MicroRNA-125b Promotes Neuronal Differentiation in Human Cells by Repressing Multiple Targets^{∇†}

Minh T. N. Le,^{1,2} Huangming Xie,^{1,2,3} Beiyang Zhou,³ Poh Hui Chia,^{4‡} Pamela Rizk,⁴
Moonkyoung Um,^{3§} Gerald Udolph,⁴ Henry Yang,⁵
Bing Lim,^{1,2,6*} and Harvey F. Lodish^{1,3,7*}

Computation and Systems Biology, Singapore-MIT Alliance, 4 Engineering Drive 3, Singapore 117576, Republic of Singapore¹;
Genome Institute of Singapore, 60 Biopolis Street, Genome, Singapore 138672, Republic of Singapore²; Whitehead Institute for
Biomedical Research, 9 Cambridge Center, Suite 601, Cambridge, Massachusetts 02142³; Institute of Medical Biology,
8A Biomedical Grove, Immunos, Singapore 138648, Republic of Singapore⁴; Bioinformatics Group,
Singapore Immunology Network, 8A Biomedical Grove, Singapore 138648, Republic of Singapore⁵;
Beth Israel Deaconess Medical Center, Harvard Medical School, CLS 442, 330 Brookline Ave.,
Boston, Massachusetts 02215⁶; and Department of Biology, Massachusetts Institute of
Technology, Cambridge, Massachusetts 02142⁷

Received 1 November 2008/Returned for modification 14 December 2008/Accepted 11 July 2009

MicroRNAs (miRNAs) are a class of small noncoding RNAs that regulate gene expression at the posttranscriptional level. Research on miRNAs has highlighted their importance in neural development, but the specific functions of neurally enriched miRNAs remain poorly understood. We report here the expression profile of miRNAs during neuronal differentiation in the human neuroblastoma cell line SH-SY5Y. Six miRNAs were significantly upregulated during differentiation induced by all-trans-retinoic acid and brain-derived neurotrophic factor. We demonstrated that the ectopic expression of either miR-124a or miR-125b increases the percentage of differentiated SH-SY5Y cells with neurite outgrowth. Subsequently, we focused our functional analysis on miR-125b and demonstrated the important role of this miRNA in both the spontaneous and induced differentiations of SH-SY5Y cells. miR-125b is also upregulated during the differentiation of human neural progenitor ReNcell VM cells, and miR-125b ectopic expression significantly promotes the neurite outgrowth of these cells. To identify the targets of miR-125b regulation, we profiled the global changes in gene expression following miR-125b ectopic expression in SH-SY5Y cells. miR-125b represses 164 genes that contain the seed match sequence of the miRNA and/or that are predicted to be direct targets of miR-125b by conventional methods. Pathway analysis suggests that a subset of miR-125b-repressed targets antagonizes neuronal genes in several neurogenic pathways, thereby mediating the positive effect of miR-125b on neuronal differentiation. We have further validated the binding of miR-125b to the miRNA response elements of 10 selected mRNA targets. Together, we report here for the first time the important role of miR-125b in human neuronal differentiation.

MicroRNAs (miRNAs) represent an emerging class of small noncoding RNAs that play important roles in the posttranscriptional regulation of gene expression (2). They are transcribed initially as long RNAs and then processed by two RNase complexes, Drosha and Dicer, into ~22-nucleotide (nt) duplexes that are subsequently loaded into RNA-induced silencing complexes (2). Mature miRNAs in the RNA-induced silencing complexes usually bind to the 3' untranslated region (UTR) of mRNAs, leading to the translational suppression or destabilization of the target mRNAs or both (10). The inter-

action between a miRNA and its target mRNA does not require perfect complementarity. Hence, a single miRNA has the potential to regulate multiple target mRNAs (10).

miRNAs have been demonstrated to be essential for neural development. Recent reports highlighted the abundant and diverse expression of miRNAs in the central nervous system (15–17, 29, 33, 36). Mammalian brain tissues express about 70% of experimentally detectable miRNAs, many of which are developmentally regulated (15–17, 29, 33, 36). In maternal-zygotic zebrafish *dicer* mutants, a deficiency in Dicer-mediated biogenesis of miRNAs leads to severe defects in brain morphogenesis (11). Similarly, the loss of Dicer in *sca3* mutant *Drosophila melanogaster* enhances neurodegeneration (4). The specific knockdown of Dicer in mouse midbrain dopaminergic neurons resulted in a progressive loss of these cells (14). Recent studies have also elucidated the contribution of individual miRNAs to various aspects of neural development. For example, miR-9a regulates the organizer function of the zebrafish midbrain-hindbrain boundary (21). In *Caenorhabditis elegans*, *lsy-6* and miR-273 determine the cell fate of chemoreceptor neurons (13). miR-7 regulates the differentiation of photoreceptor neurons in *Drosophila* (23). The miR-200 family regulates the terminal differentiation of olfactory neurons in both

* Corresponding author. Mailing address for Harvey F. Lodish: Whitehead Institute for Biomedical Research, 9 Cambridge Center, Suite 601, Cambridge, MA 02142. Phone: (617) 258-5216. Fax: (617) 258-6768. E-mail: lodish@wi.mit.edu. Mailing address for Bing Lim: Genome Institute of Singapore, 60 Biopolis Street, Genome, Singapore 138672, Republic of Singapore. Phone: 65 6478 8186. Fax: 65 6478 9005. E-mail: limb1@gis.a-star.edu.sg.

† Supplemental material for this article may be found at <http://mcb.asm.org/>.

‡ Present address: Department of Biological Sciences, Stanford University, Stanford, CA 94305.

§ Present address: Boston Biomedical Research Institute, 64 Grove Street, Watertown, MA 02472.

[∇] Published ahead of print on 27 July 2009.

mouse and zebrafish (5). In addition, miRNAs play important roles in neuronal function and survival. In *Drosophila*, the miRNA *bantam* prevents neuronal apoptosis by suppressing the proapoptotic gene *hid* (4). In mature rat neurons, miR-134 localizes to dendrites and regulates spine size (31). In *C. elegans*, miR-1 regulates MEF-2-dependent retrograde signaling at neuromuscular junctions (34). Most functional studies of miRNAs in neuronal development have been carried out using animal models, and it remains to be proven if miRNAs play the same role in human neurogenesis.

In this study we sought to understand the role of miRNAs in the differentiation of human neural cells using simple in vitro models, human neuroblastoma SH-SY5Y cells and human neural progenitor ReNcell VM (RVM) cells. When sequentially treated with all-*trans*-retinoic acid (RA) and brain-derived neurotrophic factor (BDNF), SH-SY5Y cells give rise to fully differentiated neuron-like cells (8). These differentiated SH-SY5Y cells are withdrawn from the cell cycle, express various neuronal markers, and exhibit carbachol-evoked noradrenaline release (8). Moreover, as no glial cell is derived by this process, it is a robust and homogenous model system for investigating neuronal differentiation (8). Using microarrays and Northern blots, we identified a group of miRNAs that are significantly upregulated in differentiated SH-SY5Y cells. We further showed that one of these miRNAs, miR-125b, significantly enhances the differentiation and neuronal morphogenesis of SH-SY5Y cells. In addition, this miRNA also promotes neurite outgrowth in human neural progenitor RVM cells.

miR-125b is a homolog of *lin-4*, which is the first miRNA discovered and an important regulator of developmental timing in *C. elegans* (30). miR-125b is abundantly expressed in animal brains and is upregulated during neurogenesis (17, 29, 33, 35). However, the function of miR-125b in neural development has been unclear. For the first time, our report demonstrates that miR-125b is important in regulating neuronal differentiation. Furthermore, we identified a large number of putative target genes repressed by miR-125b ectopic expression in SH-SY5Y cells. Data from computational analyses suggests that 10 of these genes antagonize several neurogenic pathways, especially extracellular signal-regulated kinase (ERK) signaling, which is known to mediate the effect of RA in neuronal differentiation.

MATERIALS AND METHODS

Cell culture and differentiation conditions. SH-SY5Y cells and HEK-293T cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 4,500 mg/liter glucose, 10% heat-inactivated fetal bovine serum (Gibco), 110 mg/liter sodium pyruvate (Gibco), 2 mM L-glutamine (Gibco), and 1% penicillin-streptomycin (Gibco). This medium will be called hereafter "growth medium" for SH-SY5Y cells. For differentiation, SH-SY5Y cells were seeded onto collagen-coated plates (BD Biosciences) at an initial density of 10^4 cells/cm². RA (Sigma) was added at a final concentration of 10 μ M the next day after plating. After 5 days, the cells were washed three times with DMEM and incubated with 50 ng/ml BDNF (Sigma) in growth medium without serum for 7 days.

RVM cells were cultured in laminin-coated plates in DMEM-F12 (1:1) medium (Invitrogen) supplemented with 10% B27 medium (Invitrogen), 10 μ g/ml gentamicin (Gibco), 10 units/ml heparin (Sigma), 20 ng/ml epidermal growth factor (EGF), and 10 ng/ml basic fibroblast growth factor (bFGF) (Invitrogen). For differentiation, the growth medium was replaced with neurobasal medium (Invitrogen) supplemented with 10% B27 medium (Invitrogen), 10 μ g/ml gentamicin (Gibco), and 10 units/ml heparin (Sigma).

miRNA expression profiling. Total RNA samples were extracted from untreated SH-SY5Y cells, cells treated with RA for 5 days, and cells treated

subsequently with BDNF in serum-free medium for 7 days. Small RNA was purified, labeled, and subjected to an oligonucleotide-based microarray as previously described (3). Briefly, two ³²P-labeled RNA markers of 18 nt and 24 nt were coloaded with total RNA samples and used as indicators to identify the small RNA population on the gel separating 100 μ g of total RNA. RNAs of 18 to 24 nt were gel purified and sequentially ligated to a 3'-end adaptor and a 5'-end adaptor. Ligated products were gel purified, reverse transcribed, PCR amplified, and labeled with Cy3. The labeled sense strand was then gel purified and applied onto the array. A set of synthetic reference oligonucleotides (with a uniform amount of oligonucleotides corresponding to every probe) was processed similarly but labeled with Cy5. These Cy5-labeled reference oligonucleotides were applied concurrently with the Cy3-labeled samples onto a DNA oligonucleotide-based array, serving as internal hybridization controls. This array (provided by the Bartel laboratory at the Whitehead Institute) contains ~600 DNA probes, including probes for 175 human miRNAs (3). The obtained signals were normalized to the total intensity of all noncognate probes (corresponding to the nematode miRNAs that are not conserved in humans). Subsequently, signals from the biological samples were normalized to the corresponding references as Cy3/Cy5 ratios. The final reading was the average normalized intensity of four replicates (two biological replicates each with two technical replicates).

Northern blot analysis. A total of 10 to 40 μ g of each total RNA sample and a ³²P-labeled Decade RNA marker (Ambion) were separated on a 15% denaturing gel, transferred onto a Genescreen Plus membrane (Perkin-Elmer), UV cross-linked, and baked at 80°C for 30 min. DNA probes with the sequences complementary to the miRNAs were synthesized (Invitrogen) and labeled with [γ -³²P]ATP (Amersham). U6 RNA and 5S RNA probes were used to determine loading equity. The probe sequences are provided in Table S1 in the supplemental material. The membrane was prehybridized in PerfectHyb buffer (Sigma) with 1 mg of freshly added sheared salmon sperm DNA (Sigma) for 2 h at 48°C. Subsequently, the labeled probes were added; hybridization was carried out overnight at 48°C. The membrane was then washed and developed according to the Bartel laboratory Northern blot protocol (<http://web.wi.mit.edu/bartel/pub/>).

Transfection of miRNA duplexes and antisense oligonucleotides. SH-SY5Y cells (passage number of less than 25) were seeded at 80,000 cells/well in a collagen-coated 12-well plate (BD Bioscience). On the next day, using 4 μ l Lipofectamine 2000 reagent (Invitrogen) per well according to the manufacturer's instructions, the cells were transfected with one of the following RNA oligonucleotides at an 80 nM final concentration: BlockIT fluorescent oligonucleotide (Invitrogen), scrambled duplex (Ambion PremiR negative control 1), miRNA duplex (Ambion PremiR), or miRNA antisense (Ambion AntimiR). After 5 h, the transfection medium was replaced with fresh growth medium either with or without 10 μ M RA. Approximately 125,000 RVM cells were transfected in suspension with 80 nM RNA oligonucleotides as described above for SH-SY5Y cells. After 5 h of transfection, the medium was changed to fresh RVM growth medium or differentiation medium, and the cells were plated into laminin-coated plates.

Immunostaining and HCS. Four days after transfection, SH-SY5Y cells or RVM cells were fixed with 4% paraformaldehyde for 15 min, followed by three washes with phosphate-buffered saline (PBS). After a 1-h blocking with 0.2% Triton X-100 and 3% goat serum in PBS, the cells were incubated with primary antibodies overnight at 4°C. The primary antibodies used in this study include mouse monoclonal β III-tubulin antibody (1:1,000 dilution; Abcam), mouse monoclonal Map2ab (1:1,000 dilution; Sigma), mouse monoclonal panaxonal neurofilament antibody (1:1,500 dilution; Covance), goat monoclonal synaptotagmin V (Syt5) antibody (1:1,000 dilution; Santa Cruz), and rabbit polyclonal Musashi-1 antibody (1:1,000 dilution; Abcam). Subsequently, the cells were washed with PBS three times and then incubated with Alexa Fluor 488 goat anti-mouse, Alexa Fluor 568 goat anti-rabbit, or Alexa Fluor 488 donkey anti-goat secondary antibody (Invitrogen) for an hour. Hoechst dye (Invitrogen) was added for 5 min. The cells were then washed with PBS three times. For high-resolution imaging, the cells were observed with a Zeiss Duo inverted confocal microscope (Carl Zeiss Vision GmbH). For quantitative imaging, fluorescent images of the cells were collected automatically by use of the Cellomics high-content screening (HCS) system using a 10 $\times$ objective lens. For neurite outgrowth assays, images of β III-tubulin and Hoechst staining were analyzed using Neuronal Profiling BioApplication software (Cellomics). Differentiated SH-SY5Y cells with neurite outgrowth were defined as β III-tubulin-positive cells with neurites longer than 30 μ m. For RVM cells, the percentage of differentiated cells with neurite outgrowth was defined as the percentage of β III-tubulin-positive cells having neurites longer than 20 μ m (RVM cells are smaller than SH-SY5Y cells, so we applied a lower threshold of neurite length). For quantifications of neuronal marker staining, the images were analyzed by Target Activation BioApplication (Cellomics). In all the HCS assays, a cell was considered

TABLE 1. Target validation summary^a

miR-125b target	Downregulated by 2-day overexpression of miR-125b in:		Predicted MRE	MREs validated by luciferase reporter assay	Specificity of miR-125b target site in 3' UTR validated by mutagenesis and luciferase reporter assay
	GM	RA			
AP1M1	No	Yes	AGCCACCTAGCCTCAGGGAC	Yes	NT
STK11IP	Yes	Yes	GAGAATGATCTGGCCTCAGGGG	Yes	NT
PSMD8	Yes	Yes	TCTGGTGGGCATTGCTCAGGGT	Yes	NT
ITCH	Yes	Yes	TTTGTCAATTTGAATTCAGGGAA	Yes	NT
TBC1D1	Yes	Yes	CGGGAAGTGTGCTTCTCAGGGA	Yes	Yes
TDG	Yes	Yes	TTGAAGTGCCTTGCATCAGGGAT	Yes	NT
MKNK2	Yes	Yes	CCAGCCCGCAGTATTTTCAGGGAC	Yes	NT
DGAT1	Yes	Yes	TCTGTCTGCACCCCTCAGGGA	Yes	Yes
GAB2	Yes	Yes	TCAAAGCACTTGACATCAGGGAC	Yes	NT
SGPL1	Yes	Yes	TGTTCCATTCCTCCATCTCAGGGA	Yes	Yes

^a Ten target genes were selected from the microarray data, target prediction, and pathway analysis. Their expression pattern after a 2-day overexpression of miR-125b in growth medium (GM) or in differentiation medium containing RA was validated by real-time PCR (Fig. 8a). The predicted MREs were validated for binding to miR-125b by luciferase reporter assays (Fig. 8b and c); in three cases, the specificity of the response to miR-125b was validated by luciferase reporter assays in which the predicted miR-125b target sites in the 3' UTRs were mutated (Fig. 8c). NT, not tested.

positive for a specific staining only if its fluorescent intensity was equal or higher than the mean intensity plus two times the standard deviation of the respective scrambled control replicates.

Quantitative real-time PCR. RNA was extracted from SH-SY5Y cells using Trizol reagent (Invitrogen) and subsequently column purified with an RNeasy kit (Qiagen). For quantitative real-time PCR of miRNA, 100 ng of total RNA was reverse transcribed and subjected to a TaqMan miRNA assay (Applied Biosystems) using primers and probes specific for individual miRNAs or for the U6 RNA internal control. For quantitative real-time PCR of mRNAs, cDNA synthesis was performed with 1 µg of total RNA using the High Capacity cDNA archive kit (Applied Biosystems) and subjected to SYBR green or TaqMan gene expression assays (Applied Biosystems) according to the manufacturer's protocol.

Gene expression microarray and data analysis. Total RNA was extracted as described above. A total of 750 µg of total RNA was reverse transcribed, converted to cRNA, labeled, purified, and applied onto the Illumina Ref-8 v2 human bead chip (Illumina) according to the manufacturer's instructions. First, the respective backgrounds were subtracted from all the raw data using Bead Studio (Illumina) and then normalized using the cross-correlation method described previously by Chua et al. (6). Subsequently, normalized data were processed for the identification of differentially expressed genes using log₂ 1.5-fold as the critical value for the mean of log₂ *n*-fold changes in expression between miR-125b duplex (125b-DP)/miR-125b antisense (125b-AS)-transfected samples and the scrambled controls.

Genes that were differentially expressed 4 days after the transfection of 125b-DP were subjected to Gene Ontology (GO) analysis by using BiNGO (25). The percentage of these genes classified into each GO process was compared with that of the whole genome. Statistically significant ($P < 0.05$) classes were selected. For the clustering of genes differentially expressed 2 days posttransfection, normalized and log₂-transformed data were subtracted from the mean values across all the arrays. Hierarchical clustering was then performed for these processed data using average linkages.

Motif analysis by MEME. We checked if the 3' UTR sequences of the 388 primary effectors of miR-125b (selected by microarray analysis) were available in the GenBank database. A total of 253 genes were found with the 3' UTR sequences. Following the removal of the poly(A) tails, sequences were masked for repeats using RepeatMasker (<http://www.repeatmasker.org/>) and analyzed by MEME with the motif width from 4 to 9 and other MEME default parameters. Sequence logos were constructed using WebLogo (<http://weblogo.berkeley.edu>).

Target prediction. The targets of miR-125b were predicted by four different methods, TargetScan 4.2 (22), miRBASE target (12), rna22 (28), and miRNA Viewer (9), using default parameters. To check the statistical significance of the enrichment, we randomly selected 388 genes from either the whole genome or all the differentially expressed genes (differentially expressed by at least one treatment) and then applied the four methods to predict the targets of miR-125b. The random selection and the target prediction were repeated 10,000 times. The average percentages of predicted targets out of the selected gene lists were then compared.

Pathway analysis. Ingenuity Pathway Analysis (IPA) (Ingenuity System) was used to link the direct targets predicted either by MEME or by conventional methods with the genes differentially expressed 4 days after the ectopic expression of miR-125b. We first compared the functional annotation of the two gene groups and subsequently considered only the networks with differentially expressed neurogenesis-related genes. We extracted only the pathway links with the direct targets as the starting points and with known functions related to neurogenesis or differentiation.

Luciferase reporter assay. miRNA response elements (MREs) (Table 1) or the whole 3' UTRs of the target genes were cloned into the psiCHECK-2 vector (Promega) between the XhoI and NotI sites immediately 3' downstream of the *Renilla* luciferase gene. The top (sense) and bottom (antisense) strands of each MRE were designed to contain XhoI and NotI sites, respectively. After synthesis, these were annealed and ligated into the psiCHECK-2 vector. A 500-bp segment containing the miR-125b MRE in the 3' UTRs of three selected target genes, TBC1D1, DGAT1, and SGPL1, were synthesized as minigenes (first base) with or without seven mismatches (CTCAGGG was mutated to GAGTCCC) in the seed region of miR-125b MREs and subcloned into the psiCHECK-2 vector.

Ten nanograms of each psiCHECK-2 construct was cotransfected with 10 nM 125b-DP or scrambled duplex into HEK-293T cells in a 96-well plate using Lipofectamine 2000 (Invitrogen). After 48 h, the cell extract was obtained, and firefly and *Renilla* luciferase activities were measured with the dual-luciferase reporter system (Promega) according to the manufacturer's instructions.

Statistical analysis. A Student's *t* test was used to determine the significance of differences between the treated samples and the controls where values resulted from quantitative real-time PCR, HCS assays, or permutation of target prediction. Statistical analysis was performed using Microsoft Excel. For GO analysis, the *P* value of any enrichment was calculated by BiNGO by using a hypergeometric distribution with Bonferroni correction (25).

Microarray data accession number. Microarray data were deposited into the Gene Expression Omnibus under accession number GSE14787.

RESULTS

Profiling miRNA expression in SH-SY5Y cells during differentiation. To understand the regulation of miRNA expression in human neuronal differentiation, we induced the differentiation of SH-SY5Y cells into neuron-like cells according to methods described previously by Encinas et al. (8) and observed the same morphological changes described by those authors. Neurite outgrowth became apparent after a 5-day treatment with RA and became profuse after a subsequent 7-day treatment with BDNF in serum-free medium (Fig. 1a).

FIG. 1. Neuronal differentiation of SH-SY5Y cells. (a) Representative pictures of undifferentiated cells (day 0) and cells undergoing differentiation (day 5 and day 12). Differentiation was induced by RA for 5 days and subsequently induced by BDNF in serum-free medium for 7 days. (b) Immunostaining of differentiating SH-SY5Y cells for the mature neuronal marker Map2ab (green) and the neural progenitor marker Musashi-1 (Msi1) (red). Nuclei were counterstained with Hoechst dye (blue). (c) Microarray and quantitative real-time PCR (qRT-PCR) analysis of representative marker genes including Syt5, cannabinoid receptor 1 (Cnr1), and GABA type B receptor 1 (Gabbr1) in differentiating SH-SY5Y cells. The microarray data were normalized as described in Materials and Methods. Quantitative real-time PCR readings were normalized to the expression level of β -actin. All the readings are presented as average changes $\pm$ standard errors of the means (SEM) relative to the gene expression level on day 0.

Treated cells lost the expression of the neural progenitor marker Musashi-1, while they increased the expression of the mature neuronal marker Map2ab (Fig. 1b). Gene expression changes profiled by cDNA microarrays indicated the acquisition of neuronal markers (upregulation of neuronal microtubule-associated proteins, ion channels, and neurotransmitter receptors), withdrawal from the cell cycle, and reduced metabolism (downregulation of proliferation and metabolic markers) (see Table S2 in the supplemental material). These results are consistent with a differentiation process from neural progenitors to mature neurons. The upregulation of the neuronal markers Syt5, cannabinoid receptor 1, and GABA type B re-

ceptor 1 (Gabbr1) was confirmed by quantitative real-time PCR (Fig. 1c).

Next, we examined the miRNA expression profiles for undifferentiated SH-SY5Y cells (day 0) and differentiated SH-SY5Y cells after 5 days of RA treatment (day 5) and after an additional 7 days of BDNF treatment in serum-free medium (day 12). We profiled 175 human miRNAs using an miRNA array designed previously by Baskerville and Bartel (3) and applied stringent normalization by subtracting the signal from a reference synthetic oligonucleotide for every miRNA (see Fig. S1 in the supplemental material). Based on the change and expression level, we selected 12 miRNAs that were significantly

FIG. 2. Analysis of miRNA expression in differentiating SH-SY5Y cells. (a) Microarray-detected absolute expression level of the miRNAs that were selected based on their significant and consistent changes in expression during differentiation. (b) Northern blot validation of selected miRNAs. The phosphorimages represent three blots (separated by solid lines), including two biological duplicates loaded onto lanes 1 to 3 and lanes 4 to 6 respectively. U6 RNA and 5S RNA are shown as loading controls. (c) Northern analysis-detected expression changes of selected miRNAs (with significant changes), normalized to the expression level of 5S RNA.

and consistently regulated during the course of differentiation (Fig. 2a). The expression of the selected miRNAs was validated by Northern blotting (Fig. 2b). We found that six miRNAs, miR-7, miR-124a, miR-125b, miR-199a, miR-199a*, and miR-214, were consistently upregulated during differentiation (Fig. 2b and c). Other miRNA candidates were not detected or showed no significant change in their levels of expression by Northern blotting (Fig. 2b).

Ectopic expression of six miRNA candidates and their effects on neurite outgrowth. Transfection using Lipofectamine 2000 was optimized to deliver double-stranded RNA into SH-SY5Y cells. To examine the transfection efficiency, we first transfected the cells with a fluorescent RNA duplex. After 1 day, more than 80% of the cells were positive for fluorescence, and fluorescence persisted until 4 days after transfection (Fig. 3a). We then transfected the cells with miRNA duplexes and

found that by 4 days after transfection, the levels of the corresponding mature miRNAs were very high compared to those of mock-transfected cells (Fig. 3b). Beyond 4 days posttransfection, the cells often became confluent and unhealthy. Hence, we performed all the experiments using cells collected 4 days after transfection. For gain-of-function (ectopic expression) studies, a duplex corresponding to each individual miRNA candidate was transfected into SH-SY5Y cells, and after 4 days, the cells were fixed and stained for β III-tubulin, a neuron-specific marker.

To quantify the degree of neuronal differentiation, neurite outgrowth was analyzed by use of the Cellomics HCS system, where a large number of images was acquired automatically, and neurites were traced and measured in a uniform manner. Since β III-tubulin is an early neuronal marker, only β III-tubulin-positive cells that possessed neurites longer than 30 μ m,

FIG. 3. Ectopic expression of candidate miRNAs and neurite outgrowth assay in SH-SY5Y cells. (a) The transfection efficiency was tested with 80 nM fluorescent double-stranded RNA in SH-SY5Y cells. Fluorescence (green) was observed every day until day 4 after transfection. (b) Relative quantification of miR-7 and miR-125b levels in SH-SY5Y cells 4 days after a transfection with 80 nM synthetic miR-7 duplex (7-DP) or 125b-DP. The obtained real-time PCR readings were normalized to the U6 RNA level and are presented as the changes $\pm$ SEM relative to the respective miRNA level in mock-transfected controls ($n \geq 4$). (c) Percentage of differentiated cells with neurite outgrowth, defined as cells staining positively for β III-tubulin and possessing neurites longer than 30 μ m. The error bars represent SEM of data from at least three biological replicates. The percentage of neuron-like cells in each biological replicate was analyzed and averaged by at least 15 images; each image of 10 $\times$ magnification typically captures 200 to 500 SH-SY5Y cells. The results of Student *t* tests are indicated as ** ($P < 0.01$ compared to the scrambled control). (d) Representative images of SH-SY5Y cells stained for β III-tubulin (green) and Hoechst dye (blue) 4 days after mock transfection or transfection with different miRNA duplexes. Images were acquired by a confocal microscope using a 20 $\times$ objective lens. Scale bar, 100 μ m.

about three times the diameter of the cell bodies, were counted as neuronal cells. The percentage of SH-SY5Y cells meeting this stringent criterion is very low, only $\sim 1\%$, in growth medium. By our stringent standards, we found that only the ectopic expression of miR-124a or miR-125b significantly increased the percentage of differentiated cells with neurite outgrowth, by approximately twofold, compared to the mock and scrambled transfection controls (Fig. 3c and d). Our subsequent analysis focused on miR-125b since the function of miR-124a in neuronal differentiation was described previously (26, 39).

miR-125b is necessary and sufficient for neurite outgrowth and neuronal marker gene expression. We assayed the effects of the miR-125b gain and/or loss of function on neuronal differentiation by measuring neurite outgrowth of SH-SY5Y cells both in growth medium and in differentiation medium containing RA. Synthetic 125b-DP was used for gain-of-function studies. For loss-of-function studies of miR-125b, we used an antisense oligonucleotide (125b-AS) that is able to reduce the level of synthetic miR-125b (when transfected together with 125b-DP at the same concentration) as well as the level of endogenous miR-125b (when transfected alone) (Fig. 4a).

FIG. 4. Function of miR-125b in SH-SY5Y cell differentiation. (a) Change in miR-125b levels in SH-SY5Y cells 4 days after transfection with scrambled duplex (scrambled-DP), 125b-DP, or 125b-AS and maintained in growth medium (GM) or in differentiation medium containing RA. Each oligonucleotide was transfected at an 80 nM final concentration. The obtained real-time PCR readings were normalized to the U6 RNA level and are presented as the changes $\pm$ SEM relative to the respective miRNA levels of the scrambled transfected control ($n \geq 4$). (b) Percentages of differentiated SH-SY5Y cells with neurite outgrowth treated as described above (a) and their average neurite lengths. Automated image acquisition and quantitative analysis were carried out by use of the Cellomics HCS system. Differentiated cells with neurite outgrowth were defined as β III-tubulin-positive cells with neurites longer than 30 μ m. (c) SH-SY5Y cells stained with β III-tubulin (green) and Hoechst dye (blue) 4 days after transfection. Transfection and treatments were applied as described above (a). The images were acquired by a confocal microscope using a 20 $\times$ objective lens. Scale bar, 100 μ m. (d) SH-SY5Y cells stained with neuronal markers (green) and Hoechst dye (blue). The cells were transfected as described above (a) and maintained in growth medium for 4 days. They were then fixed and stained for neuronal markers including Map2ab, neurofilaments, and Syt5. The bar chart presents the percentage of positively stained cells quantified by the Cellomics HCS system. (e) Relative quantification of marker gene expression. The cells were transfected as described above (a). RNA was isolated for quantitative real-time PCR 4 days posttransfection. The obtained readings were normalized to β -actin expression levels (internal control) and are presented as average changes $\pm$ SEM relative to the level of the transcripts in the scrambled control. In all cases, the error bars represent SEM of data from at least three biological replicates. In all HCS experiments, the phenotype of each biological replicate was analyzed and averaged by at least 15 images; each image of 10 $\times$ magnifications typically captures 200 to 500 SH-SY5Y cells. The results of Student *t* tests are indicated as * for a *P* value of <0.05 and ** for a *P* value of <0.01 where the variation is compared to that of the scrambled control.

Consistent with the screening results, the ectopic expression of miR-125b significantly increased the percentage of differentiated cells with neurite outgrowth in both growth medium and differentiation medium containing RA (Fig. 4b and c). Specifically, the percentage of differentiated cells with neurite outgrowth derived by spontaneous differentiation (of mock or scrambled control transfection in growth medium) was $\sim 1\%$. miR-125b ectopic expression alone (in growth medium) increased this fraction of differentiated cells by twofold, to $\sim 2\%$.

Culture in differentiation medium with RA resulted in $\sim 2.8\%$ of cells with neurite outgrowth, while miR-125b ectopic expression again doubled the percentage of cells with neurite outgrowth to 5.7%. This effect of miR-125b gain of function was titrated by cotransfection with 125b-AS in both growth medium and RA-containing medium (Fig. 4b and c). We also considered the average neurite length (of neurites selected with a minimum length of 30 μ m) as an indicator of SH-SY5Y neuronal differentiation. miR-125b ectopic expression in-

creased the average neurite length of SH-SY5Y cells by ~ 2.5 μm in growth medium and by ~ 6 μm in differentiation medium relative to the scrambled-duplex transfection control (Fig. 4b and c). Together, these results indicate that miR-125b alone is sufficient to stimulate neurite outgrowth.

Conversely, the specific knockdown of endogenous miR-125b by an antisense oligonucleotide (125b-AS) reduced the average neurite length by ~ 9 μm ($P < 0.01$), indicating that endogenous miR-125b expression is necessary for neurite outgrowth (Fig. 4b and c). The cotransfection of 125b-DP with 125b-AS abrogated the increase in average neurite length due to miR-125b ectopic expression, demonstrating the specificity of the antisense oligonucleotide. Together, the results show that miR-125b is both necessary and sufficient to stimulate neurite outgrowth.

The role of miR-125b in promoting the differentiation of SH-SY5Y cells was demonstrated further by staining for additional neuronal markers. As quantified by use of the Cellomics HCS system, miR-125b gain of function in growth medium significantly increased the percentage of cells positive for mature neuronal markers including Map2ab, neurofilament, and Syt5 (Fig. 4d). These stimulatory effects were specifically abrogated by cotransfection with the 125b-AS oligonucleotide. The transcript levels of the mature neuronal markers Map2ab and Gabbr1 were also increased by miR-125b ectopic expression (Fig. 4e). In contrast, the level of expression of the neural progenitor marker Musashi-1 was reduced significantly (Fig. 4e).

miR-125b is upregulated during differentiation of RVM cells, and miR-125b ectopic expression promotes neurite outgrowth in these cells. To elucidate the function of miR-125b in non-cancer-derived cells, we used RVM cells, a neural progenitor cell line isolated from normal human brain and immortalized by *v-myc* induction. EGF and bFGF were used to maintain the cells in the undifferentiated state. The differentiation of RVM cells into neurons and glial cells was induced by the withdrawal of the growth factors. During this process, we observed a continuous change in morphology marked by the appearance of neurite outgrowth (Fig. 5a). By quantitative real-time PCR, we found that miR-125b was gradually and significantly upregulated during the 7-day differentiation of RVM cells (Fig. 5b). The efficiency of transfection in RVM cells was comparable to that in SH-SY5Y cells: following transfection with a fluorescent RNA duplex, fluorescence was observed in more than 80% of RVM cells by day 1 and remained detectable until day 4 posttransfection (Fig. 5c).

We then transfected 125b-DP into RVM cells. Transfected cells were maintained either in growth medium (containing EGF and bFGF) or in differentiation medium (in the absence of the two growth factors), and neurite outgrowth was assayed as for SH-SY5Y cells. miR-125b ectopic expression significantly promoted the neurite outgrowth of RVM cells in both growth medium and differentiation medium, as indicated by the percentage of differentiated cells with neurite outgrowth (βIII-tubulin-positive cells with neurites longer than 20 μm) (Fig. 5d and e). In addition, miR-125b ectopic expression significantly increased the average neurite length of differentiated neurons in growth medium but not in differentiation medium (Fig. 5d and e). The effect of 125b-DP transfection was abrogated by cotransfecting 125b-AS at an equal concentration (Fig. 5d and e). Hence, the effects of miR-125b on the differ-

entiation of RVM neural progenitor cells are similar to the effects of miR-125b on neuroblastoma SH-SY5Y cells.

Profiling downstream effectors of miR-125b. To understand the mechanism of the miR-125b-dependent differentiation of neural cells, we studied the changes in the global gene expression profile of SH-SY5Y cells following miR-125b ectopic expression. First, the gene expression profile of SH-SY5Y cells 4 days after transfection with 125b-DP in growth medium was compared with that of the scrambled-duplex transfection control. We found that the genes upregulated by miR-125b ectopic expression were preferentially classified by GO into biological processes related to development, especially nervous system development, neurite growth, cell adhesion, cell morphology, motility, and cytoskeleton organization (Fig. 6a). Specifically, the percentage of miR-125b-upregulated genes classified into each of these categories was statistically higher than the percentage of the whole genome sorted into the same category (Fig. 6a). On the other hand, genes downregulated by miR-125b ectopic expression were overrepresented by those related to metabolism and transcriptional regulation (Fig. 6b). Note that the changes in gene expression due to miR-125b ectopic expression were profiled in transfected cells that had not necessarily differentiated. Since the transfection efficiency was very high, we assumed that most if not all the cells responded to the elevated level of miR-125b. Thus, these responses indicate a global transition of the cells from an undifferentiated state to a differentiated state following miR-125b overexpression. Although not all the cells expressed mature neuronal markers and/or exhibited neurite outgrowth by day 4 posttransfection, many of them appear to have already acquired the expression of neuron-related genes.

Second, we sought to identify the more direct effectors of miR-125b by examining global gene expression profiles at an earlier time point, 2 days posttransfection. Microarray profiling was performed on SH-SY5Y cells transfected with scrambled duplex and 125b-DP in growth medium or in differentiation medium containing RA. We also included two other treatments: miR-125b knockdown (125b-AS transfected) and neutralization of 125b-DP (cotransfection of 125b-AS and 125b-DP at equal concentrations) in differentiation medium. Strikingly, miR-125b ectopic expression downregulated a large number of genes, both in growth medium and in differentiation medium, forming a distinct cluster from all other treatments (Fig. 6c). Unexpectedly, the knockdown of miR-125b did not show an opposite effect. Since SH-SY5Y cells also express miR-125a, which has the same seed sequence and can target a set of genes similar to that targeted by miR-125b, knocking down miR-125b alone may be insufficient to release the repression of all its targets.

Identification of direct targets of miR-125b. In an attempt to identify the direct targets of miR-125b in neuronal differentiation, i.e., mRNAs whose expression is directly downregulated by this miRNA, we selected 388 genes that were downregulated by miR-125b ectopic expression in growth medium and in RA-containing medium relative to all other transfection conditions. To examine whether these genes might be directly regulated by miR-125b binding, we applied two different bioinformatic approaches.

The first approach was to search for a common motif in the 3' UTR of the downregulated genes by using MEME motif

FIG. 5. Expression and function of miR-125b in RVM cells during differentiation. (a) Representative pictures of undifferentiated cells (day 0) and differentiating RVM cells (day 3, day 5, and day 7). Undifferentiated cells were maintained in growth medium containing growth factors, whereas differentiation was induced by the removal of growth factors and the addition of neurobasal medium. Scale bar, 100 μ m. (b) Expression pattern of miR-125b in RVM cells during differentiation quantified by quantitative real-time PCR. The readings were normalized to U6 data and are presented as average changes $\pm$ SEM ($n = 6$) relative to the miRNA level in undifferentiated cells (day 0). The results of Student t tests are indicated as ** for a P value of <0.01 . (c) Fluorescence in RVM cells 1 to 4 days after transfection with 80 nM fluorescent duplex. Scale bar, 100 μ m. (d) Representative images of RVM cells stained with β III-tubulin (green) and Hoechst dye (blue) 4 days after (i) mock transfection or transfection with (ii) 80 nM scrambled duplex (scrambled-DP), (iii) 80 nM 125b-DP, (iv) 80 nM 125b-DP and 80 nM 125b-AS. After transfection, the cells were maintained in growth medium (GM) or in differentiation medium (DM) until they were fixed and stained. Images were acquired by a confocal microscope using a 20 $\times$ objective lens. Scale bar, 100 μ m. (e) Percentage of differentiated RVM cells with neurite outgrowth and their average neurite lengths. The cells were treated as described above (d). Automated image acquisition and quantitative analysis were carried out by use of the Cellomics HCS system. Differentiated RVM cells with neurite outgrowth were defined as β III-tubulin-positive cells with neurites longer than 20 μ m. The error bars represent SEM ($n \geq 3$). The phenotype of each replicate was analyzed and averaged by at least 15 images. Each image of 10 $\times$ magnifications typically captures 1,000 to 2,000 RVM cells. The results of two-tailed t tests are indicated as * for a P value of <0.05 and ** for a P value of <0.01 .

FIG. 6. Profiling of the downstream effectors of miR-125b. (a) GO classification of genes upregulated 4 days after transfection of 125b-DP. (b) GO classification of genes downregulated 4 days after transfection of 125b-DP. In a and b, SH-SY5Y cells were transfected with 80 nM scrambled duplex or 80 nM 125b-DP and then maintained in growth medium. RNA samples were collected 4 days after transfection and subjected to gene expression profiling using a Ref-8 v2 Illumina bead chip. The percentage of differentially expressed genes (125b-DP versus scrambled duplex by 1.5-fold) belonging to each GO processes was compared to the percentage of the whole genome sorted into the same category. Categories enriched for the differentially expressed genes are shown in the charts. *P* values (representing the difference between the percentage of differentially expressed genes and the percentage of the whole genome in each category) are indicated as * for a *P* value of <0.05 and ** for a *P* value of <0.01. (c) Heat map representation of gene expression profiles for SH-SY5Y cells 2 days after transfection with scrambled duplex, 125b-DP, and/or 125b-AS in growth medium (GM) or in the presence of RA. Each oligonucleotide was transfected at a final concentration of 80 nM. Gene expression was analyzed by use of a Ref-8 v2 Illumina bead chip. The colors indicate the average changes in intensity normalized to the mean of all arrays, ranging from one-quarter-fold (green) to fourfold (red). Only genes downregulated by 125b-DP are shown. The tree diagram represents the clustering of treatments based on their gene expression patterns.

discovery according to methods described previously by Lim et al. (24). From the 388 candidate genes, we were able to obtain the sequences of 253 3' UTRs from published data (135 candidate genes had no available 3' UTR sequence). A search by MEME identified a 6-nt motif, "TCAGGG" in 129 of these sequences, that is, 51% out of the 253 available 3' UTRs. Importantly, this motif is perfectly complementary to the seed sequence (nt 2 to 8) of miR-125b (Fig. 7a). Extensions of this

common motif to 7 to 9 nt also matched the seed sequence of miR-125b in a significant proportion of these 129 3' UTR sequences (Fig. 7a). As a control, we analyzed the 3' UTR sequences of the genes upregulated 4 days after the ectopic expression of miR-125b and found no enrichment in the "TCAGGG" motif (data not shown).

The second approach is an integrated prediction of miR-125b targets using four different conventional methods: Tar-

getScan 4.2 (22), mirBASE target (12), rna22 (28), and miRNA Viewer (9). Different prediction methods identified different numbers of targets with considerable overlap (Fig. 7b). In total, the four prediction methods identified 97 genes (25%) among the 388 downregulated genes as being the direct targets of miR-125b (Fig. 7b). When the same prediction methods were applied to genes randomly selected from the whole genome or from unfiltered differentially expressed genes, only 4% and 11% of these genes were predicted to be targets of miR-125b, respectively (Fig. 7c). Hence, our list of 388 candidate miR-125b targets is significantly enriched for predicted direct targets. Furthermore, among the 97 predicted direct miR-125b targets, we found that 81% of their 3' UTRs contain the 6-nt motif that was identified by MEME, matching the seed sequence of miR-125b. The list of targets predicted by both approaches is provided in Tables S3 and S4 in the supplemental material.

Pathway analysis and validation of direct miR-125b targets.

To understand how the predicted targets of miR-125b regulate neuronal differentiation, we examined their known functions and the signaling networks connecting them with other genes (considered indirect effectors) that were differentially regulated 4 days after the transfection of 125b-DP (shown in Fig. 6a and b). The analysis was performed using the Ingenuity System, which maps biomolecular networks based on known signaling pathways and known interactions with reliable data curation. The predicted direct miR-125b targets (resulting from MEME and the conventional predictions described above) include genes of diverse functions. Compared to these direct targets, the group of indirect effectors is significantly enriched in genes involved in nervous system development and function. Interestingly, many of the indirectly regulated neuronal genes are extensively connected to the predicted direct targets, forming a large network with hundreds of genes. To simplify the network, we selected only the direct targets that are placed upstream of the indirect effectors and filtered for pathways that are relevant to neuronal differentiation. We propose a model of regulation based on the resultant network (Fig. 7d). In this model, miR-125b directly suppresses the expression of 10 key target genes that, in turn, repress pathways that mediate neuronal differentiation. Pathways in the model encompass both signaling transduction and gene regulation with the key signaling molecules protein kinase C, JNK, ERK, mitogen-activated protein kinase, and vascular EGF and the important transcription factors SMAD2, SMAD4, and STAT3.

The final outcome of miR-125b upregulation during neurogenesis (Fig. 2) would then be the upregulation of many neurologically important genes such as SCNBA, EPHB2, KCNQ2, FLNA, SYN2, and NEFM (Fig. 7d).

Subsequently, we used real-time PCR to validate the expression of the 10 target genes used in our model. All 10 genes were downregulated by a 2-day overexpression of miR-125b in growth medium or differentiation medium except AP1M1, which was downregulated only in the presence of RA (Fig. 8a). Furthermore, the binding of miR-125b to the predicted MREs in the 3' UTR of the 10 targets was also validated by a luciferase reporter assay (Fig. 8b). In this assay, individual MREs were cloned into the 3' UTR of a luciferase reporter gene. The construct plasmids were transfected into HEK-293T cells, and luciferase activity was quantified after 2 days. The cotransfection of 125b-DP with the plasmids suppressed luciferase activity by 30 to 70% ($P < 0.01$) in comparison to a scrambled-duplex-cotransfected control. These data indicate that transfected miR-125b bound to the target MREs and repressed the expression of luciferase.

To confirm the specific interaction of miR-125b with the target MREs, we selected the top three hits, TBC1D1, DGAT1, and SGPL1, from the MRE-luciferase reporter assay. A 500-bp segment containing the miR-125b MRE in the 3' UTR of each gene was cloned after the luciferase reporter gene; in these constructs, we also made seven mismatches in the predicted seed region of the binding sites (MREs) for miR-125b. Figure 8c shows that miR-125b reduced the luciferase activity of the DGAT1, SGPL1, and TBC1D1 reporters to ~82%, 65%, and 63% of the control level, respectively. Importantly, the activity of the DGAT1 and SGPL1 luciferase reporters in the presence of miR-125b was restored to 100% by the mutation of the predicted miR-125b seed region. The mutation of the miR-125b seed segment in the reporter for TBC1D1 resulted in a partial but significant recovery of luciferase activity. These data suggest that the predicted seed region is absolutely necessary for the binding of miR-125b to the 3' UTR of DGAT1 and SGPL1 but that it is not the only factor that determines the binding of miR-125b to the 3' UTR of TBC1D1. In summary, we have shown that miR-125b is likely to directly target the 10 genes in the neurogenic pathway listed in Table 1, in particular DGAT1, SGPL1, and TBC1D1.

FIG. 7. Target prediction and pathway analysis. (a) MEME motif discovery searching for motifs containing 4 to 9 nt in the 3' UTRs of the downregulated genes. The available 3' UTR sequences were collected for 253 out of the 388 genes downregulated exclusively by 2 days of miR-125b ectopic expression. MEME identified significant motifs of 6 to 9 nt in a proportion of the 3' UTR sequences (shown as the percentage of the UTRs with the consensus). The motifs (orange text) are perfectly complementary to the seed region of miR-125b (nt 2 to 8 [shaded blue text]). MEME expectation represents the probability at which the same motifs can be found by chance. (b) Prediction of miR-125b targets by conventional methods. Targets of miR-125b among the 388 genes downregulated exclusively by miR-125b gain of function were predicted by four methods, TargetScan 4.2, mirBASE target, RNA22, and miRNA Viewer, using default settings. The numbers outside the circles represent the total numbers of targets predicted by each method. The numbers inside the circles represent the numbers of overlapped and nonoverlapped targets. (c) Statistical test elucidating the significance of our selection method. The targets of miR-125b were predicted (using the four methods described above) from a list of 388 genes randomly selected from the whole genome and another list of 388 genes randomly selected out of 6,448 genes, which are differentially expressed by at least one treatment. The random selection and prediction were repeated 10,000 times. The average percentage of enriched targets was compared with the 97 enriched targets found in b. The P values computed by a Student's one-sample t test indicate the significance of the differences. (d) Modeled downstream network of miR-125b that mediates neuronal differentiation. Connections were mapped using IPA. PI3K, phosphatidylinositol 3-kinase; IL-12, interleukin-12.

FIG. 8. Target validation. (a) Quantitative real-time PCR validating the expression pattern of miR-125b target genes 2 days after transfection of 125b-DP into SH-SY5Y cells in growth medium (GM) or in differentiation medium containing RA. The readings were normalized to the expression level of β -actin. All the readings are presented as average changes $\pm$ SEM relative to the gene expression level in the cells transfected with scrambled duplex (DP). (b) Luciferase reporter assays validating the binding of miR-125b to the 3' UTRs of the target genes. Reporter genes contain only the MREs that were predicted to bind to miR-125b. An MRE with perfect complementarity to miR-125b was used as a control. (c) Reporter genes contain 500 bp of the 3' UTRs of selected target genes with wild-type sequence or with seven mismatches in the seed region of the MREs. Plasmids carrying each reporter gene were cotransfected with 125b-DP into 293T cells. Luciferase readings were obtained 48 h after transfection and are presented here as the average percentages of luciferase activity $\pm$ SEM ($n \geq 3$) relative to the scrambled-duplex-cotransfected control (100%). The results of Student *t* tests comparing the luciferase activities of each reporter gene in the presence of 125b-DP versus the scrambled duplex are indicated as ** for a *P* value of <0.01 . The results of Student *t* tests comparing the luciferase activity of the mutant reporter genes with the activity of the corresponding wild-type reporter genes in the presence of 125b-DP are indicated as ## for a *P* value of <0.01 .

DISCUSSION

In our study, we utilized a simple in vitro model of human neuronal differentiation in which human neuroblastoma SH-SY5Y cells were differentiated into a homogenous population of cells with neuronal morphology. The advantages of this model over other in vitro systems for human neuronal differentiation include its robust differentiation capability (terminal differentiation is obtained within 2 weeks of induction) and the formation of neurons only and not other cell types such as glia (8). In comparison to previous reports of miRNAs in human neural differentiation (20, 33, 38, 40), which focused mainly on the profiling of miRNAs, we have advanced well beyond expression profiling and established a number of reliable assays to assess the biological functions of specific miRNAs in the neuronal differentiation of SH-SY5Y cells as well as of human neural progenitor RVM cells. We identified two miRNAs,

miR-124a and miR-125b, which promote neurite outgrowth. We further demonstrated how the upregulation of miR-125b during neurogenesis downregulates a set of direct mRNA targets. Since the proteins encoded by these mRNAs normally repress neurogenesis, our model (Fig. 7d) suggests how miR-125b induction causes an enhanced expression of multiple neuron-important genes.

miR-125b is expressed in many types of tissues, but its highest level of expression is in the brain, especially in mature neurons but not astrocytes (33, 35, 37). miR-125b is upregulated during mouse neurogenesis (35), during the neural differentiation of mouse embryonic stem cells (18), and upon RA treatment of embryonic carcinoma cells (33) and of neuroblastoma SK-N-BE cells (19). Adding to these studies, our data demonstrate that miR-125b is not only a marker of differentiation but also a regulator of neuronal differentiation in SH-

SY5Y cells. By quantifying the effect of miR-125b ectopic expression and miR-125b knockdown on neurite outgrowth and on the expression of neuronal markers, we demonstrate that miR-125b is both necessary and sufficient to promote the neuronal differentiation of SH-SY5Y cells.

In our functional assays, we examined the effect of miR-125b ectopic expression on differentiation over a short time frame of 4 days and found that only a fraction of the cells differentiated. Importantly, the percentage of “differentiated cells” varies depending on the criteria used for quantification. In the neurite outgrowth assay, we considered only the differentiated cells with apparent neurite outgrowth. Because we used very stringent parameters that allow us to identify only the most mature neurons, β III-tubulin-positive cells with neurites longer than 30 μ m, the percentage of the selected cells was rather small, 1 to 6% (Fig. 4b). Reducing the stringency by considering a lower minimum neurite length would increase the percentage of selected cells, but the neurite identification then becomes less accurate since cell edges can be mistaken as short neurites. In our immunostaining assay, where differentiation was determined based on the expression of the neuronal protein markers Map2ab, neurofilament, and Syt5, we observed a higher percentage of differentiated cells, 5 to 16% (Fig. 4d). Hence, the cells appeared to upregulate these markers earlier than the onset of neurite outgrowth.

Because we were concerned with the abnormal karyotype and tumor origin of SH-SY5Y cells, we examined the expression and the function of miR-125b in a more physiologically relevant cell type, human neural progenitor RVM cells. Like primary neural stem cells, RVM cells have a normal karyotype and are able to differentiate into both neurons and glial cells (7). We showed that, as in SH-SY5Y cells, miR-125b expression was gradually upregulated during the differentiation of RVM cells. miR-125b ectopic expression significantly enhanced the neurite outgrowth of RVM cells in both growth medium and differentiation medium. Thus, our data indicate that miR-125b is important for neuronal differentiation in both RVM cells and SH-SY5Y cells and suggest a common function of miR-125b in neural progenitor cells. Potentially, miR-125b gain of function may be useful to enhance the *in vitro* neuronal differentiation of primary human neural stem cells for treatments of neurodegenerative diseases. This approach would probably be more advantageous than other types of gene therapy since the miRNA is a small molecule that, in principle, can be delivered more easily into a cell.

On the other hand, we also noted several differences in the effects of miR-125b on SH-SY5Y and RVM cells. miR-125b ectopic expression exhibited a stronger effect on the average neurite length in RVM cells than in SH-SY5Y cells in growth medium, but the reverse was observed for differentiation medium. Hence, in RVM cells, miR-125b alone is sufficient to promote the extension of neurite length, but in SH-SY5Y cells, it requires the addition of RA. Furthermore, the knockdown of miR-125b in SH-SY5Y cells significantly reduced the extension of neurites induced by RA; however, the same effect was not observed when miR-125b was knocked down in RVM cells undergoing differentiation. Since the two cell lines were differentiated by two different methods, the differences in the effects of miR-125b may be more apparent than real, but it does appear as if the role of miR-125b in neurite outgrowth is more

necessary for the RA-induced differentiation of SH-SY5Y cells than it is for the differentiation of RVM cells upon the withdrawal of EGF and bFGF. Additionally, the phenotype may also be determined by the intrinsic differences between the two cell lines: as they express different mRNAs, the genes directly and indirectly affected by miR-125b regulation are likely to be different. The physiological functions of miR-125b *in vivo* may also depend on different extrinsic and intrinsic factors that are regulated in a temporal and spatial manner. Interestingly, we recently showed that the knockdown of miR-125b leads to severe defects in zebrafish brain development, including the malformation of axonal tracts in midbrain and hindbrain, suggesting that miR-125b is required for neuronal differentiation *in vivo* (our unpublished data). It would be interesting to further study the cell-specific function of miR-125b *in vivo*.

To understand the mechanism mediating miR-125b function, we conducted global profiling to identify miR-125b-responsive genes. We chose to perform this experiment primarily using SH-SY5Y cells because these cells are more responsive to the modulation of miR-125b levels than RVM cells. Using microarrays, we identified 388 genes repressed by miR-125b ectopic expression and predicted that 164 of these genes are the direct targets of miR-125b. This prediction is supported by two lines of evidences: (i) MEME motif discovery identified a 6-nt motif in the 3' UTR of 129 genes that is perfectly complementary to the seed sequence of miR-125b, and (ii) an integrative search using four conventional miRNA target prediction methods identified 97 direct targets among the 388 genes repressed by miR-125b. Moreover, we found that 57 (~35%) out of the 164 selected targets were downregulated by RA- or BDNF-induced neuronal differentiation by ≥ 1.5 -fold. The inverse expression pattern of these genes in comparison to the endogenous expression of miR-125b implies that they are targeted by miR-125b during differentiation. Although the actual number of endogenous targets is subject to a further validation of our predictions, we do expect the complex function of miR-125b to be mediated by multiple mRNA targets. Previous profiling studies of miRNA targets by microarrays and proteomics demonstrated that miRNAs usually downregulate several hundred genes; the targets are mostly repressed at both mRNA and protein levels, although a number of them are regulated only at the protein level (1, 32). Our microarray data for SH-SY5Y cells were able to identify only the targets regulated by miR-125b through mRNA degradation and/or deadenylation. In a separate study, we found that p53 is a bona fide target of miR-125b; a modulation of miR-125b largely affects the p53 protein level but did not show any significant change in the transcript level of p53 in SH-SY5Y cells (19a). Besides p53, it is possible that our microarray analysis also missed other targets that are regulated only by translational inhibition.

We next asked how miR-125b mediates neuronal differentiation by suppressing the 164 predicted targets. Data from IPA suggest that a subset of these targets is connected to the neuronal genes that were indirectly upregulated by miR-125b gain of function. We propose a simple model to explain how miR-125b enhances differentiation. In constructing the model, we assumed that the direct targets of miR-125b inhibit pathways that promote the expression of neuronal genes. Hence, from the network connecting the predicted downregulated direct mRNA targets and the upregulated indirect neuronal ef-

factors, we selected the pathways relevant to neurogenesis and the direct targets with known inhibitory effects or known binding to the components of these pathways. The model focused on 10 predicted direct targets of miR-125b, and we validated these both by real-time PCR analysis of mRNA expression after the ectopic expression of miR-125b and by a luciferase reporter assay (Table 1). IPA also revealed that many genes in the modeled pathways are regulated by RA in the same manner as by miR-125b. This relationship, and the fact that RA upregulates miR-125b during differentiation, suggests that miR-125b mediates RA-induced differentiation in SH-SY5Y cells. Our proposed model of the miR-125b network supports this hypothesis, since the ERK signaling pathway featured prominently in our model is also known to mediate RA-induced differentiation in SH-SY5Y cells (27). Indeed, the model also predicts that miR-125b exerts positive feedback on RXRA, the receptor for RA.

In addition, IPA shows that the predicted targets of miR-125b are also connected to the repressed indirect effectors (genes downregulated 4 days after the transfection of 125b-DP), mainly with positive regulatory effects. These networks are involved in metabolism, proliferation, and apoptosis; thus, in part, miR-125b may enhance differentiation by reducing cell metabolism and proliferation. Experimentally, we did not find any significant effect of miR-125b gain of function on proliferation (using Ki67 staining) (data not shown). Laneve et al. also previously found that miR-125b alone has very little effect on proliferation, although the ectopic expression of miR-125b together with miR-125a and miR-9 inhibits cell cycling in neuroblastoma cells (19). Hence, the withdrawal of SH-SY5Y cells from the cell cycle during differentiation may require a synergistic effect between miR-125b and other miRNAs. On the other hand, the negative regulation by miR-125b on a number of apoptotic genes, including the four targets BAK1, TP53INP1, PPP1CA, and PRKRA in the p53 pathway, suggests that miR-125b has an antiapoptotic effect. In a separate study, we found that miR-125b downregulates the p53 pathway and that miR-125b gain of function represses apoptosis induced by H7 in SH-SY5Y cells (19a). These results suggest that miR-125b may promote the survival of differentiated neuronal cells by suppressing apoptosis.

In conclusion, we report here several important and novel functions of miR-125b in neuronal differentiation. Our results demonstrate that this miRNA promotes the differentiation of human neuroblastoma SH-SY5Y cells and human neural progenitor VSM cells toward the neuronal phenotype. In SH-SY5Y cells, we propose a model where the action of miR-125b is mediated by 10 targets that repress multiple pathways involved in neuronal differentiation.

ACKNOWLEDGMENTS

We thank all our colleagues in Biopolis and the Whitehead Institute, especially Philip Gaughwin, Boon Seng Soh, Wai Leong Tam, Yen Sin Ang, Yvonne Tay, Yin Loon Lee, Andrew Thomson, Prakash Rao, Shilpa Hattangadi, and Cheng Cheng Zhang for fruitful discussions and Ng Shyh-Chang and Senthil Raja Jayapal for proofreading the paper. We thank David Bartel and his laboratory for providing the miRNA microarray and other reagents. We also thank the staffs at the Biopolis High Content Screening Facility for providing the HCS service and advices.

M.T.N.L. and H.X. were supported by an SMA graduate fellowship. P.H.C., P.R., G.U., and H.Y. were supported by A*STAR, Singapore. B.L. and H.F.L. were partially supported by SMA grant C-382-641-001-091. B.L. was also supported by NIH grants DK047636 and AI54973. H.F.L. and B.Z. were supported by NIH grant R01 DK068348.

REFERENCES

1. Baek, D., J. Villen, C. Shin, F. D. Camargo, S. P. Gygi, and D. P. Bartel. 2008. The impact of microRNAs on protein output. *Nature* **455**:64–71.
2. Bartel, D. P. 2004. microRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**:281–297.
3. Baskerville, S., and D. P. Bartel. 2005. Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes. *RNA* **11**:241–247.
4. Bilen, J., N. Liu, B. G. Burnett, R. N. Pittman, and N. M. Bonini. 2006. microRNA pathways modulate polyglutamine-induced neurodegeneration. *Mol. Cell* **24**:157–163.
5. Choi, P. S., L. Zakhary, W. Y. Choi, S. Caron, E. Alvarez-Saavedra, E. A. Miska, M. McManus, B. Harfe, A. J. Giraldez, R. H. Horvitz, A. F. Schier, and C. Dulac. 2008. Members of the miRNA-200 family regulate olfactory neurogenesis. *Neuron* **57**:41–55.
6. Chua, S. W., P. Vijayakumar, P. M. Nissom, C. Y. Yam, V. V. Wong, and H. Yang. 2006. A novel normalization method for effective removal of systematic variation in microarray data. *Nucleic Acids Res.* **34**:e38.
7. Donato, R., E. A. Miljan, S. J. Hines, S. Aouabdi, K. Pollock, S. Patel, F. A. Edwards, and J. D. Sinden. 2007. Differential development of neuronal physiological responsiveness in two human neural stem cell lines. *BMC Neurosci.* **8**:36.
8. Encinas, M., M. Iglesias, Y. Liu, H. Wang, A. Muhaisen, V. Cena, C. Gallego, and J. X. Comella. 2000. Sequential treatment of SH-SY5Y cells with retinoic acid and brain-derived neurotrophic factor gives rise to fully differentiated, neurotrophic factor-dependent, human neuron-like cells. *J. Neurochem.* **75**:991–1003.
9. Enright, A. J., B. John, U. Gaul, T. Tuschl, C. Sander, and D. S. Marks. 2003. microRNA targets in *Drosophila*. *Genome Biol.* **5**:R1.
10. Filipowicz, W., S. N. Bhattacharyya, and N. Sonenberg. 2008. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat. Rev. Genet.* **9**:102–114.
11. Giraldez, A. J., R. M. Cinalli, M. E. Glasner, A. J. Enright, J. M. Thomson, S. Baskerville, S. M. Hammond, D. P. Bartel, and A. F. Schier. 2005. microRNAs regulate brain morphogenesis in zebrafish. *Science* **308**:833–838.
12. Griffiths-Jones, S., R. J. Grocock, S. van Dongen, A. Bateman, and A. J. Enright. 2006. miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res.* **34**:D140–D144.
13. Johnston, R. J., and O. Hobert. 2003. A microRNA controlling left/right neuronal asymmetry in *Caenorhabditis elegans*. *Nature* **426**:845–849.
14. Kim, J., K. Inoue, J. Ishii, W. B. Vanti, S. V. Voronov, E. Murchison, G. Hannon, and A. Abeliovich. 2007. A microRNA feedback circuit in midbrain dopamine neurons. *Science* **317**:1220–1224.
15. Kim, J., A. Krichevsky, Y. Grad, G. D. Hayes, K. S. Kosik, G. M. Church, and G. Ruvkun. 2004. Identification of many microRNAs that copurify with polyribosomes in mammalian neurons. *Proc. Natl. Acad. Sci. USA* **101**:360–365.
16. Kosik, K. S. 2006. The neuronal microRNA system. *Nat. Rev. Neurosci.* **7**:911–920.
17. Krichevsky, A. M., K. S. King, C. P. Donahue, K. Kharpko, and K. S. Kosik. 2003. A microRNA array reveals extensive regulation of microRNAs during brain development. *RNA* **9**:1274–1281.
18. Krichevsky, A. M., K. C. Sonntag, O. Isacson, and K. S. Kosik. 2006. Specific microRNAs modulate embryonic stem cell-derived neurogenesis. *Stem Cells* **24**:857–864.
19. Laneve, P., M. L. Di, U. Gioia, M. E. Fiori, E. Ferretti, A. Gulino, I. Bozzoni, and E. Caffarelli. 2007. The interplay between microRNAs and the neurotrophin receptor tropomyosin-related kinase C controls proliferation of human neuroblastoma cells. *Proc. Natl. Acad. Sci. USA* **104**:7957–7962.
- 19a. Le, M. T., C. Teh, N. Shyh-Chang, H. Xie, B. Zhou, V. Korzh, H. F. Lodish, and B. Lim. 2009. MicroRNA-125b is a novel negative regulator of p53. *Genes Dev.* **23**:862–876.
20. Lee, Y. S., H. K. Kim, S. Chung, K. S. Kim, and A. Dutta. 2005. Depletion of human microRNA miR-125b reveals that it is critical for the proliferation of differentiated cells but not for the down-regulation of putative targets during differentiation. *J. Biol. Chem.* **280**:16635–16641.
21. Leucht, C., C. Stigloher, A. Wizenmann, R. Klafke, A. Folchert, and L. Bally-Cuif. 2008. microRNA-9 directs late organizer activity of the midbrain-hindbrain boundary. *Nat. Neurosci.* **11**:641–648.
22. Lewis, B. P., C. B. Burge, and D. P. Bartel. 2005. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* **120**:15–20.
23. Li, X., and R. W. Carthew. 2005. A microRNA mediates EGF receptor

- signaling and promotes photoreceptor differentiation in the *Drosophila* eye. *Cell* **123**:1267–1277.
24. Lim, L. P., N. C. Lau, P. Garrett-Engele, A. Grimson, J. M. Schelter, J. Castle, D. P. Bartel, P. S. Linsley, and J. M. Johnson. 2005. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature* **433**:769–773.
  25. Maere, S., K. Heymans, and M. Kuiper. 2005. BiNGO: a Cytoscape plugin to assess overrepresentation of gene ontology categories in biological networks. *Bioinformatics* **21**:3448–3449.
  26. Makeyev, E. V., J. Zhang, M. A. Carrasco, and T. Maniatis. 2007. The microRNA miR-124 promotes neuronal differentiation by triggering brain-specific alternative pre-mRNA splicing. *Mol. Cell* **27**:435–448.
  27. Miloso, M., D. Villa, M. Crimi, S. Galbiati, E. Donzelli, G. Nicolini, and G. Tredici. 2004. Retinoic acid-induced neuritogenesis of human neuroblastoma SH-SY5Y cells is ERK independent and PKC dependent. *J. Neurosci. Res.* **75**:241–252.
  28. Miranda, K. C., T. Huynh, Y. Tay, Y. S. Ang, W. L. Tam, A. M. Thomson, B. Lim, and I. Rigoutsos. 2006. A pattern-based method for the identification of microRNA binding sites and their corresponding heteroduplexes. *Cell* **126**:1203–1217.
  29. Miska, E. A., E. Alvarez-Saavedra, M. Townsend, A. Yoshii, N. Sestan, P. Rakic, M. Constantine-Paton, and H. R. Horvitz. 2004. Microarray analysis of microRNA expression in the developing mammalian brain. *Genome Biol.* **5**:R68.
  30. Olsen, P. H., and V. Ambros. 1999. The lin-4 regulatory RNA controls developmental timing in *Caenorhabditis elegans* by blocking LIN-14 protein synthesis after the initiation of translation. *Dev. Biol.* **216**:671–680.
  31. Schratt, G. M., F. Tuebing, E. A. Nigh, C. G. Kane, M. E. Sabatini, M. Kiebler, and M. E. Greenberg. 2006. A brain-specific microRNA regulates dendritic spine development. *Nature* **439**:283–289.
  32. Selbach, M., B. Schwanhauser, N. Thierfelder, Z. Fang, R. Khanin, and N. Rajewsky. 2008. Widespread changes in protein synthesis induced by microRNAs. *Nature* **455**:58–63.
  33. Sempere, L. F., S. Freemantle, I. Pitha-Rowe, E. Moss, E. Dmitrovsky, and V. Ambros. 2004. Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. *Genome Biol.* **5**:R13.
  34. Simon, D. J., J. M. Madison, A. L. Conery, K. L. Thompson-Peer, M. Soskis, G. B. Ruvkun, J. M. Kaplan, and J. K. Kim. 2008. The microRNA miR-1 regulates a MEF-2-dependent retrograde signal at neuromuscular junctions. *Cell* **133**:903–915.
  35. Smirnova, L., A. Grafe, A. Seiler, S. Schumacher, R. Nitsch, and F. G. Wulczyn. 2005. Regulation of miRNA expression during neural cell specification. *Eur. J. Neurosci.* **21**:1469–1477.
  36. Wheeler, G., S. Ntounia-Fousara, B. Granda, T. Rathjen, and T. Dalmay. 2006. Identification of new central nervous system specific mouse microRNAs. *FEBS Lett.* **580**:2195–2200.
  37. Wienholds, E., W. P. Kloosterman, E. Miska, E. Alvarez-Saavedra, E. Berezikov, E. de Bruijn, H. R. Horvitz, S. Kauppinen, and R. H. Plasterk. 2005. microRNA expression in zebrafish embryonic development. *Science* **309**:310–311.
  38. Yoong, L. F., G. Wan, and H. P. Too. 2006. Glial cell-line derived neurotrophic factor and neurturin regulate the expressions of distinct miRNA precursors through the activation of GFRalpha2. *J. Neurochem.* **98**:1149–1158.
  39. Yu, J. Y., K. H. Chung, M. Deo, R. C. Thompson, and D. L. Turner. 2008. microRNA miR-124 regulates neurite outgrowth during neuronal differentiation. *Exp. Cell Res.* **314**:2618–2633.
  40. Zhao, J. J., Y. J. Hua, D. G. Sun, X. X. Meng, H. S. Xiao, and X. Ma. 2006. Genome-wide microRNA profiling in human fetal nervous tissues by oligonucleotide microarray. *Childs Nerv. Syst.* **22**:1419–1425.