MicroRNA *let-7* Regulates 3T3-L1 Adipogenesis

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Differentiation of 3T3-L1 cells into adipocytes involves a highly orchestrated series of events including clonal expansion, growth arrest, and terminal differentiation. The mechanisms coordinating these different steps are not yet fully understood. Here we investigated whether microRNAs (miRNAs) play a role in this process. Microarray analysis was performed to detect miRNA expression during 3T3-L1 preadipocyte differentiation. Several miRNAs, including *let-7*, were up-regulated during 3T3-L1 adipogenesis. Ectopic introduction of *let-7* into 3T3-L1 cells inhibited clonal expansion as well as terminal differentiation. The mRNA encoding high-mobility group AT-hook 2 (HMGA2), a transcription factor that regulates growth and proliferation in other contexts, was inversely correlated with *let-7* levels during 3T3-L1 cell adipogenesis, and *let-7* markedly reduced HMGA2 concentrations. Knockdown of HMGA2 inhibited 3T3-L1 differentiation. These results suggest that *let-7* plays an important role in adipocyte differentiation and that it does so in part by targeting HMGA2, thereby regulating the transition from clonal expansion to terminal differentiation. (*Molecular Endocrinology* 23: 925–931, 2009)

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The mouse 3T3-L1 preadipocyte cell line has been used extensively to dissect the molecular mechanisms underlying adipocyte differentiation (1). After reaching confluence, 3T3-L1 cells undergo growth arrest due to contact inhibition. In response to a standard cocktail of hormones, including insulin, cAMP analogs, and glucocorticoids, the cells reenter the cell cycle for several additional rounds of division. This period of clonal expansion is followed by cell cycle exit and terminal differentiation into mature adipocytes (2, 3). The timing of this differentiation process is controlled to a large extent by an elaborate transcriptional cascade involving peroxisome proliferator-activated receptor-γ (PPARγ), CCAAT enhancer-binding protein-α (C/EBPα), C/EBPβ, and C/EBPδ, and E2F transcription factor-1 (E2F1) and -4 (E2F4), among others (3, 4).

MicroRNAs (miRNAs) are endogenous, noncoding RNAs generally 20–24 nucleotides in length that play important roles in many physiological processes including growth, differentiation, and development (5). miRNAs function by binding to the 3′ untranslated regions of target mRNAs, thereby repressing their translation and/or promoting their decay (6). Several groups have examined the expression of miRNAs during adipocyte differentiation. In experiments performed with human preadipocytes, Esau *et al.* (7) showed that the miRNA miR-143 is induced during differentiation and that its inhibition with antisense oligonucleotides blocked differentiation. In a subsequent survey experiment, Kajimoto *et al.* (8) cloned 65 miRNAs from pre- and postdifferentiated 3T3-L1 cells and showed that 21 miRNAs were either up- or down-regulated during differentiation. Finally, using a microarray approach, Wang *et al.* (9) recently identified members of the miR-17-92 cluster of miRNAs as up-regulated during 3T3-L1 preadipocyte differentiation and showed that overexpression of the miR-17-92 cluster accelerated adipocyte differentiation. Taken together, these studies indicate that miRNAs may play a prominent role in regulating adipogenesis.

In this study, we have used a microarray strategy to comprehensively assess miRNA expression during 3T3-L1 cell differentiation. We demonstrate regulation of several miRNAs including *let-7*, which is known to regulate proliferation and differentiation processes in species ranging from *Caenorhabditis elegans* to man (10, 11). Evidence is presented that *let-7* contributes to adipogenesis by governing the transition from clonal expansion to terminal differentiation.

Results

Expression of *let-7* and other miRNAs during adipogenesis

To investigate whether miRNAs are involved in adipocyte differentiation, we examined the expression of 386 miRNAs

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Abbreviations: BrdU, Bromodeoxyuridine; C/EBP, CCAAT enhancer-binding protein; DMI, dexamethasone, 3-isobutyl-1-methylxanthine, and insulin; E2F, E2F transcription factor; FBS, fetal bovine serum; miRNA, microRNA; PPARγ, peroxisome proliferator-activated receptor-γ; RT-qPCR, real-time quantitative PCR; siRNA, small interfering RNA.

during 3T3-L1 differentiation using microarray analysis. Post-confluent 3T3-L1 cells were induced to differentiate using a cocktail of dexamethasone, 3-isobutyl-1-methylxanthine, and insulin (DMI). RNA was prepared from cells at 0, 1, 4, and 7 d after adipogenic induction (Fig. 1A), and small RNAs were purified for use in microarray analysis (Fig. 1B). Among the 386 miRNAs examined, 23 were either increased or decreased more than 1.5-fold during 3T3-L1 differentiation (Fig. 1B). Induction of several of these, including *let-7*, miR-103, miR-143, miR-193, and miR-210, was confirmed by Northern blot analysis (Fig. 1C). The expression of all these miRNAs was up-regulated after 2 d differentiation and maintained at a high level in mature adipocytes. Consistent with our *in vitro* findings, *let-7*, miR-103, miR-143, miR-193, and miR-210 were all expressed in murine white adipose tissue (Fig. 1D). Additional profiling studies revealed that each of these miRNAs was expressed in multiple tissues including brown adipose tissue (Fig. 1D).

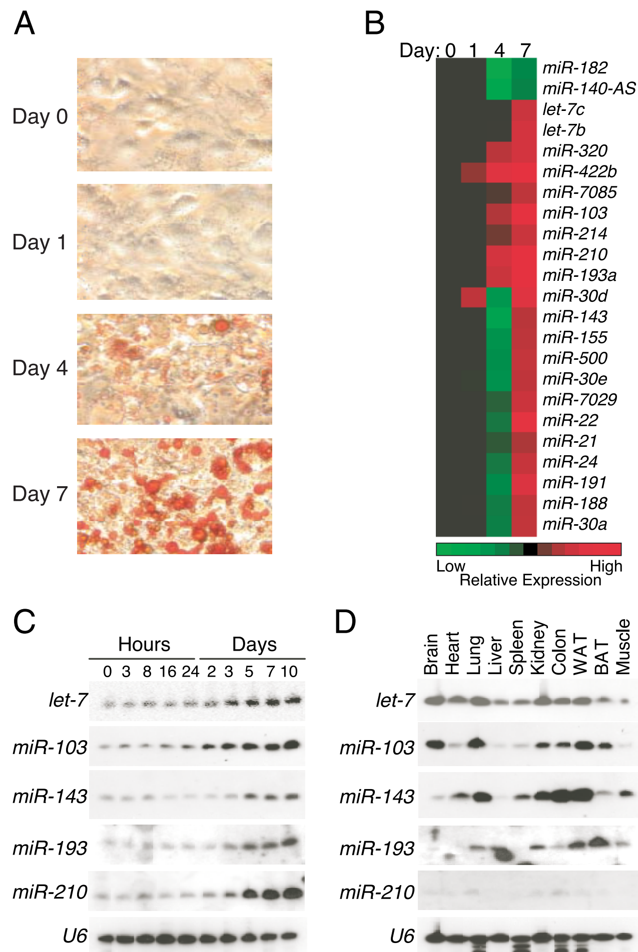

FIG. 1. Expression of miRNAs during 3T3-L1 adipogenesis. A, Lipid accumulation during 3T3-L1 cell differentiation into adipocytes was monitored by Oil Red O staining on the days shown (magnification, ×200). B, miRNAs were extracted from the cells shown in A and subjected to microarray analysis. Normalized log2 expression data of miRNAs that changed at least 1.5-fold during adipogenesis were plotted as a heat map (*red*, higher expression; *green*, lower expression relative to expression on d 0). C and D, Expression of a subset of miRNAs was surveyed further by Northern blot analysis at the time points indicated during 3T3-L1 cell differentiation (C) and in 10 different adult male mouse tissues (D). *U6* small nuclear RNA was used as a loading control.

We chose to focus further experiments on *let-7* given its recently established role in regulating cell fate decisions in *C. elegans* and *Drosophila* (10, 12, 13). *Let-7* expression was increased in 3T3-L1 cells differentiated by treatment with either the DMI cocktail or the PPARγ agonist rosiglitazone (Fig. 2A). Using a third independent assay, *let-7* levels were also increased during insulin-induced differentiation of 3T3-F442A cells into adipocytes (Fig. 2A). In agreement with these findings, *let-7* was abundant in mature adipocytes isolated from mice but barely detectable in preadipocytes (Fig. 2B). *Let-7* was not induced by DMI treatment of NIH3T3 cells, which do not differentiate into adipocytes, nor was it induced during differentiation of C2C12 cells into myotubes (Fig. 2C). These data show that *let-7* induction is not invariably associated with either DMI treatment or differentiation processes.

There are several *let-7* isoforms in the mouse genome that differ in only one to two nucleotides. Because these isoforms cannot be distinguished by Northern blot analysis, we quantified their levels by real-time quantitative PCR (RT-qPCR). Consistent with the microarray and Northern blot data presented above, the most abundant *let-7* isoforms, including *let-7a*, *let-7b*, and *let-7d*, were all up-regulated during 3T3-L1 adipogenesis (Fig. 2D). Interestingly, with the exception of *let-7b*, all the *let-7* isoforms decreased from d 0 to d 1 and then increased. We speculate that this transient dip in *let-7* expression may be permissive for clonal expansion (see below).

Let-7 inhibits 3T3-L1 differentiation

To test whether *let-7* plays a role in 3T3-L1 adipogenesis, pre-*let-7a* oligonucleotide was transfected into 3T3-L1 cells, where it was efficiently converted into mature *let-7a* as confirmed by Northern blot analysis (Fig. 3A). Introduction of ectopic *let-7a* in 3T3-L1 cells before DMI treatment inhibited their differentiation into adipocytes as measured by Oil Red O staining (Fig. 3B) and triglyceride content (Fig. 3C) on d 6 of differentiation. mRNA levels of PPARγ and C/EBPα, two transcription factors whose induction is important for adipocyte differentiation, were significantly lower in cells transfected with *let-7a* compared with cells transfected with control oligonucleotide (Fig. 3D). Likewise, mRNA levels of the mature adipocyte markers, fatty acid binding protein 4 (aP2), and adipsin, were decreased by *let-7a* transfection (Fig. 3D). In contrast, C/EBPβ and C/EBPδ, two genes whose expression is important during the early stages of adipogenesis, were not significantly affected by *let-7a* at the 6-d time point.

Overexpression of *let-7* impairs clonal expansion of 3T3-L1 cells

The treatment of 3T3-L1 cells with an adipogenic stimulus initiates a complex sequence of events including clonal expansion, cell cycle exit, and terminal differentiation (3). Because *let-7* controls exit from the cell cycle in *C. elegans* and *D. melanogaster* (10, 12, 13), we postulated that its up-regulation on d 1 of adipogenesis might play an analogous role in promoting terminal differentiation of adipocytes. To test this hypothesis, 3T3-L1 cells were transfected with either *let-7a* or control oligonucleotides and then allowed to reach confluence, at which point the adipogenic program was initiated with the DMI cock-

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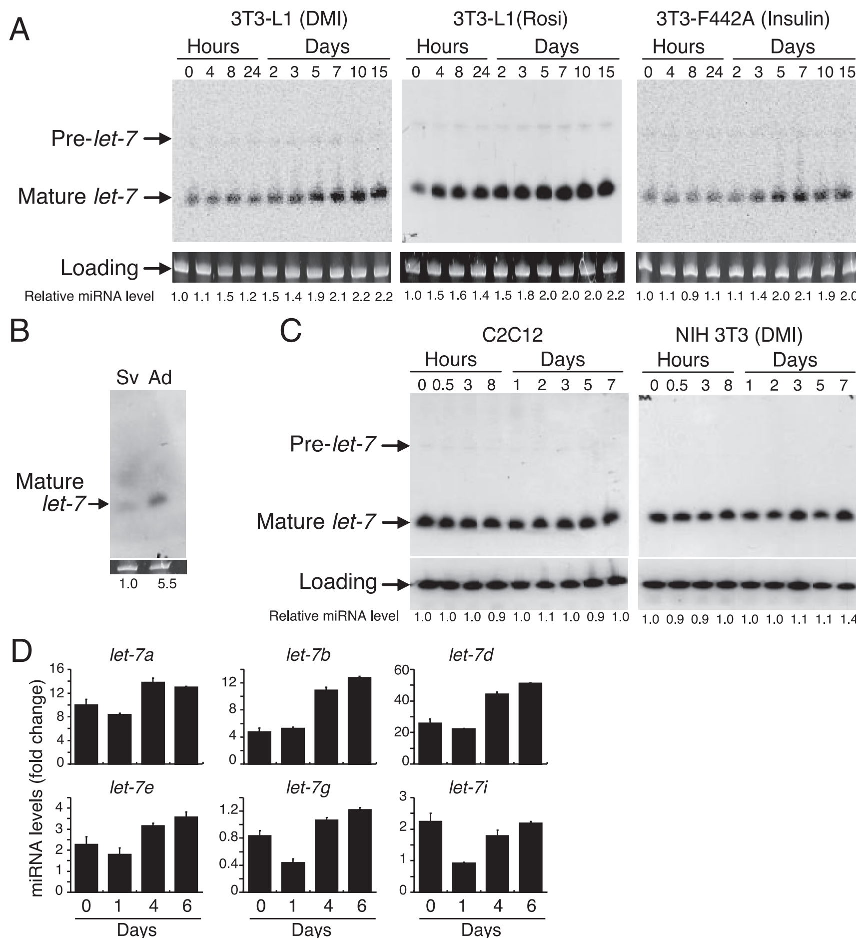

FIG. 2. Expression of *let-7* is specific to the adipogenic differentiation program. A, Postconfluent, preadipocyte cell lines were induced to differentiate by incubation with DMI or rosiglitazone (Rosi) (for 3T3-L1 cells) or with insulin (for 3T3-F442A cells). Northern blot analysis was performed at the indicated time points using a *let-7a* probe that detects both the precursor and mature miRNA. Relative levels of mature *let-7* expression were obtained by normalizing the scanned densities of each band to the *U6* loading control and are represented as fold changes compared with the level at d 0. B, Stromal vascular (SV) preadipocyte and mature adipocyte (Ad) cell fractions were isolated from adipose tissue of adult male mice and subjected to Northern blot analysis. Ethidium bromide staining of total RNA is shown as the loading control. C, C2C12 and NIH3T3 cells were induced to differentiate with a myocyte differentiation medium or with DMI, respectively. Northern blot analysis was performed as in A. D, Total RNA was isolated at the time points indicated during 3T3-L1 cell differentiation and individual subtypes of the *let-7* family were quantified by RT-qPCR normalized for loading to *U6* small nuclear RNA (from triplicate wells, ± SD).

tail. As shown in Fig. 4A, proliferation as measured by bromodeoxyuridine (BrdU) incorporation was reduced in cells transfected with *let-7a* compared with control oligonucleotide, and there was a corresponding decrease in cell number (Fig. 4B). *Let-7a* transfection increased the fraction of cells in the G0/G1 phase while decreasing the fraction in S phase (Fig. 4C), indicating that *let-7* causes cell cycle delay. Furthermore, several cell cycle genes including *cyclin A2*, *cyclin B1*, *cyclin D1* and *D2*, *cyclin E2*, *cdk4*, and *pcna* were down-regulated by *let-7* at the mRNA level (Fig. 4D). These data support a role for *let-7* in blocking clonal expansion during adipogenesis.

Let-7 may regulate clonal expansion and differentiation by targeting HMGA2

In an effort to identify genes through which *let-7* mediates its effect on adipocyte differentiation, microarray analysis was performed using mRNA prepared from 3T3-L1 cells harvested 24 h after transfection with either *let-7a* or control oligonucleotide. Genes whose expression was down-regulated more than 2-fold by *let-7a* are listed in Table 1. Several of these genes, including *Hmga2*, *E2f6*, *Cdc34*, and *Igf2bp1*, have been previously shown to be targets of *let-7* (14–17).

Hmga2 was the gene whose expression was most affected by *let-7a* in 3T3-L1 cells (Table 1). Notably, mice lacking HMGA2 have a striking reduction in adipose tissue (18). Conversely, transgenic overexpression of a truncated HMGA2 in mice resulted in a marked increase in fat tissue, adipose tissue inflammation, and a high incidence of lipomas (19, 20). Thus, HMGA2 was a strong candidate for being a *let-7* target in differentiating adipocytes. In agreement with the microarray data, ectopic *let-7* reduced HMGA2 protein concentrations more than 3-fold in 3T3-L1 cells (Fig. 5A). Interestingly, *Hmga2* mRNA was rapidly induced in 3T3-L1 cells during adipocyte differentiation, with levels peaking 4 h after treatment with the DMI cocktail and returning to basal concentrations on d 2 (Fig. 5B). There was a subsequent increase in HMGA2 protein concentrations, with levels peaking at d 2 (Fig. 5C). HMGA2 and *let-7* expression were inversely correlated during adipocyte differentiation (compare Fig. 2A, *left panel*, with Fig. 5, A and B). Finally, small interfering RNA (siRNA) knockdown of HMGA2 inhibited 3T3-L1 differentiation as measured by *Ap2* and *Pparγ* mRNA levels (Fig. 5D). Taken together, these results strongly suggest that *let-7* regulates 3T3-L1 differentiation in part by targeting HMGA2.

In complementary studies, we also examined *Hmga2* expression during 3T3-F442A cell differentiation. A marked induction of *Hmga2* mRNA occurred within 12 h of insulin treatment followed by a slow return to baseline levels at d 5 (supplemental Fig. S1, published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). Thus, as in 3T3-L1 cells, *Hmga2* and *let-7* expression (Fig. 2) are inversely correlated. Transfection of 3T3-F442A cells with *let-7* oligonucleotide before insulin induction blunted adipocyte differentiation as measured by reduced expression of *Pparg* and *aP2* (supplemental Fig. S1). Although the expression of other adipocyte marker genes including *C/ebpα*, -*β*, and -*δ* and adipsin were not significantly changed, we note that the efficiency with which 3T3-F442A cells were transfected with *let-7* oligonucleotide was low compared with 3T3-L1 cells (data not shown). Overall, these data lend further support to the importance of the *let-7*/*Hmga2* interaction during preadipocyte differentiation.

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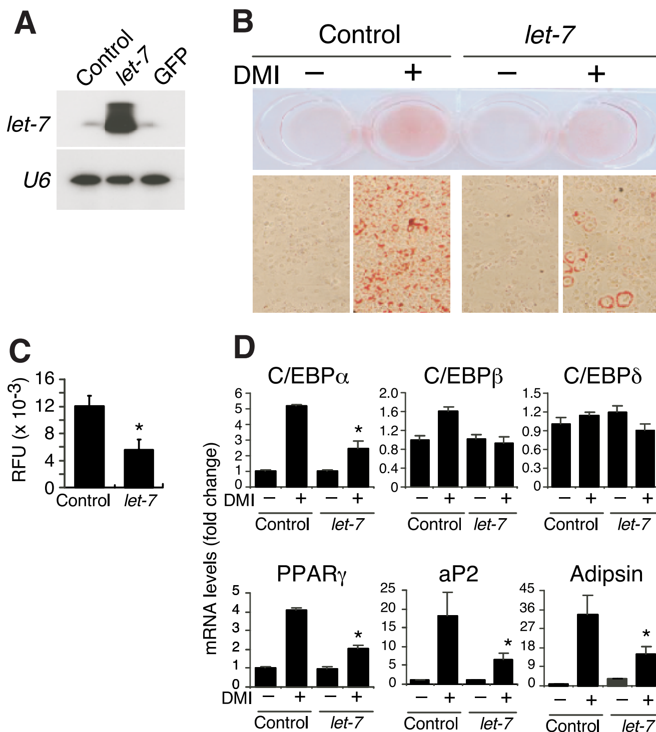

FIG. 3. Ectopic presence of *let-7* inhibits 3T3-L1 adipogenesis. A, 3T3-L1 cells were transfected by electroporation with a control oligonucleotide, *let-7a* precursor oligonucleotide, or a GFP expression plasmid (to monitor transfection efficiency). RNA was isolated 24 h later, and Northern analysis was performed using a *let-7a* probe. The same membrane was reprobed for *U6* expression as a loading control. B, After reaching confluency, transfected cells in A were incubated with (+) or without (−) DMI for 6 d and then stained with Oil Red O. Shown are representative wells of the cultured cells (*top*) and their magnification at ×200 (*bottom*). C, Triglyceride content of cells in B was measured by fluorescence as described in *Materials and Methods* (n = 6 ± SEM; *, $P < 0.01$ *vs.* control oligo). RFU, Relative fluorescence units. D, Expression of various genes from cells in B was measured by RT-qPCR (n = 3 ± SEM; *, $P < 0.01$ *vs.* control oligo).

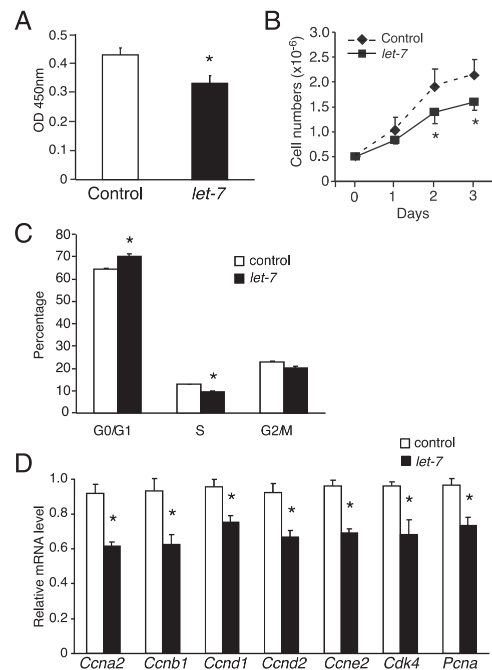

FIG. 4. Ectopic presence of *let-7* inhibits 3T3-L1 clonal expansion. A, BrdU incorporation assay of 3T3-L1 cells 24 h after control oligo or *let-7a* precursor transfection using Roche cell proliferation ELISA kit (n =10 ± SEM; *, $P < 0.01$ *vs.* control oligo). B, 3T3-L1 cells transfected with a *let-7a* precursor or control oligonucleotide were incubated with DMI (d 1), and cell growth was monitored for 3 d (n = 3 ± SEM; *, $P < 0.01$ *vs.* control oligo). C, Cell cycle analysis of 3T3-L1 cells using flow cytometry 24 h after control oligo or *let-7a* precursor transfection (n = 3 ± SEM; *, $P < 0.01$ *vs.* control oligo). D, Expression of cell cycle genes from 3T3-L1 cells transfected with *let-7a* precursor or control oligo was measured by RT-qPCR. Cyclophilin was used as a reference gene (n = 3 ± SEM; *, $P < 0.05$ *vs.* control oligo).

Discussion

The differentiation of preadipocytes into mature fat cells requires a highly orchestrated series of changes in gene expression. Although a transcription factor cascade has been identified that regulates adipocyte differentiation, the molecular mechanisms that coordinate the different phases of adipogenesis are not yet completely understood. In this report, we have identified the miRNA *let-7* as an important regulator of adipogenesis in 3T3-L1 cells. *Let-7* is up-regulated after induction of adipogenesis by either the standard DMI cocktail or the combination of rosiglitazone and insulin. Consistent with these *in vitro* findings, *let-7* is much more abundant in mature adipocytes than preadipocytes derived from mouse epididymal adipose. Notably, introduction of ectopic *let-7* blocks 3T3-L1 cell growth and completely inhibits terminal differentiation as measured by lipid accumulation and marker genes.

How does *let-7* block adipocyte differentiation? Our results demonstrate that *let-7* blocks 3T3-L1 differentiation at the clonal expansion stage. This finding is consistent with previous reports showing that *let-7* represses cell proliferation in cancer cell lines (14, 21). In microarray experiments performed with RNA from 3T3-L1 cells transfected with *let-7a*, *Hmga2* was the most strongly down-regulated RNA, and there was a corresponding decrease in HMGA2 protein concentrations. Previous studies have demonstrated that *let-7* represses HMGA2 expression by binding to six different sites in the *Hmga2* 3′-untranslated region (16). HMGA2 is an architectural transcription factor that alters chromatin structure. Rearrangements of the *HMGA2* gene are frequently observed in benign tumors of mesenchymal origin, including lipomas (22, 23). Interestingly, both gain-of-function and loss-of-function experiments in mice implicate HMGA2 in adipogenesis. Mice lacking HMGA2 have marked reductions in adipose tissue (18). Conversely, transgenic mice overexpressing either full-length or truncated derivatives of HMGA2 develop lipomatosis (19, 20, 24). These findings, together with our data showing that siRNA-mediated knockdown of HMGA2 blocks 3T3-L1 adipogenesis, suggest that HMGA2 is an important target for the effects of *let-7* on adipocyte differentiation. However, because *let-7* regulates numerous genes, its effects on adipocyte differentiation are likely to be complex and involve regulation of additional genes. In this regard, it is interesting that *let-7* reduced the mRNA levels of *E2f6* and *Stat1*. Other members of the E2F family are known to regulate adipogenesis (4), and *Stat1* has been shown to be up-regulated during adipogenesis and to regulate gene expression in mature adipocytes (25–27).

The regulation of miRNA expression during adipocyte differentiation has been examined by several groups. In a survey of miRNA regulation during 3T3-L1 cell differentiation, Kajimoto *et al.* (8) observed increased expression of the *let-7b* isoform,

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TABLE 1. Genes down-regulated by *let-7* in 3T3-L1 cells

Gene	Accession no.	Fold changes
Hmga2	NM_010441	−3.58
Isg15	NM_015783	−3.28
Nfib	NM_001113209	−2.78
Nme4	NM_019731	−2.70
Stat1	NM_009283	−2.65
Parp12	NM_172893	−2.65
Usp18	NM_011909	−2.64
Rnf213	NM_001040005	−2.62
Iigp2	NM_019440	−2.61
Apol9a	XM_128064	−2.60
Mx2	NM_013606	−2.56
Vstm2a	NM_145967	−2.54
Cdsn	NM_001008424	−2.48
Lgals3bp	NM_011150	−2.25
Apol9b	NM_173743	−2.21
Arhgap20	NM_175535	−2.20
Irgm	NM_008326	−2.18
Igtp	NM_018738	−2.17
Ube1l	NM_023738	−2.17
Samd9l	XM_620286	−2.17
E2f6	NM_033270	−2.15
Ai606181	XR_035116	−2.11
Cdc34	NM_177613	−2.09
Plagl2	NM_018807	−2.07
Igf2bp1	NM_009951	−2.00

Total RNA was extracted from 3T3-L1 cells transfected with *let-7* precursor or control oligonucleotides and analyzed 24 h after transfection using a mouse gene expression microarray. Changes of 2-fold or greater are shown.

which was robustly induced in our study. In a study of human adipocyte differentiation, Esau *et al.* (7) saw increased expression of *let-7a* and *let-7c*. These findings support a role for *let-7* in regulating fat cell differentiation in both humans and mice. Surprisingly, there is relatively little overlap in the miRNAs regulated in our study and that of Kajimoto *et al.* with only *let-7b*, miR-143, miR-182, and miR-422b showing similar patterns. The basis for this difference is not known, but it does not appear to be due to marked differences in the differentiation protocols.

Although we were able to observe a strong gain-of-function phenotype in 3T3-L1 adipocytes with *let-7a*, we did not observe a reciprocal phenotype in knockdown experiments performed with 2′-*O*-methyl oligoribonucleotide inhibitors against the various *let-7* isoforms (data not shown). Functional redundancy among miRNAs is well documented (28), and thus the presence of many *let-7* isoforms is a likely explanation for the lack of an effect we seen in these experiments. In this regard, we note that transfection of *let-7b* had the same effect as *let-7a* on 3T3-L1 cell differentiation (data not shown).

In summary, we provide evidence that *let-7* regulates adipocyte differentiation. We propose that *let-7* does this in part by targeting the transcription factor HMGA2, thereby promoting the transition of preadipocytes from clonal expansion to terminal differentiation (Fig. 6). The role of *let-7* in mediating this transition switch is reminiscent of its developmental role in *C. elegans*, where *let-7* regulates the transition from the larva to adult (10, 11). Our findings suggest that *let-7* may have important implications in obesity and other forms of metabolic disease in which there are alterations in the amount and/or function of adipose tissue.

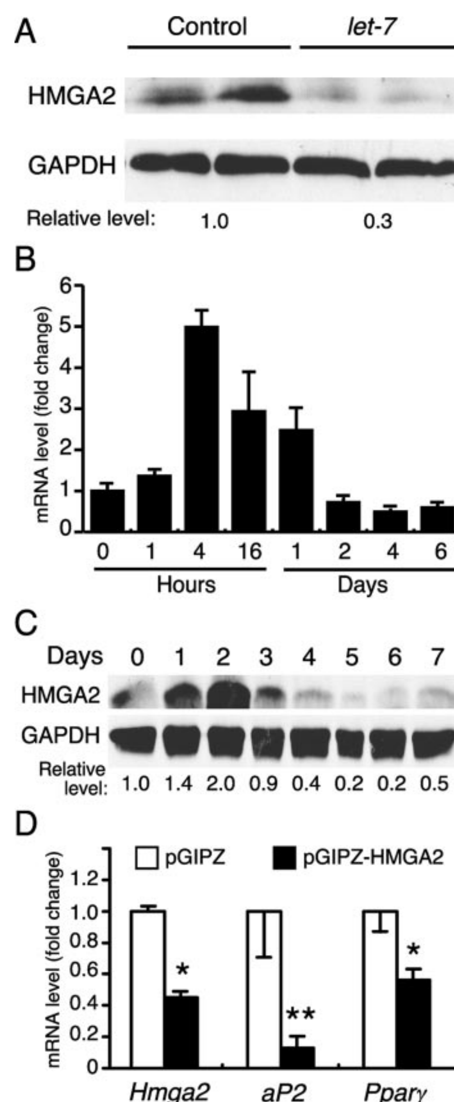

FIG. 5. *Let-7* regulates adipogenesis by decreasing HMGA2 expression. A, HMGA2 protein levels in 3T3-L1 cells were assayed by Western blot analysis 48 h after transfection with a *let-7a* precursor or control oligonucleotide. GAPDH is shown as a loading control. B, *Hmga2* mRNA levels were evaluated by RT-qPCR at the indicated time points after treatment of 3T3-L1 cells with DMI (shown are data from triplicate samples ± SD). C, HMGA2 protein levels were evaluated by Western blot analysis at the indicated time points after treatment of 3T3-L1 cells with DMI. D, mRNA expression of various adipogenic genes was assayed by RT-qPCR in differentiated 3T3-L1 adipocytes that were treated with siRNAs for *Hmga2* or a control sequence (n = 3 ± SEM; *, $P < 0.05$; **, $P < 0.01$). *Hmga2* levels were monitored 24 h after siRNA transfection and *aP2* and *Pparγ* levels were monitored at 6 d after siRNA transfection and incubation with DMI.

Materials and Methods

Cell culture and differentiation

3T3-L1 cells were cultured in a 5% CO_2 humidified atmosphere in DMEM/high glucose with L-glutamine (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin. For DMI differentiation, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 5 μg/ml insulin were added to the culture medium 2 d after the cells reached confluence. After 48 h, the culture medium was replaced with DMEM containing 10% FBS and 5 μg/ml insulin. The culture medium was replaced every 48 h until the preadipocytes differentiated into mature adipocytes. For differentiation with rosiglitazone, confluent 3T3-L1 preadipocytes were cultured in DMEM supplemented with 10% FBS and 5 μM rosiglita-

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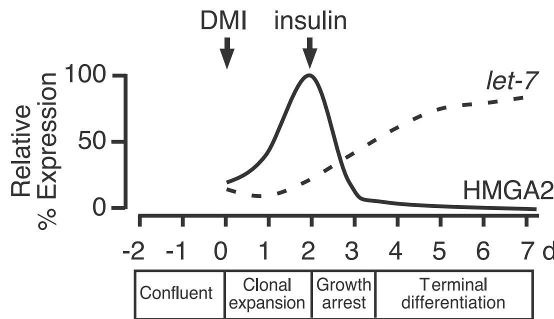

FIG. 6. Schematic model of *let-7* and HMGA2 regulation during 3T3-L1 adipogenesis. Induction of adipocyte differentiation by DMI initially results in cell growth and clonal expansion that requires HMGA2 expression. Further stimulation of these cells by insulin results in a *let-7*-dependent decrease in HMGA2 expression, which in turn leads to growth arrest and terminal differentiation.

zone. Medium was replaced every 48 h. For 3T3-F442A differentiation, confluent cells were cultured in DMEM/high glucose with 10% FBS, and differentiation induced with DMEM/high glucose supplemented with 10% FBS and 5 μg/ml insulin. For C2C12 differentiation, confluent myoblasts were cultured in DMEM supplemented with 20% FBS, and differentiation was induced by subsequently culturing the cells in DMEM supplemented with 2% horse serum.

Northern blot analysis

Each sample, consisting of 10 μg total RNA in 2 × sample loading buffer, was incubated at 65 C for 10 min, chilled on ice for 3 min, and loaded on a 15% polyacrylamide gel containing 7 M urea (Invitrogen). Electrophoresis was performed at 250 V for 1 h in 1× Tris-borate-EDTA buffer. RNA was transferred onto Hybond N+ membrane (Amersham Biosciences, Piscataway, NJ) at 20 V for 45 min in 0.5× Tris-borate-EDTA. RNA was cross-linked to the membrane with a UV-linker. Starfire probes for each miRNA were labeled with [α-^{32}P]dATP (Amersham) using the Starfire kit (IDT, Coralville, IA). Labeled probe was separated from free [α-^{32}P]dATP using Sephadex G-25 columns (Roche, Indianapolis, IN). Hybridization was done at 45 C in 0.2 M Na_2HPO_4, 7% sodium dodecyl sulfate buffer overnight. Membranes were washed with 2× standard saline citrate/0.1% sodium dodecyl sulfate twice and exposed to x-ray film. Ethidium bromide staining or U6 blotting were used as loading controls.

RT-qPCR

Extraction and quantitation of mRNA levels by RT-qPCR was performed as described using an ABI 7900HT sequence detection system and the SYBR green assay (29). The following primers were used for quantitative real-time PCR (30, 31): C/EBPα, gacatcagcgcctacatcga (forward) and tcggctgtgctggaagag (reverse); C/EBPβ, atttctatgagaaaagaggcgtatgt (forward) and aaatgtcttcactttaatgctcgaa (reverse); C/EBPδ, ttccaaccccttccctgat (forward) and ctggagggtttgtgttttctgt (reverse); Adipsin, aggacgacctcattctttttaagc (forward) and acttctttgtcctcgtattgcaa (reverse); aP2, gcctgccactttccttgtg (forward) and gacatcagcgcctacatcga (reverse); PPARγ, caagaataccaaagtgcgatcaa (forward) and gagctgggtcttttcagaataataag (reverse); CyclinA2, ttccacttggctctctacacagt (forward) and ccagtctgttgtgccaatga (reverse); CyclinB1, tggcgctcagggtcacta (forward) and cgcctgccatactgacctt (reverse); CyclinD1, gccgagaagttgtgcatctaca (forward) and tgttcaccagaagcagttccatt (reverse); CyclinD2, cgtacatgcgcaggatggt (forward) and aattcatggccagaggaaagac (reverse); CyclinE2, caccaagaagcatcagtatgagatt (forward) and gggctgattcctccagaca (reverse); Cdk4, cctgccggttgagaccat (forward) and agaaaatccaggccgcttag (reverse); Pcna, cgaaggcttcgacacatacc (forward) and ggacatgctggtgaggttca (reverse); and Hmga2, aacctgtgagccctctcctaag (forward) and gccgtttttctccaatggtc (reverse).

miRNA concentrations were measured by RT-qPCR using the TaqMan MicroRNA Assay Kit (Applied Biosystems, Foster City, CA). miRNAs were reverse transcribed using miRNA-specific primers and RT-qPCR with TaqMan probes was done to detect mature miRNAs. Reverse transcription and RT-qPCR were done according to the manufacturer's instructions with U6 as a loading control. U6 primers and probe were ctcgcttcggcagcaca (forward), aacgcttcacgaatttgcgt (reverse), and ctctgtatcgttccaattttagtat (probe).

Microarray

For miRNA microarray, miRNAs were isolated from total RNA using a FlashPAGE fractionator (Ambion, Austin, TX). Total RNA (100 μg) was loaded onto FlashPAGE precast gels and small size RNAs collected after electrophoresis at 80 V for 12 min. Small size RNA was purified using the FlashPAGE Reaction Clean Up Kit (Ambion). Small size RNA (1 μg) was used for microarray on a custom slide containing 386 probes for human, mouse, and rat miRNAs from the mirVana miRNA Probe Set (Ambion). Slides were scanned and analyzed using GeneTraffic software.

For mRNA microarray, Illumina Beadarrays (Illumina, San Diego, CA) were used. Total RNA was extracted from 3T3-L1 cells 24 h after transfection with either *let-7* or control oligonucleotides.

Western blot analysis

3T3-L1 cells were scraped, washed with PBS, and lysed with M-Per Mammalian Protein Extraction Reagent (Pierce, Rockford, IL). Proteins were separated by 15% SDS-PAGE and transferred to cellulose membranes. Membranes were immunoblotted with a 1:200 dilution of anti-HMAG2 rabbit antibody (Biocheck, Foster City, CA) followed by a 1:5000 dilution of antirabbit horseradish peroxidase secondary antibody. The same membrane was blotted with a 1:10,000 dilution of anti-GAPDH horseradish peroxidase-conjugated antibody (Sigma-Aldrich, St. Louis, MO) as a loading control.

Oil Red O staining

Oil Red O staining of 3T3-L1 cells was done as previously described (32). Briefly, cells were washed twice with PBS and fixed in 10% formaldehyde in PBS for 15 min. Cells were stained in fresh Oil Red O solution (60% Oil Red O stock solution consisting of 0.5% Oil Red O in isopropanol and 40% H_2O) for at least 1 h. After staining, the cells were washed with 60% isopropanol and then with H_2O.

Triglyceride content measurement

Triglyceride content in cells was measured using AdipoRed assay reagent (Cambrex, Walkersville, MD) according to the manufacturer's instructions. Assays were performed in a 96-well format and fluorescence measured in a Victor 1420 Multilabel Counter (PerkinElmer, Waltham, MA) at an excitation wavelength of 485 nm and an emission wavelength of 572 nm.

BrdU incorporation assay

3T3-L1 cells were transfected with control oligo or *let-7* precursor and cultured in a 96-well plate. At 24 h after transfection, BrdU was added to culture medium to a final concentration of 10 μM. After BrdU labeling for 2 h, medium was removed and BrdU incorporation was measured using the cell proliferation ELISA kit (Roche) and a 1:200 dilution of anti-BrdU-peroxidase conjugate antibody. Reactions were stopped with 1 M H_2SO_4 and absorbance measured at 450 nm.

Cell cycle analysis

3T3-L1 cells transfected with control or *let-7* precursor oligonucleotides were cultured in six-well plates. Twenty-four hours later, cells were harvested and washed with PBS. Cells were fixed with cold 70% ethanol, stained in 50 μM propidium iodide, and subjected to cell cycle analysis using FACScan (Becton Dickinson, San Jose, CA). Results were analyzed using Flowjo software.

Transfection assays

3T3-L1 cells were trypsinized and pelleted at 90 × *g* for 10 min. Cell pellets were resuspended in 100 μl Nucleofector buffer (Nucleofector

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Kit V) (Amaxa Biosciences, Gaithersburg, MD) with 2 μg of either *let-7* precursor (Ambion), control oligonucleotide, or expression plasmid. Nucleofection was performed using the Nucleofector I device (Amaxa Biosciences) and program T-30.

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