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Function of miR-125b in Zebrafish Neurogenesis

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***Abstract*—**MicroRNAs are an important class of gene expression regulators that are involved in many biological processes including embryogenesis. miR-125b is a conserved microRNA that is enriched in the nervous system. We have previously reported the function of miR-125b in neuronal differentiation of human cell lines. We also discovered the function of miR-125b in regulating p53 in human and zebrafish. Here we further characterize the brain defects in zebrafish embryos injected with morpholinos against miR-125b. Our data confirm the essential role of miR-125b in brain morphogenesis particularly in maintaining the balance between proliferation, cell death and differentiation. We identified *lunatic fringe* (*lfng*) as an additional target of miR-125b in human and zebrafish and suggest that *lfng* may mediate the function of miR-125b in neurogenesis. Together, this report reveals new insights into the function of miR-125b during neural development of zebrafish.

***Keywords*—**microRNA, miR-125b, neurogenesis, zebrafish.

I. Introduction

microRNAs (miRNAs) is a class of ~22 nucleotide RNAs that regulate gene expression at the post-transcriptional level [1]. miRNAs are present in all types of tissues and they perform various physiological functions [2]. While the functions of most miRNAs remain to be discovered, there are increasing examples of important miRNAs involved in different physiological and pathological processes [3]. The impact of miRNAs is attracting more and more attention from researchers.

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The discovery of miRNAs started with *lin-4* that suppresses *lin-14* and modulates timing of developmental stages in *C. elegans* [4]. miR-125b is a homolog of *lin-4* (82% identical) and is highly conserved from flies to humans (100% identical). The expression of miR-125b is upregulated during development. *C. elegans lin-4* and *Drosophila* miR-125 are found only in post-embryonic stages [4-5]. During mouse embryogenesis, by contrast, miR-125b expression increases gradually from embryonic day twelve till birth [6]. The highest expression of miR-125b was observed by *in situ* hybridization in the midbrain-hindbrain boundary of mouse embryos [2]. miR-125b expression is enriched in the central nervous system (CNS) including the brain and the spinal cord of zebrafish and mouse, suggesting that miR-125b may play a role in the development or function of the CNS [7-9] .

Recently, we reported the functions of miR-125b both *in vitro* and *in vivo*. First, we demonstrated the important role of miR-125b in spontaneous and induced differentiation of SH-SH5Y cells. miR-125b was upregulated during differentiation of human neural progenitor ReNcell VM cells, and miR-125b ectopic expression significantly promoted 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 many genes that were predicted by TargetScan 5.1. to be the direct targets of miR-125b [10]. Pathway analysis suggested that a subset of miR-125b-repressed targets antagonized 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 and confirmed the binding specificity for three targets by mutagenesis. Together, these data demonstrated for the first time the important role of miR-125b in human neuronal differentiation. Second, we examined the function of miR-125b in zebrafish development [11]. Morpholino-mediated knockdown of miR-125b leads to severe defects in zebrafish embryos, where neural cell death was the most apparent phenotype. This phenotype resembled Mdm2 morphants in which the p53 pathway is activated; hence, we postulated that the function of miR-125b in zebrafish embryos was mediated by the p53 pathway. Interestingly, p53 is predicted to be a target of miR-125b in both zebrafish and

humans. We validated this prediction by a luciferase reporter assay and mutagenesis. Our data revealed that miR-125b bound directly to the 3' UTR of human and zebrafish p53 mRNAs, and repressed p53 protein levels in a manner dependent on its binding site in the p53 3'UTRs. miR-125b-mediated regulation of p53 is critical for modulating apoptosis in human cells and in zebrafish embryos during development and during the stress response [11].

Here, we further demonstrate the functions of miR-125b in zebrafish neural development. We confirmed the importance of miR-125b in zebrafish embryogenesis by both knockdown and ectopic expression approaches. In addition, we showed that knockdown of miR-125b led to an accumulation of mitotic cells and blocked differentiation of neuronal cells. We also validated *lfng* as a target of miR-125b and suggested that it may be responsible for miR-125b function in neurogenesis.

II. Materials and Methods

Microinjection in Zebrafish Embryos

Zebrafish embryos were injected at 1-4 cell stage with 0.75 pmole miR-125b morpholinos (Gene Tools) or 12.5 fmole to 37.5 fmole miR-125b duplex (Ambion).

Immunostaining of Zebrafish Embryos

Embryos were fixed in 4% paraformaldehyde at 4°C overnight. After three washes with PBDT (PBS containing 2% BSA, 1% DMSO and 0.5% Triton X-100), the embryos were incubated with cold acetone at -20ºC for 20 minutes and washed with PBDT three times, blocked with blocking buffer (Roche) for one hour then incubated with mouse anti-acetylated tubulin monoclonal antibody (Sigma) or mouse anti-phospho histon-3 antibody overnight at 4ºC. After washing with PBDT six times, they were incubated with Alexa Fluor 568 goat-anti–mouse IgG antibody, 1:200 (Molecular Probe) for 4 hours. Following 5 washes in PBDT (30 minutes each), the embryos were fixed again in 4% paraformaldehyde at 4°C overnight.

Image Acquisition and Microscope Settings

Fluorescent images of staining were obtained with a LSM510 confocal laser-scanning microscope (Carl Zeiss Vision GmbH). A bright-field image was acquired at the same time as the fluorescent image. Projection of image stacks was made by the Zeiss image browser. Images were then imported into Adobe Photoshop for cropping, resizing, and orientation. Contrast and brightness were adjusted equally for all images of the same figure.

Images of live embryos were obtained by a SZX12 stereomicroscope (Olympus) and a MagnaFIRE SP camera (Olympus). The embryos were mounted in 3% methyl-cellulose. Images were acquired with a 65x objective, at a resolution of 1280x1024, with ~100 ms exposure and 8 bit depth at room temperature. The image set of each embryo was combined, resized, cropped, and oriented using Adobe Photoshop.

Quantitative Real-Time PCR

RNA was extracted from zebrafish embryos using Trizol® reagent (Invitrogen) and subsequently column-purified with RNeasy® kits (Qiagen). For qRT-PCR of miR-125b, 100 ng of total RNA was reverse-transcribed and subjected to Taqman® microRNA assay (Applied Biosystems).

Luciferase Reporter Assay

The miRNA response elements (MREs, Supplementary Table V) or the whole 3' UTR 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. They were synthesized, annealed and ligated into the psiCheck-2 vector. 10 ng of each psiCHECK-2 construct was co-transfected with 10 nM miR-125b duplex or negative control 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.

III. Results

Ectopic Expression and Knockdown of miR-125b in Zebrafish Embryos

To confirm the functions of miR-125b in zebrafish embryonic development, we followed both gain-of-function and loss-of-function approaches. Ectopic expression of miR-125b was archived by injection of a synthetic double stranded RNA mimicking miR-125b duplex into one-cell stage embryos. The synthetic duplex (125b-DP) resulted in nearly 200 fold increase in the mature miR-125b level by 24 hours post-fertilization (hpf) (Fig. 1B). By contrast, we knocked down miR-125b in zebrafish embryos using four morpholinos (Fig. 1A) that we have published [11]: MO-m125b targeted the mature miR-125b, and three MO-lo125bs targeted the three precursors of miR-125b. MO-m125b or the equal-molar mixture of three MO-lo125b morpholinos resulted in a complete suppression of mature miR-125b expression as quantified by real-time PCR at 24 hpf (Fig. 1B). Injection of a mismatch morpholino (containing five nucleotides different from MO-m125b) did not suppress the level of miR-125b (Fig. 1B).

Consistent with our prior report [11], injection of MO-m125b or the mixture of MO-lo125b caused shrinking of the head and excessive cell death in the brain of most embryos and curvation of the body axis (somite defects) in more than half of the injected embryos at 24 hpf (Fig. 2 and Table I). The phenotypes also response to the dosages of MO-m125b and MO-lo125b (Fig. 2 and Table I). More severe cell death and somite defects were observed at higher dose of the morpholinos. This suggests that the effects of the morpholinos were specific.

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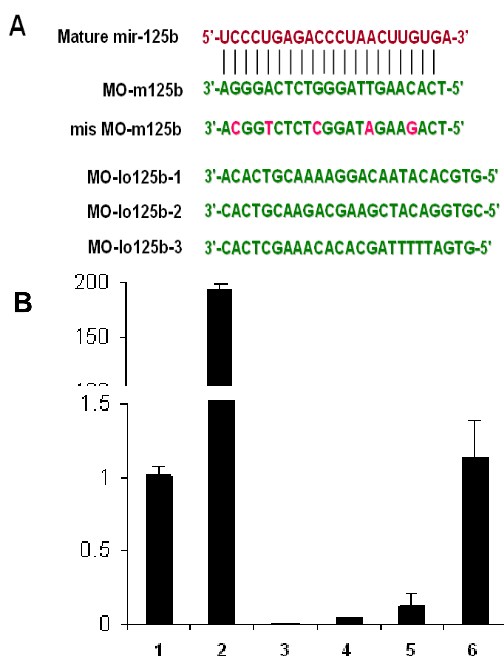

Fig. 1 Knockdown and ectopic expression of miR-125b in zebrafish embryos. (a) Sequences of morpholinos (MO) targeting either the mature miR-125b (m125b) or the loop regions of *pre-mir-125b* (lo125b). (b) Quantification of mature miR-125b level by qRT-PCR in zebrafish embryos at 24 hpf. Lane 1: mismatched MO, lane 2: miR-125b duplex (DP), lane 3: MO-m125b, lane 4: MO-lo125b mix + 37.5 fmole negative control DP, lane 5: MO-lo125b mix + 12.5 fmole 125bDP, lane 6: MO-lo125b mix + 37.5 fmole 125bDP. Total RNA was extracted from the embryos at 24 hpf. miR-125b level was normalized to 18S RNA levels and presented as average fold change ± s.e.m. ($n \geq 4$) compared to the expression of miR-125b in uninjected embryos

Interestingly, ectopic expression of miR-125b using 125b-DP led to rounded body and loss of ventricles in the brain (Fig. 2). The midbrain-hindbrain boundary (MHB), the most important organizer of the brain was lost by both knockdown and ectopic expression of miR-125b. The phenotypes were partially rescued when miR-125b DP was coinjected with MO-m125b (Fig. 2). In these embryos, MHB was present whereas cell death was less than in the knockdown.

Loss of miR-125b Caused Severe Defects in Neurogenesis

To understand the function of miR-125b in neurogenesis, we visualized the axonal tracts in MO-m125b-injected embryos by anti-acetylated tubulin immunostaining. Knockdown of mir-125b led to severe loss of mature neurons and axonal tracts in all parts of the brain (Fig. 3A-B). Particularly, we observed thinner anterior commissure, missing post-optic commissure and missing axon bundles in optic tectum. The axonal tracts of the morphants' hindbrain were developed in a wrong pattern (Fig. 3C).

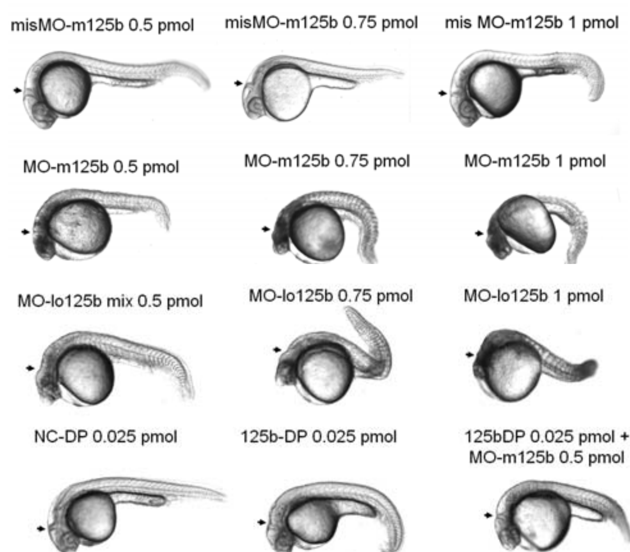

Fig. 2 Phenotypes of miR-125b knockdown and etopic expression in zebrafish embryos: Embryos were injected with MO-m125b or MO-lo125b mix or mismatched morpholino (misMO) and/or miR-125b DP or negative control duplex (NC-DP) at indicated dosage. Images of live embryos were taken at 24 hpf. Midbrain-hindbrain boundary is marked with the arrows

TABLE I
PERCENTAGE OF EMBRYOS WITH DEVELOPMENTAL DEFECTS INCLUDING SHRINKING BRAIN WITH DEATH CELLS, ABSENCE OF MIDBRAIN-HINDBRAIN BOUNDARY AND CURVATION OBSERVED AT 24 HPF

	misMO-m125b 0.5 pmol	MO-m125b 0.5 pmol	MO-m125b 0.75 pmol	MO-m125b 0.5 pmol + 125bDP 0.025 pmol	MO-lo125b mix 0.5 pmol	MO-lo125b mix 0.75 pmol
Total embryos	71	180	39	42	55	57
Normal embryos	100%	8%	5%	71%	11%	5%
Shrinking brain with dead cells	0%	92%	95%	21%	82%	95%
No midbrain-hindbrain boundary	0%	6%	97%	2%	38%	86%
Curvation or abnormal somites	0%	22%	90%	10%	29%	77%

We further analyzed the brain phenotypes by staining with phospho-histon-3, a proliferation marker. Knockdown of mir-125b resulted to a dramatic accumulation of mitotic cells (Fig. 4). At 24hpf, the mitotic cells accumulate especially at the olfactory placode, the pituitary gland and the hindbrain of the morphants. At 48 hpf, mitotic cells spread everywhere in the

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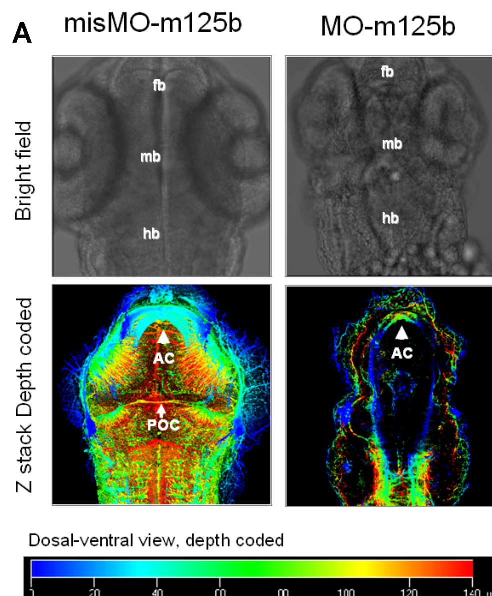
morphants' brains while in control embryos, they appear only in the midline and surrounding the midbrain-hindbrain boundary (Fig. 4). These results suggest that the loss of mir-125b arrest neural cells in a mitotic precursor state, blocking their differentiation into mature neurons.

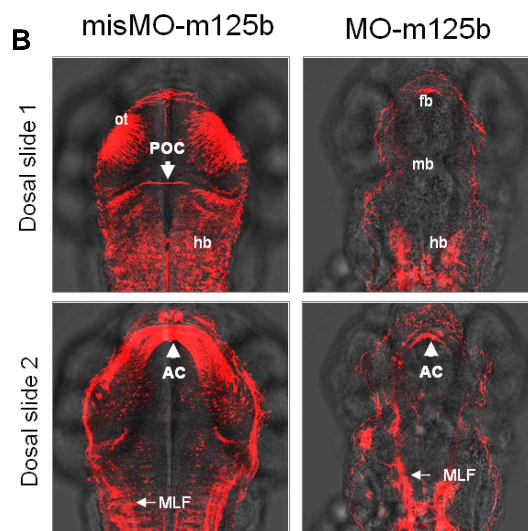
miR-125b Targets Lunatic Fringe

To identify a target that may be responsible for the function of miR-125b in neurogenesis, we searched the Microcosm target database for miR-125b's conserved targets in both zebrafish and humans and examined the expression pattern documented by Zfin database, particularly for targets expressed in zebrafish brain during development. We found that *lunatic fringe* (*lfng*) was the most promising target that contains a binding site for miR-125b in both humans and zebrafish and it is expressed in the zebrafish brain. We validated the binding of miR-125b to the predicted miRNA response element (MRE) by a luciferase reporter assay (Fig. 5). The results showed that miR-125b was able to bind to the MRE in *lfng* mRNAs of both humans and zebrafish and suppressed the luciferase activity to less than 40% relative to that of the controls.

IV. Discussion

We previously demonstrated the role of miR-125b in differentiation of human neural progenitor cells and in regulating p53-mediated apoptosis in humans and zebrafish [10-11]. The function of miR-125b in zebrafish neurogenesis was not elucidated before this report. Here, we performed an analysis of miR-125b function in neural development of zebrafish embryos. By loss-of-function and gain-of-function approaches, we showed that miR-125b is essential for brain morphogenesis. Loss of miR-125b resulted in an accumulation of mitotic cells, an increase in cell death and a reduction in differentiation. By contrast, ectopic expression of miR-125b affected the formation of ventricles, probably due to overproliferation and/or decrease in physiological apoptosis. Therefore, miR-125b plays a vital role in the balance between proliferation, cell death and differentiation.





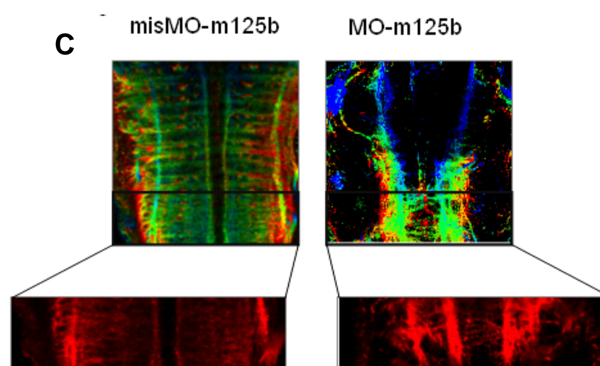


Fig. 3 Acetylated tubulin staining for mature neurons and axonal tracts in zebrafish brain at 48 hpf. The embryos were injected with misMO or m125bMO. (A) A Z stack of multiple optical slides in bright field or with color coding for depth from dosal to ventral. (B) Two representative dosal slides. (C) Magnified Z stack of the hindbrain. Abbreviations: AC, anterior commissure; fb, forebrain; hb, hindbrain; mb, midbrain; MLF, medial longitudinal fasciculus; ot, optic tectum; POC, postoptic commissure

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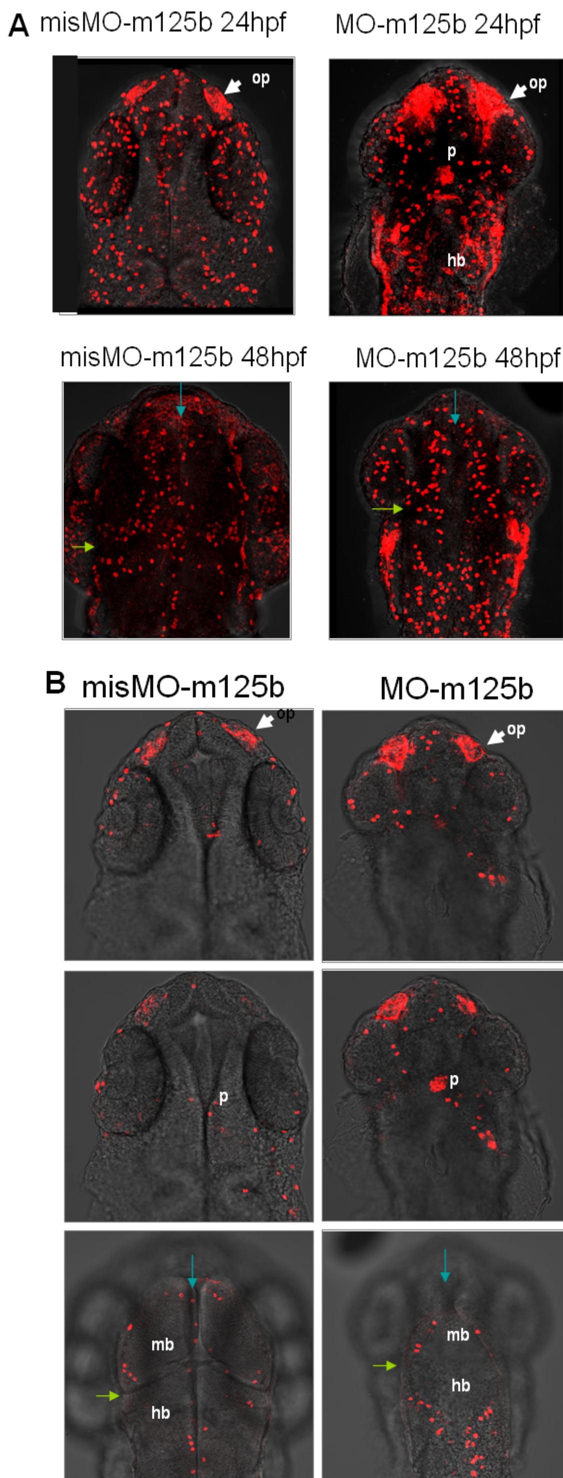

Fig. 4 Phospho histone 3 staining for proliferating cells in zebrafish brain at 24 or 48 hpf: (A) Projection view of multiple optical slides, (B) Representative optical slides. At 24hpf, the mitotic cells accumulate especially at the olfactory placode (op), the pituitary gland (p) and the hindbrain (hb) of the morphants

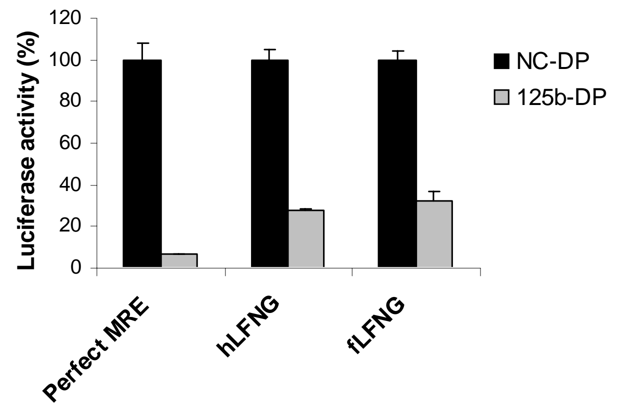

Fig. 5 – Luciferase reporter assay demonstrating binding of miR-125b to the microRNA response elements (MREs) in the 3' UTR of human and zebrafish *lfng* mRNAs. The MREs were cloned into the 3'UTR of a *renilla* luciferase gene and cotransfected with NC-DP or 125b-DP into 293T cells. *Renilla* luciferase readings were obtained 48 hours after transfection, normalized to the level of the control firefly luciferase and presented as percentage ± s.e.m (n = 6), relative to NC-DP

The function of miR-125b in regulating apoptosis was well explained by the interaction of this microRNA with p53 mRNA [11]. As shown previously, the cell death phenotype in miR-125b morphants was rescued almost completely by p53 depletion. However, loss of mature neurons and axonal tract was not rescue completely by this approach. Hence, the function of miR-125b in neurogenesis was also mediated by other targets. The targets of miR-125b that we identified earlier in human neurolastoma SH-SY5Y cells are not well conserved in zebrafish [10]. However, there are a number of putative targets, predicted by Microcosm target, with known function in zebrafish neurogenesis.

One of the most promising candidate targets is *lunatic fringe* (*lfng*) which encodes a glycosyltransferase that regulates Notch signalling. It is expressed in zebrafish brain especially in the midline precursor cells. Since miR-125b knockdown affect the midline cells in the brain, as shown by our phospho histone-3 staining, the function of miR-125b may associate with *lfng*. Interestingly, human *lfng* mRNA also has a binding site for miR-125b. We have performed a luciferase reporter assay to confirm the binding of miR-125b to the predicted microRNA reponse elements (MREs) in both humans and zebrafish. Indeed, miR-125b also targets another gene in the human Notch signalling pathway, *musashi 1 (msi1)*, which is the marker of neural stem cells. Overexpression of miR-125b reduces the expression of *msi1* in neuroblastoma cells [10]. Zebrafish *musashi* mRNA homolog also contains several seed matches for miR-125b. So this gene is likely to be the target of miR-125b in zebrafish. Together, our results suggest that miR-125b may promote differentiation of zebrafish neural cells by suppressing the neuronal-inhibitory effect of the Notch signaling pathway.

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In addition, *zin5* is also an interesting putative target of miR-125b. It is the marker of the midbrain hindbrain boundary (MHB) which is absent in both miR-125b knockdown and ectopic expression. Probably, the physiological level of miR-125b is essential to maintain the right level of *zin5* expression to define the MHB in zebrafish brain. The function of miR-125b in neural patterning may be associated with several targets that are important for brain morphogenesis, including *desert hedgehog (dhh), Indian hedgehog B (ihhb), hoxa9b, hoxd3a, gli1, pax5* and *her9*. These targets are good candidates for further study of miR-125b function in zebrafish neurogenesis.

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