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Ectopic *Six3* expression in the dragon eye goldfish

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Abstract

For goldfish (*Carassius auratus*), there are many varieties with different eye phenotypes due to artificial selection and adaptive evolution. Dragon eye is a variant eye characterized by a large-size eyeball protruding out of the socket similar to the eye of dragon in Chinese legends. In this study, anatomical structure of the goldfish dragon eye was compared with that of the common eye, and a stretching of the retina was observed in the enlarged dragon eye. Moreover, the homeobox-containing transcription factor *Six3* cDNAs were cloned from the two types of goldfish, and the expression patterns were analyzed in both normal eye and dragon eye goldfish. No amino acid sequence differences were observed between the two deduced peptides, and the expression pattern of Six3 protein in dragon eye is quite similar to common eye during embryogenesis, but from 2 days after hatching, ectopic Six3 expression began to occur in the dragon eye, especially in the outer nuclear layer cells. With eye development, more predominant Six3 distribution was detected in the outer nuclear layer cells of dragon eye than that of normal eye, and fewer cell-layers in outer nuclear layer were observed in dragon eye retina than in normal eye retina. The highlight of this study is that higher *Six3* expression occurs in dragon eye goldfish than in normal eye goldfish during retinal development of larvae.
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Keywords: Homeobox; Eye development; *Six3* gene; Outer nuclear layer; Goldfish (*C. auratus*); Immunofluorescence localization

1. Introduction

Diverse varieties of the domesticated goldfish originate from wild goldfish (*Carassius auratus*) in China (Chen, 1928), suggesting that artificial selection and adaptive evolution should play significant roles in their evolution (Wu and Fu, 1997). Goldfish eyes vary greatly among the different varieties. Normal eye, dragon eye, telescope eye, celestial eye, bubble eye, and cinnabar eye are main types in several hundred varieties. Especially, the dragon eye is large and protrudes out of eye-socket, and therefore is designated as describing as eye of dragon in Chinese legends. The molecular basis underlying the morphologically different eye is extremely interesting, and will be a fascinating theme in fish comparative physiology and evolutionary biology.

Six3 (*sine oculis homeobox 3*), homologous gene to *so* (*sine oculis*) in *Drosophila melanogaster*, has been identified in many species, such as *Xenopus laevis* (Zuber et al., 1999), *Oryzias latipes* (Loosli et al., 1998), *Danio rerio* (Seo et al., 1998), *Gallus gallus* (Bovolenta et al., 1998), *Mus musculus* (Oliver et al., 1995), *Homo sapiens* (Granadino et al., 1999) and jellyfish *Cladonema radiatum* (Stierwald et al., 2004). The *Drosophila so* and murine *Six3* were observed to play a key role in the process of eye formation (Cheyette et al., 1994; Serikaku et al., 1994; Oliver et al., 1995), and ectopic lens and retina induction was also demonstrated in transgenic *Oryzlas latipes*, zebrafish and *Xenopus leavis* embryos in response to the murine *Six3* (Oliver et al., 1996; Oliver and Gruss, 1997; Kobayashi et al., 1998; Loosli et al., 1999; Bernier et al., 2000). Moreover, *Six3* was determined to be a direct negative regulator of Wnt1 expression in chick and fish embryos (Lagutin et al., 2003). In contrast, inactivation of *Six3* function resulted in the lack of forebrain and eyes in *O. latipes* (Carl et al., 2002), and the targeted disruption of mouse *Six3* and *Six6* caused anterior truncation of forebrain and retinal hypoplasia, respectively (Li et al., 2002; Liu et al., 2006). Additionally, as a transcriptional repressor (Kobayashi et al., 2001), *Six3* was shown to play a very crucial role in stimulating cell proliferation of *D. rerio* and *X. leavis* eye development (Tessmar et al., 2002). Furthermore,

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the antagonistic action of geminin and Six3 and a delicate balance that leads to the precise control of organ size during eye development were revealed (Bene et al., 2004), and some interactive molecules with Six3 were also identified in the developing eye of medaka embryos (Lopez-Rios et al., 2003). The data imply that physiological function of Six3 is related to forebrain development and lens formation, and further comparative study is necessary in vertebrates.

The expression pattern of *Six3* has been well described in early embryogenesis for several vertebrates, such as mouse (Oliver et al., 1995), human (Granadino et al., 1999), chicken (Bovolenta et al., 1998), medaka (Loosli et al., 1998), zebrafish (Seo et al., 1998) and *X. leavis* (Ghanbari et al., 2001), but has not been described in later embryogenesis and larval development. In order to reveal the relationship between the *Six3* expression and the dragon eye development in goldfish, we have cloned the goldfish *Six3* homologue, and analyzed its expression pattern and developmental behavior during embryogenesis and larval development. In comparison with wild type of grass goldfish with normal eye, ectopic expression of *Six3* was revealed in the dragon eye formation.

2. Materials and methods

2.1. Source of fish and embryos

In the reproductive season, two strains of goldfish including grass goldfish *C. auratus* (normal eye goldfish) and dragon eye goldfish were provided by TungHoi Aquarium Company in Guangdong, China. Fish were inbred respectively by artificial spawning and fertilization. Embryos and larva were incubated at 22 °C, and sampled at different stages. Eye development was observed under light microscope at 22 °C.

2.2. Anatomical comparison between normal eye and dragon eye

Normal eye and dragon eye excised from freshly killed adult goldfish were immediately embedded in O.C.T. (Optimal Cutting Temperature, Germany) and frozen in liquid nitrogen. The samples were sectioned 8 μm thick in transverse planes at −25 °C in a microtome (Leica) and mounted on 0.1% poly-L-lysine-coated slides and air-dried. After Hematoxylin–Eosin staining and Permount (Fisher) coverslipping, the sections were checked under light microscope.

2.3. Cloning and sequencing of goldfish Six3 full-length cDNA

Normal eye and dragon eye SMART (switch mechanism at the 5′ end of RNA templates) cDNA libraries were synthesized using cDNA Library Construction Kit (Clontech). According to the conserved regions of the *H. sapiens* (AF083891), *M. musculus* (BC094426) and *D. rerio* (AB004881) *Six3* genes, two degenerate primers, P1 (5′-TACCACATCCTGGAARMCCAC-3′) and P2 (5′-TAAACCARTTGCCYACTTGWGT-3′) were designed and used for PCR screening. After denaturation at 94 °C 4 min, 35 cycle amplifications were performed at 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min, and then incubated at 72 °C for 7 min. The template was 1 μL of 1:50 diluted goldfish dragon eye SMART cDNAs or normal eye SMART cDNAs. The PCR products were purified with DNA Extraction Kit (Fermentas) and cloned into pGEM-T vector (Promega) for sequencing on ABI Prism 377.

RACE-PCR was used to clone full-length *Six3* cDNAs. One μM of P2 primer and 0.3 μM of 5′ adaptor primer (Clonetech) were used for 5′ RACE. Similarly, 3′ RACE was performed with a P1 primer and 3′ adaptor primer (Clonetech). In both RACE reactions the annealing temperature was 59 °C and 36 cycles were performed. The RACE products were analyzed further by cloning and sequencing. Similar sequences were searched using BLAST at web servers of the National Center of Biotechnology Information. Multiple sequence alignments were performed using the Vector NTI suite 8.0 program. Motifs in proteins were predicted by software ScanProsite on http://ca.expasy.org/tools/scanprosite/. In addition, *Six3* 5′UTR and 3′UTR sequences from different individuals were compared between dragon eye goldfish and normal eye goldfish using Vector NTI suite 8.0 program.

2.4. Preparation of polyclonal antibody

The goldfish *Six3* cDNA fragment (262–1143 nt) coding 293 amino acids was PCR amplified using the primers PE5: 5′-GCGAATTCATGGTTTTCAGGTCTCCT-3′ and PE3: 5′-GCCTCGAGTCATACATCGAAATCAG-3′ with the SMART cDNAs as template. Following digestion with *Eco*R1 and *Xho*1 the fragment was inserted into the expression vector pET-32a and transformed into BL21 (DE3) strain. One mmol/L IPTG induction and SDS-PAGE analysis indicated that the recombinant bacteria produced about 50 kDa soluble fusion protein. After purification by metal chelation chromatography with His-Bind kit (Novagen), the protein was used to immunize a rabbit for 4 times at the dose of 500 μg each time as described (Dong et al., 2004).

2.5. RT-PCR and Western blot detection

RT-PCR was used to detect the *Six3* mRNA in various tissues and embryos at different developmental stages. Total RNAs were extracted from heart, liver, spleen, kidney, brain, muscle, eye and eggs of the adult dragon eye goldfish and goldfish embryos at different developmental stages (unfertilized mature eggs, fertilized eggs, blastula embryos, gastrula embryos, optic vesicle embryos, tail bud embryos, lens embryos, heartbeat embryos, hatching larvae and hatched larvae of normal eye goldfish and dragon eye goldfish) using SV Total Tissue RNA Isolation System (Promega) respectively. The first strand DNA was synthesized using SuperScript II Reverse Transcriptase (GIBRCO) with oligo (dT) at 37 °C for 1 h. β-Actin was amplified for 26 cycles as positive control with primers β-actin-F: 5′-CACTGTGCCCATCTACGAG-3′ and β-actin-R: 5′-CCATCTCCTGCTCGAAGTC-3′ to adjust the concentrations of the reverse-transcribed first stand cDNAs (Zhang and Gui, 2004). PCRs were performed with the primers P3 (5′-CGACTCCATGGTTTTCAGGTC-3′) and P4 (5′-GCCTCGTTCATTCATACTG-3′) for tissue samples, P1 and P2 for embryo samples. After denaturation at 94 °C for 2 min, PCR

was performed for 35 cycles (30 s at 94 °C, 30 s at 56 °C, and 1 min at 72 °C) and then further incubated for 7 min at 72 °C. Water was used for the RT-PCR negative control template to exclude any contamination. The PCR products were electrophoresed on 0.8% agarose gel containing 0.5 μg/mL ethidium bromide.

The soluble proteins from eye, heart, liver, spleen, brain, muscle and kidney of dragon eye goldfish were isolated, respectively. Embryo extracts were prepared at different developmental stages including unfertilized mature eggs, fertilized eggs, blastula embryos, gastrula embryos, optic vesicle embryos, tail bud embryos, lens embryos, heartbeat embryos, hatching larvae, hatched larvae and 1 day old larvae from normal eye goldfish and dragon eye goldfish, respectively. A total of 100 embryos were homogenized in 200 μL of extract buffer (EB) (100 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES pH 7.7, 50 mM sucrose, 1 mM DTT, 0.1 mM PMSF, 25 μg/mL aprotinin, 10 μg/mL cytochalasin B, and 50 μg/mL cycloheximide), or 10 mg tissues in 500 μL of EB respectively. In addition, each single eye was extracted and analyzed from 20 normal goldfish and 20 dragon eye goldfish at 1 day, 2 days, 5 days, 15 days and 1 month old, respectively. After SDS-PAGE, the proteins were electrophoretically blotted to a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in TBST buffer (100 mM NaCl, 25 mM Tris–Cl, 0.1% Tween-20, pH 7.5) at room temperature for 1 hour, and incubated in the rabbit antiserum at the dilution of 1:500 in TBST buffer containing 1% nonfat dry milk at room temperature for 1 hour. The membrane was washed 5 times for 10 min each in TBST buffer, and incubated with 1:2000 diluted alkaline phosphatase conjugated goat anti-rabbit IgG (Sino–American) for 1 h at room temperature. After the nitrocellulose membrane was washed 4 times for 10 min in TBST buffer, the detection was performed using BCIP/NBT staining. Moreover, the Western blot detections for single eye were repeated three times in three independent experiments, and the positive bands were quantified for statistical analysis by software Band Leader Version 3.0.

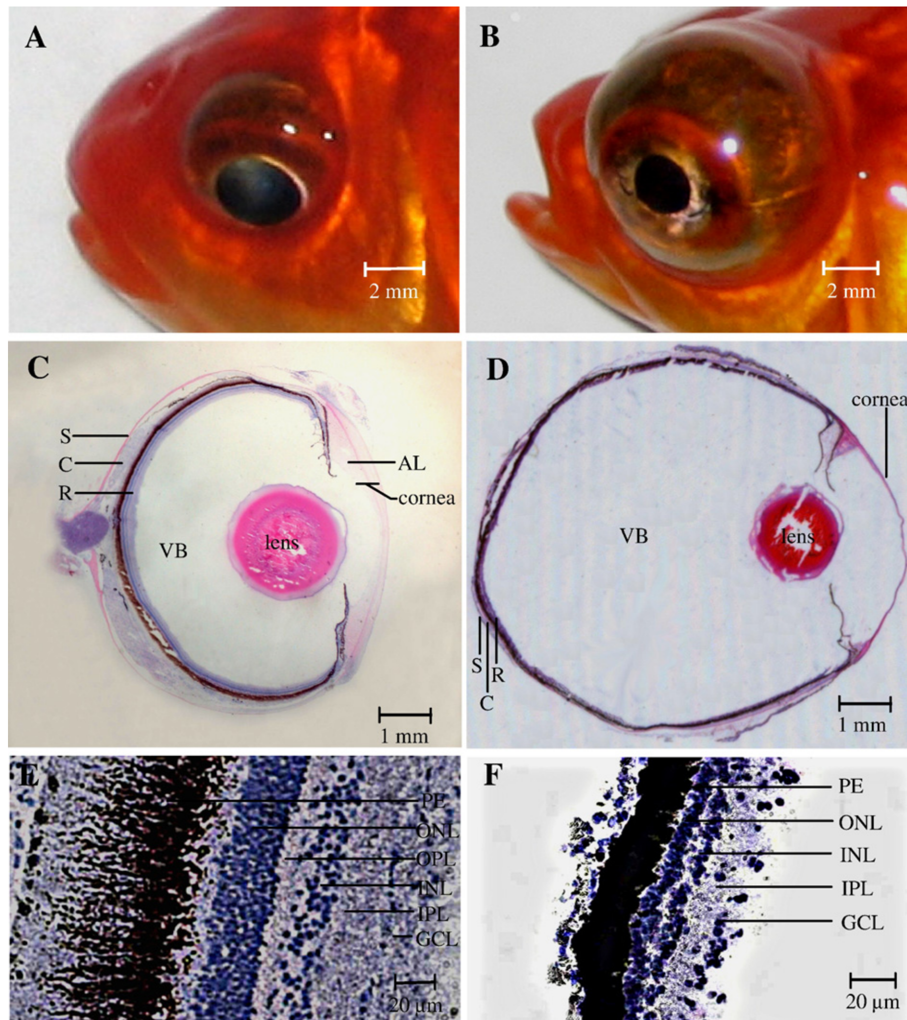


Fig. 1. Comparative and anatomic observation of normal eye and dragon eye. (A) and (B) show lateral observation of normal eye (A) and dragon eye (B). (C) and (D) show transversal section of normal eye (C) and dragon eye (D), stained by hematoxylin–eosin. (E) and (F) show retina of normal eye (E) and dragon eye (F). The scales are indicated in lower right corner. VB, vitreous body; AL, autochthonous layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PE, pigmented epithelium; S, sclera; C, choroids; R, retina.

2.6. Immunofluorescence localization

To investigate *Six3* expression pattern and developmental behavior, we performed immunoflourescence localization for the goldfish eyes at different developmental stages. The eyes were embedded in O.C.T. and immediately frozen in liquid nitrogen. The embryos were freshly immersion-fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.0) at 4 °C overnight and then protected by immersion in 30% sucrose solution in PBS buffer overnight at 4 °C. After embedding in O.C.T. at −25 °C, all the samples were sectioned 8–12 μm thick using frozen microtome (Leica) and mounted on 0.1% poly-L-lysine-coated slides and air-dried. The sections were rehydrated in PBS for 30 min, and incubated in 5% nonfat dry milk in PBS at room temperature for 1 h to prevent nonspecific binding of antibodies. The sections were then incubated in 1% nonfat dry milk in PBS containing the rabbit antiserum (1:100 dilution) at 4 °C overnight, and washed 5 times for 5 min with PBST. After incubating in FITC-conjugated secondary antibody (goat anti-rabbit IgG, 1:100 dilution, Pierce) in darkness for 1 h, it was washed 5 times for 10 min with PBS. Then, the sections were stained with propidium iodide for 10 min and washed 4 times for 5 min with PBS to show cell nuclear localization. Finally, the sections were observed with a Leica confocal fluorescence microscope.

3. Results

3.1. Structural difference between normal eye and dragon eye in goldfish

In comparison with normal eye (Fig. 1A), dragon eye (Fig. 1B) is characterized as enlarged eye protruding out of eye-

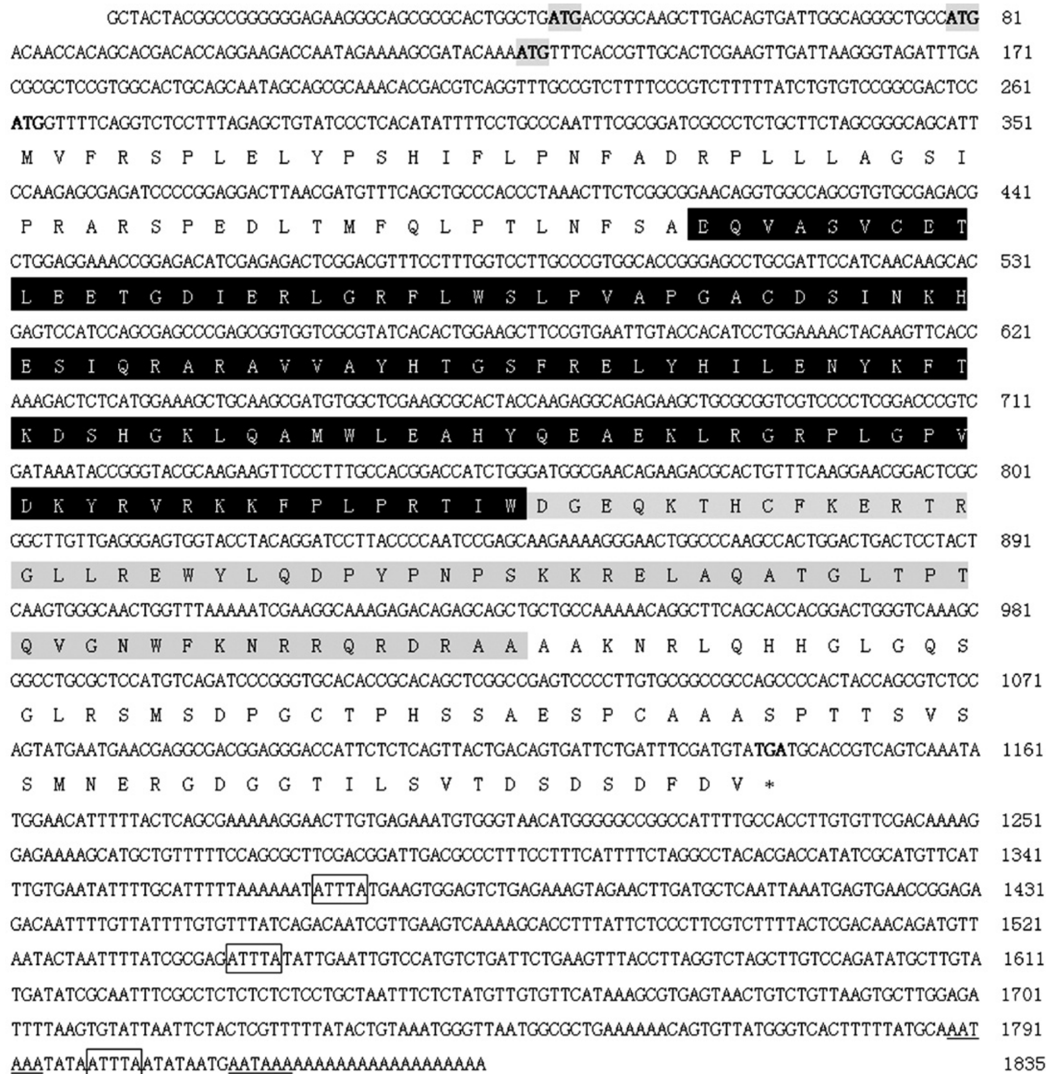


Fig. 2. Nucleotide sequence and the deduced amino acid sequence of goldfish *six3*. The boldfaces show the start codon (ATG) and the terminal codon (TGA). The first 3 ATG sequences in the 5′-terminus of *Six3* cDNA are also indicated in both shadows and boldface, but they are incapable of initiating long enough ORFs. The polyadenylation signals (AATAAA) are indicated with underlines and the motifs associated with mRNA instability (ATTTA) are shown in boxes. The *Six* domains (residues 52–166) and the homeodomain (residues 167–226) are shown in black and gray, respectively.

socket. The transversely anatomical observation revealed significant structural difference between the two types of eyes. As shown in Fig. 1C and D, the size of dragon eye is larger than that of common eye mainly because the vitreous body (VB) is expanded and the eyeball wall becomes thinner. The cornea in dragon eye is similar to that in normal eye, but the cornea curvature increases in dragon eye. In contrast to the eyeball enlargement, the lens volume of dragon eye becomes smaller than that of normal eye, and the configuration also appears abnormal in dragon eye.

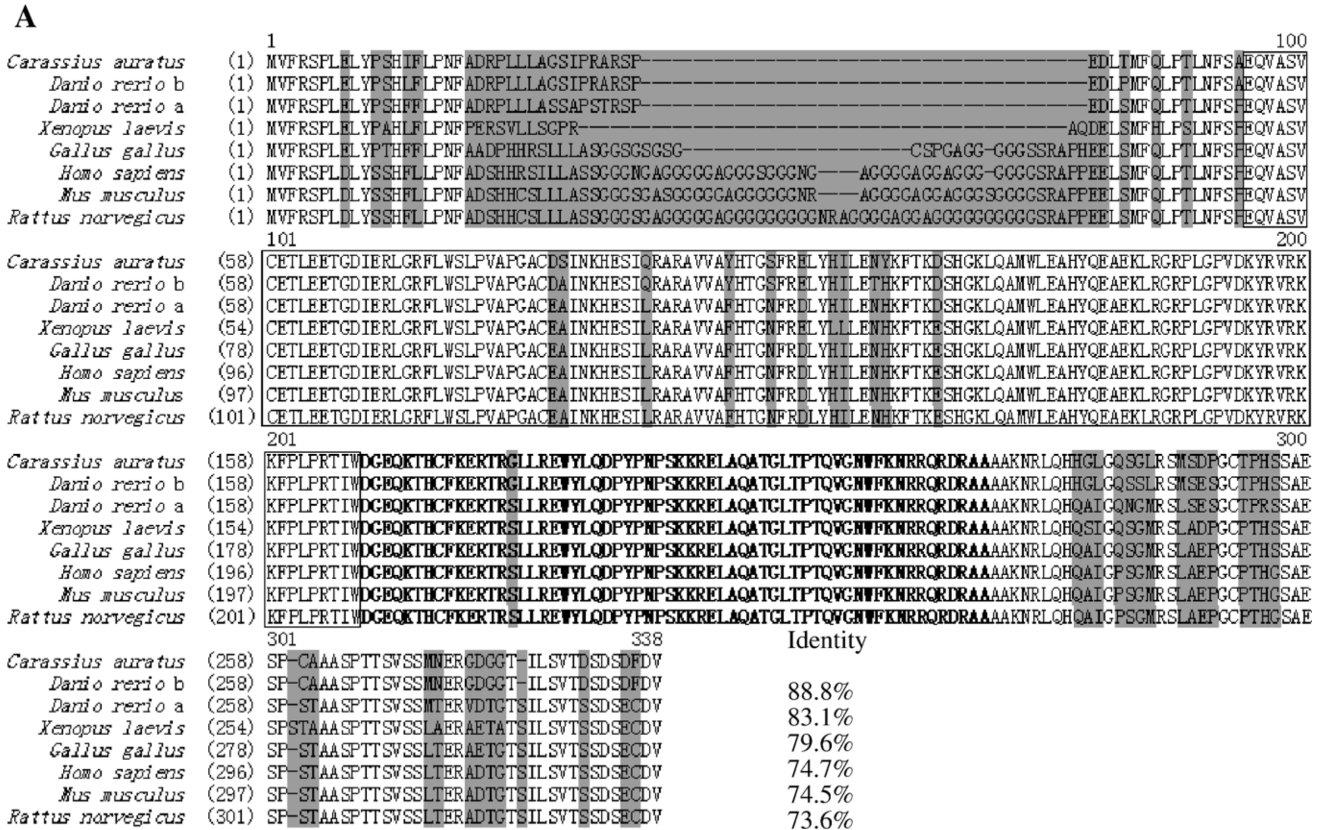


Fig. 3. (A) Multiple alignment of the deduced amino acid sequences of goldfish Six3 (accession number EF174420) with seven known vertebrate Six3 sequences [*D. rerio 3b* (BC059425), *D. rerio 3a* (BC059414), *G. gallus* (Y15106), *Xenopus laevis* (AF183571), *H. sapiens* (NM_005413), *M. musculus* (NM_011381), *Rattus norvegicus* (NM_023990)] from GenBank by Vector NTI 8.0 software. The putative *Six* domain is shown in boxes and the homeodomain in boldface. The diverse amino acid sites are indicated in shadow. (B) Diagrammatic representation of the percentages of amino acid homologies of the seven different Six3 proteins when compared with the goldfish. The homeodomain is shown in black (60 amino acids), while the conserved upstream *Six* domain is in grey (115 amino acids). The hollow boxes represent other coding sequence of these genes.

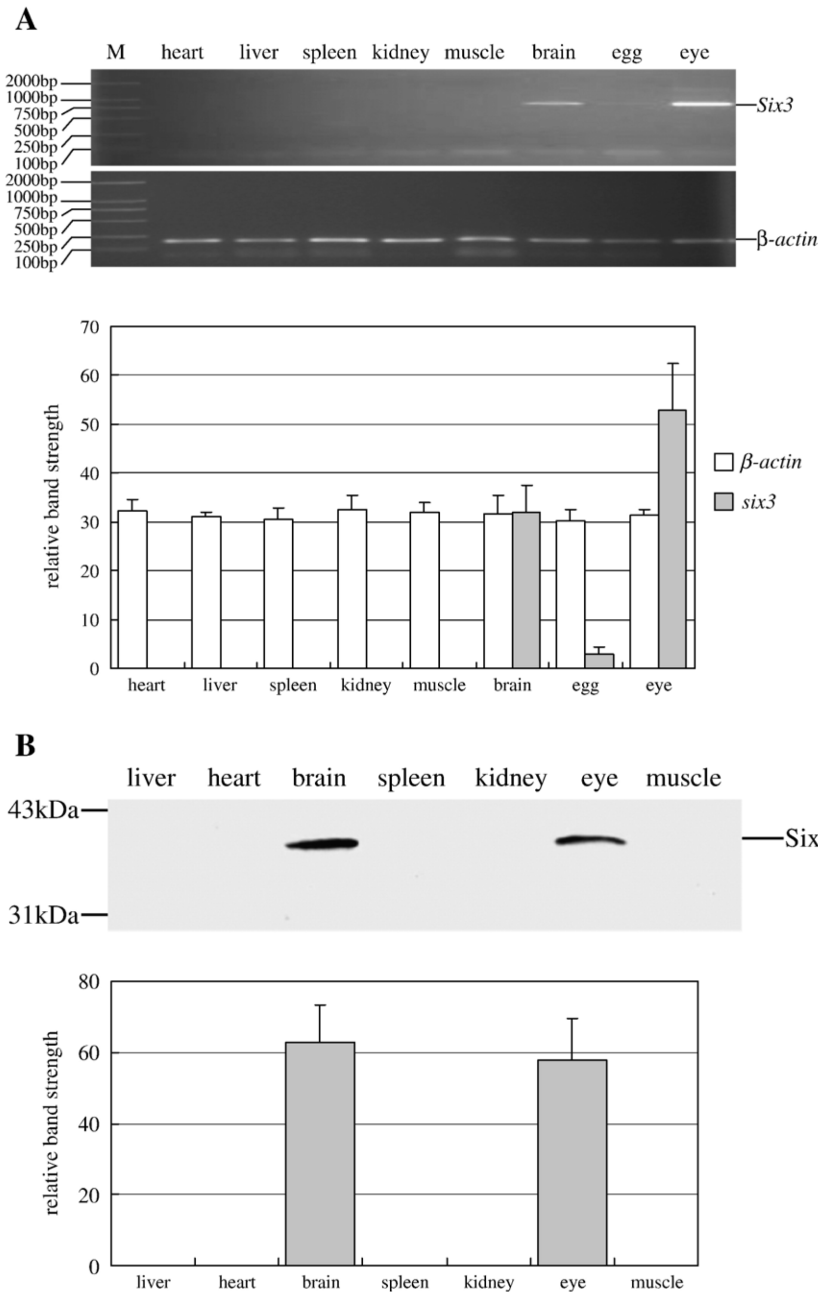
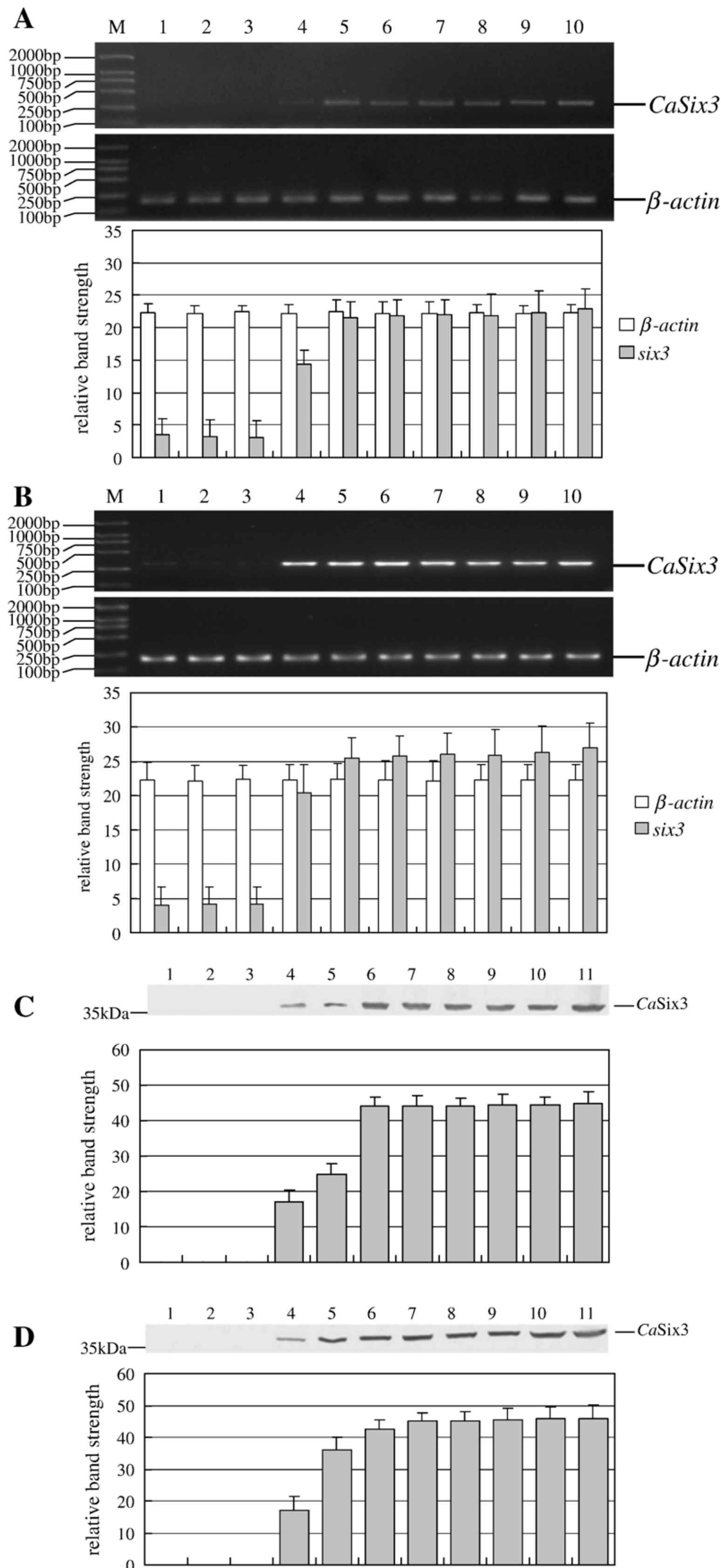


Fig. 4. RT-PCR (A) and Western blot (B) analysis of *Six3* expression in various tissues of dragon eye goldfish. β-actin was used as control to adjust the concentration of the template. M is DL2000. The histograms are the quantification data from three individuals for both RT-PCR and Western blot analysis. Relative band strengths were calculated by statistical analysis of software Band Leader Version 3.0. The values represent the means ± standard deviation, and the standard deviation was shown by the bar.

Further magnification of the dragon eye retina observed quite abnormal cytoarchitecture. As shown in Fig. 1E and F, the retina in dragon eye is obviously thinner than that in normal eye, and the outer nuclear layer is so close to the inner nuclear layer that the outer plexiform layer is difficult to distinguish (Fig. 1F). Moreover, there are fewer cell-layers in ONL, INL and GCL of

Fig. 5. RT-PCR and Western blot analysis of *Six3* mRNA and protein during embryogenesis. (A) shows *Six3* and β-actin (as a control) transcripts in different stage embryos of normal eye goldfish. (B) shows *Six3* and β-actin (as a control) transcripts in different stage embryos of dragon eye goldfish. (C) and (D) show Six3 proteins in different stage embryos of normal eye goldfish (C) and dragon eye goldfish (D). M, DL2000. 1 to 11 indicate 11 samples for mature eggs (1), fertilized eggs (2), blastula embryos (3), gastrula embryos (4), optic vesicle embryos (5), tail bud embryos (6), lens embryos (7), heartbeat embryos (8), hatching larvae (9), and hatched larvae (10) and 1 day old larvae (11). The histograms are the quantification data from three independent experiments for both RT-PCR and Western blot analysis. Relative band strengths were calculated by statistical analysis of software Band Leader Version 3.0. The values represent the means ± standard deviation, and the standard deviation was shown by the bar.

dragon eye retina (Fig. 1F) than in the normal eye retina (Fig. 1E). The data imply that the dragon eye enlargement might have resulted from a stretching of the retina, because the dragon eye retina was thinner, and the retina area was larger. In contrast to normal eye (Fig. 1E), the extending of the pigmented epithelium and the outer segments of rod cells and cone cells



(photoreceptor cells) were arrayed in disorder in dragon eye (Fig. 1F).

3.2. Molecular characterization of the goldfish Six3

Goldfish full-length *Six3* cDNAs were cloned from dragon eye (accession number EF174420) and normal eye (accession number EF174421) goldfish, respectively. As shown in Fig. 2, the full-length *Six3* cDNA is 1835 bp. The open reading frame is composed of 882 bp encoding 293 amino acids, and is identical between dragon eye and normal eye goldfish. It has a 261 bp 5′ untranslated region (UTR) and a 692 bp 3′UTR containing two canonical polyadenylation signal sequences (AATAAA). Three motifs associated with mRNA instability (ATTTA) were also found in the 3′ UTR.

Multiple alignments show that the goldfish Six3 amino acid sequence exhibits high homology to other vertebrate Six3 sequences (Fig. 3A). The mid 175 amino acids consist of a highly divergent putative homeodomain and a highly conserved *Six* domain shared by all *Six* family members. As shown in Fig. 3B, there is 100% identity in the homeodomain between goldfish Six3 and *D. rerio* Six3b, and 98.3% to the homeodomains of other 6 species. The identities of the *Six* domain homologies between goldfish and the other seven species range from 97.4% (*D. rerio* Six3b) to 93.0% (*G. gallus* Six3, *H. sapiens* Six3, *M. musculus* Six3, *Rattus norvegicus* Six3, *X. laevis* Six3), indicating that goldfish *Six3* belongs to the *Six3* family, similar to *D. rerio Six3*b.

Moreover, the *Six3* 5′UTR and 3′UTR sequences were compared between dragon eye goldfish and normal eye goldfish. The 3′UTR sequences from 3 normal eye goldfish and 3 dragon eye goldfish had 99.4% identity between them. However, in the 5′UTR sequences, the identity of 8 sequences from normal eye goldfish was 91.0%, and 8 dragon eye goldfish 90.3%, and a lower identity value 89.5% exhibited among all of them (data not shown).

3.3. Expression patterns of Six3 in various tissues and embryogenesis

RT-PCR analysis revealed predominant *Six3* transcription in eye and brain, and lower transcription level in eggs, but not in heart, liver, spleen, kidney and muscle of the goldfish (Fig. 4A). Western blot also detected a specific Six3 protein in eye and brain, but not in heart, liver, spleen, kidney and muscle (Fig. 4B). The Six3 protein band was slightly larger than the estimated molecular weight of 33 kDa, suggesting that some kinds of protein modification might occur in the Six3 protein. The expression pattern was similar between the dragon eye goldfish and the normal eye goldfish (data not shown).

Following the above observation, *Six3* expression pattern was analyzed in goldfish embryos at different developmental stages. As shown in Fig. 5A and B, a small quantity of maternal *Six3* mRNAs exist in mature eggs, and the zygotic *Six3* begins to transcribe from gastrula embryos. Then, the transcripts keep a stable level during embryogenesis, which is consistent with the known earlier expression pattern of *Six3* gene in other vertebrates. Similar to mRNA transcription pattern, *Six3* protein is also detected first in gastrula embryos (Fig. 5C, D) by Western blot. And no significant difference was revealed between the normal eye goldfish and the dragon eye goldfish.

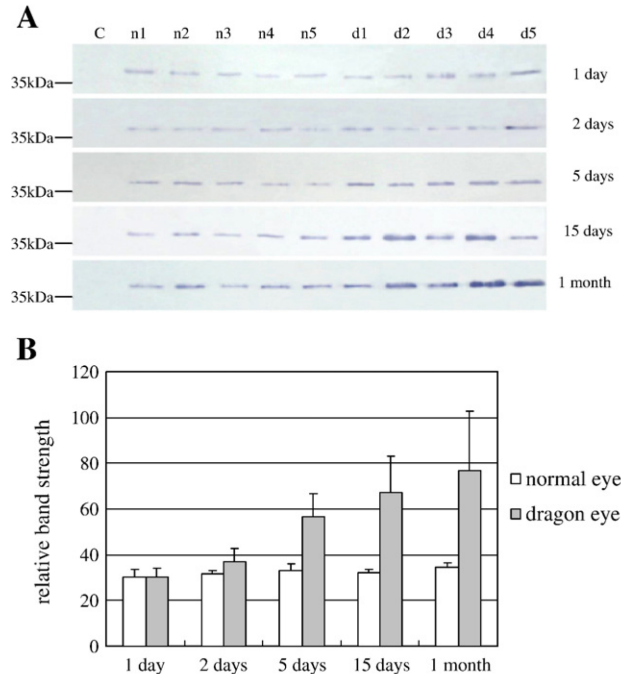


Fig. 6. (A) Western blot analysis of Six3 protein in single eye of the hatched larvae of dragon eye goldfish and normal eye goldfish at different time points, such as 1 day, 2 days, 5 days, 15 days and 1 month. C, loading buffer without protein extraction; n, normal eye (n1 to n5); d, dragon eye (d1 to d5). Equivalent protein extract from each eye was detected. The left shows standard protein molecular weight marker, and the right shows different time points. (B) Quantitative and statistical data of 20 single eye Six3 protein bands from the hatched larvae of dragon eye goldfish and normal eye goldfish at different time points. *X*-axis shows different time points of the hatched larvae, and *Y*-axis shows relative band strength. Relative band strengths were calculated from three independent experiments by statistical analysis of software Band Leader Version 3.0 based on the band strength values of normal eyes at 1 day old goldfish. The values represent the means ± standard deviation, and the standard deviation was shown by the bar.

3.4. Higher expression of Six3 in dragon eye than in normal eye

To reveal the association between *Six3* expression and dragon eye development, the eyes were individually isolated from the hatched larvae of dragon eye goldfish and normal eye goldfish at 1 day, 2 days, 5 days, 15 days and 1 month, and subjected to Western blot analysis. As shown in Fig. 6A, the Six3 expression level of dragon eyes is similar to that of the normal eyes in 1 day larvae, but higher expression is observed from the second day in dragon eye larvae than in normal eye larvae. For example, in 5 days old larvae, 90% of dragon eyes show higher expression content. From 15 days to 1 month, the higher expression levels are detected in dragon eyes than in normal eyes. Quantitative and statistical analysis further confirmed the above description. As shown in Fig. 6B, the average Six3 contents of dragon eyes are about 1.70, 2.10 and

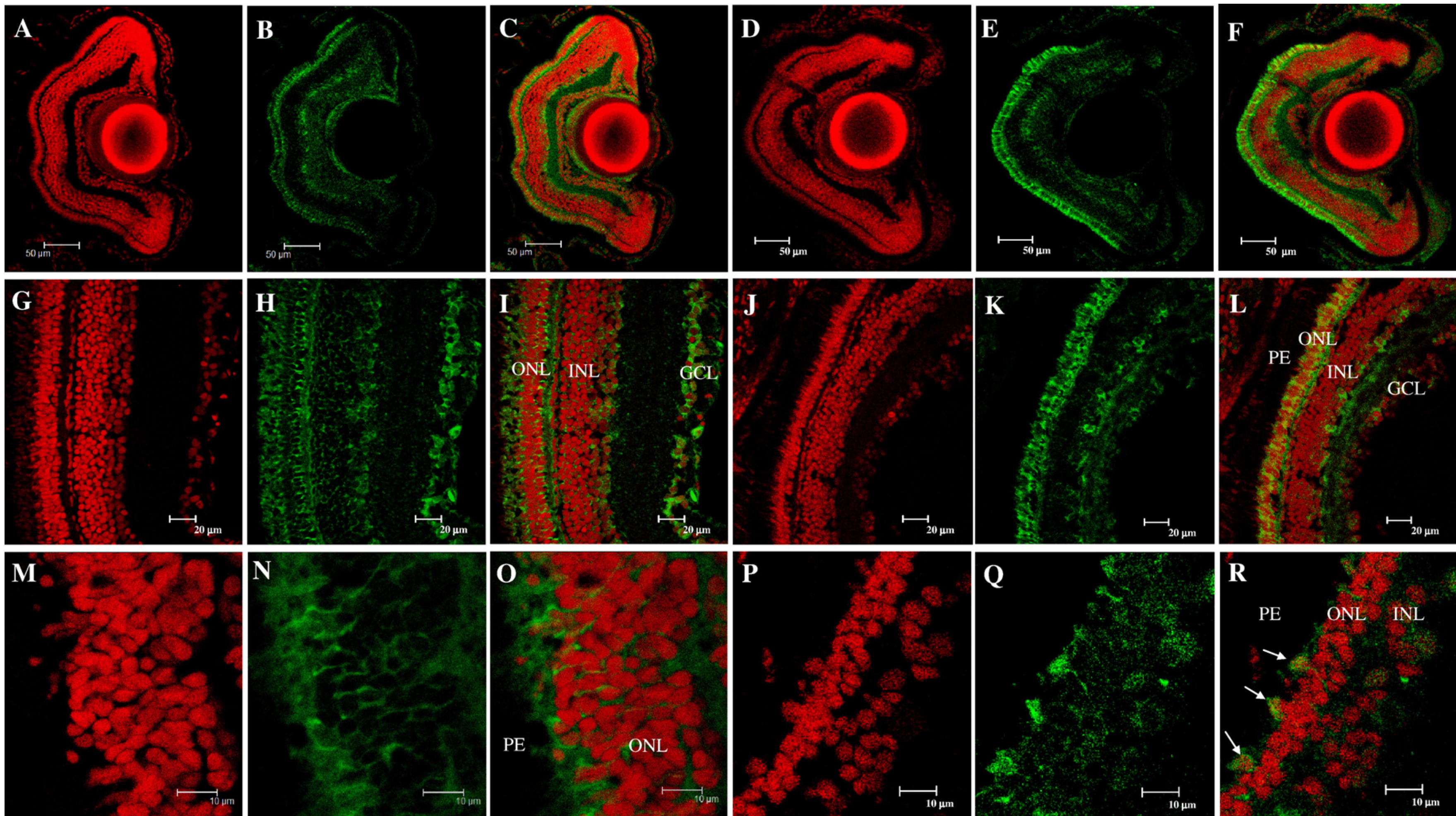


Fig. 7. Immunofluorescence localization of Six3 protein in normal (A–C, G–I, M–O) and dragon eye (D–F, J–L, P–R) at 5 day old (A–F), 1 month old (G–L), and 2 month old (M–R)) larvae. The scales are indicated in lower corner. From Fig. A to R, three panels are divided in a group. In each group, red in first panel is PI staining for nuclei, and green in second panel is FITC showing the localization of Six3 protein, and the last one merge the two previous ones. Note: GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PE, pigmented epithelium. The arrows in the panel R show Six3 positive cone cells.

2.22 times higher than that of normal eyes in the 5 day, 15 day and 1 month goldfish larvae respectively.

The significant Six3 expression difference between dragon eye and normal eye goldfish was further confirmed by double staining localization of propidium iodide for nucleus and green immunofluorescence for Six3 protein. In 5 day old larva of normal eye goldfish, Six3 protein distributed mainly in outer nuclear layer cells, ganglion cell layer cells and surrounding cells of lens (Fig. 7A–C). In comparison with the normal eye, ectopic high expression of Six3 protein was detected predominantly in the outer nuclear layer cells of dragon eye (Fig. 7D–F), whereas there was no obvious difference in other cell layers between normal eye and dragon eye. Along with development progress, more predominant Six3 distribution was observed in the outer nuclear layer cells of dragon eye (Fig. 7J–L) than that of normal eye (Fig. 7G–I) at 1 month old larvae, and significant anatomical difference was detected between dragon eye and normal eye, even though no phenotypic difference appeared at that stage. In comparison with normal eye (Fig. 7G–I), the dragon eye retina stretched and thinned, and predominant Six3 distribution was significantly observed in the outer nuclear layer cells of dragon eye (Fig. 7J–L). Moreover, when morphological difference appeared in the dragon eye at two months after hatching, more significantly cellular distribution changes were detected in the retina. Similarly to the differences observed previously in adults (Fig. 1), fewer cell-layers in outer nuclear layer were observed in dragon eye retina (Fig. 7P–R) than in normal eye retina (Fig. 7M–O), and some Six3 positive cone cells were found to scatter between outer nuclear layer and pigmented epithelium of the dragon retina (Fig. 7R).

4. Discussion

In this study, goldfish *Six3* was cloned and analyzed, and the putative Six3 protein exhibits 97.3% identity to *D. rerio* Six3b, with a homeodomain (residues 167–226) and a *Six* domain (residues 52–166). The same Six3 protein sequences exist in dragon eye goldfish and normal eye goldfish. Their 3′ UTR sequences are very conserved, and the identity reaches 99.4%. However, their 5′ UTR sequences are variable. The highlight of this study is that higher *Six3* expression exists in dragon eye goldfish than in normal eye goldfish during retinal development of larvae. Previous reports indicated that low concentration of Six3 could induce *X. leavis* retina enlargement (Bernier et al., 2000), and that direct interaction of Six3 and geminin could adjust cell proliferation and differentiation, and regulate the delicate balance that leads to the precise control of organ size during eye development (Bene et al., 2004). In comparison with previous transgenic and knock-down data in model animals including *O. latipes*, zebrafish and *X. leavis* (Oliver et al., 1996; Oliver and Gruss, 1997; Kobayashi et al., 1998; Loosli et al., 1999; Bernier et al., 2000; Lagutin et al., 2003), the current study revealed higher expression of *Six3* in the dragon eye formation than in the normal eye development, and thereby confirmed the functional role of Six3 in the process of eye development and formation.

During embryogenesis, anatomic structure and Six3 expression pattern of the dragon eye is similar to that of the normal eye. After two days of hatching, retina cells begin to differentiate, and consequently to form outer nuclear layer, inner nuclear layer and ganglion cell layer. Just at the same time, ectopic *Six3* expression happens predominantly in the outer nuclear layer cells of dragon eye. With eye development, more predominant Six3 distribution was detected in the outer nuclear layer cells of dragon eye than that of normal eye, and fewer cell-layers in outer nuclear layer were observed in dragon eye retina than in normal eye retina. It is well known that teleostean retinal growth continues postembryonically at the interface between neural retina and ciliary epithelium, a region called ciliary marginal zone (CMZ), and the CMZ activity is largely responsible for the growth of the eye in larvae and adults (Hitchcock and Raymond, 2004). As shown in Fig. 7, some Six3 positive cone cells are indeed found to scatter between outer nuclear layer and pigmented epithelium of the dragon retina. Therefore, the current Western blot and immunofluorescence localization data suggest that the ectopic high expression of Six3 in outer nuclear layer of dragon eye might be related to the dragon eye formation. Of course, further investigation, such as BrdU labeling during the stages of increase in the size of the eye, will be necessary and might provide interesting data for the issue.

Six3 has been revealed to have two paralogues in teleosts, suggesting that the two *Six3* paralogues might cover *Six3* as well as part of the mammalian *Six6* functions (Conte and Bovolenta, 2007). In the current study, we only characterized a single *Six3* paralogue. Therefore, additionally molecular work will be required to exploit the interesting and fascinating potentials in the goldfish system.

Acknowledgment

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References

Bene, F.D., Tessmar-Raible, K., Wittbrodt, J., 2004. Direct interaction of geminin and Six3 in eye development. Nature 427, 745–749.

Bernier, G., Panitz, F., Zhou, X.L., Hollemann, T., Gruss, P., Pieler, T., 2000. Expanded retina territory by midbrain transformation upon overexpression of Six6 (Optx2) in Xenopus embryos. Mech. Dev. 93, 59–69.

Bovolenta, P., Mallamaci, A., Puelles, L., Boncinelli, E., 1998. Expression pattern of cSix3, a member of the Six/sine oculis family of transcription factors. Mech. Dev. 70, 201–203.

Carl, M., Loosli, F., Wittbrodt, J., 2002. Six3 inactivation reveals its essential role for the formation and patterning of the vertebrate eye. Development 129, 4057–4063.

Chen, S.C., 1928. Transparency and mottling: a case of Mendelian inheritance in the goldfish, *Carassius auratus*. Genetics 13, 434–452.

Cheyette, B.N.R., Green, P.J., Martin, K., Garren, H., Hartenstein, V., Zipursky, S.L., 1994. The Drosophila sine oculis locus encodes a homeodomain-containing protein required for the development of the entire visual system. Neuron 12, 977–996.

Conte, I., Bovolenta, P., 2007. Comprehensive characterization of the cis-regulatory code responsible for the spatio-temporal expression of olSix3.2 in the developing medaka forebrain. Genome Biol. 8, R137.

Dong, C.H., Yang, S.T., Yang, Z.A., Zhang, L., Gui, J.F., 2004. A C-type lectin associated and translocated with cortical granules during oocyte maturation and egg fertilization in fish. Dev. Biol. 265, 341–354.

Ghanbari, H., Seo, H.C., Fjose, A., Brändli, A.W., 2001. Molecular cloning and embryonic expression of Xenopus Six homeobox genes. Mech. Dev. 101, 71–277.

Granadino, B., Gallardo, M.E., López-Ríos, J., Sanz, R., Ramos, C., Ayuso, C., Bovolenta, P., Rodríguez de Córdoba, S., 1999. Genomic cloning, structure, expression pattern, and chromosomal location of the human SIX3 gene. Genomics 55, 100–105.

Hitchcock, P.F., Raymond, P.A., 2004. The teleost retina as a model for development and regeneration biology. Zebrafish 1, 257–271.

Kobayashi, M., Toyama, R., Takeda, H., Dawid, I.B., Kawakami, K., 1998. Overexpression of the forebrain-specific homeobox gene six3 induces rostral forebrain enlargement in zebrafish. Development 125, 2973–2982.

Kobayashi, M., Nishikawa, K., Suzuki, T., Yamamoto, M., 2001. The homeobox protein Six3 interacts with the Groucho corepressor and acts as a transcriptional repressor in eye and forebrain formation. Dev. Biol. 232, 315–326.

Lagutin, O.V., Zhu, C.C., Kobayashi, D., Topczewski, J., Shimamura, K., Puelles, L., Russell, H.R.C., McKinnon, P.J., Solnica-Krezel, L., Oliver, G., 2003. Six3 repression of Wnt signaling in the anterior neuroectoderm is essential for vertebrate forebrain development. Genes Dev. 17, 368–379.

Li, X., Perissi, V., Liu, F., Rose, D.W., Rosenfeld, M.G., 2002. Tissue-specific regulation of retinal and pituitary precursor cell proliferation. Science 297, 1180–1183.

Liu, W., Lagutin, O.V., Mende, M., Streit, A., Oliver, G., 2006. Six3 activation of Pax6 expression is essential for mammalian lens induction and specification. EMBO J. 25, 5383–5395.

Loosli, F., Köster, R.W., Carl, M., Krone, A., Wittbrodt, J., 1998. SIX3, a medaka homologue of the Drosophila homeobox gene sine oculis is expressed in the anterior embryonic shield and the developing eye. Mech. Dev. 74, 159–164.

Loosli, F., Winkler, S., Wittbrodt, J., 1999. Six3 overexpression initiates the formation of ectopic retina. Genes Dev. 13, 649–654.

Lopez-Rios, J., Tessmar, K., Loosli, F., Wittbrodt, J., Bovolenta, P., 2003. Six3 and Six6 activity is modulated by members of the groucho family. Development 130, 185–195.

Oliver, G., Gruss, P., 1997. Current views on eye development. Trends Neurosci. 20, 415–421.

Oliver, G., Loosli, F., Köster, R., Wittbrodt, J., Gruss, P., 1996. Ectopic lens induction in fish in response to the murine homeobox gene Six3. Mech. Dev. 60, 233–239.

Oliver, G., Mailhos, A., Wehr, R., Copeland, N.G., Jenkins, N.A., Gruss, P., 1995. Six3, a murine homologue of the sine oculis gene, demarcates the most anterior border of the developing neural plate and is expressed during eye development. Development 121, 4045–4055.

Seo, H.C., Drivenes, Ø., Ellingsen, S., Fjose, A., 1998. Expression of two zebrafish homologs of the murine Six3 gene demarcates the initial eye primordia. Mech. Dev. 73, 45–57.

Serikaku, M.A., O'Tousa, J.A., 1994. Sine oculis is a homeobox gene required for Drosophila visual system development. Genetics 138, 1137–1150.

Stierwald, M., Yanze, N., Bamert, R.P., Kammermeier, L., Schmid, V., 2004. The Sine oculis/Six class family of homeobox genes in jellyfish with and without eyes: development and eye regeneration. Dev. Biol. 274, 70–81.

Tessmar, K., Loosli, F., Wittbrodt, J., 2002. A screen for cofactors of Six3. Mech. Dev. 117, 103–113.

Wu, H.S., Fu, Y.Y., 1997. Chinese Goldfish. Tianjin Science and Technology Press. (in Chinese).

Zhang, Y.B., Gui, J.F., 2004. Molecular characterization and IFN signal pathway analysis of *Carassius auratus* CaSTAT1 identified from the cultured cells in response to virus infection. Dev. Comp. Immunol. 28, 211–227.

Zuber, M.E., Perron, M., Philpott, A., Bang, A., Harris, W.A., 1999. Giant eyes in *Xenopus laevis* by overexpression of XOptx2. Cell 98, 341–352.