose tissues was examined with quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR; n=3, normalized to β-actin). IL indicates interleukin; TNF-α, tumor necrosis factor-α; and MCP1, monocyte chemotactic protein-1. 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 with anti- bodies against Akt1/2 and phosphorylated Akt (Ser473). E, Adipokine expression levels were examined by qRT-PCR (n=3, normalized to β-actin). *P<0.05; **P<0.001.(A is contributed by Guoqing Zhuang, B and D are contributed by Xin Guo)

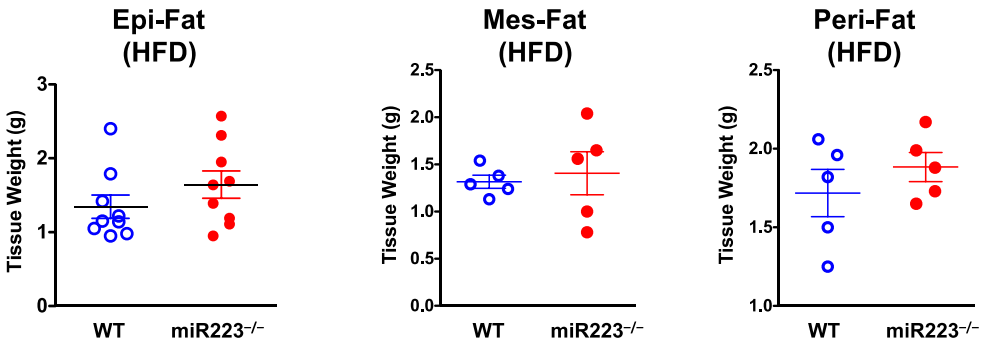


Figure 11. Adipose tissues weight. Adipose tissues were collected from wild type (WT) or miR-223$^{-/-}$ mice after 12 weeks of HFD feeding. Tissue weight was measured and analyzed between two groups. (Contributed by Guoqing Zhuang)

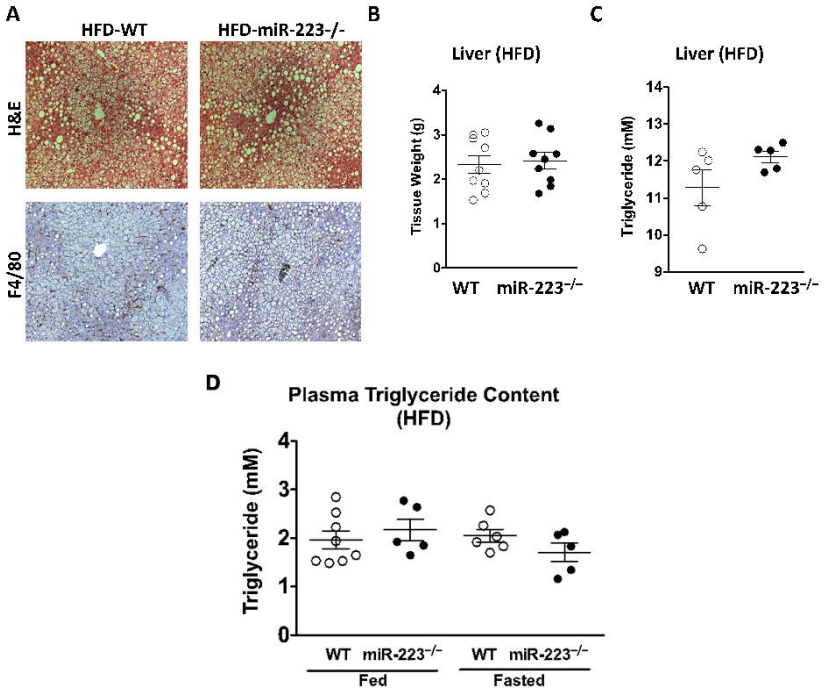


Figure 12. Lipid accumulation in liver, liver weight and plasma triglyceride levels in blood samples from WT or miR-223$^{-/-}$ mice. Livers were collected from wild type (WT) or miR-223$^{-/-}$ mice after 12 weeks of HFD feeding regimen. Tissues were fixed and used for H&E staining and immunohistochemical 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. Also, (D) blood samples were collected from mice after feeding (Fed) or after 16 hours of fasting (Fasted). (Contributed by Chaodong Wu)

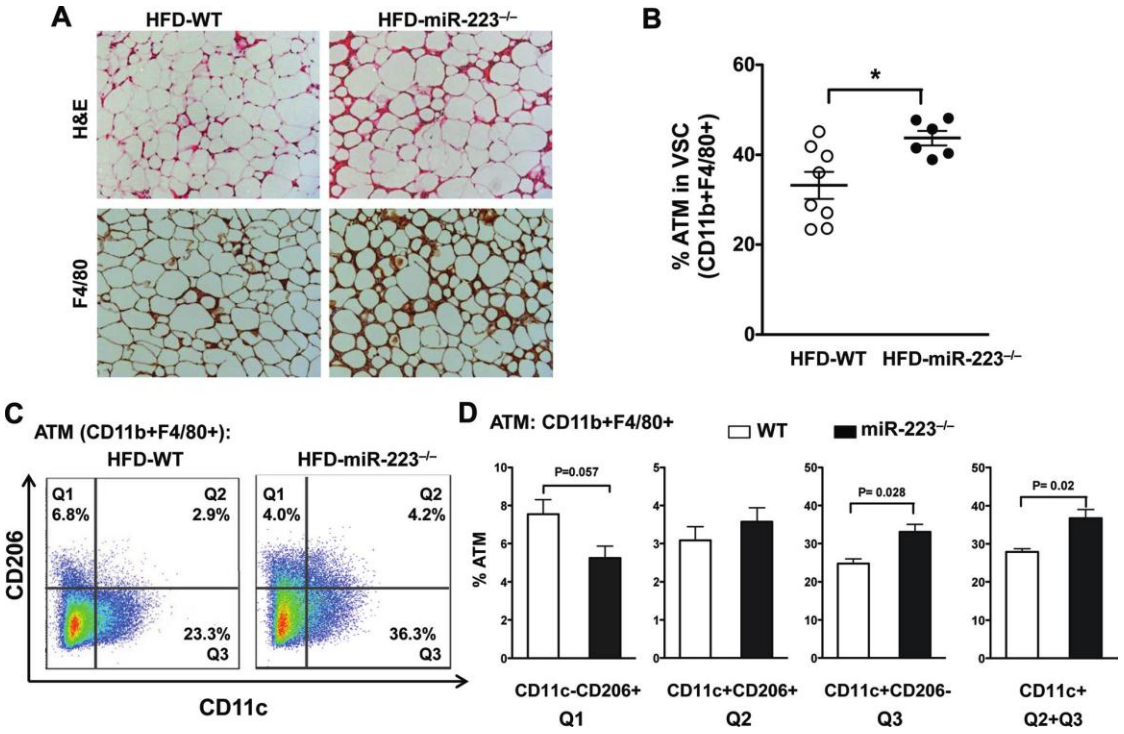


Figure 13. MicroRNA-223 (miR-223) deficiency enhances macrophage infiltration. A, Adipose tissue sections of high-fat diet (HFD)-fed mice were stained with hematoxylin and eosin (H&E; top) or F4/80 for macrophage infiltration (bottom). B, The adipose tissue macro- phages (ATMs; $CD11b^{+}F4/80^{+}$) were examined by flow cytometry analysis from visceral stromal cells (VSCs). C and D, Macrophage subtypes were further analyzed by fluorescence-activated cell sorter analysis with antibodies against CD11c and CD206. Data in B and D are presented as mean±SEM; n=5. (A is contributed by Chaodong Wu; B, C and D are contributed by Guoqing Zhuang)

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 trans-plantation 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 14A and 14B). 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 15). 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 14C), suggesting a successful long-term stem cell repopulation in the recipients (Figure 16). In addition, BMT-miR-223$^{-/-}$ mice exhibited increased severity of glucose intolerance (Figure 14D) and insulin resistance (Figure 14E) compared with BMT-WT mice. As expected, elevated pro-inflammatory cytokines (Figure 14F) accompanied by enhanced nuclear factor κ B activation (Figure 14G) 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 17). Taken together, our results suggest that exacerbated adipose tissue inflammation and insulin resistance in miR-223-deficient mice are due mainly to enhanced pro-inflammatory response of myeloid cells with miR-223 ablation.

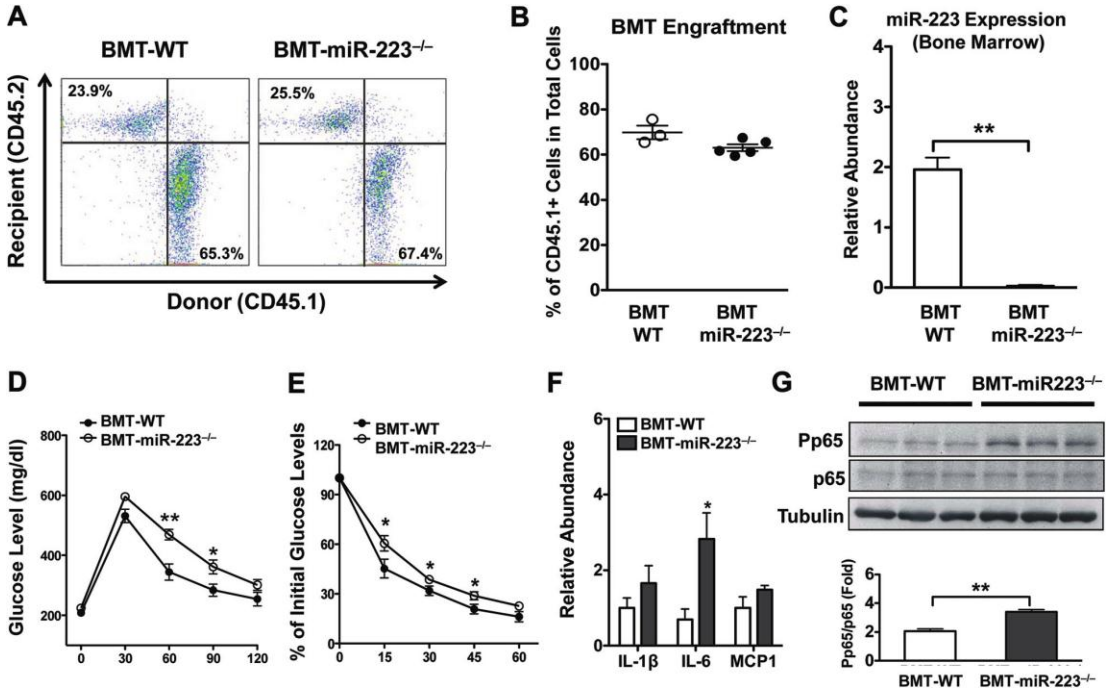


Figure 14. Bone marrow transplantation analysis. A and B, The engraftment was examined through the use of flow cytometry analysis with antibodies against CD45.1 (donor) and CD45.2 (recipient). WT indicates wild type. C, After 3 months, microRNA-223 (miR-223) expression in the bone marrow of transplanted 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 κ B 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. $^{*}P<0.05$; $^{**}P<0.001$. (G is contributed by Xin Guo)

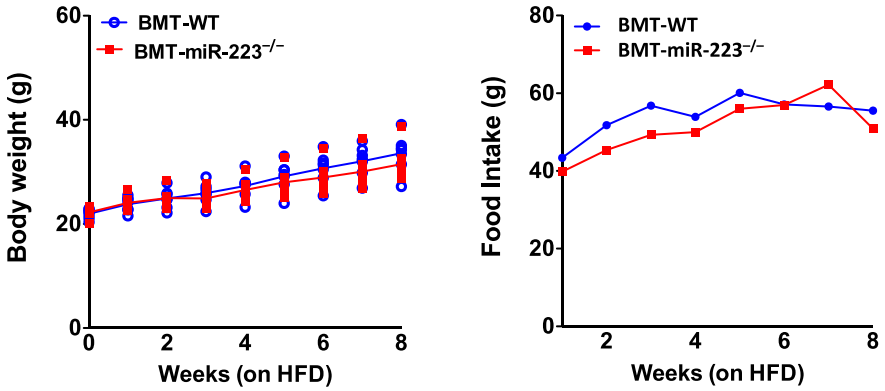


Figure 15. Body weight and food intake in wild type donor (BMT-WT) or miR-223$^{-/-}$ mice (BMT-miR-223$^{-/-}$). After engraftment was confirmed, mice received either bone marrow cells from either wild type donor (BMT-WT) or miR-223$^{-/-}$ mice (BMT-miR-223$^{-/-}$) were fed on a HFD (60% fatty acid). 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. (Contributed by Guoqing Zhuang)

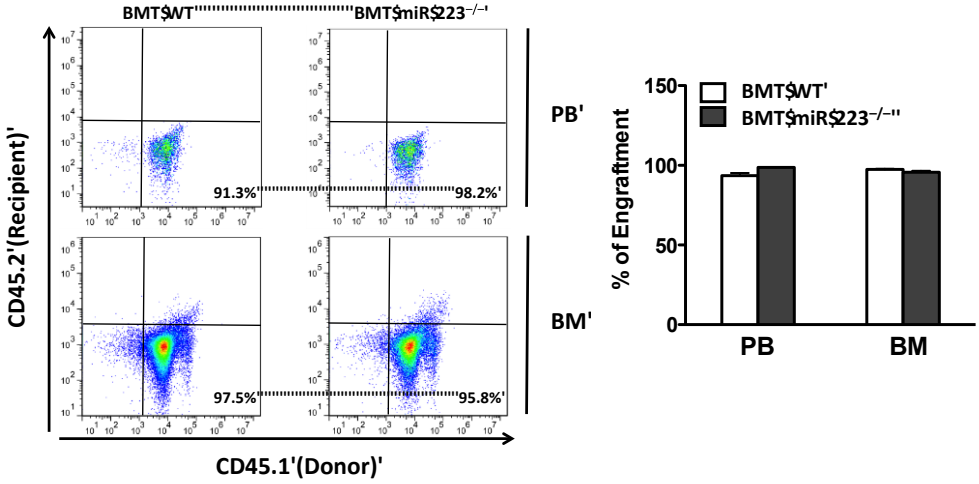


Figure 16. Donor cell engraftment analysis after transplantation. Three months after transplantation, peripheral blood (PB) samples(n=4) were collected from recipient mice to evaluate the donor cell engraftment. After mice were sacrificed for the tissue collection, the engraftment was further examined in the bone marrow samples using flow cytometry (n=4).(Contributed by Guoqing Zhuang)

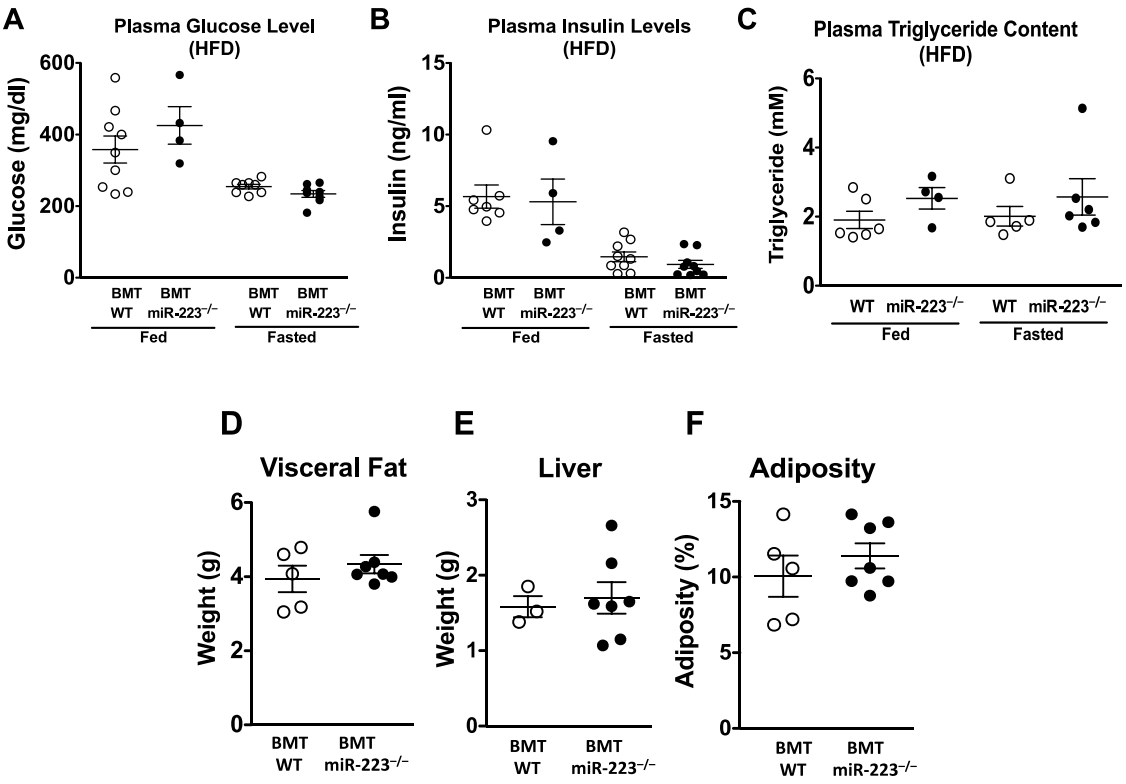


Figure 17. Metabolites measurement in the plasma of transplanted mice after HFD feeding. Metabolites in the plasma of transplanted mice were measured after mice were fed or fasted for 16 hours after 8 week of HFD feeding: glucose (A), insulin (B) and triglyceride (C). The weight of visceral fat (D) and liver (E) were measured after mice were sacrificed. Visceral fat adiposity were calculated with respect to the body weight (F) (A, B and C are contributed by Chaodong Wu; D,E and F are contributed by Guoqing Zhuang)

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 18). 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 19), 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 20A), whereas LPS stimulation slightly decreased miR-223 levels in BMDMs (Figure 20A). 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 20B). Quantitative RT-PCR analysis showed that pro-inflammatory 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 20C). 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 20D).

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 κ B p65 phosphorylation compared with control adipocytes (Figure 21A). Additionally, in miR-223$^{-/-}$ BMDM-treated WT adipocytes, there was a decrease in insulin-stimulated Akt (Ser473) phosphorylation (Figure 21B) and an increase in pro-inflammatory cytokines on LPS stimulation (Figure 21C). These results recapitulated adipose tissue inflammatory and metabolic responses of HFD-fed miR-223$^{-/-}$ mice (Figure 10B and 10D) and BMT- miR-223$^{-/-}$mice (Figure 14F and 14G) and clearly demonstrate that miR-223 is indeed an important regulator of macrophage polarization.

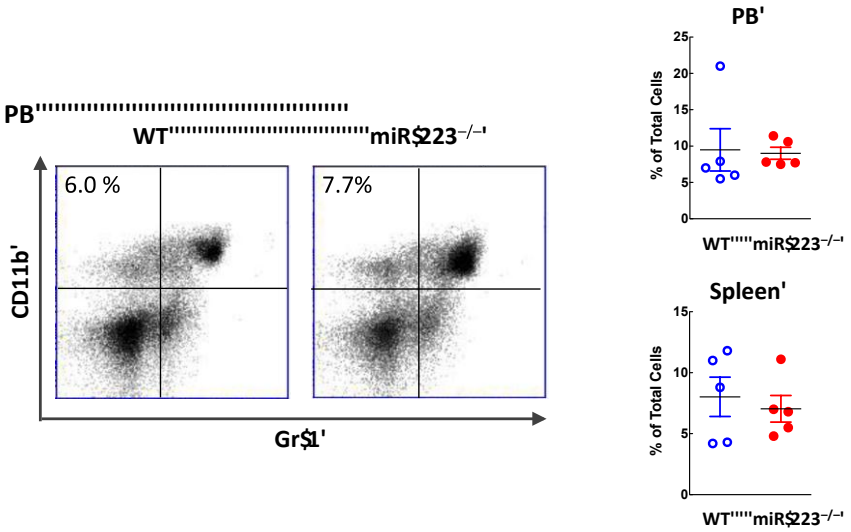


Figure 18. Monocytes analysis from peripheral blood and spleen. The percentage of monocytes (CD11b^{+}Gr-1^{-}) were analyzed from peripheral blood samples (PB) or spleen of wild type (WT) and miR-223$^{-/-}$ mice using flow cytometry. No significant differences were observed between two groups in the proportion of monocytes. (Contributed by Guoqing Zhuang)

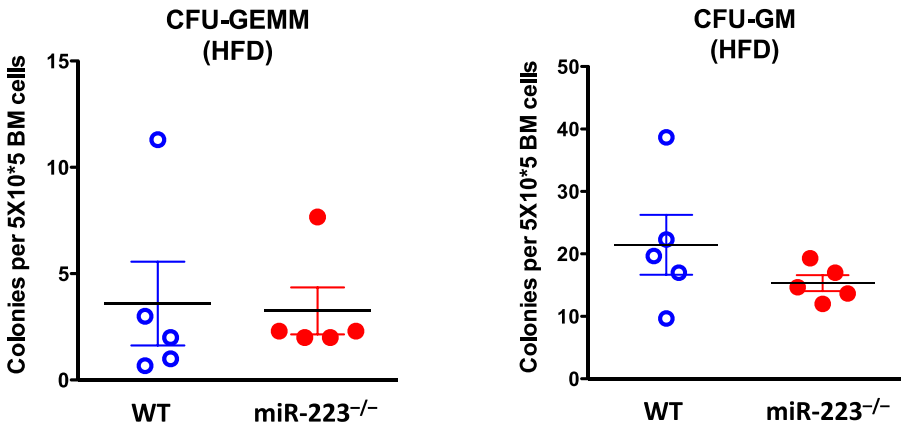
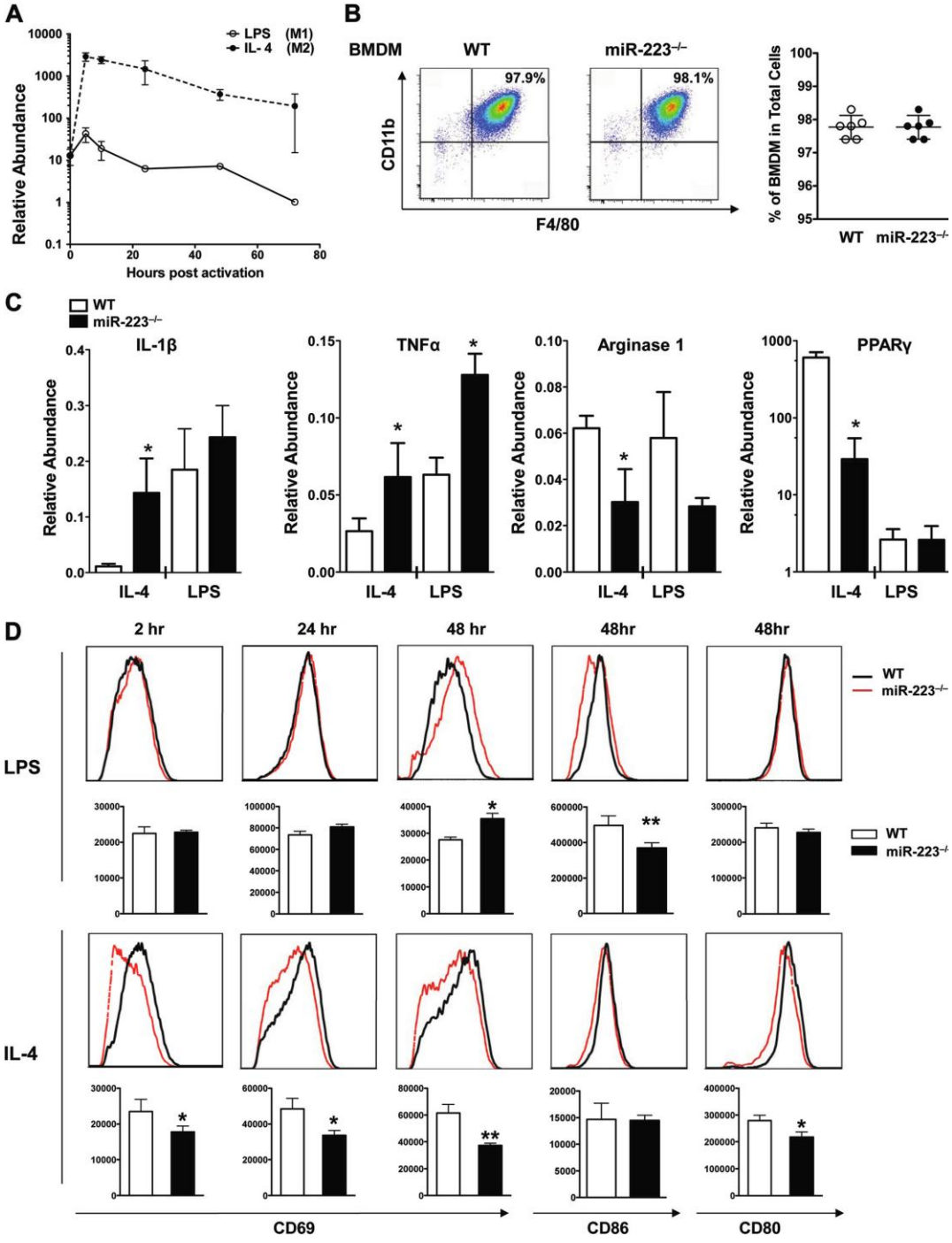


Figure 19. Colony forming assay. Bone marrow cells were extracted from wild type (WT) or miR223$^{-/-}$ mice after 12 weeks of HFD feeding. Red blood cells were lysed with NH4Cl (StemCell Technologies) and plated in the methocult® GF 3434 (StemCell Technologies). CFU-GEMM and CFU-GM were counted after 10 days culture and analyzed to compare the effects of miR-223 on myeloid progenitors. Each group contained cell preparations from five mice. (Contributed by Gang Wang)

Figure 20. 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). TNF-α 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.


A
LPS (M1)
IL-4 (M2)
Relative Abundance
Hours post activation
B
BMDM
WT
miR-223−/−
97.9%
98.1%
CD11b
F4/80
% of BMDM in Total Cells
C
WT
miR-223−/−
IL-1β
TNFα
Arginase 1
PPARγ
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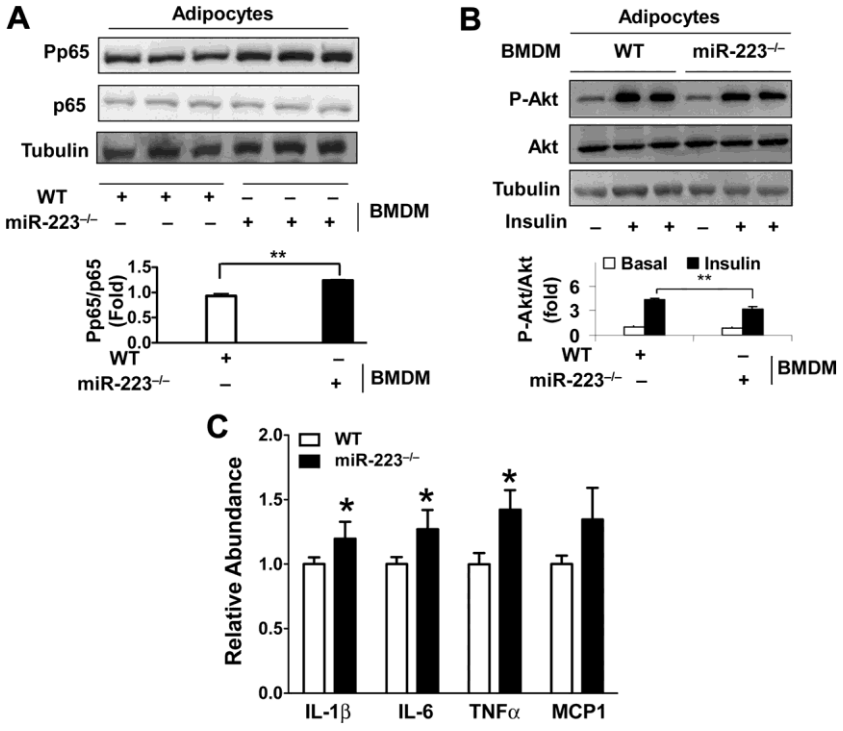


Figure 21. Macrophage effects on adipocyte insulin signaling. Bone marrow-derived macrophages (BMDMs) derived from wild-type (WT) or microRNA-223-deficient (miR-223$^{-/-}$) mice were cultured with differentiated 3T3-L1 cells at a ratio of 10:1. A, At 48 hours after co-culture, cells were collected and examined for activation of nuclear factor κ B. B, Cells were treated with insulin (100 nmol/L) for 30 minutes before harvest. The activation of Akt was examined with antibodies against Akt and phosphorylated (p) Akt (Ser473). C, The cytokine expression levels in co-cultured cells were examined with quantitative reverse transcriptase-poly-merase chain reaction assays (n=3, normalized to β-actin). Data are presented as mean±SEM. IL indicates interleukin; TNF-α, tumor necrosis factor-α; and MCP1, monocyte chemotactic protein-1. $^{*}P<0.05$; $^{**}P<0.001$. (A and B are contributed by Xin Guo)

Pknox1 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, Pknox1 was identified as a genuine target of miR-223 (Figure 22A). 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 22B and 22C). Pknox1 expression was higher in miR-223$^{-/-}$ BMDMs stimulated with LPS (Figure 22D and Figure 23) compared with BMDMs from control mice. Consistent with in vitro observations, Pknox1 protein levels in the adipose tissues collected from HFD-fed mice were inversely correlated with miR-223 expression levels (Figure 22E).

The importance of miR-223-mediated suppression of Pknox1 in macrophage polarization was further investigated with the use of gene-specific short interfering RNAs to knock down elevated levels of Pknox1 (Figure 24A). Knockdown of Pknox1 (siPknox1) in miR-223$^{-/-}$ BMDMs decreased pro- inflammatory cytokine production (IL-1β; Figure 24B) and partially blocked M1 response as indicated by fluorescence-activated cell sorter analysis (Figure 24C). M2 activation was also partially restored in miR-223$^{-/-}$ BMDMs with siPknox1 knockdown as judged by elevated arginase 1 levels (Figure 24B). To further confirm the function of Pknox1 in macrophage polarization, we introduced ectopic expression of this protein in BMDMs by lentiviral infection (Figure 25). Pknox1 overexpression partially recapitulated the miR-223$^{-/-}$ macrophage response to LPS with a significantly enhanced shift in surface markers (Figure 24D) and elevated inflammatory cytokine production (Figure 24E). These results demonstrated that Pknox1 is a bona fide target of miR-223 and plays a role in regulating macrophage polarization.

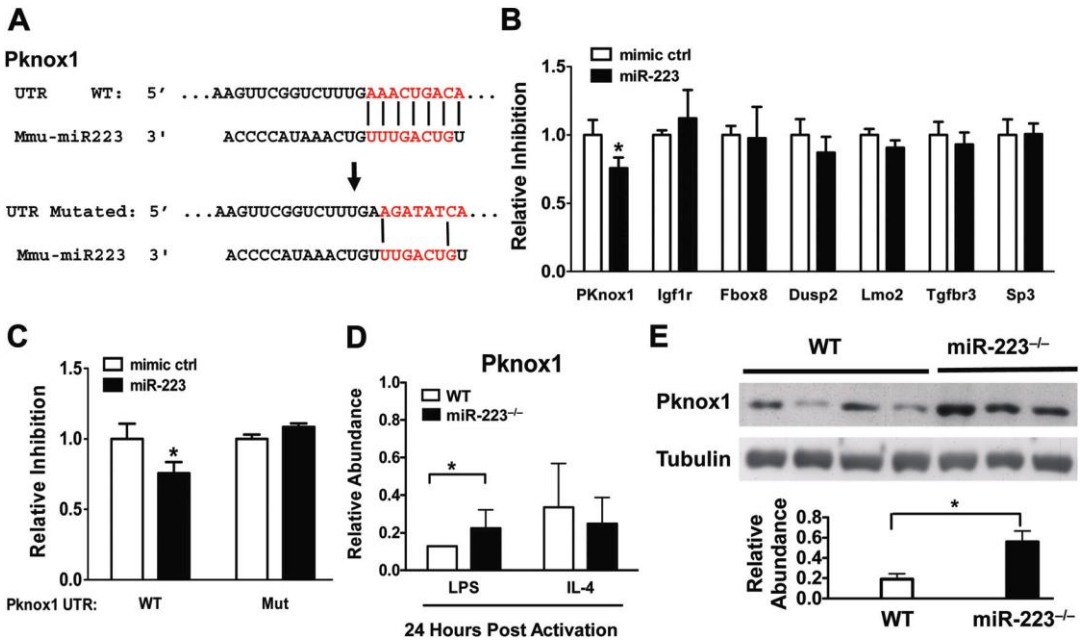


Figure 22. Pknox1 is a microRNA-223 (miR-223) target. A, Predicted miR-223 binding site in the 3' untranslated region (UTR) of Pknox1 and a mutated version of the seed match region (red). Reporter constructs containing a 3' UTR region with wild-type (WT; B) or mutated miR-223 binding site of Pknox1 (mut) (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. (E is contributed by Xin Guo)

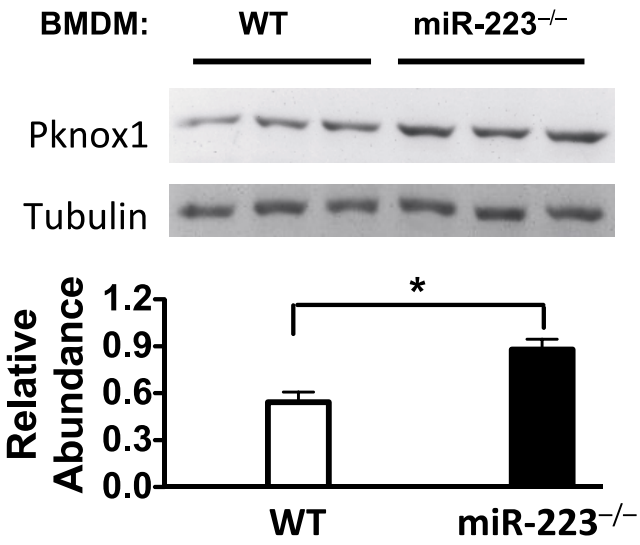
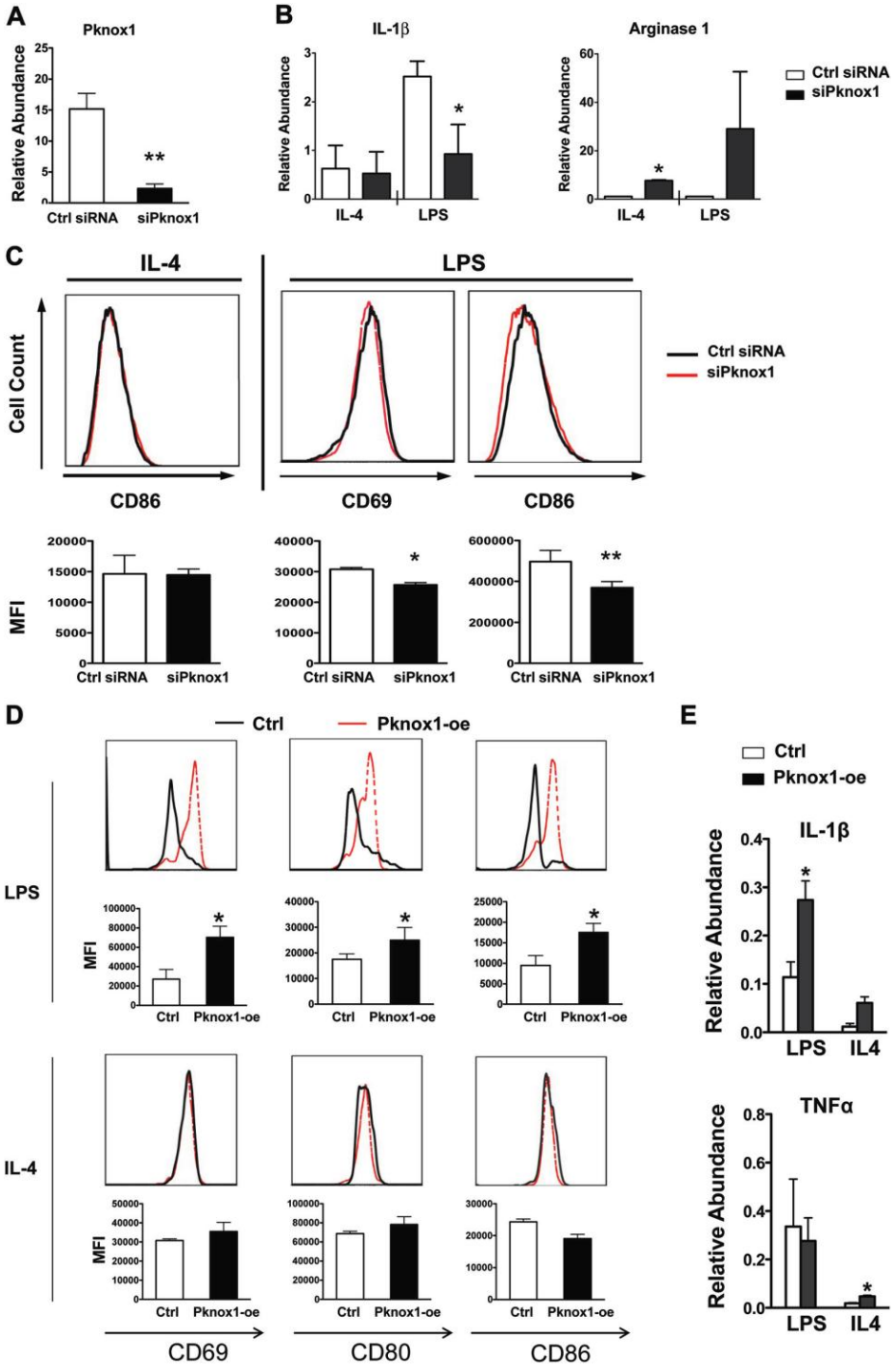


Figure 23. Pknox1 expression level in BMDMs. BMDMs derived from HFD fed wild type or miR-223$^{-/-}$ mice were stimulated with LPS (100 ng/ml). The protein levels of Pknox1 were examined using Western blots. The intensity of Pknox1 bands in each sample were normalized to the tubulin loading Controls and calculated for statistic analysis using student t test. *, $p<0.05$. (Contributed by Xin Guo)

Figure 24. 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 lipopolysac-charide (LPS; 100 ng/mL) or interleukin (IL)-4 (10 ng/mL) for 24 hours. B, IL-1β production and arginase 1 expression were measured with qRT-PCR (normalized to β-actin). C, Surface markers (CD86 and CD69) were examined by fluorescence-activated cell sorter; n=3. MFI indicates median florescence intensity. BMDMs from wild-type mice were lentivirally infected to introduce ectopic Pknox1 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). TNF-α indicates tumor necrosis factor-α
$^{*}P<0.05$; $^{**}P<0.001$.


A
Pknox1
Relative Abundance
Ctrl siRNA
siPknox1
B
IL-1β
Arginase 1
IL-4
LPS
Ctrl siRNA
siPknox1
C
IL-4
LPS
Cell Count
CD86
CD69
CD86
MFI
D
Ctrl
Pknox1-oe
LPS
IL-4
CD69
CD80
CD86
E
IL-1β
TNFα
LPS
IL4

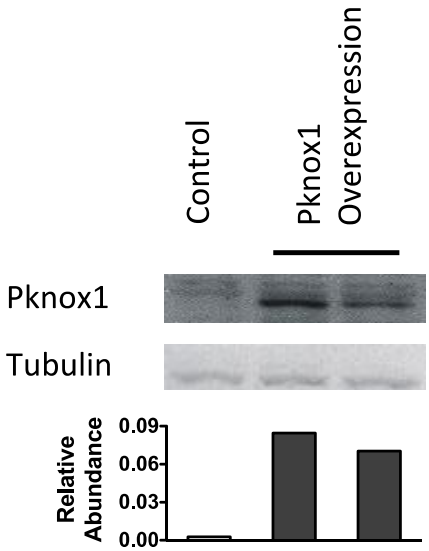


Figure 25. Confirmation of over-expressed Pknox1 level. Ectopic expression of Pknox1 was introduced by lentiviral infection (pLen66.2- Pknox1) followed by confirmation with Western blots. An empty lentiviral was used as a control. The intensities of each Pknox1 band were normalized to the loading control (Tubulin). (Contributed by Xin Guo)

Discussion

Macrophage polarization is a critical component of the inflammatory response in metabolic tissues and is of particular importance in adipose tissue.[23,27,41,56,57] The present study provides evidence for the first time to support an essential role for miRs in regulating macrophage polarization. Notably, miR-223 is differentially expressed during macro- phage polarization, and miR-223-deficient macrophages were hypersensitive to LPS stimulation and exhibited delayed responses to IL-4 compared with controls (Figure 20). 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 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 κ B 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 12). 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,[72] a chemokine marker of macrophage infiltration, into adipose tissue in both adipose tissue and primary adipocytes isolated from miR-223$^{-/-}$

mice (Figure 24C). Adipose tissue inflammation is well documented as an important contributor to systemic insulin resistance.[1,40,42] 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, pro-inflammatory cytokine expression, and nuclear factor κ B 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 9). Thus, increased adipose tissue inflammation resulting from miR-223 deficiency contributed, in large part, to systemic insulin resistance in miR-223-deficient mice.

miRs 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 Pknox1 as a genuine target of miR-223. The expression of Pknox1 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.

Conclusion

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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