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Depletion of Human Micro-RNA miR-125b Reveals That It Is Critical for the Proliferation of Differentiated Cells but Not for the Down-regulation of Putative Targets during Differentiation*

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Micro-RNAs are small non-coding RNAs that regulate target gene expression post-transcriptionally through base pairing with the target messenger RNA. Functional characterization of micro-RNAs awaits robust experimental methods to knock-down a micro-RNA as well as to assay its function *in vivo*. In addition to the recently developed method to sequester micro-RNA with 2′-*O*-methyl antisense oligonucleotide, we report that small interfering RNA against the loop region of a micro-RNA precursor can be used to deplete the micro-RNA. The depletion of miR-125b by this method had a profound effect on the proliferation of adult differentiated cancer cells, and this proliferation defect was rescued by co-transfected mature micro-RNA. This technique has unique advantages over the 2′-*O*-methyl antisense oligonucleotide and can be used to determine micro-RNA function, assay micro-RNAs *in vivo*, and identify the contribution of a predicted micro-RNA precursor to the pool of mature micro-RNA in a given cell. miR-125b and *let-7* micro-RNAs are induced, whereas their putative targets, *lin-28* and *lin-41*, are decreased during *in vitro* differentiation of Tera-2 or embryonic stem cells. Experimental increase or decrease of micro-RNA concentrations did not, however, affect the levels of the targets, a finding that is explained by the fact that the down-regulation of the targets appears to be mostly at the transcriptional level in these *in vitro* differentiation systems. Collectively these results reveal the importance of micro-RNA depletion strategies for directly determining micro-RNA function *in vivo*.

Micro-RNAs (miRNAs)[1] are a class of small non-coding RNAs that are processed by Dicer from precursors with a characteristic hairpin secondary structure (1). It is now known that there are hundreds of evolutionarily conserved miRNAs in plants and animals, but their functions are not well understood (2–16). Current knowledge of miRNA function is mainly based on the result from the founding members of miRNA, *lin-4* (2) and *let-7* (17) in *Caenorhabditis elegans*. Both are heterochronic genes whose mutations result in developmental defects (for review see Ref. 18). Up-regulation of *lin-4* during the first larval stage (L1) triggers the subsequent developmental stages by translational repression of *lin-14* and *lin-28*, both of which contain sites of partial complementarity to *lin-4* in the 3′-untranslated region (19, 20). Likewise, *let-7* is induced during development and translationally repress *lin-41* through partially complementary base-pairing between *let-7* and target sequences in the 3′-untranslated region of *lin-41* (17, 21–23).

The study of a miRNA function has been hampered by the lack of an efficient method to deplete a miRNA, especially in mammals where genetic experiments are hard to perform. Kawasaki and Taira (24) reported a knock-down of a miRNA by a siRNA against the loop region of the miRNA precursor, but the paper was retracted (25). Recently, 2′-*O*-methyl antisense oligonucleotide against a miRNA was reported to specifically knock-down the miRNA (26, 27). Despite the successful knock-down of a miRNA *in vitro* and *in vivo*, this method has several limitations. First, a direct measurement of the depletion of a miRNA is difficult, because 2′-*O*-methyl antisense oligonucleotide binds to the miRNA and sequesters it from its target rather than induces its degradation. In addition, even a slight contamination of 2′-*O*-methyl antisense oligonucleotide in an RNA preparation interferes with assays to measure the miRNA level, such as Northern blot, primer extension assay, or RNase protection assay.[2] Therefore, the only possible way to confirm the decrease of a miRNA is to employ a very indirect and cumbersome assay, measuring the level of expression of a reporter gene containing the target sequence of the miRNA. In addition, although rescuing the depletion effect by adding back miRNA is the most stringent control against the nonspecific effects of miRNA depletion, the knock-down phenotype cannot be rescued in the presence of the 2′-*O*-methyl antisense oligonucleotide.

In addition to 2′-*O*-methyl antisense oligonucleotides to miRNAs, we now show that siRNA against the loop region of a pre-miRNA can be used to specifically down-regulate miRNAs. The unique advantages of this method over 2′-*O*-methyl antisense oligonucleotide will be discussed. Using both these methods, we show that miR-125b, putative homolog of *lin-4* in *C. elegans* (9), is critical for the proliferation of differentiated human cell lines, providing a new example of the biological role of a mammalian miRNA. In addition, we could restore miRNA function in the miRNA-depleted cells by the introduction of single-stranded RNA representing the miRNA sequence.

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[1] The abbreviations used are: miRNA, micro-RNA; siRNA, small interfering RNA; NP, neuronal precursor; EC, embryonic carcinoma; ES cell, embryonic stem cell; *gapdh*, glyceraldehyde-3-phosphate dehydrogenase gene; EB, embryoid bodies.

[2] Y. S. Lee and A. Dutta, unpublished data.

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Although the exact regulatory mechanism of mammalian *lin-28* and *lin-41* in *in vitro* differentiation systems or the role of the miRNAs in this regulation has not yet been described, a translational control of mammalian Lin-28 has been hypothesized similar to that seen in *C. elegans* (28–30). The hypothesis is based on the following observations; 1) the evolutionary conservation of *lin-4* and *let-7* miRNAs and their targets *lin-28* and *lin-41*, 2) the presence of putative target sequences with imperfect complementarity to miR-125b and *let-7* within the 3′-untranslated region of *lin-28* and *lin-41*, and 3) induction of miR-125b and *let-7* and the attendant decrease of Lin-28 protein during differentiation. This assumption was supported by an experiment with artificial luciferase construct fused to the *let-7* target sequence that is naturally found in the 3′-untranslated region of mammalian *lin-28* mRNAs (30). Using the methods developed above to decrease or increase miR-125b or *let-7*, we, however, find that the decrease of *lin-28* and *lin-41* during differentiation appears independent of the levels of these miRNAs, demonstrating the importance of miRNA depletion strategies to identify real targets of miRNAs *in vivo*. The negative result is probably explained by the fact that the decrease of *lin-28* and *lin-41* is mainly at the transcriptional stage, suggesting a variation in the paradigm of *lin-28* and *lin-41* regulation during differentiation of mammalian cells *in vitro*.

EXPERIMENTAL PROCEDURES

RNA Isolation, Northern Blotting, and Primer Extension Assay—Total RNAs from various cell lines were isolated with Trizol reagent (Invitrogen) according to the manufacturer's instructions. Total RNAs from human cervix, prostate, and brain were purchased from Ambion. Multiple tissue Northern blot (human MTN blot II) was purchased from Clontech.

Primer extension assay (31) was performed with Superscript II reverse transcriptase (Invitrogen) as per the manufacturer's instructions, with minor modifications. 20 μl of extension reaction contained 0.1 pmol of the γ-^{32}P-labeled primer and 10 μg of total RNA. In the case of the 5 S rRNA extension reaction, 1 μg of total RNA was used. Extension reaction at 50 °C for 50 min was quenched by the addition of 20 μl of sequencing gel loading buffer (32). Extension products were resolved from the primer by electrophoresis in an 18% polyacrylamide gel with 7 M urea.

The sequences of the primers are as follows: let-7a-3-ext, 5′-GCCCCAAACTATACAACCTACTAC-3′; miR-125a-ext, 5′-TCACAGGTTAAAGGGTCTCA-3′; miR-125b-ext, 5′-CATCACAAGTTAGGGTCTCA-3′; 5 S rRNA-ext, 5′-GATCGGGCGCGTTCAGGGTGGTAT-3′; miR-16-ext, 5′-ACTACGCCAATATTTACGTGCT-3′; miR-15a-ext, 5′-AATCCACAAACCATTATGTGCT-3′.

siRNAs, 2′-O-Methyl Oligonucleotides, miRNA, and Transfections—siRNA duplexes and 2′-*O*-methyl oligonucleotides were synthesized by Dharmacon. Transfection was performed with Oligofectamine reagent (Invitrogen) as per the manufacturer's instructions. Unless indicated, 960 nM of siRNA duplexes designed against the loop regions of miRNAs was used for the transfection into Tera-2 or PC-3 cell lines (ATCC number CRL-1435). In the case of 2′-*O*-methyl antisense oligonucleotides against miR-125b or GL2, 160 nM oligonucleotides were transfected into PC3 or HeLa cells.

The target sequences for siRNA duplexes are as follows: siRNA against miR-125b-1 (si125b-1), 5′-AACCGUUUAAAUCCACGGGUU-3′; siRNA against miR-125b-2 (si125b-2), 5′-AAGGUAUUUUAGUAACAUCAC-3′; siRNA against miR-16–1 (si16–1), 5′-AAGAUUCUAAAAUUAUCUCCA-3′; siRNA against miR-16–2 (si16–2), 5′-GGCGUAGUGAAAUAUAUAU-3′; siRNA against miR-15a (si15a), 5′GUGGAUUUUGAAAAGGUGC3′.

RNA oligonucleotides of mature let-7a-3 and miR-125b sequences were synthesized by Dharmacon. Transfection was performed as described above but at the final concentration of 2 μM. During long-term experiment with multiple transfections, we performed the first three transfections every day and the transfections from the fourth and later every other day.

Cell Proliferation Assay—Cell growth was measured with CellTiter 96 non-radioactive cell proliferation assay kit (Promega).

Cell Culture, Retinoic Acid Treatment, and Stem Cell Differentiation—Tera-2 cells (ATCC number HTB-106) were treated with 5 μM all-trans-retinoic acid (Sigma) on a gelatin-coated tissue culture dish (33). Culture medium with retinoic acid was refreshed every other day. The cells were cultured in the presence of retinoic acid for 20 days, with subsequent cultivation for an additional 40 days without retinoic acid.

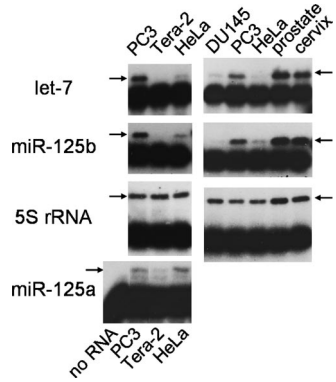

FIG. 1. **Quantitative measurement of miRNAs from various cell lines and tissues.** Primer extension assays were performed with RNAs from various cell lines and tissues as described under "Experimental Procedures" to detect *let-7*, miR-125a, miR-125b, and 5S rRNA as a control. The extension products are indicated by *arrows*.

Neural induction from mouse embryonic stem (ES) cell line D3 (ATCC number CRL-11632) was maintained and differentiated using the five-stage procedure as described (34). Briefly, undifferentiated ES cells were cultured on gelatin-coated dishes in supplemented Dulbecco's modified Eagle's medium (Invitrogen) and differentiated into embryoid bodies (EBs) on nonadherent bacterial dishes. The EBs were then plated onto an adhesive tissue culture surface, and neuronal precursor (NP) cells were selected in serum-free medium. After 6–10 days of selection, cells were trypsinized, and nestin$^+$ NP cells were expanded in N2 medium supplemented with 1 μg/ml laminin (Sigma) and 10 ng/ml bovine fibroblast growth factor (R & D Systems). After expansion for 4 days, bovine fibroblast growth factor was removed to induce differentiation to neuronal phenotypes.

Nuclear Run-on Assay—ORFs of *lin-28* and *lin-41* were PCR-amplified from Tera-2 cDNA. 0.5 μg of each fragment was slot-blotted onto a positively charged Nylon membrane (Nytran from Schleicher & Schuell).

Nucleus isolation and run-on transcription reaction were performed as described (35) with modifications. After the transcription reaction, Trizol reagent (Invitrogen) was added, and an RNA probe was prepared according to the manufacturer's instructions for Trizol reagent. Membranes were probed with ^{32}P-labeled run-on RNA for 16 h at 68 °C in hybridization buffer containing 0.25 M Na_2HPO_4 (pH 7.2), 1 mM EDTA, and 7% (w/v) SDS. Membranes were washed twice in 2× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate) at room temperature for 15 min each followed by a wash in 0.2× SSC and 1% (w/v) SDS at 65 °C for 20 min. The blot was exposed to a PhosphorImager Storm 860 (Amersham Biosciences) and quantitated.

Recombinant Lin-28 and Antiserum—cDNA containing full-length human Lin-28 protein (209 amino acids) was cloned into pET-23a(+) (Novagen). Recombinant Lin-28 with hexahistidine tag was expressed in BL21(DE3), purified through nickel-charged chelating Sepharose column (Amersham Biosciences), and used for raising antiserum in rabbit. Antibody was proven specific by the following criteria. It recognized the recombinant Lin-28 and a single protein band of expected size of Lin-28 in Tera-2 cell extract, whereas preimmune serum did not detect a protein of appropriate size (data not shown). In addition, it failed to detect protein in the extract of PC-3 cells where *lin-28* mRNA was not detectable.

RESULTS

Expression of let-7 and miR-125b in Differentiated Cells—Primer extension assays were employed to quantitate miR-125a and miR-125b, miRNAs that are the putative *lin-4* homologs in mouse and human (9). miR-125a is detectable in PC-3, HeLa, Tera-2 (Fig. 1), and other cell lines (data not shown). Two other miRNAs, miR-15 and miR-30, are present in a constant amount in these three cell lines (data not shown). In contrast, miR-125b level is highest in PC-3, moderate in HeLa and other cell lines, and undetectable in Tera-2 (Fig. 1; data not

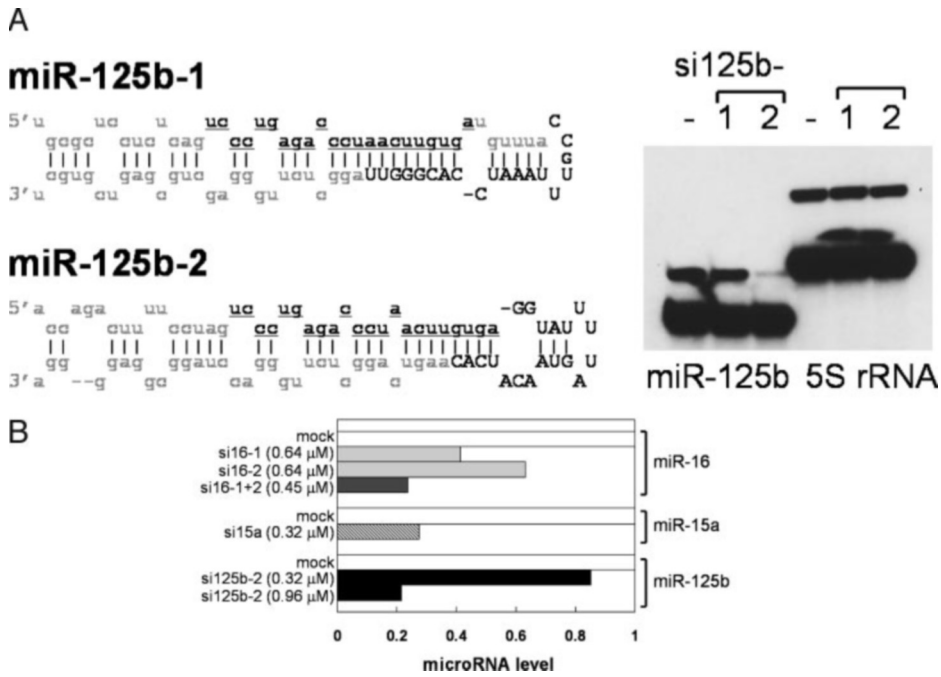

FIG. 2. **Depletion of miR-125b and two other miRNAs by siRNAs against the precursors.** *A*, the two hairpin precursors from predicted for miR-125b are shown in the *left panel*. *Underlined black*, mature miR-125b sequence. *Capitals black*, the target sequences of siRNAs. The *right panel* shows the primer extension assays to detect miR-125b or 5S rRNA control after three rounds of transfection at 24-h intervals with each siRNA. *B*, quantitation of miRNAs (*right*) by primer extension after transfection of the siRNAs (*left*). The concentration of each siRNA during the transfection is indicated in *parentheses*. The relative intensity of the miRNA primer extension product was normalized to that of the 5 S rRNA control and compared with the corresponding signal from mock-transfected cells.

shown). Tera-2 is an embryonic carcinoma (EC) cell line representing undifferentiated cells. The higher expression of miR-125b in differentiated cells compared with undifferentiated cells is also seen with *let-7* (9, 21, 36–38). Tissues from prostate, cervix, and brain have higher levels of these miRNAs than the corresponding cell lines (Fig. 1). Thus, in all cell lines and tissues examined, human *let-7* and miR-125b are expressed specifically in differentiated cells or tissues, reminiscent of the higher expression seen later in development in flies and worms.

The Depletion of miR-125b-2 in Differentiated Cancer Cells Inhibits Cell Proliferation—We first attempted to decrease miR-125b by siRNAs against the precursor of the mature miRNA. The hairpin-shaped pre-miRNA is generated in the nucleus by the action of Drosha (39) but is exported to the cytoplasm (40), where it is processed by Dicer to release the mature miRNA (31, 41–44). The pre-miRNA is, therefore, expected to be susceptible to siRNAs against the loop of the hairpin. There are two predicted precursors of miR-125b in humans, miR-125b-1 and -2. Both precursors share identical mature miRNA sequence, but the sequences flanking the mature miRNA are different (Fig. 2*A*, *left panel*). Two synthetic siRNAs were designed against the loop regions of miR-125b-1 and -2 (si125b-1 and si125b-2; Fig. 2*A*, *left panel*). After transfection of these siRNAs into PC-3 cells, the miR-125b level was measured by a primer extension assay. miR-125b was greatly reduced by the si125b-2 but not si125b-1 (Fig. 2*A*, *right panel*). This observation suggests that most of the mature miR-125b is derived from the precursor miR-125b-2 in PC-3 cells.

To test if this strategy to knock-down miRNAs can be applied to other miRNAs, siRNAs against additional pre-miRNAs were transfected into PC-3 cells. miR-16 has two predicted precursors, miR-16–1 and -2. Transfection of siRNA against miR-16–1 and -2 (si16–1 and -2) decreased the mature miRNA to 40 and 60%, respectively (Fig. 2*B*). Thus, ~60% of mature miR-16 is derived from the precursor -1, and the rest is from precursor -2. Consistent with this interpretation, co-transfection of the two siRNAs against miR-16 decreased the level of miR-16 to ~20%, which is close to the background level. Efficient knock-down of miR-15a was also achieved by the transfection of siRNA against the loop region of the corresponding pre-miRNA.

Titration of siRNA dose revealed that siRNA concentration of 0.32–1 μM was required to decrease the miRNA levels (Fig. 2*B*). This dose is higher than the amount of siRNA required to knock down a typical mRNA (0.02–0.3 μM). The lower the efficiency of the siRNA-directed degradation of a miRNA precursor could be due to the short length and/or transient nature of miRNA precursors. Indeed, the precursors of miRNA were never detected in our primer extension assays, presumably because miRNA precursors are quickly processed into mature miRNA. The inefficiency of siRNA against pre-miRNA might account for the failure of this strategy in two other cell lines, HeLa and DU 145. Nevertheless, the results dictate that an siRNA can be used to deplete a miRNA in at least some cell lines like PC-3.

To investigate the biological effect of miR-125b, cell proliferation was measured after depletion of miR-125b by RNA interference (Fig. 3*A*). si125b-2, but not si125b-1, reduced proliferation of PC-3 cells. Growth was restored by the co-introduction of a synthetic mature miR-125b that was designed not to anneal to si125b-2, demonstrating that growth suppression is specifically caused by the depletion of miR-125b. This result also indicates that miRNA function can be provided to cells by transient transfection of single-stranded RNA. Tera-2 EC cells did not express miR-125b, and accordingly, si125b-2 did not suppress growth of Tera-2 cells. To eliminate a trivial explanation, we demonstrated that the RNA interference machinery is intact in Tera-2 cells by transfecting siRNA against *ORC2* in this cell line and measuring Orc2 protein level (Fig. 3*B*). Thus, the failure of si125b-2 to suppress Tera-2 cell proliferation is not due to absence of the siRNA machinery and provides an additional control against nonspecific effects of si125b-2 in PC-3 cells.

To confirm that the proliferation defect in Fig. 3*A* is specifically caused by miR-125b depletion and to test if this defect is seen in cell lines other than PC-3, the experiment was repeated after transfection of 2′-*O*-methyl antisense oligonucleotide against miR-125b. Again, depletion of miR-125b by 2′-*O*-methyl oligonucleotide resulted in the reduction of proliferation, whereas the transfection of 2′-*O*-methyl oligonucleotide against GL2 did not (Fig. 3*C*). In addition, this proliferation defect was observed not only in PC-3 cells but also in HeLa cells. Because identical results were obtained from two independent methods and each experiment was performed with stringent controls, it is clear that miR-125b is essential for the

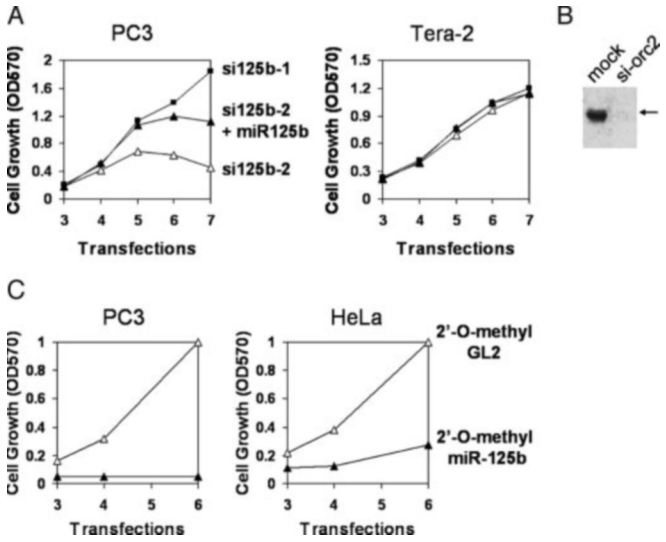

FIG. 3. **The effect of miR-125b depletion on the proliferation of differentiated cells.** *A*, after transfection of miR-125b and/or siRNA against miR-125b into PC-3 and Tera-2 cells, cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The first three transfections were done at 24-h intervals, and the fourth transfection onward was at 48-h intervals. The *x* axis indicates the number of transfections, and the *y* axis indicates the absorbance value at 570 nm. *B*, immunoblot of Orc2 protein (indicated by the *arrow*) from Tera-2 cells transfected without (*mock*) or with siRNA against *orc2* (*si-orc2*). *C*, after transfection of 2′-*O*-methyl antisense oligonucleotide against miR-125b and GL2 control, cell proliferation of PC-3 and HeLa was measured as described in *panel A*.

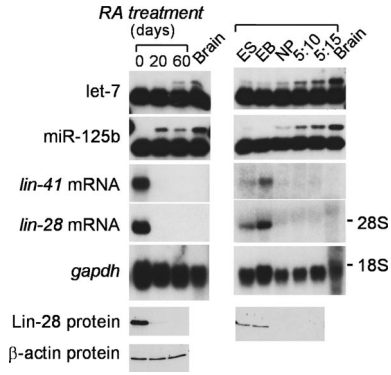

FIG. 4. **Expression of miR-125b, *let-7*, and their targets during differentiation of cells in culture.** *Left panel*, primer extension assay (*let-7*, miR-125b), Northern hybridization (*lin-41*, *lin-28*, *gapdh*) and immunoblot (Lin-28, β-actin) were performed with samples prepared from Tera-2 embryonic carcinoma cells before (0 days) or after 20 and 60 days of retinoic acid (*RA*) treatment. *Right panel*, same assays were performed with samples from mouse ES cells subjected to *in vitro* differentiation. 5:10 and 5:15, 10 and 15 days of the final neuronal differentiation stage (stage 5). In the Lin-28 immunoblot, equal loading of protein in each lane was confirmed by Ponceau S staining of the blot (not shown). Total RNA from brain was included as a positive control for differentiated tissue.

proliferation of differentiated cells.

miR-125b and let-7 Increase, Whereas lin-28 and lin-41 Decrease at mRNA Levels during Differentiation—Taken together with the expression profiles of the human miRNAs in Fig. 1, the previous results in *C. elegans* (17, 19–23) suggest that *let-7* and miR-125b miRNAs are induced during differentiation, and this up-regulation of the miRNAs post-transcriptionally repress their putative targets, which contain partially complementary target sequences in their 3′-untranslated region. To test whether this regulation could be recapitulated in mammalian *in vitro* differentiation systems, we induced the differentiation of Tera-2 cells in the neuronal direction by retinoic acid (45). miR-125b became pronounced after 20 days of retinoic acid treatment (Fig. 4, *left panel*). *let-7* increased to a level barely detectable after 20 days and to a higher level after 60 days. This time pattern was reminiscent of *C. elegans* in which *lin-4* is expressed earlier than *let-7* during development (17, 19). In contrast to the miRNAs, Lin-28 protein decreased to barely detectable levels after 20 days. Also, the mRNA of *lin-28* and *lin-41* disappeared completely by 20 days of retinoic acid (Fig. 4, *left panel*). Thus, the regulation of the miRNAs and their targets was recapitulated in the differentiation of EC cells *in vitro*.

To confirm the changes in expression of miRNAs and their targets in a second differentiation system, we induced the differentiation of mouse ES cells to the neuronal fate using the five-stage *in vitro* differentiation procedure (34). Both *let-7* and miR-125b miRNAs were weakly expressed in ES cells, slightly decreased in EBs, and then increased with differentiation through NP stage into mature neurons (Fig. 4, *right panel*). RNA from adult brain is shown for comparison. Lin-28 protein was expressed in ES and EB stages but absent in NP stage and later on. Notably, it was the mRNA level of *lin-28* and *lin-41* that is exactly anti-proportional to that of *let-7* and miR-125b (Fig. 4, Northern blots in the *right panel*). This suggests that miRNAs might regulate the mRNA level of *lin-28* and *lin-41* through posttranscriptional cleavage of mRNA as seen in plants rather than regulate *lin-28* at the translational stage. It is worth noting that although there is a slight increase in *lin-28* mRNA and decrease in miR-125b miRNA between ES cells and EBs, there was no increase in Lin-28 protein level in EBs despite the decreased miRNA to target ratio. This, too, argues against translational control of *lin-28* by the miRNAs.

miR-125b and let-7 Are neither Sufficient nor Necessary for the Transcriptional Down-regulation of lin-28 and lin-41 during Differentiation—Next, we attempted to provide direct evidence for the effect of the miRNAs on the expression of their putative targets by using our experimental tools to deplete or introduce a miRNA as described above. We performed a finer time course on the differentiating Tera-2 cells to test if there is an intermediate stage when the expression of *lin-28* and *lin-41* is regulated post-transcriptionally and is affected by experimental changes in miRNA levels. The steady-state mRNA levels of *lin-28* and *lin-41* started decreasing by 4–6 days and were almost completely reduced by 12–14 days after retinoic acid treatment (Fig. 5).

Because siRNA to the pre-miRNA did not work in the differentiating cells, we depleted miR-125b in this system by transfecting 2′-*O*-methyl antisense oligonucleotides (Fig. 5*A*). In addition, RNA oligonucleotides with miR-125b and *let-7* sequence were transfected to assess the effect of premature induction of the miRNAs during differentiation (Fig. 5*B*). Neither the depletion of miR-125b nor the premature introduction of mature miR-125b or *let-7* miRNA resulted in a striking change in the mRNA profile of *lin-28* and *lin-41* in the differentiating Tera-2 cells (Fig. 5). Depletion of miR-125b produced a slight increase of *lin-28* mRNA at 8 and 14 days (Fig. 5*A*, *top panel*, *lanes 9* and *12*), whereas transfection of miR-125b and *let-7* miRNA produced a slight decrease in *lin-28* mRNA (Fig. 5*B*, *top panel*, *lanes 9*, *10*, *12*, and *13*). Although these effects might suggest that the miRNAs repress the target transcriptionally or by post-transcriptional cleavage, the effects are marginal compared with the overall change of target message during differentiation.

Lin-28 protein remained relatively stable until 12–14 days of retinoic acid treatment (Fig. 5, *A* and *B*, *bottom panel*) followed by a significant reduction at 20 days and complete disappearance at 60 days (Fig. 4, *left panel*). Thus, the Lin-28 protein

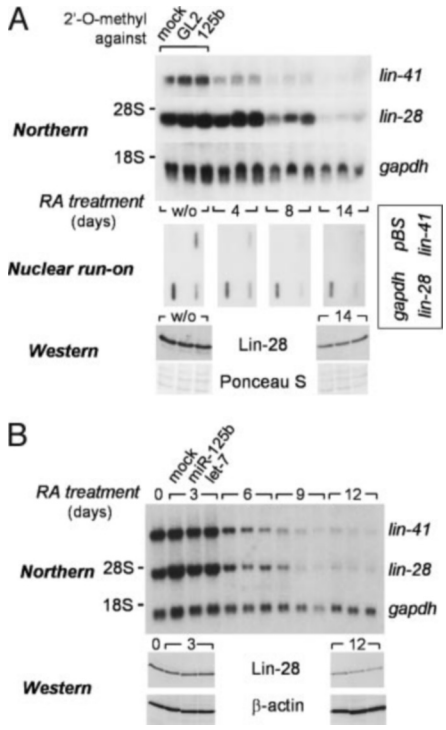

FIG. 5. **Effect of miRNAs on putative targets during *in vitro* differentiation.** *A*, Northern hybridization (*top panel*), nuclear run-on (*middle*), and immunoblot (*bottom*) were performed with samples prepared from Tera-2 cells after 4 days without retinoic acid (*RA*) treatment (*w/o*) or after 4, 8, and 14 days of retinoic acid treatment. The cells were not transfected (−) or were transfected with 2′-*O*-methyl antisense oligonucleotide against GL2 (*GL2*) and miR-125b (*125b*). The first three transfections were done at 24-h intervals, and the fourth transfection onward was done at 48-h intervals. A nuclear run-on experiment was performed at the same time points during differentiation with untransfected Tera-2 cells. In Lin-28 immunoblot, Ponceau S staining of the blot shows equal loading of protein in each lane. *B*, Northern hybridization (*top panel*) and immunoblot (*bottom*) were performed with samples prepared before (0 days) or after 3, 6, 9, and 12 days of retinoic acid treatment combined with the transfections of RNA oligonucleotides encoding miR-125b or *let-7*.

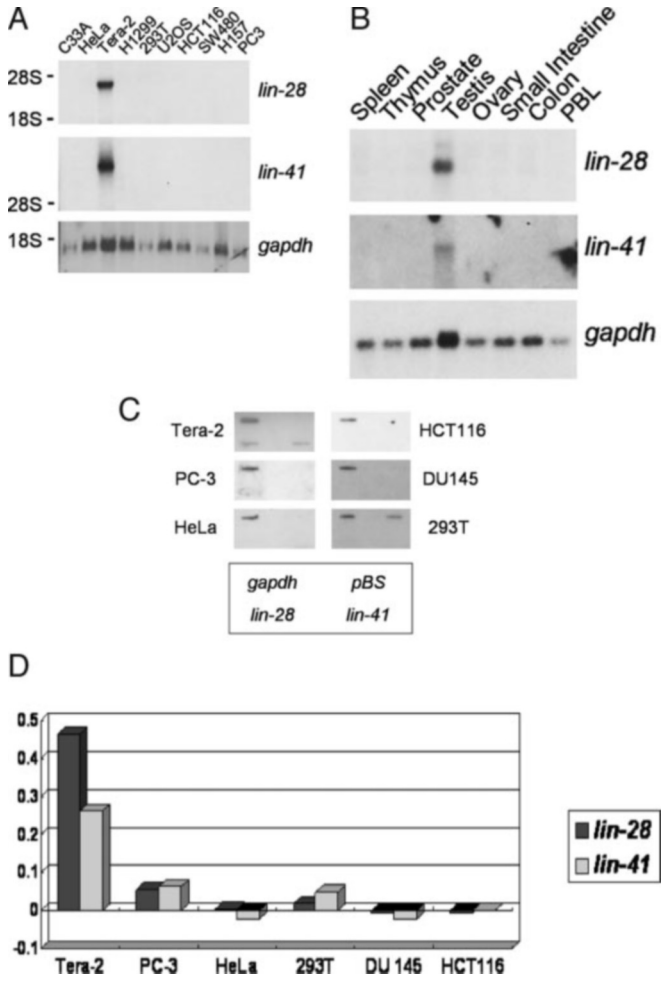

FIG. 6. **Expression of *lin-28* and *lin-41*, putative targets of the miRNAs, in various cell lines and tissues.** *A*, mRNA levels of *lin-28* and *lin-41* as well as *gapdh* as a control in various cell lines were detected by Northern hybridizations. The locations of 18 S and 28 S ribosomal RNA are indicated in the *left*. *B*, Northern hybridizations were performed on a tissue blot (human MTN blot II from Clontech). *PBL*, peripheral blood leukocytes. *C*, nuclear run-on experiments were performed with nuclei from the indicated cell lines. The identities of each band are marked in a *box below*. *pBS* indicates the pBluescript vector negative control. 293T cells have an integrated pBluescript. *D*, each band in the blot of *panel E* was quantitated by autoradiography. The signal from pBluescript vector negative control was subtracted, and then the intensities were normalized to that for the *gapdh* gene. In the case of 293T, the signal from the blank area was used as background, whereas normalization instead was from pBluescript vector.

expression seems to simply follow the mRNA level during differentiation. Lin-28 protein level was also not affected by the depletion or introduction of miRNAs in the differentiating Tera-2 cells (Fig. 5, *A* and *B*, *bottom panels*). Transfection of *let-7* and miR-125b miRNAs did not decrease *lin-28* mRNA or protein in non-differentiating Tera-2 cells (data not shown). Conversely, depletion of miR-125b in PC-3 cells failed to induce *lin-28* mRNA or protein (data not shown). Translational regulation of Lin-41 by *let-7* is not plausible either, since *let-7* miRNA is barely detectable at 20 days, by which time *lin-41* mRNA is fully down-regulated.

Taken together, these results suggest that at least in two mammalian *in vitro* differentiation models, down-regulation of *lin-41* and *lin-28* is not effected by translational suppression by *lin-4* and *let-7*. Although there is a decrease in Lin-28 protein during differentiation, the decrease correlates with a decrease in mRNA abundance of the *lin-28* gene.

lin-28 and lin-41, Specifically Expressed in Undifferentiated Cells, Are Regulated at the Transcriptional Stage—Next, we investigated the expression of *lin-28* and *lin-41* in a number of human cell lines (Fig. 6*A*) to generalize our result in Tera-2 differentiation. Among the cell lines tested, *lin-28* and *lin-41* mRNAs were observed only in Tera-2, which expressed no detectable level of *let-7* and miR-125b. All other cell lines have variable, but detectable levels of *let-7* and miR-125b (data not shown) but with complete absence of *lin-28* and *lin-41* mRNA. Given the negative correlation in the expression of the miRNAs and their target mRNAs, it is not plausible that the miRNAs regulate the protein expression by annealing to the target mRNAs. Consistent with the abundance of *lin-28* and *lin-41* in undifferentiated cells, only testis expressed detectable levels of *lin-28* and *lin-41* mRNA among various tissues in a Northern blot (Fig. 6*B*).

The low levels of *lin-28* and *lin-41* mRNAs could be due to decreased transcription or due to mRNA degradation directed by the miRNAs as seen in plants. Nuclear run-on assays were performed to address this question. Fig. 6, *C* and *D*, show that *lin-28* and *lin-41* are suppressed at the transcriptional level in all tested cell lines relative to the undifferentiated Tera-2 cells. Consistent with this, the decrease of *lin-41* mRNA on day 4 and of *lin-28* mRNA on day 8 in differentiating Tera-2 cells coincided with a reduction of transcription as measured by nuclear run-on assays (*middle panel* of Fig. 5*A*). Therefore, the *in vitro* differentiation program appears to regulate the two genes

mostly at the transcriptional level, accounting for the unresponsiveness to any decrease or increase of miR125b or *let-7* miRNAs.

DISCUSSION

siRNA to Pre-miRNA to Assess miRNA Function—With the discovery of hundreds of miRNAs in plants and animals, methods that can quantitatively inactivate miRNAs are needed to assess their function. 2′-*O*-methyl oligoribonucleotides have been recently reported to specifically inactivate the RNAi activity of miRNAs in cultured cells and in *C. elegans* (26, 27). Here we demonstrate that an siRNA against the loop region of the precursor to a miRNA can selectively down-regulate the mature miRNA. The decrease of miRNA was monitored directly by primer extension assay, whereas an inhibition of the miRNA can be monitored only through an indirect reporter-based assay when 2′-*O*-methyl antisense oligoribonucleotide is used.

The depletion of miR-125b specifically resulted in the proliferation defect, and this proliferation defect was rescued by synthetic mature miRNA. Our result not only gives clues to the miRNA function but also provides an assay for miRNA function. Functional evaluation of a co-transfected mature miRNA is a particularly attractive aspect of this approach because such a rescue is hard to achieve when miRNAs are directly neutralized by 2′-*O*-methyl antisense oligonucleotides. This assay should allow us to evaluate sequence features of a miRNA that are critical for its function.

siRNA to miRNA Precursor to Assess miRNA Biogenesis—For many miRNAs computational methods have predicted multiple potential precursor genes in the genome. It is not clear whether all the precursor genes contribute equally to the mature miRNA pool. Because the loop regions of different pre-miRNAs are not conserved, the use of siRNA against the loop region has a unique advantage over 2′-*O*-methyl antisense oligonucleotide in determining which miRNA precursor gene contributes to the mature miRNA pool in the cell. In the case of miR-16, both precursors contribute to the pool of mature miRNA in PC-3 cells. In contrast, miR-125b-2 is the primary source of the mature miR-125b in this cell, because si125b-2 but not si125b-1 depleted most of the mature miR-125b. The genes for miR-125b-1 and -2 are located in chromosomes 11 and 21, respectively. miR-125b-2 is located in the intron of a gene, whereas miR-125b-1 is located in the exon of a gene (NT_033899.473; Genscan). There was no significant difference in density the of genes or of repetitive elements between these genomic loci. The Mfold program indicates that both precursors form hairpins with nearly identical stability. The difference in precursor use could be due to a difference in the transcription of the two genes containing the precursors. Alternatively, a miRNA precursor in the intron of a gene may be more available for processing into a miRNA than a precursor contained in the exon of a gene. Further work is necessary to test these hypotheses, but the siRNA strategy allows us to begin addressing these questions.

Biological Role of miR-125b in Prostate Cancer Cell Line, PC-3—The depletion of miR-125b through multiple transfections of si-125b2 resulted in a significant proliferation defect in PC-3 cells. The growth defect of PC-3 cells was not accompanied by an aberrant accumulation of cells in one stage of the cell cycle or by apoptosis (data not shown). The reduction of proliferation is not caused by a nonspecific effect mediated by activation of PKR by the double-stranded siRNA (46) because it was not seen with si125b-1 nor was it seen in Tera-2 cells that did not express the miRNA. Furthermore, depletion of miR-125b by 2′-*O*-methyl oligonucleotide resulted in the same phenotype, supporting the specific effect of miR-125b on the cell proliferation. Several genes besides *lin-28* have been reported with mRNAs containing sequences that can form an RNA-RNA duplex with miR-125b (47, 48). Possible involvement of these candidate genes is currently under investigation to account for the miR-125b knock-out phenotype.

Conserved Pattern of Expression of miRNA Targets but Diverged Mechanism of Regulation—We showed that *lin-28* and *lin-41* from human and mouse are specifically expressed in EC cells or ES cells in which miR-125b and *let-7* are not detectable. As differentiation proceeds, *lin-28* and *lin-41* decrease, whereas miR-125b and *let-7* increase. This reciprocal expression pattern recapitulates the expression profile during *C. elegans* development. Our results are consistent with recently published reports suggesting that mammalian Lin-28 protein is down-regulated, whereas the miRNAs miR-125b and *let-7* orthologs are up-regulated during differentiation (28, 29),. The conservation of sequence and expression pattern from nematode to humans suggests that the regulatory events mediated by *lin-4*, *let-7*, *lin-28*, and *lin-41* are also conserved.

There is, however, a marked divergence in the regulation of *lin-28* and *lin-41* between *C. elegans* and the mammalian *in vitro* differentiation systems studied here. In *C. elegans*, translational repression is the favored mechanism for the down-regulation of heterochronic genes by *let-7* and *lin-4*. As detailed in the introduction, a significant body of evidence leads us to expect a similar translational control of mammalian Lin-28 (28–30). This expectation is particularly well supported by an experiment with an artificial luciferase construct fused to the *let-7* target sequence, naturally found in the 3′-untranslated region of mammalian *lin-28* mRNAs (30). This construct is subject to *let-7*-dependent translational repression, strongly suggesting that the miRNAs can translationally repress the mammalian *lin-28* gene.

Our results, however, demonstrate that the regulation of mammalian *lin-28* and *lin-41* in the systems we studied is at the stage of transcription and is not affected by *let-7* and miR-125b. The nuclear run-on experiment during Tera-2 differentiation suggested that the down-regulation is transcriptional. In a panel of six cell lines the mammalian *lin-28* and *lin-41* appear regulated at the transcriptional stage. The mutual exclusivity in the expression of the miRNAs and their targets also supports the notion that the miRNAs do not regulate their targets at the translational stage. In addition, the artificial introduction or depletion of the miRNAs in differentiating or undifferentiated Tera-2 cells or PC-3 cells did not change the level of Lin-28 protein, *lin-28* mRNA, or *lin-41* mRNA, arguing against the possibility that the target mRNA is cleaved by the miRNA. This does not, of course, rule out the possibility of such mRNA cleavage in other situations. In our experiments, mouse ES cell was the only system in which the miRNAs and their targets exist together. However, here too, there was no increase in Lin-28 protein level in the subsequent EB stage relative to ES despite the decreased miRNA to *lin-28* mRNA ratio.

Thus, siRNAs can down-regulate miRNAs in mammalian cells in culture, and we use them to (*a*) demonstrate an essential role of miR-125b in differentiated cells, (*b*) develop a functional assay for miR-125b, and (*c*) identify which precursor gene is used to give rise to a miRNA. We also show that although the miRNAs miR-125b and *let-7* are induced during differentiation of EC or ES cells in culture, whereas their targets *lin-28* and *lin-41* are repressed, the mechanism of the repression is different from the classical annealing of miRNA to target message and translation inhibition noted in *C. elegans*. *Lin-28* and *lin-41* are transcriptionally repressed, and we have no evidence that the repression is mediated by the direct effect

of miR-125b or *let-7*. Of course, these results do not imply that mammalian miR-125b and *let-7* never regulate their mammalian targets translationally. This question has to be re-visited if future experiments reveal additional examples (like the differentiating ES cells) where the target mRNAs and the miRNA are co-expressed, using the methods of miRNA depletion reported here.

In conclusion, we demonstrate that the miRNA depletion strategies used here are invaluable for discovering unexpected functions of miRNAs like the importance of miR125b in the viability of differentiated cells. In addition, such experiments are essential for objectively evaluating functions of miRNAs predicted from genetics in lower eukaryotes or from computational studies.

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