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MECHANISMS OF DEVELOPMENT

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.

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*.

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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 *PTBP1* (*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