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.

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 zebrafish 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...
Quantitative Real-Time PCR
RNA was extracted from zebrafish embryos using Trizol® reagent (Invitrogen) and subsequently column-purified with RNasy® 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 Xhol 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 Xhol 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.
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 ≥ 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).

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 |
|---|---|---|---|---|
| miR-125b | miR-125b | miR-125b | miR-125b |
| 0.5 pmol | 0.75 pmol | 1 pmol | 0.5 pmol + 0.025 pmol 125bDP |
| 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 24 hpf, 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
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. 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 \textit{lfng} mRNAs. The MREs were cloned into the 3'UTR of a \textit{renilla} luciferase gene and cotransfected with NC-DP or 125b-DP into 293T cells. \textit{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 rescued 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 neuroblastoma 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 \textit{lunatic fringe} (\textit{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 \textit{lfng}. Interestingly, human \textit{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, \textit{musashi 1} (\textit{msi1}), which is the marker of neural stem cells. Overexpression of miR-125b reduces the expression of \textit{msi1} in neuroblastoma cells [10]. Zebrafish \textit{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.
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.

REFERENCES