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Targeting microRNAs in obesity

Huangming Xie, BSc(Hons), Ph.D.^{1,2}[graduate student], Lei Sun, Ph.D.¹[Post-doctoral fellow], and Harvey F. Lodish, Ph.D.^{1,2,3,*}[Professor of Biology, Member, Whitehead Institute]

¹Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, Massachusetts 02142, USA

²Computation and Systems Biology, Singapore-MIT Alliance, National University of Singapore, 4 Engineering Drive 3, Singapore 117576

³Departments of Biology and Biological Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA

Abstract

Obesity is a serious health problem worldwide associated with an increased risk of life-threatening diseases such as type 2 diabetes, atherosclerosis, and certain types of cancer. Fundamental for the development of novel therapeutics for obesity and its associated metabolic syndromes is an understanding of the regulation of fat cell development. Recent computational and experimental studies have shown that microRNAs (miRNAs) play a role in metabolic tissue development, lipid metabolism and glucose homeostasis. In addition, many miRNAs are dysregulated in metabolic tissues from obese animals and human, which potentially contributes to the pathogenesis of obesity associated complications. In this review, we will summarize the current state of understanding of the roles of miRNAs in metabolic tissues under normal development and obese conditions, and discuss the potential use of miRNAs as therapeutic targets.

Keywords

adipogenesis; adipose tissue; biomarker; diabetes; glucose homeostasis; insulin resistance; lipid metabolism; liver; microRNAs; obesity; pancreas; therapeutics

1. Introduction

Gene expression in human is precisely controlled in a cell, temporal, and condition specific manner. Therefore, completely understanding the regulatory mechanisms of gene expression is important in genomic medicine. One of the major discoveries in the last decade is microRNAs (miRNAs), which constitute an abundant and evolutionarily conserved class of post-transcriptional regulators of gene expression^{1–3}. miRNAs are small endogenous noncoding RNAs that base pair to sites within target mRNAs, triggering either a block in translation or mRNA degradation or both^{4, 5}. The expression of miRNAs is often tissue-specific or developmental-specific^{1, 2}. The post-transcriptional programs controlled by miRNAs affect diverse biological processes, including development, cell differentiation, apoptosis, immune responses, metabolism and many diseases including various cancers, cardiovascular disease, viral infection and neurodegenerative diseases^{6–14}. As example, signature miRNA expression patterns differentiate many types of related cancers better than

*Correspondence: Harvey F. Lodish: lodish@wi.mit.edu, Phone: 617-258-5216, Fax: 617-258-6768.

more classical analyses of mRNA profiles or protein markers, and allow better prognosis of disease progression¹⁵.

As outlined in Figure 1, miRNAs are transcribed by polymerase II or polymerase III to primary transcripts (pri-miRNAs) in the nucleus. Endonuclease Drosha and its cofactor DGCR8 process pri-miRNA by cutting it at the bottom of its stem loop to ~70-nucleotide precursors (pre-miRNAs). Upon export to the cytoplasm by exportin 5 and Ran-GTP, Dicer generates a ~22 nucleotide miRNA duplex. One strand (mature miRNA) is then preferentially retained in the RNA-induced silencing complex (RISC) and base pairs with specific sequences in their target mRNAs. Depending on the level of complementarity, silencing of the transcript can occur via Argonaut-dependent mRNA cleavage or translational repression or both. The translationally repressed mRNA is either stored in P-bodies or enters the mRNA decay pathway for destruction¹⁶.

miRNA targeting is primarily through seed-matched sites located within favorable predicted contexts in 3' untranslated regions (UTRs). Both computational and experimental studies show that each miRNA likely targets ~400 mRNAs and that almost half of the mRNAs in mammalian cells are targeted by one or more miRNAs³. As a group, miRNAs may directly regulate expression of over 30% of human and mouse genes and more than 60% of human protein-coding genes have been under selective pressure to maintain pairing to miRNAs^{3, 17}. Furthermore, miRNAs are attractive candidates to be involved in complex diseases such as obesity because the simultaneous coordination of a large number of target genes, potentially accomplished by a single miRNA, may be key to defining specific pathogenic cell states.

Although miRNA expression profiles and functions have been extensively investigated in the hematopoietic and neuronal systems^{18, 19}, little is known about the role of miRNAs in metabolic tissues, particularly adipose tissue¹⁰. More recently, several intriguing studies have uncovered critical roles of miRNAs in the development of metabolic tissues such as adipose tissue and pancreas. Many miRNAs are also dysregulated in metabolic tissues from obese animals and humans, and possibly involved in the tissue dysfunction and secondary complications associated with obesity and diabetes. The purpose of this review is to summarize the most recent progress in understanding the roles of miRNAs in metabolic tissues under normal development and obese conditions and to provide a perspective to future research directions in order to utilize miRNAs as diagnostic markers or therapeutic targets.

2. miRNAs in metabolic tissues

2.1 Adipose tissue

Adipose tissue is not only a storage depot of triglycerides, but also an endocrine organ and an important regulator of whole-body energy homeostasis²⁰⁻²². Adipocyte-specific proteins induced during differentiation, such as adiponectin, resistin, and leptin regulate many aspects of lipid and glucose metabolism in adipose tissue, muscle, and liver, and *via* actions on the brain that affect food intake^{23, 24}. The development of obesity depends on the coordinated interplay of adipocyte hypertrophy (increased fat cell size), adipocyte hyperplasia (increased fat cell number), and angiogenesis²⁵. Both adipocyte hypertrophy and hyperplasia occur during normal growth phases and the development of obesity. As discussed below, miRNAs are important regulators of fat cell development and insulin sensitivity (Table 1). Many miRNAs are differentially expressed in different fat depots and between normal and obese adipose tissue, likely associated with proper function of adipose tissue.

2.1.1 miRNA and fat cell development—Adipocytes are derived from multipotent mesenchymal precursor cells that commit to preadipocytes and then either remain quiescent or proceed to become differentiated adipocytes²⁶. The differentiation process is tightly controlled by a combination of multiple transcription factors including peroxisome proliferator-activated receptor gamma (PPAR γ) and extracellular hormones such as insulin^{27–29}. The first and best characterized model of adipogenesis *in vitro* is the 3T3-L1 cell line, a substrain of the Swiss 3T3 mouse cell line³⁰. When treated with a combination of chemicals, post-confluent 3T3-L1 cells undergo clonal expansion, growth arrest and terminal differentiation. Differentiated adipocytes accumulate lipid droplets and express genes associated with lipid metabolism (FABP4), glucose homeostasis (GLUT4), and endocrine functions (adiponectin).

2.1.1.1 miRNA expression: Attempts to catalogue miRNA expression in adipose tissue and during adipogenesis have been carried out using different profiling platforms. Using a miRNA cloning strategy, Gu et al cloned 45 known and 2 novel miRNAs from bovine adipose tissue³¹. More recently, Liang et al further compared the expression of 345 miRNAs in 40 normal human tissues by quantitative reverse transcription-polymerase chain reaction (RT-PCR)³². Unlike neuronal and muscular tissues, their result did not identify any miRNA expressed exclusively in adipose tissue. One caveat of such tissue profiling studies is that one could easily get fat contamination when isolating other tissues such as breast. Interestingly, miRNA profiling in purified mouse primary adipocytes identified many adipocyte-enriched miRNAs including miR-103 and miR-143, in comparison to chondrocytes and osteoblasts, which are also derived from mesenchymal progenitor cells^{33–35}.

Using Northern blot analyses, Kajimoto et al profiled ~100 miRNAs in mouse preadipocyte 3T3-L1 cells before and after differentiation and showed that 21 miRNAs were either up- or down-regulated during differentiation³⁶. To provide better sensitivity and coverage, more recent studies have profiled miRNA expression at different stages of 3T3-L1 differentiation using a miRNA microarray approach^{33, 37, 38, 39, 40}. In addition, Xie et al compared the expression of 12 selected miRNAs by RT-PCR in enriched mouse primary preadipocytes and adipocytes and suggested that similar changes in miRNA expression occur during *in vitro* and *in vivo* adipogenesis³³. These studies offer an overview of miRNA regulation during fat cell development and identify many candidate miRNAs for further investigation. Of particular relevance, many miRNAs including let-7, miR-30, 103, 143 and 422b are upregulated during adipogenesis as suggested by multiple independent studies and confirmed by either Northern blots or RT-PCR^{33, 36, 37, 40}. Many of these differentially regulated miRNAs are important regulators of adipogenesis, as discussed below.

2.1.1.2 miRNA function: To infer the global functional role of miRNA in adipose biology, one can disrupt miRNA processing machinery (Figure 1) by conditional knock-out of Dicer or DGCR8 in the adipose tissue, similar to studies performed in skin and lung^{41–43}. So far, such an animal model has not been reported. Nevertheless, in cell culture, knock-down of Droscha by siRNA two days before induction of differentiation abolished 3T3-L1 adipogenesis³⁸.

The first evidence for participation of individual miRNAs in adipogenesis and lipid metabolism came from a genetic screen in *Drosophila*. Xu et al⁴⁴ found that deletion of miR-14 results in increased levels of triacylglycerol and diacylglycerol, whereas increases in miR-14 copy number have the opposite effect. Using a similar method, Teleman et al⁴⁵ demonstrated that homozygous mutations in the miR-278 gene, which is prominently expressed in the fat body of flies, causes a smaller fat body and reduced ratio of total body triglycerides to total protein. This phenotype could be rescued by miR-278 expression.

Mutant flies also exhibited hyperglycemia in spite of elevated insulin-like peptide levels due to insulin resistance of the fat body. Thus, miR-278 regulates insulin sensitivity. However, miR-14 and miR-278 have so far been found only in insects and there are no known homologues of these miRNAs in mammals.

More compelling evidence for the role of miRNA in adipogenesis has been obtained in studies of mammalian cells. To screen candidates that have regulatory roles in adipogenesis, Esau et al³⁷ transfected antisense oligonucleotides targeting 86 human miRNAs into primary human preadipocytes. Blocking miR-143 effectively inhibited all five adipocyte differentiation markers by at least 40%, suggesting a pro-adipogenic role of miR-143. Consistent with this, miR-143 expression increased during mouse adipogenesis in vitro and in vivo³³. In addition, ectopically expressing miR-143 in 3T3-L1 preadipocytes accelerated fat cell formation³³, thus confirming its important role in modulating adipogenesis.

A computational study predicted that the miRNA paralogs miR-103 and miR-107 affect multiple mRNA targets in pathways that involve cellular acetyl-CoA and lipid metabolism⁴⁶. Interestingly, these miRNAs are derived from introns of the pantothenate kinase gene family members that activate pantothenate for the biosynthesis of coenzyme A. Experimentally, ectopic expression of miR-103 in 3T3-L1 preadipocytes accelerated adipogenesis, as measured both by the upregulation of many adipogenesis markers including PPAR γ and FABP4, and by an increase in triglyceride accumulation at an early stage of adipogenesis³³. Based on mRNA profiling and computational predictions, potentially important mRNA targets for miR-103 includes several anti-adipogenic factors such as ARNT, FZD1, and RUNX1T1/ETO/MTG8, whose levels are normally downregulated during adipogenesis³³.

Many signaling pathways have an effect on adipogenesis²⁸. For example, insulin and BMP2 promotes adipogenesis while Wnt-family proteins and TGF β repress adipogenesis. Therefore, miRNAs activating or inhibiting any of these signaling pathways are likely to affect adipogenesis. Kennell et al⁴⁷ identified miR-8 as a negative regulator of Wnt signaling in *Drosophila*. They further demonstrated that the miR-200 family, homologues of *Drosophila* miR-8 in mammals, promoted adipogenesis. Retroviral expression of miR-200 cluster in mouse ST2 marrow stromal cells increased lipid accumulation and expression of FABP4 and partially rescued the block of differentiation caused by treatment with recombinant Wnt3a.

Since clonal expansion is one of the key events taking place in early adipogenesis of 3T3-L1 cells in vitro, any miRNAs affecting this process could have a profound effect on adipogenesis. Wang et al³⁸ reported that the miR-17-92 cluster, which promotes cell proliferation in various cancers, is upregulated 2–3 fold during the early clonal expansion stage of 3T3-L1 adipogenesis. Stable overexpression of these miRNAs moderately accelerates adipocyte differentiation in vitro after hormonal stimulation. Additionally, this study provided convincing evidence that Rb2/p130 is a *bona fide* target of miR-17-92 during adipogenesis. This is not totally unexpected because previous observations suggested a p130:p107 switch during the first 24h after hormonal induction of adipogenesis⁴⁸.

Another miRNA related to clonal expansion is let-7, which is well known to regulate cell proliferation and differentiation processes in species ranging from *C. elegans* to human^{49, 50}. Expression of let-7 slightly decreased from day 0 to day 1 and then increased during terminal adipogenesis⁴⁰. Ectopic expression of let-7 by transfecting pre-let-7 oligonucleotide into 3T3-L1 cells prior to induction of differentiation inhibited clonal expansion as well as terminal differentiation, in part by targeting HMGA2⁴⁰. Interestingly, mice lacking HMGA2 have marked reductions in adipose tissue⁵¹. However, the involvement of the miR-17-92

cluster and let-7 during *in vivo* fat cell development awaits further investigation since clonal expansion is not required for primary preadipocyte differentiation.

Among many downregulated miRNAs during adipogenesis, the miR-27 gene family have been functionally characterized using 3T3-L1 preadipocytes and OP9 mouse bone marrow mesenchymal stem cells³⁹. Overexpression of miR-27 by transfecting miR-27 precursors before adipogenic stimulation specifically inhibited adipocyte formation. Mechanistically, miR-27 prevents the induction of PPAR γ and C/EBP α , the two master transcriptional regulators of adipogenesis. Interestingly, although PPAR γ contains a putative binding site for miR-27, miR-27 does not repress the level of PPAR γ protein in differentiating 3T3-L1 cells if miR-27 was transfected after 2 days after adipogenic stimulation. Therefore, it is plausible that miR-27 inhibits adipogenesis by targeting an unknown gene that prevents the transcriptional induction of PPAR γ .

It is interesting to note that overexpression of miRNAs that are upregulated during adipogenesis, namely miR-143, miR-103, miR-17-92, alone without hormonal induction cannot trigger adipogenesis, suggesting that they are not sufficient to initiate differentiation^{33, 38}. Similarly, inhibition of endogenous miR-27 using antisense oligonucleotides is not sufficient to promote adipogenesis³⁹.

2.1.2 miRNA and insulin sensitivity—To identify miRNAs associated with insulin sensitivity, He et al⁵² examined the miRNA expression profile by miRNA microarray analysis of skeletal muscles from healthy and Goto-Kakizaki rats, a model of type 2 diabetes. miR-29 family members are upregulated in diabetic animals compared to control animals. Northern blot analysis further revealed their upregulation in all three insulin-responsive tissues including adipose tissue, muscle and liver of diabetic rats. Adenovirus-mediated overexpression of miR-29 in 3T3-L1 largely repressed insulin-stimulated glucose uptake, presumably through inhibiting Akt activation. High levels of miR-29 led to insulin resistance mimicking the insulin resistance in cells incubated with high glucose and high insulin. Interestingly, the miR-29 level was upregulated in the presence of high glucose (hyperglycemia) and high insulin (hyperinsulinemia) in 3T3-L1 adipocytes. Two candidate genes *Insig1* (insulin-induced gene 1) and *Cav2* (caveolin 2) were validated as targets of miR-29 but their participation in insulin signaling pathway is not clear. Further studies are needed to test whether higher level of miR-29 in insulin-responsive tissues result in lower levels of target proteins.

With the observation that individual miRNAs moderately repress many targets, it is tempting to hypothesize that a miRNA family or a miRNA cluster targeting multiple components of the same signaling pathway may be more effective and functionally important than a single miRNA. Xu and Wong performed a computational screen to identify mouse signaling pathways targeted by miRNA clusters⁵³. Most strikingly, one miRNA cluster, *mmu-mir-183-96-182* targets *Irs1*, *Rasa1*, and *Grb2*, all of which are located in the insulin signaling pathway. These predictions were further supported experimentally by luciferase report assays. This supports the notion that different members of one miRNA cluster target different components along a signaling pathway and can coordinately control the signal transduction process.

Studies in cancer cells also suggested insulin receptor substrate (IRS)-1 can be repressed at the translation level by miR-145 and miR-126^{54, 55}. Since IRS is an important component of the insulin signaling pathway, it merits further investigation whether these interactions are physiologically relevant and play a role in the pathogenesis of insulin resistance.

2.1.3 miRNAs in different fat depots—Adipose tissue consists of white adipose tissue (WAT) and brown adipose tissue (BAT). While WAT stores excess energy, BAT is very active in energy expenditure and has an anti-obesity function^{56, 57}. This property relies on the expression of a BAT specific protein, UCP-1, which is a proton transporter located in the inner mitochondrial membrane and allows dissipation of the proton electrochemical gradient in the form of heat instead of ATP⁵⁸.

While BAT persists through life in rodent, it has traditionally been considered insignificant in adult human. In human fetuses and newborns, BAT is found in axillary, cervical, perirenal, and periadrenal regions, but decreases shortly after birth⁵⁶. However, using morphological studies and positron-emission tomography scanning studies, three independent groups have recently provided conclusive evidence for the existence of active BAT in adults^{59–61}. Importantly, the presence of BAT negatively correlates with both BMI and percentage of body fat, whereas it positively correlates with resting metabolic rate, implicating a role of BAT in preventing obesity in adult human.

White adipose tissue can be further subdivided into subcutaneous fat and visceral fat, which is most closely related to insulin resistance⁵⁶. Global miRNA expression comparison between subcutaneous and omental fat, a type of visceral fat, from 15 human individuals suggested little differences in these two fat depots overall⁶²; this supports the notion that subcutaneous and visceral fat are indeed two locations of a developmentally homogeneous adipose organ.

Recently, exciting research suggested that white and brown adipocytes originate from different precursors with brown preadipocytes being more closely related to skeletal muscle cells than to white preadipocytes⁵⁷. Investigation of miRNAs in brown fat has just begun. miR-455, which is low in white pre- and mature adipocyte, was enhanced during brown adipocyte differentiation, similarly to the expression pattern of the brown adipocyte differentiation marker UCP-1⁶³; thus it may play a role during brown adipocyte differentiation or contribute to the mature brown adipocyte function. Three classical “myogenic” miRNAs, miR-1, miR-133a and miR-206 were absent from white adipocytes but were specifically expressed both in brown pre- and mature adipocytes⁶³. More comprehensive high-throughput expression profiling studies should identify more miRNAs specifically enriched in white fat, brown fat and muscle. Brown fat enriched miRNAs may be related to energy expenditure and/or thermogenesis. In vitro and in vivo manipulation of these brown fat enriched miRNAs, independently or together with transcription factors, may help confer the brown adipocyte properties to white adipocyte or muscle cells, which may be used clinically to treat obesity.

2.1.4 miRNA in obese adipose tissue—Adipose tissue undergoes a dramatic expansion in obesity, which eventually results in adipose tissue dysfunction. As adipose tissue expands, macrophage infiltration in adipose tissue occurs⁶⁴. Chronic inflammation and hypoxia are two principal features of obese adipose tissue in animals and humans^{64, 65}. Inflammatory cytokines including TNF- α are largely responsible for suppressing many adipocyte-specific genes including PPAR γ and adiponectin and reactivating expression of many cell cycle genes, resulting in insulin resistance in obese adipose tissue⁶⁶.

To compare miRNA expression levels in normal and obese states at a genome-wide scale, Xie et al³³ profiled the expression of more than 370 miRNAs in enriched epididymal adipocytes from leptin deficient ob/ob and diet-induced obese (DIO) mice using miRNA microarrays. A total of 71 miRNAs were expressed at significantly different levels in adipocytes from wild type and ob/ob mice of the same gender and age whereas 35 miRNAs were differentially expressed between control and DIO mice. Table 2 lists miRNAs either

up- or down-regulated significantly regardless of the obesity model. There was a global positive correlation of miRNA regulation in these two different models of obesity³³, suggesting that the changes in the adipocyte miRNA expression profile of ob/ob mice cannot result from leptin deficiency alone, but likely associated with obesity in general.

Most importantly, miRNAs that were induced during adipogenesis were decreased in adipocytes from both types of obese mice and *vice versa*³³. For example, miR-422b, miR-103, miR-30c were induced during adipogenesis but were downregulated in obese adipocytes. Conversely, miR-221 and miR-222 were decreased during adipogenesis but were upregulated in obese adipocytes. These changes are likely associated with the chronic inflammatory environment and elevated TNF- α levels in obese adipose tissue, since they were mimicked by TNF- α treatment of differentiated 3T3-L1 adipocytes³³. The remarkable inverse regulatory pattern for many miRNAs during adipogenesis and obesity has important implications for understanding adipose tissue dysfunction in obese mice and humans and the link between chronic inflammation and obesity with insulin resistance.

Independently, Lin et al³⁹ observed the moderate increase of miR-27, a regulator of adipogenesis discussed in the previous section, in epididymal fat tissue of ob/ob mice. They found that miR-27 expression could be regulated by hypoxia, an important extracellular stress associated with obesity. Whether miR-27 is regulated by inflammatory cytokines is still an open question.

In human, significantly lower expression of miR-17-5p, miR-132 and miR-134 was found in omental fat of overweight and obese individuals with newly diagnosed type 2 diabetes compared to those with normal glucose tolerance, whereas the opposite pattern was found for miR-181a⁶². Expression of miR-17-5p and miR-132 was negatively associated with visceral fat area⁶². Most strikingly, lower expression of omental miR-132 was correlated with higher macrophage infiltration whereas higher expression of miR-181a was correlated with lower circulating adiponectin⁶². One possible caveat is that the unfractionated adipose tissues profiled in their study contain many different cell types; thus the difference of certain miRNAs may represent a decrease in the fraction of mature adipocytes in visceral adipose tissue in type 2 diabetes patients, possibly as a result of infiltrating macrophages. Further studies are necessary to dissect causal from correlative relationships and the association needs to be confirmed in larger cohorts.

2.2 Liver

miR-122, a predominant miRNA in the liver, is involved in the regulation of several cholesterol biosynthesis pathway genes⁶⁷. In vivo inhibition of miRNA-122, in normal and a diet induced obesity mouse model, resulted in a significant decrease in plasma cholesterol levels and improvement in liver steatosis^{67, 68}. In addition, the miR-30 family was recently shown to be required for vertebrate hepatobiliary development, as knockdown of miR-30a in zebrafish larva resulted in defective biliary morphogenesis⁶⁹. Interestingly, hepatic function was preserved up to 100 days old when Dicer1 was knocked out at birth in the differentiated liver⁷⁰. This was rather surprising considering the striking effect on cholesterol metabolism when just miR-122 was knocked down. The authors suspected that additional miRNAs contributed to the regulation of metabolic pathways and that global loss of all miRNAs masks the phenotypes uncovered by the unbalanced inhibition of a single component of miRNA-mediated regulation⁷⁰.

Recently, Li et al⁷¹ analyzed the expression of miRNAs in livers of ob/ob mice, streptozotocin (STZ)-induced type 1 diabetic mice and normal C57BL/6 mice by miRNA microarray. Compared to normal C57BL/6 mice, ob/ob mice showed up-regulation of 8 miRNAs (miR-34a, miR-31, miR-103, miR-107, miR-194, miR-335-5p, miR-221, and

miR-200a) and down-regulation of 4 miRNAs (miR-29c, miR-451, miR-21 and miR-122) in fatty livers. Up-regulation of miR-34a and down-regulation of miR-122 was found in livers of STZ-induced diabetic mice.

2.3 Pancreas

The role of miRNAs in insulin production (miR-30d), insulin secretion (miR-375, miR-9 and miR-96) and pancreatic islet development (miR-375 and miR-124a) have been reported and extensively reviewed previously⁷²⁻⁷⁵. More recently, Poy et al⁷⁶ reported that mice lacking miR-375 are hyperglycemic, and exhibit increased total pancreatic alpha-cell numbers, decreased beta-cell mass, higher fasting and fed plasma glucagon levels, and increased gluconeogenesis and hepatic glucose output. More interestingly, increased expression of miR-375 is observed in pancreatic islets of leptin-deficient ob/ob mice, which also have increased beta-cell mass⁷⁶. Genetic deletion of miR-375 from ob/ob mice profoundly diminished the proliferative capacity of the endocrine pancreas and resulted in a severe diabetic state⁷⁶.

Another study by Lovis et al⁷⁷ investigated the contribution of miRNAs to fatty acid induced pancreatic beta-cell dysfunction. Prolonged exposure of the beta-cell line MIN6B1 and pancreatic islets to palmitate caused a time- and dose-dependent increase of miR-34a and miR-146. Elevated levels of these miRNAs were also observed in islets of diabetic db/db mice⁷⁷. Blocking miR-34a or miR-146 activity partially protects palmitate-treated cells from apoptosis⁷⁷. Therefore, at least part of the detrimental effects of palmitate on beta-cells is caused by alterations in the level of specific miRNAs.

3. Challenges and perspectives

3.1 Functional validation in animal models

As discussed so far, most gain-of-function or loss-of-function studies on miRNA have been carried out in cell lines or primary cell cultures. The efficiency of knocking down miRNAs in cell culture is still not optimized; thus, specific miRNA or miRNA cluster knockout mice, especially conditional knockout mice, are the most powerful loss-of-function approach to assess their role in development and obesity. Fortunately, the Sanger Institute in Cambridge has started an initiative to create a library of knockouts of each of the 500 miRNAs identified in the mouse genome⁷⁸. The resource will eventually be available to all researchers. Meanwhile, researchers may find some clues about miRNA function from current knockout mice models for protein-coding genes because it is estimated that expression of a miRNA may have been disrupted along with another gene in about 200 knockout cases⁷⁹. However, it is challenging to knockout all miRNA family members that are regarded to be functionally redundant if these family members are not on the same chromosome. For example, miR-103/107 family members, miR-103-1, miR-103-2 and miR-107, reside on three different chromosomes.

3.2 Combinatorial effect of miRNAs

Beside the fact that each mammalian miRNA regulates a large number of target genes, several different miRNAs can act additively or synergistically at multiple target sites of a single mRNA⁸⁰. The potential interaction networks connecting miRNAs and mRNAs are enormous and can be further expanded by feedback or feed-forward loops⁸¹. The effect of single miRNA may be small and difficult to detect; nonetheless each probably makes an important contribution to the robustness of the development of metabolic tissues and pathogenesis of obesity and associated disorders. Ideally, one should manipulate multiple candidate miRNAs in different combinations rather than changing one miRNA at a time for functional characterization. However, this is labor intensive and technically challenging.

3.3 Therapeutic strategies targeting miRNAs

With experimental evidence beginning to provide functional roles of miRNA in development and metabolism, the need has become apparent to test miRNAs as viable therapeutic targets. Some lessons learned from existing antisense technology and gene therapy approaches can be adapted to manipulate miRNA levels in vivo.

Enhancement of miRNA activity can be accomplished by transfecting synthetic miRNA mimetics or by using plasmids to transcribe miRNAs from endogenous or viral promoters. Repression of miRNA activity can be performed with antisense oligonucleotides with modifications to increase stability and binding specificity. Three major types of modifications includes 2'-O-methyl (2'-O-Me), 2'-O-methoxyethyl (2'-O-MOE) oligonucleotides and locked nucleic acids (LNAs)⁸². Initial studies exploiting these modified antisense oligonucleotides to repress miRNA expression have shown promising results in animals and non-human primates.

Of relevance to metabolism, Esau et al⁶⁸ antagonized miR-122, a liver specific miRNA, with 2'-O-MOE oligonucleotides in normal and diet-induced obese mice. When injected intraperitoneally, miR-122 inhibition resulted in reduced plasma cholesterol levels in both normal and diet-induced obese mice. Strikingly, miR-122 inhibition in a diet-induced obesity mouse model significantly improved liver steatosis. Independently, Krutzfeldt et al⁶⁷ used cholesterol-conjugated 2'-O-Me oligonucleotides (also known as antagomirs) to silence miRNAs. Intravenous administration of antagomirs against miR-122 reduced plasma cholesterol level significantly. The silencing of endogenous miRNAs by antagomirs seems to be specific, efficient, and long-lasting. Most recently, Elmen et al⁸³ used unconjugated LNAs to antagonize miR-122 in non-human primates. Acute administration by intravenous injections in African green monkeys resulted in uptake of LNAs in the cytoplasm of primate hepatocytes and dose-dependent lowering of plasma cholesterol. Silencing of miR-122 was efficient, long-lasting, and reversible without any evidence for LNA-associated toxicities or histopathological changes. Additional examples of the use of antisense oligonucleotides targeting miRNAs have been tabulated in a previous review article⁸². An alternative approach of silencing miRNAs is the use of miRNA sponges, which contain multiple tandem binding sites for targeted miRNAs, thereby acting as decoys to titrate miRNAs away from their natural targets⁸⁴. When miRNA sponges are expressed in a plasmid, the effect can be long lasting. Two recent studies have utilized this strategy to inhibit miR-223 and miR-31 in vivo^{85, 86}. One can potentially inhibit multiple miRNAs with a single plasmid by tandem decoy sequences against different miRNAs of interest.

In the future, and with better understanding of the upstream factors controlling miRNA expression, we may be able to therapeutically increase or decrease expression of specific miRNA(s) in disease tissue and thus ameliorate certain disease symptoms.

3.4 Delivery of therapeutic agents

Currently, there is an intense effort to identify agents capable of targeted delivery of nucleic acids to specific tissues and cells⁸⁷⁻⁹². One approach is conjugating a lipophilic moiety or receptor ligand, such as cholesterol in the case of antagomirs, to the oligonucleotide. Alternatively, oligonucleotides can be packed into liposomes, polymers, or nanoparticles that facilitate endocytosis. Recently, combinatorial chemistry has yielded a novel class of lipidoids that may allow for the development of new classes of delivery reagents⁹³.

One challenge is the delivery method to transfer the miRNAs or its inhibitors into the desired tissue. So far, much of the success has been obtained in liver. Some metabolic tissues may be easier accessed and targeted than others in this regard. Circumventing this

technical difficulty may involve development of different chemical modifications and conjugates designed to specifically interact with cell-specific membrane proteins.

4. Conclusion

Emerging evidence from both gain- and loss-of-function studies suggests that miRNAs play an important role in metabolic tissue development and function. Expression profiling studies have revealed that some miRNAs are dysregulated in metabolic tissues under the obese state, and possibly involved in the pathogenesis of various metabolic disorders. Further functional characterization of metabolically important miRNAs *in vitro* and *in vivo* and better understanding of their mechanism of action may allow for identification of novel therapeutic targets and strategies. The safe, effective and targeted delivery of RNA therapeutics remains an important challenge for clinical development.

5. Expert opinion

The complexity of post-transcriptional regulation by miRNA is still far from understood. Further characterization of metabolically important miRNAs *in vivo* is likely to shed more light on their functions. Large scale proteomics will help identify targets of a specific miRNA since the effect of miRNAs is often more pronounced at the protein level than at the mRNA level. Improved computational algorithms predicting miRNA targets will aid in this effort as well. The potential interaction networks connecting miRNAs and mRNAs are enormous and complex, thus a system biology approach is necessary to understand the entire RNA regulatory network.

High-throughput miRNA profiling studies have and will continue to reveal miRNA dysregulation in metabolic tissues. However, it is hard to dissect whether change of certain miRNA cause metabolic tissue dysfunction or miRNA is dysregulated as a consequence of metabolic tissue dysfunction. Establishment of the causal relationship will enable better selection of therapeutically potential targets.

Recently, many genome-wide association studies have identified susceptibility loci and gene polymorphisms for obesity and type 2 diabetes⁹⁴⁻⁹⁸. Some of these polymorphisms may create or destroy a putative miRNA target site responsible for the phenotypic variation. As an example, Villuendas et al⁹⁹ showed that the ACAA-insertion/deletion polymorphism at the 3'UTR of the insulin-like growth factor II receptor (IGF2R) was associated with type 2 diabetes and surrogate markers of insulin resistance. Most strikingly, using luciferase reporter assays, Lv et al¹⁰⁰ showed that hsa-miR-657 acts directly at the 3'UTR of the IGF2R and the repression was greater when ACAA was deleted. This finding raise the possibility that the ACAA-insertion/deletion polymorphism may result in the change of IGF2R expression levels at least in part by hsa-miR-657-mediated regulation, contributing to the pathogenesis of type 2 diabetes.

Since miRNAs have been shown to be differentially expressed in healthy and disease states¹⁰¹, it is tempting to use miRNA as biomarkers for diagnosis. Excitingly, Chen et al¹⁰² demonstrated that miRNAs were present in serum and plasma of humans and other animals. miRNA levels in serum are stable, reproducible and consistent among individuals of the same species. Using high-throughput sequencing, they identified specific expression patterns of serum miRNAs for lung cancer, colorectal cancer, and diabetes¹⁰². In addition, 17 miRNAs were exclusively detected in serum compared to blood cells from diabetic patients¹⁰². Although the source of these miRNAs is not yet clear, a serum miRNA fingerprint could complement the current biomarker, C-reactive protein (CRP) for the diagnosis of diabetes. It would be interesting to find out when these serum miRNA expression change respect to disease progression.

In conclusion, miRNAs are promising therapeutic targets for management of obesity and related metabolic disorders. With this great promise, many biotech firms are springing up to develop miRNA-based products that can diagnose, treat, or predict the course of disease¹⁰³. However, to realize the full potential of miRNA-based therapeutics, more efficient and specific silencing of miRNA and targeted delivery are needed.

Abbreviations

miRNA	microRNA
RISC	RNA-induced silencing complex
UTRs	untranslated regions
PPARγ	peroxisome proliferator-activated receptor gamma
RT-PCR	reverse transcription-polymerase chain reaction
IRS	insulin receptor substrate
WAT	white adipose tissue
BAT	brown adipose tissue
DIO	diet-induced obese
2'-O-Me	2'-O-methyl
2'-O-MOE	2'-O-methoxyethyl
LNAs	locked nucleic acids
IGF2R	insulin-like growth factor II receptor

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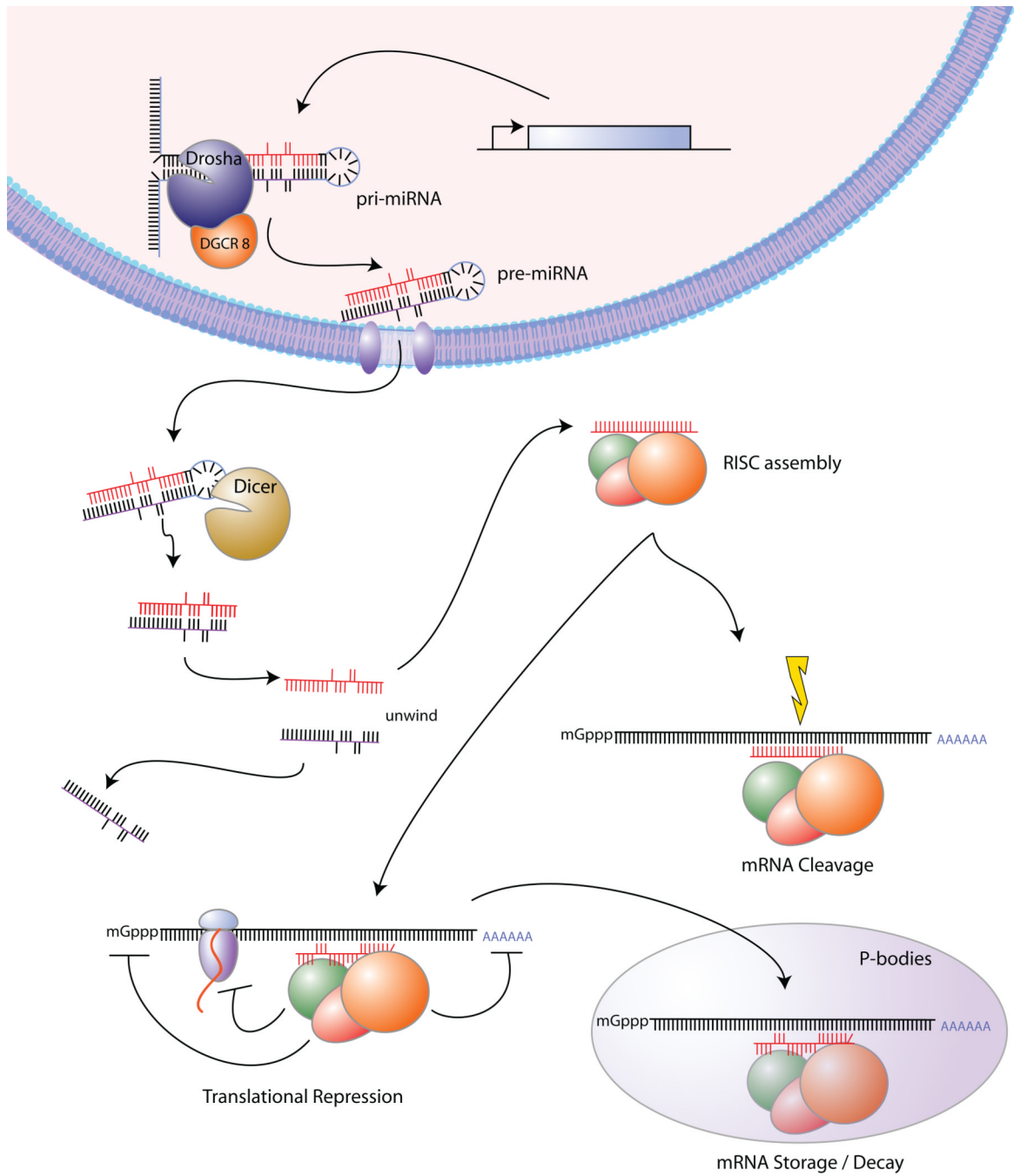


Figure 1.
miRNA biogenesis and mechanisms of action.

Table 1

Mammalian miRNAs regulating adipogenesis and insulin sensitivity.

miRNA	Function	Model system	Targets	References
miR-143	Pro-adipogenic	Primary human preadipocytes, 3T3-L1	ERK5/MAPK7 ^(?)	33, 37
miR-103	Pro-adipogenic	3T3-L1		33
miR-200	Pro-adipogenic	ST2		47
miR-17-92 cluster	Pro-adipogenic	3T3-L1	Rb2/p130	38
Let-7	Anti-adipogenic	3T3-L1	Hmga2	40
miR-27	Anti-adipogenic	3T3-L1, OP9		39
miR-29	Inhibitor of glucose uptake	3T3-L1	Insig1 ^(?) , Cav2 ^(?)	52

3T3-L1, mouse preadipocyte cell line; ST2 and OP9, mouse bone marrow stromal cells

[?] Validated targets but their participation in adipogenesis or insulin signaling pathway is not clear

Table 2

Principal miRNAs differentially regulated in enriched mouse epididymal adipocytes from both types of obese mice (*ob/ob* and *DIO*) compared to wild type mice. Analysis is performed on the miRNA profiling data described by Xie et al ³³.

Up-regulated in obese mice	Down-regulated in obese mice
miR-16 miR-24 miR-221 miR-222 miR-223 miR-146b miR-23b miR-27a miR-27b miR-342-3p	let-7d(*) miR-103 miR-107 miR-145 miR-320 miR-30a*

Mature miRNAs are referred to by their names in miRBase version 10.1