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MicroRNA-143 Regulates Adipocyte Differentiation*[S]

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MicroRNAs (miRNAs) are endogenously expressed 20–24 nucleotide RNAs thought to repress protein translation through binding to a target mRNA (1–3). Only a few of the more than 250 predicted human miRNAs have been assigned any biological function. In an effort to uncover miRNAs important during adipocyte differentiation, antisense oligonucleotides (ASOs) targeting 86 human miRNAs were transfected into cultured human pre-adipocytes, and their ability to modulate adipocyte differentiation was evaluated. Expression of 254 miRNAs in differentiating adipocytes was also examined on a miRNA microarray. Here we report that the combination of expression data and functional assay results identified a role for miR-143 in adipocyte differentiation. miR-143 levels increased in differentiating adipocytes, and inhibition of miR-143 effectively inhibited adipocyte differentiation. In addition, protein levels of the proposed miR-143 target ERK5 (4) were higher in ASO-treated adipocytes. These results demonstrate that miR-143 is involved in adipocyte differentiation and may act through target gene ERK5.

The first miRNA[1] was identified in *Caenorhabditis elegans* as a gene important for timing of larval development (5). miRNAs have since been implicated in many processes in invertebrates, including cell proliferation and apoptosis (6, 7), fat metabolism (6), and neuronal patterning (8). As many miRNAs are conserved across species (9–11), they are likely to be involved in developmental processes in all animals. Only a few mammalian miRNAs have been assigned any function, and at least two of these are involved in developmental processes: miR-181 promotes B cell development in mice (12) and miR-196a regulates several Hox genes (13), which code for a family of transcription factors involved in various developmental programs in animals (14).

We hypothesized that miRNAs may play a role in maturation of human adipocytes. Understanding the molecular events involved in adipocyte differentiation is of interest for development of therapeutics for metabolic diseases such as obesity and diabetes. *In vitro* cell culture systems, such as human primary subcutaneous pre-adipocytes, have been crucial in uncovering signaling pathways important for adipocyte differentiation (15). These cells can be cultured with differentiation-promoting hormonal stimuli, causing them to develop into cells that morphologically and functionally resemble mature adipocytes. In this study we have inhibited a panel of miRNAs in pre-adipocytes using antisense oligonucleotides and evaluated the effect on adipocyte differentiation. Combined with expression analysis of miRNAs in differentiating adipocytes by microarray, one miRNA, miR-143, was identified which normally promotes adipocyte differentiation. These results indicate that miRNAs do play a role in adipocyte differentiation and are potential therapeutic targets for obesity and metabolic diseases.

EXPERIMENTAL PROCEDURES

Oligonucleotide Synthesis—Oligonucleotides were prepared using conventional phosphoramidite chemistry and DNA synthesis equipment (Applied Biosystems). The 2′-*O*-methoxyethyl phosphoramidites and succinate-linked solid support were prepared as described previously (16). The purity of all samples was >85% as monitored by electrospray ionization-mass spectrometry analysis following precipitation. ASO sequences were complementary to the published mature miRNA sequence, with the length of the ASO varying accordingly (see supplemental Fig. S1)

Culture and Differentiation of Pre-adipocytes—Human white pre-adipocytes (Zen-Bio Inc.) were grown in pre-adipocyte media (Zen-Bio Inc.). One day before transfection, the pre-adipocytes were seeded into 96-well plates. Transfection of 250 nM oligonucleotide in Lipofectin (Invitrogen) was performed in triplicate, according to manufacturer's instructions. A positive control peroxisome proliferator-activated receptor-γ2 (PPAR-γ2) ASO, and a negative control ASO were also included in at least 6 wells in each experiment. After the cells reached confluence, they were exposed to differentiation medium (Zen-Bio Inc.) containing rosiglitazone, 3-isobutyl-1-methylxanthine, dexamethasone, and insulin for 3 days. Cells were then fed adipocyte medium (Zen-Bio Inc.), which was replaced at 2–3-day intervals. Ten days after the induction of differentiation, cells were lysed and adipocyte endpoints were examined. Triglyceride accumulation was measured using the Infinity triglyceride reagent kit (Sigma).

Real-time RT-PCR—Adipocyte total RNA was harvested from cells in 96-well plates using RNeasy 96 columns (Qiagen) on a Bio Robot 3000 (Qiagen). Real-time RT-PCR was performed (Applied Biosystems, Prism 7700) using the following primer/probe sets: adipocyte lipid-binding protein (aP2), 5′-GGTGGTGGAATGCGTCATG-3′ (forward) and 5′-CAACGTCCCTTGGCTTATGC-3′ (reverse); probe, 5′-FAM-AAGGCGTCACTTCCACGAGAGTTTATGAGA-TAMRA-3′; glucose transporter 4 (GLUT4), 5′-GGCCTCCGCAGGTTCTG-3′ (forward) and 5′-TTCGGAGCCTATCTGTTGGAA-3′ (reverse); probe, 5′-FAM-TCCAGGCCGGAGTCAGAGACTCCA-TAMRA-3′; hormone-sensitive lipase (HSL), 5′-ACCTGCGCACAATGACACA-3′ (forward) and 5′-TGGCTCGAGAAGAAGGCTATG-3′ (reverse); probe, 5′-FAM-CCTCCGCCAGAGTCACCAGCG-TAMRA-3′; PPAR-γ2, 5′-AAATATCAGTGTGAATTACAGCAAACC-3′ (forward) and 5′-GGAATCGCTTTCTGGGTCAA-3′

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Methods, Table S1, and Fig. S1.

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[1] The abbreviations used are: miRNA, microRNA; aP2, adipocyte lipid-binding protein; ASO, antisense oligonucleotide; GLUT4, glucose transporter 4; HSL, hormone-sensitive lipase; MOE, methoxyethyl; PPAR-γ, peroxisome proliferator-activated receptor-γ; RT, reverse transcription; FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine; ERK, extracellular signal-regulated kinase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; snRNA, small nuclear RNA.

(reverse); probe, 5′-FAM-TGCTGTTATGGGTGAAACTCTGGGAGATT-CT-TAMRA-3′. Expression levels were normalized to total RNA, determined by Ribogreen (Molecular Probes).

Microarray—254 oligonucleotides (5′-amino-modifier-C6) corresponding to human and mouse mature sense miRNA sequences were spotted on three-dimensional CodeLink slides (GE Health/Amersham Biosciences) with a final concentration of 20 μM and processed according to manufacturer's recommendations. First strand cDNA synthesized from 20 μg TRIzol-purified total RNA was labeled with biotinylated ddUTP using the Enzo BioArray end labeling kit (Enzo Life Sciences Inc.) Hybridization, staining, and washing were performed according to a modified Affymetrix Antisense genome array protocol. Axon B-4000 scanner and Gene-Pix Pro 4.0 software were used to scan images. Non-positive spots after background subtraction, and outliers detected by the ESD procedure (17) were removed. The resulting signal intensity values were normalized to per-chip median values and then used to obtain geometric means and standard errors for each miRNA. Each miRNA signal was transformed to log base 2, and a one-sample *t* test was conducted. For the adipocyte experiment shown here, two independent hybridizations for each sample were performed on chips with each miRNA spotted four times. Two sample Welch's *t* test was used for comparison of adipocyte and pre-adipocyte miRNA expression levels. Further details are included in the supplemental Methods.

Northern Blotting—RNA from human tissues (Ambion) or RNA isolated from differentiating adipocytes (TRIzol, Invitrogen) was separated on a 10% acrylamide TBE-urea mini-gel (Invitrogen) and then electroblotted onto Hybond N+ nylon filter (Amersham Biosciences). An end-labeled (Promega) oligonucleotide probe for miR-143 (5′-TGAGCTACAGTGCTTCATCTCA-3′) was hybridized to the filter in Rapidhyb buffer (Amersham Biosciences). The blot was reprobed for U6 to control for equal loading. Quantitation was done using a Storm 860 PhosphorImager (Amersham Biosciences) and ImageQuant software.

Luciferase Assay—pGL3-miR-143 sensor plasmid was constructed by insertion of two miR-143 binding sites in the XbaI site of the pGL3-Control vector (Promega). The pCR3-pri-mir-143 plasmid was constructed by T/A cloning of a 430-bp miR-143 PCR product into the pCR3 eukaryotic expression vector (Invitrogen). The PCR primers used were: 5′-AGG TTT GGT CCT GGG TGC TCA AAT GGC AGG-3′ (forward) and 5′-TGC CCA GAC TCG TGA AGC AGA TCG TGG CAC-3′ (reverse). For transfection, adherent HeLa cells were seeded in 24-well plates in minimal essential medium + 10% fetal bovine serum and then transfected using Lipofectamine 2000 (Invitrogen) the following day, according to the manufacturer's instructions. 30 ng of pGL3-miR-143 sensor plasmid, 10 ng of pRL-CMV (Promega), 100 ng of pCR3-pri-mir-143 expression plasmid, and the indicated dose of ASO were transfected into each well, and each treatment was done in triplicate. 24 h after transfection, firefly and *Renilla* luciferase activities were measured using a dual luciferase assay (Promega). The sequence of scrambled control ASO is 5′-AGACTAGCGGTATCTTTATCCC-3′. The sequence of miR-129 ASO is 5′-GCAAGCCCAGACCGCAAAAAG-3′.

Western Blotting—Adipocytes were lysed in radioimmune precipitation assay buffer with protease inhibitor mixture (Roche Applied Science). Proteins were separated on an 8% SDS-PAGE gel under reducing conditions, electroblotted onto polyvinylidene difluoride membrane, and blotted with anti-ERK5 antibody (Cell Signaling). The blot was reprobed with anti-G3PDH antibody (Abcam). Protein levels were quantitated on a Bio-Rad Chemidoc system.

RESULTS

Identification of miRNAs Involved in Adipocyte Differentiation through Combined Functional and Expression Analysis—To identify miRNAs important for modulating adipocyte differentiation, primary human pre-adipocytes were transfected prior to induction of differentiation with 2′-*O*-methoxyethyl (2′-*O*-MOE) phosphorothioate-modified antisense RNA oligonucleotides targeting miRNAs. In each experiment, a negative control ASO was included, as well as a PPAR-γ2 ASO, which inhibits adipocyte differentiation. Three days after ASO transfection, when the cells reached confluence, they were exposed to differentiation-inducing medium containing dexamethasone, the PPAR-γ agonist rosiglitazone, 3-isobutyl-1-methylxanthine, and insulin. Ten days later, differentiation was assessed by measuring triglyceride accumulation in the cells, as well as the expression of four adipocyte-specific genes: the insulin-responsive glucose transporter GLUT4, HSL, fatty acid-binding protein aP2, and PPAR-γ2 by quantitative RT-PCR.

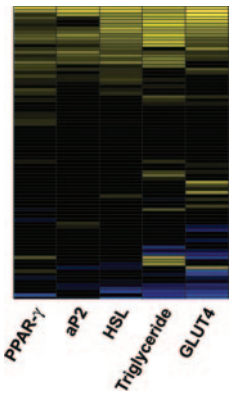

FIG. 1. **Identification of miRNAs that modulate adipocyte differentiation using 2′-O-MOE antisense RNA oligonucleotides.** Heat map showing levels of triglyceride accumulation and mRNA levels of adipocyte-specific genes GLUT4, aP2, HSL, and PPAR-γ2 in primary human differentiated adipocytes transfected as pre-adipocytes with 250 nM 2′-*O*-methoxyethyl-modified ASOs. *Yellow* depicts inhibition of an end point relative to a negative control ASO, and *blue* depicts an increase in an end point. Each row represents a targeted miRNA. The sequences of the ASOs and the data used to generate the heat map are contained in supplemental Table S1.

The heat map in Fig. 1 summarizes the data from ASOs targeting 86 miRNAs. Two ASOs, targeting miR-9* and miR-143, inhibited all five adipocyte differentiation markers by at least 40%, when compared with control ASO-treated adipocytes. Five ASOs inhibited three adipocyte endpoints, but none of these affected PPAR-γ2, which is the most significant marker of adipocyte differentiation. While no ASO increased all five end points, three ASOs, targeting miR-196, let-7c, and miR-145, increased three endpoints by 40% or more. However, only let-7c caused an increase in PPAR-γ2 expression.

To correlate this functional data with expression patterns of miRNAs in the developing adipocytes, a microarray-based technique was used to profile miRNA expression in the human pre-adipocytes and differentiated cells. The miRNA array was first used to profile miRNA expression in different cell lines and organs in human and mouse, and the data showed a good correlation with both published Northern blotting data (18) and our own Northern blotting data. Using this technique, miRNAs that are differentially expressed in adipocytes compared with pre-adipocytes were identified and are shown in Table I. miR-143, which was a hit in the adipocyte differentiation functional assay, also appeared to be up-regulated in differentiated adipocytes, further supporting a role for it in modulating adipocyte differentiation.

Concentration-dependent Inhibition of Adipocyte Differentiation by miR-143 Antisense Oligonucleotide—The effects of the miR-143 ASO were further characterized in pre-adipocytes. The reduction in triglyceride accumulation by the miR-143 ASO was concentration-dependent, with a maximum inhibition of 75% at 300 nM ASO (Fig. 2*A*). The negative control ASO failed to significantly reduce triglyceride accumulation at any concentration tested, while the positive control PPAR-γ2 ASO effectively inhibited triglyceride accumulation. Expression of the four adipocyte hallmark genes was also inhibited by the miR-143 ASO in a concentration-dependent manner, while the negative control ASO had no significant effect on their expression (Fig. 2*A*). ASO inhibition of miR-143 prevents adipocyte-specific gene expression and the accumulation of triglycerides, suggesting that miR-143 is normally involved in promoting adipocyte differentiation or function.

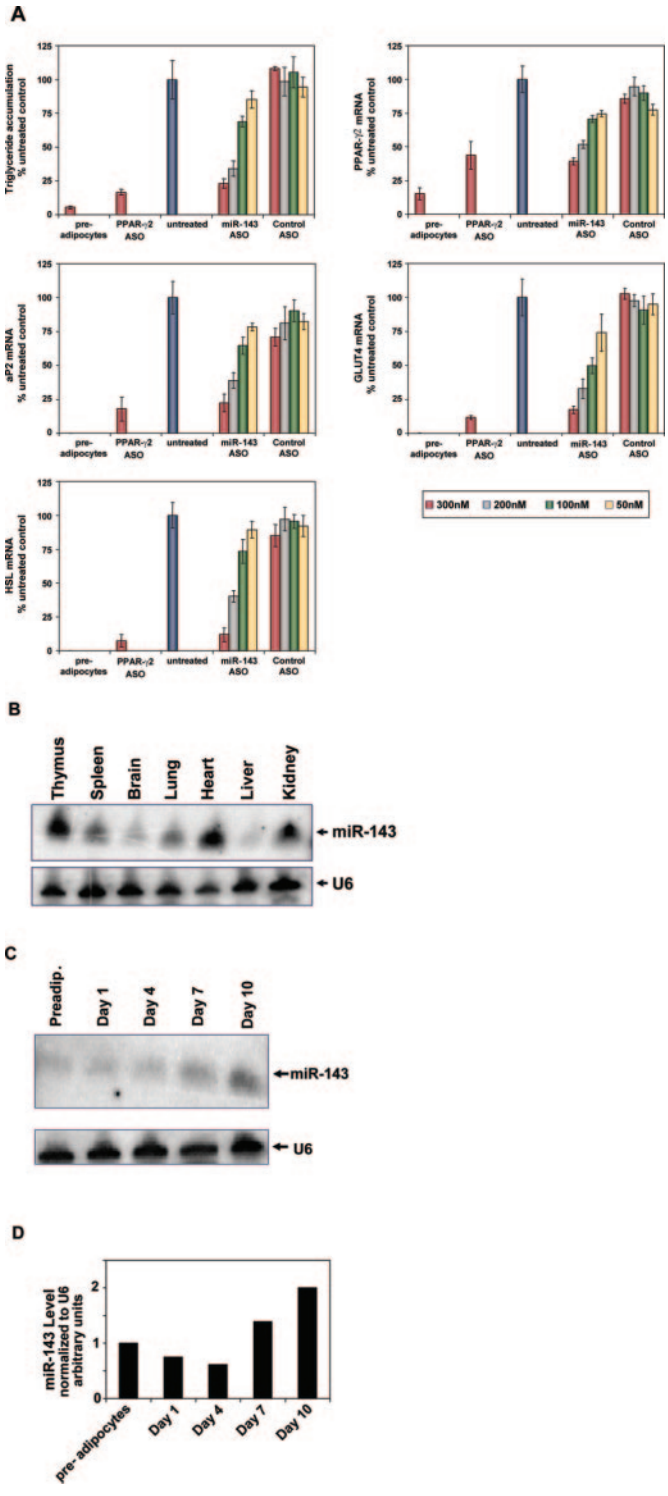

FIG. 2. **Expression levels and ASO inhibition of miR-143 in differentiating adipocytes.** *A*, concentration-dependent inhibition of adipocyte differentiation end points by miR-143 ASO. The level of each adipocyte differentiation end point in pre-adipocytes and in adipocytes treated with PPAR-γ2 ASO (which inhibits adipocyte differentiation) are shown for comparison. *B*, Northern blotting of human tissue RNA for mature miR-143. The blot was reprobed for U6 snRNA as a loading control. *C*, Northern blotting for mature miR-143 in differentiating primary human pre-adipocytes at various times post-induction. The blot was reprobed for U6 snRNA as a loading control. *D*, quantitation of Northern blot.

Northern Blot Analysis of miR-143 Expression—The expression pattern of miR-143 in a panel of human tissues by northern blotting was examined. The highest levels of miR-143 were found in heart, kidney, and thymus, while modest levels were observed in spleen, brain, and liver (Fig. 2*B*). Expression of miR-143 was also examined in a similar panel of mouse tissues, and the expression pattern correlated well with the human tissues (data not shown). Although the human tissue panel did not include adipose tissue, white adipose tissue from the *ob*/*ob* mouse strain had the highest level of miR-143 of any tissues examined. Northern blot experiments confirmed that expression of miR-143 is increased in adipocytes relative to pre-adipocytes. (Fig. 2, *C* and *D*). MiR-143 expression increased between day 4 and day 10 by almost 4-fold (Fig. 2, *C* and *D*).

TABLE I

miRNAs differentially expressed during adipocyte differentiation

Data from microarray analysis of miR expression was used to identify miRs with differential expression in human cultured adipocytes compared to preadipocytes. Only miRs with a statistically significant ($p \leq 0.05$) differential expression are listed. Welch's two-sample t test was used to compute p values. -Fold change is the change in expression in adipocytes relative to pre-adipocytes.

miRNA	-Fold change	p value
miR-130b	3.86	0
miR-30c	3.74	0.0154
miR-191	3.22	0.0040
miR-30a*	2.83	0.0329
miR-130a	2.76	0.0299
miR-143	2.61	0.0171
miR-30d	2.54	0.0169
miR-196	2.17	0.0356
miR-107	2.17	0.0007
miR-30b	2.02	0.0429
miR-19b	1.99	0.0480
miR-103	1.98	0.0267
miR-296	1.86	0
miR-92	1.74	0
let-7c	1.48	0.0467
miR-99b	1.45	0.0089
let-7a	1.39	0.0025
miR-198a	1.34	0.0104
miR-100	−1.14	0.0175
miR-138	−2.18	0.0069
miR-93	−2.96	0.0086
miR-20	−3.15	0.0474

2′-O-MOE Antisense Oligonucleotides Are Effective Inhibitors of MicroRNA Activity—Antisense inhibition of miRNAs using 2′-*O*-methyl-modified ASOs in cell culture, *C. elegans*, and *Drosophila* embryos has been reported (19–21). To verify that 2′-*O*-MOE ASOs were also able to effectively inhibit miRNA activity, a miRNA activity assay using a luciferase expression plasmid containing two perfectly complementary miR-143 binding sites was established (pGL3-miR-143 sensor, Fig. 3*A*). Transfection of the pri-mir-143 expression plasmid (pCR3-pri-mir-143, Fig. 3*A*) into HeLa cells caused a 14-fold inhibition of luciferase activity compared with pGL3-miR-143 sensor transfection alone (Fig. 3*B*). Addition of miR-143 ASO was able to prevent the repression of luciferase activity by miR-143 in a concentration-dependent manner. Addition of a scrambled control ASO, or an ASO against an unrelated miRNA, miR-129, had no effect on luciferase repression by miR-143 (Fig. 3*B*). Thus, 2′-*O*-MOE ASOs are effective and specific inhibitors of miRNA activity.

The effect of miR-143 ASO treatment on miR-143 levels in differentiating adipocytes was also examined. Northern blotting for miR-143 in ASO-treated adipocytes revealed a 60% decrease in mature miR-143 levels compared with untreated control 7 days after the induction of differentiation. A negative control ASO did not cause a reduction in miR-143 (Fig. 3*C*). A reduction in miR-143 levels was not necessarily expected, as 2′-*O*-MOE ASOs do not support RNaseH-mediated cleavage of the target. However, in other experiments we have observed that 2′-*O*-MOE ASOs targeting mature miRNA can inhibit

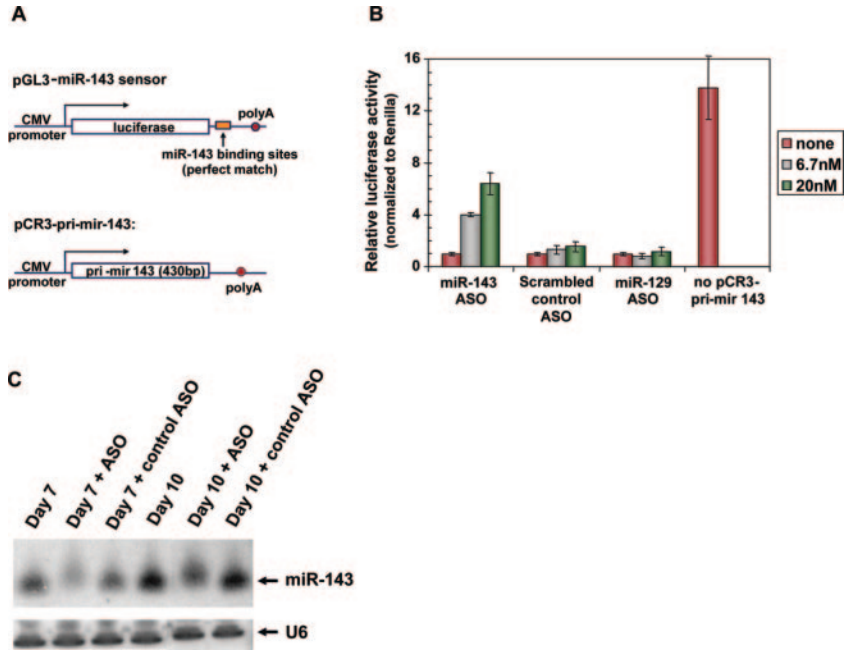

FIG. 3. **2′-MOE antisense oligonucleotides are effective inhibitors of miRNA activity in cultured cells.** *A*, expression plasmids used in luciferase assay for miR-143 activity. *B*, HeLa cells were transiently transfected with pRL-CMV *Renilla* luciferase plasmid (Promega), pGL3-miR-143 sensor, and unless indicated, pCR3-pri-mir-143. ASOs were transfected at the indicated doses and evaluated for their ability to interfere with miR-143 inhibition of pGL3-miR-143 sensor luciferase expression. Luciferase activity was assayed 24 h post-transfection and for each sample was normalized to *Renilla* luciferase activity. *C*, Northern blotting for miR-143 in differentiating adipocytes treated with 250 nM ASO targeting miR-143 at days 7 and 10 after induction of differentiation. The blot was reprobed for snRNA U6 as a loading control.

Drosha processing of pri-miRNA transcripts and subsequently reduce mature miR levels.[2]

Increased Protein Levels of Putative miR-143 Target ERK5 in miR-143 ASO-treated Adipocytes—There have been several efforts to use bioinformatics techniques to identify target mRNAs regulated by miRNAs (4, 22–26). We wanted to test whether protein levels of any predicted miR-143 targets were altered in the miR-143 ASO-treated adipocytes. However, of the miR-143 targets proposed by Lewis *et al.* (4), only antibodies for ERK5/BMK1 were commercially available. Levels of ERK5 protein in normal adipocytes were compared with adipocytes that had been treated with miR-143 ASO before induction of differentiation. After 7 or 10 days of differentiation, the level of ERK5 protein, normalized to G3PDH levels, was 2-fold higher in the miR-143 ASO-treated cells (Fig. 4). The levels of ERK5 protein were unchanged in adipocytes treated with an ASO targeting miR-23b, which also inhibited adipocyte differentiation in this assay (data not shown). These data suggest that the up-regulation of ERK5 is specific to the miR-143 ASO treatment and not a general result of inhibition of adipocyte differentiation, consistent with the hypothesis that ERK5 is a miR-143 target gene.

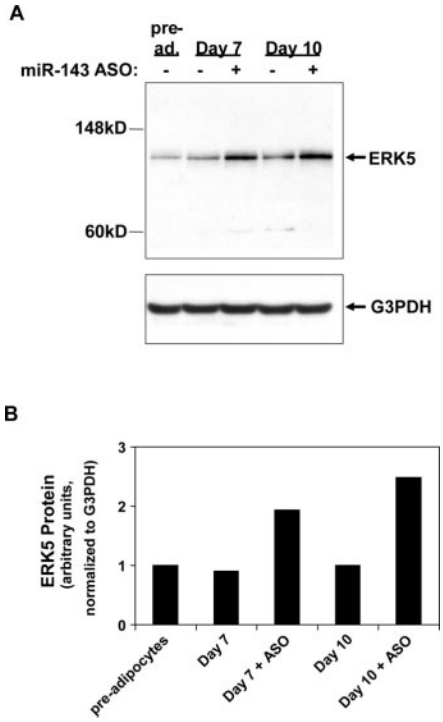

FIG. 4. **Predicted miR-143 target ERK5/BMK1 is up-regulated in miR-143 ASO-treated adipocytes** *A*, Western blotting for ERK5/BMK1 in differentiating adipocytes treated with 250 nM ASO targeting miR-143, at days 7 and 10 after induction of differentiation. The blot was reprobed for G3PDH as a loading control. *B*, quantitation of Western blot.

DISCUSSION

In this study we have screened ASOs targeting miRNAs for their ability to modulate adipocyte differentiation in a cultured primary human adipocyte model system. These data, together with a global analysis of miRNA expression patterns, allowed us to identify several miRNAs involved in adipocyte differentiation. 2′-*O*-MOE ASOs were a useful tool for dissecting the biological importance of miRNAs, and our library of miRNA ASOs will be of use in a variety of *in vitro* and *in vivo* systems to rapidly uncover the biological roles of miRNAs. In addition, ASOs targeting miRNA may be valuable as therapeutics in a variety of disease indications.

One miRNA identified in the screen, miR-143, was of particular interest. The observation that miR-143 is up-regulated as adipocytes differentiate, taken together with the inhibition of adipocyte differentiation by miR-143 ASO, indicates that miR-143 normally promotes adipocyte differentiation. Interestingly, miR-143 levels were previously reported to be decreased in colorectal adenocarcinomas compared with normal tissue (27), and the authors were unable to detect miR-143 expression in a variety of human cancer cell lines. Our own efforts to find a cancer cell line expressing miR-143 were also unsuccessful (data not shown). This is consistent with a role for miR-143 in promoting differentiation and suggests that its down-regulation in cancer cell lines may be a cause or a consequence of the undifferentiated state.

Numerous bioinformatics approaches to predicting miRNA targets have been put forward, but direct verification of a

[2] B. Lollo, C. Esau, E. Peralta, J. Baroldi, R. Quiambao, R. Jain, and S. M. Freier, manuscript in preparation.

miRNA-regulated target in a biological system has so far only been accomplished in *C. elegans* (28, 29). ERK5/BMK1 was predicted to be a miR-143 target in one such study, and we found ERK5 protein levels up-regulated in miR-143 ASO-treated adipocytes. However, as each miRNA is expected to regulate multiple target genes, it is difficult to know whether the effect of miR-143 inhibition on adipocyte differentiation is primarily mediated through ERK5 or through other unknown miR-143 target genes. ERK5 is known to promote cell growth and proliferation in response to tyrosine kinase signaling (30), but its role in adipocyte differentiation has not been examined. It may be involved in regulating the balance between proliferation and differentiation of adipocytes. In this case, miR-143 inhibition of ERK5 protein in differentiated adipocytes, either directly, through binding of the ERK5 3′UTR, or indirectly through another target gene, may be one way to fine tune the MAP kinase signaling pathways to maintain the differentiated state. Further experiments will be necessary to dissect the role of ERK5 in adipocyte differentiation. Regardless, identification of miR-143 as an important regulator of adipocyte differentiation suggests that miRNAs are potential therapeutic targets for obesity and metabolic disease.

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