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MicroRNAs in adipogenesis and as therapeutic targets for obesity

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Abstract

Introduction—Obesity and obesity-related disease have reached pandemic proportions and are prevalent even in developing countries. Adipose tissue is increasingly being recognized as a key regulator of whole-body energy homeostasis and consequently as a prime therapeutic target for metabolic syndrome. This review discusses the roles of miRNAs, small endogenously expressed RNAs that regulate gene expression at a post-transcriptional level, in the development and function of adipose tissue and other relevant metabolic tissues impacted by obesity. Several high-throughput studies have identified hundreds of miRNAs that are differentially expressed during the development of metabolic tissues or as an indication of pathophysiology. Further investigation has functionalized the regulatory capacity of individual miRNAs and revealed putative targets for these miRNAs. Therefore, as with several other pathologies, miRNAs are emerging as feasible therapeutic targets for metabolic syndrome.

Areas covered—This review provides a comprehensive view of miRNAs involved in adipogenesis, from mesenchymal stem cell lineage determination through terminal adipocyte differentiation. We also discuss the differential expression of miRNAs among adipose depots and the dysregulation of miRNAs in other metabolic tissues during metabolic pathophysiology. Finally, we discuss the therapeutic potential of targeting miRNAs in obesity and give a perspective on the challenges and advantages of miRNA-based drugs.

Expert opinion—miRNAs are extensive regulators of adipocyte development and function and are viable therapeutic targets for obesity. Despite the broad-spectrum and redundancy of miRNA–target interactions, sophisticated bioinformatic approaches are making it possible to determine the most physiologically relevant miRNAs to target in disease. In vivo delivery of miRNAs for therapeutic purposes is rapidly developing and has been successful in other contexts. Additionally, miRNAs can be used as prognosis markers for disease onset and progression. Ultimately, miRNAs are prime therapeutic targets for obesity and its consequent pathologies in other metabolic tissues.

Keywords

adipogenesis; adipose tissue; diabetes; glucose homeostasis; lipid metabolism; metabolic syndrome; metabolic tissues; microRNAs; obesity; therapeutics

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Declaration of interest

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1. Introduction

Metabolic syndrome, which is characterized by overweight and obesity and their associated non-communicable diseases (NCDs), has become pandemic in scale and now poses an immense threat on global health. Overweight (body mass index (BMI) 25 – 30) and obesity (BMI > 30) are highly associated with type II diabetes, hypertension, cardiovascular disease, and several cancers and are largely attributable to poor diet (i.e., increased dietary energy intake) and sedentary lifestyle [1]. In 2000, poor diet and physical inactivity were recorded as the second leading cause of death in the USA (16.6%) [2]. This is a rapidly increasing epidemic in the USA as mortality associated with poor metabolic health only accounted for 14% of annual deaths 10 years previously and is expected to soon become the leading cause of death [2]. According to the Center for Disease Control (CDC), in 2003 the costs for medical expenses related to overweight and obesity in the USA were approximately $75 billion and were largely financed by Medicare and Medicaid. Over 50% of American adults are overweight or obese [3], and, according to the World Health Organization (WHO), of over a billion adults worldwide who are overweight at least 300 million are obese.

The growing obesity pandemic is not exclusive to the industrialized world; developing countries are heavily affected by this trend due to increased sweetening of foods, the rapid increase of consumption of animal-source foods, dramatic shifts of work forces from agriculture and other physically-demanding jobs to service sector jobs, and various other factors [4]. This is also facilitated by the increase in distribution of affordable motorized transportation and labor-saving mechanized devices by large, multinational companies [4]. Developing countries are ill equipped to counter such trends, which are conducive to the development of poor metabolic health.

miRNAs are a class of small non-coding RNAs (approximately 22 nucleotides in length) that are increasingly being recognized as viable therapeutic targets for a host of diseases. miRNAs can be transcribed by either RNA polymerase II or III into primary transcripts called pri-miRNAs. These transcripts are cleaved in the nucleus by the enzyme Drosha and its cofactor Pasha at the bottom of their stem loops to make ~70 nucleotide precursors called pre-miRNA. Pre-miRNAs are exported to the cytosol and are processed by other enzymes such as Dicer to generate an approximately 22 nucleotide mature miRNA duplex that can be incorporated into an RNA-induced silencing complex (RISC) to be active as a post-transcriptional regulator. miRNA biogenesis is reviewed in [5]. miRNAs silence transcripts by facilitating Argonaut-dependent cleavage of mRNAs and/or by acting as a physical barrier to translation, depending on the level of complementarity with their targets [6]. The 5′ proximal seed sequence of miRNAs hybridizes with a sequence in the 3′ untranslated region of the mRNA [6]. Computational and experimental analyses have revealed that individual miRNAs each target approximately 400 mRNAs and almost half of the mammalian transcriptome is targeted by miRNAs [7].

miRNAs have been proposed as therapeutic targets against cancer, HCV, HIV, and cardiovascular disease [8–11]. This review discusses miRNAs involved in the development and function of adipose tissue and suggests their novel therapeutic potentials in mitigating obesity and its associated NCDs. Adipose tissue is increasingly being recognized as a key regulator of energy homeostasis not only because it acts as a chemical energy store but also due its production of multiple cytokines with activities that influence whole-body metabolism [12]. The increase in adiposity associated with weight gain causes chronic inflammation of adipose tissue, which has been linked to insulin resistance and hyperlipidemia that causes detrimental steatosis in other tissues. Several studies have elucidated the roles of miRNAs in adipocyte development and metabolic function and thus
implicate them as therapeutic targets for re-establishing proper energy homeostasis and countering the pathological consequences of adipose tissue expansion.

2. MicroRNAs and adipocyte development

There are two key steps to mature adipocyte development: determination and terminal differentiation [13]. In the determination step, multipotent mesenchymal stem cells (MSCs) are induced to become preadipocytes, which are morphologically similar to MSCs but have lost the ability to differentiate into non-adipocyte cell types [13]. MSCs can also develop into osteoblasts (bone forming cells) and chondrocytes (cartilage forming cells) and express discrete amounts of factors specific to each of the three lineages that inhibit each other to maintain the undifferentiated state. For example, the adipocyte-specific PPARγ protein binds the runt related (RUNX2) protein, a transcription factor that transactivates osteogenic genes, to inhibit osteogenesis [14]. Lineage determining conditions cause factors of one lineage to overshadow the others and commit the MSC to that cell fate.

In addition, several evolutionarily conserved signaling pathways, such as wingless-related MMTV integration site (WNT), bone morphogenetic protein (BMP), and TGF-β signaling, regulate this determination step. The WNT and TGF-β signaling pathways disfavor adipogenic fate in MSCs. As an example, ectopic expression of Wnt10b in the ST2 murine stromal cell line favors osteogenesis at the expense of adipogenesis [15]. TGF-β signaling activates small and mothers against decapentaplegic (Smad) proteins, transcriptional regulators that migrate to the nucleus and activate osteogenic and chondrogenic genes. Accordingly, RNA interference-mediated repression of Smad3 in human adipose-tissue-derived mesenchymal stem cells (hASCs) promotes adipogenesis [16]. Unlike the activity of WNT and TGF-β signaling in MSC development, which elicits a more or less binary response, other signaling pathways operate through more complex mechanisms. Of these, BMPs are notable. Like TGF-β proteins, BMPs activate Smad transcription factors. BMPs have been reported to regulate MSC determination depending on the concentration and type of the BMP involved. For instance, BMP-2 promotes adipogenesis in C3H10T1/2, a murine MSC cell line, at low concentrations, whereas higher concentrations of BMP-2 favor osteogenic and chondrogenic phenotypes [17].

Following commitment to the adipocyte lineage, preadipocytes undergo terminal adipocyte differentiation, acquire a mature adipocyte morphology, and express mature adipocyte markers such as fatty acid binding protein 4 (FABP4) and glucose transporter type 4 (GLUT4). Hormonally induced preadipocytes reenter the cell cycle and may undergo a few more rounds of mitotic expansion before acquiring the cellular machinery necessary for lipid storage and metabolism. PPARγ is a master transcriptional regulator of adipogenesis that activates genes involved in terminal differentiation and mature functions of adipocytes. Continued expression of PPARγ is necessary for maintaining the differentiated state, and adenoviral infection of a dominant negative PPARγ in 3T3-L1 adipocytes results in ‘anti-differentiation’, or a loss of distinctive adipocyte morphology and function [18]. An isoform of PPARγ, PPARα, also contributes to adipogenesis but performs a more ancillary role [19]. Members of the CCAAT-enhancer-binding protein (C/EBP) family are also important in adipogenesis. C/EBPα is transcriptionally activated by PPARγ, [20] and genome-wide analysis by Lefterova et al. [21] revealed that C/EBPα DNA binding in mature adipocytes greatly overlaps with the binding of PPARγ, suggesting that its transcriptional activity is also necessary for adipogenesis. Additionally, C/EBPβ and C/EBPδ contribute to the transcriptional activation of PPARγ in early adipogenesis [21], and C/EBPβ redundantly activates adipogenic genes targeted by C/EBPα [22]. While knockdown of PPARγ or both C/EBPα and C/EBPβ markedly decreases the expression of several adipocyte genes,
knockdown of all three results in an even more dramatic decrease in the expression of these genes [21]. These data suggest that the three transcription factors function synergistically to regulate adipogenesis. The activity of C/EBPs is further regulated by the extracellular-signal-regulated kinase (ERK) signaling cascade which, when activated, results in the phosphorylation and inactivation of C/EBPβ [23]. Recently, it has been demonstrated that miRNAs are another class of factors regulating adipocyte development (Table 1), which are discussed in detail below.

### 2.1 miRNAs involved in MSC fate determination

miRNAs play critical roles in regulating in the lineage fate of MSCs. For example, miR-204 targets and downregulates Runx2 and thereby promotes the adipogenic lineage [24]. Huang et al. determined that knockdown of miR-204 by a miR-204 sponge increased the expression of osteogenic markers in C3H10T1/2 cells and murine bone marrow stromal cells (BMSCs). Conversely, overexpression of miR-204 and miR-211, its human homologue, using either retroviral infection or by transfection of microRNA oligonucleotides promoted adipogenesis as confirmed by the increased expression of mature adipocyte markers and lipid droplet accumulation [24].

Multiple studies have shown that miRNAs are extensively involved in WNT signaling. A genetic screen in Drosophila revealed that miR-8 is a negative regulator of WNT signaling that directly targets the mRNAs encoding two pathway elements, the wntless and CG32767 genes [25]. Additionally, the mammalian miR-8 homologues miR-141, miR-200a/b/c, and miR-429 demonstrated a conserved regulation of WNT signaling in ST2 cells. These homologues are transcribed from two genomic clusters, one containing miR-200c and miR-141 and the other containing miR-200a/b and miR-429. Ectopic expression of either cluster in ST2 cells promoted lipid droplet formation and a marked increase in the production of FABP4, a crucial indicator of mature adipocyte function. Additionally, Kennell et al. found that the over-expression miR-8 homologues partially rescued an adipogenic phenotype in ST2 cells expressing recombinant Wnt3a [25].

In a related study by Qin et al. [26], 18 additional miRNAs were shown to be possible repressors of WNT signaling via high-throughput microarray analysis in 3T3-L1 cells, and 29 others were identified as possible activators of WNT signaling. Glycogen synthase kinase (GSK)-3β inhibits WNT signaling by phosphorylating β-catenin, a transcriptional activator of WNT target genes, and thus promoting its degradation. LiCl inhibits GSK-3β and consequently activates intracellular WNT signaling elements. The rationale of Qin et al.’s study was that miRNAs differentially expressed between control and LiCl-treated cells are probably involved in WNT signaling. The probable targets of these miRNAs were determined by bioinformatic analysis. MiR-210, which was confirmed to target the Tcf712 gene via a luciferase reporter assay, was chosen for further study. TCF712 protein is a transcription factor that activates WNT target genes in association with β-catenin. Accordingly, overexpression of miR-210 was also shown to accentuate an adipogenic phenotype in hormonally induced 3T3-L1 preadipocytes.

Similarly, miR-21 has been implicated as a mediator of TGF-β signaling in hASCs [16]. MiR-21 was found to downregulate a TGFBR2 luciferase reporter construct, and therefore is a likely negative regulator of TGF-β signaling. Supporting this notion, levels of phosphorylated Smad3 protein were inversely correlated to miR-21 levels when the microRNA was overexpressed via lentiviral transduction or suppressed by antisense oligonucleotides. Phosphorylated Smad3 forms a complex with Smad4 and travels to the nucleus where it activates gene transcription [27]. Therefore, miR-21 affects TGF-β signaling by impairing the phosphorylation of Smad3. Accordingly, miR-21 was transiently

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upregulated upon hormonal induction of adipogenesis in hASCs, and overexpression of miR-21 enhanced adipogenesis [16].

Lastly, Lin et al. [28] found that ectopic expression of miR-199a in either C3H10T1/2 or ATDC5 mouse prechondrogenic cell lines inhibits early chondrogenesis by targeting Smad1, a downstream target of BMP-2. These findings were supported by the reduced expression of chondrogenic markers (cartilage oligomeric matrix protein (COMP), type II collagen, and sexdetermining region on the Y chromosome-related homebox 9 (Sox9)) and an increased expression of these markers upon antisense suppression of miR-199a. These experiments demonstrate that miRNAs are intricately involved in MSC fate determination through both regulation of lineage-specific transcription factors and evolutionarily conserved signaling pathways.

2.2 miRNAs in terminal differentiation and mature adipocyte function

A preponderance of evidence shows that miRNAs are indispensable for terminal adipocyte differentiation and function. One such study by Mudhasani et al. [29] revealed that miRNAs are globally important for adipogenesis in vitro. This group affected a total knockdown of miRNAs in mouse embryonic fibroblast (MEFs) and primary preadipocytes isolated from the subcutaneous adipose depot. This system involved the use of a conditional Dicer gene that is floxed by loxP sequences; such LoxP-floxed genes are excised from the genome upon adenoviral infection of Cre recombinase [30]. As Dicer is an essential enzyme for miRNA maturation, without it pre-miRNAs cannot be further processed to become functional miRNAs. Homozygous ablation of Dicer in preadipocytes before induction dramatically impaired lipogenesis and downregulated several fold adipocyte markers such as PPARγ, Fas, GLUT4, and FABP4. [29] Furthermore, Mudhasani et al. showed that the observed impairment of adipogenesis was not due to a non-specific repression of cellular proliferation: knockout of the cyclin-dependent kinase 4 inhibitor A (ink4a) locus in Dicer-ablated cells prevented premature cellular senescence but did not rescue an adipogenic phenotype, thus indicating that miRNA knockdown in preadipocytes specifically obstructs adipocyte differentiation. In a more recent study from the same group, Mudhasani et al. generated Dicer<sup>Lox/Lox</sup>: Ap2-Cre transgenic mice (Cre recombinase is expressed under the control of the adipocyte protein 2, also known as Fabp4, promoter) to explore the role of Dicer in vivo [31]. These mice displayed a severe depletion of adipose tissue. This study provides genetic evidence for the physiological importance of miRNAs in regulating adipocyte development. Unfortunately, most of the adipose conditional Dicer knockout mice succumbed between one to three weeks postnatally, preventing further characterization of the roles of miRNAs in adult murine adipose tissue.

To profile the global changes of miRNA expression during adipogenesis, Ortega et al. performed a miRNA expression array study using total RNA extracted from human-derived preadipocytes and adipocytes [32]. This study showed that of 799 miRNAs assayed, the expression of approximately 70 miRNAs (8.8%) was significantly changed between non-induced and induced cells, implying the importance of miRNAs in adipocyte development.

Several individual miRNAs have been implicated in adipogenic processes. For instance, Let7 and miR-17-92 have been shown to regulate the clonal expansion of preadipocytes preceding the acquisition of mature adipocyte morphology [33,34]. Let-7 is upregulated during 3T3-L1 adipogenesis, and its ectopic expression inhibits both the clonal expansion and terminal differentiation of 3T3-L1 preadipocytes [33]. Let-7 targets cyclin-dependent kinase 4 inhibitor A (Hmga-2), which regulates growth and proliferation of other cell types, and when ectopically expressed reduces HMGA-2 protein levels as much as three-fold. Hmga-2 knockout in mice leads to markedly less adiposity [35], while transgenic overexpression of truncated HMGA-2 is conducive to uncontrolled clonal expansion of
preadipocytes characterized by increased adipose mass and higher incidence of lipomas [36]. Thus, Let-7 regulates adipogenesis by negatively regulating the clonal expansion of preadipocytes. The miR-17-92 cluster, which in several cancers promotes cell proliferation, targets tumor suppressor retinoblastoma2 (Rb2/p130) mRNA and accelerates adipogenesis when ectopically expressed in 3T3-L1 cells [34]. The cluster is upregulated during the preadipocyte clonal expansion stage, and siRNA-mediated knockdown of Rb2/p130 at this stage correspondingly accelerates adipocyte differentiation similar to that seen with miR-17-92 overexpression.

Another pertinent study by Yang et al. [37] showed that miR-138 targets EP300 interacting inhibitor of differentiation 1 (Eid-1), which is thought to be involved in coupling re-entry into the cell cycle with transcriptional activation of genes responsible for cell differentiation, and is a negative regulator of adipogenesis that is downregulated upon hormonal induction of adipogenesis in hASCs. Eid-1 binds the retinoblastoma protein and promotes cell cycle re-entry in skeletal muscle [38], so miR-138-mediated repression of Eid-1 impedes the development of growth-arrested preadipocytes. Also, overexpression of miR-138 in hASCs reduced lipid droplet accumulation, inhibited expression of key adipogenic transcription factors C/EBPα and PPARγ2 (one of the two PPARγ isoforms found in humans and mice), and also blocked induction of several other adipogenic markers including FABP4 and lipoprotein lipase (LPL). Knockdown of Eid-1 by RNA interference recapitulated this phenotype [37]. Thus, miR-138 is a negative regulator of adipogenesis at least partially through targeting Eid-1.

miR-27a and miR-27b are negative regulators of adipogenesis, and both have been shown to directly target PPARγ mRNA [39–41]. Both miRNAs are downregulated upon hormonal induction of adipogenesis in vitro. Transfection of either miRNA in 3T3-L1 or OP9 cells, a mouse stromal cell that undergoes adipogenesis after treatment with the same adipogenic stimulants as 3T3-L1 cells, inhibited adipocyte formation as characterized by a blockage of the expression of adipogenic markers, [39]; overexpression of miR-27b in hASCs has similar effects [41]. miR-27a is expressed more abundantly in the stromal vascular fraction of murine adipose tissue than in mature adipocytes [40].

miR-378/378*, two miRNAs transcribed from the same locus, promote lipogenesis [42]. The miR-378/378* locus is contained within the intron of PPARγ-coactivator-1 (PGC-1) β, and both are highly induced during adipogenesis. Overexpression of miR-378/378* in ST2 cells enlarged lipid droplets and increased the incorporation of [14C] acetate into triglycerides. Quantitative RT-PCR analysis revealed that levels of PPARγ and C/EBPs were not appreciably affected by miR-378/378* overexpression; however, there was upregulation of PPARγ2 and lipogenic genes. Oddly, this overexpression appeared to increase the transcriptional activity of C/EBPα and -β on adipogenic target promoters. Also, miRNAs that positively and negatively impact C/EBP activity have been identified. miR-31 directly targets C/EBPα, and levels of this miRNA are downregulated in adipogenesis as assessed by microarray analysis and qRT-PCR in hASCs [43]. In the context of macrophage studies, miR-155 directly targets C/EBPβ [44], miR-143 targets ERK5 and thus accelerates adipogenesis in 3T3-L1 cells [45,46] presumably by preventing the phosphorylation and inactivation of C/EBPβ. Additionally, miR-143 enrichment in mature murine adipose tissue is several fold higher than in 3T3-L1 adipocytes [46]. Also, miR-448 negatively regulates adipogenesis by targeting Kruppel-like factor 5 (Klf5) [47], a transcription factor that is induced by C/EBPβ and -δ and drives adipogenesis by contributing to the induction of PPARγ [48].
There are several miRNAs that have been implicated as regulators of mature adipocyte metabolic functions. A comprehensive study by Kajimoto et al. [49] found 21 miRNAs that are differentially expressed between 3T3-L1 preadipocytes and 3T3-L1 adipocytes 9 days post induction (well after terminal differentiation occurs in vitro). Upregulated miR-NAs include miRs-10b, -15, -26a, -34c, -98, -99a, -101, -101b, -152, -183, -185, and -224; miRs-103, -181a, and -182 were downregulated in the day 9 samples. However, none of these were differentially expressed at 1, 2, or 5 days post induction. Also, antisense inhibition of the upregulated miRNAs at day 3 and day 5 post induction did not detectably impair the upregulation of adipocyte markers or lipid droplet formation associated with adipogenesis. The authors therefore proposed that these miRNAs are somehow involved in mature adipocyte function and not in differentiation.

Indeed, some miRNAs have been implicated in the specialized metabolic functions of mature adipocytes. miR-14 is important in lipid metabolism in *Drosophila* [50]. miR-14 knockout *Drosophila* displayed a roughly two-fold increase in triglyceride content, and diglyceride content was also noticeably increased but to a lesser extent. Consequently, the lipid droplets from the adipocytes of these knockout flies were significantly larger as triglycerides are the major component of adipose lipid droplets. Interestingly, the levels of several other classes of lipids, including free fatty acids (FFAs), cholesterol esters, lysophosphatidylcholine, phosphatidylcholine, sphingomyelin, and total phospholipids were not significantly affected. These data indicate that miR-14 is specifically a dose dependent regulator of triglyceride and diglyceride content in *Drosophila*.

miR-278, which has also been characterized in *Drosophila*, regulates energy homeostasis and insulin sensitivity [51]. miR-278 targets the expanded transcript, and miR-278-knockout flies display a large reduction in total body triglyceride content and fat body mass. Expanded regulates cell growth; knockout of expanded results in tissue overgrowth and overexpression conversely causes a decrease in tissue mass [52]. Intriguingly, miR-278 mutants were insulin-resistant and had higher levels of insulin and circulating sugar mobilized from adipose tissue stores [51]. Although miR-14 and miR-278 have been identified in *Drosophila* as crucial regulators of adipose tissue, their mammalian homologues have yet to be discovered.

Lastly, a recent computational study predicted that miR-103 and -107 human miRNA paralogs participate in regulating several metabolic pathways [53]. In vertebrates, these miRNAs are located in the intronic regions of the pantothenate kinase (PANK) genes, and bioinformatic analysis reveals that they probably act synergistically with PANK in regulating Acetyl CoA and lipid metabolism. Another study showed that miR-103 exhibits nine-fold upregulation in early 3T3-L1 adipogenesis and also accelerates lipid droplet formation when ectopically expressed [46]. The possible role of miR-107 in adipogenesis has yet to be experimentally validated.

### 3. MiRNAs in obesity

Obesity occurs when energy intake exceeds energy expenditure. Excess energy is stored as triglycerides in adipocytes, which in obesity are increased in both cell number (hyperplasia) and cell size (hypertrophy) via increased recruitment of MSCs to the adipocyte lineage and accumulation of larger lipid droplets in mature adipocytes. In addition, excess lipid is demobilized from adipose tissue and deposited in other organs, such as muscle and liver, which in part causes the systemic insulin resistance associated with obesity.

Invasion of adipose depots by macrophages is a key feature of obese adipose tissue that is conducive to its adaption to adverse conditions of such as hypoxia and mechanical strain from hypertrophy. Macrophage infiltration clears dead cells and also exposes adipocytes to
inflammatory cytokines that promote ‘anti-adipogenesis’, or the disassembly of distinct adipocyte morphology and function to relieve the strain of excessive lipid uptake [54]. Macrophage infiltration also promotes the vascularization of adipose tissue [55]. Stimulation of increased angiogenesis by adipose tissue during severe obesity is macroscopically detectable in human visceral and subcutaneous adipose depots via angiography [56].

TNF-α is a prominent inflammatory cytokine secreted by macrophages that acts as a negative adipogenic regulator. It prevents adipogenic differentiation in 3T3-L1 preadipocytes and causes ‘anti-adipogenesis’ in 3T3-L1 adipocytes [54]. Ectopic expression of TNFα simulates the chronic inflammatory environment of obese adipose tissue by blocking the expression of adipogenic genes, notably PPARγ, C/EBPα and FABP4, [57] and inducing the expression of preadipocyte markers [58].

Adipose tissue remodeling during obesity has several pathological consequences. Insulin resistance may be largely a consequence of the chronic inflammatory environment of obese adipose tissue; consequently, several recent studies have proposed to address obesity-induced insulin resistance through anti-inflammatory methods (reviewed in [59]). Also, the demobilization of lipid content from adipose depots leads to hyper-lipidemia and steatosis in other tissues. Recent studies have identified several miRNAs expressed in metabolic organs that could be used as feasible therapeutic targets for obesity and its consequent pathologies. These are summarized in Table 2 and will be discussed in the subsequent sections.

### 3.1 MicroRNAs in obese adipose tissue

A groundbreaking study by Xie et al. [46] revealed that miRNAs implicated in adipogenesis are largely inversely expressed in obese adipose tissue. For instance, miR-422b, 148a, 103, 107, 30c, 30a-5p and 143 are normally upregulated in adipogenesis but are markedly downregulated in adipocytes taken from the diet-induced obesity (DIO) mouse model; conversely, miR-221 and 222 levels normally decrease in adipogenesis but are upregulated in obesity. The study also comprehensively showed that of a set of 79 miRNAs 31 out of 41 that are upregulated in 3T3-L1 adipogenesis and 26 out of 38 that are downregulated displayed expression profiles that were inversely correlated with those of adipocytes taken from ob/ob mice. Consistently, after induction of anti-adipogenesis by ectopic expression of TNF-α in 3T3-L1 cells for 24 h, Xie et al. observed that the aforementioned miRNAs displayed expression patterns resembling those of adipocytes from DIO and ob/ob mice, thus indicating that the dysregulation of adipogenic miRNA expression in obese mice is due to the chronic inflammatory environment of obese adipose tissue.

In another high-throughput study using human subcutaneous adipose tissue samples, Ortega et al. [32] found that approximately 50 of 799 miRNAs tested (6.2%) were appreciably deregulated in samples taken from obese subjects in comparison with samples taken from lean subjects. Of these, the expression levels of 17 were highly correlated with BMI and metabolic parameters. Most of the observed deregulated miRNAs had also been previously identified as being differentially expressed during adipogenesis [32].

In addition to these high-throughput studies, several recent studies provided functional analyses of individual miRNAs in obese adipose tissue. MiR-519d is aberrantly expressed in subcutaneous adipose tissue taken from obese subjects [60]. MiR-519d targets PPARα, which was noted earlier to be an activator of adipogenesis subsidiary to PPARγ. PPARα transcriptionally regulates genes required for maintaining the redox balance of the oxidative catabolism of fatty acids. Essentially, enrichment of miR-519d in adipocytes disrupts fatty acid metabolism and contributes to cellular hypertrophy.
Other miRNAs function as inducers of inflammatory signaling in obese adipose tissue. Strum et al. [60] showed that miR-132 promotes the production of pro-inflammatory secretion factors in human adipose tissue in response to nutritional stress. Using primary human adipose tissue-derived preadipocytes and adipocytes differentiated in vitro, they showed that miR-132 is rapidly induced when cells are switched to serum-free medium, which mimics the nutrient-deficient microenvironment of obese adipose tissue. Cells also displayed an increased secretion of inflammatory factor IL-6 and monocyte chemoattractant protein-1 (MCP-1) under these conditions. MiR-132 was shown to target sirtuin 1 (SirT1) and thereby reduce the deacetylation of p65. This induces NF-κB-mediated activation of IL-6 and MCP-1 expression.

Microarray expression profile studies have identified several miRNAs that are differentially expressed between normoglycemic rats and Goto-Kakizaki (GK) spontaneous type II diabetic rats in insulin target tissues [61–63]. In particular, He et al. [61] demonstrated that miRNA paralogs miR-29a/b/c promote insulin resistance by antagonizing AKT signaling. In addition to being upregulated in diabetic insulin target tissues, overexpression of any of these miRNAs in 3T3-L1 adipocytes repressed insulin-mediated glucose uptake, and miR-29a/b were upregulated in 3T3-L1 adipocytes upon insulin treatment. Similarly, miR-125a is overexpressed in the liver and adipose tissue of GK rats [62]. In silico analysis of probable miR-125a target genes revealed an overrepresentation of genes involved in MAPK signaling and lipid and glucose metabolism. miR-222, -27a, -195, -103, and 10b are also differentially expressed between normoglycemic and GK rats in insulin target tissues and in cultured adipocytes in response to increases in intracellular glucose [63].

3.2 Differential expression of miRNAs among different adipose depots and its implications for obesity

Clinical studies have shown that the risk of obesity-associated disease is specifically correlated with increased adiposity of particular adipose depots. An illuminating study by O’Connell et al. [64] demonstrated that a subset severely obese subjects, deemed ‘metabolically healthy obese’ (MHO), had essentially normal metabolic profiles in comparison with other obese subjects. The MHO subjects had a significantly lower mean omental adipocyte size than other subjects, and yet the size of subcutaneous adipocytes was similar between the groups. While both the size of omental and subcutaneous adipocytes positively correlated with fatty liver, only omental adipocyte size was correlated with the progression of hepatic steatosis to fibrosis and the degree of insulin resistance.

With regard to this, a study by Klöting et al. [65] showed that differential miRNA expression between these depots in humans may contribute to the observed differences in metabolic profiles. Of 106 miRNAs assayed, none were exclusively expressed in human omental or subcutaneous adipose tissue, indicating a common developmental origin of these adipocytes. However, 16 of these miRNAs (most notably miRs -17-5p, -132, -99a, -134, 181a, -145 and -197) exhibited depot-specific expression patterns that correlate with adipocyte size, visceral fat area, fasting glucose level and concentration of adipose-secreted cytokines. Such differential miRNA expression between adipose depots can be exploited therapeutically by identifying and targeting intrinsic differences among adipose depots that contribute the most to pathology during obesity.

In addition to white adipose tissue (WAT), brown adipose tissue (BAT), is also detectable in adult humans [66]. BAT dissipates the stored chemical energy of lipids in a process termed adaptive thermogenesis [66], and brown adipocytes are therefore morphologically very distinct from white adipocytes; they contain several, small lipid droplets instead of one large droplet and significantly more mitochondria for energy-dissipative metabolism [67]. BAT is closer in lineage to skeletal muscle than it is to WAT, and these two cell types arise from a
myogenic factor 5 (Myf5)-positive precursor [68]. While the primary function of adaptive thermogenesis is necessary for warmth in infants and small mammals [69] the process known as diet-induced thermogenesis may occur as a defense against obesity [70]. BAT ablation in mice via transgenic, tissue-specific expression of diphtheria toxin under the control of the promoter of uncoupling protein 1 (UCP1), a highly BAT-enriched mitochondrial proton transporter and the chief facilitator of adaptive energy dissipation, significantly increases the propensity for diet-induced obesity [71]. However, whether BAT has similar anti-obese properties in humans is not yet clear [72].

Revisiting Mudhasani et al.’s study [31] where Dicer was genetically ablated in the adipose tissue of mice, the group found that while WAT depots were depleted no apparent depletion of BAT was observed; however, the brown adipocytes from these mice displayed a decreased expression of genes involved in thermoregulation. Also, Walden et al. [73] have identified several miRNAs that are differentially expressed between WAT and BAT. miR-143, mentioned previously as being highly enriched in murine WAT, [46] is not enriched in murine BAT [73]. Furthermore, three myogenic miRNAs, miRs-1, -133a and -206, are also expressed in both brown preadipocytes and mature adipocytes but not WAT [73], supporting the notion that BAT and skeletal muscle arise from a common precursor. Also, miR-455 expression was specifically upregulated during brown adipogenesis, and its expression pattern correlates with UCP1 [73]. MiR-455 is one of several miRNAs that show over twofold upregulation in skeletal muscle as a consequence of various myopathies [74]; miR-455 may accelerate brown adipogenesis in a fashion similar to miR-17-92’s acceleration of 3T3-L1 adipogenesis [73].

3.3 Relevant miRNAs in other metabolic tissues affected by obesity

The increase in circulating lipid levels associated with obesity-induced demobilization of adipose tissue causes improper lipid storage in other tissues, notably the liver. Non-alcoholic fatty liver disease leads to cirrhosis of liver tissue. miRNAs have been identified as regulators of liver cell development, [75] but also, more pertinently, in processes indicative of fatty liver disease such as steatosis, inflammation and cell apoptosis [76–78].

Cheung et al. [76] determined that of miRNAs differentially expressed between patients with both metabolic syndrome and nonalcoholic steatohepatitis (NASH) and control patients with normal liver histology, the targets of the most deregulated miRNAs affect apoptosis, inflammation, oxidative stress and metabolism. The mRNA and protein levels of several hepatic lipogenic proteins were mostly upregulated in NASH subjects. Notably, miR-122 displayed a dramatic downregulation (> 60%) in NASH subjects and was shown to largely target hepatic lipogenic genes. Also, miR-122 has been implicated in cholesterol biosynthesis [77,78]. Antisense oligonucleotide inhibition of miR-122 in diet-induced obese mice decreased plasma cholesterol levels and enhanced liver steatosis [78].

Another high-throughput study by Whittaker et al. [79] revealed miRNAs that are involved in hepatic lipid droplet formation. In this study human hepatocytes were transiently transfected with 327 individual miRNAs. Cell cultures were fixed, stained for lipid content, and analyzed via automated microscopy for characteristics of lipid droplet formation on a cell-by-cell basis. 11 miRNAs were shown to alter lipid droplet formation, and in particular miR-181d strikingly reduced droplet size by approximately 60%.

Additionally, miR-34a and -205 levels are appreciably increased (over threefold) in murine liver in response to obesity, whereas miR-151, -133a, -329, -201, -330, -17-3p, -298, -328, and -380-5p levels were downregulated [80]. In the same study, profiling analysis of 290 liver samples taken from F2 ob/ob mice revealed 21 miRNAs that displayed significant linkage (LOD > 5.3 and genome-wide p-value < 0.005). Strikingly, approximately 10% of
the miRNAs profiled in the liver across distinct obese mice strains showed heritability and a high copy number ratio in comparison to lean mice.

Hyperlipidemia also causes fatty acid-induced β cell dysfunction, which entails dysregulation of insulin production and secretion and promotes apoptosis. Lovis et al. [81] found that levels of miR-34a and -146 are enriched in the pancreatic islets of db/db diabetic mice. These miRNAs were also upregulated in a time- and dose-dependent manner in MIN6B1 β cells after chronic exposure to palmitate. Upregulation of miR-34a is associated with activation of p53 by targeting bcel leukemia-lymphoma associated gene 2 (BclII), consequent sensitization to apoptosis, and an impaired capacity for nutrient-induced secretion. Transient overexpression of miR-146 does not appreciably influence insulin secretion but contributed to apoptosis. miR-146 negatively regulates NF-kB signaling by targeting IL-1 receptor-associated kinase 1 (Irak1) and TNF receptor-associated factor 6 (Traf6) and thus sensitizes β-cells to apoptosis. Additionally, of the glucose-responsive miRNAs discovered in a screen of MIN6B1 cells, miR-30d has been revealed as a positive regulator insulin transcription [82]; also, miR-124a and miR-96 modulate insulin secretion by targeting proteins involved in insulin exocytosis [83].

Importantly, β cell mass has been shown to vary in adult life in response to metabolic stresses. Such processes are regulated at the transcriptional level and influence anti- or pro-apoptotic signaling cues [84,85]. miR-375 has been implicated as a regulator of β cell mass and proliferation [86]. β cells taken from miR-375-knockout mice display decreased size and an impaired proliferative capacity. Intriguingly, miR-375 was enriched in the pancreatic islets taken from ob/ob mice, and genetic deletion of miR-375 in obese mice caused a severe diabetic response.

Other miRNAs involved in insulin signaling include miR-126, -145 and the miR-183-96-182 cluster [87–89]. miR-126 and -145 target insulin receptor substrate-1 (IRS-1) in breast and colon cancer cells respectively [87,88]. The miR-183-96-182 cluster targets insulin signaling pathway elements IRS-1, RAS p21 protein activator (Rasa1), and growth factor receptor-bound protein 2 (Grb2) as determined by a computational screen of mouse signaling pathways [89]. This demonstrates the novel nature of miRNA action in that members of a single cluster can coordinately target components of a signaling pathway and therefore exert a more significant influence on signal transduction.

4. Conclusion

miRNA microarrays and other high-throughput assays have revealed hundreds of miRNAs that are differentially expressed as a consequence of metabolic tissue development or dysfunction. miRNAs are crucial regulators of adipogenesis and are involved in both the fate determination of pluripotent MSCs and the terminal differentiation of preadipocytes. miRNA expression profiling reveals that miRNA levels are significantly inversely correlated between the adipose tissue of control and obese mouse models. miRNAs are also involved in obesity-induced pathologies in adipose tissue such as disruption of fatty acid metabolism, cellular hypertrophy, inflammation and systemic insulin resistance. miRNAs contribute to liver development and, as a consequence of adipose tissue demobilization by inflammatory processes, are deregulated in hepatic steatosis. Lastly, miRNAs regulate pancreatic β cell development, insulin transcription and secretion and are differentially expressed between normoglycemic and diabetic rats. Additional characterization of pertinent miRNAs in vivo will be highly conducive to the further development of the field and will provide more insight into feasible therapeutic strategies that target miRNAs.
5. Expert opinion

Significant progress has been achieved in understanding the physiological roles of miRNAs in adipocytes and other metabolic cell types and in pathological roles in obesity and metabolic syndrome. However, how miRNAs affect metabolism in vivo, particularly in mammals, remains poorly understood because only a few such animal models are currently available. Fortunately, the Sanger Institute in Cambridge, UK announced that they would generate a library of knockouts of each of the ~500 miRNAs identified in the mouse genome, which will be eventually available to all researchers [90]. An alternative approach employs intravenous injection of antagoniRs, antisense oligonucleotide constructs, to block a specific miRNA or a combination of miRNAs [77]. This approach is still very expensive, but we believe that it will become more affordable to more labs, as RNA technologies develop very quickly.

The dysregulation of miRNAs as a result of hypoxic stress in obese adipose tissue has yet to be studied. Several hypoxia-regulated miRNAs (HRMs) have been studied in carcinogenesis and have been shown to help cells adapt to oxygen-poor microenvironments [91–93]. miR-210 responds to hypoxia by antagonizing pro-apoptotic signaling, decreasing mitochondrial activity, and promoting a transition to glycolysis [91,92]. Also, studies have elucidated the roles of miRNAs involved in angiogenesis (reviewed in [94]). In vivo tissue-specific knockdown of endothelial miRNA in mice via Dicer ablation reduces angiogenic responses to a variety of stimuli including ectopic VEGF expression, tumors, and wound healing [95]. Recent studies have proposed that modulating the angiogenesis of adipose tissue can be used as a therapy for obesity [96]. Because adipose tissue expansion is dependent on angiogenesis, studies have suggested that anti-angiogenic therapeutics may prove effective. Endogenous anti-angiogenic factors, such as angiostatin and endostatin, have been shown to reduce the body of weight of obese mice when ectopically expressed [97]. Perhaps further investigation of hypoxia-regulated miRNAs and angiomiRs in the specific context of obesity can illuminate their roles as potential targets for angiogenesis-targeting therapies.

A huge challenge lies in developing safe and efficient methods of delivering miRNA-targeting therapeutics, which has been pioneered to some extent in cancer and hepatitis C infection. For instance, delivery of miR-26a via an adeno-associated viral vector (AAV) halts tumor progression in hepatocellular carcinoma (HCC) mice [98]. AAV is a burgeoning therapeutic tool that has no apparent pathology in humans [99], and the miR-26a vector proved to be an effective therapeutic in the HCC mice that not only promoted tumor apoptosis in a highly specific manner but also displayed little toxicity [98]. However, such delivery strategies have a long way to go before they are clinically viable. Also, the drug SPC3649 contains a locked nucleic acid (LNA) inhibitor against miR-122 [100], a miRNA that has been shown to aid hepatitis C virus replication in the liver [101]. SPC3649 is currently undergoing Phase I clinical trials [100].

In addition to using miRNAs as potential therapeutic targets in metabolic disease, recent findings have shown that circulating miRNAs can be used as novel prognosis tools. Zampetaki et al. [102] found that plasma expression levels of miRs-20b, -21, -24, -15a, -126, -191, -197, -223, -320 and -486 were lower in a diabetic cohort when compared with normal subjects and that miR-28-3p was moderately upregulated. Significantly, changes in the expression of several of these miRNAs preceded the development of disease, and most of the expression patterns observed in the diabetic cohort were observed in serum taken from the leptin-deficient obese mouse model when compared with normoglycemic mice.
Another inevitable issue that needs to be addressed is how to integrate the effects of multiple miRNAs that are dysregulated in the disease state. Each miRNA targets multiple mRNAs, which may coordinate or antagonize each other’s functions. In addition, miRNA-target interactions depend not only on the sequence of the target site but also on the cellular context in which the interactions occur. In light of such complexity, sophisticated bioinformatic methods must be used to determine the combined effects of multiple altered miRNAs in a tissue specific manner, and a recent study by Gallagher et al. [103] bridged this gap. To investigate the roles of miRNAs in muscle insulin resistance in type 2 diabetes, they compared the gene expression profiles of both mRNAs and miRNAs between 71 patients versus 47 controls. The tissue-specific gene and miRNA expression data were combined with miRNA target site prediction data from the TargetScan database (available at: http://www.targetscan.org/) to generate a weighted cumulative context score (wCCS) for miRNA-target interactions that accounts for physiological context. Gene ontology analysis showed that the genes most strongly targeted by the collective net changes in miRNA expression are enriched for six canonical pathways, five of which are related to insulin resistance or muscle metabolism. Such a ranking system that takes into account miRNA and mRNA activity and cellular context can be a useful tool for future studies.

The broad-spectrum and redundancy of miRNA–target interactions seems to pose a great difficulty in developing miRNA therapeutics, and the modulation of a single micro-RNA changes the expression of hundreds of genes. However, if dysregulation of a microRNA significantly contributes to a disease in which all miRNA targets are consequently dysregulated, restoring the normal expression of that miRNA would be the best method, if not the only method, of therapy.

Taken together, these innovations illustrate that while the development of miRNA-based therapeutics is in its infancy, there is an inherent potential to the field because of well-characterized miRNA overexpression and inhibition methods and the potency of miRNAs as genetic regulators. miRNAs can be used as prognosis markers for disease progression and in the case of metabolic syndrome can be used synergistically with other treatments (such as anti-inflammatory and anti-angiogenic therapies) to mitigate obesity and its consequent pathologies. The development of miRNA-targeting therapeutics for metabolic syndrome is warranted not only because of innovations mentioned in this review but also because of the central problem that metabolic disease poses to society.

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Article highlights

- MiRNAs are key and extensive regulators of mesenchymal stem cell lineage determination and regulate both lineage-specific transcriptional networks and evolutionary conserved signaling pathways.

- MiRNAs are globally important for terminal adipocyte differentiation and mature adipocyte function.

- MiRNAs are largely dysregulated in obesity as a result of the chronic inflammatory environment of obese adipose tissue.

- Different adipose depots display distinct miRNA expression patterns, which may have implications for the different metabolic properties of these depots during obesity.

- Several miRNAs are dysregulated in other metabolic tissues during obesity-related diseases such as fatty liver, pancreatic β cell dysfunction, and systemic insulin resistance.

This box summarizes key points contained in the article.
Table 1

MicroRNAs involved in adipogenesis and mature adipocyte function.

| MiRNAs                        | System       | Direct targets | Function                                                                 | Ref.     |
|-------------------------------|--------------|----------------|--------------------------------------------------------------------------|----------|
| miR-204, -211                 | C3H10T1/2, BMSC | Runx2          | Pro-adipogenic; blocks osteogenesis in MSCs                               | [24]     |
| miR-8, -210                   | ST2, 3T3-L1  | wntless, CG32767, Tcf712 | Pro-adipogenic; antagonize WNT signaling                                   | [25,26] |
| miR-21                        | hASC         | Tgfbr2         | Pro-adipogenic; antagonizes TGF-β signaling                                | [16]     |
| miR-199a                      | C3H10T1/2, ATDC5 | Smad1         | Pro-adipogenic; blocks chondrogenesis                                      | [28]     |
| Let7, miR-17-92               | 3T3-L1       | Hmga-2, Rb2/p130 | Regulate the clonal expansion of preadipocytes                           | [33,34] |
| miR-138                       | hASC         | Eif-1          | Anti-adipogenic; conducive to growth arrest                               | [37]     |
| miR-27a/b                     | 3T3-L1, OP9, hASC | Pparγ         | Anti-adipogenic                                                           | [39–41] |
| miR-378/378*                  | ST2          | –              | Pro-adipogenic; promotes lipogenesis                                       | [42]     |
| miR-31                        | hASC         | C/ebpα         | Anti-adipogenic                                                           | [43]     |
| miR-155                       | Macrophage   | C/ebpβ         | Not verified in adipogenesis                                             | [44]     |
| miR-143                       | 3T3-L1       | Erk5           | Pro-adipogenic; highly enriched in murine adipose tissue in vivo          | [45,46] |
| miR-448                       | 3T3-L1       | Klf5           | Pro-adipogenic                                                           | [47]     |
| miRs-10b, -15, -26a, -34c, -98, -99a, -101, -101b, -152, -183, -185, -224 | 3T3-L1 | –             | Up-regulated after terminal adipogenesis; likely involved in mature adipocyte function | [49]     |
| miRs-103, -181a, -182         | 3T3-L1       | –              | Down-regulated after terminal adipogenesis; likely involved in mature adipocyte function | [49]     |
| miR-14                        | Drosophila   | –              | Dose-dependent regulator of intracellular triglyceride and diglyceride content | [50]     |
| miR-278                       | Drosophila expanded | –            | Involved in insulin sensitivity and energy homeostasis                    | [51]     |
| miRs-103, -107                | In silico, 3T3-L1 | –             | Predicted to regulate acetyl coA and lipid metabolism. MiR-103 experimentally confirmed as pro-adipogenic | [46,53] |

3T3-L1: Murine preadipocyte cell line; ATDC5: Murine prechondrogenic cell line; BMSC, ST2, and OP9: Murine bone marrow stromal cell lines; C3H10T1/2: Murine mesenchymal stem cell line; hASC: Human adipose-derived stem cells.
### Table 2

Relevant miRNAs in obesity and its related diseases.

| MiRNAs                      | Tissue               | Direct targets | Function/dysregulation                                                                 | Ref. |
|-----------------------------|----------------------|----------------|---------------------------------------------------------------------------------------|------|
| miRs-422b, -148a, -103, -107, -30c, -143, -30u-5p, -221, -222 | Adipose              | –              | Display inverse expression patterns during obesity and analogously after TNFα treatment in 3T3-L1 cells | [46] |
| miR-519d                    | Adipose              | Pparα          | Overexpression disrupts fatty acid metabolism; promotes cellular hypertrophy          | [59] |
| miR-132                     | Adipose              | Sirt1          | Promotes production of inflammatory cytokines in response to nutritional stress       | [60] |
| miR-29α/b/c                 | Adipose              | –              | Contribute to insulin resistance by antagonizing AKT signaling                         | [61] |
| miR-125a                    | Adipose, Liver       | –              | Predicted to target genes involved in MAPK signaling and lipid and glucose metabolism; enriched in diabetic mice | [62] |
| miRs-222, -27a, -195, -103, -10b | Adipose, Liver, Muscle | –              | Differentially expressed between normoglycemic and diabetic rats                      | [63] |
| miRs-17-5p, -132, -99a, -134, -181α, -145, -197 | Adipose            | –              | Differentially expressed between subcutaneous and omental depots in humans           | [65] |
| miRs-1, -133α, -206         | Brown adipose        | –              | Myogenic miRNAs that are expressed in brown but not white preadipocytes and adipocytes | [73] |
| miR-455                     | Brown adipose        | –              | Up-regulated in brown adipogenesis                                                   | [73] |
| miR-122                     | Liver (see [77])     | –              | Targets hepatic lipogenic genes. Downregulated in fatty liver disease. Involved in cholesterol biosynthesis | [76–78] |
| miR-181d                    | Liver                | –              | Negatively regulates hepatic lipid droplet formation                                 | [79] |
| miR-205, -151, -34α, -133a, -329, -201, -330, -17-3p, -298, -328, -380-5p | Liver              | –              | Dysregulated in response to obesity                                                   | [80] |
| miR-34a                     | Pancreas             | BclII, Vamp2   | Promotes apoptosis in response to fatty acid-induced β cell dysfunction; impairs insulin secretion | [81] |
| miR-146                     | Pancreas             | Irak1, Traf6   | Promotes apoptosis in response to fatty acid-induced β cell dysfunction              | [81] |
| miR-30d                     | Pancreas             | –              | Positive regulator of insulin transcriptional activation                             | [82] |
| miR-124α, -96               | Pancreas             | Rab27A         | Negatively regulate insulin exocytosis                                              | [83] |
| miR-375                     | Pancreas             | (see [86])     | Positive regulator of increased size and proliferative capacity of β cells           | [86] |
| miR-183-96-182              | In silico            | Irs-1, Rasα1, Grb2 | Negatively regulates insulin signaling                                              | [89] |
| miRs-20b, -21, -24, -15α, -126, -191, -197, -223, -320, -486, -28-3p | In circulation | –              | Differentially expressed between normoglycemic and diabetic cohorts. Feasible prognosis markers | [102] |