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Research report

Misexpression of miR-196a induces eye anomaly in *Xenopus laevis*

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ABSTRACT

miR-196a is located in the posterior trunk and plays a role in limb development. Here we show that miR-196a is able to induce eye anomaly in *Xenopus laevis*. Microinjection of synthetic miRNA precursor molecule for mammalian miR-196a into *Xenopus* embryo is sufficient for miR-196a overexpression during early development. The misexpression of miR-196a in anterior embryo led to dose-dependent eye anomalies, especially size reduction. In addition, the expression of *ET*, *Rx1*, *Six3*, *Pax6*, *Lhx2*, *Optx2* and *Ath5* in eye field or optic cup was also down-regulated. These results indicate that miR-196a can target gene(s) in the genetic network involved in eye formation, providing a potential tool for studying the mechanisms of eye development and diseases.

1. Introduction

MicroRNAs (miRNAs) are short (20–25 nucleotides) non-coding RNA molecules recently identified in many organisms as genetic regulators at the posttranscriptional level. In most cases, miRNA regulates gene expression through translational repression or degradation of the target mRNA. Computational methods predict that miRNA could have multiple targets and may regulate up to one third of human genes [5,14,26]. Currently, miRNAs have been found to be involved in multiple events of development, and miRNA misexpression is related to diseases like several types of cancer [4,17]. However, the roles of most miRNA remain unclear. In an extensive screening of the miRNAs involved in eye development and diseases, we found that microinjection of mammalian miR-196a precursors in anterior part of *Xenopus* embryo led to eye anomalies. These results suggest that spatial and temporal regulation of the endogenous miR-196a, especially maintaining its expression pattern in early stages, plays an important role in the eye development.

miR-196 is a group of highly conserved miRNAs detected in the genome of many species and has been validated in human, mouse, rat, *Xenopus* and zebrafish (miRBase) [24]. There are at least two groups of miR-196: miR-196a and miR-196b. miR-196a was first identified in vertebrates [13] and is localized in the Hox clusters of mammals [27]. It was first reported to be expressed in the posterior trunk of mouse embryos [16]. This pattern has also been observed in zebrafish by whole mount *in situ* hybridization [25]. miR-196a has been shown to degenerate *HoxB8* transcripts by its interaction with a conserved 3′-UTR complementary region [16,27], and to function in a 'fail-safe mechanism' to assure the right expression profile required for proper limb formation [9]. However, the detailed expression pattern of miR-196a and its other roles during development remain to be elucidated, especially in *Xenopus laevis*.

In this study, we examined expression pattern and carried out gain-of-function analysis of miR-196a in *X. laevis*. Our results show that miR-196a interacts with multiple genes involved in eye formation, and induces eye anomaly. These results have implications for mechanisms of eye development.

2. Materials and methods

2.1. X. laevis

X. laevis was purchased from Nasco International Inc. and raised according to standard methods [21]. Induction of ovulation in females, in vitro fertilization, embryo culture and staging were carried out as described [18,19,21].

2.2. In situ hybridization

The sequence of LNA (locked–nucleic acid) oligo for antisense mature miR-196a was 5′-CCAACAACATGAAACTACCTA-3′, and was synthesized from EXIQON (miRCURY™ detection probe, Vedbaek, Denmark). The oligo was labeled using DIG oligonucleotide 3′-end labeling kit (Roche, Basel, Switzerland), and purified

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using Sephadex G25 MicroSpin columns (Amersham Biosciences, Piscataway, USA). For *in situ* hybridization, approximately 100 ng/ml of labeled probe was used [12,25].

Whole mount *in situ* hybridization for detecting miRNA expression in *Xenopus* embryos was performed essentially as described [7], with the following modifications for the hybridization with LNA probe [12]: embryos at earlier than stage 33, and stages 33–46 were treated with proteinase K for 10 and 45 min, respectively. The temperature for hybridization and subsequent washing steps was adjusted to approximately 22 °C below the predicted melting temperature of the LNA-modified probe. In addition, a digoxigenin (DIG)-labeled oligonucleotide homologous to the lacZ′ region in pUC and M13 plasmids (30 mer, 5′-p TTGGGTAACGCCAGGGTTTTCCCAGTCACG OH-3′) was used as the negative control.

In situ hybridization on whole embryos of *Xenopus* was performed as previously described [7] to examine the expression of transcriptional factors including *ET*, *Rx1*, *Pax6*, *Six3*, *Lhx2*, *Optx2* and *Ath5*.

2.3. Microinjection

miR-196a precursor RNA and 2′-O-methyl antisense RNA oligonucleotide for misexpression and rescue experiment were designed to target the mature sequence of miR-196a (5′-UAGGUAGUUUCAUGUUGUUGG-3′), and obtained from Ambion (Austin, USA), such as Anti-miR™ miRNA Inhibitor for hsa-miR-196a-1; Pre-miR™ miRNA Precursor molecules for hsa-miR-196a-1 and mmu-miR-196a-2. The negative controls for precursor molecule and inhibitor were from Ambion Cat. No. 17110 and No. 17010, respectively. Pre-miR™ miRNA Precursor molecule for hsa-miR-198 (Ambion) was used as an additional negative control. Beta-galactosidase (β-gal) was used as a lineage tracer.

Embryos were co-injected with 100–500 pg β-gal mRNA and stained as previously described [1]. Capped mRNAs were synthesized from linearized plasmid templates using mMESSAGE mMACHINE kits (Ambion). Embryos were injected at one dorsal-animal blastomere at 8-cell stage with Eppendorf FemtoJet (Hamburg, Germany), and then grown up as previously described [15].

2.4. Quantitative analysis of eye phenotype

The injected and control embryos for phenotype analysis were grown to late tadpole (stages 45–46) and stored in 100% ethanol after being fixed in 4% paraformaldehyde in 0.1 M phosphate buffer. The diameter of eye (D) was measured under microscope with 10 μm microruler. The eye size reduction (R) was defined as the decrease of the eye volume: $R = (D_a^3 - D_b^3)/D_a^3$ (D_a is the eye diameter at uninjected side or that of the bigger eye in the wild-type control embryo, while D_b is the diameter at the injected side or that of the smaller eye in control). Statistical analyses were performed using one-way ANOVA followed with Duncan test. Comparisons of reduction (R) were made between the wild-type control embryos and those with different applied injections, and between embryos with different injections. At least 3 independent sets of experiments were performed, with the number of embryos in each group no less than 30. The difference between two compared groups was defined to be significant when P value <0.05.

2.5. Histology and imaging

The injected and control embryos for phenotype analysis were grown to stage 46 and stored in 100% ethanol after being fixed in 4% formaldehyde in MEM (MOPS/EGTA/Magnesium Sulfate phosphate buffer). For sectioning analysis, samples were embedded in paraffin, serially sectioned in 10 μm, and stained with hematoxylin–eosin solution. Embryos for phenotype analysis were photographed on an OLYMPUS SZX12 (Japan) stereomicroscope using a digital acquisition system (Olympus C4040). Sections were photographed on a compound microscope (Nikon FXA, Japan) with a microscope digital color camera (Olympus DP71).

2.6. Bioinformatics

miRNA precursor and mature sequences of mouse, human, zebrafish, *Xenopus tropicalis* are downloaded from **the miRNA Registry database** (http://www.sanger.ac.uk/Software/Rfam/mirna/), and mature sequence of *X. laevis* is from Watanabe et al. [24].

The predicted binding sites of miR-196a in the candidate targets of *X. laevis* were obtained by searching using RNAhybrid [20]. The mRNA sequences of *Rx1*, *Pax6*, *Six3*, *Ath5* and *HoxC8* were from NCBI. The mature sequences of both *Xenopus* and mammalian (human or mouse) miR-196a were used in target prediction.

3. Results and discussion

3.1. The expression pattern of miR-196a in the developing *Xenopus* embryo

To determine if the eye anomaly induced by microinjected miR-196a precursors is due to an endogenous role or an ectopic disturbance, we examined the expression of miR-196a during *Xenopus* development. The homology analysis was performed using the validated and predicted miR-196a precursor and mature sequences of human, mouse, zebrafish, *X. tropicalis* and *X. laevis*. MiR-196a-1 and miR-196a-2 share identical mature sequence. Twenty base-pair core mature sequences from the second nucleotide of 5′ termini are identical in all species (Fig. 1). In addition, conserved nucleotides are observed in regions flanking the core sequences. Therefore, miR-196a is highly conserved during evolution. An antisense probe and an inhibitor for miR-196a were designed to target the shortest mature molecule with the 20 conserved nucleotides (UAGGUAGUUUCAUGUUGUUGG).

The expression pattern of miR-196a was determined by whole mount *in situ* hybridization using LNA-modified antisense DNA oligonucleotide probe (Fig. 2A). miR-196a begins to be expressed in tailbud at a low level from stage 26 (early tailbud stage) in *X. laevis*, with the expression level increasing from stages 30 to 46. By stage 32, miR-196a expression is also detected in the posterior spinal cord. As the embryos develop, localization of miR-196a expression in the spinal cord gradually extends into a more anterior region. This pattern has been confirmed in zebrafish and mouse [12,25]. No signal was detected in the predicted eye field and developing eye, neither did the embryos hybridized with probe for negative control (Fig. 1, and data not shown). The specific location of miR-196a in posterior trunk suggests that the eye anomaly induced by miR-196a microinjection in head is due to an exogenous disturbance and that

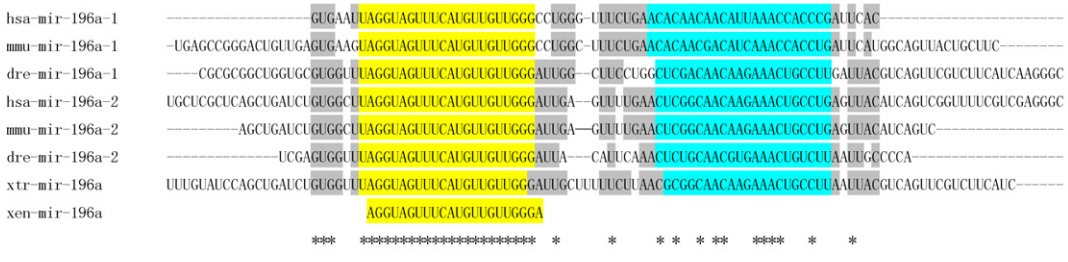

Fig. 1. The homologous analysis of miR-196a from different species. The miR-196a precursors and mature sequences from human (hsa), mouse (mmu), zebrafish (dre), *Xenopus tropicalis* (xtr) and *Xenopus laevis* (xen) are shown in ClustalW alignment format. The sequences of mature molecules are in yellow, the complementary sequences in blue, and the nucleotides conserved in the flanking regions in grey. The nucleotide positions conserved in all miR-196a sequences are indicated with stars. The sequences are all from miRBase Sequences Database (the predicted sequences of miRNA precursors in the database include some flanking sequence from the presumed primary transcript), except that of xen-miR-196a is from Watanabe et al. [24]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

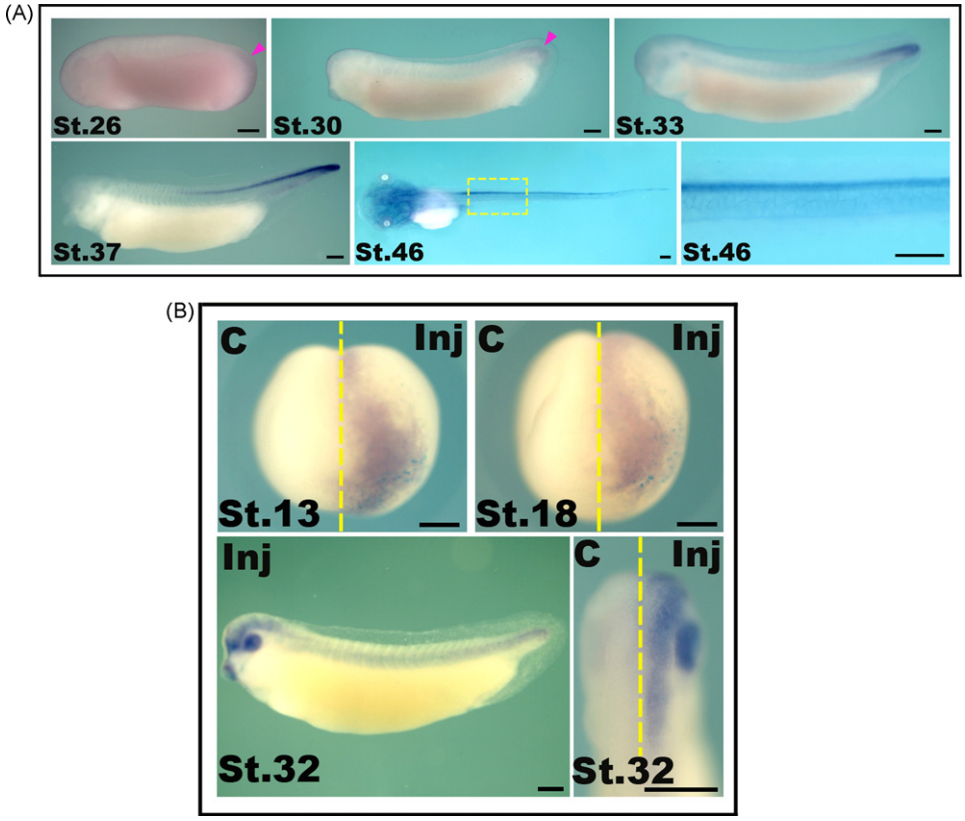

Fig. 2. Expression of miR-196a in the control and the miR-196a precursor-injected *Xenopus* embryos. The expression of miR-196a was detected by *in situ* hybridization with DIG-labeled antisense LNA probe on both wild-type (A) and miR-196a precursor-injected (B) *Xenopus* embryos. (A) Expression of miR-196a at stages (st.) 26, 30, 33, 37 and 46. Embryos of st. 26–37 are shown from the lateral view, and those of st. 46 are shown from the dorsal view. The trunk of st. 46 embryos in the frame of yellow dash line is also shown at a higher magnification. miR-196a expression signals in tailbuds of st. 26 and 30 embryos are indicated by a pink arrow head. (B) Embryos were injected with 0.025 pmol synthetic precursor molecule for mmu-miR-196a in one dorsal-animal blastomere at 8-cell stage. Ectopic expression (purple or dark blue) of miR-196a was detected at st. 13, 18 and 32. Beta-galactosidase (β-gal) was co-injected as a tracer with β-gal signal shown in light blue. The st. 13 and 18 embryos are shown from the anterior view, whereas that of st. 32 is shown from both lateral (injected side) and dorsal views. Yellow dash lines indicate the midlines of body to separate the injected (Inj) and uninjected control (C) side. Scale bars: 300 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

the ectopic miR-196a could target genes regulating eye development.

3.2. Microinjection of synthetic precursor molecules leads to misexpression of miR-196a during early development of Xenopus embryo

To verify that the synthetic Pre-miR™ miRNA precursor molecules for mammalian-miR-196a (Ambion) were properly processed in *Xenopus*, miR-196a expression in injected embryos was detected by *in situ* hybridization with LNA probe. The synthetic precursor molecules for human (hsa) or mouse (mmu) miR-196a, which shows identical core mature sequence as miR-196a of *Xenopus* (Fig. 1), were injected into *Xenopus* embryos with different dosages. Precursor molecules were injected into one dorsal-animal blastomere at 8-cell stage to induce ectopic expression in dorsal head including the eye region in which no endogenous miR-196a was detected. As shown in Fig. 2B, ectopic miR-196a expression was detectable at different stages in the prospective anterior neural plate (stages 13 and 18) or dorsal heads (stage 32) at the 0.025 picomoles (pmol) mmu-miR-196a injected side but not at the control side. Similar results were obtained in microinjection with hsa-miR-196a precursor molecule (data not shown). These results indicate that synthetic miRNA precursor molecules lead to misexpression of miR-196a during early development, and microinjection of the Pre-miR™ molecules is an efficient approach for miRNA overexpression in *X. laevis*.

3.3. Misexpression of miR-196a leads to specific eye anomalies in a dose-dependent manner

To study effects induced by ectopic expression of miR-196a, different doses of miR-196a precursor (pre-miR-196a) or controls (pre-control or pre-miR-198) were injected into one dorsal-animal blastomere at 8-cell stage. The injected embryos were grown to stage 46 for morphological analysis. Microinjection of pre-miR-196a at a high dose (0.05 pmol) led to a reduction of the anterior head including the eye at the injected side. In addition, most embryos showed other eye defects such as pigmented and enlarged optic nerve, ventral coloboma, and reorientation of the optic fissure (Fig. 3A–C and data not shown). At a lower concentration (0.025 pmol), pre-miR-196a microinjection caused eye size reduction without obvious morphological defects in other structures (Fig. 3F). This effect was specific, because embryos microinjected with pre-miR-198 or other control from Ambion did not cause any morphological defects at the same dose or even at dosage higher than 0.5 pmol (Fig. S1 and data not shown). As shown in Fig. 3J, the average difference in the eye volume (*R*%) is 5%, 7%, 1% and 5% in the wild-type control, 0.05 pmol pre-control, 0.5 pmol pre-198 and 0.025 pmol miR-196a inhibitor (anti-196a) injected embryos, respectively. No significant difference ($P > 0.05$) was observed between the *R*% values in these groups. In contrast, in embryos injected with 0.025 or 0.05 pmol pre-miR-196a, the average *R*% values were 46% and 55%, respectively, showing significant ($P < 0.005$) eye size reduction at the injected side. In addition, the

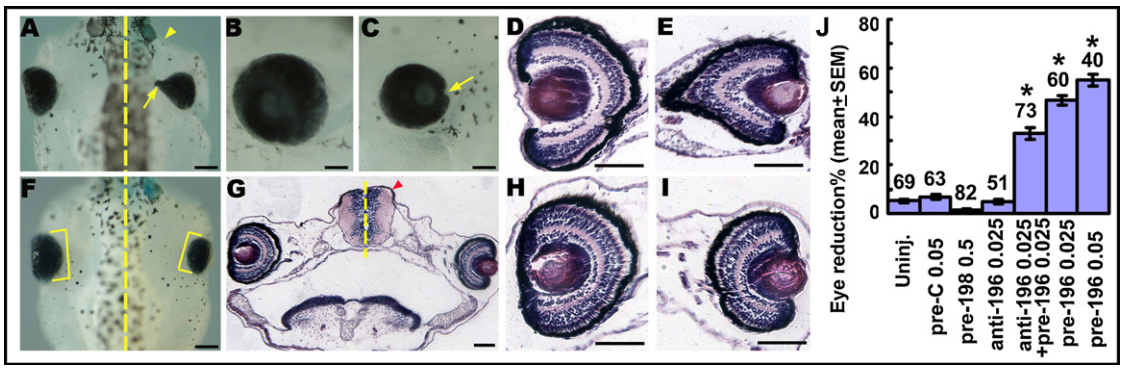

Fig. 3. Dose-dependent eye anomalies induced by miR-196a injection. miR-196a was injected at 0.05 (A–E) or 0.025 (F–I) pmol in one dorsal-animal blastomere at 8-cell stage. Beta-galactosidase was co-injected as a tracer of the injected side and stained in light blue. The heads of injected embryos grown to stage 46 are shown from the dorsal view (A and F). The head reduction and optic nerve anomaly at the injected side are indicated with a yellow arrowhead and an arrow, respectively. In brackets are the diameters of eyes for size calculation. The lateral views (B and C) of the eyes with higher magnification in (A) show additional ventral defects (yellow arrow) of the eye. Panels (D), (H) and (E), (I) show the transversal sections stained with hematoxylin (blue)–eosin solution (pink), respectively. Panels (H) and (I) show higher magnifications of the eyes in (G). Slight enlargement in the brain (red arrow head) is detected along with eye reduction at the injected side. Yellow dash lines indicate the midlines of the embryos. Scale bars: 200 μm (A and F), 100 μm (B–E and G–I). The bar graph in panel (J) shows the averages of relative eye difference/reduction (*R*%) in uninjected control embryos (uninj.) and those injected with 0.05 pmol pre-control (pre-C 0.05), 0.5 pmol pre-miR-198 (pre-198 0.5), 0.025 pmol miR-196a inhibitor (anti-196 0.025), 0.025 pmol miR-196a precursor molecule together with 0.025 pmol inhibitor (anti-196 0.025 + pre-196 0.025), and 0.025 and 0.05 pmol miR-196a precursor molecules (pre-196 0.025, pre-196 0.05). Numbers above the bars are the number of embryos scored in each group. Data were collected from three independent experiments and shown as the mean value with the standard error of mean (±S.E.M). Asterisks indicate statistical significance of $P < 0.05$ (one-way ANOVA followed by Duncan test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

eye size reduction phenotype became stronger in a dose-dependent manner. The effect of eye reduction induced by 0.025 pmol pre-miR-196a was partially rescued by co-injection with the same dose of miR-196a inhibitor, and the average eye change was significantly ($P < 0.001$) reduced from 46% to 33%. Increasing the dose of anti-miR-196a to 0.05 pmol did not further decrease the level of eye reduction but led to gastrulation defects, possibly due to nonspecific effects. The above results suggest that misexpression of miR-196a leads to specific dose-dependent eye anomalies, especially eye size reduction.

To further characterize the eye phenotype caused by miR-196a misexpression, we sectioned the injected embryos at the level of eye. The formation of the optic cup with the retina and the lens was not affected in most of the injected embryos, and the cells in the retina were also well organized as in the control with five layers of pigmented retina epithelium (PRE), the out nuclear (ONL), the outer plexiform (OPL), the inner nuclear (INL), the inner plexiform (IPL) and retinal ganglion cell (RGC) (Fig. 3G–I). At the low dose, miR-196a ectopic expression affected the eye size but not the layer organization of the retina. In addition, slight expansion of the neural tube was observed at the miR-196a injected side (Fig. 3G), suggesting that some eye anlagen cells may be transformed into the neural tube cells. In more severely affected embryos with reduction in both eye and other anterior regions, the central retina expanded into the optic nerve region forming a large pigmented optic stalk-like structure connected to the brain vesicle, and the eye became closer to the midline of the body (Fig. 3D and E). Therefore, a high level of miR-196a expression affected not only the eye size, but also the formation of the optic cup and nerve.

3.4. *miR-196a reduces eye field and interacts with the genetic network regulating eye development*

The control of eye size is determined by the early specification of eye field from the anterior neural plate and the later cell proliferation in the ciliary marginal zone (CMZ) of the retina [6,29,30]. The morphological changes of the anterior neural plate in miR-196a injected embryos were visible at the neurula stage, and the size reduction of optic vesicle was evident as early as stage 26 before the formation of optic cup (data not shown). This suggests an early effect of the injected miR-196a on eye development. To reveal which gene(s) might be involved in the eye defects induced by miR-196a, we investigated the effects of miR-196a on the expression of several eye-field transcriptional factors (EFTFs), *ET*, *Rx1*, *Pax6*, *Six3* and *Lhx2*. These five EFTFs play important roles in mapping the eye domain in the anterior neural plate [29]. The pre-miR-196a-injected (0.025 pmol) embryos were collected at stage 12.5/13 and the mid-neurula (stage 15/18) stage. The expression of *ET*, *Rx1*, *Pax6*, *Six3* and *Lhx2* was examined by whole mount *in situ* hybridization. As shown in Fig. 4, at stage 13, *ET*, *Rx1*, *Pax6*, *Six3* and *Lhx2* all began to show expression in the eye primordium with overlapping expression patterns. Expression of *ET*, *Rx1* and *Lhx2* was restricted in the eye field, whereas *Pax6* and *Six3* expression domains extended to the presumptive telencephalon [29]. Ectopic expression of miR-196a down-regulated the expression of all these EFTFs, with the effects detectable from stage 13 and more prominent at stage 18. These results indicate that the small eye phenotype induced by miR-196a is associated with regulation of genes controling the eye field.

To investigate whether miR-196a ectopic expression exerts a later effect on the retinogenesis and the optic nerve development, we examined the expression of the above EFTFs, as well as *Ath5* and *Optx2*, at early optic cup stage (st. 33) when the retina and optic stalk had formed (Fig. 4). *Ath5* is important for retinogenesis and is required for retinal ganglion cell and optic nerve formation [10,11]. *Optx2* is involved in the size control of *Xenopus* eye [30]. As shown in Fig. 4, the expression of *ET* is restricted in dorsal retina at stage 33. At the side injected with miR-196a, *ET* expression pattern was the same as in the control side, indicating that the dorsal-ventral polarity/subdivision of the retina was not altered by the exogenous miR-196a. At this stage, the *Rx1*, *Pax6*, *Six3*, *Lhx2*, *Optx2* and *Ath5* are all expressed in the neural retina [2,8,11,23,28,30] covering the entire retina region at the control side from a lateral view. At miR-196a injected side, the expression domains of *Rx1*, *Pax6*, *Lhx2* and *Optx2* were all reduced with reduced eye size but the *in situ* signal levels not much changed. On the other hand, *Six3* and *Ath5* expression level was strongly down-regulated in miR-196a injected side as compared to the control side. Furthermore, the expression domain of *Ath5* in the small eye embryos was more restricted to the central retina, with expression not detectable in some of injected embryos.

We further searched for potential target sites for miR-196a using software RNA hybrid [20] and identified potential binding sites in

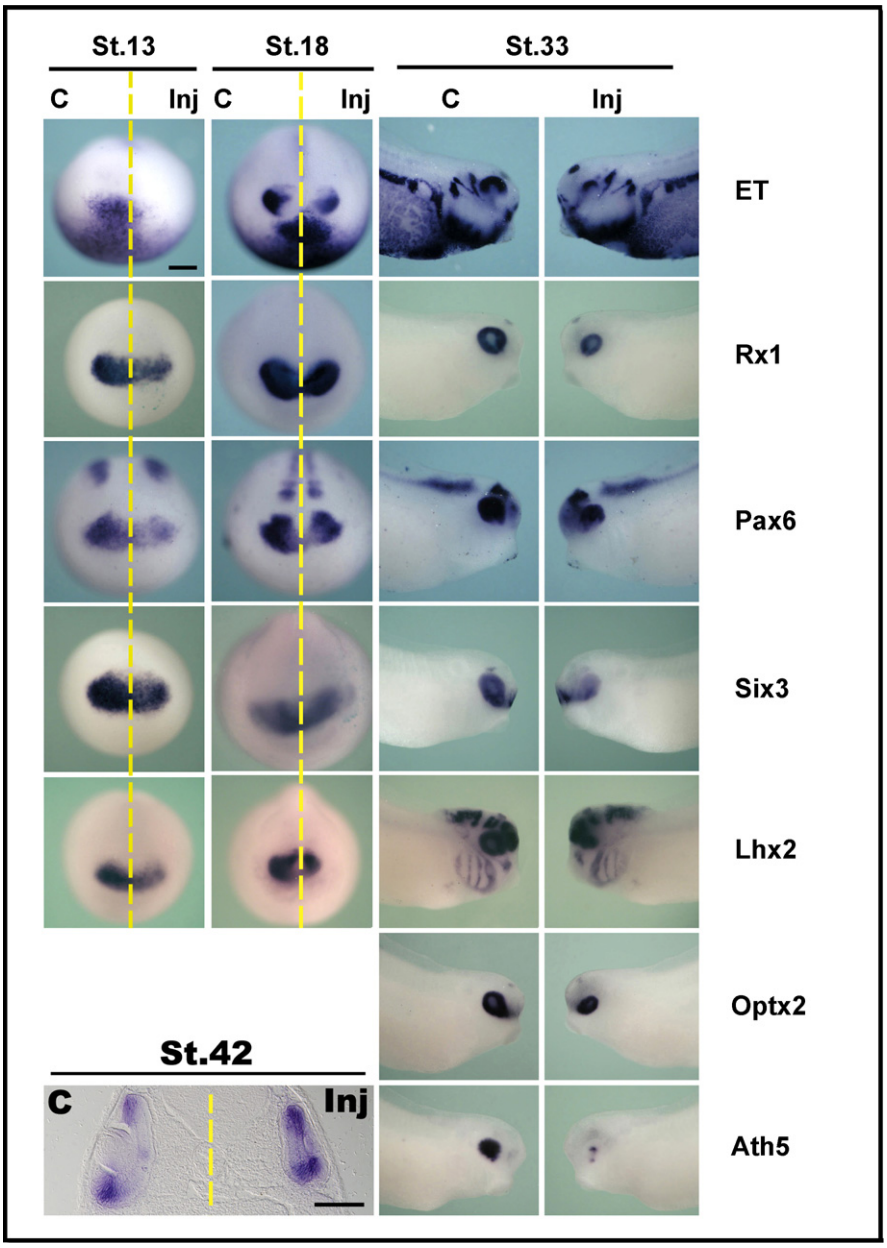

Fig. 4. MiR-196a expression reduces the eye field and expression of genes regulating eye development. The expression of the transcription factors *ET*, *Rx1*, *Pax6*, *Six3*, *Lhx2*, *Optx2* and *Ath5* was detected by *in situ* hybridization. Embryos were injected with 0.025 pmol precursor molecule for mmu-miR-196a in one dorsal-animal blastomere at 8-cell stage and collected at stage 13 (anterior view), stage 18 (anterior view), stage 33 (lateral view) or stage 42 (transection, 15 μm). The yellow dash lines indicate the midlines of the embryos. Scale bars: 300 μm for the whole mount embryos, 100 μm for the transection. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

the 3′-UTR of *X. laevis Ath5* transcript (Table 1). This suggests that down-regulation of *Ath5* may be due to direct degeneration of *Ath5* by miR-196a. In *Rx1*, *Pax6*, *Six3* and *Optx2*, the predicted miR-196a binding sites were not found in 3′-UTR but in the coding region (CDS), suggesting that down-regulation of these genes by miR-196a might be caused by interactions in the CDS, similar to some other miRNAs [3,22]. However, as the reported sequences of these genes might not include the entire 3′-UTR regions, we could not exclude the possibility that there were additional miR-196a binding sites involved.

As the expression of *Ath5* in retina coincides with the onset of neural differentiation [11], the strong effect on *Ath5* at st. 33 might be also due to the eye size reduction and/or the delay in the eye development in addition to the direct effect on *Ath5* mRNA degradation. We examined *Ath5* expression at later stages (st. 41/42), and show that *Ath5* down-regulation was not as strong as at st. 33. Sectioning of miR-196a injected embryos at st. 41/42 (Fig. 4) revealed that the down-regulation of *Ath5* expression accompanied eye size reduction instead of decrease in its expression level. Moreover, in the injected eye, *Ath5* expression was less restricted to the CMZ and similar to a pattern of *Ath5* at earlier stages of eye development [11]. Therefore, the strong reduction of *Ath5* at st. 33 might be due to a combination of direct mRNA degradation, the eye size reduction and the delay of eye development. At later stage, the injected miR-196a might also be diluted or cleared, with its effect reduced.

The above results suggest that the miR-196a-induced eye reduction may be a result of an early reduction of eye field and of its interaction with its target genes at later stages. However, further investigation is necessary to determine whether these interactions reduce cell proliferation and/or increase apoptosis in the eye. Study-

Table 1
The predicted binding sites of miR-196a in the candidate targets of *Xenopus laevis* by RNAhybrid.

Name	Accession number, full length (CDS)	Position (Mfe, kcal/mol), xen/hsa
ET	AF173940, 4310 bp (548–2698)	282 (−27.1)/299 (−25.1) in 5′-UTR
Rx1	AF017273, 1485 bp (259–1227)	496 (−24.9)/170 (−21.8), in CDS/5′-UTR
Pax6	XLU77532, 2487 bp (573–1841)	645 (−25.2)/1583 (−21.2), in CDS
Six3	AF183571**, 2298 bp (122–997)**	**918 (−27.2)/920 (−24.3), in CDS**
Optx2	AF081352, 1593 bp (365–1099)	825 (−27.7)/1073 (−24.2), in CDS
Ath5	XLU93170**, 2123 bp (207–623)**	**1905 (−23.7)/1906 (−23.2), in 3′-UTR**
HoxC8	AF001596.1, 2380 bp (450–1220)	−31.9 (2187)/−29.0 (2188), in3′-UTR

Notes: Mfe represents the minimum free energy of the duplex between the miRNA and the predicted binding site. Position denotes the location of the binding site in the target mRNA. Xen/hsa means whether the target position was predicted with the mature sequence of *Xenopus laevis* (xen) or human sequence (hsa). Bold fonts indicate the cases where both miRNAs share the same conserved biding sites. *HoxC8* is shown as a positive control. CDS, coding region and UTR, untranslated region.

ing regulation of these genes by miR-196a should shed light on the regulatory mechanism of eye formation. In addition, although endogenous expression of miR-196a is restricted to the spinal cord, its target genes might be expressed in both the eye and the spinal cord, like *Pax6*; therefore those miR-196a target genes in eye may also function in regulating of the development of the spinal cord.

In conclusion, our study has shown that endogenous miR-196a is expressed at the posterior trunk during *Xenopus* development and its expression level increases in later stages. Microinjection of synthetic pre-miR-196a into *Xenopus* embryo is sufficient for miR-196a overexpression during early development. Ectopic expression of miR-196a in the dorsal anterior region of *Xenopus* embryo induces eye anomalies in a dose-dependent manner. These effects are associated with down-regulation of *ET*, *Rx1*, *Six3*, *Pax6*, *Lhx2*, *Optx2* and *Ath5* in the eye field or optic cup. These results suggest that miR-196a may interact with the genetic network regulating eye development, providing a useful tool in studying the mechanisms of eye development and possibly retinal diseases.

Conflict of interest

No.

Acknowledgments

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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.brainresbull.2008.12.009.

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