MiR-124 regulates early neurogenesis in the optic vesicle and forebrain, targeting NeuroD1

Kaili Liu1,2,3, Ying Liu1,2,* Weichuan Mo1,3, Rong Qiu1, Xiumei Wang1,2, Jane Y. Wu1,4 and Rongqiao He1,3,5,*

1The State Key Laboratory of Brain and Cognitive Science, 2Key Laboratory of Noncoding RNA, Institute of Biophysics, Chinese Academy of Sciences, 15 Datun Road, Chaoyang District, Beijing 100101, 3Graduate University of Chinese Academy of Sciences, Beijing 100049, China, 4Department of Neurology, Lurie Comprehensive Cancer Center, Center for Genetic Medicine, Northwestern University Feinberg School of Medicine, Chicago, IL 60611, USA and 5Key Laboratory of Mental Health, Chinese Academy of Sciences, Beijing 100101, China

Received April 23, 2010; Revised September 5, 2010; Accepted September 23, 2010

ABSTRACT

MicroRNAs (miRNAs) are involved in the fine control of cell proliferation and differentiation during the development of the nervous system. MiR-124, a neural specific miRNA, is expressed from the beginning of eye development in Xenopus, and has been shown to repress cell proliferation in the optic cup, however, its role at earlier developmental stages is unclear. Here, we show that this miRNA exerts a different role in cell proliferation at the optic vesicle stage, the stage which precedes optic cup formation. We show that miR-124 is both necessary and sufficient to promote cell proliferation and repress neurogenesis at the optic vesicle stage, playing an anti-neural role. Loss of miR-124 upregulates expression of neural markers NCAM, N-tubulin while gain of miR-124 downregulates these genes. Furthermore, miR-124 interacts with a conserved miR-124 binding site in the 3'-UTR of NeuroD1 and negatively regulates expression of the proneural marker NeuroD1, a bHLH transcription factor for neuronal differentiation. The miR-124-induced effect on cell proliferation can be antagonized by NeuroD1. These results reveal a novel regulatory role of miR-124 in neural development and uncover a previously unknown interaction between NeuroD1 and miR-124.

INTRODUCTION

Although many of the coding genes involved in eye development have been known for decades, the post-transcriptional mechanisms controlling their expression are poorly understood. In recent years, systematic studies in zebrafish and mouse have determined specific microRNAs (miRNAs) expressed in the developing eye and brain (1,2). MiR-7 and let-7 have been shown to be involved in Drosophila eye development (3,4). In Xenopus, miR-24a has been reported to play an essential role in repressing apoptosis in the developing neural retina (5). However, the functions of most miRNAs in eye development are still unclear.

Eye development starts from the specification and splitting of the eye field in the anterior neural plate, followed by the formation of the optic vesicle and optic cup which are laterally protruded from the ventral forebrain. The eye retina, a derivative of the primary brain vesicle which has limited cell types, has been used as a simplified model of the central nervous system for studying the molecular control of neurogenesis during development (6,7).

MiR-124 is a group of well conserved miRNAs and has been reported to be abundantly expressed in the brain and retina of the mouse (8), rat (9), chick (10,11), Xenopus laevis (12,13) and zebrafish (14). Recently, based on their analysis of the first miR-124 mutant, Clark et al. (15) discovered that Caenorhabditis elegans miR-124 is expressed in a subset of sensory neurons. Many reports show that miR-124 can promote neuronal differentiation. For example, ectopic expression of miR-124 in HeLa cells shifts the expression profile toward a brain-like pattern (16). In mouse embryonic development, miR-124 promotes the differentiation of progenitor cells into mature neurons by directly targeting PTBP1 (PTB/hnRNP I) mRNA which encodes a global repressor of alternative pre-mRNA splicing in non-neuronal cells (17). In adult regeneration, miR-124 increases neuron formation by...
targeting sox9 (18). However, the functions of miR-124 in neural development are also controversial. For instance, Cao et al. (19) showed that neither inhibition nor overexpression of miR-124 alone significantly alters neuronal fate. Viswanathan et al. (20) using the same model, found that miR-124 helps modestly promote neuronal differentiation.

We have previously reported that miR-124 is expressed in the developing and adult nervous system of Xenopus laevis, and that its overexpression results in an abnormal eye phenotype with decreased cell proliferation in the optic cup, while its downregulation leads to no morphological defects (13). As the expression of Xenopus miR-124 in the brain and eye fields initiates at the mid-neurula stage (12,13), a developmental period at the beginning of optic vesicle formation and retinogenesis, it is necessary to investigate the role of miR-124 in the early neurogenesis of the eye in order to fully understand its role during eye development.

Here, we studied the effect of miR-124 on cell proliferation and differentiation in early optic vesicle development using both loss- and gain-of-function experiments. We found miR-124 is both necessary and sufficient for cell proliferation and the repression of neurogenesis in the optic vesicle and forebrain. This role is distinct from that observed in later developmental stages and in adults. In addition, we have shown that NeuroD1 is targeted by miR-124 and can restore miR-124-induced cell proliferation. These results indicate that the role of miR-124 in neurogenesis varies in a stage-dependent manner during eye development, and that the NeuroD1-miR-124 interaction is involved in the early regulation of both genes.

**MATERIALS AND METHODS**

**Microinjection**

Oligonucleotides or mRNAs were injected into one or two dorsal-animal blastomere(s) of an eight-cell stage embryo using an Eppendorf FemtoJet (Hamburg, Germany) and embryos were then cultured as previously described (21). For the loss-of-function study, 0.2 pmol 2'-O-methyl antisense RNA oligonucleotides for miR-124 (Anti-124) and a control inhibitor (Anti-ctrl) were used (Ambion, USA) according to our previously published method (13). For the gain-of-function study, 0.025 pmol miR-124 precursor (Pre-124) and a control precursor (Pre-ctrl) were used (Ambion, USA). Capped mRNAs of NeuroD1 (22) were synthesized from linearized plasmid templates using mMESSAGE mMACHINE kits (Ambion, USA). Embryos were co-injected with 100–500 pg β-gal or 200–400 pg GFP mRNA as a lineage tracer. Embryos injected with β-gal were stained as previously described (23).

**Bromodeoxyuridine (BrdU) incorporation and immunohistochemistry**

Both BrdU and phosphohistone-H3 (pH3) staining were used for cell proliferation assays. BrdU (Sigma B9285) was incorporated as described by Qiu et al. (13), Quick and Serrano (24). Embryos were fixed with 4% paraformaldehyde in PBS and cryoprotected with 20% sucrose in PBS overnight at 4°C, before embedding in OTC and storing at −70°C. The cryosections (12 μm) were immunostained with mouse anti-BrdU (1:200 Santa Cruz) or rabbit anti-phosphohistone-H3 (1:200, Upstate Biotechnology). TRITC-conjugated goat anti-mouse IgG (1:100, Sigma) and TRITC-conjugated goat anti-rabbit IgG (1:200, Santa Cruz) were used as secondary antibodies. All cell nuclei were counterstained with Hoechst 33258 (Sigma). Images were taken using a compound microscope (Nikon FXA, Japan).

Counts of BrdU-positive (N_BrdU), pH3-positive (N_pH3) and Hoechst-labeled cells (N_Hoechst) were obtained from embryo sections by tracing digitized images projected on a computer monitor. The ratio of proliferating cells in the eye was calculated as: N_BrdU or pH3 / N_Hoechst × 100%.

**In situ hybridization**

Whole mount in situ hybridization was performed on Xenopus embryos as previously described (13,25). The cRNA probe for NeuroD1 and the LNA probe for mature miR-124 were prepared separately according to methods described previously (13). Embryos were fixed with MEMFA and stored in ethanol at −20°C before use. For paraffin sections, samples were embedded in paraffin after being rehydrated. Images of whole-mount embryos were taken using a stereomicroscope (Olympus SZX12, Japan) with a digital acquisition system (Olympus C4040, Japan). Sections were photographed on an inverted microscope (Olympus IX71, Japan) or a compound microscope (Nikon FXA, Japan) using DIC optics or fluorescent filters.

**RNA extraction, RT–PCR and real-time PCR**

Total RNA was extracted from the heads of embryos at the optic vesicle stage using an RNeasy Micro Kit (Qiagen, Germany) according to the manufacturer’s instructions. The first-strand cDNA synthesis was performed with M-MLV Reverse Transcriptase (Promega, USA). The following primers were used for PCR, NCAM (Forward: 5’-CACAGTTCCACCAATGC-3’, Reverse: 5’-GGAATCAAGCGGTACAGA-3’), N-tubulin (Forward: 5’-ACACGGCATTTGATCCTAC-3’, Reverse: 5’-AGCTCCTTCCGTGTAATTGAC-3’), NeuroD1 (Forward: 5’-TTATGTTACCCATGC-3’, Reverse: 5’-CTTCCAAGAACCACAAC-3’), Lhx2 (Forward: 5’-GTTGGAAAGCTTGTCATTGC-3’, Reverse: 5’-AGCTCCTTCCGTGTAATTGAC-3’), NeuroD1 (Forward: 5’-AGATTCATACATCCCCGTATC-3’, Reverse: 5’-CTCTAAGCAAACTCAATAC-3’), elrC (Forward: 5’-AGATCCAGATCTATAGATCTATC-3’, Reverse: 5’-TGTTCCGAAACTCTAATCT-3’), evx1 (Forward: 5’-AGAATCATACATCCCCGTATC-3’, Reverse: 5’-AGCAGCTTTTGTGCCTTACT-3’), xtwi (Forward: 5’-AGCCGAAAAGTTGCAG-3’, Reverse: 5’-AGAATCATACATCCCCGTATC-3’, Reverse: 5’-AGAATCATACATCCCCGTATC-3’, Reverse: 5’-AGAATCATACATCCCCGTATC-3’, Reverse: 5’-AGAATCATACATCCCCGTATC-3’, Reverse: 5’-AGAATCATACATCCCCGTATC-3’, Reverse: 5’-AGAATCATACATCCCCGTATC-3’, Reverse: 5’-AGAATCATACATCCCCGTATC-3’, Reverse: 5’-AGAATCATACATCCCCGTATC-3’, Reverse: 5’-AGAATCATACATCCCCGTATC-3’), PCR was conducted using normalized amounts of template. The number of PCR cycles performed varied from 24 to 30 depending on the individual gene. An annealing temperature of 52°C was used for the NeuroD1 primer set while other primer pairs were annealed at 56°C.
For real-time PCR, the resultant cDNA was diluted 1:20. The PCR reactions were performed with a TransStart Green qPCR SuperMix UDG kit (Transgen, China) on an MJ Research Chromo4 detector (Biorad) using a SYBR green fluorescence quantification system. The relative expression level was calculated by the $2^{-\Delta\Delta C_{T}}$ method. Means ± SEM are from three independent experiments.

**MiRNA target prediction**

The predicted miR-124 recognition elements (MRE) in NeuroD1 were analyzed by RNAhybrid (26) using the highly conserved mature sequence of miR-124 and the NeuroD1 3′-untranslated region (UTR) of human (NM_002500), mouse (NM_010894), rat (NM_019218), Xenopus tropicalis (Xt7.1-XZT30819.5, an EST with a longer 3′-UTR than the Refseq NM_001097399) and Xenopus laevis (NM_001092127). RNAhybrid was operated with either perfect (no U:G allowed in the seed) or imperfect (U:G allowed in the seed) seed match, and the helix constraint in the seed was set from positions 2 to 7 of the miRNA sequence. MiR-124 target candidates in other species were retrieved from Targetscan (27), Pictar (28) and miRbase (29).

**Luciferase reporter assay**

The firefly luciferase reporter genes were constructed using the pCS2-Luc vector and the 3′-UTR sequences of Xenopus NeuroD1 (NeuroD1 3′-UTR), the primers for PCR amplification of the 3′-UTP fragment were as follows: 5′-CGTGAATTCC TTAATTGACCATGCGG-3′ (forward) and 5′-TCACCTGAGGTCTCTAAGGCACACACA-3′ (reverse). The underlined sequences are introduced EcoRI and XhoI sites, respectively. Constructs with mutated 3′-UTR of NeuroD1 (NeuroD1-Mut) were used as negative controls. Mutations in positions 2–7 of the miR-124 seed were introduced using a QuikChange mutagenesis kit (Stratagene, USA). The 293T cells were cultured in DMEM supplemented with 10% fetal bovine serum. A total of 5 × 10^4 cells/well were seeded in 24-well plates. After 24 h in culture, the cells were transfected using Lipofectamine 2000 (Invitrogen, USA) with a mixture containing 1 μg/ml of firefly luciferase reporter plasmid, 20 nM miR-124 or control precursor and 20 ng/ml of Renilla reniformis luciferase encoding plasmid (pRL-TK, Promega, USA). Cells transfected without the precursor served as controls for normalization. Luciferase activity was measured 24–48 h post-transfection using a dual-luciferase assay system (Promega, USA). All transfections were repeated independently at least three times.

**Statistical analysis**

At least three independent experiments were performed in each case. Statistical analysis was performed using one-way ANOVA followed by the Duncan test. Differences among groups were considered to be significant when $P < 0.05$.

**RESULTS**

MiR-124 is both necessary and sufficient for cell proliferation in the optic vesicle and forebrain

In Xenopus, miR-124 is expressed from the beginning of eye formation (around stage 18) in the retinal progenitors of the eye anlagen (12–13). To investigate the roles of miR-124 on the early stages of eye development, we used BrdU incorporation in a loss-of-function study to examine cell proliferation in the early optic vesicle and forebrain of Xenopus embryos at around stages 22/24 (when most of the embryonic retinal progenitors have already formed and the first population of retinal neurons has just begun to differentiate). Effects on the differentiating optic cup were also examined at around stages 33/34. 2′-O-methyl antisense oligonucleotides for miR-124 (anti-124, 0.2 pmol) were injected into dorsal-animal blastomer(s) of an eight-cell stage frog embryo to block miR-124 expression. This approach has previously been shown to be efficient for miR-124 downregulation, at least to stage 33 (13). As a result, the proliferating cell ratio decreased significantly ($P < 0.01$) in the optic vesicle and forebrain at stages 20–24 (22.7%), compared with that in the uninjected control (45.2%). Embryos injected with a control inhibitor (negative control, Anti-ctrl) showed no significant change in cell proliferation (39.3%). When embryos were at stage 33 and the optic vesicle had developed into an optic cup, the difference between the ratios of proliferating cells in the anti-124 injected embryos and controls was insignificant (Figure 1A), indicating that miR-124 is necessary for maintaining proliferation of neural progenitors in the early optic vesicle but not in the optic cup.

We then tested whether loss of miR-124 could influence neurogenesis while repressing proliferation. NCAM and N-tubulin (neural-specific class II β-tubulin) were used as neuronal markers (30–32). RT–PCR and real-time RT–PCR experiments indicated that loss of miR-124 significantly enhanced the expression of NCAM and N-tubulin at the early optic vesicle stage ($P < 0.01$ and $P < 0.05$, respectively) (Figure 1B). This suggests that miR-124 is required for the maintenance of proper cell proliferation and the repression of neural differentiation during early eye development.

In order to investigate whether miR-124 is sufficient to promote cell proliferation and repress neurogenesis during the optic vesicle stages, we performed a miR-124 gain-of-function study by microinjecting 0.025 pmol miR-124 precursor (pre-124) at the eight-cell stage and detected cell proliferation as above. At stages 20–24, upregulation of miR-124 led to a significant increase ($P < 0.01$) in the proliferating cell ratio in the forebrain and optic vesicle (71.6%), compared with that of the uninjected control (45.2%). Application of pre-ctrl did not appear to alter cell proliferation (43.2%) (Figure 1A). RT–PCR and real-time RT–PCR results show that expression of NCAM and N-tubulin were significantly decreased on miR-124 overexpression ($P < 0.01$ and $P < 0.05$, respectively) (Figure 1B). At stage 33, the opposite effect on cell proliferation was observed in the optic cup (Figure 1A) in agreement with results previously.
reported (13). These knockdown and overexpression effects show that miR-124 plays differential roles during eye development, and that the level of miR-124 is positively correlated to cell proliferation and negatively correlated to neurogenesis during early optic vesicle stages.

**MiR-124 negatively regulates expression of NeuroD1**

To investigate whether the proliferating cells which had increased or decreased in the optic vesicle in response to the gain or loss of miR-124 were specified neuronal progenitors, the effects of miR-124 on proneural genes were also considered. Since *NeuroD1* is a well-known neurogenic factor and proneural marker in both the embryonic and adult central nervous systems (30,33–36), and is implicated as a candidate target of miR-124 (13,37), we investigated whether *NeuroD1* is negatively regulated by miR-124 and whether it is involved in the early role of miR-124 during the optic vesicle stage. Therefore, we

---

**Figure 1.** MiR-124 regulates cell proliferation and neurogenesis in the optic vesicle and forebrain. (A) Proliferating cells were detected with a BrdU (red) incorporation assay. Hoechst (blue) was applied to label the nuclei of all cells. The dashed line in the schematic diagram [images from Nieuwkoop and Faber, 1994, *Normal Table of Xenopus laevis* (Daudin)] indicates the location of the transverse sections in the developing eye. In the optic vesicle (arrow) and forebrain (arrow head) of embryos at stage (st.) 22/23, the BrdU-positive cell ratio was significantly reduced when a miR-124 inhibitor (Anti-124) was injected, but significantly increased when an miR-124 precursor (Pre-124) was applied. In the optic cup (st.33/34), injection of either control inhibitor (Anti-ctrl) or precursor (Pre-ctrl) molecules gave no significant change in cell proliferation compared with the uninjected control (Uninj). The bar graph illustrates the BrdU-positive ratio of the transverse sections (mean ± SEM, 24 sections from six embryos). Scale bar: 100 μm. (B) Expression of *NCAM* and *N-tubulin* are significantly upregulated with downregulation of miR-124, but significantly downregulated with overexpression of miR-124. *ODC* and –RT are the internal and negative controls, respectively, for the RT–PCR procedure. The bar graph illustrates the gene expression level analyzed by real-time RT–PCR. Means ± SEM are from three independent experiments. The values of injected groups were compared with those of uninjected controls by one-way ANOVA followed by the Duncan test. *P* and *#*P < 0.05; **P and ##*P < 0.01.
performed both further loss- and gain-of-function studies to verify the functional effects of miR-124 on NeuroD1. Expression of Lhx2, a target of miR-124 in the eye, verified in our previous work (13), was also analyzed.

By whole-mount in situ hybridization, loss of miR-124 was shown to contribute to the increase in expression of NeuroD1 but not Lhx2 from stages 20 to 22/23 compared with that on the control side (Figure 2A). However, NeuroD1 expression was no longer significantly affected by loss-of-miR-124 at the optic cup stage (stage 33) (Figure 2A). This stage-dependent upregulation of NeuroD1 was confirmed by RT–PCR and real-time RT–PCR (Figure 2B).

In addition, we detected the expression of two genes downstream of NeuroD1, elrC (38) and xtwi (30), which are activated and inhibited by NeuroD1, respectively. ElrC expression markedly increased, whereas xtwi expression was downregulated, indicating that NeuroD1 activity is also upregulated within miR-124-silenced embryos (Figure 2C). These results confirm that loss of miR-124 promotes NeuroD1 expression and that miR-124 is necessary for controlling NeuroD1 expression at the optic vesicle stage.

In agreement with the results of the loss-of-function assay, the level of NeuroD1 at stage 22/23 was downregulated when miR-124 was overexpressed (Figure 3A). Expression of the two downstream genes elrC and xtwi was correspondingly reduced and increased, respectively (Figure 3B). However, expression of Lhx2 was also significantly downregulated (Figure 3A), indicating that miR-124 is sufficient for repressing Lhx2 transcription at both the optic vesicle and optic cup stages. This result is consistent with our previous in situ hybridization results (13). Interestingly, real-time RT–PCR

Figure 2. Downregulation of miR-124 increases the expression of NeuroD1 in the optic vesicle. (A) Expression of NeuroD1 increased in stages 20 and 22/23 embryos but not in stages 33/34 embryos injected (Inj.) with Anti-124, as indicated by in situ hybridization, while expression of Lhx2 was not affected at any of these stages. Yellow dashed lines indicate the midlines of Stage 20 embryos. Red dots circle the position of the optic vesicle/cup. Scale bar: 500 μm. (B) RT–PCR and real-time PCR quantification confirm that expression of NeuroD1, but not Lhx2, in stage 22/23 embryos is upregulated on loss of miR-124. (C) Correspondingly, the expression of erC increased and that of xtwi decreased. *P and **P < 0.05; ***P and ****P < 0.01.
results showed that the NeuroD1 expression level decreased to 42.4% and was much lower than the expression level of Lhx2 (59.4%) (Figure 3A), suggesting that miR-124 is a strong inhibitor of NeuroD1 expression specifically at the optic vesicle stage in Xenopus laevis. Taken together, the above loss- and gain-of-function studies provide both direct and indirect evidence that NeuroD1 is negatively regulated by miR-124 at least at the optic vesicle stage. These results also indicate that miR-124 represses both proneural and neuronal properties at these stages, thus playing an anti-neural role.

MiR-124-enhanced cell proliferation is rescued by NeuroD1

To investigate whether miR-124 promotes cell proliferation by repressing NeuroD1, the miR-124 precursor was co-injected with NeuroD1 mRNA and effects on cell proliferation in the optic vesicle were compared with the effects of injecting NeuroD1 or pre-124 alone (Figure 4). Stimulation of cell proliferation by miR-124 overexpression was confirmed with a pH3 staining assay. As expected, co-expression of 10 pg NeuroD1 mRNA drastically reduced (P < 0.05) the increased cell proliferation resulting from miR-124 upregulation. Expression of NeuroD1 alone at the same dose led to no significant changes in the pH3-positive cell ratio compared to the blank control. These results indicate that NeuroD1 counteracts miR-124-induced cell proliferation in early eye development, suggesting that NeuroD1 may be a key factor involved in the regulation of neurogenesis by miR-124 during the optic vesicle stages.

NeuroD1 is a direct target of miR-124

The above results strongly suggest that NeuroD1 is a functionally important target of miR-124. To test this possibility, we analyzed vertebrate NeuroD1 sequences in silico. We found that NeuroD1 is evolutionarily conserved from amphibians to humans both in its coding region (data not shown) and in its 3'-UTR (Figure 5A), and that it is a candidate target of miR-124 as predicted computationally by RNAhybrid (26), Targetscan (27) and Pictar (28).

Next, we used luciferase reporter assays to check whether NeuroD1 is a target of miR-124. The 3'-UTR of NeuroD1 containing the predicted MRE was inserted downstream from the luciferase-coding region in the reporter vector. Two constructs containing the antisense sequence of miR-124 (Anti-124) or the 3'-UTR of Lhx2 were employed as positive controls (13). Two other constructs with 3'-UTRs of Pax6, which contains no MRE (13), or a mutated 3'-UTR of NeuroD1 (NeuroD1-Mut) (Figure 5A) were used as negative controls. Each reporter construct was separately co-transfected into 293T cells with the miR-124 precursor or the control precursor molecules. Consistent with our previous results (13), the luciferase activity of the positive controls, the Anti-124 and Lhx2 reporters, was reduced to 3.6% and 64.5%, respectively, while the Pax6 3'-UTR negative control did not significantly alter the luciferase activity (Figure 5B). The incorporation of the NeuroD1 3'-UTR in the reporter resulted in a significant (P < 0.01) decline in luciferase activity to 58.3%. In contrast, the incorporation of the NeuroD1-Mut fragment did not change expression.

Figure 3. MiR-124 overexpression reduces the expression of NeuroD1. RT–PCR (gel panel) and real-time PCR (bar graph) conditions are the same as those in Figure 2. (A) Gain of miR-124 resulted in downregulation of both NeuroD1 and Lhx2 at Stages 22/23. (B) Expression of elrC decreased and that of xtwi increased. *P and **P < 0.05; ***P and ****P < 0.01.
NeuroD1

Pre-124 group compared with the uninjected blank control. Injection of proliferating cells in the brain and optic vesicle were detected in the graph (Mean ± SEM, 16 sections from four embryos). More

The pH3-positive cell ratio of transverse sections was shown in the bar (pH3) antibody (red). Nuclei were labeled with Hoechst33258 (blue).

Co-injection with Pre-124 plus NeuroD1 mRNA alone had no obvious effect on cell proliferation. Co-injection with Pre-124 plus NeuroD1 restored cell proliferation to the level of the blank control. Scale bar: 100 μm. *P < 0.05; **P < 0.01.

DISCUSSION

Using loss- and gain-of-function studies, we have provided the first evidence that miR-124, a neuronal-specific miRNA, antagonizes NeuroD1 and plays an anti-neuronal role by promoting cell proliferation and repressing neurogenesis in early eye development (Figure 6).

MiR-124 is a highly conserved and CNS-enriched miRNA that has been reported in a range of species from C. elegans to humans (4,8,17,19–20,39–44). Previous work by in vitro analysis has shown that miR-124 overexpression represses cell proliferation and promotes neuronal differentiation (17,45–46). However, results reported from in vivo analyses are somewhat divergent. It has been shown that neither inhibition nor overexpression of miR-124 alone significantly alters neuronal fate in chick embryonic development (19).

At the same time, miR-124 has been found to moderately enhance neuronal differentiation in chicks (20). Recently, Maiorano et al. (47) reported that miR-124 promotes embryonic cortico-cerebral neuronogenesis in mice and we also found that miR-124 overexpression decreases retinal cell proliferation in the Xenopus optic cup (13). MiR-124 is both necessary and sufficient for adult neurogenesis in mice, regulating the progression from progenitor cells to neurons (18). These results indicate that miR-124 plays a role in repressing cell proliferation and/or inducing neurogenesis. However, all of the above results were obtained from analyses after the optic vesicle stages. While miR-124 starts to be expressed at the onset of neurogenesis in the neurula stage, its role during this period of early neurogenesis is unclear. Our work shows that at the Xenopus optic vesicle stage, miR-124 is both required and sufficient for cell proliferation and repression of neurogenesis in the forebrain and optic vesicle, playing an anti-neural role distinct from that in later developmental and adult stages.

During the dynamic embryonic development stages, miR-124 is expressed in different cells of the central nervous system (13). The diverse roles of miR-124 in vivo are likely to be developmental stage dependent. In contrast to results reported here for the optic vesicle stage, we previously found that at the optic cup stage when the level of miR-124 has increased to a high level and miR-124-expressing cells have become more specified, gain of miR-124 decreases cell proliferation in the retina (13). In experiments with divergent results from chicks (19–20), overexpression of miR-124 was carried out by electroporation at stage HH13, when the optic cup stage starts to form from the optic vesicle; and the effect of miR-124 was investigated at stage HH25. The developmental stage selected in these chick experiments was later than those used in our experiments. In Maiorano’s work in mice (47), the role of miR-124 was analyzed at a developmental period (E12.5–E14.5) even later than those in the chick experiments. Therefore, miR-124 might act as an ‘enhancer’ for cell proliferation and an inhibitor for neurogenesis in the less-specified earlier cells, while playing a reverse role in the later stages. A recent report showed that miR-124 transgenic embryonic mice have enlarged body sizes and increased weight compared with controls (48). In this case, transformation with miR-124 significantly (Figure 5B). The above results demonstrate that miR-124 can directly target the MRE in the 3′-UTR of the NeuroD1 to repress gene expression.

To confirm the interaction of miR-124 with NeuroD1 in vivo, we performed whole-mount in situ hybridization on wild-type embryos at stage 22/23. The expression level of miR-124 in the optic vesicle and forebrain was quite low compared with the strong expression of NeuroD1. In these embryos, NeuroD1 signals were observed to be restricted in the dorsal region of the anterior forebrain and the peripheral optic vesicle, areas where miR-124 shows relatively low levels of expression (Figure 5C). These results show that the expression of miR-124 and NeuroD1 are somewhat, though not completely, complementary to each other in the optic vesicle and anterior forebrain, supporting the hypothesis that NeuroD1 can act as a direct target of miR-124 in vivo to control cell proliferation and neurogenesis.
was conducted by microinjection into mouse blastomeres and led to an increased growth rate as determined by BrdU labeling. This result provides more evidence that miR-124 overexpression enhances cell proliferation during early embryonic development. Therefore, we propose that miR-124 may act as either a positive or a negative regulator of neurogenesis depending on developmental stage.

Different genes have been identified as targets of miR-124 in the developing central nervous system (13,17,19–20), pancreas (37) and adult brain (18,49). Recent studies using high-throughput techniques have also shown that miR-124 has hundreds of targets (14–15,50). The existence of multiple targets implies that miR-124 has multiple roles. However, these roles are as yet largely unexplored. NeuroD1, a candidate miR-124 target (13,37) that has yet to be verified, is well known as a proneural bHLH transcription factor due to its critical role in promoting neuronal differentiation, and has been shown to be downregulated by miR-124 overexpression (13). In this study, we show that NeuroD1 is specifically upregulated by knocking down miR-124 at the optic vesicle stage, and that miR-124 can repress gene expression by targeting the 3'-UTR of NeuroD1, thus providing both in vitro and in vivo evidence that NeuroD1 is a target of miR-124. Systematic research on miRNA and its targets in zebrafish has shown that NeuroD1 is upregulated in the MZDicer mutant which does not contain miR-124 (14). Our conclusions are strongly supported by this recent genetic evidence in zebrafish.

NeuroD1 (NeuroD) was identified in mouse and Xenopus simultaneously, and acts as one of the earliest transcription factors promoting neuronal differentiation (30). Knockout of NeuroD leads to neuronal deficits in the granule layers of the cerebellum and hippocampus (51,52), and its overexpression has been shown to inhibit cell proliferation and promote neurogenesis (53,54). These effects are coincident with those of gain and loss of function of miR-124 at the optic vesicle stage. However, excessive cell death has also been observed in the NeuroD-deficient mice (51,52). This is in contrast to the significant

![Figure 5. MiR-124 targets NeuroD1. (A) An evolutionarily conserved miR-124 target element (red) is located at the 3'-UTR of NeuroD1 mRNAs in humans (hsa), mice (mmu), rats (rno), Xenopus tropicalis (xtr) and Xenopus laevis (xla). The minimal free energy (Mfe) of xla-NeuroD1 pairing to xla-miR-124, xtr-miR-124 and mmu-miR-124 was below –20 kcal/mol. A mutant NeuroD1 plasmid (xla-NeuroD1-Mut) was constructed with mutations in the underlined positions which pair with the miR-124 seed sequence (2–7 nt). Homologous sites are marked by asterisks. (B) Luciferase assays were carried out in the 293T cell line using pCS2-Luc-NeuroD1 3'-UTR reporters (in xla). Positive (Anti-miR-124, Lhx2) and negative (Pax6, NeuroD1-Mut) controls were set. Anti-miR-124 almost completely blocks luciferase activity. The relative luciferase activity of the NeuroD1 group is significantly lower than that of the negative control groups and similar to that of the Lhx2 positive control. Means ± SD are from three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001. (C) In situ hybridization of miR-124 and NeuroD1 at stage 22/23. MiR-124 is weakly expressed in the eye (arrow) in comparison with expression of NeuroD1 as shown in the lateral view. Transverse sections at the level of the eye showed that the expression patterns of miR-124 and NeuroD1 were partially complementary in the forebrain (arrow head) and optic vesicle (arrow).]
Figure 6. A putative scheme of the regulatory effect of miR-124 on NeuroD1 in early eye and brain development. NeuroD1, which represses cell proliferation and promotes neuronal differentiation, is one of the target genes of miR-124. The arrow represents promotion; the right angle represents repression.

increase in proliferating cells in miR-124-injected embryos in this study, indicating that expression changes in other miR-124 targets are also involved in the miR-124 overexpression effects. As the upregulation of NeuroD1 by miR-124 knockdown is significant only at the optic vesicle stage but not at the later optic cup stage, and high levels of miR-124 and NeuroD1 co-localize in the central retina at the early optic cup stage (st.32–st.41) (13,55), other regulators may also be involved in the regulation of NeuroD1 expression. The differential interaction of miR-124 with its targets may be key in determining its changing roles during neural development.

No significant morphological defects have been observed in miR-124 downregulated embryos (13), suggesting possible compensatory events and complex regulation of overall embryonic development. This observation is consistent with recent results from C. elegans, showing that miR-124 mutant worms show no obvious morphological changes (15). However, cell proliferation and differentiation are closely related events during neurogenesis, and the timing of cell cycle exit has been shown to be critical to cell fate determination (56–58). NeuroD1 is also known to play multiple roles in neuronal development and to influence the fate of specific neuronal cells (30,33–34,51,54,59–62). For example, in chicks and mice, NeuroD1 regulates photoreceptor cell formation and is involved with other bHLH transcription factors in controlling retinal subtype specification (60,62–65). Recent findings on zebrafish retina development show that NeuroD1 is dynamically expressed in the proliferating cells that give rise to the photoreceptor cell lineage and that its overexpression inhibits retinal cell proliferation and promotes neuronal differentiation (53,54). Our identification of the conserved miR-124 binding site in the 3′-UTR of NeuroD1, together with the known functional conservation of miR-124 and NeuroD1, suggests that the novel post-transcriptional regulation of NeuroD1 by miR-124 described here may also be conserved in other species, modulating multiple functional roles of both genes.

ACKNOWLEDGEMENTS

The authors are grateful to Prof. William A Harris/Christine Holt’s labs for the NeuroD1 constructs. The authors thank Haihong Ye and Yaobo Liu for their helpful discussions.

FUNDING

National Sciences Foundation of China (NSFC, 30771129); CASNN-GWPPS-2008; National Basic Research Program of China (973 Project, 2005CB522804 2009CB825402 and 2010CB912303); QCAS Biotechnology Fund 2010; (2006CB911003). Funding for open access charge: National Sciences Foundation of China (NSFC, 30771129).

Conflict of interest statement. None declared.

REFERENCES

1. Xu,S., Witmer,P.D., Lumayag,S., Kovacs,B. and Valle,D. (2007) MicroRNA (miRNA) transcriptome of mouse retina and identification of a sensory organ-specific miRNA cluster. J. Biol. Chem., 282, 25053–25066.
2. Wienholds,E., Kloosterman,W.P., Miska,E., Alvarez-Saavedra,E., Berezikov,E., de Bruijn,E., Horvitz,H.R., Kauppinen,S. and Plasterk,R.H. (2005) MicroRNA expression in zebrafish embryonic development. Sciences, 309, 310–311.
3. Li,X. and Carthew,R.W. (2005) A microRNA mediates EGF receptor signaling and promotes photoreceptor differentiation in the Drosophila eye. Cell, 123, 1267–1277.
4. O’Farrell,F., Esfahani,S.S., Engstrom,Y. and Kylsten,P. (2008) Regulation of the Drosophila lin-41 homologue dappled by let-7 reveals conservation of a regulatory mechanism within the LIN-41 subclade. Dev. Dyn., 237, 196–208.
5. Walker,J.C. and Harland,R.M. (2009) microRNA-24a is required to repress apoptosis in the developing neural retina. Genes Dev., 23, 1046–1051.
6. Adler,R. and Canto-Soler,M.V. (2007) Molecular mechanisms of optic vesicle development: complexities, ambiguities and controversies. Dev. Biol., 305, 1–13.
7. Harada,T., Harada,C. and Parada,L.F. (2007) Molecular regulation of visual system development: more than meets the eye. Genes Dev., 21, 367–378.
8. Deo,M., Yu,J.Y., Chung,K.H., Tippets,M. and Turner,D.L. (2006) Detection of mammalian microRNA expression by in situ hybridization with RNA oligonucleotides. Dev. Dyn., 235, 2538–2548.
9. Frederikse,P.H., Donnelly,R. and Partyka,L.M. (2006) miRNA and Dicer in the mammalian lens: expression of brain-specific miRNAs in the lens. Histochem. Cell Biol., 126, 1–8.
10. Sweetman,D., Rathjen,T., Jefferson,M., Wheeler,G., Smith,T.G., Wheeler,G.N., Munsterberg,A. and Dalmay,T. (2006) FGF-4 signaling is involved in mir-206 expression in developing somites of chicken embryos. Dev. Dyn., 235, 2185–2191.
11. Darnell,D.K., Kaur,S., Stanislaw,S., Konieczka,J.H., Yatskievych,T.A. and Antin,P.B. (2006) MicroRNA expression during chick embryo development. Dev. Dyn., 235, 3156–3165.
2878 Nucleic Acids Research, 2011, Vol. 39, No. 7

12. Watanabe, T., Takeda, A., Mise, K., Okuno, T., Suzuki, T., Minami, N. and Imai, H. (2005) Stage-specific expression of microRNAs during Xenopus development. FEBs Lett., 579, 318–324.

13. Qi, R., Liu, K., Liu, Y., Mo, W., Flynt, A.S., Patton, J.G., Kar, A., Wu, J.Y. and He, R. (2009) The role of miR-124a in early development of the Xenopus eye. Mech. Dev., 126, 804–816.

14. Shikumata, A., Stark, A., Sive, H. and Bartel, D.P. (2009) Coherent but overlapping expression of microRNAs and their targets during vertebrate development. Genes Dev., 23, 466–481.

15. Clark, A.M., Goldstein, L.D., Telvin, M., Tavare, S., Shaham, S. and Miska, E.A. (2010) The microRNA miR-124 controls gene expression in the sensory nervous system of Caenorhabditis elegans. Nucleic Acids Res., 38, 3780–3793.

16. Lim, L.P., Lau, N.C., Garrett-Engele, P., Schelter, J.M., Castle, J., Bartel, D.P., Linsley, P.S. and Johnson, B.J. (2005) Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature, 433, 769–773.

17. Makeyev, E.V., Zhang, J., Carrasco, M.A. and Maniatis, T. (2007) The MicroRNA miR-124 promotes neuronal differentiation by triggering brain-specific alternative pre-mRNA splicing. Mol. Cell, 27, 435–448.

18. Cheng, L.C., Pattraniva, E., Tavazoie, M. and Doetsch, F. (2009) miR-124 regulates adult neurogenesis in the subventricular zone stem cell niche. Nat. Neurosci., 12, 399–408.

19. Cao, X., Pfaff, S.L. and Gage, F.H. (2007) A functional study of miR-124 in the developing neural tube. Genes Dev., 21, 531–536.

20. Visvanathan, J., Lee, S.E., Lee, B. and Lee, S.K. (2007) The microRNA miR-124 antagonizes the anti-neural REST/SC1 pathway during embryonic CNS development. Genes Dev., 21, 744–749.

21. Lupo, G., Liu, Y., Quir, R., Chandraratna, R.A., Barsacchi, G., He, R.Q. and Harris, W.A. (2005) Dorsoventral patterning of the Xenopus eye: a collaboration of Retinoid, Hedgehog and FGF receptor signaling. Development, 132, 1737–1748.

22. Moore, K.B., Schneider, M.L. and Vetter, M.L. (2002) Posttranslational mechanisms control the timing of BHLH function and regulate retinal cell fate. Neuron, 34, 183–195.

23. Andreazzoli, M., Gestri, G., Angeloni, D., Menna, E. and Barsacchi, G. (1999) Role of Zic1 in Xenopus eye and anterior brain development. Development, 126, 2451–2460.

24. Quick, Q.A. and Serrano, E.E. (2007) Cell proliferation during the early compartmentalization of the Xenopus laevis inner ear. Int. J. Dev. Biol., 51, 201–209.

25. Harland, R.M. (1991) In situ hybridization: an improved whole-mount method for Xenopus embryos. Methods Cell Biol., 36, 685–695.

26. Rehmsmeier, M., Steffen, P., Hochsmann, M. and Giegerich, R. (2004) Fast and effective prediction of microRNA/target duplexes. RNA, 10, 1507–1517.

27. Lewis, B.P., Burge, C.B. and Bartel, D.P. (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell, 120, 15–20.

28. Krek, A., Dunker, A.K., food, M.N., Wolff, R., Rosenberg, L., Epstein, E.J., MacMenamin, A., Davis, D., Gunalus, K.C., Stoffel, M. et al. (2005) Combinatorial microRNA target predictions. Nat. Genet., 37, 495–500.

29. Griffiths-Jones, S., Saini, H.K., van Dongen, S. and Enright, A.J. (2008) miRBase: tools for microRNA genomics. Nucleic Acids Res., 36, D154–D158.

30. Lee, J.E., Hollenberg, S.M., Snider, L., Turner, D.L., Lipnick, N. and Weintraub, H. (1995) Conversion of nervous system-specific, class II beta-tubulin gene from Xenopus laevis. Nucleic Acids Res., 17, 8000.

31. Lee, J.K., Cho, J.H., Hwang, W.S., Lee, Y.D., Reu, D.S. and Suk-Him, H. (2000) Expression of neuroD/BETA2 in mitotic and postmitotic neuronal cells during the development of nervous system. Dev. Dyn., 217, 361–367.

32. Schlosser, G. and Northcutt, R.G. (2000) Development of neurogenic placodes in Xenopus laevis. J. Comp. Neurol., 418, 121–146.

33. Chae, J.H., Stein, G.H. and Lee, J.E. (2004) NeuroD: the predicted and the surprising. Mol. Cells, 18, 271–288.

34. Gao, L., Ure, K., Abelis, J., Lague, D.C., Nave, K.A., Goebl, S., Eisch, A.J. and Hsieh, J. (2009) NeuroD is essential for the survival and maturation of adult-born neurons. Nat. Neuronosci., 12, 1090–1092.

35. Baroukh, N., Ravier, M.A., Loder, M.K., Hill, E.V., Bounacera, A., Scharff, R.A. and Van Obberghen, E. (2007) MicroRNA-124a regulates Foxa2 expression and intracellular signaling in pancreatic β-cell lines. J. Biol. Chem., 282, 19575–19588.

36. Logan, M.A., Steele, M.R., Van Raay, T.J. and Vetter, M.L. (2005) Identification of shared transcriptional targets for the proneural bHLH factors Xath5 and XNeuroD. Dev. Biol., 285, 570–583.

37. Aboobaker, A.A., Tomancak, P., Patel, N., Rubin, G.M. and Lai, E.C. (2005) Drosophila microRNAs exhibit diverse spatial expression patterns during embryonic development. Proc. Natl. Acad. Sci. USA., 102, 18017–18022.

38. Krchekvsky, A.M., Sonntag, K.C., Isacson, O. and Kosik, K.S. (2006) Specific microRNAs modulate embryonic stem cell-derived neurogenesis. Stem Cells, 24, 857–864.

39. Nelson, P.T., Baldwin, D.A., Klooosterman, W.P., Kauppinen, S., Plasterk, R.H. and Mourelatos, Z. (2006) RAKE and LNA-ISH reveal microRNA expression and localization in adult human brain. RNA, 12, 187–191.

40. Sempere, L.F., Freemantle, S., Pitta, J., Mass, E., Dmitrovsky, E. and Ambros, V. (2004) Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. Genome Biol., 5, R13.

41. Persson, J., Hostager, B., Staden, R. and Vihakkar, R. (2008) Regulation of cyclin dependent kinase 6 by microRNA 124 in medulloblastoma. J. Neurooncol., 90, 1–7.

42. Li, K.K., Pang, J.C., Ching, A.K., Kong, X., Wang, Y., Zhou, L., Chen, Z. and Ng, H.K. (2009) miR-124 is frequently down-regulated in medulloblastoma and is a negative regulator of survival and maturation of adult-born neurons. Genes Dev., 23, 1218–1223.

43. Rajasethupathy, P., Fiumara, F., Sheridan, R., Betel, D., Puthanveetil, S.V., Russo, J., Sander, C., Tuschl, T. and Kandel, E. (2009) Characterization of small RNAs in aplysia reveals a role for miR-124 in constraining synaptic plasticity through CREB. Neuron, 63, 803–817.

44. Chi, S.W., Zang, J.B., Mele, A. and Darnell, R.B. (2009) Argonaute HITS-CLIP decodes microRNA-mRNA interaction maps. Nature, 460, 479–486.

45. Miyata, T., Maeda, T. and Lee, J.E. (1999) NeuroD is required for differentiation of the granule cells in the cerebellum and hippocampus. Genes Dev., 13, 1647–1652.

46. Liu, M., Pleasure, S.J., Collins, A.E., Noelbs, J.L., Naya, F.J., Tsai, M.J. and Lowenstein, D.H. (2000) Loss of BET2/NeuroD leads to malformation of the dentate gyrus and epilepsy. Proc. Natl. Acad. Sci. USA., 97, 865–870.

47. Ochocinska, M.J. and Hitchcock, P.F. (2007) Dynamic expression of the basic helix-loop-helix transcription factor neuroD in the...
rod and cone photoreceptor lineages in the retina of the embryonic and larval zebrafish. J. Comp. Neurol., 501, 1–12.
54. Ochocinska,M.J. and Hitchcock,P.F. (2009) NeuroD regulates proliferation of photoreceptor progenitors in the retina of the zebrafish. Mech. Dev., 126, 128–141.
55. Wang,J.C. and Harris,W.A. (2005) The role of combinational coding by homeodomain and bHLH transcription factors in retinal cell fate specification. Dev. Biol., 285, 101–115.
56. Cepko,C.L., Austin,C.P., Yang,X., Alexiades,M. and Ezzeddine,D. (1996) Cell fate determination in the vertebrate retina. Proc. Natl Acad. Sci. USA, 93, 589–595.
57. Ohnuma,S., Hopper,S., Wang,K.C., Philpott,A. and Harris,W.A. (2002) Co-ordinating retinal histogenesis: early cell cycle exit enhances early cell fate determination in the Xenopus retina. Development, 129, 2435–2446.
58. Rapaport,D.H., Wong,L.L., Wood,E.D., Yasumura,D. and LaVail,M.M. (2004) Timing and topography of cell genesis in the rat retina. J. Comp. Neurol., 474, 304–324.
59. Mueller,T. and Wullimann,M.F. (2002) Expression domains of neuroD (nrd) in the early postembryonic zebrafish brain. Brain Res. Bull., 57, 377–379.
60. Akagi,T., Inoue,T., Miyoshi,G., Bessho,Y., Takahashi,M., Lee,J.E., Guillemot,F. and Kageyama,R. (2004) Requirement of multiple basic helix-loop-helix genes for retinal neuronal subtype specification. J. Biol. Chem., 279, 28492–28498.
61. Hitchcock,P. and Kakuk-Atkins,L. (2004) The basic helix-loop-helix transcription factor neuroD is expressed in the rod lineage of the teleost retina. J. Comp. Neurol., 477, 108–117.
62. Liu,H., Eitter,P., Hayes,S., Jones,I., Nelson,B., Hartman,B., Forrest,D. and Reh,T.A. (2008) NeuroD1 regulates expression of thyroid hormone receptor 2 and cone opsins in the developing mouse retina. J. Neurosci., 28, 749–756.
63. Yan,R.T. and Wang,S.Z. (2004) Requirement of neuroD for photoreceptor formation in the chick retina. Invest. Ophthalmol. Vis. Sci., 45, 48–58.
64. Ma,W., Yan,R.T., Xie,W. and Wang,S.Z. (2004) A role of ath5 in inducing neuroD and the photoreceptor pathway. J. Neurosci., 24, 7150–7158.
65. Inoue,T., Hojo,M., Bessho,Y., Tanou,Y., Lee,J.E. and Kageyama,R. (2002) Math3 and NeuroD regulate amacrine cell fate specification in the retina. Development, 129, 831–842.