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MicroRNAs Induced During Adipogenesis that Accelerate Fat Cell Development Are Downregulated in Obesity
Huangming Xie,1,2,3 Bing Lim,2,3,4 and Harvey F. Lodish1,2,5

OBJECTIVE—We investigated the regulation and involvement of microRNAs (miRNAs) in fat cell development and obesity.

RESEARCH DESIGN AND METHODS—Using miRNA microarrays, we profiled the expression of >370 miRNAs during adipogenesis of preadipocyte 3T3-L1 cells and adipocytes from leptin deficient ob/ob and diet-induced obese mice. Changes in key miRNAs were validated by RT-PCR. We further assessed the contribution of the chronic inflammatory environment in obese adipose tissue to the dysregulated miRNA expression by tumor necrosis factor (TNF-α) treatment of adipocytes. We functionally characterized two adipocyte-enriched miRNAs, miR-103 and miR-143, by a gain-of-function approach.

RESULTS—Similar miRNAs were differentially regulated during in vitro and in vivo adipogenesis. Importantly, miRNAs that were induced during adipogenesis were downregulated in adipocytes from both types of obese mice and vice versa. These changes are likely associated with the chronic inflammatory environment, since they were mimicked by TNF-α treatment of differentiated adipocytes. Ectopic expression of miR-103 or miR-143 in preadipocytes accelerated adipogenesis, as measured both by the upregulation of many adipogenesis markers and by an increase in triglyceride accumulation at an early stage of adipogenesis.

CONCLUSIONS—Our results provide the first experimental evidence for miR-103 function in adipose biology. 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. Diabetes 58:1050–1057, 2009

Adipose tissue is not only a storage depot of triglycerides, but it is also an endocrine organ and an important regulator of whole-body energy homeostasis (1–3). Abnormal fat accumulation in obesity increases risk of life-threatening diseases such as type 2 diabetes, atherosclerosis, and certain types of cancer (4,5). Fundamental for the development of novel therapeutics for obesity and its associated metabolic syndromes is an understanding of the regulation of adipogenesis, which is tightly controlled by a combination of multiple transcription factors and extracellular hormones such as insulin (6–8).

Potential regulators of adipogenesis include microRNAs (miRNAs), which encode an abundant class of ~22 nucleotide evolutionarily conserved RNAs that control gene expression at the posttranscriptional level by targeting miRNAs for degradation or translational repression or both (9–11). Computational and experimental analyses suggest that miRNAs may regulate expression of ~30% of human and mouse genes (12). Furthermore, miRNAs are attractive candidates for regulating cell fate decisions and 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 differentiated or pathogenic cell states.

Although miRNA expression profiles and functions have been extensively investigated in the hematopoietic system and neuronal and muscle tissues (13–15), little is known about the role of miRNAs in metabolic tissues, particularly adipose tissue (16). Of particular relevance, miR-14 and miR-278 in the fat body of flies regulate lipid metabolism (17,18), miR-122 in mouse liver controls triglyceride metabolism and cholesterol biosynthesis (19,20), and experiments using antisense oligonucleotides transfected into cultured human preadipocytes suggested that miR-143 is involved in adipocyte differentiation (21). Using Northern blot analyses, Kajimoto et al. (22) profiled ~100 miRNAs including three novel miRNAs in 3T3-L1 cells before and after differentiation, and Gu et al. (23) cloned 45 known and 2 novel miRNAs from bovine adipose tissue. Moreover, as based on computational analysis of miRNA target sites in their 3’ untranslated region (UTR) sequences, 71% (282 out of 395) of expressed sequence tags with unique 3’UTRs differentially expressed during 3T3-L1 differentiation are potentially regulated by miRNAs (24).

However, few adipocyte miRNAs have been analyzed, in part because of the low sensitivity and coverage of cloning and Northern blot analyses. Except for miR-143, none of the candidate adipocyte–important miRNAs overlapped in the studies of Esau et al. (21) and Kajimoto et al. (22). Previous studies also used unfractionated primary adipose tissue, which consists of a heterogeneous mixture of cell types (23,25–28). And except for knocking down expression of miR-143, there have been no functional characterizations of adipocyte miRNAs. Additionally, there has been no systematic comparison of miRNA expression levels in normal and obese states, despite the fact that many adipose-important miRNAs are dysregulated in adipocytes from obese animals and humans (25–28).
RESEARCH DESIGN AND METHODS

Cell culture, differentiation, and chemical treatments. 3T3-L1 cells (American Type Culture Collection) were cultured and differentiated as described earlier (29). For tumor necrosis factor (TNF)-α treatment, 1 µmol/l human TNF-α (PeproTech) was added to the growth medium on day 8 after induction of differentiation and incubated for 24 h as described previously (30).

Mouse studies. Male C57BL/6J and B6.V-CD3/3 (ob/ob) mice (Jackson Laboratory) were maintained at the animal facility of the Whitehead Institute for Biomedical Research. All animal experiments were performed with the approval of the Massachusetts Institute of Technology Committee on Animal Care. For the ob/ob model, all mice were provided with a normal diet. Epididymal fat pads were harvested from mice at 16–17 weeks of age. For the diet-induced obesity (DIO) model, we randomized 10 6-week-old male C57BL/6J littermates to either normal diet or high-fat diet (50% fat, Teklad). After 3 months, epididymal fat pads were harvested. Fat tissues from two to four mice of the same group were pooled together as one biological sample for microarray or RT-PCR experiments. Primary adipocytes were obtained after collagenase/dispase digestion, and en-...
accumulation, the same miRNA microarrays were used to compare miRNA levels in normal adipocytes with those of obese mice. We first used leptin-deficient \( \text{ob} / \text{ob} \) mice; at 4 weeks of age, \( \text{ob} / \text{ob} \) mice are obese and exhibit hyperglycemia, glucose intolerance, and insulin resistance, phenotypes that resemble the pathophysiology of human type 2 diabetes (33). A total of 71 miRNAs were expressed at significantly different levels (Fig. 2A, \( P < 0.01 \), Student’s \( t \) test). Twelve regulated miRNAs, including 9 downregulated and 3 upregulated in \( \text{ob} / \text{ob} \) mice, were validated by RT-PCR assays (Fig. 2B).

We also used DIO mice as a model of obesity. After being fed with a 55% high-fat diet for 3 months (from weeks 6 to 18), DIO mice became significantly heavier than normal mice and had almost twice the mass of epididymal fat compared with littermate controls (Fig. 3A and B, \( P < 0.01 \), Student’s \( t \) test). Twelve regulated miRNAs, including 9 downregulated and 3 upregulated in \( \text{ob} / \text{ob} \) mice, were validated by RT-PCR assays (Fig. 2B).

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miRNA expression profiling in adipocytes from control and DIO mice. A: Weight of control and DIO mice (n = 5). DIO mice were fed with a 55% high-fat diet starting from week 6 to week 18. Controls were fed with a normal diet. Data are expressed as means ± SE. **P < 0.01. B: Weight of epididymal fat pads from control and DIO mice (n = 5) at week 18. ***P < 0.001. C: Intensity scatter plot showing comparison of miRNA profiles in adipocytes from control and DIO mice. Mirtron miR-1224, Novel_miR#5 (novel miRNA cloned from bovine adipose tissue described in the text), and some miRNAs used in our detailed analyses are highlighted in red and indicated by a label. D: Scatter plot showing the positive correlation of miRNA regulation in two disease models for obesity: ob/ob and DIO mice. A total of 78 miRNAs, whose levels are above the mean expression of all miRNAs in at least one of the four samples (enriched adipocytes from wild-type [WT], ob/ob, control, and DIO mice), are shown. Fold-changes are based on array results and plotted in a log2 scale. Some miRNAs used in our detailed analyses are highlighted in red and indicated by a label. Pearson’s correlation coefficient: r = 0.51, P < 0.0001.

FIG. 3. miRNA expression profiling in adipocytes from control and DIO mice. A: Weight of control and DIO mice (n = 5). DIO mice were fed with a 55% high-fat diet starting from week 6 to week 18. Controls were fed with a normal diet. Data are expressed as means ± SE. **P < 0.01. B: Weight of epididymal fat pads from control and DIO mice (n = 5) at week 18. ***P < 0.001. C: Intensity scatter plot showing comparison of miRNA profiles in adipocytes from control and DIO mice. Mirtron miR-1224, Novel_miR#5 (novel miRNA cloned from bovine adipose tissue described in the text), and some miRNAs used in our detailed analyses are highlighted in red and indicated by a label. D: Scatter plot showing the positive correlation of miRNA regulation in two disease models for obesity: ob/ob and DIO mice. A total of 78 miRNAs, whose levels are above the mean expression of all miRNAs in at least one of the four samples (enriched adipocytes from wild-type [WT], ob/ob, control, and DIO mice), are shown. Fold-changes are based on array results and plotted in a log2 scale. Some miRNAs used in our detailed analyses are highlighted in red and indicated by a label. Pearson’s correlation coefficient: r = 0.51, P < 0.0001.

miRNA regulation by TNF-α in adipocytes from obese mice. Chronic inflammation by macrophages is a principal feature of obese adipose tissue (33,34–36); TNF-α is a major macrophage-produced cytokine involved in chronic inflammation and is largely responsible for inducing insulin resistance in obese adipose tissue (37). Previously, we reported that in 3T3-L1 adipocytes, TNF-α treatment suppressed expression of many adipocyte-specific genes and reactivated expression of preadipocyte genes, inducing insulin resistance (30). Figure 5 shows, using quantitative RT-PCR assays, that treatment of differentiated 3T3-L1 adipocytes for 24 h with TNF-α reduced the expression of the same miRNAs, including miR-103 and miR-143, which were downregulated in adipose tissue from ob/ob mice. Conversely, TNF-α induced the expression of three
miRNAs were downregulated (Ob_Down). 26 miRNAs were upregulated during obesity (Ob_Up), whereas 12 were downregulated during differentiation (Diff_Down), among which were 10 miRNAs were upregulated (Ob_Up). A total of 38 miRNAs which 31 miRNAs were downregulated during obesity (Ob_Down), miRNAs were upregulated during differentiation (Diff_Up), among

A

FIG. 4. Inverse correlation of miRNA expression during adipogenesis and obesity. A: Scatter plot showing the inverse correlation of miRNA regulation during adipogenesis and obesity. A total of 79 miRNAs, whose levels are above the mean expression of all miRNAs in at least one of the four samples (undifferentiated 3T3-L1 preadipocytes [day 0], differentiated 3T3-L1 adipocytes [day 9], and adipocytes from wild-type [WT] and ob/ob mice) are shown. Fold-change is based on array result and plotted in a log2 scale. The 12 miRNAs that are the focus of our detailed analyses are highlighted in red and indicated by a label. Pearson’s correlation coefficient: \( r = -0.51 \), \( P < 0.0001 \). B: Fisher’s exact test for 79 miRNAs is shown in Fig. 3A. A total of 41 miRNAs were upregulated during differentiation (Diff_Up), among which 31 miRNAs were downregulated during obesity (Ob_Down), whereas 10 miRNAs were upregulated (Ob_Up). A total of 38 miRNAs were upregulated during differentiation (Diff_Up), whereas 26 miRNAs were downregulated (Ob_Down). \( P = 0.0001 \) by Fisher’s exact test.

miR-103 and miR-143 accelerate adipogenesis when expressed ectopically. To determine whether the changes in miRNA expression we observed in adipocytes from both ob/ob and DIO mice likely were caused by the enhanced expression of TNF-α seen in obese adipose tissue.

miR-103 and miR-143 accelerate adipogenesis when expressed ectopically. To determine whether the changes in miRNA expression we observed might affect adipogenesis, we focused on two miRNAs, miR-103 and miR-143, which were upregulated during adipogenesis and downregulated during obesity. Both miRNAs are highly conserved and are abundant in adipocytes. The level of mature miR-143 increased slightly during 3T3-L1 adipogenesis, but the rise is much more dramatic in vivo (Figs. 6A and 1C). Expression of miR-103 was induced approximately ninefold during adipogenesis, with the increase occurring mainly after day 2 (Fig. 6A).

To evaluate the effects of these miRNAs on preadipocyte growth and differentiation, we used a retroviral vector to stably express them in 3T3-L1 preadipocytes. GFP was used as a reporter so that infected 3T3-L1 cells could be easily selected by fluorescence-activated cell sorting. We obtained four independent batches of miRNA overexpressing cells. GFP+ cells expressed three- to fourfold higher levels of mature miRNA than control preadipocytes, which expressed the empty vector alone (Fig. 6B). Thus, miR-103 and miR-143 were ectopically expressed at physiological levels.

FIG. 5. miRNAs differentially expressed in adipocytes from ob/ob mice compared with wild-type (WT) mice are regulated similarly by TNF-α treatment of 3T3-L1 differentiated adipocytes for 24 h. miRNA levels are measured by RT-PCR, normalized to internal control and plotted as fold-change in log2 scale. x-axis, ob/ob vs. WT, same data as Fig. 2B; y-axis, differentiated adipocyte after TNF-α treatment for 24 h versus untreated control \( (n = 4) \). Pearson’s correlation coefficient: \( r = 0.90 \), \( P < 0.0001 \).

Figure 6C shows that ectopic expression of miR-103 or miR-143 has little effect on 3T3-L1 cell growth. Without adipogenic stimuli, there were no evident morphological changes in 3T3-L1 cells ectopically expressing these miRNAs (data not shown). However, ectopic expression of miR-103 or miR-143 did accelerate the rate of 3T3-L1 differentiation, as measured by triglyceride accumulation (Fig. 6D) and by the upregulation of many adipocyte-important genes at early stages of adipogenesis (Fig. 6E). Figure 6D shows that cells ectopically expressing either miR-103 or miR-143 accumulated twice the normal level of triglyceride at day 4 of differentiation but a normal level of triglyceride at day 7.

The accelerated differentiation was accompanied by increased expression of key transcription factors such as PPARγ2, key cell cycle regulators such as G0/G1 switch 2 (G0s2), and molecules associated with lipid metabolism (FABP4), glucose homeostasis (GLUT4), and endocrine functions (adiponectin) of adipocytes. Quantitative RT-PCR showed that ectopic expression of miR-103 or miR-143 caused little change in the low level of expression of these genes before the onset of differentiation (day 0). Importantly, ectopic expression of miR-103 or miR-143 did hasten the expression of these pro-adipogenic factors at early stages of adipogenesis (Fig. 6E and F). At day 2, for example, expression of PPARγ2 was doubled by ectopic expression of either
miR-103 or miR-143 hastens expression of miR-103 or miR-143. miR-103 or miR-143. miR-103 increased the expression level of FABP4 and adiponectin approximately ninefold and fourfold, respectively (Fig. 6F). Gene expression profiling by microarrays (data not shown) also suggested a significant enrichment of other proteins known to be important during adipogenesis or in lipid metabolism pathways in day 2 cells, in which miR-103 or miR-143 was ectopically expressed compared with control cells at day 2. Notably, neither miR-103 nor miR-143 modulated early adipogenic markers such as LPL at day 0 (Fig. 6F and data not shown). Levels of miR-103 and miR-143 are normally induced early in differentiation, and they likely downregulate expression of unknown mRNAs whose encoded protein(s) normally slow the process of adipogenesis.

**DISCUSSION**

In this study, we have generated a comprehensive database of the expression patterns of over 370 known mouse mature miRNAs during normal fat cell development and, for the first time, in adipocytes from normal and obese mice. Similar changes in miRNA expression occur during in vitro and in vivo adipogenesis. Many of these differentially regulated miRNAs are likely to be important in adipocyte biology, as we showed that ectopic expression of two normally upregulated miRNAs, miR-103 or miR-143, in preadipocytes accelerates the rate of fat cell formation. Importantly, miRNAs that were induced during adipogenesis were decreased in adipocytes from both types of obese mice and vice versa. We suggest that these changes are linked to the chronic local inflammation environment and enhanced TNF-α levels in obese adipose tissue, since similar changes in the pattern of miRNA expression occurred after TNF-α treatment of differentiated adipocytes. Our work provides an important first step toward construction of the entire RNA regulatory network underlying fat cell development and adipose dysfunction in obesity. An understanding of the role of miRNAs in adipocyte biology may lead to novel RNA-based therapies that complement current anti-obesity treatments.

**Regulated expression of functionally important miRNAs.**

Our genome-wide miRNA profiling study identified many differentially regulated miRNAs, including miR-103 and miR-143. miR-143 is likely to be important in adipocyte biology, since transfection of antisense miR-143 oligonucleotides in cultured human preadipocytes inhibits all five adipocyte differentiation markers by at least 40% (21). Our experiments ectopically expressing miR-143 resulted in the opposite effect, upregulation of adipogenesis markers, thus confirming its important role in modulating adipogenesis.

Wang et al. (38) reported that the miR-17-92 cluster is upregulated twofold only during the early clonal expansion stage of adipogenesis and that these miRNAs accelerate adipocyte differentiation by negatively regulating p130. Because we focused only on miRNAs upregulated during adipocyte differentiation per se, our microarray analyses did not identify the miR-17-92 cluster. miR-103 or miR-143 effect on 3T3-L1 cell growth by MTT assay. Data are expressed as mean ± SE (n = 6). D: Ectopic expression of miR-103 or miR-143 increases triglyceride accumulation at day 4 (D4) but not day 7 (D7). Triglycerides are normalized to total protein and plotted relative to their respective levels in preadipocyte (day 0). Data are expressed as mean ± SE (n = 3). E: Expression level of miR-103 or miR-143 in infected and sorted cells compared with vector control. Data are expressed as mean ± SE (n = 3). C: Ectopic expression of miR-103 or miR-143 has little effect on 3T3-L1 cell growth by MTT assay. Data are expressed as mean ± SE (n = 6).
cluster as significantly upregulated. We did not focus on miRNA changes during the clonal expansion phase in part because there are no primary cell samples corresponding to this stage. In addition, according to our microarray data, the miR-17-92 cluster is not abundantly expressed in differentiated adipocytes.

Importantly, we also studied purified primary adipocytes, which are more homogeneous than the unfractonated primary adipose tissue used in an earlier study (23); this enabled us to identify adipocyte-enriched miRNAs. Comparing the 44 miRNAs most highly expressed in epididymal adipocytes from normal mice with the 29 miRNAs most highly expressed in chondrocytes and osteoblasts (39), expression of 18 miRNAs, including let-7, miR-23, miR-26, and miR-30, are common to all three lineages. This suggests that they are functionally important for differentiation of all three cell types (adipocytes, chondrocytes, and osteoblasts) that are formed from mesenchymal stem cells (40). In contrast, miRNAs preferentially enriched in one particular mesenchymal lineage may have roles in cell fate determination and lineage differentiation of mesenchymal stem cells or be required for the function of these differentiated cells. As an example, miR-140 is enriched in osteoblasts and chondrocytes, and miR-140 was reported previously to be important for bone development by downregulating histone deacetylase 4 (41).

**Downstream effectors and targets for miR-103 and miR-143.** 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 metabolism (42). Supplementary Tables S1 and S2 suggest many potentially important miRNA targets for miR-103 and miR-143, including several anti-adipogenic factors such as ARNT, FZD1, and RUNX1T1/ETO/MTG8, whose levels are normally downregulated during adipogenesis. These genes were selected based on meeting all of the following criteria: 1) expressed in 3T3-L1 cells at day 0 or day 2, 2) a lower mRNA expression level in day 2 cells in which miRNA is ectopically expressed compared with control cells at day 2, and 3) predicted targets based on TargetScan v4.12 (12,43).

Some targets not listed here may have decreased protein levels but unchanged mRNA levels; some may be predicted by computational softwares other than Targetscan. Importantly, miRNAs likely target many mRNAs (10,11), and defining one major target of either mRNA will be difficult and likely impossible. For example, many miR-143 targets shown in supplementary Table S2 are equally if not more interesting than MAPK7/ERK5, which was previously suggested as an miR-143 target (21). Validation of potential targets by luciferase reporter assays and Western blots is ongoing but beyond the scope of this article.

**Functionally important adipocyte miRNAs are down-regulated in obesity, likely because of macrophage infiltration and TNF-α.** Most strikingly, several miRNAs, including miR-103 and miR-143, exhibit inverse patterns of regulation during adipogenesis compared with those during obesity (Fig. 4A), indicating that obesity leads to a loss of miRNAs that characterize fully differentiated and metabolically active adipocytes. Earlier studies reported that many adipogenic genes whose expression increases during adipogenesis, displayed markedly decreased expression in adipocytes from epididymal fat pads of ob/ob and DIO mice (25–27).

In general, we observed a greater extent of miRNA dysregulation in adipocytes from ob/ob compared with DIO mice. In parallel, several groups reported a greater change of altered mRNA expression in adipocytes from ob/ob mice than DIO mice (25–27). This is likely because ob/ob mice have increased adiposity compared with DIO mice of the same age (data not shown). Alternatively, 3 months of a high-fat diet might not be long enough to induce the same changes as seen in adipocytes from ob/ob mice. Nevertheless, the positive correlation of miRNA regulation we observed in these two different models of obesity (Fig. 3D) suggests that the changes in the adipocyte miRNA expression profile in ob/ob mice cannot result from leptin deficiency alone.

More likely these changes are associated with the chronic inflammatory environment in obese adipose tissue. Increased expression of inflammation-related genes was found in isolated abdominal subcutaneous adipocytes and cultured stromal vascular cells from obese Pima Indians compared with nonobese control subjects (44,45). We hypothesize that one or more miRNAs upregulated upon TNF-α treatment of adipocytes are involved in destabilizing PPARD or CCAAT/enhancer-binding protein α or another mRNA encoding an adipocyte-important transcription factor.

Ruan et al. (30) reported that TNF-α suppressed adipocyte-specific genes and activated expression of preadipocyte genes in 3T3-L1 cells. In parallel, we showed here that, in 3T3-L1 adipocytes, TNF-α repressed miRNAs that are normally upregulated during adipogenesis. Thus, elevated levels of TNF-α in obese adipose tissue are likely the cause of the changes in miRNA levels we observed in obesity. Our results (Fig. 5) also suggested that obesity had a much stronger impact on the expression of these miRNAs than did exposure of 3T3-L1 cells to TNF-α. These differences are probably due to the relatively long half-life of miRNAs and some delay in transcription in response to TNF-α treatment. Additionally, the chronic inflammatory state of obese adipose tissue likely depends on other inflammatory cytokines such as IL-6, leading to impaired adipose tissue function.

**miRNAs are highly connected nodes in regulatory networks underlying adipogenesis and adipose dysfunction in obesity.** Only a small fraction of known miRNAs is significantly regulated during adipogenesis (Fig. 1A). However, each miRNA is thought to regulate, on average, about 200 target genes and has widespread impact on protein output (10,11). Multiple miRNAs can act additively or synergistically at multiple target sites on a single mRNA (46). The potential interaction networks connecting miRNAs and mRNAs are enormous and can be further expanded by feedback or feed-forward loops (47). Whereas miR-103 and miR-143 have been suggested to be important for adipogenesis, little is known about the specific mRNA targets of these miRNAs. Their effects on adipogenesis could be achieved by significant downregulation of one or two “primary” target mRNAs or by more modest downregulation of perhaps hundreds of preadipocyte-important miRNAs. Clearly, we need to identify biologically relevant targets for these key miRNAs. In the reconstructed regulatory network, each miRNA will likely link to multiple target genes and serve as a controlling point and potential target for therapeutic intervention.

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