Roles for miRNA-378/378* in adipocyte gene expression and lipogenesis

Isabelle Gerin,1,4 Guido T. Bommer,2,4 Colin S. McCoin,1 Kyle M. Sousa,1 Venkatesh Krishnan,3 and Ormond A. MacDougald1,2

Departments of 1Molecular and Integrative Physiology, and 2Internal Medicine, University of Michigan, Ann Arbor, Michigan; 3Musculoskeletal Research, Lilly Research Laboratories, Indianapolis, Indiana; and 4de Duve Institute, Université Catholique de Louvain, Brussels, Belgium

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Gerin I, Bommer GT, McCoin CS, Sousa KM, Krishnan V, MacDougald OA. Roles for miRNA-378/378* in adipocyte gene expression and lipogenesis. Am J Physiol Endocrinol Metab 299: E198–E206, 2010. First published May 18, 2010; doi:10.1152/ajpendo.00179.2010.—In this study, we explored the roles of microRNAs in adipocyte differentiation and metabolism. We first knocked down Argonaute2 (Ago2), a key enzyme in the processing of micro-RNAs (miRNAs), to investigate a potential role for miRNAs in adipocyte differentiation and/or metabolism. Although we did not observe dramatic differences in adipogenesis between Ago2 knock-down and control 3T3-L1 cells, incorporation of [14C]glucose or acetate into triacylglycerol, and steady-state levels of triacylglycerol were all reduced, suggesting a role for miRNAs in adipocyte metabolism. To study roles of specific miRNAs in adipocyte biology, we screened for miRNAs that are differentially expressed between preadipocytes and adipocytes for the 3T3-L1 and ST2 cell lines. Distinct subsets of miRNAs decline or increase during adipocyte conversion, whereas most miRNAs are not regulated. One locus encoding two miRNAs, 378/378*, contained within the intron of adipocyte conversion, whereas most miRNAs are not regulated. One locus encoding two miRNAs, 378/378*, contained within the intron of PGC-1α is highly induced during adipogenesis. When overexpressed in ST2 mesenchymal precursor cells, miRNA378/378* increases the size of lipid droplets and incorporation of [14C]acetate into triacylglycerol. Although protein and mRNA expression levels of C/EBPα, C/EBPβ, C/EBPδ, and PPARγ1 are unchanged, microarray and quantitative RT-PCR analyses indicate that a set of lipogenic genes are upregulated, perhaps due to increased expression of PPARγ2. Knockdown of miRNA378 and/or miRNA378* decreases accumulation of triacylglycerol. Interestingly, we made the unexpected finding that miRNA378/378* specifically increases transcriptional activity of C/EBPα and C/EBPβ on adipocyte gene promoters. PPARγ coactivator-1β; micro-RNA

Adipogenesis; PPARγ coactivator-1β; micro-RNA

It has been estimated that miRNAs might regulate expression of one-third of human genes. Posttranscriptional RNA silencing is a conserved regulatory mechanism present in almost all eukaryotic organisms. More specifically, the micro-RNA (miRNA) pathway regulates gene expression by inducing degradation and/or translational repression of target mRNAs. Most animal miRNAs are partially complementary to their targets and mediate their silencing effects via translational repression. miRNAs are transcribed as primary transcripts (pri-miRNAs) that are afterward processed within the nucleus into premiRNAs by complexes containing the core RNAse III enzyme Drosophila. Those pre-miRNAs are exported to the cytoplasm and cleaved by Dicer to produce miRNA/miRNA* duplexes. miRNAs associate with Argonaute (Ago) proteins to form the core effector complex known as RISC (for RNA-induced silencing complex). This complex is capable of recognizing mRNAs and inhibiting protein translation. In 2006, Schmitter and colleagues analyzed HEK293 cell lines depleted of Dicer or individual Ago proteins (20). Their results indicated that Ago2 was the most important Ago protein acting in the miRNA pathway in HEK293 cells and that knocking down Ago2 had a similar effect to that of Dicer. More recently, Su et al. demonstrated that mammalian Agos all contribute to miRNA silencing, and individual Agos have overlapping functions in this process (22). In addition to the role of Ago2 in the action of miRNAs, Diederichs and colleagues have shown that Ago2 also plays an important role in the processing of the mature form of several miRNAs (3).

A number of miRNAs have been reported to be expressed and regulated during adipocyte differentiation. Esau et al. (5) were the very first ones to show that miRNA-143 regulated adipocyte differentiation. More recently, Xie et al. (26) showed that miRNA-103 and miRNA-143 are induced during adipogenesis and accelerate fat cell development. Oskowitz and colleagues analyzed miRNA expression during human multipotent stromal cells differentiation (18). They identified 19 miRNAs that were upregulated during osteogenesis and 20 additional miRNAs induced during adipogenesis. Overexpression of miRNA-27 inhibits adipocyte formation by blocking expression of peroxisome proliferator-activated receptor (PPARγ) and C/EBPα, without affecting myogenic differentiation (16). miRNA let-7 (24) also plays a role in adipogenesis. Expression of several others have been described as expressed and regulated in the adipogenic differentiation of adipose-derived stem cells (25) or in human omental and subcutaneous adipose tissue (12).

Materials and Methods

Cell culture. Maintenance and adipogenesis of 3T3-L1 preadipocytes were as described previously using methylisobutylxanthine, dexamethasone, and insulin (MDI) (7). For adipogenesis, cells that had been confluent for 2 days (day 0) were treated with 10% fetal calf serum (FCS), 1 μM dexamethasone, 0.5 mM methylisobutylxanthine, 1 μg/ml insulin, and 5 μg/ml troglitazone. On day 2, cells were fed with 1 μg/ml insulin in 10% FCS media, and on day 4 and every 2 days thereafter, cells were refed with 10% FCS. Lipid accumulation in adipocytes was visualized by staining with Oil Red-O (14). Mature-derived ST2 cells were incubated at 37°C and 5% CO2 in α-MEM supplemented with 10% FCS (Atlanta Biologicals, Lawrenceville, GA).

Plasmids and transfections. A genomic fragment of 2.6 kb and encompassing miRNA378/378* was amplified with the primers 5'-gcttcgtggtgtcatcatc-3' and 5'-ctggcaggagctcataag-3' with Pfu TurboHotstart polymerase (Stratagene, La Jolla, CA), and subsequently subcloned into pMSCV PURO. Plasmids expressing wild-type C/EBPα and C/EBPβ were described previously (4).
promoter-luciferase reporter construct for GLUT4 was provided by Sven Enerbäck (Göteborg University) (8).

Retroviral transduction of cells. 293T cells (10-cm plates) were translectected by calcium phosphate coprecipitation with the viral packaging vectors SV\epsilon-E-MLV-env and SV\omega-E-MLV in addition to retroviral vectors as indicated in the figure legends (7.5 g of each). Virus-containing medium was collected 16 h after transfection and passed through a 0.45-μm syringe filter. Polybrene (hexadimethrine bromide; Sigma) was added to a final concentration of 8 g/ml. This medium was then applied to subconfluent (30–50%) cells in 10-cm plates. The infection protocol was repeated every 8–16 h until cells were 80% confluent. Cells were then trypsin-treated and replated in DMEM supplemented with 10% FCS and 2 μg/ml puromycin (Sigma) for pSUPERIOR/pMSCV-based vectors.

Transient transfection and luciferase assay. NIH-3T3 cells were transfected using Fugene 6 (Roche, Basel, Switzerland). Cells were transfected with the indicated amount of luciferase reporter gene, 50 ng of pRL-SV40 Renilla (Promega, Madison WI), and the indicated amounts of expression plasmid in 6-well plates. To correct for variance in transfection efficiency, luciferase values were normalized against relative light units from Renilla activity.

Stable knock-down of Ago2 and Dicer in 3T3-L1 cells. Twenty-one nucleotide short hairpin RNA loops were used to knock down Ago2 mRNA and Dicer mRNA. The sequences were as reported by Schnitter et al. (20): shAgo2: forward, 5'-GATCCCCGAGGACAAAGATGTAT-TATTCAGAGATAATACATTTTTGTTTGGAAA-3' and reverse 5'-AGCTTTTCACAAAAAGCAGGACAAAGATGTAT-TATTCAGAGATAATACATTTTTGTTTGGAAA-3' and reverse 5'-AGCTTTTCACAAAAAGCAGGACAAAGATGTAT-TATTCAGAGATAATACATTTTTGTTTGGAAA-3' and reverse 5'-AGCTTTTCACAAAAAGCAGGACAAAGATGTAT-TATTCAGAGATAATACATTTTTGTTTGGAAA-3'. The forward and reverse oligo strands were annealed and cloned into the pSUPERIORretro.puro vector (OligoEngine, Seattle, WA), which had been linearized with BglII and HindIII.

Transient transfections of antagomirs in 3T3-L1 cells. Control antagomirs 378, 378*, and 132 (Applied Biosystems/Ambion, Austin, TX) at a concentration of 50 nM were added to adipocyte differentiation medium at day 3. Medium was changed at day 4 as usual. Cells were harvested or used for metabolic assays at day 7.

Quantitative RT-PCR. One microgram of total RNA was transcribed to cDNA using the TaqMan system (Applied Biosystems, E199MIRNA-378/378* AND ADIPOCYTES

Fig. 1. Ago2 is required for efficient incorporation of [14C]glucose into triacylglycerol. A: knockdown of Ago2 in 3T3-L1 adipocytes. After retroviral transduction with shRNAs, Ago2 expression levels in day 7 3T3-L1 adipocytes were measured by quantitative RT-PCR, and are shown relative to cyclophilin. Expression is means ± SD. B: Ago2 deficiency does not alter morphology in 3T3-L1 adipocytes. Phase contrast pictures shown were taken 11 days after induction of adipocyte differentiation. C and D: knockdown of Ago2 does not change C/EBPγ, PPARγ, and FABP4 expression in 3T3-L1 adipocytes. C/EBPγ mRNA expression is shown relative to cyclophilin as means ± SD. D: at day 8, cells were lysed, and expression of C/EBPγ, PPARγ, and FABP4 proteins were analyzed by immunoblot analysis. E and F: Ago2 is required for efficient incorporation of [14C]acetate and [14C]glucose into triacylglycerol. E: day 11 adipocytes were incubated in presence of [14C]acetate for 1 h. Cells were washed once in PBS, and total cellular lipids were extracted by a mixture of methanol and chloroform (1:2), followed by separation by thin layer chromatography. F: day 11 adipocytes were starved for 3 h in PBS. After incubation with 2 μCi [14C]glucose for 2.5 h, cells were harvested and analyzed by thin-layer chromatography. G and H: quantification of indicated spots. Counts are shown as means ± SD in arbitrary units. Significant difference: * P < 0.05; ** P < 0.01.
Foster City, CA). Quantitative RT-PCR was performed according to the manufacturer’s protocol. SYBR Green I was used to monitor amplification of DNA on MyiQ quantitative PCR detection system (Bio-Rad, Hercules, CA). After amplification, melting curve analysis was performed as described by the manufacturer. Gene expression was normalized to 18S, cyclophilin, or TATA-box binding protein mRNAs, depending on the relative abundance of mRNA. Oligonucleotide primers for amplification of 18S (10), cyclophilin (10), C/EBPβ (10), PPARγ (10), and TATA-box binding protein (21) have been reported. Primers for the other genes were designed using PRIMER EXPRESS software (Applied Biosystems, Foster City, CA) and validated for quantitative RT-PCR. Sequences are available on request.

Lipid synthesis and extraction. Cellular lipids were extracted essentially by the Folch method (6). Briefly, cells were incubated for 2 h at 37°C in the presence of [14C]acetic acid or [14C]glucose, washed once in cold PBS, and then lysed in 0.5 ml of ethanol. One milliliter of chloroform was added and mixed by vortexing. After addition of 0.5 ml of HCl (0.1 N), lysates were mixed by gentle inversion. Tubes were centrifuged at 500 × g for 10 min. The upper phase and interface were discarded, and the organic phase was washed twice with 0.5 ml of PBS. Extracts were dried under N2, and the pellet was resuspended in the appropriate volume of chloroform-methanol (2:1).

Extracts were separated by thin-layer chromatography with diethyl ether-hexane-glacial acetic acid (35:65:1, vol/vol/vol) as carrier solvent. Phospholipids and triacylglycerol were identified with standards. β-Oxidation assay-mitochondrial β-oxidation of [9,10(n)H]-palmitic acid (GE Healthcare Life Sciences, Piscataway, NJ) in 3T3-L1 adipocytes was assayed by the degree of incorporation of [3H] into H2O, as described by Keller et al. (11).

Western blots. Immunoblots were performed with antibodies specific for C/EBPβ, C/EBPα, PPARγ (Santa Cruz Biotechnology), and FABP4 (Cell Signaling). Bound horseradish peroxidase-coupled secondary antibody was visualized with Pierce Super Signal or Super Signal Ultra enhanced chemiluminescence substrates (Thermo Fisher Scientific, Rockford, IL).

RNA purification and microarray analyses. Total RNA was isolated from 3T3-L1 cells and ST2 stromal vascular cells with RNA Stat60 (Tel-Test B). The fraction containing miRNAs was further purified on a gel according to Ambion’s protocol. The Ambion miRNA probe set was spotted in triplicate on Nexterion E slides and subsequently hybridized to enriched small RNA fraction labeled by miRNA probe set was spotted in triplicate on Nexterion E slides and subsequently hybridized to enriched small RNA fraction labeled by miRNA probe set was spotted in triplicate on Nexterion E slides and subsequently hybridized to enriched small RNA fraction labeled by miRNA probe set was spotted in triplicate on Nexterion E slides and subsequently hybridized to enriched small RNA fraction labeled by miRNA probe set was spotted in triplicate on Nexterion E slides and subsequently hybridized to enriched small RNA fraction labeled by miRNA probe set was spotted in triplicate on Nexterion E slides and subsequently hybridized to enriched small RNA fraction labeled by miRNA probe set was spotted in triplicate on Nexterion E slides and subsequently hybridized to enriched small RNA fraction labeled by miRNA probe set was spotted in triplicate on Nexterion E slides and subsequently hybridized to enriched small RNA fraction labeled by miRNA probe set was spotted in triplicate on Nexterion E slides and subsequently hybridized to enriched small RNA fraction labeled by miRNA probe set was spotted in triplicate on Nexterion E slides and subsequently hybridized to enriched small RNA fraction labeled by miRNA probe set was spotted in triplicate on Nexterion E slides and subsequently hybridized to enriched small RNA fraction labeled by miRNA probe set was spotted in triplicate on Nexterion E slides and subsequently hybridized to enriched small RNA fraction labeled by miRNA probe set was spotted in triplicate on Nexterion E slides and subsequently hybridized to enriched small RNA fraction labeled by miRNA probe set was spotted in triplicate on Nexterion E slides and subsequently hybridized to enriched small RNA fraction labeled by miRNA probe set was spotted in triplicate on Nexterion E slides and subsequently hybridized to enriched small RNA fraction labeled by miRNA probe set was spotted in triplicate on Nexterion E slides and subsequently hybridized to enriched small RNA fraction labeled by miRNA probe set was spotted in triplicate on Nexterion E slides and subsequently hybridized to enriched small RNA fraction labeled by miRNA probe set was spotted in triplicate on Nexterion E slides and subsequently hybridized to enriched small RNA fraction labeled by miRNA probe set was spotted in triplicate on Nexterion E slides and subsequently hybridized to enriched small RNA fraction labeled by miRNA probe set was spotted on AJP-Endocrinol Metab • VOL 299 • AUGUST 2010 • www.ajpendo.org

Fig. 2. Regulated expression of micro-RNAs (miRNAs) during adipocyte differentiation. A: downregulated miRNAs during 3T3-L1 cell differentiation. The table shows the ratio of the level of expression in adipocytes vs. preadipocytes, as well as the coefficient of variation for two independent hybridizations with dye-swap. B: Northern blot analysis of preadipocytes (PA) and adipocytes (Ads) derived from 3T3-L1 and ST2 mesenchymal precursor cells for miRNA199b and miRNA378. C: upregulated miRNAs during 3T3-L1 cell differentiation. Data are presented as in A. D: Northern blot analysis of preadipocytes (PA) and adipocytes (Ads) derived from 3T3-L1 and ST2 mesenchymal precursor cells for miRNA10a, miRNA103, and miRNA378.
differentiation of adipocytes is not substantially influenced by knockdown of Ago2.

To assess a potential role of Ago2 in lipid metabolism, we examined effects of Ago2 knock-down on lipogenesis. After metabolic labeling with \[^{14}C\]acetate for 45 min or with \[^{14}C\]glucose for 2 h, lipids were extracted and separated by thin-layer chromatography (Fig. 1, E and F, respectively). Knock-down of Ago2 resulted in a significant reduction in triacylglycerol and phospholipids newly synthesized from radiolabeled acetate (Fig. 1G). When incubated in presence of radiolabeled glucose, we also saw a significant reduction in newly synthesized triacylglycerol but not in phospholipids (Fig. 1H). These results suggest that impairing biosynthesis of miRNAs and action of miRNAs negatively influences adipocyte lipid production.

Microarray analysis of miRNAs in preadipocytes and adipocytes. Given our results showing an impairment in adipocyte lipid synthesis following alterations in global miRNA processing, we next sought to identify specific miRNAs that modulate adipocyte function. To screen for miRNAs that were differentially regulated during adipocyte differentiation, we analyzed two different experimental systems: the 3T3-L1 preadipocyte line and the ST2 mesenchymal precursor line. Both cell lines can be efficiently induced to undergo adipocyte differentiation. The Ambion miRNA probe set was spotted on Nexterion glass slides and hybridized with small RNA fractions isolated from 3T3-L1 and ST2 preadipocytes and differentiated adipocytes. MiRNAs whose expression decreases during the transition between 3T3-L1 preadipocytes and adipocytes are ranked in Fig. 2A. By Northern blot, we confirmed that miRNA-199b (Fig. 2B), miRNA-197 (data not shown), and miRNA-34b (Fig. 2H) are downregulated during adipocyte differentiation. In contrast, miRNAs induced during adipogenesis were not as numerous. The most upregulated miRNAs were miRNA-378/378*, let-7c, miRNA-103, miRNA-107, and miRNA-210 (Fig. 2C). We confirmed by Northern blots using preadipocyte and adipocyte RNAs from two different cell lines (3T3-L1 and ST2) that miRNA-378/378*, miRNA-10a, miRNA-103, and miRNA-107 are induced during adipogenesis (Fig. 2D).

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**Fig. 3.** miRNA378/378* is derived from an intron of the PGC-1β gene and is coexpressed with PGC-1β during adipocyte differentiation. A: schematic representation of the genomic localization of miRNA378/378*. miRNA378/378* (red) is located in the first intron of the PGC-1β gene. miRNA378* is derived from the 5' half and miRNA378 (formerly named miRNA422b) is derived from the 3' half of the precursor hairpin. B: tissue distribution of miRNA378/378* and PGC-1β. Expression of miRNA378/422b and miRNA378* was analyzed by Northern blot of total RNA of the indicated tissues. PGC-1β miRNA expression was measured by quantitative RT-PCR and is shown relative to the expression of cyclophilin. P-WAT, perirenal white adipose tissue; E-WAT, epididymal white adipose tissue. C: level of expression of miRNA378/378* and PGC-1β during adipocyte differentiation. RNA from 3T3-L1 cells was harvested at the indicated times, and level of expression of miRNA378/378* was measured by Northern blot. The PGC-1β mRNA levels were measured at the same times by quantitative RT-PCR.
To establish whether some of these miRNAs might play a role in adipocyte differentiation, we generated retroviral vectors that contain an ~500-bp genomic region encompassing the indicated miRNAs. In one instance, inhibition of proliferation by miRNA34b overexpression hampered its analysis during adipogenesis (1). Morphological changes such as increased lipid droplet size during adipocyte differentiation were only observed on overexpression of miRNA 378/378*. We therefore decided to focus our further analysis on these miRNAs.

Induction of miRNA 378/378* during adipogenesis. Among the miRNAs identified as regulated during preadipocyte differentiation, miRNA 378/378* was of particular interest due to its peculiar genomic localization, since these miRNAs are localized in the first intron of PGC-1β (Fig. 3A, indicated by the filled square) and are highly conserved between species. Hence, we started by analyzing the abundance of miRNA 378 and miRNA 378* in different mouse tissues (Fig. 3B) by Northern blot. In parallel, we measured the levels of PGC-1β mRNA in the same tissues by quantitative RT-PCR. Expression of the 378/378* miRNAs and PGC-1β were highly concordant and showed peak levels in brown adipose tissue, heart, muscle, and skeletal muscle, respectively. Given the role of PGC-1β in mitochondrial biogenesis, it is notable that all these tissues are very rich in mitochondria. Lower levels of these miRNAs were found in other tissues, including white adipose tissue. We then investigated the expression of miRNA 378/378* during 3T3-L1 adipocyte differentiation. Total RNA was harvested at the indicated times, and Northern blot analysis was performed for miRNA 378 and miRNA 378*. In parallel, quantitative RT-PCR was performed to determine expression of PGC-1β mRNA. As shown in Fig. 3C, we observed that expression of miRNA 378/378* and PGC-1β are all induced during adipogenesis. Thus miRNA 378/378*, which are localized in the first intron of PGC-1β, are regulated similarly to the PGC-1β mRNA itself.

Expression of miRNA 378/378* stimulates lipogenesis without effects on β-oxidation or adipogenesis. To investigate the roles of miRNA 378/378* in adipocyte differentiation and/or metabolism, we ectopically expressed a longer precursor transcript that comprises the miRNA 378/378* locus in ST2 mesenchymal precursors. We harvested total RNA at the indicated times and checked by Northern blot analysis the level of overexpression of miRNA 378 during ST2 adipocyte differentiation. As shown in Fig. 4A, in control ST2 cells, expression of miRNA 378 increases by approximately twofold between preadipocytes and day 6 adipocytes. In contrast, in ST2 cells
infected with a miRNA378/378* retrovirus, the expression of miRNA378 in preadipocytes already surpasses the highest expression observed in control cells. With differentiation, expression increases a further 10-fold (Fig. 4A). Phenotypically, at day 3 after induction of adipocyte differentiation, lipid droplets are already well defined in ST2 cells overexpressing miRNA378/378*, whereas they are still budding in control cells (Fig. 4B). Adipogenesis is not influenced by enforced expression of miRNA378/378* as assessed by quantification of adipocyte number with phase-contrast microscopy (Fig. 4C). Western blot analyses for adipocyte markers revealed only marginal changes in the levels of expression of C/EBPβ, C/EBPδ, PPARγ1, and FABP4; however, PPARγ2 and GLUT4 proteins show a slight but statistically significant increase in expression (Fig. 4D).

To confirm our morphological observation that ectopic expression of miRNA378/378* increases lipid droplet size, we measured the total cellular content of triacylglycerols and found them elevated in miRNA378/378* adipocytes compared with controls (Fig. 4E). To assess potential roles for miRNA378/378* on lipid metabolism, we examined effects on 3T3-L1 adipocytes at day 3 of differentiation. Selected gene sets enriched in upregulated and downregulated genes are shown. B: qualified RT-PCR validation of selected genes shows increased expression of KLF15 as a potential mediator of the observed phenotype. *Significant difference (P < 0.05).

Gene expression profiles in control and miRNA378/378* day 3 ST2 adipocytes. We used microarray analysis to better understand the effects of miRNA378/378* on global gene expression in ST2 cells. RNA was purified from control and miRNA378/378*-expressing ST2 cells at day 3 of adipocyte differentiation. The experimental design is illustrated in Fig. 5A. Using gene set enrichment analysis (GSEA), we profiled changes in gene expression that occur in response to overexpression of miRNA378/378* (17, 23). Eight sets of genes appeared to be preferentially upregulated by expression of miRNA378/378* (Fig. 5A). Among those eight sets of genes, three are related to adipocyte differentiation ("upregulated during adipocyte differentiation", "troglitazone up", and "fatty acid metabolism"). Interestingly, four additional sets were linked to mitochondria and their function like "mitochondria," "oxidative phosphorylation," "electron transport chain," and "Krebs cycle up." Finally, a "PGC1-activated genes" set was also upregulated on overexpression of miRNA378/378* (Fig. 5A). Only one group of genes was downregulated on overexpression of miRNA378/378*: "ribosomal proteins" (Fig. 5A). To validate whether predicted genes were indeed up- or downregulated on overexpression of miRNA378/378*, we performed quantitative RT-PCR on RNA samples that had been harvested at day 3 of differentiation. Figure 5B shows that several genes related to adipocyte differentiation and lipid synthesis are indeed upregulated, including KLF15, FABP4, FAS, SCD-1, and resistin.

What are the targets of miRNA378/378* during adipogenesis? To investigate the potential mechanisms by which miRNA378/378* induces adipocyte gene expression and lipid accumulation, we amplified >20 3′-untranslated regions (UTRs) of target genes predicted either by TargetScan4.2 (15) or by Srivastava (unpublished data) and cloned them adjacent to luciferase to create a miRNA378/378* reporter construct. However, none of the predicted 3′UTRs were inhibited on translation by miRNA378/378* (data not shown). Endogenous miRNA378/378* in lipid accumulation. Because ST2 adipocytes have very low endogenous levels of miRNA378/378*, we assessed the consequences of antagonizing miRNA378/378* in day 7 3T3-L1 adipocytes. We used 3′-end-cholesterol-modified chemically engineered oligonucleotides [termed antagonirs (13)] to knock down miRNA378 and/or 378*. 3T3-L1 cells at day 3 of differentiation were
treated with 50 nM of antagonirs 378, 378*, or both for 24 h, and RNA was harvested at day 7. Northern blot analysis shows that, in presence of antagonir 378 or both antagonirs, microRNA 378 is not detectable (Fig. 7A). The same is true for expression levels of miRNA378* when knocked down specifically with antagonist miRNA378* or both (Fig. 7B), suggesting that these antagonirs are useful tools for dissecting downstream effects of miRNA378/378*. Although antagonirs to miRNA378/378* did not cause an observable change to adipocyte morphology by day 7 of differentiation, metabolic labeling of day 7 adipocytes with [14C]acetic acid for 1 h revealed that synthesis of triacylglycerols is decreased by antagonir 378* or antagonirs 378 and 378* compared with a control antagonir (Fig. 7C) by 24 and 20%, respectively. In addition, a trend toward decreased TAG synthesis with antagonir 378 was observed. The amount of phospholipids

![Fig. 6. Effects of miRNA378/378* overexpression on luciferase reporter constructs carrying 3' UTR of predicted target genes. NIH-3T3 cells were transiently transfected with luciferase miRNA sensor genes containing the 3' UTRs of a total of 24 predicted targets. Luciferase activity was normalized to a cotransfected SV40 promoter renilla luciferase construct. Control plasmid or miRNA378/378* retroviral plasmid was cotransfected as indicated. Error bars represent standard deviations (n = 3). The perfect reverse complement sequence of miRNA378 (378WT) was used as a positive control. Differences between control and miRNA378/378* were determined using Student's t-test. **Significant difference (P < 0.01).](http://ajpendo.physiology.org/)

![Fig. 7. Knock-down of miRNA 378/378* and knock-down of Dicer. A: 50 nM 3'-end-cholesterol-modified antisenese oligonucleotides (called antagonirs) were added to 3T3-L1 cells at day 3 of differentiation for 24 h. RNA was harvested at day 7. Northern blot analysis of day 7 adipocytes transfected with control, antagonir 378, antagonir 378*, or both antagonirs, and probed for miRNA378. B: same as in A but probed for miRNA378*. C: day 7 3T3-L1 adipocytes transfected with the indicated antagonirs at day 3 were incubated in presence of [14C]acetic acid for 1 h. Cells were washed once in PBS, and total cellular lipids were extracted by a mixture of methanol and chloroform (1:2), followed by separation by thin-layer chromatography. Quantification of triacylglycerol and phospholipids is shown relative to the control. Data are the average of at least four experiments. *Significant difference (P < 0.05). D: 15 ug of RNA harvested from day 3 ST2 adipocytes overexpressing miRNA378/378*, from control 3T3-L1 day 7 adipocytes, from 3T3-L1 adipocytes where Ago2 or Dicer were knocked down were loaded on a gel for Northern blot analysis. Membrane was probed for miRNA378, miRNA378*, miRNA103, and miRNA21.](http://ajpendo.physiology.org/)
synthesized was also reduced by antagomirs 378 or 378* alone or in combination. In presence of antagomir 132, no change was observed (data not shown).

We also considered whether miRNA 378/378* might act through a mechanism independent of the classical miRNA machinery. In fact, the miRNA378/378* locus represents the head-to-head insertion of two common vertebrate genomic repeats, that form a very long, very stable, and perfectly complementary hairpin loop. Thus we evaluated expression of miRNA378 and miRNA378* in 3T3-L1 adipocytes where Ago2 or Dicer had been knocked down. As expected, miRNA103 and miRNA 21 levels are significantly reduced in cells with decreased expression of Ago2 or Dicer (Fig. 7D). Surprisingly, levels of miRNA378 or miRNA378* are unchanged when expression of Ago2 or Dicer is deficient (Fig. 7D), suggesting that a different processing mechanism is involved in the formation of these miRNAs. It should be noted that genetic inactivation of Dicer in other cellular systems does not completely abolish the generation of mature miRNAs (2). Similarly, Ago2 is not required for the generation of all miRNAs (3).

Transactivation of C/EBP transcription factors by miRNA378/378*. As part of our efforts to better understand the mechanism of miRNA378/378* action, we investigated other potential activities for these miRNAs. Interestingly, we observed that cotransfection of a miRNA378/378* expression vector increased transactivation of the GLUT4 promoter by C/EBPα and C/EBPβ (Fig. 8, A and B). Although expression of miRNA378/378* also increases transactivation of the GLUT4 promoter by C/EBPβ (Fig. 8B), coactivation properties appear specific to the C/EBPs since we did not observe interaction between miRNA378/378* and PPARγ on a PPRE reporter gene (data not shown). Also, no activation of the GLUT4 promoter by miRNA378/378* was observed in the absence of cotransfected C/EBP. These data suggest that effects of miRNA378/378* on lipid synthesis and adipocyte gene expression are mediated, at least in part, through transcriptional coactivation of C/EBP transcription factors.

DISCUSSION

There are a number of published reports asserting that miRNAs modulate adipogenesis (5, 12, 16, 18, 24–26). Several miRNAs have been reproducibly observed to be altered during adipogenesis in vitro and in vivo both in human and mouse systems (such as miRNA103, miRNA107, and miRNA378/378*). Although prior data have suggested that some of these miRNAs play a role in adipocyte differentiation, our data suggest that miRNAs are in addition likely to play a critical regulatory role in adipocyte metabolism. For example, knocking down Ago2 leads to slight increases in adipocyte gene expression but greatly impairs conversion of glucose or acetate to triacylglycerol (Fig. 1). In addition, among several miRNAs that are increased during adipocyte differentiation, we provide evidence that miRNA378/378* may play a regulatory role in lipid metabolism. Located in the first intron of PGC-1β, miRNA378/378* are coordinately expressed during adipogenesis and in the same tissues as PGC-1β (Fig. 3, B and C). Coordinate regulation of expression between an intronic miRNA and the parent gene has been observed previously for miRNA107 and pantothenate kinase, a key regulatory enzyme

in the CoA biosynthetic pathway (26). We show that overexpression of miRNA378/378* during adipogenesis increases triacylglycerol (Fig. 4E) accumulation due to increased de novo lipogenesis (Fig. 4H). Adipogenesis per se did not appear to be influenced by miRNA378/378*; however, increased expression of PPARγ2 and GLUT4 was observed (Fig. 4, B–D).

We introduced point mutations into miRNA 378 or 378* that in theory should have allowed us to express functional 378 and 378* miRNAs singly. However, despite our efforts, we were not able to distinguish whether effects on adipocyte gene expression or lipogenesis were solely due to overexpression of microRNA378 or microRNA378* because disruption of one miRNA influenced the expression and/or stability of the other (data not shown). Knock-down with antagonirs suggests though that both miRNAs make independent but complementary contributions to effects on lipid metabolism (Fig. 7C).

Gene profiling of RNA harvested from adipocytes that overexpressed microRNA378/378* showed that increased fatty acid synthesis could be in part due to elevated expression of fatty acid synthase, which was then confirmed by qRT-PCR (Fig. 5B). We also showed that microRNA378 and 378* could be knocked down in 3T3-L1 adipocytes (Fig. 7, A and B), which resulted in a reduction in triacylglycerol and phospholipids synthesis, at least in presence of antagonir 378* or antagonirs 378 and 378* (Fig. 7C). Interestingly, when Dicer was knocked down, we could still detect microRNAs 378 or 378*, whereas several other microRNAs, as expected, were
essentially not detectable (Fig. 7D). In a traditional search for potential targets for miRNA378 or miRNA378*, we observed that none of the 24 predicted 3’UTRs tested was downregulated in response to miRNA378 or miRNA378* (Fig. 6). Surprisingly, some of the suggested target genes showed an increase in reporter gene expression. Although this observation is consistent with an indirect effect on the transcriptional or translational efficiency of the reporter constructs, it is worthwhile to note that Kahai and colleagues similarly observed an increase in the activation of the predicted miRNA378* target gene nephronecрин (9). Therefore, we considered whether these miRNAs might exert their effects in adipocytes through an atypical mechanism, such as transcriptional coactivators. We observed that, in the presence of microRNA378/378*, C/EBPβ and C/EBPδ activity on the GLUT4 promoter was increased (Fig. 8). Although the mechanism might be through translational repression of a co-repressor, it could also be that the pre- or mature miRNAs directly bind C/EBPs or their transcriptional coregulators to influence transcriptional activity (see supplemental materials available online at the Am J Physiol Endocrinol Metab website). RNAs have been demonstrated to serve as transcriptional coactivators (14, 19), and this possibility is also consistent with our inability to detect a classical target for these miRNAs among the predicted targets tested.

GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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