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

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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

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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 over-expression of miR-124 alone significantly alters neuronal fate. Visvanathan *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 33 258 (Sigma). Images were taken using a compound microscope (Nikon FXA, Japan).

Counts of BrdU-positive (N_{BrdU}), pH3-positve (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: $N_{BrdU\ or\ pH3} / N_{Hoechst} \times 100\%$.

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 refixed. 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′-CACAGTTCCACCAAATGC-3′, Reverse: 5′-GGAATCAAGCGGTACAGA-3′), *N-tubulin* (Forward: 5′-ACACGGCATTGATCCTACAG-3′, Reverse: 5′-AGCTCCTTCGGTGTAATGAC-3′), *NeuroD1* (Forward: 5′-GTTATTGTACCCATGCCG-3′, Reverse: 5′-GTCTCTA AGGCAACACAAC-3′), *Lhx2* (Forward: 5′-GTTGGAAAGCTTGTCATTGC-3′, Reverse: 5′-CCTTCGGAAACTCAAATCAG-3′), *elrC* (Forward: 5′-AGAATCATCACATCCCGTATC-3′, Reverse: 5′-CAGGCTTTGTGCTGTTTACT-3′), *xtwi* (Forward: 5′-AGTCCGATCTCAGTGAAGGGC-3′, Reverse: 5′-TGTGTGTGGCCTGAGCTGTAG-3′), and *ODC* (Forward: 5′-AATGGATTTCAGAGACCA-3′, Reverse: 5′-CCAAGGCTAAAGTTGCAG-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 $2^{-\Delta\Delta Ct}$ 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 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*. The primers for PCR amplification of the 3′-UTP fragment were as follows: 5′-CGTGAATTCG TTATTGTACCCATGCCG-3′ (forward) and 5′-TCACT CGAGGTCTCTAAGGCAACACAAC-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 blastomere(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

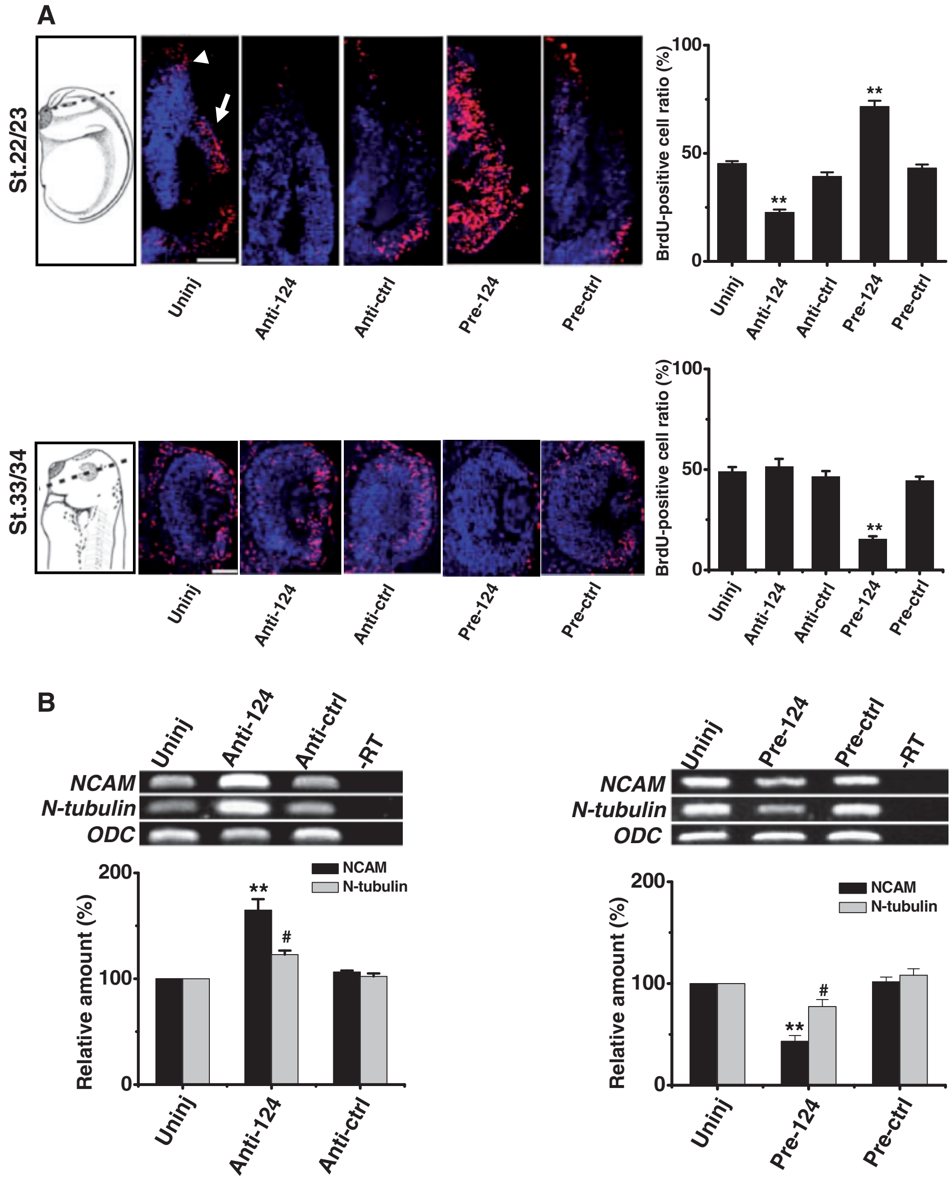

Figure 1. MiR-124 regulates cell proliferation and neurogenesis in the optic vesicle and forebrain. (**A**) Proliferating cells were detected with a BrdU (red) incorporation assay. Hoechst (blue) was applied to label the nuclei of all cells. The dashed line in the schematic diagram [images from Nieuwkoop 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 $^{\#\#}P < 0.01$.

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 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

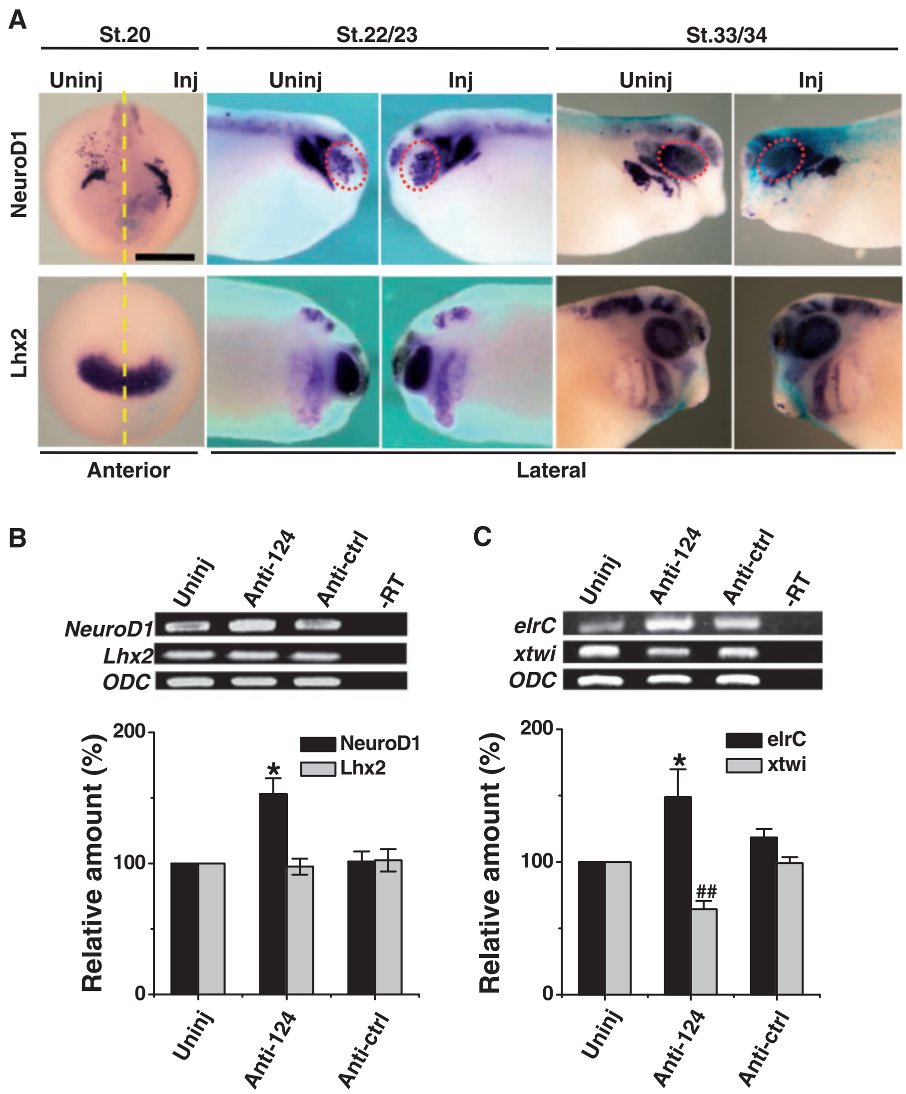

Figure 2. Downregulation of miR-124 increases the expression of *NeuroD1* in the optic vesicle. (**A**) Expression of *NeuroD1* increased in stages 20 and 22/23 embryos but not in stages 33/34 embryos injected (Inj.) with Anti-124, as indicated by *in situ* hybridization, while expression of *Lhx2* was 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 $^{\#\#}P < 0.01$.

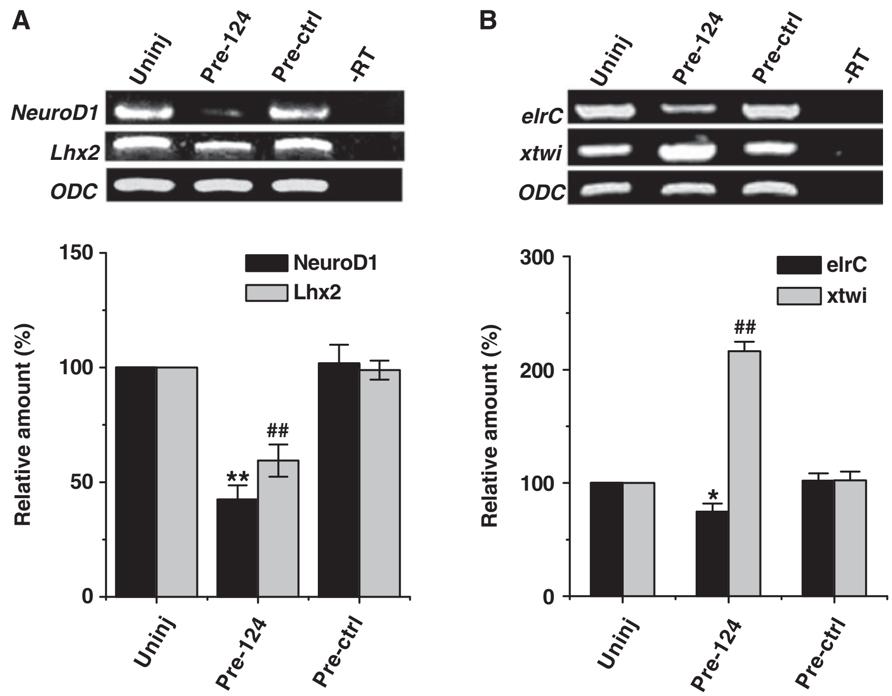

Figure 3. MiR-124 overexpression reduces the expression of *NeuroD1*. RT–PCR (gel panel) and real-time PCR (bar graph) conditions are the same as those in Figure 2. (**A**) Gain of miR-124 resulted in downregulation of both *NeuroD1*and *Lhx2* at Stages 22/23. (**B**) Expression of *elrC* decreased and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

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

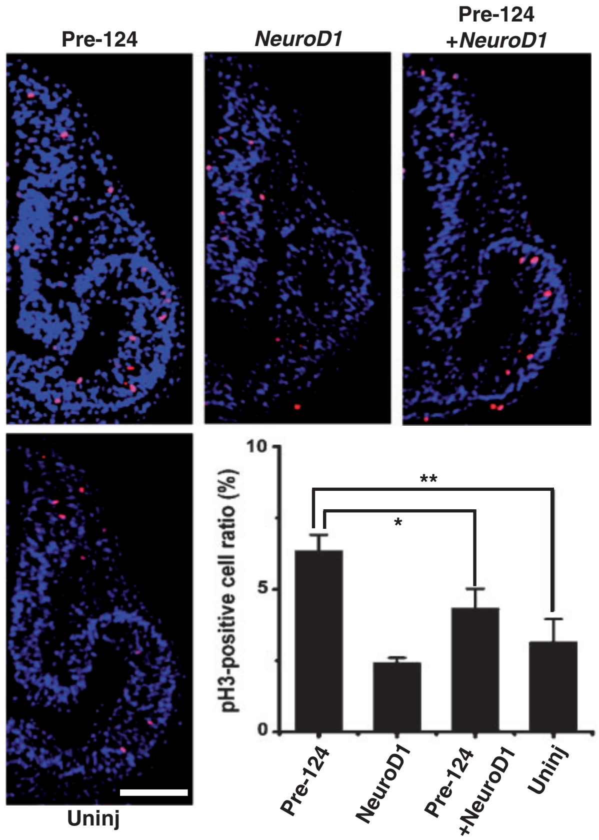

Figure 4. *NeuroD1* antagonizes miR-124-induced cell proliferation. Transverse sections of the optic vesicle in stage 22/23 embryos injected with Pre-124 (0.025 pmol) and/or *NeuroD1* (10 pg). Proliferzating cells were immunohistochemically stained with phosphohistone-H3 (pH3) antibody (red). Nuclei were labeled with Hoechst33 258 (blue). The pH3-positive cell ratio of transverse sections was shown in the bar graph (Mean ± SEM, 16 sections from four embryos). More proliferating cells in the brain and optic vesicle were detected in the Pre-124 group compared with the uninjected blank control. Injection of *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. $*P < 0.05$; $**P < 0.01$.

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.

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 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

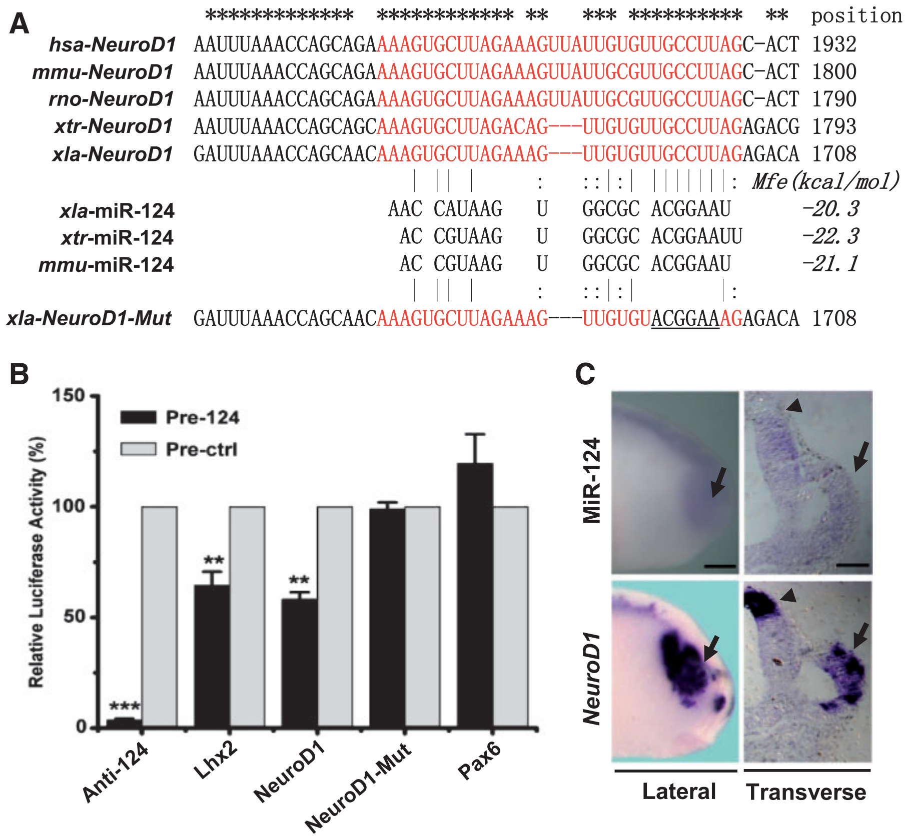

Figure 5. MiR-124 targets *NeuroD1*. (**A**) An evolutionarily conserved miR-124 target element (red) is located at the 3′-UTR of *NeuroD1* mRNAs in humans (*hsa*), mice (*mmu*), rats (*rno*), *Xenopus tropicalis* (*xtr*) and *Xenopus laevis* (*xla*). The minimal free energy (Mfe) of *xla-NeuroD1* pairing 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).

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 *NeuroD1* 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

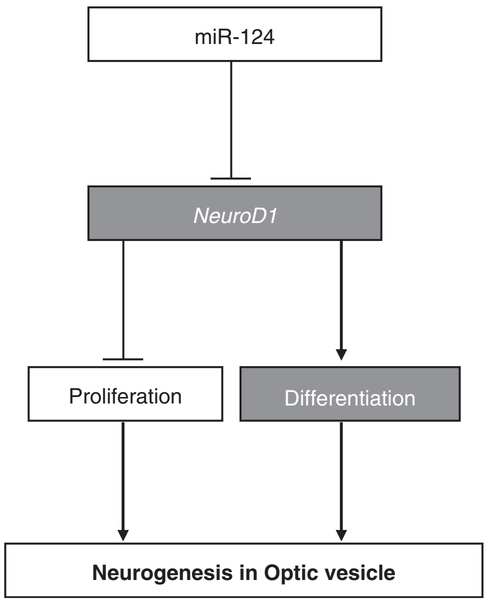

Figure 6. A putative scheme of the regulatory effect of miR-124 on *NeuroD1* in early eye and brain development. *NeuroD1*, which represses cell proliferation and promotes neuronal differentiation, is one of the target genes of miR-124. The arrow represents promotion; the right angle represents repression.

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.

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