Homeobox transcription factor Six7 governs expression of green opsin genes in zebrafish

Yohey Ogawa†, Tomoya Shiraki†, Daisuke Kojima and Yoshitaka Fukada

Department of Biological Sciences, Graduate School of Science, The University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033, Japan

†These authors contributed equally to this study.

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1. Introduction

In vertebrates, vision is triggered by absorption of photons by visual opsins localized in outer segments of retinal photoreceptor cells. The photoreceptor cells are classified into rod and cone cells, which are distinct from each other in morphology and photoreponse [1–3]. Rods are responsible for twilight vision, while cones function under the daylight condition. Combination of multiple cone subtypes (i.e. UV-, blue-, green- and red-sensitive cones) confers many vertebrates with high-acuity colour vision [4,5], which is based on mutually exclusive expression of one subtype of the opsin in single cells. One of the key vertebrate species that helps us understand visual system evolution is the Southern Hemisphere lamprey, Geotria australis: it is a jawless vertebrate whose ancestor diverged from the lineage leading to jawed vertebrates probably before 500 Ma [6], and it has four subtypes of the cone opsins [7], namely UV (short wavelength sensitive 1: SWS1), blue (short wavelength sensitive 2: SWS2), green (medium wavelength sensitive: RH2) and red (long wavelength sensitive: M/LWS). The ancestral species of the jawed vertebrate is thus thought to have acquired four spectrally distinct cone subtypes by two rounds of chromosome duplications and appears to have had the tetrachromatic colour vision system [8].

The developmental process of visual photoreceptors has been well studied in mice, and important transcription factors governing the development of the photoreceptors have been identified [9,10]. In particular, cone-rod homeobox (Crx) is a master transcriptional regulator for differentiation of retinal progenitor cells into photoreceptor cells [11–13]. Crx determines the cell fate of the photoreceptor progenitor cells in combination with other transcription factors such as thyroid hormone receptor beta (Thrb), which regulates development of the cone photoreceptors expressing M/LWS opsin gene [14]. On the other hand, transcriptional regulatory mechanisms of SWS2 and RH2 opsin genes remain elusive because
mammals lost SWS2 and RH2 opsin genes during evolution. Hence, the vertebrate species having all four cone subtypes provide an excellent platform to study detailed mechanisms of the development of the four cone photoreceptors.

Zebrafish (Danio rerio) is a diurnal teleost having four cone subtypes, and it is a valuable animal model to study genetic mechanisms of vertebrate retinal development and diseases [15]. Recent studies isolated the promoter and/or enhancer subtypes, and it is a valuable animal model to study genetic development of the four cone photoreceptors.

Sine oculis homeobox homolog 7 (six7) is a group of evolutionarily conserved transcription factors found in diverse organisms ranging from worms to humans [22]. Previous studies reported that six7 is expressed during gastrulation and early segmentation [23], and that combined inactivation of two Six3/6/7 group members, Six7 is found in ray-finned fish but not in mammals and vertebrate species share a mechanism underlying exclusive expression of the opsin genes in cone cells. Despite these advances, studies so far have been unable to identify any transcription factors responsible for expression of SWS2 and RH2 in vertebrates.

To understand regulation of six2 and rh2 opsin genes, we searched for cone-specific transcription factors in zebrafish and found that sine oculis homeobox homolog 7 (six7) is predominantly expressed in cone photoreceptors. The Six family is a group of evolutionarily conserved transcription factors found in diverse organisms ranging from worms to humans [22]. Previous studies reported that six7 is expressed during gastrulation and early segmentation [23], and that combined inactivation of two Six3/6/7 group members, six7 and six3b, results in loss of the eyes [24]. Despite the involvement of six7 in early eye development, it still remains unclear whether and how six7 is engaged in cone photoreceptor development. We investigated roles of six7 in cone development and in cone opsin expression by generating six7 knock-out (KO) zebrafish with TAL effector nucleases (TALENs). The six7 KO zebrafish lost expression of the green opsin at both larval and adult stages, suggesting that six7 is indispensable for the development of the green cones.

2. Material and methods

Full methods and any associated references are available in the electronic supplementary material, methods.

(a) Zebrafish

Zebrafish were treated in accordance with the guidelines of the University of Tokyo. The wild-type (WT) zebrafish strain Ekk-Will were raised in a 14:1:10 D cycle, and fed twice per day with living baby brine shrimp. Embryos were raised at 28.5°C in egg water (artificial seawater diluted 1.5:1000 in water).

(b) Construction of TAL effector nuclease targeting-vector

We designed TAL effector repeats recognizing exon 1 of zebrafish six7 gene with the Golden Gate method [25]. These repeats were cloned into our original vectors (pTAL7DD and pTAL7RR), which were modified from pTAL3 vector (Addgene plasmid 31034), based on pCS2TAL3DD and pCS2TAL3RR [26]. Information about construction of pTAL7DD and pTAL7RR vectors is in the electronic supplementary material, methods.

(c) Immunohistochemistry and in situ hybridization

Immunohistochemistry and in situ hybridization were performed as described previously [27–30] with some modifications. Detailed information about the methods is in the electronic supplementary material, methods.

(d) Isolation of rods and cones

EGFP-positive rods and cones were isolated by fluorescence activating cell sorter (FACS) from the adult retinas of the two lines of transgenic zebrafish, Tg(rho:egfp)ja2 [31] and Tg(gnat2:egfp)ja23, respectively. These lines express green fluorescent proteins in rods and all the cone subtypes, respectively. Tg(gnat2:egfp)ja23 was generated according to the method described in the previous study [32]. Detailed procedures for isolation of photoreceptors are described in the electronic supplementary material, methods.

(e) RNA extraction and RT-qPCR

Total RNA was extracted and purified using RNeasy Micro Kit (74004, Qiagen) from the eyes of adult or larval zebrafish. The ocular RNA (450 ng for the adult eye or 90 ng for the larval eye) was reverse-transcribed into cDNA using the oligo (dT)15 primer with GoScript Reverse Transcriptase (A5003, Promega). The cDNA was subjected to quantitative PCR using GoTaq qPCR Master Mix (A6001, Promega) with the StepOnePlus Real-time PCR system (Applied Biosystems), following the manufacturer’s protocol. The gene-specific primers used for RT-qPCR are listed in the electronic supplementary material, table S3. In all the figures, the mRNA levels were normalized to beta-actin 2 (actb2) mRNA levels.

3. Results

(a) Cone-specific expression of six7

In microarray analysis, we compared gene expression profiles between rod and cone cells purified from the adult zebrafish retina (yet to be published). Among approximately 500 genes that were found to be abundantly expressed in cones (more than 10 times higher than in rods), we focused on sine oculis homeobox homolog 7 (six7), whose signal intensity in cones was the highest among the cone-enriched transcription factors in our microarray analysis. Six7 belongs to Six family, and Six7 is found in ray-finned fish but not in mammals (figure 1). The phylogenetic tree showed that, among Six3/6/7 group, Six7 subfamily was separated before the divergence between Six3 and Six6 subfamilies, both of which are widely conserved in vertebrate species (figure 1).

We investigated the expression pattern of six7 in zebrafish. RT-qPCR assay confirmed that six7 was far more abundantly expressed in cones than in rods (more than 10 times; figure 2a). Among the adult tissues, six7 was predominantly expressed in the retina (figure 2b). In situ hybridization analysis of the retina indicated that six7 was expressed specifically in the photoreceptor layer (figure 2f). In particular, the six7-positive cells were mainly located in the layer of the red and green cones (figure 2e), suggesting that six7 regulates transcription in the green and red cones.

We then analysed expression profiles of six7 during the retinal development. At 4 days post fertilization (4 dpf), six7 expression was detected in the anterior regions of the
latter (including the eyes), but not in the posterior region (figure 2c). Eye-specific expression of six7 was confirmed by whole-mount in situ hybridization (figure 2f). The six7 expression in the eye began at 1.5 dpf, when the retinal development just starts, suggesting the six7 expression in the retinal progenitor cells. In situ hybridization using 3.5 dpf zebrafish ocular sections revealed that six7-positive cells were localized in the photoreceptor cell layer, in which the positive cells were distributed uniformly across the whole retina (figure 2g). Considering that rods are tightly clustered together in the ventral retina at this developmental stage [33] (see also figure 3c), the expression pattern of six7 was similar to that of the cone-specific phototransduction component genes. Although we cannot rule out the possibility that six7 is also expressed in rods at the larval stages, these results imply that six7 is predominantly expressed in cones at 3.5 dpf and suggest that six7 plays a role in the cone development.

(b) Decrease of green and blue cones in the larva of six7 knock-out zebrafish

To examine the role of six7 in photoreceptor development, we generated KO zebrafish with TALENs. The KO zebrafish had a mutation in the six7 coding region; the ja51 mutation caused a frameshift of the amino acid sequence of Six7 by 8 bp loss, thereby introducing an immature stop codon (figure 3a,b). The KO zebrafish apparently had the normal body shape throughout the developmental stages (data not shown).

We examined expression levels of phototransduction component genes in the eyes of the WT zebrafish and six7ja51/ja51 KO zebrafish larvae by RT-qPCR. Notably, mRNA levels of the middle wavelength-sensitive opsins, green opsins (rh2-1 and rh2-2) and blue opsin (sws2) were all reduced in 3.5 dpf six7ja51/ja51 larvae (figure 3d). Like other teleosts, zebrafish has multiple copies of green opsin genes, rh2-1, rh2-2, rh2-3 and rh2-4, among which rh2-3 and rh2-4 are expressed at extremely low levels during larval stage in WT [34] and also in six7ja51/ja51 (data not shown).

In situ hybridization analysis (figure 3f) revealed that green opsin expression was hardly detected in the eye of six7ja51/ja51 larvae, while blue opsin expression was reduced approximately by half in six7ja51/ja51 larvae, suggesting decreases in the numbers of middle wavelength-sensitive cones (i.e. green and blue cones). Consistently, six7ja51/ja51 larvae exhibited reduced mRNA levels of arrestin 3a (arr3a) and arrestin 3b (arr3b) (figure 3d), which are expressed in the double cones (green and red cones) and the single cones (UV and blue cones), respectively [35]. This observation further supports the reduction of the number of blue and green cones in six7ja51/ja51 larvae at 3.5 dpf.

The reduction in larval mRNA levels of blue and green opsins was also observed in another six7 mutant line, six7ja52/ja52 (electronic supplementary material, figure S1b). Using this line, we generated six7ja52/ja52, six7ja53/ja53 double KO larvae and found that the double mutant had extremely small eyes (electronic supplementary material, figure S1c); the loss of the eyes recapitulated the morpholino-induced phenotypes of six7/six3b-deficient larvae reported in the previous study [24], supporting that the six7 deficiency is caused by the ja52 mutation. The expression profiles of opsin genes in six7ja52/ja52 larvae were quite similar to those in six7ja51/ja51 larvae, suggesting that the phenotype of six7 mutants did not arise from any off-target effect of TALENs but from the six7 deficiency. In the following experiments, we used six7ja51/ja51 zebrafish, which we refer to as six7 KO.

(c) Increase of rod cells in the larva of six7 knock-out zebrafish

In contrast to the remarkable reduction in the number of blue and green cones, the mRNA levels of rod-specific
phototransduction component genes, rhodopsin (rho) and rod transducin α-subunit (gnat1), were upregulated in six7 KO larvae as compared with WT (figure 3d). The number of Gnat1-positive cells was increased in KO larvae when assessed by immunohistochemistry with anti-Gnat1 antibody (figure 3c). It is also evident that six7 KO exhibited increased mRNA levels of rod-specific transcription factors, neural retina leucine zipper (nrl) and nuclear receptor subfamily 2, group E, member 3 (nr2e3) (figure 3e). These two transcription factors are both essential for rod development in mice [36,37]. Together, these data support the notion that KO larvae had many more rods than WT larvae at 3.5 dpf.

(d) Decrease of green cones in adult six7 knock-out zebrafish

To examine whether the number of photoreceptors expressing green opsins are reduced throughout developmental stages, we analysed expression profiles of opsin genes in adult zebrafish. The number of green cones expressing rh2-1, rh2-2, rh2-3 or rh2-4 was severely reduced in six7 KO at the adult stage (figure 4a,b), indicating that transcription of all the green opsin genes was suppressed throughout developmental stages in KO. The reduction of the number of green cone cells in KO at both adult and larval stages suggests that six7 is indispensable for development of green cones.

(e) Developmental stage-dependent phenotypes of blue cones and rods in six7 knock-out zebrafish

The mRNA levels of rod-specific genes, rho and gnat1, were upregulated in six7 KO larvae (figure 3d), but at the adult stage, they were not significantly different between WT and KO (figure 4a). Similarly, the suppressed expression of blue opsin gene (srs2) in six7 KO was observed only at the larval stage (figure 3d) but not at the adult stage (figure 4a). The number of blue cones was reduced in the adult KO central retina, where early differentiated photoreceptors were located (figure 4b; electronic supplementary material, figure S2), suggesting that six7 is necessary for the development of blue cones only during the larval stage.
Figure 3. Analysis of the WT zebrafish and the six7 KO zebrafish at the larval stage. (a) Top: exon–intron organization and partial nucleotide sequences of zebrafish six7. The binding sites of the left and right TALENs are highlighted in blue. The recognition site of the endonuclease XhoI is highlighted in green. Middle: the nucleotide sequence of the KO (ja51 mutant) fish is compared with the WT sequence. Deletions are indicated by dashes. Bottom: schematic of Six7 and its mutant protein. (b) Genotyping of the six7 KO zebrafish. See details in the electronic supplementary material, methods. (c) Immunofluorescence of ocular sections of the WT and KO larvae at 3.5 dpf. The ocular sections were immunostained with the anti-Gnat1 antibody for rods (green) and with the zpr1 antibody for double cones (i.e. red and green cones; magenta). DAPI staining identified all the cell nuclei (blue). Scale bar, 40 μm. (d,e) Expression profiles of the phototransduction component genes (d) and the transcription factors contributing to the photoreceptor development in mouse and/or zebrafish (e) in the WT and six7 KO larval eyes at 3.5 dpf. The mRNA levels were quantified by RT-qPCR. The data were represented by mean ± s.e.m. (n = 5). Statistical significance between WT and six7 KO data is shown by the asterisks (*p < 0.05 by Student’s t-test). (f) Expression profiles of the cone opsin genes by in situ hybridization using the larval eyes of the WT and KO at 3.5 dpf. The lws probe was designed to recognize both of the lws1 and lws2 opsin genes. D, dorsal side; V, ventral side. Scale bar, 50 μm. (Online version in colour.)
The loss of 

and with anti-Gnat2 antibody to stain all the cone sub-

nohistochemistry with 1D4 antibody as a red cone marker

loss of six7 supplementary material, figure S2). Considering that the 

the larval stage (figure 3) and that 

the total number of the red cones. These results suggest that six7 also plays an important role in spatio-temporally regulated expression of red opsin subtypes.

(f) Ectopic expression of red opsin gene lws1 in the central retina of adult six7 knock-out zebrafish

Zebrafish have two duplicated red opsin genes, lws1 and lws2, and their expression is spatially regulated in the retina [19,34]. In the eyes of adult six7 KO, the lws1 mRNA level was markedly upregulated (figure 4a), in contrast to its normal expression in six7 KO larvae (figure 3d). Spatial expression pattern of lws1 in the adult KO retina was also distinct from that in the adult WT retina. In WT, the lws1 expression was confined to the peripheral retina, while the lws2 expression was more localized in the central retina (figure 4b; electronic supplementary material, figures S2 and S4b). In six7 KO, the lws1-expressing cells were observed across the whole retina (figure 4b; electronic supplementary material, figures S2 and S4b). At this adult stage (3 mpf), the number of lws2-expressing cells in the central retina was mostly unaffected by the loss of six7 (figure 4b), as is consistent with the RT-qPCR analysis (figure 4e), but surprisingly, at the later stage (4 mpf), lws2 showed a severely reduced expression in the central retina of KO (figure 4b; electronic supplementary material, figure S2). Considering that the loss of six7 does not affect the development of red cone, at the larval stage (figure 3) and that six7 KO maintains the laminar structure of the retina (figure 4), we suppose that six7 controls only the expression pattern of the duplicated red opsin genes in the red cones. We further validated the number of the red cones in six7 KO by whole-mount immunohistochemistry with 1D4 antibody as a red cone marker [38] and with anti-Gnat2 antibody to stain all the cone subtypes. The loss of six7 caused reduction in the number of the Gnat2-positive cells in the double cone layer, while apparent increase or decrease in the red cones was not observed in six7 KO retina at 6 mpf (electronic supplementary material, figure S4c). Collectively, the deficiency of six7 is likely to cause gradual switching of the expression of red opsins from lws2 to lws1 in the central retina without affecting the total number of the red cones. These results suggest that six7 also plays an important role in spatio-temporally regulated expression of red opsin subtypes.

(g) Expression levels of transcription factors responsible for photoreceptor development in six7 knock-out zebrafish

Two transcription factors (tbxb2b and thrb) are known to contribute to development of UV and red cone cells in zebrafish, respectively [20,21]. The mRNA levels of these transcription factors were mostly unaffected by the loss of six7 at both the larval and adult stages (figure 3e; electronic supplementary material, figure S3). The unaltered expression of these transcription factors indicates that the developmental process of the green cones might be independent of that of the UV and red cones. Importantly, no significant change was observed between WT and KO in the mRNA levels of crx and orthodenticle homolog 5 (otx5) (figure 3e; electronic supplementary material, figure S3), the master transcriptional regulators of photoreceptor cells in zebrafish [39,40]. It should be noted that the loss of six7 did not affect the laminar structure of the larval retina (figure 3c,f), nor the positions and the morphologies of each photoreceptor cells in the adult retina (figure 4e; electronic supplementary material, figure S2). Together, our results indicate that six7 governs the expression of green opsin genes without affecting gene expression of the key transcription factors.
4. Discussion

We investigated the role of six7, which is predominantly expressed in cone photoreceptors throughout the development of zebrafish (figure 2). We generated six7 KO zebrafish and found that six7 was essential for the expression of all subsets of the green opsin genes (figures 3 and 4), which have been duplicated and diverged in the teleost lineage. The loss of six7 caused expression switching between the two red opsin genes, from lws2 to lws1, in the central retina at the adult stage (figure 4). These results demonstrate that six7 is important for the effective detection of the middle- and long-wavelength light by controlling the expression of the green and red opsins in zebrafish. In six3b KO larvae, on the other hand, no significant alteration of mRNA levels of the opsin genes was observed (electronic supplementary material, figure S5), suggesting that six7 plays specialized roles in regulating the expression of these opsin genes, in contrast to the redundant role of six3b and six7 in eye formation [24] (electronic supplementary material, figure S1).

We observed an increase in the number of rods and reduced number of blue cones in six7 KO at the larval stage (figure 3). Recently, Saade et al. [41] also reported an increased number of rods due to the loss of six7. In their report, a forward genetic screen of the genes involved in rod development led to isolation of a mutant called lots-of-rods jr (lfr), which was characterized by an increased number of rods at the larval stage. They mapped the lfr mutation on chromosome 7, in which six7 is located. The morpholino-mediated knockdown of six7 phenocopied the lfr mutation [41]. In this study, by using the six7 KO zebrafish, we analysed the role of six7 in the photoreceptor development at the adult stage. The six7 KO caused the increase in number of rods as well as the suppression of the mRNA level of the blue opsin only at the larval stage (figure 3d), while the number of the green cones was reduced by six7 KO at both the larval and adult stages (figures 3d and 4a). By contrast, the ectopic expression of the red opsin lws1 in the central retina of six7 KO was observed only at the adult stage (figure 4a). These phenotypes observed at either the larval or the adult stage suggest that the differentiation programmes of the retinal progenitor cells change as zebrafish grow, and that six7 contributes to stage-dependent differentiation in photoreceptor development.

So far, six7 has been found widely in the ray-finned fish genomes but not in birds nor mammals. The emergence of the Six7 subfamily at the early evolutionary stage (figure 1; electronic supplementary material, figure S9a) suggests that six7 is lost in some vertebrate species. We noticed a gene termed SIX7 in the Ensembl Genome database of reptilian species, green anole and Chinese soft-shell turtle, and our subsequent tBLASTn searches identified SIX7-like sequences in the NCBI genome database of green sea turtle and Indian python. These reptilian SIX7 sequences are quite diverged from those of ray-finned fish Six7, as is evident from too many amino acid substitutions and gaps in the sequence alignment (electronic supplementary material, figure S6b). Additional genome information will be required to make clear whether these SIX7 genes are true orthologues of ray-finned fish six7.

Although it is likely that six7 directs expression of rh2 (the green opsin) genes in ray-finned fish, SIX7 is lost in birds and elephant shark, both of which retain RH2 genes. In these species, expression of the RH2 gene could be regulated by a six7-independent mechanism. To gain insight into evolutionary aspects of the regulatory mechanisms, we compared the gene synteny around the RH2 gene locus among various vertebrate species (electronic supplementary material, figure S7). Intriguingly, the gene synteny can be classified into two types: (i) the non-teleost type and (ii) the teleost type (electronic supplementary material, figure S7). The non-teleost type was found in the species ranging from elephant shark to tetrapods, suggesting that this synteny represents an ancient type. In the teleost lineage, the genes around RH2 are likely to have been rearranged into the teleost type, in which six7 could have acquired the important role in the expression of rh2 genes.

Previously, Tsujimura et al. [18] identified a 0.5 kbp enhancer region (termed locus control region; LCR) located 15 kbp upstream of the tandem array of rh2 genes, as a regulator essential for expression of all the duplicated rh2 genes in zebrafish [18]. Interestingly, LCR contains the binding sites of both Six7 (TAATGTC) [42] and Crx (TAATC) [11]. The loss of expression of all subsets of rh2 genes in the six7 KO zebrafish (figure 4a,b) suggests that six7 may activate expression of the rh2 genes through the LCR. Teleosts possess a variable number of duplicated rh2 genes [43] (electronic supplementary material, figure S8), whose spectral sensitivities are diverged from each other [44]. The six7 regulation of the duplicated rh2 expression may enable teleosts to adapt to diverse aquatic environments.

Ethics. All research described here adhered to local guidelines and all appropriate ethical approval and licences were obtained.

Data accessibility. The datasets supporting this article have been uploaded as part of the electronic supplementary material.

Authors’ contributions. T.S. and Y.F. conceived and designed the research. Y.O., supported by T.S., carried out the molecular laboratory work, participated in data analysis and carried out sequence alignments. T.S. performed flow cytometry. D.K. and Y.F. supervised the project. Y.O., T.S., D.K. and Y.F. wrote the manuscript. All authors approved the manuscript for publication.

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