RESEARCH COMMUNICATION

microRNA-24a is required to repress apoptosis in the developing neural retinaJames C. Walker and Richard M. Harland¹

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Programmed cell death is important for the proper development of the retina, and microRNAs (miRNAs) may be critical for its regulation. Here, we report that *miR-24a* is expressed in the neural retina and is required for correct eye morphogenesis in *Xenopus*. Inhibition of *miR-24a* during development causes a reduction in eye size due to a significant increase in apoptosis in the retina, whereas overexpression of *miR-24a* is sufficient to prevent apoptosis. We show that *miR-24a* negatively regulates the proapoptotic factors *caspase9* and *apaf1*, demonstrating a role for miRNAs in the regulation of apoptosis during normal development.

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Programmed cell death, or apoptosis, regulates the size and morphology of developing tissues and organs, and this is particularly true in the developing neural retina. The neural retina gives rise to the photoreceptors, retinal ganglion cells, and the optic nerve, and as many as 90% of newborn retinal ganglion cells die during rat retinal development (de la Rosa and de Pablo 2000; Vecino et al. 2004). In *Xenopus*, spatiotemporal elimination of retinal cells is a key factor in maturation (Gaze and Grant 1992). In the chick, Caspase-dependent apoptosis has been demonstrated in the retina, and inhibition of Caspases results in an enlargement of the ganglion cell layer (Mayordomo et al. 2003). Similarly, in mice, apoptotic factors are highly expressed in the early retina and down-regulated as development proceeds (O'Driscoll et al. 2006; Wallace et al. 2006), and knockdown of Caspases results in an overgrown retina (Hakem et al. 1998). However, the factors important for the regulation of Caspases and other apoptotic factors in the eye are unknown, although some transcriptional regulation has been postulated (Wallace et al. 2006).

microRNAs (miRNAs), a class of regulatory noncoding RNA genes, have evolutionarily conserved roles during the development of many organs, including the heart and nervous system (Zhao et al. 2005; Makeyev et al. 2007). miRNAs are transcribed by RNA Polymerase II as pri-

mary miRNAs (pri-miRNAs). These transcripts are then processed by the enzymes Drosha and Dicer to generate the mature single-stranded miRNA of ~22 nucleotides, which is then incorporated into the RNA-induced silencing complex (RISC), characterized by the presence of the Argonaute family of proteins (Pasquinelli et al. 2005). This complex is responsible for the regulatory function of the miRNAs, leading to translational repression or degradation of target mRNAs.

Several miRNAs have been implicated in the regulation of apoptosis in *Drosophila* (Xu et al. 2004). In various forms of cancer, miR-21 has been shown to be an anti-apoptotic factor (Chan et al. 2005; Cheng et al. 2005) and miR-34 has been shown to be a downstream target of p53 and an inducer of cell death (He et al. 2007). However, knowledge is still lacking about the in vivo roles of most miRNAs during vertebrate development. Recent work in mice has shown that Dicer inactivation, specifically in the retina, results in neuronal degeneration (Damiani et al. 2008). Therefore, miRNAs may be important for the regulation of cell death pathways in the retina during development.

In this study, we show through loss-of-function experiments that *miR-24a* is necessary for proper neural retina development. We further demonstrate that knockdown of *miR-24a* results in an increase in apoptosis, while proliferation, patterning, and differentiation of the eye remain unchanged. We find that loss of *miR-24a* function leads to an increase in Caspase9 protein levels without altering mRNA levels, and that the miRNA knockdown phenotype is dependent on the function of Caspase9. In addition, we establish that *miR-24a* is capable of repressing the proapoptotic factors *apaf1* and *caspase9* by interaction with their 3' untranslated regions (UTRs). Finally, we show that *miR-24a* is able to prevent Caspase-dependent apoptosis when overexpressed. Together these data indicate that *miR-24a* is an essential regulator of *caspase9* and programmed cell death in the developing neural retina.

Results and Discussion*miR-24a is expressed in the developing neural retina*

Previously, we had reported the pri-miRNA expression patterns for several miRNAs during development of *Xenopus tropicalis* (Walker and Harland 2008). Particularly intriguing was expression of *miR-24a*, which appeared in the eye anlage and the posterior mesenchyme during late neurula stages (Fig. 1A). In *Xenopus laevis*, neural retina development begins when the eye anlagen evaginate from the anterior portion of the neural tube and continues as the optic cup invaginates and later differentiates into three nuclear layers (Nieuwkoop and Faber 1994). Further analysis of the expression of *miR-24a* revealed that this miRNA is expressed specifically in the neural retina throughout the development of the eye until at least stage 40 (Fig. 1B–D'; data not shown). Results obtained by in situ hybridization were confirmed by RT-PCR (Fig. 1E).

Knockdown of miR-24a results in reduced eye size

To determine the function of *miR-24a* during development, we used an antisense morpholino oligonucleotide

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Figure 1. *miR-24a* is expressed in the developing neural retina. In situ hybridization shows expression of *pri-miR-24a* in the embryonic eye anlagen and posterior mesenchyme (stage 19 [A] and stage 23 [B]). Expression continues in the neural retina as the optic cup develops and invaginates (stage 28 [C] and stage 31 [D]). (C, inset) Sense probe shows no expression. (E) RT-PCR for the primary miRNA from stages 7 to 31 shows that *miR-24a* expression begins at stage 19 and is highly expressed throughout maturation.

designed to complement both the mature *miR-24a* sequence and some sequence of the adjacent loop in the predicted pri-miRNA structure (24aMO). This design was predicted to prevent processing of the primary miRNA transcript as well as to inhibit the function of the mature miRNA (Yin et al. 2008). Injection of 20 ng of 24aMO in one cell at the two-cell stage resulted in a reduction in eye size (Fig. 2A–F'), which was significant after stage 28 ($n = 94$ sections). Eyes containing 24aMO were reduced 50% in size compared with the uninjected control eye at stage 40 (data not shown). To ensure the specificity of the morpholino, we performed several control experiments. We were able to rescue the morpholino by coinjecting 2 ng of duplex *miR-24a* RNA, but not by coinjecting *miR-133b* RNA (Fig. 2G), showing that the rescue is not due to general miRNA activity. In addition, a mismatch morpholino with four base substitutions in the “seed” region (24ammMO) did not have a phenotype at an equivalent dose (Fig. 2G).

To further ensure that 24aMO was blocking *miR-24a* function, we constructed several GFP reporters, where the coding region of a destabilized GFP was fused to a 3'UTR containing miRNA recognition elements (MREs) (see the Supplemental Material). If the miRNA functionally interacts with the recognition element, then the miRNA would repress translation of the GFP and the embryo would lack green fluorescence. RNA coding for RFP without MREs was always coinjected, so that a measure of the repression of GFP by the miRNA could be generated by quantification of the green:red fluorescence ratio. A reporter GFP with two perfect *miR-24a* recognition elements in its 3'UTR (24aMRE), when coinjected with 500 pg of duplex *miR-24a*, was translationally repressed (Fig. 2H). However, when 10 ng of 24aMO were also coinjected, it was able to block the miRNA-induced repression (Fig. 2H). This repression was dependent on the *miR-24a* recognition elements, because mutating

these elements (mut24aMRE) resulted in no repression by the miRNA (Fig. 2H). Together, these results show that 24aMO is able to block the function of *miR-24a* in vivo, and that this leads to a reduction in eye size.

Figure 2. *miR-24a* knockdown results in a reduction in eye size. (A–F') Hematoxylin and eosin staining of 12- μ m sections in embryos injected with 20 ng of 24aMO in one cell at the two-cell stage, injected half on the right. The knockdown of *miR-24a* causes a reduction in eye size that does not occur until after stage 26 (A), first becoming prevalent at stage 28 (B). The reduction in eye size becomes obvious in stage 31 (C), stage 34 (D), stage 40 (E), and stage 45 (F,F') embryos (see also Supplemental Fig. 1). (G) The size of the eye was measured at stage 40 and the ratio of the injected eye to the uninjected eye in each embryo was used to classify the severity of phenotypes. *miR-24a* knockdown caused many embryos to have a smaller eye, an effect that was rescuable by coinjection of *miR-24a* duplex RNA, but not of *miR-133b* duplex RNA. A mismatch morpholino also had no effect on eye size ratio ($n = 121$). (H) 24aMO functionally represses *miR-24a*. A GFP construct with two *miR-24a* recognition elements (24aMRE) when injected alone strongly fluoresces, but shows significantly lower levels of fluorescence when coinjected with duplex *miR-24a* RNA ($n = 9$; error bars are the standard error of the mean [SEM]). This effect is dependent on the interaction between *miR-24a* and the *miR-24a* recognition elements, because mutating either abrogates the effect. 24aMO is able to rescue fluorescence of 24aMRE by blocking the function of *miR-24a* when coinjected. Similar results were obtained with multiple experiments. RFP without *miR-24a* recognition elements was always coinjected, so that a measure of GFP repression was expressed as the relative fluorescence of GFP/RFP.

Knockdown of *miR-24a* leads to an increase in apoptosis

We first analyzed the gene expression patterns of patterning and differentiation genes in the eye, as well as terminal differentiation markers. There was no change in the levels of expression for any of these genes, indicating that patterning and differentiation were unperturbed (Supplemental Fig. S1). Instead, eye size may be affected by a decrease in proliferation or an increase in apoptosis. Analysis of phosphorylated histone-3, a marker of dividing cells (Saka and Smith 2001), showed no significant difference between the 24aMO-injected side and the uninjected side in the number of proliferating cells in the eye (Fig. 3A). In contrast, TUNEL staining on 24aMO-injected embryos clearly demonstrated that apoptosis was significantly increased in the eye of *miR-24a* knockdown embryos (Fig. 3B). Injection with the mismatch morpholino (24ammMO) had no such increase (data not shown). Furthermore, the increase in apoptosis due to *miR-24a* knockdown began even before a morphological phenotype was observed, as early as stage 24, and continued through the development of the eye to tadpole stages (Fig. 3B; data not shown). These results indicate that *miR-24a* may be a potent negative regulator of members of the apoptotic pathway during eye development.

Figure 3. *miR-24a* knockdown has no effect on proliferation, but leads to an increase in apoptosis. (A) Immunohistochemistry for phosphorylated histone3 (PH3, in red) shows that there is no significant difference in proliferation between control eyes and 24aMO-injected eyes ($n = 6$ embryos per stage; 18 sections per embryo; sections are 12 μm ; error bars are SEM). (B) Knockdown of *miR-24a* results in a significant increase in apoptosis at all stages assayed, measured by the number of TUNEL-positive nuclei per 20 ($n = 6$ embryos per stage; 11 sections per embryo; error bars are SEM).

Knockdown of *miR-24a* causes up-regulation of Caspase9 protein and inhibition of Caspase9 rescues the knockdown phenotype

One of the most important members of the apoptotic pathway during neural development is *caspase9* (Cecconi et al. 2008). Therefore, we examined both *caspase9* mRNA levels by RT-PCR and protein levels by Western blot. In *miR-24a* knockdown embryos, the levels of *caspase9* mRNA were the same as uninjected controls or embryos injected with the mismatch morpholino 24ammMO (Fig. 4A). However, Caspase9 protein levels were increased significantly in embryos with reduced *miR-24a* function, relative to controls (Fig. 4B). These data demonstrate that *caspase9* is regulated post-transcriptionally and may be a direct target of *miR-24a*.

To test whether Caspase9 function was necessary for the *miR-24a* knockdown phenotype, we coinjected several specific Caspase inhibitors with 24aMO and analyzed their effect on eye size. Significantly, the only specific inhibitor we tested that was able to fully rescue the 24aMO phenotype was the Caspase9-specific inhibitor Ac-LEHD-CMK (Fig. 4C). An inhibitor to all Caspases was also able to rescue the *miR-24a* knockdown phenotype (Fig. 4C). Together, these data suggest that *miR-24a* regulates apoptosis by repressing protein translation of *caspase9* mRNA.

miR-24a can negatively regulate the proapoptotic factors caspase9 and apaf1

We investigated the possibility that members of the apoptotic pathway were direct targets of *miR-24a*. First, we analyzed the 3'UTRs of proapoptotic genes for putative *miR-24a*-binding sites from both *X. tropicalis* and *X. laevis* using miRbase (Griffiths-Jones et al. 2008) as well as RNAhybrid analysis of EST databases (Kruger and Rehmsmeier 2006). Both *caspase9* and *apoptosis protease-activating factor 1* (*apaf1*) were identified by this method as potential *miR-24a* targets (Fig. 5A). Caspase9 and Apaf1, along with Cytochrome C from the mitochondria, form a complex in the cytosol known as the apoptosome, which initiates a catalytic cascade that leads eventually to cell death (Penalzo et al. 2008). *Caspase9* and *apaf1* are both expressed in the neural retina during the time of development when *miR-24a* functions (Supplemental Fig. S2).

To test whether *miR-24a* is able to regulate *caspase9* and *apaf1* directly, we made reporter constructs with the *X. laevis* 3'UTR of these genes fused to a destabilized GFP. In addition, we mutated two putative *miR-24a* recognition elements in the 3'UTRs as controls (mutCasp9 and mutApaf1). *miR-24a* was able to significantly repress the expression of GFP only when *miR-24a* sites in the 3'UTR of *caspase9* or *apaf1* were present (Fig. 5B). This demonstrates that *miR-24a* can specifically target the *miR-24a*-binding sites in the 3'UTRs of both *caspase9* and *apaf1*. The *caspase9* 3'UTR may have more *miR-24a*-binding sites than the two we identified, as the mutated version is still slightly repressed relative to the "no UTR" control.

miR-24a is able to prevent hydroxyurea-induced apoptosis

Our loss-of-function studies determined that *miR-24a* is necessary in the eye to prevent apoptosis from occurring,

Figure 4. Knockdown of *miR-24a* causes up-regulation of Caspase9 protein and is rescuable by its inhibition. (A) RT-PCR of *caspase9* on stage 28 heads shows no change in mRNA levels between uninjected and *miR-24a* knockdown embryos. (B) Western blot for Caspase9 on stage 28 heads shows a significant increase in protein levels when *miR-24a* function is blocked ($n = 3$ experiments; error bars are SEM). (C) Coinjection of Caspase inhibitors to rescue the *miR-24a* knockdown phenotype. Only a pan-Caspase inhibitor (All) or an inhibitor specific to Caspase9 rescued the reduction in eye size caused by injection of 24aMO ($n \geq 47$ for each treatment; error bars are SEM).

and that depletion of *miR-24a* leads to the development of a smaller eye. To test whether *miR-24a* is sufficient to regulate other modes of apoptosis, we induced apoptosis by soaking embryos in 15 mM hydroxyurea; this causes large amounts of Caspase-dependent apoptosis, visible as cell autolysis and loss of adhesion, but only at the onset of zygotic transcription (Stack and Newport 1997; Takayama et al. 2004). To assess *miR-24a*-mediated rescue, we injected 2 ng of *miR-24a* into both cells of a two-cell stage embryo and incubated them in 15 mM hydroxyurea until early gastrula stages, then analyzed the number of embryos undergoing apoptosis. We found that the addition of *miR-24a* significantly reduced the number of embryos undergoing apoptosis, and that this reduction was not seen when embryos were injected with equal amounts of a 4-base-pair mismatch RNA (*miR-24amm*) (Fig. 5C). These results indicate that *miR-24a* is able to prevent apoptosis more generally than in the eye, most likely by targeting components of the apoptosis pathway including *caspase9* and *apaf1*.

Conclusions

We found that *miR-24a* represses apoptosis in the neural retina, likely by down-regulating *apaf1* and *caspase9*.

These two proteins are the major components of the apoptosome, the first complex in the protease cascade deployed during programmed cell death. Normally, Cytochrome *c* released from the mitochondria binds Apaf1 and induces the recruitment of Caspase9 to the apoptosome. The interaction between these proteins leads to the activation of Caspase9, which then cleaves and activates downstream Caspases, including Caspase3, Caspase6, and Caspase7 (Guerin et al. 2006). Our data implicate miRNAs as important regulators of the apoptosome during vertebrate development. Other genes also may be regulated by *miR-24a* in this process and make a contribution to the knockdown phenotype. It is intriguing that *miR-23b* and *miR-27b*, the other miRNAs in the cluster with *miR-24a*, are predicted to regulate other genes important for apoptosis, such as the low-affinity NGF receptor or *diablo*, an antagonist of inhibitor

Figure 5. *miR-24a* targets the 3' UTR of *caspase9* and *apaf1*. (A) The 3'UTRs of *X. laevis caspase9* and *apaf1* have two putative *miR-24a*-binding sites, as measured by RNAhybrid (Kruger and Rehmsmeier 2006). (B) The addition of *miR-24a* causes GFP reporters with the 3'UTR of *caspase9* and *apaf1* show repression by *miR-24a*. Mutating the putative *miR-24a*-binding sites in these constructs (mutCasp9 and mutApaf1) abolishes the repression ($n = 4$ experiments; error bars are SEM). (C) Embryos injected with 2 ng of *miR-24a* or *miR-24amm* or nothing (control) were cultured in 15 mM hydroxyurea to induce apoptosis at the onset of gastrulation. Only *miR-24a* was able to significantly repress or delay the onset of apoptosis ($n = 3$ experiments; error bars are SEM).

of apoptosis proteins (IAPs) (Guerin et al. 2006; Griffiths-Jones et al. 2008).

This study also makes clear the importance of proper regulation of apoptosis in the neural retina in *X. laevis*. Mouse knockouts of *caspase9* have an excessive accumulation of neurons throughout the CNS, including the retina (Hakem et al. 1998). Knockouts of *apaf1* also have retinal overgrowth in addition to forebrain overgrowth and thickening of the hindbrain walls (Cecconi et al. 1998). Here, we show that disrupting a negative regulator of these genes has the opposite effect to their elimination, resulting in higher levels of apoptosis and a smaller eye. Previous reports have disrupted the transcription of *apaf1* by interfering with histone deacetylation, causing an increase in apoptosis in the developing retina (Wallace and Cotter 2008). Our data argue that post-transcriptional regulation by miRNAs also may be a factor in the correct regulation of *apaf1* during normal amphibian development.

Mouse knockouts of *caspase3* can have a similar phenotype to those of *caspase9*, though the phenotype occurs in far fewer animals (10% vs. 97%), and cell death is still seen in the retina (Cecconi et al. 2008). This agrees with our experiments using Caspase inhibitors: While the Caspase3 inhibitor was able to partially rescue the phenotype caused by depletion of *miR-24a*, the rescue was not significant, indicating that it probably has only a small role in the downstream activity of the Caspase cascade.

Remarkably, the loss of a single miRNA is sufficient to release members of the apoptotic pathway from inhibition, resulting in a significant increase in apoptosis and severe morphological disruption of eye structures. This occurs without any disruption in early patterning or specification. This indicates that *miR-24a* functions primarily as a negative regulator of apoptosis in the neural retina. Similar miRNA regulation may be important for other neural structures that also require precise levels of apoptosis during development, including the brain and spinal cord (De Pietri Tonelli et al. 2008). Further characterization of the functions of neuronally expressed miRNAs should lead to an understanding of how general this mechanism is during development.

Materials and methods

pri-miRNA in situ hybridization and RT-PCR

X. tropicalis in situ hybridizations for primary miRNA transcripts were done as in Walker and Harland (2008) and RT-PCRs were performed as in Sive et al. (2000). The primers used were as follows: for *pri-miR-24a*, 5'-GGACCTGTCTCTTGTGCC-3' and 5'-GCTCAAGGGCCCGACTC-3'; for *otx2*, 5'-CGGGATG GATTTGTTGCA-3' and 5'-TTGAACCAGACCTGGACT-3'; for *ornithine decarboxylase*, 5'-GTCAATGATGGAGTGTAT-3' and 5'-TTCCATTCCGCTCTCCTGAG-3'; for *caspase9*, 5'-CAG AATGCGCTCCTTTCACTTTG-3' and 5'-AAGTAGCTCAGC ATTCTCTCTAC-3'; and for *ef1a* as in Krieg et al. (1989).

24aMO injections and eye size measurements

X. laevis eggs were collected and fertilized and embryos were cultured by standard procedures (Sive et al. 2000). An antisense oligonucleotide morpholino designed to block *miR-24a* was ordered from GeneTools, LLC, with the sequence 5'-CTGTTCTCTGCTGAAGTGAAGCAGTG-3' (24aMO), as well as a mismatched morpholino with the sequence 5'-CTGTTCTGCTGAAGTGAAGTGAAGCAGTG-3' (24aMMO). The dose injected throughout our analyses was 20 ng in one cell at the two-cell stage, except where noted otherwise.

Eye size was quantified by measuring the anterior-posterior diameter of the eye at its greatest point at stage 40 (staging according to Nieuwkoop and Faber [1994]) using a Zeiss Stemi SV6 dissecting scope with an eyepiece reticle.

Western blots

Embryos were injected and cultured until stage 28. Heads were dissected away from bodies and prepared for Westerns as in Sive et al. (2000). Actin antibody (Santa Cruz Biotechnologies, #H3105) was used at 1:4000 and Caspase9 antibody (Abcam, ab25758) was used at 1:200 overnight. A secondary antibody (Jackson ImmunoResearch, HRP-conjugated donkey anti-rabbit IgG) was used at 1:5000. Embryo ran at ~45 kDa and full-length Caspase9 at ran ~47 kDa.

Fluorescent protein injection and quantification

Katushka RFP RNA was coinjected with every GFP experiment at 200 pg per cell. All RNA for injection was made using SP6 RNA polymerase mMessage mMachine kit (Ambion) and AscI-digested template. RNA for GFP constructs was injected at a dose of 1 ng per cell into both cells of the two-cell stage embryo. *miR-24a* and *miR-133b* RNA mimicking the endogenous hairpin (Integrated DNA Technologies) were *miR-24a*Top (5'-GUGCC UACUGAACUGAUUACAGU-3'), *miR-24a*Bot (5'-UGGCUCU GUUCAGCAGGAACAG-3'), *miR-133b*Top (5'-UUGGUCCCC UUCAACCAGCUA-3'), and *miR-133b*Bot (5'-GCUGGUCAAA CGGAACCAAUU-3'). RNAs were combined and diluted to a stock concentration of 1 µg/µL, heated for 1 min to 80°C, and then allowed to cool to room temperature to form duplexes. The same was done for *miR-24mm*Top (5'-GUGGUACUGAA CUGAUUAGAGU-3') and *miR24mm*Bot (5'-UCUCUCAGUU CAGCACCAACAG-3'). All photos from a particular experiment were identically batch-processed using Photoshop (Adobe), and ImageJ1.34S (Wayne Rasband, NIH, Bethesda, MD) was used to measure average pixel intensity of RFP and GFP. The ratio between GFP and RFP was then used for comparison. All statistical significance tests were done using the unpaired Student *t*-test.

Immunohistochemistry and TUNEL

Cryosections were as in Zaghloul and Moody (2007). Phosphorylated *histone3* (Upstate Biotechnologies) staining was done as in Ruan et al. (2006). TUNEL staining was performed as in Hensley and Gautier (1997), using BM Purple for the staining (Roche).

Caspase inhibitors

Caspase inhibitors (CalBiochem) were caspase-1 inhibitor I (Ac-YVAD-CHO), caspase-3 inhibitor II (Z-DEVD-FMK), caspase inhibitor I (Z-VAD-FMK), and caspase-9 inhibitor III (Ac-LEHD-CMK), all used at 1 ng per injection. Stocks were diluted in DMSO or distilled water.

Hydroxyurea treatment

Hydroxyurea (Sigma) was diluted in 1/3× MR solution to a final concentration of 15 mM and embryos were treated from 2 h post-fertilization until stage 10.5, then fixed in MEMFA (Sive et al. 2000).

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