microRNA-125b Promotes Neuronal Differentiation in Human Cells by Repressing Multiple Targets

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microRNAs are a class of small non-coding RNAs that regulate gene expression at the post-transcriptional level. Research on microRNAs has highlighted their importance in neural development but the specific functions of neural-enriched microRNAs remain poorly understood. We report here the expression profile of microRNAs during neuronal differentiation in the human neuroblastoma cell line SH-SY5Y. Six microRNAs were significantly upregulated during differentiation induced by all-trans-retinoic acid and brain-derived neurotrophic factor. We demonstrated that 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 spontaneous and induced differentiation of SH-SH5Y cells. miR-125b is also upregulated during differentiation of human neural progenitor ReNcell VM cells, and miR-125b ectopic expression significantly promotes 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 microRNA and/or predicted to be direct targets of miR-125b by conventional methods. Pathway analysis suggests that a subset of miR-125b-repressed targets antagonize 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 microRNA response elements of ten selected mRNA targets. Together, we report here for the first time the important role of miR-125b in human neuronal differentiation.
INTRODUCTION

MicroRNAs (miRNAs) represent an emerging class of small non-coding RNAs that play important roles in post-transcriptional regulation of gene expression (2). They are transcribed initially as long RNAs and then processed by two RNase complexes, Drosha and Dicer, into ~22 nucleotides (nt) duplexes that are subsequently loaded into the RNA-induced silencing complexes (RISCs) (2). Mature miRNAs in the RISCs usually bind to the 3’ UTR of messenger RNAs (mRNAs), leading to translational suppression or destabilization of the target mRNAs or both (10). Interaction 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 have highlighted the abundant and diverse expression of miRNAs in the central nervous system (CNS) (15-17,30,34,37). Mammalian brain tissues express about 70% of experimentally detectable miRNAs, many of which are developmentally regulated (15-17,30,34,37). In maternal-zygotic zebrafish dicer mutants, deficiency in Dicer-mediated biogenesis of miRNAs leads to severe defects in brain morphogenesis (11). Similarly, the loss of Dicer in sca3-mutant Drosophila enhances neurodegeneration (4). Specific knockdown of Dicer in mouse midbrain dopaminergic neurons resulted in progressive loss of these cells (14). Recent studies have also elucidated the contribution of individual miRNAs in various aspects of neural development. For example, mir-9a regulates the organizer function of the zebrafish midbrain-hindbrain boundary (21). In C. elegans, lsy-6 and mir-273 determine the cell fate of chemoreceptor neurons (13). miR-7 regulates differentiation of
photoreceptor neurons in *Drosophila* (23). The mir-200 family regulates the terminal differentiation of olfactory neurons in both 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 (32). In *C. elegans*, mir-1 regulates MEF-2 dependent retrograde signaling at the neuromuscular junctions (35). Most functional studies of miRNAs in neuronal development have been carried out in 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 differentiation of human neural cells using simple *in vitro* models, human neuroblastoma SH-SY5Y cells and human neural progenitor ReNcell VM 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 differentiation and neuronal morphogenesis of SH-SY5Y cells. In addition, this miRNA also promotes neurite outgrowth in human neural progenitor ReNcell VM 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* (31). miR-125b is abundantly expressed in animal brains and is upregulated during neurogenesis (17,29,34,36). 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. Computational analysis suggests that ten of these genes antagonize several neurogenic pathways, especially ERK signaling that is known to mediate the effect of retinoic acid in neuronal differentiation.
MATERIALS AND METHODS

Cell culture and differentiation condition

SH-SY5Y cells and HEK-293T cells were maintained in Dulbecco's Modified Eagle medium (DMEM) containing 4500 mg/L glucose, 10% heat-inactivated fetal bovine serum (GIBCO), 110 mg/L 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 on collagen-coated plates (BD Biosciences) at an initial density of $10^4$ cells/cm$^2$. All-trans-retinoic acid (Sigma) was added at a final concentration of 10 µM on the next day after plating. After five days, the cells were washed three times with DMEM and incubated with 50 ng/ml brain-derived neurotrophic factor (Sigma) in growth medium without serum for seven days.

ReNcell VM (RVM) cells were cultured in laminin-coated plates in DMEM/F12 (1:1) medium (Invitrogen), supplemented with 10% B27 medium (Invitrogen), 10 µg/ml Gentamycin (GIBCO), 10 units/ml Heparin (Sigma), 20 ng/ml epidermal growth factor and 10 ng/ml basic fibroblast growth factor (Invitrogen). For differentiation, the growth medium was replaced with the Neurobasal medium (Invitrogen), supplemented with 10% B27 medium (Invitrogen), 10 µg/ml Gentamycin (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 from 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 $^{32}$P-labeled RNA markers of 18 nt and 24 nt were co-loaded with total RNA samples and used as indicators to identify the small RNA population on the gel separating 100 µg of total RNA.

18-24 nt RNAs were gel-purified and sequentially ligated to 3’- and a 5’-end adaptors. Ligated products were gel-purified, reverse transcribed, PCR-amplified and labeled with Cy3. The labeled sense strand was then gel-purified and applied to 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 to a DNA oligonucleotide-based array, serving as internal hybridization controls. This array (provided by the Bartel laboratory at Whitehead institute) contains ~600 DNA probes, including probes for 175 human miRNAs (3). The obtained signals were normalized to the total intensity of all non-cognate probes (corresponding to the nematode miRNAs that are not conserved in human). 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). Microarray data was deposited into Gene Expression Omnibus (accession number: GSE14787).
Northern blot analysis

10 – 40 µg of each total RNA sample and a 33P –labeled Decade® RNA marker (Ambion) were separated on a 15% denaturing gel, transferred to a Genescreen Plus membrane (PerkinElmer), UV-cross-linked and baked at 80ºC for 30 minutes. DNA probes with the sequences complementary to the miRNAs were synthesized (Invitrogen) and labeled with 32P-γ-ATP (Amersham). U6 RNA and 5S RNA probes were used to determine loading equity. The probe sequences are provided in Supplementary Table 1. The membrane was prehybridized in PerfectHyb buffer (Sigma) with 1 mg of freshly added sheared salmon sperm DNA (Sigma) for two hours at 48 ºC. Subsequently, the labeled probes were added; hybridization was carried out overnight at 48 ºC. 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 less than 25) were seeded as 80,000 cells/well in collagen-coated 12-well plate (BD Bioscience). On the next day, using 4 µl lipofectamine™2000 reagent (Invitrogen) per well according to the manufacturer’s instruction, the cells were transfected with one of these RNA oligos at 80 nM final concentration: BlockIT™ fluorescent oligo (Invitrogen), scrambled duplex (Ambion PremiR negative control #1), miRNA duplex (Ambion PremiR) or miRNA antisense (Ambion AntimiR). After five hours, the transfection medium was replaced by fresh growth medium either with or without 10 µM RA. 125,000 ReNcell VM (RVM) cells were transfected in suspension with 80 nM RNA oligos in the same manner as for SH-SY5Y cells. After
five hours of transfection, the medium was changed to fresh RVM growth medium or differentiation medium and the cells were plated in laminin-coated plates.

**Immunostaining and high-content screening**

Four days after transfection, SH-SY5Y cells or RVM cells were fixed with 4% paraformaldehyde for 15 minutes, followed by three washes with phosphate buffer saline (PBS). After one-hour blocking with 0.2% TritonX100 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 βIII-tubulin antibody (Abcam, 1:1000 dilution), mouse monoclonal Map2ab (Sigma, 1:1000 dilution), mouse monoclonal pan-axonal neurofilament antibody (Covance, 1:1500 dilution), goat monoclonal synaptotagmin V antibody (Santa Cruz 1:1000 dilution), rabbit polyclonal musashi-1 antibody (Abcam 1:1000 dilution). Subsequently, the cells were washed with PBS for 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 five minutes. The cells were then washed with PBS for 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 the Cellomics® high content screening system using a 10x objective lens. For neurite outgrowth assays, images of βIII-tubulin and Hoechst staining were analyzed using the Neuronal Profiling BioApplication software (Cellomics). Differentiated SH-SY5Y cells with neurite outgrowth were defined as βIII-tubulin positive cells with neurites longer than 30 µm. For RVM cells, the percentage of differentiated cells with neurite outgrowth was defined as the percentage of
βIII-tubulin positive cells having neurites longer than 20 µm (RVM cells are smaller than SH-SY5Y cells so we applied a lower threshold of neurite length). For quantification of neuronal marker staining, the images were analyzed by Target Activation BioApplication (Cellomics). In all the high content screening assays, a cell was considered as positive for a specific staining only if its fluorescent intensity was equal or higher than the mean intensity plus two times the standard deviations 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 RNeasy® kit (Qiagen). For quantitative real-time PCR of miRNA, 100 ng of total RNA was reverse-transcribed and subjected to 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) following the manufacturer’s protocol.

Gene expression microarray and data analysis

Total RNA was extracted as described above. 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) following the manufacturer’s instructions. First, from all the raw data the respective backgrounds were subtracted using Bead Studio (Illumina) and then normalized using the cross-correlation method according to Chua et al (6). Subsequently, normalized data were processed for identification of differentially expressed genes using log21.5 as the critical value for the mean of log2 fold changes between miR-125b duplex(125b-DP)/antisense(125b-AS)-transfected samples and
the scrambled controls. Microarray data was deposited into Gene Expression Omnibus (accession number: GSE14787).

Genes that were differentially expressed four-day post-transfection of the miR 125b-DP were subjected to Gene Ontology (GO) analysis, 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 clustering of genes differentially expressed two-day post-transfection, normalized and log₂ transformed data were subtracted from the mean value across all the arrays. Hierarchical clustering was then performed for these processed data using average linkage.

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 at GeneBank. 253 genes were found with the 3’UTR sequences. Following removal of the polyA tails, sequences were masked for repeats using RepeatMasker (http://www.repeatmasker.org/) and analyzed by MEME with the motif width from four to nine 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), miRNA Viewer (9) using the default parameters. To check the statistical
significance of the enrichment, we randomly selected 388 genes from either the whole genome or from all the differentially expressed genes (differentially expressed by at least one treatment) then applied the four methods to predict the targets of miR-125b. The random selection and the target prediction were repeated for 10,000 times. The average percentages of predicted targets out of the selected gene lists were then compared.

Pathway analysis

Ingenuity Pathway Analysis (Ingenuity® System) was used to link the direct targets predicted either by MEME or by conventional methods with the genes differentially expressed four-days after 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

The 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 site, 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-base-pair segment containing the miR-125b MRE in the 3’ UTRs of three selected target genes, TBC1D1, DGAT1 and SGPL1, were synthesized as minigenes (1st 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.
10 ng of each psiCHECK-2 construct was co-transfected with 10 nM miR-125b duplex or scrambled duplex into HEK-293T cells in a 96-well plate using lipofectamin-2000 (Invitrogen). After 48 hours, the cell extract was obtained; firefly and Renilla luciferase activities were measured with the Dual-Luciferase® reporter system (Promega) according to the manufacturer’s instructions.

**Statistical analysis**

Student’s t-test was used to determine the significance of differences between the treated samples and the controls where values were resulted from quantitative real-time PCR, high content screening assays, or permutation of target prediction. Statistical analysis was performed using Microsoft Excel. For Gene Ontology (GO) analysis, the p-value of any enrichment was calculated by BiNGO, using a hypergeometric distribution with bonferroni correction (25).
RESULTS

Profiling miRNA expression in SH-SY5Y cells during differentiation

To understand the regulation of miRNA expression in human neuronal differentiation, we induced differentiation of SH-SY5Y cells into neuron-like cells according to Encinas et al (8) and observed the same morphological changes as described. Neurite outgrowth became apparent after a five-day treatment with all-trans-retinoic acid (RA) and became profuse after a subsequent seven-day treatment with brain-derived neurotrophic factor (BDNF) in serum-free medium (Fig. 1a). Treated cells lost expression of the neural progenitor marker Musashi-1 (Msi1) while they increased expression of the mature neuronal marker Map2ab (Fig. 1b). Gene expression changes profiled by cDNA microarrays indicate acquisition of neuronal markers (upregulation of neuronal microtubule associated proteins, ion channels, neurotransmitter receptors), withdrawal from the cell cycle, and reduced metabolism (downregulation of proliferation and metabolic markers) (Supplementary Table 2). These results are consistent with a differentiation process from neural progenitors to mature neurons. The upregulation of the neuronal markers synaptotagmin V (Syt5), cannabinoid receptor 1 (Cnr1), GABA type B receptor 1 (Gabbr1) was confirmed by quantitative RT-PCR (Fig. 1c).

Next we examined the miRNA expression profiles in undifferentiated SH-SY5Y cells (day 0) and in differentiated SH-SY5Y cells after five days of RA treatment (day 5) and after additional seven days of BDNF treatment in serum-free medium (day 12). We profiled 175 human miRNAs using a miRNA array designed by Baskerville et al (3) and applied a stringent normalization by subtracting the signal from a reference synthetic oligonucleotide for every miRNA (Supplementary Fig. 1). Based on the fold change and expression level, we selected 12 miRNAs that were significantly and consistently
regulated during the course of differentiation (Fig. 2a). Expression of the selected miRNAs was validated by Northern blot (Fig. 2b). We found that six miRNAs, including miR-7, miR-124a, miR-125b, miR-199a, miR-199a* and miR-214, were consistently upregulated during differentiation (Fig. 2b, c). Other miRNA candidates were not detected, or showed no significant change in their expression by Northern blot (Fig. 2b).

Ectopic expression of six miRNA candidates and their effects on neurite outgrowth

Transfection using lipofectamine\textsuperscript{TM}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 one day, more than 80% of the cells were positive for fluorescence and fluorescence persisted until four days after transfection (Fig. 3a). We then transfected the cells with miRNA duplexes and found that by four days after transfection, the levels of the corresponding mature miRNAs were very high compared to those of mock transfected cells (Fig. 3b). Beyond four days post-transfection, the cells often became confluent and unhealthy. Hence, we performed all the experiments using the cells collected four 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 four days, the cells were fixed and stained for βIII-tubulin, a neuron-specific marker.

To quantify the degree of neuronal differentiation, neurite outgrowth was analyzed by the Cellomics\textsuperscript{®} high-content screening (HCS) system where a large number of images were acquired automatically, and neurites were traced and measured in a uniformed manner. Since βIII-tubulin is an early neuronal
marker, only βIII-tubulin positive cells that possess neurites longer than 30 µm, 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 ~1%, in growth medium. By our stringent standards, we found
that only ectopic expression of miR-124a or miR-125b significantly increased the percentage of
differentiated cells with neurite outgrowth, by ~2 fold, compared to the mock and scrambled
transfection controls (Fig. 3c, d). Our subsequent analysis focused on miR-125b since the function of
miR-124a in neuronal differentiation has been described previously (26,40).

miR-125b is necessary and sufficient for neurite outgrowth and neuronal marker gene
expression

We assayed the effects of miR-125b gain/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 miR-125b duplex (125b-DP) was used for gain-of-function. For loss-of-function
studies of miR-125b, we used an antisense oligonucleotide (125b-AS) which 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).

Consistent with the screening results, miR-125b ectopic expression significantly increased the
percentage of differentiated cells with neurite outgrowth in both growth medium and differentiation
medium containing RA (Fig. 4b, c). Specifically, the percentage of differentiated cells with neurite
outgrowth derived by spontaneous differentiation (of mock or scrambled control transfection in
growth medium) was ~1%. miR-125b ectopic expression alone (in growth medium) increased this
fraction of differentiated cells by two fold, to ~2%. Culture in differentiation medium with RA
resulted in ~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 a cotransfection with miR-125b antisense in both growth medium and RA-containing
medium (Fig. 4b, c). We also considered the average neurite length (of neurites selected with the
minimum length of 30 µm) as an indicator of SH-SY5Y neuronal differentiation. miR-125b ectopic
expression increased 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, c).
Together, these results indicate that miR-125b alone is sufficient to stimulate neurite outgrowth.

Conversely, 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, c). 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 differentiation of SH-SY5Y cells was demonstrated further by
staining for additional neuronal markers. As quantified by 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 synaptotagmin V (Fig. 4d). These stimulatory
effects were specifically abrogated by cotransfection with the miR-125b antisense 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 expression of the neural progenitor marker Musashi1 (Msi1) was reduced significantly (Fig. 4e).

miR-125b is upregulated during differentiation of ReNcell VM 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 ReNcell VM (RVM) cells, a neural progenitor cell line isolated from normal human brain and immortalized by v-myc induction. Epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) were used to maintain the cells in the undifferentiated state. Differentiation of RVM cells into neurons and glial cells was induced by 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 seven-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 one and remained detectable until day four post-transfection (Fig. 5c).

We then transfected mir-125b duplex (125b-DP) into RVM cells. Transfected cells were maintained either in the growth medium (containing EGF and bFGF) or in the 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 neurite outgrowth of RVM cells in both growth
medium and in 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, e). In addition, miR-125b ectopic expression significantly increased the average neurite length of differentiated neurons in the growth medium but not in the differentiation medium (Fig. 5d, e). The effect of 125b-DP transfection was abrogated by cotransfecting 125b-AS at an equal concentration (Fig. 5d, e).

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

Profiling the downstream effectors of miR-125b

To understand the mechanism of 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 four days after transfection with 125b-DP in growth medium was compared with that of the scrambled-duplex (DP) transfection control. We found that the genes upregulated by miR-125b ectopic expression were preferentially classified by Gene Ontology (GO) into biological processes related to development, especially nervous system development, neurite growth, cell adhesion, cell morphology, and 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 four post-transfection, many of them appear to have already acquired the expression of neuronal-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, two days post-transfection. Microarray profiling was performed on SH-SY5Y cells transfected with scrambled-DP and 125b-DP in growth medium (GM) or in differentiation medium containing RA. We also included two other treatments: miR-125b knockdown (125b-AS transfected) and neutralization of the 125b-DP (cotransfection of 125b-AS and 125b-DP at equal concentration) 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, 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 that can target a similar set of genes as 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 the MEME motif discovery according to Lim et al (24). From the 388 candidate genes we were able to obtain from published data the sequences of 253 3’ UTRs (135 candidate genes had no available 3’ UTR sequence). A search by MEME identified a 6-nucleotide 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 (nucleotides 2-8) of miR-125b (Fig. 7a). Extensions of this common motif to 7-9 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 four days after 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: TargetScan 4.2 (22), mirBASE target (12), ma22 (28), 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 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 as 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-nucleotide motif that was identified by MEME, matching the seed sequence of miR-125b. The list of targets predicted by both approaches is provided in Supplementary Table 3 and Supplementary Table 4.

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 as indirect effectors) that were differentially regulated four days after transfection of 125b-DP (shown in Fig. 6a-b). The analysis was performed using the Ingenuity® System that maps biomolecular networks based on known signaling pathways and known interactions with reliable data curation. The predicted direct miR-125b targets (resulted from the MEME and the conventional predictions 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 ten 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 PKC, JNK, ERK, MAPK, VEGF 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 neuronal- 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 ten target genes used in our model. All ten genes were downregulated by a two-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, binding of miR-125b to the predicted microRNA response elements (MREs) in the 3’ UTR of the ten 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 two days. Cotransfection of miR-125b duplex with the plasmids suppressed luciferase activity by 30-70% (P < 0.01) in comparison to a scrambled-duplex cotransfected control. This data indicates that the 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-base-pair 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 mutation of the predicted miR-125b seed region. 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 target directly the ten genes in the neurogenic pathway listed in Table 1, in particular DGAT1, SGPL1, and TBC1D1.

**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 two weeks of induction) and the formation only of neurons and not other cell types such as glia (8). In comparison to previous reports on miRNAs in human neural differentiation (20,34,39,41) which mainly focused on profiling of miRNAs, we have advanced well beyond expression profiling and established a number of reliable assays to assess the biological function of specific miRNAs in neuronal differentiation of SH-SY5Y cells as well as of human neural progenitor ReNcell VM cells. We identified two miRNAs, miR-124a and miR-125b, which promote neurite outgrowth. We further demonstrated how 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 enhanced expression of multiple neuron-
important genes.

miR-125b is expressed in many types of tissues but its highest expression is in the brain, especially
in mature neurons but not astrocytes (34,36,38). miR-125b is upregulated during mouse
neurogenesis (36), during neural differentiation of mouse embryonic stem cells (18), and upon RA
treatment of embryonic carcinoma cells (34) and of neuroblastoma SK-N-BE cells (19). Adding to
these studies, our data demonstrates 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
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 four 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 - βIII-tubulin positive cells with neurites longer than 30 µm - the
percentage of the selected cells was rather small, 1-6% (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 neuronal protein markers Map2ab, neurofilament and Syt5, we observed a higher percentage of differentiated cells, 5-16\% (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 differentiation of RVM cells. miR-125b ectopic expression significantly enhanced neurite outgrowth of RVM cells in both growth medium and differentiation medium. Thus, our data indicates that miR-125b is important for neuronal differentiation in both RVM cells and SH-SY5Y cells, and suggests a common function of miR-125b in neural progenitor cells. Potentially, miR-125b gain-of-function may be useful to enhance in vitro neuronal differentiation of primary human neural stem cells for the 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 in 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 retinoic acid (RA). Furthermore, 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 RA-induced differentiation of SH-SY5Y cells than it is for differentiation of RVM cells upon 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 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 (Le et al, manuscript in preparation). 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 a global profiling to identify miR-125b-responsive genes. We chose to perform this experiment primarily in SH-SY5Y cells because these cells are more responsive to the modulation of miR-125b levels in comparison to 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 evidence: i) MEME motif discovery identified a 6-nucleotide motif in the 3' UTR of 129 genes that is perfectly complementary to the seed sequence of miR-125b; 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 \( \geq 1.5 \) 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 subjected to a further validation of our predictions but 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 demonstrate 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 (1,33). Our microarray data in SH-SY5Y cells was 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; modulation of miR-125b largely affects p53 protein level but did not show any significantly change in the transcript level of p53 in SH-SY5Y cells (Le et al, manuscript in preparation). Beside 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. Ingenuity Pathway Analysis (IPA) suggests 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 effectors, 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 ten predicted direct targets of miR-125b, and we validated these both by real-time PCR analysis of mRNA expression after ectopic expression of miR-125b and by a luciferase reporter assay (Table 1). IPA also reveals 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 a 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 four 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 found that the effect of miR-125b alone has very little effect on proliferation although ectopic expression of miR-125b together with miR-125a and miR-9 inhibit 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 anti-apoptotic effect. In a separate study, we found that miR-125b downregulates the p53 pathway and miR-125b gain-of-function represses apoptosis induced by H7 in SH-SY5Y cells (Le et al, manuscript in preparation). 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 differentiation of the human neuroblastoma SH-SY5Y cells and the human neural progenitor RVM cells towards the neuronal phenotype. In SH-SY5Y cells, we propose a model where the action of miR-125b is mediated by ten targets that repress multiple pathways involved in neuronal differentiation.

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REFERENCES


FIGURE LEGENDS

Figure 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 all-trans-retinoic acid (RA) for five days and subsequently by brain-derived neurotrophic factor (BDNF) in serum-free medium for seven days.

(b) Immunostaining of differentiating SH-SY5Y cells for the mature neuronal marker Map2ab (green) and the neural progenitor marker Msi1 (red). Nuclei were counterstained with Hoechst (blue).

(c) Microarray and quantitative real-time PCR (qRT-PCR) analysis of representative marker genes including Synaptotagmin V (Syt5), Cannabinoid receptor 1 (Cnr1) and GABA type B receptor 1 (Gabbr1) in differentiating SH-SY5Y cells. The microarray data was normalized as described in the method section. Quantitative real-time PCR readings were normalized to the expression level of β-actin. All the readings were presented as average fold change ± standard error (s.e.m.) relative to the gene expression level on day 0.

Figure 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 phospho-images represent three blots (separated by solid lines), including two biological duplicates loaded on lane 1-3 and lane 4-6 respectively. U6 RNA and 5S RNA were shown as loading controls.

(c) Northern-detected expression fold change of selected miRNAs (with significant changes), normalized to the expression of 5S RNA.

Figure 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 four days after transfection.

(b) Relative quantification of miR-7 and miR-125b level in SH-SY5Y cells four days after a transfection with 80 nM synthetic miR-7 duplex (7-DP) or miR-125b duplex (125b-DP). The obtained real-time PCR readings were normalized to U6 RNA level and presented as log2 fold change ± s.e.m. relative to the respective miRNA level in mock transfected control (n ≥ 4).

(c) The percentage of differentiated cells with neurite outgrowth, defined as cells stained positively for βIII-tubulin and possess neurites longer than 30 μm. The error bars represent standard error (s.e.m.) of 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 10x-magnification typically captures 200-500 SH-SY5Y cells. The results of student’s t-test are indicated as ** P < 0.01, compared to the scrambled control.
(d) Representative images of SH-SY5Y cells stained for βIII-tubulin (green) and Hoechst (blue) four days after transfection with mock or with different miRNA duplexes. Images were acquired by a confocal microscope using a 20x objective lens. Scale bar, 100 µm.

Figure 4. The function of miR-125b in SH-SY5Y cell differentiation

(a) Log$_2$ fold change of miR-125b level in SH-SY5Y cells four days after a transfection with scrambled duplex (scrambled-DP), miR-125b duplex (125b-DP) or miR-125b antisense (125b-AS) and maintained in growth medium or in differentiation medium containing RA. Each oligonucleotide was transfected at 80 nM final concentration. The obtained real-time PCR readings were normalized to U6 RNA level and presented as log$_2$ fold change ± s.e.m. relative to the respective miRNA level in scrambled transfected control (n ≥ 4).

(b) The percentage of differentiated SH-SY5Y cells with neurite outgrowth treated as in (a) and their average neurite length. Automated image acquisition and quantitative analysis was carried out by the Cellomics® HCS system. Differentiated cells with neurite outgrowth were defined as βIII-tubulin positive cells with neurites longer than 30 µm.

(c) SH-SY5Y cells stained with βIII-tubulin (green) and Hoechst (blue) four days after transfection. Transfection and treatments were applied as in (a). The images were acquired by a confocal microscope using a 20x objective lens. Scale bar, 100 µm.

(d) SH-SY5Y cells stained with neuronal markers (green) and Hoechst (blue). The cells were transfected as in (a) and maintained in growth medium for four days. They were then fixed and stained for neuronal markers including Map2ab, Neurofilaments and Synaptotagmin V (Syt5). The bar chart presents the percentage of positive-stained cells as quantified by the Cellomics® HCS 36
Relative quantification of marker gene expression. The cells were transfected as described in (a). RNA was isolated for quantitative real-time PCR four days post-transfection. The obtained readings were normalized to \( \beta \)-actin (internal control) and presented as average fold change \( \pm \) s.e.m. relative to the level of the transcripts in scrambled control.

In all cases, the error bars represent s.e.m. of at least three biological replicates. In all high content screening experiments, the phenotype of each biological replicate was analyzed and averaged by at least 15 images; each image of 10x-magnifications typically captures 200-500 SH-SY5Y cells. The results of student’s t-test are indicated as * \( P < 0.05 \) and ** \( P < 0.01 \) where the variation is compared to that of the scrambled control.

**Figure 5. Expression and function of miR-125b in ReNcell VM cells during differentiation**

(a) Representative pictures of undifferentiated cells (day 0) and differentiating ReNcell VM (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 \( \mu \)m.

(b) The expression pattern of miR-125b in RVM cells during differentiation, quantified by quantitative real-time PCR. The readings were normalized to U6 and presented as average fold change \( \pm \) s.e.m. (n = 6) relative to the miRNA level in undifferentiated cells (day 0). The results of student’s t-test are indicated as ** \( P < 0.01 \).

(c) Fluorescence in RVM cells one to four days after transfection with 80 nM fluorescent duplex. Scale bar, 100 \( \mu \)m.
(d) Representative images of RVM cells stained with βIII-tubulin (green) and Hoechst (blue) four days after the transfection with (i) mock, (ii) 80 nM 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 20x objective lens. Scale bar, 100 µm.

(e) The percentage of differentiated RVM cells with neurite outgrowth and their average neurite length. The cells were treated as in (d). Automated image acquisition and quantitative analysis was carried out by the Cellomics® HCS system. Differentiated RVM cells with neurite outgrowth were defined as βIII-tubulin positive cells with neurites longer than 20 µm. The error bars represent s.e.m. (n≥3). The phenotype of each replicate was analyzed and averaged by at least 15 images. Each image of 10x-magnifications typically captures 1000-2000 RVM cells. The results of two tail t-test are indicated as * P < 0.05 and ** P < 0.01.

Figure 6. Profiling the downstream effectors of miR-125b

(a) Gene ontology (GO) classification of genes upregulated four days after transfection of miR-125b duplex.

(b) Gene ontology (GO) classification of genes downregulated four days after transfection of miR-125b duplex.

In (a) and (b), SH-SY5Y cells were transfected with 80 nM scrambled duplex or 80 nM miR-125b duplex (125b-DP) then maintained in growth medium. RNA samples were collected four 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-DP 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. The P
values (represents the difference between the percentage of differentially expressed genes and the
percentage of the whole genome in each category) are indicated as * P < 0.05 and ** P < 0.01.

(c) Heat map representation of gene expression profile in SH-SY5Y cells two days after a
transfection with scrambled-DP, 125b-DP and/or 125b-AS in growth medium (GM) or in the
presence of retinoic acid (RA). Each oligo was transfected at the final concentration of 80 nM. Gene
expression was analyzed by Ref-8 v2 Illumina bead chip. The colors indicate the average fold
change of the intensity normalized to the mean of all arrays, ranging from ¼ (green) to 4 fold (red).
Only genes downregulated by 125b-DP are shown. The tree diagram represents clustering of
treatments based on their gene expression pattern.

Figure 7. Target prediction and pathway analysis
(a) MEME motif discovery searching for motif containing 4-9 nucleotides in the 3’ UTR of
downregulated genes. The available 3’ UTR sequences were collected for 253 out of the 388 genes
downregulated exclusively by two days of miR-125b ectopic expression. MEME identified
significant motifs size 6-9 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 (nucleotide 2-8, 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 circle represent the total numbers of targets predicted by each method. The numbers inside the circles represent the number of overlapped and non-overlapped targets.

(c) Statistic test elucidating the significance of our selection method. The targets of miR-125b were predicted (using the four methods above) from a list of 388 genes randomly selected from the whole genome and another list of 388 genes randomly selected out of 6448 genes which are differentially expressed by at least one treatment. The random selection and the prediction were repeated for 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) A modeled downstream network of miR-125b that mediates neuronal differentiation. Connections were mapped using Ingenuity Pathway Analysis.

Figure 8. Target validation

(a) qRT-PCR validating the expression pattern of miR-125b target genes two days after the transfection of miR-125b duplex (125b-DP) into SH-SY5Y cells in growth medium (GM) or in differentiation medium containing retinoic acid (RA). The readings were normalized to the expression level of β-actin. All the readings were presented as average log₂ fold change ± standard error (s.e.m.) relative to the gene expression level in the cells transfected with scrambled duplex.

(b) Luciferase reporter assays validating the binding of miR-125b to the 3’ UTRs of the target genes. Reporter genes contain only the microRNA response elements (MREs) that were predicted to bind to miR-125b. An MRE with perfect complementary to miR-125b was used as 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 encoding each reporter gene were cotransfected with miR-125b duplex into 293T cells. Luciferase readings were obtained 48 hours after transfection and presented here as the average percentage of luciferase activity ± s.e.m. (n ≥ 3) relative to the scrambled duplex cotransfected control (100%). The results of student’s t-test comparing the luciferase activity of each reporter gene in the presence of miR-125b duplex versus the scrambled duplex are indicated as ** P < 0.001. The results of student’s t-test comparing the luciferase activity of the mutant reporter genes versus the corresponding wild type reporter genes in the presence of miR-125b duplex are indicated as ## P < 0.001.
Table 1. Target validation summary

Ten target genes were selected from the microarray data, target prediction, and pathway analysis. Their expression pattern after two-day overexpression of miR-125b in growth medium (GM) or in differentiation medium containing all-trans-retinoic acid (RA) was validated by real-time PCR (Fig. 8a). The predicted miRNA response elements (MREs) were validated for binding to miR-125b by luciferase reporter assays (Fig. 8b-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” means “not tested”.

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<th>miR-125b targets</th>
<th>Downregulated by 2-day overexpression of mir-125b</th>
<th>Predicted MREs</th>
<th>MREs validated by luciferase reporter assay</th>
<th>Specificity of the miR-125b target site in the 3’UTR validated by mutation and luciferase reporter assay</th>
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Figure 1

(a) Day 0, Day 5, Day 12

(b) Day 0, Day 5, Day 12

(c) Log2 fold change in expression level

- Yir5
- Cnr1
- Gabbr1
Figure 2

(a) Normalized expression level (array analysis)

- miR-106
- let-7b
- miR-199a
- miR-124a
- miR-143
- miR-125b
- miR-7
- miR-189
- miR-199a
- miR-27a
- miR-21
- miR-214

- Day 0
- Day 5
- Day 12

(b) Normalized expression fold change (Northern blot)

- let-7b
- miR-106
- miR-124a
- miR-189
- miR-7
- 5S RNA
- U6 RNA
- miR-125b
- miR-143
- miR-199a
- miR-199a
- 5S RNA
- U6 RNA
- miR-214
- miR-27a
- miR-21
- 5S RNA
- U6 RNA
Figure 3

(a) Images showing cell differentiation over time (Day 1, Day 2, Day 3, Day 4).

(b) Bar graph showing Log2 fold change in mature miRNA level for miR-125b and miR-7.

(c) Bar graph showing percentage of differentiated cells with neurite outgrowth for different conditions:
- Mock
- Scrambled duplex
- miR-7 duplex
- miR-124a duplex
- miR-125b duplex
- miR-199a duplex
- miR-199a* duplex
- miR-214 duplex

(d) Fluorescence images comparing cell differentiation and neurite outgrowth under different conditions with various miRNA duplexes.
**FIGURE 5**

a) Images showing the cellular behavior over time from Day 0 to Day 7, transitioning from Growth medium to Differentiation medium.

b) Graph showing the fold change in mir-125b expression level for each day.

c) Images illustrating the cellular morphology on Day 1, Day 2, Day 3, and Day 4.


e) Bar charts depicting the percentage of differentiated cells with neurite outgrowth and the average neurite length for each condition.
Figure 6

a

- Neurite development
- Nervous system development
- Cell adhesion
- Cell morphogenesis & motility
- Cytoskeleton organization & biogenesis
- Developmental processes

b

- Metabolism
- Transcription regulation

Legend:
- Percentage of the whole genome
- Percentage of upregulated genes

Legend:
- Percentage of the whole genome
- Percentage of Downregulated genes

- GM Scrambled
- RA Scrambled
- RA 125b-AS
- RA 125b(DP-AS)
- GM 125b-AS
- RA 125b-AS