MicroRNAs Induced During Adipogenesis that Accelerate Fat Cell Development Are Downregulated in Obesity

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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 mRNAs 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-275 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 Essau et al. (21) and Kajimoto et al. (22). Previous studies also used unfractionated primary adipocyte 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 adipocyte-important miRNAs are dysregulated in adipocytes from obese animals and humans (25–28).

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RESULTS

**miRNA expression during in vitro adipogenesis of 3T3-L1 preadipocytes.** We profiled the expression of miRNAs during 3T3-L1 cell adipogenesis using miRNA microarrays detecting 373 mouse mature miRNAs (miRBase v9.0). Data from two biological replicates were consistent (data not shown). Most miRNAs fell along the diagonal line in the intensity scatter plot, indicating that they were not regulated during the 9 days of adipogenesis (Fig. 1A). Based on criteria described in RESEARCH DESIGN AND METHODS, 28 miRNAs were expressed at significantly different levels between undifferentiated 3T3-L1 preadipocytes (day 0) and differentiated 3T3-L1 adipocytes (day 9) (Fig. 1A, P < 0.01, Student’s t test). Expression profiles of miRNA isolated during 3T3-L1 differentiation indicated that major changes occurred as early as days 2–4 (data not shown). Of the miRNAs differentially expressed, the eight miRNAs significantly upregulated during differentiation, namely miR-422b, 148a, 107, 103, 30c, 30a-5p, 146b, and 143, and the four downregulated miRNAs, namely miR-125b, 99b, 222, and 221, were selected for validation by quantitative RT-PCR assays using samples derived from an independent set of experiments. The results confirmed our findings from the microarrays (Fig. 1B).

**miRNA expression during primary fat cell development.** To further examine the expression profiles during in vivo fat cell development, enriched epididymal adipocytes were purified by flotation after collagenase/dispase digestion and dissociation; enriched preadipocytes (CD11b+CD90+CD31−) isolated in the stromal vascular fraction were depleted of fibroblasts, endothelial cells, macrophages, and erythrocytes (supplementary Fig. S1). Differentiation of these enriched preadipocytes showed enhanced formation and accumulation of lipid droplets compared with the total population of cells in the stromal vascular fraction (data not shown). Expression levels of the 12 same selected miRNAs shown in Fig. 1B were measured by RT-PCR in RNAs from enriched preadipocytes and mature primary adipocytes. As shown in Fig. 1C, 9 of 12 miRNAs exhibited a similar regulation pattern compared with in vitro differentiation. Noteworthy is the fact that miR-143 expression increases to a larger extent in vivo compared with adipogenesis of 3T3-L1 cells; this is one of the miRNAs we selected for overexpression in 3T3-L1 preadipocytes (Fig. 6).

**Coexpression of intronic miRNAs with “host” genes.** Some of the differentially regulated miRNAs originated from introns of known genes. For example, miR-422b is located in the sense orientation in the first intron of PANK1 (Fig. 6). Further, miR-143 expression increases to a larger extent in vivo compared with adipogenesis of 3T3-L1 cells; this is one of the miRNAs we selected for overexpression in 3T3-L1 preadipocytes (Fig. 6).
accumulation, the same miRNA microarrays were used to compare miRNA levels in normal adipocytes with those of obese mice. We first used leptin-deficient ob/ob mice; at 4 weeks of age, ob/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 ob/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 ob/ob mice, were validated by RT-PCR assays (Fig. 2B).

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To compare changes in miRNA expression between these two models of obesity, we focused on a set of 78 miRNAs, whose expression levels were above the mean expression of all miRNAs in at least one of the four samples analyzed: enriched adipocytes from wild-type and ob/ob mice (Fig. 2) and adipocytes from control and DIO mice (Fig. 3C). As shown in Fig. 3D, there was a strong positive correlation between changes in miRNA expression levels in the two obesity models ($r = 0.51, P < 0.0001$, Pearson’s correlation). This suggested that similar sets of miRNAs are up- or downregulated in these two models of obesity, although the extent of regulation was usually more striking in adipocytes from ob/ob mice than DIO mice.

**Inverse correlation of miRNA expression during adipogenesis and obesity.** Importantly, we noticed that miRNAs upregulated during adipogenesis tended to be downregulated in the obese state, and vice versa. For example, miR-422b, 148a, 107, 103, 30c, 30a-5p, and 143 were induced during adipogenesis but were downregulated in obese adipocytes. Conversely, miR-222 and -221 were decreased during adipogenesis but were upregulated in obese adipocytes (Figs. 1B and 2B).

To test our observation more rigorously and on a larger scale, we focused on a set of 79 miRNAs, whose expression levels were above the mean expression of all miRNAs in at least one of the four samples analyzed: undifferentiated 3T3-L1 preadipocytes (day 0), differentiated 3T3-L1 adipocytes (day 9), and adipocytes from wild-type and ob/ob mice. The regulation patterns of these 79 miRNAs are shown as a scatter plot in Fig. 4A ($r = -0.51, P < 0.0001$, Pearson’s correlation) and categorized in Fig. 4B. Among the 41 miRNAs upregulated during differentiation, 31 miRNAs decreased during obesity. In contrast, among the 38 miRNAs downregulated during differentiation, 26 miRNAs were elevated during obesity. The inverse correlation between miRNAs implicated in adipogenesis and obesity was statistically significant (Fig. 4B, $P = 0.0001$, Fisher’s exact test).

**miRNA regulation by TNF-α in adipocytes from obese mice.** Chronic inflammation by macrophages is a principal feature of obese adipose tissue (3,34–36); 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 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 which were ectopically expressed. 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. 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 downregulated during differentiation (Diff_Down), among which 26 miRNAs were upregulated during obesity (Ob_Down), whereas 12 miRNAs were downregulated (Ob_Down). $P = 0.0001$ by Fisher’s exact test.

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miR-103 or miR-143 hastens expression of 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. 6E 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.
clustering 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 v4.2 (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 miRNA 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 downstream 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 PPARγ or CCAAT/enhancer-binding protein α or another miRNA 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 miRNAs 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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