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The role of miR-124a in early development of the *Xenopus* eye

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Abstract

It has been reported that miR-124a is abundant in the central nervous system including the eye, and is related to neurogenesis in several species. However, the role of miR-124a in the eye remains unclear. In this study, we show that the expression of miR-124a in *Xenopus laevis* begins along the neural fold, including the protruding eye anlagen, at a low level at around stage 18; its expression level gradually increases in the neural tube and the eye as embryos develop into later stages and then maintains at a high level in eye to adult stages. Microinjection of a miR-124a precursor at the 8-cell stage leads to malformation of the optic nerve and optic cup, indicating the importance of maintaining low levels of miR-124a during early embryonic development. In addition, miR-124a overexpression markedly down regulates the expression of its predicted targets *Lhx2*, *Hairy2*, *Gli3*, *NeuroD1* and *Otx2* in/around the eye anlagen, and the interaction of miR-124a with the 3' UTR of *Lhx2* represses gene expression as shown by luciferase assays. Moreover, excess miR-124a inhibits cell proliferation in the eye of *Xenopus* embryos during retinogenesis. These results indicate that miR-124a acts as a post-transcriptional regulator in the genetic network controlling eye morphogenesis and neurogenesis. The mechanism of miR-124a's early interaction with the genetic network may also persist in its later role in the maturing and adult eye and brain.

Keywords

miR-124a; Eye; *Lhx2*; Morphogenesis; Cell proliferation; *Xenopus laevis*

1. Introduction

MicroRNAs (miRNAs) are recently identified small non-coding RNA molecules which regulate gene expression at the post-transcriptional level by either repressing translation or promoting mRNA degradation (Lewis et al., 2003; Eulalio et al., 2007; Hofacker, 2007). Thousands of miRNAs have been identified in vertebrate genomes and they have been estimated to regulate up to 30% of the genes in the genome (Wienholds and Plasterk, 2005; Lewis et al., 2005). Many mammalian miRNAs are highly or specifically expressed in neural tissues and approximately 70% of experimentally detectable miRNAs are expressed in the brain (Cao et al., 2006), suggesting that miRNAs play important roles in neural development and regulation of the adult nervous system. However, the function of most miRNAs remains unclear, and an extensive analysis is necessary to reveal their precise roles *in vivo*.

To reveal the role of miRNAs in the central nervous system (CNS), especially in the eye, we carried out a microarray screening for miRNAs expressed in the eye using retinal small RNAs isolated from adult mice, rats and zebrafish. miR-124a is one of the miRNAs identified in retinas from all three species (unpublished data). In a further screening using a functional assay in *Xenopus laevis*, we found that microinjection of miR-124a precursors in the anterior part of the *Xenopus* embryo led to eye anomalies (unpublished data), suggesting that miR-124a plays an important role in eye development.

miR-124a is a group of highly conserved microRNAs abundant in the CNS including the eye (Deo et al., 2006; Darnell et al., 2006; Wienholds et al., 2005; Kloosterman et al., 2006; Sweetman et al., 2006). *In situ* hybridization with a miR-124a probe on coronal sections of the adult mouse brain shows that miR-124a is expressed throughout most parts of the brain, including the cerebral cortex and hippocampus. However, its signal is absent from the white matter and appears to localize primarily to the cytoplasm (Deo et al., 2006). In the chick, miR-124a is also expressed strongly in the brain, especially in the hindbrain, midbrain, lateral regions of the spinal cord and the pituitary rudiment (Darnell et al., 2006; Sweetman et al., 2006). miR-124a has also been shown to be expressed in the eye. It is detected strongly in most cells in the neural retina but not in the pigmented epithelium (RPE) (Deo et al., 2006). Northern blotting has demonstrated that miR-124a is also expressed in the mouse lens (Frederikse et al., 2006). Another study of embryonic development using Northern blotting showed that miR-124a expression emerges at the end of the neurula (after stage 18) and remains continuously detectable till the tadpole stage (stage 42) in *X. laevis* (Watanabe et al., 2005). Furthermore, its expression level increases as embryos develop into later stages (Krichevsky et al., 2003; Miska et al., 2004). However, a detailed report of the location of miR-124a in the CNS, especially in the eye, during development is still unavailable.

The prevalence of miR-124a expression in the developing and adult CNS suggests that miR-124a plays a pivotal role in the CNS and neurogenesis. Introducing miR-124 into a human cell line causes the expression profile to shift towards that of the brain (Lim et al., 2005), and its overexpression together with that of miR-9 in neural progenitors prevents gliogenesis (Krichevsky et al., 2006). RE1 silencing transcription factor (REST), a transcriptional repressor, inhibits the expression of neuronal genes and miR-124a in non-

neuronal cells, allowing the persistence of non-neuronal transcripts (Conaco et al., 2006). On the other hand, *REST* and its cofactor complex are also targets of miR-124a, suggesting a double-negative feedback loop between *REST* and miR-124a in stabilizing and maintaining neuronal gene expression (Wu and Xie, 2006). In addition to *REST*, small C-terminal domain phosphatase 1 (*SCP1*) is another anti-neural factor expressed in non-neuronal tissues, which is reported to be an miR-124 target during neurogenesis of the developing chick neural tube (Visvanathan et al., 2007). Another recent finding also reports that miR-124 promotes the differentiation of progenitor cells to mature neurons by directly targeting *PTBPI* (*PTB/hnRNP I*) mRNA, which encodes a global repressor of alternative pre-mRNA splicing in non-neuronal cells (Makeyev et al., 2007). However, it has also been reported that neither inhibition nor overexpression of miR-124 alone significantly altered neuronal fate (Cao et al., 2007; Conaco et al., 2006). Therefore, the exact role of miR-124a in neurogenesis remains to be elucidated. Moreover, although a high level of miR-124a expression has been detected in the retina in several species, the role of miR-124a in retina has not yet been revealed.

In this paper, using *X. laevis* as an animal model, we have studied the role of miR-124a in the developing eye and brain. Our results indicate that miR-124a is able to act on the genetic network involved in the early morphogenesis of the eye, and that maintaining a low level of miR-124a at early stages is necessary for proper cell proliferation and eye morphogenesis. We also show that *Lhx2*, a gene reported to be involved in eye development (Porter et al., 1997; Zuber et al., 2003; Ando et al., 2005; Seth et al., 2006), is a potential endogenous target for miR-124a.

2. Results

2.1. miR-124a shows a dynamic expression pattern in the developing and adult eye of *X. laevis*

Sequence analysis of validated and predicted miR-124a precursor and mature molecules of human, mouse, zebrafish, *Xenopus tropicalis* and *X. laevis* showed that the mature sequence of miR-124 is highly conserved between species. Except for miR-124 from *X. laevis*, which has a single nucleotide difference, all the other species share identical mature miR-124 sequences (Fig. S1). To check if its expression is different from that reported for other species (Deo et al., 2006; Frederikse et al., 2006) and to reveal its potential function in the *Xenopus* retina, we examined miR-124a expression in *X. laevis*. Whole mount *in situ* hybridization with a LNA-probe showed that the earliest miR-124a expression was detectable along the entire neural fold including the anterior eye anlagen at around stage 18 (Fig. 1A). During the optic vesicle stage (stage 23) (Fig. 1B), the expression of miR-124a was stronger in the brain than in the optic vesicle and posterior neural tube. Starting from stage 33 (optic cup stage) (Fig. 1C), a high level of miR-124a expression was observed in the anterior CNS which includes the brain, eye and anterior spinal cord, and miR-124a remained highly expressed in the entire CNS from stage 37 (Fig. 1D) to late tadpole stages (Fig. 1E and data not shown). This expression pattern suggests that miR-124a plays a pivotal role in the development of the central nervous system.

Sections of the embryos at eye-level showed that miR-124a was exclusively expressed in the neural retina with almost no signal detected in the most peripheral ciliary marginal zone (CMZ) at all the optic cup stages checked from stages 33 to 46 (Fig. 1H–K, data not shown). From stage 40 (Fig. 1J and K), the region where miR-124a was highly expressed was restricted further to the peripheral retina around the CMZ. In the brain, miR-124a was mainly expressed at the periphery of the olfactory bulb, telencephalon, mesencephalon and rhombencephalon (Fig. 1G and L).

In the adult, miR-124a expression was detectable in the brain and the neural retina. As shown in Fig. 1, miR-124a was highly expressed in the dissected neural retina (Fig. 1F) and in brain regions including the olfactory bulb, dorsal and rostroventral telencephalon, thalamus, hypothalamus and cerebellum (Fig. 1G and data not shown). No signal was detected in the adult retina hybridized with the probe for miR-133a, a negative control which is not expressed in retina (data not shown).

2.2. Down-regulation of miR-124a in the *Xenopus* eye

To investigate the function of miR-124a in the eye, we first used 2'-*O*-methyl antisense oligonucleotides (anti-miR-124a) to block miRNA function. Different doses (0.1–0.5 pmol) of 2'-*O*-methyl anti-miR-124a were injected into one dorsal-animal blastomere at the 8-cell stage to restrict the change in miR-124a level to one side of the dorsal head that included the eye region. Beta-galactosidase (beta-gal) was co-injected as a tracer. An inhibitor of miR-198, which is not expressed in the eye, or a control molecule (Ambion), was injected into the embryo at the same doses as negative controls. The injected embryos were grown to stages 33 or 46 to check the efficiency of inhibition and the phenotype (Fig. 2). We used *in situ* hybridization to assess changes in miR-124a levels in the eye. Results showed that 0.2 pmol 2'-*O*-methyl anti-miR-124a markedly reduced the miR-124a signal in the eye and brain at stage 33 (Fig. 2E), while at stage 46 only a very few embryos showed slight decreases in miR-124a expression in the brain (Fig. 2D). The same dose of miR-198 or a control inhibitor did not affect the expression of miR-124a (Fig. 2I and data not shown), indicating that 2'-*O*-methyl anti-miR-124a could specifically and efficiently reduce or mask endogenous miR-124a during early developmental stages. However, this inhibition of miR-124a did not lead to any obvious morphological changes in the development of *Xenopus* embryos, including the eye (Fig. 2A–C). Further histological analysis using transversal sections of the injected embryos showed no observable differences between the eye structures on the injected and control sides (Fig. 2F–H), as was the case for embryos injected with miR-198 or control inhibitor (data not shown). Embryos injected with either the miR-124a inhibitor or the two controls at a higher dosage showed similar developmental defects, suggesting non-specific phenotypes induced by overdose toxicity.

We then attempted to inhibit miR-124a using LNA-modified antisense oligonucleotides (Cao et al., 2007). Injection of 0.0675 pmol LNA-anti-miR-124a was sufficient to reduce the endogenous miR-124a signal and induce eye defects (size reduction) (Fig. S2). However, these effects were also observed in embryos injected with the control LNA inhibitor of miR-198. Lower dosage of either of the above LNA molecules gave no morphological effects on injected embryos (Fig. S2, and data not shown). These results indicate that the

LNA-anti-miR-124a was not a specific inhibitor for miR-124a under the experimental conditions used here. Further experiments are necessary to develop a more efficient strategy for knocking down miR-124a expression without non-specific toxicity.

2.3. Overexpression of miR-124a leads to dose-dependent eye anomalies

We examined the effects of miR-124a overexpression in *Xenopus* embryos by injecting at the early embryonic stage. As miR-124a expression is low at early stages and becomes high later, miR-124a microinjection would lead to a relative stronger change of miR-124a level at early stages than at later stages therefore should give a stronger overexpression effect at early stages. Precursor molecules of miR-124a (pre-miR-124a) were injected into one dorsal-animal blastomere at the 8-cell stage and the injected embryos were grown up to stage 46 for morphological analysis. Microinjection of 0.025 pmol pre-miR-124a induced eye anomalies, especially defects of the optic nerve (Fig. 3A). As shown in Table 1, approximately half (54%) of the injected embryos showed enlarged and pigmented optic nerves on the injected side, and these eyes were located closer to the midline of the body than those on the control side. With a lower dose of pre-miR-124a (0.0125 pmol), markedly fewer (14%) injected embryos showed abnormal optic nerves, while with a higher dosage (0.05 pmol), the proportion of *Xenopus* embryos with this phenotype increased to 73%, indicating that the eye defect induced by miR-124a overexpression was dose-dependent. In addition, when 0.025 pmol pre-miR-124a was co-injected with the same dose of 2'-O-methyl anti-124a, the percentage of embryos with optic stalk anomalies was reduced from 54% to 0%, while the inhibitor alone did not affect normal eye morphogenesis (Table 1). Our results indicate that eye anomalies induced by miR-124a precursor molecules could be rescued by its inhibitor. Embryos microinjected with precursor molecules of the control miRNA, miR-198 (pre-miR-198), or another negative control (pre-control) from Ambion did not cause any morphological defects at the above doses (Fig. 3A and data not shown). These results demonstrate that miR-124a overexpression specifically induced malformation of the eye.

To further characterize the eye defects induced by miR-124a overexpression, the injected embryos were sectioned at eye-level. As shown in Fig. 3B, primary eye morphogenesis with a distinct lens, retina and optic nerve was already complete on the control side. Retina cells were well organized from proximal to distal as the retinal pigment epithelium (RPE), the outer nuclear layer (ONL), the inner nuclear layer (INL) and the retinal ganglion cells (RGC). On the side injected with pre-miR-124a, the eye still kept the primary structures of the RPE, neural retina and lens, but the RPE and neural retina expanded into the prospective optic nerve region and formed a significantly larger and shorter optic stalk-like structure. Moreover, sequential transversal sections showed that this enlarged 'optic stalk' was located more anterior to the optic cup, but not at the middle-level of the eye as on the control side (Fig. 3B, indicated by pink arrows). The distal retinas in the reduced optic cup, which showed disorganization of cell layers in the neural retina and a significant reduction of distal cell layers (including INL and RGC), were also less invaginated than controls (Fig. 3B, red arrowhead). In more severely affected embryos, distal retinas were less or even not invaginated, and lens formation was also inhibited in uninvasinated retinas (data not shown). These defects in optic nerve and retina formation suggest that excess miR-124a

expression during the early embryonic stage disturbs eye morphogenesis, including the formation of the optic vesicle and cup, which in turn affects subsequent lens formation and layer organization in the neural retina.

As defects in the division of the optic stalk and retina and the subsequent formation of the optic cup and lens suggest the disturbance of proximodistal patterning of the eye, we examined expression of genetic markers for the proximal and distal eye to further characterize the timing and properties of the effects induced by miR-124a overexpression. *Pax2* is a commonly used marker of the optic stalk and fissure, while *Rx1* and *Pax6* mark the developing retina which is distally located to the optic stalk (Liu et al., 2001; Lupo et al., 2005). When retina layers are formed, the expression domains of *Rx1* and *Pax6* become restricted to the proximal (ONL, outer part of INL) and distal layers (inner part of INL and RGC), respectively (Zuber et al., 2003; Wang and Harris, 2005). The expression of these genes was detected in 0.05 pmol pre-miR-124a-injected embryos at stages 13, 20 and 33, corresponding to early eye-field specification, optic vesicle and optic cup stages, respectively (Fig. 3C, Table S1). At stage 13, when miR-124a expression was not yet detectable, *Pax2* was not clearly expressed in the eye field. The expression of *Rx1* and *Pax6*, which were localized in the eye field at this stage, were not markedly changed in most of the injected embryos, indicating that excess miR-124a does not disturb eye-field specification. At stage 20 when miR-124a starts to be expressed in the entire neural tube (including the eye anlagen), the size of the *Pax2* expression domain in the eye, including the ventral protruding optic vesicle, was not obviously affected, while distinct reduction was observed in the *Rx1*- and *Pax6*-marked retina domains in around 90% of the embryos. Moreover, the expression of *Pax2* (in 60% embryos) and *Pax6* in the presumptive eye and brain was more posterior on the injected side than on the control side. Therefore, miR-124a overexpression represses the extension of the neural tube, especially the protrusion of the optic vesicle. The repression of extension might become more evident in structures anterior than posterior to the eye during later development, which in the end led to a relative anterior 'shift' of the optic stalk observed in the miR-124 injected side at stage 46. At stage 33 when miR-124a was strongly expressed in both the eye and the brain, the expression of *Pax2* in the optic stalk and fissure was still not markedly changed on the injected side, while the *Rx1* and *Pax6* domains that mark the retina region were still reduced in around 80% of embryos. Moreover, the expression domain of *Pax6*, which is normally restricted to the most distal retina, was more strongly reduced than that of *Rx1*. These effects are consistent with results from the histological analyses on retinal sections discussed above, and indicate that the inner cell layers of the distal retina are more severely affected than the outer layers. The lack of effect on *Pax2* expression suggests that the optic fissure may have expanded with the abnormal retina into the optic stalk region. As *Pax2* is also expressed in the optical fissure, the lack of effect of miR-124a on *Pax2* expression observed here may be due to a masking effect of expression in the optical fissure.

2.4. miR-124a overexpression changes expression patterns of candidate target genes in the eye

In order to identify the target(s) of miR-124a in the eye, we screened the candidate target genes predicted by MiRanda (John et al., 2004), miRBase (Griffiths-Jones et al., 2006),

Target-Scan (Lewis et al., 2005) and PicTar (Krek et al., 2005) in vertebrates. Genes involved in eye development and neurogenesis were selected and used to search for potential target sites in the 3' UTR of *X. laevis* transcripts by RNAhybrid with a requirement of 7-nt match to the seed of miRNA (position 2–8) (Lewis et al., 2003; Xie et al., 2005). To increase the reliability of prediction results, at least one perfect (no U:G pair) 6-nt match to position 2–7 of miRNA seed was also required for transcripts considered to be potential targets here (Rehmsmeier et al., 2004; Lewis et al., 2005). Our results of online searching showed that *Lhx2*, *Gli3*, *Hairy2*, *Otx2* and *NeuroD1* are predicted targets of miR-124a in mammal and *X. tropicalis*. Moreover, mRNA transcripts of *X. laevis Lhx2*, *Gli3* and *Hairy2* also contained potential target sites for miR-124a as predicted by RNAhybrid (Table S2). In particular, one conserved candidate binding site of miR-124a was found in the 3' UTR of human, mouse, rat, dog and *X. laevis Lhx2* (Fig. 4A).

To test whether expression of these five candidate genes could be affected by miR-124a overexpression at the mRNA level, we performed *in situ* hybridization at the stages indicated above following injection of miR-124a precursor molecules (0.05 pmol) (Fig. 4B and Table S1). At stage 13, expression of all the candidate genes on the control side became clear in or around the eye field located in the anterior neural plate, except that *NeuroD1* was not yet expressed. On the injected side, *Lhx2* and *Otx2* were down-regulated in 82% and 57% of embryos, but miR-124a overexpression did not affect the expression of *Hairy2* and *Gli3* at stage 13. At the neural tube stage (stage 20) when endogenous miR-124a and the transcripts of all five genes were detectable in or around the normal primary optic vesicle, the expression domains of these candidate miR-124a targets were all reduced in more than half of the injected embryos. In addition, the expression of *Lhx2* and *Hairy2* was also reduced in the arch-encephalon. However, changes in expression of all the candidates at this stage tended to be restricted to specific regions instead of an overall reduction of the expression level throughout the miR-124a-overexpressed region traced by beta-gal staining. At the optic cup stage (stage 33), the reduction in expression of *Lhx2*, *Hairy2*, *NeuroD1* and *Otx2* in the eye was still observed in more than 70% of the embryos, whereas it was difficult to distinguish the change in *Gli3* expression between the injected and control sides due to its low expression in the normal eye. In addition, the expression domain of *Otx2* in the eye, which will represent the distal central retina later (Wang and Harris, 2005), was dramatically reduced on the injected side, indicating an inhibition of distal retina formation coincident with the results of the above analysis at both the histological and molecular levels. The expression of *Lhx2* and *Hairy2* in the brain was also significantly reduced or distorted, which is consistent with the decrease of their expression in the arch-encephalon induced by miR-124a overexpression at st.20.

The above results indicate that miR-124a overexpression leads to dynamic changes in down-regulation of the expression of these five candidate genes. The expression of these genes is significantly inhibited at stage 20, when endogenous miR-124a expression starts. These observations suggest that miR-124a is able to regulate the genetic network involved in eye development. Moreover, *Lhx2* and *Otx2* mRNAs are negatively regulated by miR-124a even at stage 13, and this early effect indicates inhibition by the degradation of these two mRNAs even though the target site of *Otx2* was not predicted in the reported 3' UTR of *Xenopus*

transcripts. The temporal and spatial restriction of the effects on other candidate target genes also implies that miR-124a may act alongside other mechanisms to regulate the expression of these genes.

2.5. miR-124a targets the 3' UTR of *Lhx2* mRNA

To determine if miR-124a can act directly on the predicted targets discussed above, we performed a luciferase reporter assay using the *Xenopus* genes *Lhx2* and *Gli3*, the two with the best seed match (Table S2, perfect 2–8 seed match). The 3' UTRs of these two genes containing the predicted miR-124a binding sites were separately fused to the luciferase coding region. The 3' UTR of *Pax6*, which does not contain perfect seed match and is not predicted to be targeted by miR-124a (Table S2), was fused to the luciferase reporter gene as a negative control and the antisense sequence of miR-124a (anti-124a) was used as a positive control. As shown in Fig. 4C, transfection of control miRNA precursor did not reduce the luciferase activity of any of the four constructs. When the miR-124a precursor was introduced, the luciferase activity of the positive control (anti-124a) and *Lhx2* reporters was down-regulated to 4% and 43%, respectively. Expression of the *Gli3* reporter and the negative control (*Pax6*), on the other hand, was not significantly changed. These results demonstrate that miR-124a can interact with the 3' UTR of *Lhx2* and downregulate its expression.

To explore the functional relationship between miR-124a and *Lhx2*, we compared their expression patterns in *Xenopus* embryos at different stages by *in situ* hybridization. As shown in Fig. 4D, while miR-124a showed low expression at early stages and high expression at late stages in the eye, *Lhx2* showed almost the reverse expression pattern during eye development. At stage 13 before miR-124a was expressed, *Lhx2* mRNA was clearly detected in the eye field. At stage 20 when the miR-124a signal became detectable in the eye anlagen, *Lhx2* expression appeared to be lower in the overlapping ventrodorsal region. At stage 33 when the miR-124a signal became strong in the central retina, *Lhx2* expression was more restricted and exhibited a low level of expression in this location. On the other hand, in the peripheral CMZ, where miR-124a was absent, *Lhx2* was expressed at a higher level. These temporal and spatial complementary expression patterns of *Lhx2* and miR-124a suggest that *Lhx2* could be an endogenous target of miR-124a during eye development, and that the expression of miR-124a leads to degradation of *Lhx2* transcripts.

2.6. miR-124a overexpression inhibits cell proliferation during retinogenesis

It has been shown that overexpression of *Lhx2* in the head results in increased cell proliferation, and that knockdown of *Lhx2* leads to deficient cellular proliferation resulting in retarded development of the eye and brain (Ando et al., 2005; Monuki et al., 2001). It is interesting therefore to see whether miR-124a overexpression affects cell proliferation in the eye. We carried out BrdU incorporation analysis at stage 33 of retinogenesis in *Xenopus* embryos injected with 0.025 pmol pre-miR-124a or pre-control, and found a significant decrease in the number of dividing (BrdU-positive) cells on the injected side of the embryo compared with the control side (Fig. 5). As shown in Fig. 5B, the average ratio of proliferating cells in all retina cells (Hoechst stained) of embryos injected with pre-miR-124a, was $49.1 \pm 2.1\%$ (mean \pm SE) in the uninjected control side at stage 33, while

that in the injected side decreased significantly to $15.5 \pm 1.3\%$. In embryos injected with the control precursor, however, the average ratio of BrdU-positive cells in the injected retina was $44.4 \pm 2.0\%$, not significantly different from that ($43.8 \pm 2.6\%$) in the control side. These results indicate that excessive miR-124a inhibits retina cell proliferation in *Xenopus* embryos, and suggests a role for miR-124a in controlling the proliferation of retinal progenitors during eye development.

3. Discussion

3.1. The role of miR-124a in *Xenopus* eye development

In this paper, we have used the *Xenopus* eye as a model system to study the role of miR-124a. Although there have been reports that miR-124a is expressed in the adult brain and eye in several species including *X. tropicalis* (Deo et al., 2006; Darnell et al., 2006; Wienholds et al., 2005; Kloosterman et al., 2006; Sweetman et al., 2006), our functional analysis data provides the first evidence that confirms the involvement of miR-124a in eye development and identifies its potential targets in *X. laevis*.

The role of miR-124a in the eye was first revealed by an expression analysis of miR-124a. *In situ* hybridization experiments showed that the expression pattern of miR-124a is dynamic throughout the developing eye and brain, beginning at a low level along the entire neural fold at around mid-neurula and increasing to a high level in the mature brain and eye. The onset of miR-124a expression observed here and its location in the mature CNS (including the eye) is similar to previous results obtained by other methods or in other species (Aboobaker et al., 2005; Darnell et al., 2006; Kloosterman et al., 2006; Watanabe et al., 2005; Wienholds et al., 2005). The presence of miR-124a in the developing neural tube and eye suggests it has an early role in the CNS development of *X. laevis*. This hypothesis is also supported by our observation that early introduction of excess miR-124a leads to malformation of the optic nerve and retina. Moreover, these eye defects are related to an early reduction of the eye anlagen and a later repression of the distal structures of the eye, as shown by the effects of a set of eye markers on expression and reduction of retinal cell proliferation during retinogenesis. These results indicate that miR-124a is able to act on the genetic network involved in the early morphogenesis of the eye, and that maintaining a low level of miR-124a at early stages is necessary for proper cell proliferation and eye formation.

In this work, the loss-of-function analysis showed no significant disturbance of eye morphogenesis, though endogenous miR-124a had clearly been inhibited at least until the early optic cup stage. This result is consistent with two previous reports: miR-124a knockdown does not have any significant effect on differentiating neural precursors (Krichevsky et al., 2006) and does not affect neuronal differentiation in the developing chick neural tube (Cao et al., 2007). It has been reported that less than 10% of individual miRNA knockouts result in clear developmental or morphological defects in *Caenorhabditis elegans* (Miska et al., 2007), and most miRNAs might act redundantly with other miRNAs as fine-tuning regulators (Cao et al., 2007; Baroukh et al., 2007). Our results support the hypothesis that miR-124a might not act as a crucial regulator of early developmental events, and it will

be interesting to determine if some fine structures or characteristics of the eye are altered by miR-124a down-regulation.

Although our results tend to indicate that miR-124a may not be necessary for early eye development in *Xenopus*, we cannot rule out the possibility that the early expression of miR-124a is not inhibited completely by the miR-124a inhibitor and that only a small amount of miR-124a is enough to maintain normal function *in vivo*. Furthermore, our loss-of-function approach using antisense oligonucleotides did not generate significant knockdown effects at the late embryonic stage (Fig. 2), so we cannot exclude the possibility that miR-124a might be necessary during late eye development in *Xenopus*. Our results of both gain- and loss-of-function analyses also reveal that early microinjection of miRNA precursors or inhibitors is efficient for early but not for late disturbance of miR-124a levels, which are present at low levels during early stages and at higher levels during later stages of development.

The LNA-modified miRNA inhibitor showed a strong non-specific effect in this study, in contrast to previously reported experiments (Cao et al., 2007; Visvanathan et al., 2007; Corsten et al., 2007; Elmén et al., 2008). This might be due to the lower incubating temperature (22 °C) used for growth of *Xenopus* embryos than that (no lower than 37 °C) for chick and mouse embryos, or for cultured cells. In our experience with *in situ* hybridization, an appropriate hybridization temperature is critical for the specific binding of the antisense LNA probe to its target. In a similar way, the interaction of antisense LNA inhibitors and endogenous miRNAs may be sensitive to temperature fluctuations. A more effective inhibitor and a better approach for disturbing the strong expression of miR-124a at late developmental stages are needed to better characterize miR-124a function in *X. laevis*.

3.2. The mechanism of the role of miR-124a in eye development

The effects of miR-124a overexpression suggest a critical role for its target(s) in retinogenesis and eye morphogenesis, especially in the events involved in protrusion of the optic vesicle and in the formation of the optic stalk and optic cup. Results of computer predictions imply that the mechanism underlying the role of miR-124a in the eye involves multiple targets. Our results for miR-124a target identification show that the 3' UTR of *Lhx2* could be directly targeted by miR-124a *in vitro*, and that its expression *in vivo* was downregulated by miR-124a overexpression. Moreover, the location of *Lhx2* transcripts and miR-124a in the eye are somewhat complementary throughout early eye development, and early effects of miR-124a overexpression down regulates *Lhx2* RNA level at stage 13. These results suggest that *Lhx2* could be an endogenous target for miR-124a in the eye, and down-regulation of the expression of *Lhx2* by miR-124a might be due to, or at least include, mRNA degradation. It has been reported that in the *Lhx2* knockout mouse, eye development arrests prior to formation of an optic cup although specification of the optic vesicle occurs (Porter et al., 1997). In Zebrafish, *bel* (*Lhx2*) mutant eyes are shorter in the dorsoventral axis and wider in the mediolateral axis than wild-type eyes (Seth et al., 2006). In addition, *Lhx2* can regulate eye size by regulating cell proliferation in zebrafish embryos (Ando et al., 2005). These results are consistent with the effects of miR-124a overexpression in *Xenopus*, implying that down-regulation of *Lhx2* by miR-124a is involved in malformation of the eye.

For the other predicted targets, the 3' UTR of *Gli3* did not show significant interaction with miR-124a to downregulate gene expression *in vitro*. *Hairy2*, *Otx2* and *NeuroD* in *X. laevis* either contain weaker or no potential target sites within their reported 3' UTR sequences (Table S1), making it less likely for them to be inhibited by miR-124a in this region. However, the effect of miR-124a overexpression on *Otx2* expression was similar to that on *Lhx2* expression and even stronger at stage 33 (Fig. 4). It is possible that some target sites have not been identified by the target prediction algorithms (including the transcript identification) used here, or that the analyzed transcript do not include all the 3' regulatory regions of these genes, or that miR-124a might interact with other regions of these genes to exert its regulatory role (Lytle et al., 2007; Duursma et al., 2008). In addition, as miRNAs can mediate down-regulation of target gene activity by either translational inhibition or target mRNA cleavage, we cannot exclude the possibility that miR-124a repressed their translation. Other factors may also interact with miR-124a to regulate the expression of these genes in restricted regions during development. Furthermore, as *Otx2*, *Pax6* and *Lhx2* are present in a genetic network controlling eye-field specification (Zuber et al., 2003), cross regulation might also be involved in the change of their expression profiles. Extensive rescue experiments might be needed to specify the key factors responsible for the phenotype induced by miR-124a overexpression.

Some other genes have been reported to be targeted by miR-124, such as the anti-neural factors *REST* (Conaco et al., 2006) and *SCP1* (Wu and Xie, 2006), neural progenitor enriched *laminin_1* and *integrin_1* (Cao et al., 2007) in the spinal cord, and a pancreatic-related *Foxa2* (Baroukh et al., 2007). Since the roles of these targets of miR-124 in the eye are still not clear, it will be interesting to determine whether these genes are involved in the malformation of the eye by miR-124a overexpression in *Xenopus*. These reports also suggest that miR-124a represses both non-neuronal and neuronal progenitor fates, and hence helps to induce neurogenesis/differentiation and maintain neuronal fate in the CNS. In addition to *Lhx2*, *NeuroD1* and *Hairy2a*, which are involved in neurogenesis, were predicted to be targets of miR-124a and the expression of these two genes was down-regulated by miR-124a overexpression. *NeuroD1* is expressed in the primary neuron and promotes its differentiation (Cho and Tsai, 2004), and *Hairy2a* has been shown to repress the expression of pro-neuronal genes in non-neuronal cells surrounding developing neurons and play a role similar to *REST* and *SCP1* (Klose and Bird, 2003). Our BrdU analysis results show that miR-124a overexpression inhibited cell proliferation in the eye during retinogenesis, which is consistent with the loss-of-function effects of *Lhx2* and *Hairy2a* (Ando et al., 2005; Monuki et al., 2001; Nichane et al., 2008). In addition, as *Lhx2*, *Gli3*, *Otx2* and *NeuroD1* are also involved in regulation of retinal cell fate (Vicizian et al., 2006; Perron et al., 2003; Wang and Harris, 2005), miR-124a may be involved in retinal cell determination by its interaction with these genes. The above evidence suggests that miR-124a might also play an important role in neurogenesis during eye development. Moreover, miR-124a might act in a similar manner in other parts of the central nervous system. Further investigation is needed to confirm these hypotheses and a more extensive screening is also necessary to identify other possible targets of miR-124a in the eye.

In summary, we have provided evidence that miR-124a antagonizes *Lhx2* and is involved in the early development of the vertebrate eye. Early overexpression of miR-124a causes decreased cell proliferation during retinogenesis and malformation of *Xenopus* eyes. The mechanism by which miR-124a interacts with the genetic network at early stages of development may persist through later development into adult stages, and may be involved in other parts of the central nervous system.

4. Experimental procedures

4.1. LNA-modified probe preparation

The sequence of the LNA probe for mature miR-124a was 5'-TGGCATTACCGCGTGCCTTAA-3'. Probes were synthesized using EXIQON (miRCURY™ detection probe) and labeled with a DIG oligonucleotide 3'-end labeling kit (Roche), then purified with Sephadex G25 MicroSpin columns (Amersham Biosciences). Approximately 100 ng/ml of labeled probe was used in the *in situ* hybridization experiments (Wienholds et al., 2005; Kloosterman et al., 2006).

4.2. In situ hybridization

Embryos were obtained and staged as previously described (Nieuwkoop and Faber, 1967). *In situ* hybridization of the transcription factors *Otx2*, *Rx1*, *Pax6*, *Pax2*, *Gli3*, *NeuroD1*, *Hairy2* and *Lhx2* was performed on whole embryos of *Xenopus* as previously described (Harland, 1991), except for the following modifications: embryos which were pre-stage 33, those from stage 33 to stage 46, and dissected adult brain and eye, were treated with proteinase K for 10, 45, 60 min, respectively. The temperature of hybridization and subsequent washing steps was adjusted to approximately 22 °C below the predicted melting temperatures of the LNA-modified probes. In addition, a digoxigenin (DIG)-labeled oligonucleotide corresponding to the lacZ' region in the pUC and M13 plasmids (30-mer, 5'-p TTGGGTAACGCCAGGGTTTTCCAGTCACG OH-3') was used as a negative control.

4.3. Microinjection

Both LNA antisense DNA and 2'-*O*-methyl antisense RNA oligonucleotides were used for loss-of-function analysis. The sequences were designed to target the conserved mature sequence of miR-124a (5'-UAAGGCACGCGGUGAAUGCC-3') or that of a control miRNA, miR-198 (5'-GGUCCAGAGGGGA GAUAGG-3'). The LNA antisense DNA was obtained from the same sources as the probes mentioned above. The 2'-*O*-methyl antisense RNA (Anti-miR™ miRNA Inhibitor) and the other negative control for the 2'-*O*-methyl oligonucleotide were obtained from Ambion (Cat. Nos. 17000 and 17010, respectively).

Pre-mmu-miR-124a-2 shares higher sequence identity than other miR-124a homologues to the reported *Xenopus* miR-124a (Xtr-miR-124) (Fig. S1), so the synthetic Pre-miR™ miRNA precursor molecule for pre-mmu-miR-124a-2 (Ambion Cat. No. 17110) was used for overexpression analysis. The Pre-miR™ miRNA precursor molecule for hsa-miR-198 (Ambion Cat. No. 17100) and a control sequence (Ambion Cat. No. 17110) were used as negative controls.

In both analyses, embryos were co-injected with 100–500 pg beta-gal or 200–400 pg GFP mRNA as a lineage tracer; and embryos injected with beta-gal were stained as previously described (Andreazzoli et al., 1999). Capped mRNAs were synthesized from linearized plasmid templates using mMESSAGE mMACHINE kits (Ambion). Oligonucleotides or mRNAs were injected into one *Xenopus* dorsal-animal blastomere at the 8-cell stage using an Eppendorf FemtoJet (Hamburg, Germany), and embryos were then cultured as previously described (Lupo et al., 2005).

4.4. Bromodeoxyuridine (BrdU) administration

BrdU (Sigma B9285) was administered to *X. laevis* embryos injected with 0.025 pmol pre-miR-124a or pre-control molecules, by immersion in a solution of 10 mM BrdU in phosphate buffered saline (PBS) for 30–60 min at room temperature (19–22 °C) as described by Quick and Serrano (2007). Three hours after exposure to BrdU, embryos were grown to the optic cup stage (stage 33) and stored in 20% sucrose after being treated with 4% para formaldehyde (PFA) (Quick and Serrano, 2007; Zuber et al., 1999). For sectioning analysis, samples were embedded in OTC, serially sectioned at a thickness of 12 µm and stained immunohistochemically using mouse anti-BrdU monoclonal antibodies. Hoechst33258 (Sigma) was used for counter-staining of all cell nuclei.

Counts of BrdU-positive and Hoechst-labeled cells in the optic cup were obtained from twelve sections of four injected embryos by tracing of digitized images projected on a computer monitor. The ratio of proliferating cells in the eye was calculated by dividing the number of BrdU-positive cells by all the cells counted (Hoechst-labeled cells) in the eye on either the injected side or the control side. Statistical analysis was performed using one-way ANOVA followed by the Duncan test. Comparisons of means of proliferating cell ratios were made between the uninjected and injected eye of either pre-miR-124a or pre-control injected embryos. Differences between groups were considered significant when $P < 0.05$. At least three independent experiments were performed.

4.5. Histology and imaging

The injected and control embryos for phenotype analysis were grown to late tadpole stage (about stage 46) and stored in 100% ethanol after being fixed in 4% formaldehyde in MEM (MOPS/EGTA/magnesium sulfate phosphate buffer) (Harland, 1991). For sectioning, samples were embedded in paraffin, serially sectioned at a thickness of 5 µm for histological analysis of the overexpression phenotype, or 15 µm for expression analysis, and then stained with hematoxylin–eosin solution.

Images were taken using an OLYMPUS SZX12 (Japan) stereomicroscope with a digital acquisition system (Olympus C4040). Sections were photographed on an inverted microscope (OLYMPUS IX71, Japan) or a compound microscope (Nikon FXA, Japan) using DIC optics or fluorescent filters. The fluorescent images taken with different filters were merged by Adobe Photoshop CS.

4.6. Luciferase assays

The firefly luciferase reporter genes were constructed using the pCS2-Luc vector and the 3' UTR sequences of *Xenopus Lhx2*, *Gli3* and *Pax6*, following PCR amplification using the primers listed in Table S3. The reporter construct of *Pax6* and that with the antisense sequence of mature miR-124a were constructed as negative and positive controls, respectively. HEK293 cells were cultured in DMEM supplemented with 10% fetal bovine serum. $5 \cdot 10^4$ cells/well were seeded in 24-well plates. After 24 h in culture, the cells were transfected using Lipofectamine 2000 (Invitrogen) with a mixture containing 1 μ g/ml of firefly luciferase reporter plasmid, 20 nM miR-124a or control precursor, and 20 ng/ml of *Renilla reniformis* luciferase encoding plasmid (pRL-TK, Promega). Cells transfected without precursor served as a control for normalization. Luciferase activity was measured 24–48 h post-transfection using a Dual-luciferase assay system (Promega). All transfections were repeated independently at least three times.

4.7. Informatics

Mouse, human, zebrafish and *X. tropicalis* miRNA precursor and mature sequences were downloaded from the miRNA Registry database (<http://www.sanger.ac.uk/Software/Rfam/mirna/>). The *X. laevis* sequence was obtained from Watanabe et al. (2005). The mRNA sequences of *Lhx2* (AY141037), *Gli3* (NM_001087971), *Hairy2* (AF139914), *Otx2* (NM_001090691, U19813), *NeuroD* (U28067) and *Pax6* (U77532) were obtained from the NCBI nucleotide database.

The predicted binding sites of miR-124a in transcripts of *X. laevis* were analyzed by RNAhybrid (Rehmsmeier et al., 2004) using the mature sequence of *X. laevis* miR-124a. RNAhybrid was run with either perfect (no U:G in seed) or imperfect (U:G allowed in seed) seed match; helix constraint in seed was set either from position 2 to 7 or 2 to 8 of the miRNA sequence. miR-124a target candidates in other species were searched from the online sources of MiRanda (www.microrna.org), mirBase (microrna.sanger.ac.uk/targets/v5/), Target-Scan (www.targetscan.org/vert_50/) and PicTar (www.pictar.org).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.mod.2009.08.002.

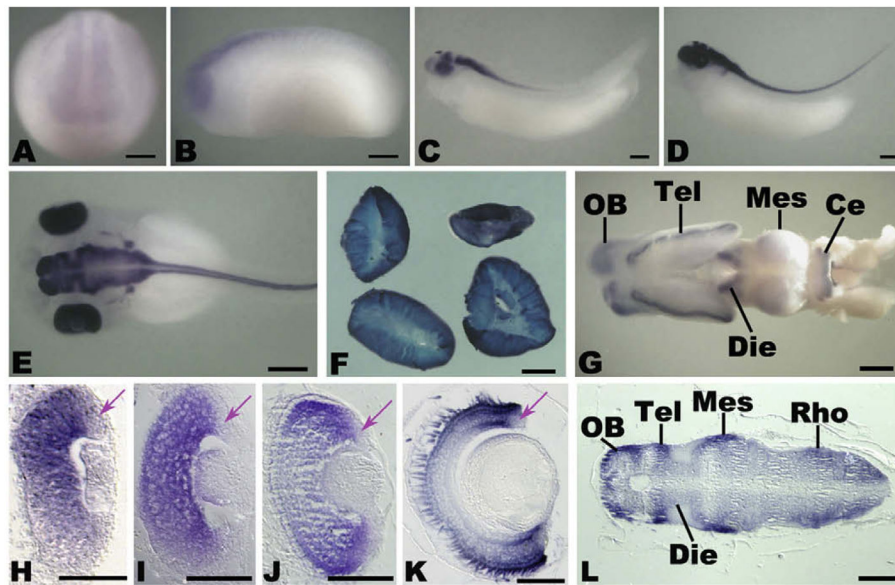


Fig. 1. Expression pattern of miR-124a during the development of *Xenopus laevis*. (A–G) Expression of miR-124a (dark blue) as detected by whole mount *in situ* hybridization in *Xenopus* embryos at stage 18 (A), stage 23 (B), stage 33 (C), stage 37 (D), stage 46 (E), and dissected adult retinas (F) and brain (G), shown from the anterior (A), lateral (B–D), or dorsal views (E and G). (H–L) Restricted location of miR-124a in the eye (H–K) and brain (L) is shown on transversal (H–K) or coronal sections (L) at stage 33 (H), stage 37 (I), stage 40 (J), and stage 46 (K and L). The pink arrow indicates the most peripheral part of the dorsal ciliary marginal zone. Scale bars: 300 μ m (A–E); 1mm (F and G); 100 μ m (H–L). Ce, cerebellum; Die, diencephalon; Mes, mesencephalon; OB, olfactory bulb; Rho, rhombencephalon; Tel, telencephalon.

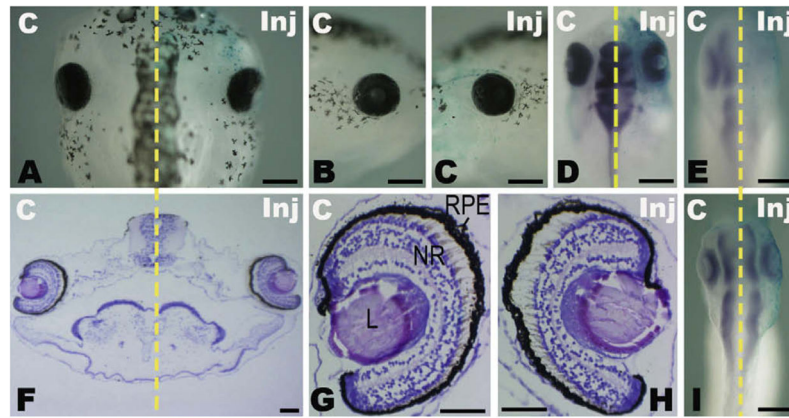


Fig. 2.

Down-regulation of miR-124a shows no obvious effect on the morphogenesis of the *Xenopus* eye. Embryos were unilaterally injected with 0.2 pmol 2'-*O*-methyl antisense oligonucleotides against miR-124a (A–H) or a control miRNA, miR-198 (I). (A–C) The eyes/head of an injected *Xenopus* embryo at stage 46 shown from the dorsal (A) or lateral (B and C) view. (D and E) The expression of miR-124a (dark blue) detected by whole mount *in situ* hybridization at stage 46 (D) and stage 33 (E). The eyes/heads of embryos are shown from the dorsal view. (F–H) The structure of the eyes of an injected embryo at stage 46 shown in a transversal section (5 μ m) stained with hematoxylin (blue)–eosin solution (pink). (G) and (H) show the eyes in (F) under a higher magnification. (I) Expression of miR-124a (dark blue) was not affected by the microinjection of miR-198 inhibitor at stage 33. Scale bars: 300 μ m (A–E, I); 100 μ m (F); 50 μ m (G and H). The yellow dashed line indicates the midline of the embryos separating the uninjected control side (C) and the injected side (Inj). The injected side is traced by the light blue staining of beta-galactosidase co-injected with miR-124a or miR-198. L, lens; NR, neural retina; RPE, retina pigmented epithelium.

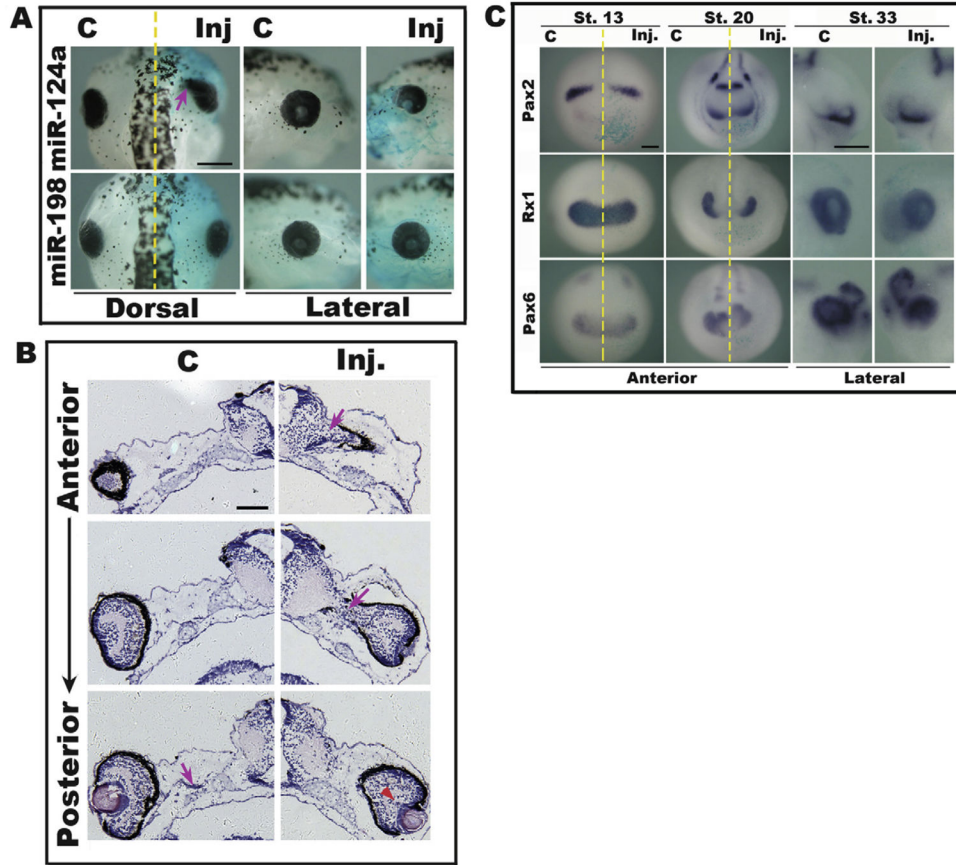


Fig. 3. Effects of miR-124a overexpression on eye development in *Xenopus*. miR-124a or control (miR-198) miRNA precursors (0.025 pmol) were microinjected into one *Xenopus* dorsal-animal blastomere at the 8-cell stage. (A) The head region of the injected *Xenopus* embryo grown to stage 46 is shown from the dorsal or lateral view. The pink arrow marks an enlarged and pigmented optic stalk-like structure induced by miR-124a injection. (B) The structure of wild-type and abnormal eyes of a stage 46 embryo injected by miR-124a is shown on a series of transverse sections (5 μ m) from anterior to posterior. The sections were stained with hematoxylin (blue)–eosin solution (pink). Pink arrows indicate the optic nerve. The red arrowhead shows the reduced retina ganglion layers. (C) The expression (dark blue) of *Pax2*, *Rx1*, and *Pax6* after miR-124a overexpression was detected by whole mount *in situ* hybridization at stages 13, 20, and 33. Stage 13 and 20 embryos are shown from the anterior view, and the eyes of stage 33 embryos are shown from the lateral view. Scale bars: 300 μ m (A and C); 100 μ m (B).

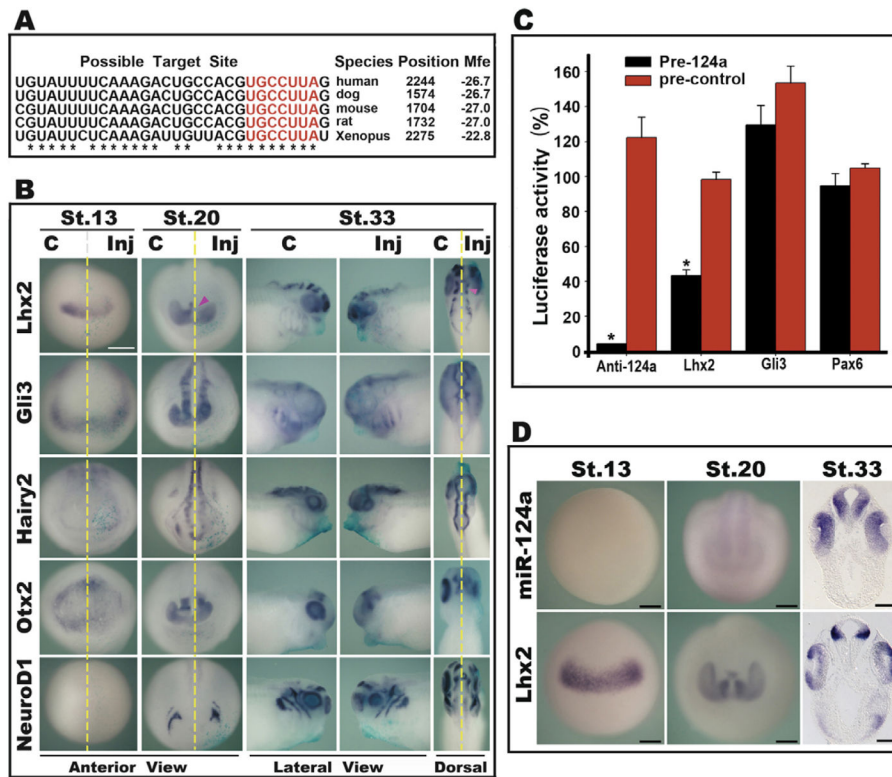


Fig. 4. miR-124a and its candidate targets. (A) An evolutionarily conserved miR-124 target site is found at the 3' UTR of *Lhx2* mRNAs in different species. Seed sequences are marked in red. Homologous sites are marked by asterisks. (B) Expression of the predicted targets of miR-124a: *Lhx2*, *Hairy2*, *Gli3*, *NeuroD1* and *Otx2* were detected by whole mount *in situ* hybridization. Embryos were injected with 0.05 pmol miR-124a precursor molecules into one dorsal-animal blastomere at the 8-cell stage and collected at stage 13 (anterior view), stage 20 (anterior view) or stage 33 (lateral view and dorsal view). The pink arrowhead indicates the reduced expression of *Lhx2* on the injected side. Scale bars: 500 μ m. (C) Luciferase assays were carried out in HEK293 cells using the pCS2-Luc-3' UTR reporters of *Lhx2*, *Gli3*, and the positive (anti-miR-124a) and negative (*Pax6*) controls. Luciferase activity after transfection of the miR-124a precursor (pre-124a) or control precursor (pre-control) was normalized by that of the control cells without precursor transfection. Data are expressed as the means of three independent transfections \pm SD, each carried out in triplicate. Asterisks indicate where the miR-124a transfected group was significantly different from the control precursor group ($P < 0.05$). (D) *In situ* hybridization detection of miR-124a and *Lhx2* expression in *Xenopus* embryos at stage 13 (anterior view), stage 20 (anterior view) and stage 33 (transsection at the eye-level). Scale bars: 300 μ m (stage 13, stage 20); 100 μ m (stage 33).

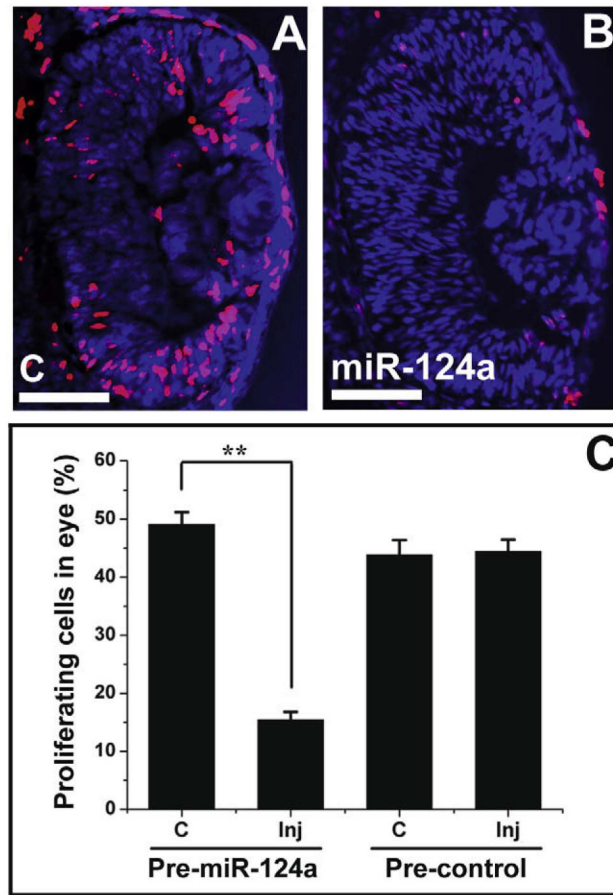


Fig. 5. miR-124a overexpression inhibits cell proliferation during retinogenesis. miR-124a or control miRNA precursor (0.025 pmol) was co-injected with GFP mRNA into one dorsal-animal blastomere of a *Xenopus* embryo at the 8-cell stage, and cell proliferation was detected by BrdU incorporation. (A and B) Transversal section of a stage 33 embryo at the level of the eye showing that the number of proliferating cells (BrdU-positive, red) on the miR-124a overexpressed side (miR-124a) was much lower than that on the control side (C). All cell nuclei were counterstained with Hoechst 33258 (blue). Scale bar: 100 μ m. (C) The ratio of proliferating retinal cells (BrdU-positive/Hoechst-labeled cells) in either the injected side (inj) or the control side (C) of embryos injected with miR-124a (pre-miR-124a) or control (pre-control) precursors. Data are expressed as means \pm SE from twelve sections of four embryos. A significant difference (** $P < 0.01$ by one-way ANOVA followed by Duncan's test) between the mean ratios between the injected and control sides was only detected for pre-miR-124a injected embryos.

Table 1

miR-124a overexpression induced specific optic nerve anomalies with a dose-dependent effect.

RNA injected	Total number of injected embryos	Percentage of embryos with an abnormal optic nerve (%)
0.05 pmol pre-miR-124a	66	73
0.025 pmol pre-miR-124a	63	54
0.0125 pmol pre-miR-124a	59	14
0.025 pmol pre-miR-124a + 0.025 pmol anti-miR-124a	92	0
0.025 pmol anti-miR-124a	75	0
0.025/0.05 pmol control precursor (negative control 1)	50	0
0.025/0.05 pmol pre-miR-198 (negative control 2)	50	0

Abbreviations: pre-miR-124a, precursor molecules of miR-124a; pre-miR-198, precursor molecules of miR-198; anti-miR-124a, 2'-O-methyl antisense oligonucleotides of miR-124a.

Note: Data are representative of three independent experiments.