MicroRNA-150 Regulates Lipid Metabolism and Inflammatory Response

Nanlan Luo1, W. Timothy Garvey2,3, Da-Zhi Wang1 and Yuchang Fu4*
1Department of Nutrition Sciences, University of Alabama at Birmingham, Birmingham, AL 35294-3360, USA
2Birmingham VA Medical Center, Birmingham, AL 35233, USA
3Department of Cardiology, Boston Children’s Hospital, Harvard Medical School, Boston, MA 02115, USA

Keywords: MiR-150; Macrophage foam cells; Lipid metabolism; Inflammation

Introduction

Metabolic syndrome is a powerful risk factor not only for the future development of type 2 diabetes but also cardiovascular disease [1-3]. Alterations of lipid metabolism and inflammatory response in macrophage cells represent a complex pathophysiological process that plays a crucial role in development of metabolic syndrome because macrophage cells accumulate large amounts of lipid and convert to foam cells, which both initiate and actively participate in atherosclerotic lesion development and insulin resistance, as well as other metabolic disorders [4]. Thus, the transformation of macrophages into foam cells is a critical component in the pathophysiological process. A well characterized model system to study the transformation of macrophages to foam cells is the THP-1 human monocytic cell line [5]. THP-1 monocytic cells can be induced to differentiate into macrophages by administration of Phorbol Myristate Acetate (PMA), and resulting macrophages can then be converted to foam cells following treatment with oxidized Low-Density Lipoprotein (oxLDL). The molecular determinants responsible for the transformation of macrophages to foam cells have not been fully elucidated. Here, we decided to capitalize on the advantages of this model system to identify microRNA molecules that are differentially expressed during this cell transformation [6,7]. Toward this end, we performed a microRNA qPCR array utilizing microRNAs isolated from oxLDL-treated and control THP-1 macrophages. One of the microRNA genes which we defined in this screen as being significantly up-regulated in oxLDL-treated macrophage foam cells is microRNA-150 (miR-150).

MiR-150 has been reported mainly expressing in the lymph nodes and spleen and is highly upregulated during the development of mature T and B cells; over expression of miR-150 in hematopoietic stem cells had little effect on the formation of either mature T cells or granulocytes or macrophages, but the formation of mature B cells was greatly impaired [8]. Studies have found that opposite expression pattern of miR-150 and c-Myb, a transcription factor controlling multiple steps of lymphocyte development, was detected during B cell differentiation and miR-150 could inhibit c-Myb gene expression and function in vitro [9,10]. Furthermore, studies have also shown that deletion of miR-150 in mice caused increased c-Myb expression and B1 cell expansion, conversely, overexpression of miR-150 in mice would reduce c-Myb expression levels and B1 cell population in vivo [11]. These results from both loss- and gain-of-function of miR-150 clearly indicate that miR-150 is responsible for B1 cell differentiation in vivo through c-Myb regulation [12].

Recent studies have shown that in human blood cells and cultured THP-1 cells, miR-150 is selectively packaged into microvesicles (MVs) and actively secreted into the blood or the culture medium; and these THP-1-derived MVs can enter and deliver miR-150 into Human Microvascular Endothelial Cells (HMEC-1), effectively reducing c-Myb expression and enhancing cell migration in HMEC-1 cells [13]. Moreover, these studies have found that MVs isolated from the plasma of patients with atherosclerosis contained higher levels of miR-150, and they more effectively promoted HMEC-1 cell migration by blood circulation than MVs from healthy donors.

Our present studies demonstrate for the first time that miR-150 can physiologically modulate metabolic activities and inflammatory...
response both in cells and animals by regulating lipid metabolism and inflammatory response. These current studies have revealed a new regulatory role of miR-150 which is related to the mechanism for lipid metabolism and inflammation.

Methods

Human THP-1 cell culture

Human monocytic leukemia THP-1 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). These cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (Tissue Culture Biologicals, Tulare, CA), penicillin (100 U/ml) and streptomycin (100 mg/ml) at 37°C in 5% CO₂. THP-1 monocytes were treated with 100 nm PMA for 24 hr to facilitate differentiation into macrophages. After treatment, the adherent macrophages were washed three times with Phosphate-Buffered Saline (PBS) and incubated with cell culture medium for 24 hr at 37°C until addition of 100 µg/mL oxLDL which was purchased from Kalen Biomedical (Montgomery Village, MD).

MicroRNA qPCR array

Human THP-1 macrophage foam cells (treated with 100 µg/ml of oxLDL for 24 hr) were harvested for microRNA isolation using a complete miRNA qPCR Array System from Qiagen (Austin, TX). Three RT2 miRNA PCR Arrays were performed and analyzed according to the manufacturer's protocols and software. The selected miRNA molecules were confirmed in oxLDL treated THP-1 macrophage foam cells and the control cells using the real-time PCR methods.

Determination of cholesterol and triglyceride concentrations in THP-1 macrophage foam cells

The concentrations of cholesterol and triglyceride in THP-1 macrophage foam cells were determined using enzymatic colorimetric assays (Wako, Richmond, VA) according to the manufacturer's protocols. The concentrations of cellular proteins from these macrophage cells were measured with a protein assay kit from Bio-Rad (Hercules, CA).

Overexpression and knockdown of miR-150 in THP-1 cells

GFP hsa-mir-150 microRNA and inhibitory hsa-mir-150 microRNA lentiviruses (10⁵ pfu/ml) were purchased from the Applied Biological Materials (ABM) (Richmond, BC, Canada). These LentimiaRa-GFP-hsa-mir-150 and LentimiaRa-Off-hsa-mir-150 lentivirus were used to infect 2x10⁶ THP-1 cells/well for 24 hr according to the manufacturer's protocols with 53% to 62% of efficiency for the infection of cells. These infected THP-1 cells were then treated with 100 nm PMA for 24 h to differentiate into macrophages. Then, these macrophages were treated with 100 µg/mL oxLDL for 24 hr to transform them into macrophage foam cells for using in the experiments.

Experimental animals

Wild type and whole body miR-150 knockout mice were purchased from the Jackson Laboratory for the experiments. To investigate the metabolism of these mice, a chow diet or a high fat diet (60% kcal% fat) from the Research Diets Company (New Brunswick, NJ) was fed to these mice from 4 weeks to 20 weeks for measuring mouse body weight and analyzing lipid accumulation and gene expression in adipose tissues.

All of these animals were housed in a specific pathogen-free facility with 12-hours light/dark cycles and received a standard laboratory chow diet except for the high fat diet experiments. Only male mice were used for the experiments. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Animal Resources Program (ARP) at the University of Alabama at Birmingham.

Mouse glucose and insulin tolerance testing

Glucose Tolerance Testing (GTT) and Insulin Tolerance Testing (ITT) in miR-150 knockout and control mice were performed as described previously [14]. Mice were injected with glucose or insulin at 20 or 22 weeks of age after consuming the high fat diet for 16 or 18 weeks. To determine glucose tolerance, animals were first fasted overnight and then given an intraperitoneal injection of glucose solution with 1.0 ml/kg (1M glucose solution) and glucose concentration was determined in mouse tail blood collected at baseline (prior to injection), and at 30, 60, 90 and 120 minutes post-injection using a HemoCue glucose 201 glucometer (HemoCue USA). To determine insulin tolerance, mice were fasted for 6 hours in the morning of the measurement and then administered an intraperitoneal injection of insulin solution (0.5U insulin/kg body weight). Glucose levels were measured in blood samples collected as above described for the glucose tolerance testing.

Gene expression in THP-1 macrophage foam cells and mouse adipose tissues

To determine the expression levels of genes coding for lipid metabolism and inflammatory cytokines, total RNA was extracted from THP-1 foam cells or wild type and transgenic mouse adipose tissues using a commercially available TRizol reagent from Invitrogen (Carlsbad, CA) according to the manufacturer's instructions. The quantitative real-time PCR analysis was using an ABI StepOnePlus Real-Time PCR System. Reactions were carried out in triplicate in a total volume of 20 µl using and a SYBR Green QPCR Master Mix (Applied Biosystems). Quantification was calculated using the starting quantity of the cDNA of interest relative to that of 18S ribosomal cDNA in the same sample.

Statistics

Experimental results are reported as the mean ± SEM. Statistical analyses were conducted using the unpaired Student's t-test assuming unequal variance unless otherwise indicated. Significance was defined as the p<0.05.

Results

miR-150 is highly induced by oxLDL in macrophage foam cells

To identify miRNAs and investigate their functional roles in lipid accumulation and inflammatory response in macrophage cells, we treated human THP-1 macrophages with 100 µg/mL of oxLDL to transform these cells into macrophage foam cells with accumulated lipid content inside of these cells. We performed miRNA PCR arrays to identify miRNA molecules that are induced by the lipid accumulation and inflammatory stimulation. Our experimental results showed that miR-150 (average over 4-fold levels, p<0.01) was one of the miRNAs which were significantly induced by oxLDL treatment in macrophage foam cells (Figure 1). Increased miR-150 expression was further confirmed with real-time PCR (data not shown).

Overexpression and knockdown of miR-150 in THP-1 cells

To identify functional roles of this microRNA in mice and to investigate the role of miR-150 in the development of atherosclerosis, we performed experiments with overexpression or knockdown of miR-150 in THP-1 cells using lentiviral vectors.
Figure 1: miR-150 is highly induced by oxLDL in THP-1 macrophage foam cells.

Human monocytic leukemia THP-1 cells were treated with 100 nM PMA for 24 h to facilitate differentiation into macrophages and the macrophages were then treated with 100 µg/mL oxLDL for 24 h to transform into foam cells. MicroRNAs were isolated by using an RT2 miRNA First Strand Kit (Qiagen) from control THP-1 macrophages (WT) and THP-1 macrophage foam cells (OxLDL) to convert to cDNAs and three RT2 miRNA PCR arrays were performed and analyzed using the complete miRNAqPCRArray System from Qiagen. The expression of miR-150 represented as three separate experiments. Error bars represented the ± SEM, **p<0.01.

Figure 2: (A) Overexpression and knockdown of miR-150 gene in macrophage cells

LentimRα-GFP-hsa-mir-150 and LentimRα-Off-hsa-mir-150 lentiviruses were used to infect 2×10⁵ THP-1 cells/well for 24 h and treated with 100 nM PMA for another 24 h. These macrophages were transformed into foam cells by adding 100 µg/mL oxLDL for 24 h. (A) Expression of miR-150 gene were examined by QPCR analyses in control THP-1 macrophage foam cells (WT), overexpression and knockdown of miR-150 macrophage foam cells. Error bars represented the ± SEM, *p<0.05, **p<0.01. (B) Determination of triglyceride and cholesterol concentrations in macrophage foam cells

The concentrations of triglyceride and cholesterol in THP-1 (WT), overexpression and knockdown of miR-150 macrophages foam cells were determined using enzymatic colorimetric assays. The concentrations of cellular proteins from these macrophage foam cells were measured with protein assay kits. Error bars represented the ± SEM, **p<0.01.

(C) and (D) Gene expression related to lipid metabolism and inflammatory response in macrophage foam cells

Total RNA was isolated from control THP-1, overexpression and knockdown of miR-150 macrophages foam cells, cDNAs were synthesized, and QPCR was performed with ACAT-1, ALBP, and HSL gene primers for lipid metabolism; IL-6, MCP-1, and TNF-α for inflammatory response. Mean ± SEM from three separate experiments with triplicate samples were presented, *p<0.05 and **p<0.01.
The above observations prompted us to investigate the in vivo function of miR-150 in lipid metabolism and inflammatory response. MiR-150 has been implicated in the regulation of B and T cell development and function and miR-150 knockout mice have been reported viable and fertile [11]. We first examined the levels of miR-150 in plasma of wild type and miR-150 knockout mice and we confirmed the absence of miR-150 expression in knockout mice (Figure 3A). Next, we assessed whether growth and development were affected in miR-150 knockout mice compared to wild type mice. There were no significant differences in reproduction, food consumption, or development between control and knockout mice when these animals were fed normal chow or the high fat diet (data not shown). However, compared to wild type mice, miR-150 knockout mice had significantly lower body weight (average 12%) when these mice were fed with a high fat diet (60% kcal% fat) for 16 weeks at age of 20 weeks (Figure 3B).

To confirm these data, we dissected wild type and miR-150 knockout mice and found significantly reduced abdominal adipose masses in knockout mice when compared to control wild type animals (Figure 3C). Our results suggest that deficiency of miR-150 in mice can significantly affect adiposity, especially adipose tissue accumulation under the high fat diet condition.

Decreased lipid accumulation and pro-inflammatory gene expression in miR-150 knockout mice

To determine the function of miR-150 on lipid metabolism and inflammatory response, mouse adipose tissues were isolated from (triglyceride and cholesterol) was significantly increased in the miR-150 overexpressed macrophage foam cells. In contrast, in the miR-150 knockdown cells, the lipid accumulation was significantly decreased when compared to control THP-1 macrophage foam cells (Figure 2B). We further examined the expression of several key genes related to lipid metabolism, such as, acyl-coenzyme A: Cholesterol acyl transferase 1 (ACAT-1), Adipocyte Lipid-Binding Protein (ALBP/aP2/FABP4) and Hormone Sensitive Lipase (HSL), and to inflammation, including Interleukin 6 (IL-6), Monocyte Chemoattractant Protein 1 (MCP-1) and Tumor Necrosis Factor alpha (TNF-α) in miR-150 overexpressed or knockdown cells. Our data demonstrated that miR-150 strongly induced the expression of pro-inflammatory cytokines and increased the expression of lipid accumulation genes in the THP-1 macrophage foam cells (Figures 2C and 2D).
20-week-old control (WT) and miR-150 knockout (miR-150-KO) mice and the cellular contents of cholesterol and triglyceride were determined. Both cholesterol (maximal average 16%) and triglyceride (maximal average 33%) levels in adipose tissues were significantly reduced (p<0.01) in miR-150 knockout mice under high fat feeding condition for 16 weeks (Figures 4A and 4B). We also examined the impact of miR-150 gene knockout on lipid metabolism and inflammatory response gene expression in adipose tissues and we found a reduced expression of ALBP, which is for lipid loading and pro-inflammatory cytokines, IL-6, MCP-1 and TNF-α, in miR-150 knockout knockouts. However, we observed an increased expression of HSL, which is for lipid hydrolyzing and anti-inflammatory cytokine IL-10 in adipose tissues of miR-150 knockout mice (Figure 5A).

To further investigate whether the reduced lipid accumulation in the metabolically active adipose tissues of miR-150 knockout mice could influence the change of macrophage subtype specific markers, we next examined the expression of macrophage-specific F4/80, macrophage galactose N-acetyl-galactosamine specific lectin 1 (Mgl 1), a marker for alternatively activated Macrophage (M2) phenotype, and Chemokine Receptor 2 (CCR2), a marker for classically activated macrophage (M1) phenotype, in adipose tissues of miR-150 knockout mice. As expected, the percentage of these pro-inflammatory markers, F4/80 (average 26%, p<0.05) and CCR2 (average 61%, p<0.01), were markedly lower in adipose tissues of miR-150 knockout mice (Figure 5B). In contrast, the anti-inflammatory marker, Mgl 1, was significantly higher (average 82%, p<0.01) in adipose tissues of miR-150-KO mice than those from control wild type mice. Thus, elimination of miR-150 resulted in favorable metabolic macrophage subtype changes in vivo.

**Improved glucose tolerance and insulin sensitivity in miR-150 knockout mice**

To investigate the function of miR-150 in systemic insulin sensitivity in vivo, we performed Glucose Tolerance Tests (GTT) and Insulin Tolerance Tests (ITT) in control and miR-150-KO mice fed with high fat diet for 16 weeks at age of 20 weeks. Experiments were performed in fasting overnight male mice. Glucose solutions were injected into peritoneal cavity at the dose of 1.0 ml/kg (1M glucose solution). Blood was collected via tail vein at the indicated time points. Glucose concentration in plasma was measured using a glucometer (Precision); n=10 in each group of mice. Insulin tolerance tests were performed on control wild type and miR-150 knockout mice under high fat diet condition for 16 weeks at age of 22 weeks. Experiments were conducted similar as the described above for the glucose tolerance tests but fasting 6 hours before the injections and insulin solutions were injected into peritoneal cavity at the dose of 0.5 U/kg body weight. Blood was collected via tail vein at the indicated time points, and glucose levels were measured; n=10 in each group of animals. Error bars represented the ± SEM, *p<0.05 and **p<0.01.

Finally, we attempted to determine miR-150 targets in adipose tissues. Based on the miRDB database for miRNA target prediction and functional annotations [15,16], AdipoR2 is one of the miR-150 target genes in both human and mouse. We experimentally examined adiponectin receptor 2 (AdipoR2) expression in adipose tissues of both miR-150 knockout and wild type control mice. AdipoR2 expression was significant increased (average 33%, p<0.01) in adipose tissues of...
miR-150 knockout mice when compared to those in control wild type mice (Figure 7). Thus, the knockout of miR-150 in mice altered the expression of one important adiponectin receptor gene in metabolically active adipose tissues in a manner that would tend to promote lipid metabolism and reduce inflammation in these tissues.

**Discussion**

As previously demonstrated, miR-150 was involved in regulating B cell development [8] and megakaryocyte differentiation [17,18], although the evidence has shown that except of lymphocytes miR-150 does not influence on the formation of either mature T cells or granulocytes or macrophages in hematopoietic stem cells, considering that interactions among these immune cells to respond to pathogens, miR-150 should have a vital role in the immune system [19]. Since lymphoid blood cells tightly are correlated with corresponding white blood cell counts, as one of the cancer biomarkers which have high stability in plasma, association with disease states, and ease of sensitive measurement, miR-150 has been investigated in numerous cancer studies [20,21]. In addition of cancer, disorders of the immune system can also result in autoimmune and inflammatory diseases [22,23].

It is well known that overnutrition or high fat diets are associated with autoimmune and inflammatory diseases such as diabetes and obesity; here, high fat diets exert multiple deleterious effects to key metabolic tissues in vivo, and immune cells, such as macrophages, T cells, and B cells, have all been implicated to play prominent roles in the inflammatory processes [24]. Recent studies have revealed that miR-150 secretion was significantly increased in ob/ob diabetic mice and obese/diabetic mice to promote angiogenesis in these animals [25]. Recent studies have also suggested that microRNAs play an important role in regulating lipid and glucose metabolism [26].

Previous studies have identified two cell-surface trans-membrane receptors, AdipoR1 and AdipoR2, for adiponectin action [27], and the adiponectin action is known to signal through these receptors and the docking protein ADPR1 [28]. Simultaneous disruption of both AdipoR1 and AdipoR2 abolished adiponectin binding and actions, resulting in increased triglyceride content, inflammation and oxidative stress in adipose tissue, and thus leading to insulin resistance and marked glucose intolerance [29]. Although recent studies have indicated that microRNAs play an important role in metabolic syndrome [26], currently, the interactions among microRNAs and AdipoR2 are completely undefined yet. Here, we have demonstrated that AdipoR2 was regulated by miR-150 as its one of the potential target genes and the interactions between miR-150 and AdipoR2 may play a critical role in physiological metabolic activities in cells/tissues; further investigations should reveal more detailed regulatory mechanism of the function of miR-150 involved in metabolism and inflammation.

Our current studies have for the first time identified that miR-150 was highly induced by oxLDL stimulation in human THP-1 macrophage foam cells; and its expression and function were related to lipid accumulation in these cells by regulating genes that are involved in lipid metabolism, such as, ACAT-1, ALBP, and HSL. Since the lipid metabolic activity and miR-150 itself are highly related to immune/inflammatory response in cells, cytokines, including IL-6, IL-10, MCP-1, and TNF-a, are also regulated by the expression of miR-150 in these cells. We have further used miR-150 knockout mice to confirm the important roles of miR-150 in regulating these key gene expression related to lipid metabolism, inflammatory response, glucose tolerance and insulin resistance in vivo. Our results have indicated that miR-150 was involved in high fat diet induced obesity/diabetes through regulating lipid metabolism and inflammatory response, probably through one of its target genes, AdipoR2, in vivo.

In conclusion, our current studies suggest that miR-150 can physiologically modulate metabolic activities and inflammatory response both in cells and animals by mainly regulating lipid metabolism and inflammatory response. These studies have revealed a new regulatory role of miR-150 and the related mechanism for lipid metabolism and inflammation, suggesting that miR-150 maybe a potential pharmaceutical candidate for prevention and therapy of metabolic disorders in the future.

**Acknowledgments**

We thank the UAB Diabetes Research and Training Center for providing outstanding core services (NIH P30 DK-56336). This work was supported by grants from American Diabetes Association (1-13-IN-19) to YF, a grant from NIH (DK-083562) to TG and a grant from NIH (HL085635) to DZW.

**Reference**

10. Thomas MD, Kremer CS, Ravichandran KS, Rajewsky K, Bender TP (2005) c-Myb is critical for B cell development and maintenance of follicular B cells. Immunity 23: 275-286.


