The generation and characterization of a transgenic zebrafish line with lens-specific Cre expression

Xuyan Peng,[1] Xiaolin Jia,[1] Guohui Shang,[2] Mengjiao Xue,[1] Mingjun Jiang,[1] Dandan Chen,[1] Fengyan Zhang,[1] Yanzhong Hu[1,3,4]

(The first two authors contributed equally to this work.)

[1]*The Laboratory of Ophthalmology and Vision Science, Department of Ophthalmology, The First Affiliated Hospital of Zhengzhou University, Zheng Zhou, China;* [2]*Department of Medical Genetics and Cell Biology, School of Basic Medical Sciences, Zhengzhou University, Zheng Zhou, China;* [3]*Joint National Laboratory for Antibody Drug Engineering, Henan University School of Basic Medical Sciences. Kaifeng, China;* [4]*Kaifeng Key Lab for Cataract and Myopia, Institute of Eye Disease, Kaifeng Central Hospital, Kaifeng, China*

Purpose: Danio rerio zebrafish constitute a popular model for studying lens development and congenital cataracts. However, the specific deletion of a gene with a Cre/LoxP system in the zebrafish lens is unavailable because of the lack of a lens-Cre-transgenic zebrafish. This study aimed to generate a transgenic zebrafish line in which Cre recombinase was specifically expressed in the lens.

Methods: The pTol2 *cryaa*:Cre-polyA-*cryaa*:EGFP (enhanced green fluorescent protein) plasmid was constructed and co-injected with Tol2-transposase into one-to-two-cell-stage wild-type (WT) zebrafish embryos. Whole-mount in situ hybridization (ISH), tissue section, hematoxylin and eosin staining, a Western blot, a split-lamp observation, and a grid transmission assay were used to analyze the Cre expression, lens structure, and lens transparency of the transgenic zebrafish.

Results: In this study, we generated a transgenic zebrafish line, zTg(*cryaa*:Cre-*cryaa*:EGFP), in which Cre recombinase and EGFP were driven by the lens-specific *cryaa* promoter. zTg(*cryaa*:Cre-*cryaa*:EGFP) began to express Cre and EGFP specifically in the lens at the 22 hpf stage, and this ectopic Cre could efficiently and specifically delete the red fluorescent protein (RFP) signal from the lens when zTg(*cryaa*:Cre-*cryaa*:EGFP) embryos were injected with the *loxP*-flanked RFP plasmid. The overexpression of Cre and EGFP did not impair zebrafish development or lens transparency. Accordingly, this zTg(*cryaa*:Cre-*cryaa*:EGFP) zebrafish line is a useful tool for gene editing, specifically with zebrafish lenses.

Conclusions: We established a zTg(*cryaa*:Cre-*cryaa*:EGFP) zebrafish line that can specifically express an active Cre recombinase in lens tissues. This transgenic zebrafish line can be used as a tool to specifically manipulate a gene in zebrafish lenses.

The ocular lens is an avascular sensory organ without any innervation, and it is responsible for transmitting and focusing light on the retina. It comprises an anterior epithelium and abundant fibers that are derived from the ectoderm visual epithelium [1]. Genetic variation, which affects the development and transparency of the crystalline lens, is a common pathological cause of congenital cataracts [2,3]. Approximately 22.3% of congenital cataracts are caused by genetic variation [4]. Moreover, there are nearly 200 loci and more than 100 genes whose mutations are associated with hereditary congenital cataracts [5]. Some of them have been suggested to be cataractous genes in mouse or zebrafish models using gene editing technology, such as *Pax6a* [6],

Foxe3 [7], *Hsf4* [8], and *αA-crystallin* [9,10]. Cre-mediated conditional knockout technology is a popular tool used to delete gene expression from a specific tissue in mouse models, and this tool has been used to manipulate and determine gene function in lens development and among patients with congenital cataracts [11]. However, the deletion of a specific gene in the zebrafish lens could not be achieved due to the lack of lens-specific Cre-expressing transgenic zebrafish.

Over 70% of annotated human genes have at least one obvious zebrafish ortholog [12]. Therefore, zebrafish became a time- and cost-efficient model to study human disease at the end of the last century because of their multiple advantages compared to established vertebrate genetic models, such as mice models [13]. Many human-disease-associated genes, including cataracts, are expressed and modeled in zebrafish [14]. For example, the conventional deletion of *pax6* in zebrafish impairs the development of the brain, retina, and

Correspondence to: Yanzhong Hu, Department of Cell Biology and Genetics, Henan University School of Basic Medical Sciences, Jin-Ming Road, Kaifeng, China, 475014; Phone: 86-18503781944; email: hyz@henu.edu.cn

lens, which clinically imitates *PAX6*-mutant-associated Aniridia diseases [15]. Conventional gene knockout via CRISPR-Cas9 or TALENs and gain-of-function via creating transgenic lines are widely used to manipulate genes in zebrafish lenses [8,16]. The limitation of conventional gene knockout is that some gene mutations lead to embryonic lethality or affect multiple organs, affecting the definition of a gene's precise function during lens development.

As in the human lens, α-crystallin is the dominant crystallin in zebrafish lenses. The α-crystallin content of the zebrafish lens has been reported with a proportion of 22% in the total lens protein [17]. α-crystallin contains two isoforms, αA-crystallin and αB-crystallin [18]. They act as chaperone proteins to regulate crystallin folding in the lens and protect lens proteostasis from divergent stresses [19]. Their chaperone activity and stability in zebrafish have been examined and compared with those in mammals [20]. Missense mutations in both the *cryaa* and *cryab* genes cause congenital cataracts and other diseases [18]. αA-crystallin is predominantly expressed in lens tissues, while αB-crystallin is expressed in other tissues (e.g., the brain, muscle, lung, liver, and heart) in addition to the lens. Therefore, the promoter of *cryaa* has been used to specifically initiate the expression of extrinsic proteins in lenses within mouse models [21]. Transgenic mice with *αA-crystallin*-promoter-driven Cre can specifically express Cre recombinase in the lens, and these Cre-Tg mice have been widely used to conditionally delete genes from the mouse lens [11]. Zebrafish have one *cryaa* gene and two duplicated *cryaba* and *cryabb* genes [22]. The expression of *cryaa* occurs uniquely in the lens for both larval [23] and adult zebrafish [24]. By contrast, the *cryaba* and *cryabb* genes are almost entirely expressed in non-lens tissues at the larval stage. During this stage, *cryaba* is expressed predominantly in non-lens tissues; for example, *cryaba*'s mRNA is mainly detected in the tissues of the lateral line, the hindbrain, muscle progenitors, and olfactory system. Conversely, *cryabb*'s mRNA is detected in hair cells and otic capsule cells, according to single-cell transcriptomic analysis [23]. As zebrafish mature, their expression of *cryaba* is restricted to the lens [25], while their *cryabb* is expressed in multiple tissues, including the lens, muscles, and the brain [22]. Additionally, cataract-linked mutants of αA-crystallin and αB-crystallin have caused cataracts in zebrafish [26]. Transgenic zebrafish with *cryaa*-promoter-driving green fluorescent protein (GFP) can specifically express GFP in their lenses [27]. These results suggest that the *cryaa* promoter is an ideal driver of extrinsic protein expression in zebrafish lenses.

The Cre-*loxP* recombination system is a powerful genetic tool for conditionally achieving gene deletion, and this system has been widely used in mice [28]. In this system, two recombinant mice are needed: *loxP*-carrying mice, in which *loxP* sequences are introduced to a targeting gene, and tissue-specific Cre-expressing transgenic mice. Cre recombinase recognizes and cleaves the *loxP*-flanked sequences to delete the target gene [29]. Tg:*cryaa*-Cre mice have been successfully used to conditionally delete the gene of *Rac1* from the mouse lens [30]. In zebrafish, Cre-mediated gene deletion has been successfully used to inactivate the *supv3l1* and *shha* genes in hepatocytes [31] and the epicardium [32], respectively. This use suggests that the Cre-*loxP* system could efficiently work in zebrafish. Furthermore, loxP-flanked transgenic zebrafish have been successfully created via homologous recombination-mediated gene editing [33] or the UFlip alleles generated by the CRISPR-Cas9 targeted integration strategy, which provide robust conditional inactivation and rescue [34]. Several Cre-Tg-zebrafish lines have been generated using Tol2-mediated transgenesis [35]. However, transgenic zebrafish with lens-specific Cre expression are still unavailable.

In this study, we generated the transgenic zebrafish line zTg*(cryaa:Cre-cryaa:EGFP)*, in which Cre and EGFP cDNA were subcloned downstream of the *cryaa* promoter. The zTg*(cryaa:Cre-cryaa:EGFP)* zebrafish exclusively expressed Cre and EGFP in the lens but not in their other tissues, and their expression patterns completely recapitulates the endogenous *cryaa* expression pattern during lens development. This ectopic Cre can specifically and efficiently delete the red fluorescent protein (RFP) signal from the lens when zTg*(cryaa:Cre-cryaa:EGFP)* embryos are injected with plasmids containing *loxP*-flanked RFP. These results suggest that zTg*(cryaa:Cre-cryaa:EGFP)* is a powerful tool for the manipulation of a specific gene in zebrafish lenses.

METHODS

Zebrafish maintenance: Danio rerio zebrafish and transgenic zTg*(cryaa:Cre-cryaa:EGFP)* zebrafish were maintained at 26–28.5 °C in a circulating water system with a light–dark cycle of 14:10 h according to the zebrafish protocol [36]. The fish were fed three times daily with newly hatched brine shrimp. Embryos were obtained via natural spawning and kept in egg water at 28.5 °C. The developmental stages were determined using days post-fertilization (dpf) or months post-fertilization (mpf) [37]. The use of all the animals in this project followed the guidelines on the use of animals by the Association for Research in Vision and Ophthalmology (Baltimore, MD). The procedures used in our study were

approved by the ethics committee of The First Affiliated Hospital at Zhengzhou University, Zheng Zhou, China.

Plasmids:

pTol **cryaa**:*Cre-polyA*-**cryaa**:*EGFP plasmid:*—A 1,028 bp DNA fragment of the *cryaa* promoter (−1 to −1,028 bp) upstream of the ATG (the protein translation initiation site) site was amplified via PCR using the wild type (WT) zebrafish genome as a template [27]. The fragment of the *cryaa* promoter was subcloned into the pGEMT-easy vector (A1360, Promega) and used as a template. Fragments of the *cryaa* promoter, Cre recombinase cDNA, and SV40-ployA were amplified via primers, respectively, and then subcloned into the PstI site of the pTol2-MCS vector (CZP10, China Zebrafish Resource Center) via homologous recombination using the ClonExpress MultiS One Step Cloning Kit (C113–02, Vazyme Biotech Co. Ltd., Nanjing, China), generating pTol *cryaa*:Cre-polyA plasmids. To generate the pTol *cryaa*:Cre-polyA-*cryaa*:EGFP plasmid, a fragment of the *cryaa* promoter, EGFP/cDNA, and SV40 ploy A was amplified with the primers and subcloned into the XmaI site of pTol *cryaa*:Cre-polyA via homologous recombination.

*The plasmids p***cryaa:RFP-cmlc:EGFP** *and p***cryaa:loxP-RFP-loxP-cmlc:EGFP***:*—DNA fragments containing the *cryaa* promoter, RFP cDNA, and the *cmlc* promoter (specifically controlling the expression of the cardiac myosin light chain (CMLC) protein in the heart tissue)-EGFP cDNA, or containing the *cryaa* promoter-*loxP* -RFP cDNA- *loxP*- *cmlc* promoter-EGFP cDNA, were synthesized in vitro and subcloned into the pUC57 vector (Dongxuan Jiyin Jiangsu Technology Co., Ltd., Jiangsu, China). The primer sequences are listed in Table 1. All recombinant plasmids were verified via DNA sequencing.

Whole-mount in situ hybridization and cryosectioning: For the whole-mount in situ hybridization of embryos older than 24 hpf, at 12 hpf, the embryos were incubated in egg water containing 0.003% 1-phenyl-2-thiourea (PTU, Sigma) to prevent pigmentation. To make sense and antisense probes for *cryaa*, *egfp*, and *cre*, the cDNA fragments of *cryaa*, *egfp*, and *cre* were amplified using the primers with T7-RNA-polymerase-recognizing sequences. The sense and antisense probes for *cryaa*, *egfp*, and *cre* were transcribed in vitro using T7 RNA polymerases, and they were labeled with digoxigenin-UTP, following the protocol provided with the TranscriptAid T7 High Yield Transcription kit (catalog no. K0441, Thermo Scientific, Waltham, MA), with the modification of the replacement of the nucleoside triphosphates (NTPs) mix with nucleic acid labeling (catalog no. 11277073910, Roche, offered by Sigma-Aldrich, Darmstadt, Germany).

Whole-mount in situ hybridization was performed following previous reports [38]. The sense probes were used as negative controls. The signals were tested with the anti-digoxigenin antibody conjugated to alkaline phosphatase (Anti-Digoxigenin-AP Fab fragments, catalog no. 11,093,274,910, Roche). Then, they were developed in the NBT/BCIP solution (catalog no. 11,681,451,001, Roche) and photographed under a DM4/Leica light microscope (M205 FA, Leica, Germany). To test the exact distribution of ISH signaling in the lens, after ISH, the embryos were cryosectioned at an 8 µm thickness, and the ISH signals were photographed under a Leica DM4 light microscope. The primer sequences are listed in Table 1.

Paraffin section and H&E staining: The zebrafish were anesthetized and fixed in 4% paraformaldehyde for 24 h at 4 °C and then washed in 50% ethanol. The fixed fish were dehydrated and embedded in paraffin, sectioned (to a thickness of 10 µm), and stained with hematoxylin and eosin.

RNA isolation and quantitative real-time PCR: The total RNA was isolated from the embryos at various developmental stages using the TRIzol Reagent (Life Technology, Carlsbad, CA), following the manufacturer's instructions for the kit. Fifty WT embryos at each stage (0, 3, 6, 12, 24, 36, 48, 60, 72, and 96 hpf) were collected and pooled together for RNA extraction. An equal amount of RNA was retrotranscripted to the first strand of cDNA using the First Strand cDNA Synthesis Kit (AE341, TransGen Biotech, Beijing, China). Quantitative real-time PCR was performed using the TransStart Green qPCR SuperMix (AQ101, TransGen Biotech) on the QuantStudio 3 Real-Time PCR System (Applied Biosystems by Thermo Fisher Scientific). According to the manufacturer's instruction, the following program was engaged: 30 s at 94 °C (initial denaturation), 5 s at 94 °C, 15 s at 60 °C, 10 s at 72 °C, and 40 cycles. *actb2* was used as the internal reference gene [39]. The experiments were repeated independently three times. The primer sequences are presented in Table 1.

Western blot: Proteins were extracted from the lens, lens-excluded eye tissues, and eyes-excluded body tissues of zTg (transgenic zebrafish) and WT zebrafish at 15 dpf using protein extraction buffer (Beyotime). The antibodies used in this study were anti-Cre recombinase (15036S, Cell Signaling Technology, Danvers, MA) and anti-Gapdh (10,494-1-AP, Proteintech, Wuhan, China) at a dilution of 1:1,000. The extracted proteins were separated using 10% SDS–PAGE (sodium dodecyl sulfate-PAGE) and then transferred onto polyvinylidene fluoride (PVDF) membranes. After blocking in 5% dry milk/phosphate buffer solution and Tween-20 (PBST), the membranes were incubated in a solution with appropriate primary antibodies. Next, they were incubated with a secondary antibody conjugated with horseradish

peroxidase. The signal was developed in the ECL buffer and detected using a charge coupled device (CCD)-camera-based imager (Amersham Imager 680, Amersham, CA; GE).

Statistical analysis: The Statistical Product and Service Solutions (SPSS) software 24.0 version was used for data analysis. The unpaired Student's *t*-test was used for statistical analysis of the data on the zebrafish's standard length and eye area, and one-way analysis of variance (ANOVA) was used for the data analysis of quantitative real-time PCR. The results were expressed as means ± standard deviations (SDs), and $p < 0.05$ was considered statistically significant.

RESULTS

The generation of zTg(cryaa:Cre-cryaa:EGFP) zebrafish: To generate a transgenic zebrafish with Cre overexpressing specifically in the lens, about 1.5 nL of a solution containing the plasmid of pTol *cryaa*:Cre-polyA-*cryaa*:EGFP (80 ng/ul) and Tol2-transposase mRNA (80 ng/ul) were microinjected into one-to-two-cell-stage embryos (Figure 1A). Tol2-transposase is responsible for catalyzing the recombination of the DNA fragment of *cryaa*:cre-polyA-*cryaa*:EGFP in the plasmid of pTol *cryaa*:Cre-polyA-*cryaa*:EGFP into the zebrafish genome. Cryaa is a lens-specific protein [27]. We used the promoter of the *cryaa* gene to drive Cre and GFP expression in this plasmid. The specific expression of GFP in zebrafish lenses was used as a selective marker for transgenic zebrafish. After injection, the embryonic zebrafish

TABLE 1. THE PRIMERS USED IN THIS STUDY.

Purpose	Symble	Forward	Reverse	length (bp)
PGEMT-easy-cryaaPro	*cryaa*-promoter	TGGAGACCCCTGATTAATAA	AATGTCAGACCTGGTAACTCCT	1028
pTol *cryaa*:Cre-polyA	*cryaa*-promoter*	<u>GGAGATCACTTAGATCTAAC</u>TGGAGACCCCTGATTAATAA	<u>ACGGTCAGTAAATTGGCCATA</u>AATGTCAGACCTGGTAACTCCT	1068
	Cre*	<u>GAGTTACCAGGTCTGACATT</u>ATGGCCAATTTACTGACCGTACA	<u>TATGGCTGATTATGATCTAG</u>CTAATCGCCATCTTCCAGCAG	1072
	SV40-ployA*	<u>TGCTGGAAGATGGCGATTAG</u>CTAGATCATAATCAGCCATACCA	GTTTCCCGGGTTCGTACGTTctgcagACGCCTTAAGATACATTGATGA	283
pTol *cryaa*:Cre-polyA -*cryaa*:EGFP	*cryaa*-promoter*	<u>CGTCTGCAGAACGTACGAAC</u>TGGAGACCCCTGATTAATAA	<u>CCCTTGCTCACCATGGTGGCA</u>ATGTCAGACCTGGTAACTCCT	1068
	EGFP-ploy A*	<u>GAGTTACCAGGTCTGACATT</u>GCCACCATGGTGAGCAAGGGC	TTTTCTCGAGTTACGCGTTcccgggACGCCTTAAGATACATTGATGA	1016
in situ *hybridization*	*cryaa*-antisense probe**	GGACCCAAGACAAGTGGCAT	<u>TAATACGACTCACTATAG</u>CAGGTTAGGGAGAAAGCGGAG	757
	cryaa-sense probe**	<u>TAATACGACTCACTATAG</u>GGACCCAAGACAAGTGGCAT	CAGGTTAGGGAGAAAGCGGAG	757
	e*gfp*-antisense probe**	GCCACCATGGTGAGCAAGGGC	<u>TAATACGACTCACTATAG</u>TGGTATGGCTGATTATGATCTAG	780
	e*gfp*-sense probe**	<u>TAATACGACTCACTATAG</u>GCCACCATGGTGAGCAAGGGC	TGGTATGGCTGATTATGATCTAG	780
	cre-antisense probe**	ATGGCCAATTTACTGACCGTACA	<u>TAATACGACTCACTATAG</u>CTTCTCTACACCTGCGGTGCTAA	651
	cre-sense probe**	<u>TAATACGACTCACTATAG</u>ATGGCCAATTTACTGACCGTACA	CTTCTCTACACCTGCGGTGCTAA	651
qRT-PCR	*cryaa*	TACCCCACCCGACTCTTTGA	AGTCCAGGATGTTGCGGAAG	124
	actb2	GATGATGAAATTGCCGCACTG	ACCAACCATGACACCCTGATGT	135

*The underline sequences are homogenous sequences, and the lower-case letter sequences are PstI restrictive endonuclease site. ** The underlined sequence was T7 RNA polymerases promoter sequences.
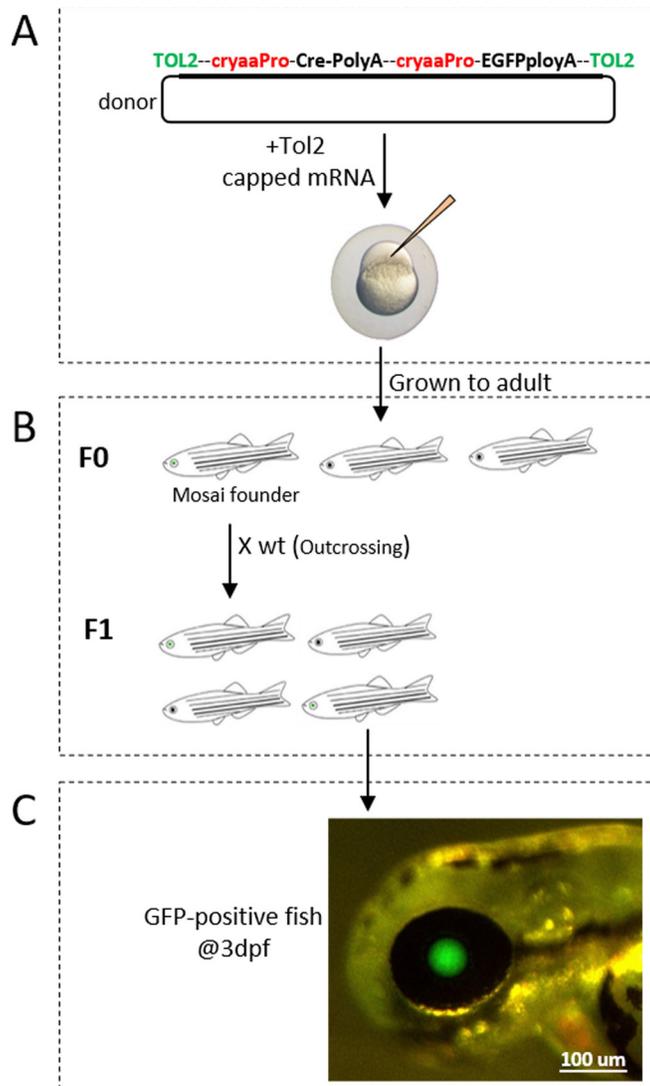
Figure 1. Generation of zTg*(cryaa:Cre-cryaa:EGFP)* zebrafish. **A**: Schematic map of the plasmid pTol2-cryaaPro-cre-polyA-cryaaPro-EGFPE-tol2. **B**: Schematic map of zebrafish breeding to generate F0 and F1 offspring. **C**: GFP fluorescent signals in 3 dpf lenses of F1 zTg*(cryaa:Cre-cryaa:EGFP)* transgenic zebrafish.

showed GFP-positive signals at 3 dpf under a fluorescence microscope, and these zebrafish were defined as F0 founders (Figure 1B). Adult GFP-positive F0 founders were bred with WT zebrafish to generate heterozygous F1 offspring (Figure 1B). The F1 GFP-positive zebrafish were selected using a fluorescence microscope and considered zTg*(cryaa:Cre-cryaa:EGFP)* individuals for further use.

zTg(cryaa:Cre-cryaa:EGFP) specifically expresses Cre protein in embryonic lenses at the 22 hpf stage: Before we determined when Cre was expressed in zebrafish lenses, we analyzed *cryaa* transcripts during the zebrafish's development in qRT-PCR and in situ hybridization (ISH) assays. The qPCR results indicated that the expression of *cryaa* mRNA was regulated during zebrafish development. The *cryaa* mRNA was expressed in the 0 hpf embryos at a relatively high level (with the relative value of 1 ± 0.24), followed by

a decrease to the lowest level (with the relative value of 0.07 ± 0.03) at the 12 hpf stage. After this, the *cryaa* mRNA expression again increased at the 24 hpf stage, and thereafter, the expression level increased gradually with embryo growth (Figure 2A). Accordingly, we postulated that the tested *cryaa* mRNA in the embryos from 0 to 24 hpf originated from maternity and that this maternal *cryaa* mRNA decreased with embryo development. The zygotic *cryaa* started to express its own mRNA in the embryos after 12 hpf. The ISH results indicated that the relative expression level of *cryaa* mRNA was consistent with the qPCR results (Figure 2B–D). As the results indicated, the maternal *cryaa* mRNA was expressed in whole embryo tissues during the early embryo stages (3 hpf to 24 hpf; Figure 2B–D); the *cryaa* mRNA was detected in the eye at 18 hpf and solely expressed in lens tissues at the 48 and 72 hpf embryo stages (Figure 3A). The

expression level and pattern of the *cryaa* mRNA showed no difference between the WT and zTg*(cryaa:Cre-cryaa:EGFP)* zebrafish, suggesting that the overexpression of Cre or EGFP did not impair *cryaa* mRNA expression in the lens. Next, we performed an ISH assay to determine the expression of *cre* and *egfp* in zTg*(cryaa:Cre-cryaa:EGFP)*. The results showed that zTg*(cryaa:Cre-cryaa:EGFP* began to express Cre and EGFP was weak in other position of the embryos (such as the trunk part) at 16 hpf (Figure 2C). After this, both Cre and EGFP were entirely expressed in lens tissues in a similar pattern (Figure 2D and Figure 3A). Like *cryaa*, *cre* and *egfp* were expressed in lens cortical fiber tissue (Figure 3B), and this finding was consistent with previous reports that *cryaa* is expressed almost exclusively in 2 and 5 dpf lens fiber cells [23]. No expression of Cre or EGFP was detectable in the WT zebrafish (Figure 2D and Figure 3A). These results indicated that Cre is predominantly expressed in the lens fibers and that the EGFP signal could represent the expression pattern of Cre during lens development. The EGFP fluorescence analysis results indicated that EGFP was constitutively expressed in the zTg*(cryaa:Cre-cryaa:EGFP)* lenses at various stages, including the larval stage (20 dpf and 1 mpf) and the adult stage (3 mpf, 6 mpf, 9 mpf, and 12 mpf; Figure 3C). Furthermore, we performed immunoblots to test the expression of Cre protein in zTg*(cryaa:Cre-cryaa:EGFP)* lenses. The 15 dpf WT and zTg*(cryaa:Cre-cryaa:EGFP* zebrafish were dissected into lenses, lens-excluded eye tissue, and eyeless zebrafish bodies. The results showed that Cre was expressed only in the lens tissues and not in the other eye tissues or body tissues of the zTg*(cryaa:Cre-cryaa:EGFP)* individuals (Figure 3D). No expression of Cre protein was detected in WT zebrafish (Figure 3D). Accordingly, we proposed that two sets of *cryaa* mRNA occurred in early embryo development that originated from maternal transportation or new zygotic synthesis. The zygotic embryo started to express *cre* at 16 hpf, which implies that the zygotic embryo starts to express its own *cryaa* at 16 hpf. The expression patterns of *cryaa*, *egfp*, and *cre* at each stage were summarized in Table 2.

The ectopic expression of Cre and EGFP does not affect zebrafish development or lens transparency: To determine whether ectopic Cre and EGFP impair zebrafish development, including lens development, we compared the development of WT zebrafish to zTg*(cryaa:Cre-cryaa:EGFP)* zebrafish. No differences in standard length from the snout to the origin of the tail fin or the eye area were observed between the WT and zTg*(cryaa:Cre-cryaa:EGFP)* zebrafish (Figure 4A,B). Histology analysis showed that ectopic Cre and GFP in the zTg*(cryaa:Cre-cryaa:EGFP)* lenses did not impair the lens

structure compared to that of WT lenses (Figure 4C). We further analyzed the transparency of 2 mpf and 9 mpf WT and zTg*(cryaa:Cre-cryaa:EGFP)* lenses via split-lamp microscopy and grid transmission images. The results revealed no cataract phenotype in the zTg*(cryaa:Cre-cryaa:EGFP)* zebrafish (Figure 4D), though EGFP signals were observed. The transparency of the zTg*(cryaa:Cre-cryaa:EGFP)* lens was similar to that of the WT lens (Figure 4D). These results indicate that lens development is not affected in this Cre-transgenic zebrafish.

zTg(cryaa:Cre-cryaa:EGFP) expresses an active Cre recombinase in the lens: To test Cre activity in the zTg*(cryaa:Cre-cryaa:EGFP)* lens, the plasmids p*cryaa:RFP-cmlc:EGFP* or p*cryaa:Flox-RFP-Flox-cmlc:EGFP* were constructed and microinjected into one-cell-stage embryos of zTg*(cryaa:Cre-cryaa:EGFP)* and WT zebrafish, respectively. Within these two constructs, the *cmlc* (cardiac myosin light chain) promoter was used to drive EGFP expression specifically in heart tissue, and it was used to indicate the successful administration of p*cryaa:RFP-cmlc:EGFP* and p*cryaa:Flox-RFP-Flox-cmlc:EGFP* into the embryos. The cDNAs of RPF and Flox-RFP-Flox were cloned downstream of the *cryaa* promoter, which specifically expresses fluorescent RFP in lens tissue. To determine Cre activity, 3 dpf embryos with GFP-positive hearts were collected to analyze RFP's expression in the lens. As the results in Figure 5 indicate, the plasmid p*cryaa:RFP-cmlc:EGFP* constitutively expressed RPF in the lens of WT zebrafish and zTg(cryaa:Cre-cryaa:EGFP) zebrafish (Figure 5A). In contrast, the p*cryaa:Flox-RFP-Flox-cmlc:EGFP* plasmid, which expresses RFP and GFP in WT zebrafish lenses and hearts, respectively (Figure 5B's upper panel) did not express RFP in the zTg*(cryaa:Cre/cryaa:EGFP)* zebrafish lenses (Figure 5B's lower panel). These results suggested that Cre exhibited recombinase activity toward Flox-RFP-Flox when p*cryaa:Flox-RFP-Flox-cmlc:EGFP* was injected into the zTg*(cryaa:Cre-cryaa:EGFP)* embryos. To determine Cre efficiency, we quantitated the RFP-positive lenses versus the GFP-positive hearts in the zTg*(cryaa:Cre-cryaa:EGFP)*:p*cryaa:Flox-RFP-Flox-cmlc:EGFP* zebrafish. Two of the 126 heart-EGFP positive zTg*(cryaa:Cre-cryaa:EGFP)*: *Flox-RFP-Flox-cmlc:EGFP* embryos exhibited lens-RFP-positivity; therefore, 98.4% of Cre-Tg-zebrafish exhibit Cre recombinase activity in the lens (Table 2). These results suggest that this novel transgenic line, zTg*(cryaa:Cre-cryaa:EGFP)*, expresses active Cre in the lens. Therefore, it could be used as a tool to specifically target gene deletion in the zebrafish lens.
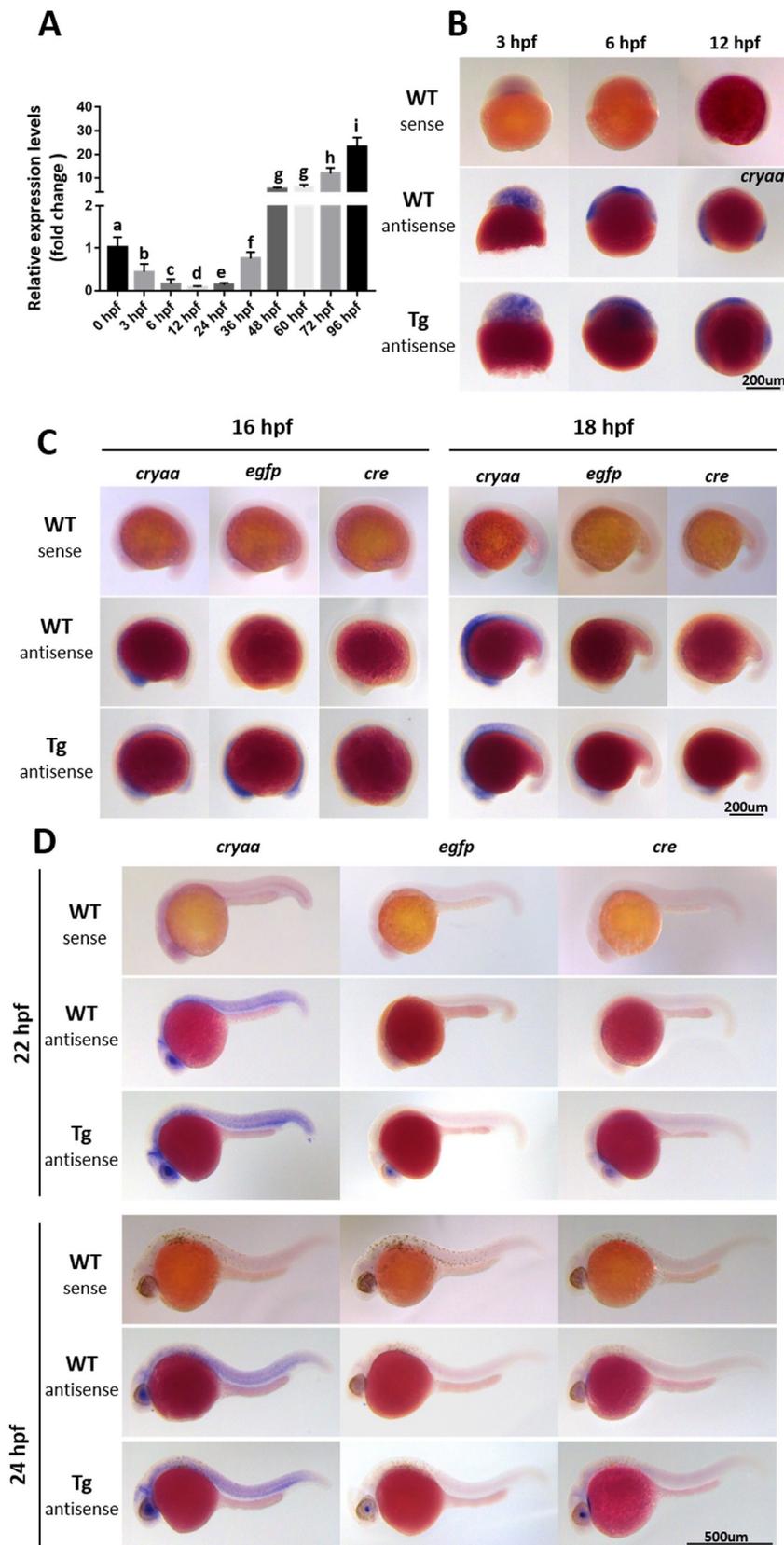
Figure 2. Developmental expression of *cryaa, cre*, and *egfp* mRNA in embryonic stages of zTg*(cryaa:Cre-cryaa:EGFP)* and WT zebrafish. **A**: qRT-PCR to measure the expression of *cryaa* mRNA in WT zebrafish during development. Error bars indicate means ± SDs. The different letters above the bars indicate significant differences. n = 3. **B**: Whole-mount in situ hybridization (WISH) of *cryaa* mRNA at 3 hpf, 6 hpf, and 12 hpf stages in WT and zTg*(cryaa:Cre-cryaa:EGFP*; Tg) zebrafish. **C**, **D**: WISH of *cryaa*, *egfp*, and *cre* mRNA expression at 16 hpf, 18 hpf, 22 hpf, and 24 hpf in WT and zTg*(cryaa:Cre-cryaa:EGFP*; Tg) zebrafish.

TABLE 2. THE EXPRESSION PATTERN OF *CRYAA*, *EGFP* AND *CRE* IN WT AND TG ALONG THE DEVELOPMENTAL STAGES.

Genes/ Stage	3 hpf		6 hpf		12 hpf		16 hpf		18 hpf		22 hpf		24 hpf		48 hpf		72 hpf	
	WT	Tg	WT	Tg	WT	Tg	WT	Tg	WT	Tg	WT	Tg	WT	Tg	WT	Tg	WT	Tg
cryaa	U	U	U	U	U	U	U	U	U	U	U	U	U	U	L	L	L	L
egfp	-	-	-	-	-	-	-	U	-	A	-	L	-	L	-	L	-	L
cre	-	-	-	-	-	-	-	U	-	A	-	L	-	L	-	L	-	L

U: ubiquitous; A: anterior body; L: lens.
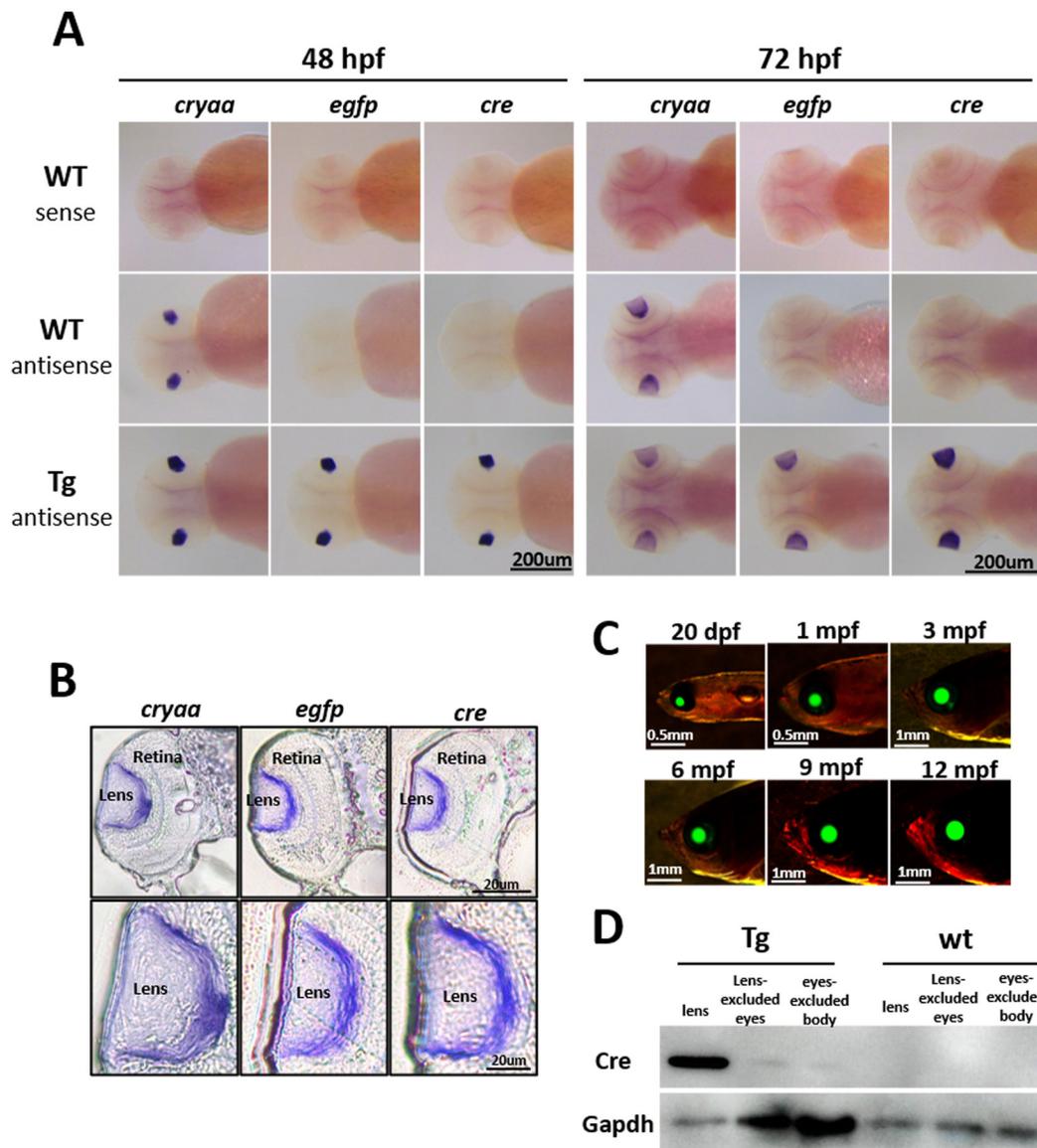
Figure 3. Expression of Cre and EGFP in zTg*(cryaa:Cre-cryaa:EGFP)* zebrafish. **A**: Whole-mount in situ hybridization (WISH) of *cryaa*, *egfp*, and *cre* mRNA in 48 and 72 hpf WT and zTg*(cryaa:Cre-cryaa:EGFP*; Tg) zebrafish. **B**: Cryosection of 72 hpf Tg embryos of **A**. **C**: Fluorescent EGFP was photographed in the lenses of zTg*(cryaa:Cre-cryaa:EGFP)* zebrafish at the 20 dpf, 1 mpf, 3 mpf, 6 mpf, 9 mpf, and 12 mpf stages. (D) Immunoblot showing the expression of Cre protein and Gapdh in the lens, lens-excluded eye tissue, and eyes-excluded body tissues of WT and zTg*(cryaa:Cre-cryaa:EGFP)* zebrafish at 15 dpf.
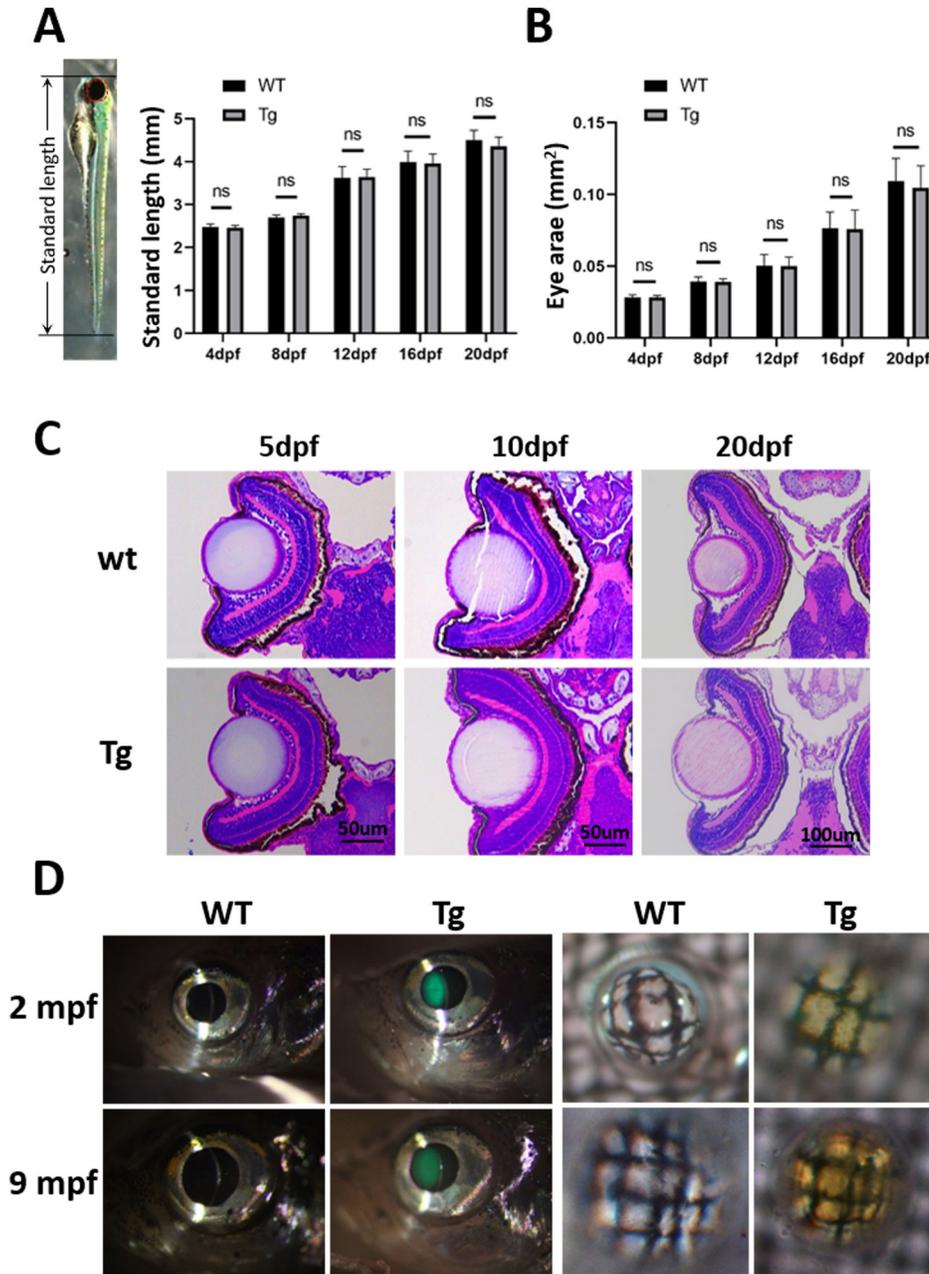
Figure 4. Ectopic expression of Cre and EGFP does not impair zTg*(cryaa:Cre-cryaa:EGFP)* development. **A**, **B**: The standard length from the snout to the origin of the tail fin (**A**) and the eye area (**B**) of WT and zTg*(cryaa:Cre-cryaa:EGFP)* zebrafish were measured using Image J (Wayne Rasband, National Institutes of Health) and analyzed by increased age. The eye area calculation range is within the dotted red circle shown in **A**. Error bars indicate means ± SDs; *ns* indicates no significant differences; *n* = 20. **C**: H&E staining of paraffin-embedded eye sections of 5 dpf, 10 dpf, and 20 dpf WT and Tg-zebrafish. The standard length of the samples was 2.5, 3.1, and 4.4 mm at 5, 10, and 20 dpf, respectively, and the eye area was 0.032, 0.043, and 0.108 mm^2 long, respectively. **D**: Photographs of the lens transparency of WT and Tg zebrafish at the stages of 2 mpf and 9 mpf taken with a split-lamp microscope and a grid transmission image assay. The standard length of the samples was 20.8 and 33.9 mm at 2 and 9 mpf, respectively, and the eye area was 2.37 and 4.26 mm^2 long, respectively.
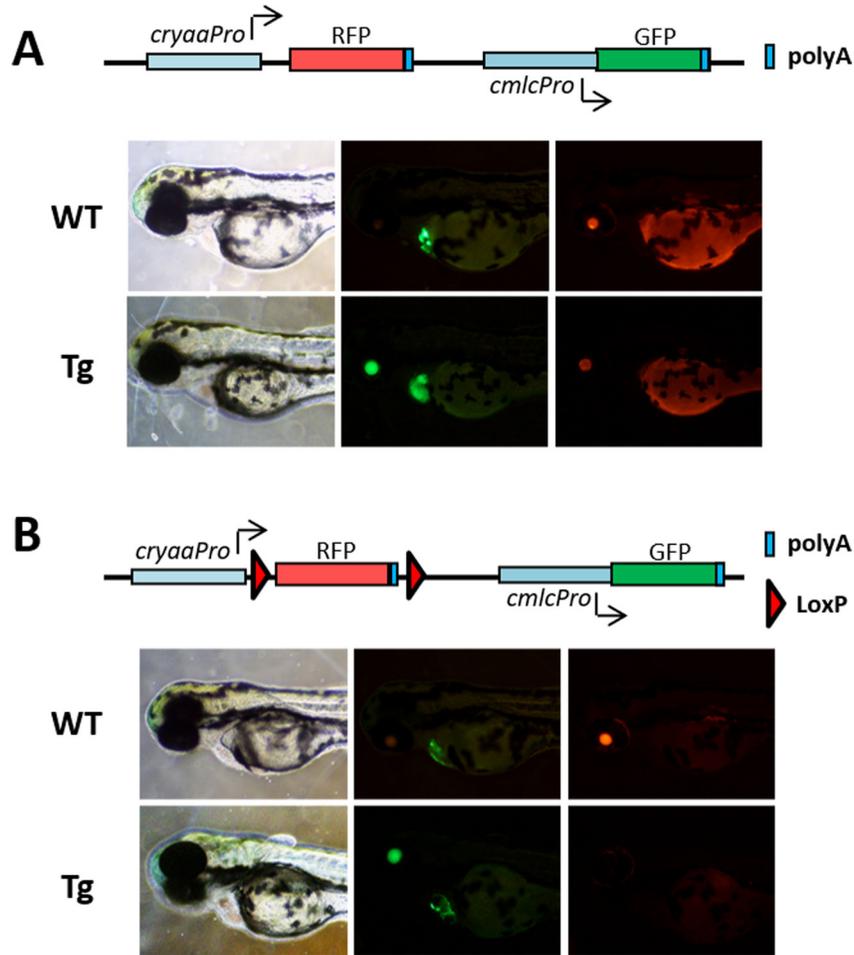
Figure 5. Measurement of Cre activity in the lens of zTg*(cryaa:Cre-cryaa:EGFP)*. **A**: Schematic map of the p*cryaa:RFP-cmlc:EGFP* plasmid and images of 2 dpf WT and zTg*(cryaa:Cre-cryaa:EGFP*; Tg) zebrafish that carried the p*cryaa:RFP-cmlc:EGFP* plasmids under bright-phase and fluorescence microscopy. RFP fluorescence was specifically expressed in the lens, while GFP was expressed in the heart. **B**: Schematic map of the p*cryaa:loxP-RFP-loxP-cmlc:EGFP* plasmid and images of the 2 dpf lenses of WT and zTg*(cryaa:Cre-cryaa:EGFP*; Tg) zebrafish that were injected with p*cryaa:loxP-RFP-loxP-cmlc:EGFP*. RFP was specifically expressed in WT zebrafish lenses (upper panel) but not in zTg*(cryaa:Cre-cryaa:EGFP*) lenses (lower panel).

TABLE 3. THE PERCENTAGE OF RFP+ EMBRYOS AMONG EGFP+ EMBRYOS IN EACH GROUP.				
Group		**RFP+**	**EGFP+**	**Percentage (%)**
cryaa:RFP-cmlc:EGFP				
	WT	78	79	98.7
	Tg	117	119	98.3
cryaa:loxP-RFP-loxP-cmlc:EGFP	WT	96	98	97.9
	Tg	2	126	1.6

132

DISCUSSION

We successfully generated a transgenic zebrafish, zTg*(cryaa:Cre-cryaa:EGFP)*, which specifically expresses a functional Cre recombinase in the lens. In this Tg-cre zebrafish, the expression of Cre and EGFP is driven by the *cryaa* promoter, and the expression patterns of EGFP and Cre recombinase faithfully recapitulate the endogenous expression pattern of *cryaa* in the lens (Figure 2 and Figure 3A and 3B). The expression pattern of EGFP was the same as that of Cre in lens tissues at 16 hpf, 2 dpf, 3 dpf, 1 mpf, 3 mpf, 6 mpf, 9 mpf, and 12 mpf for the zTg(cryaa:Cre-cryaa:EGFP) zebrafish (Figure 2 and Figure 3). Interestingly, the whole-mount ISH results showed that *cre* and *egfp* were weakly detected in the whole bodies of Tg-zebrafish at 16 hpf, and with the embryo's growth, *cre* and *egfp* were only visualized in lens tissues. The weak expression of *cryaa*-promoter-controlled Cre in the tissues outside the lens during early embryo development might have caused tissue specificity problems when it was used to edit gene expression specifically in the lens. To avoid this problem and increase lens specificity, the promoters of *cryaa* were tested at different lengths in our laboratory. Taken together, our results demonstrate that zTg*(cryaa:Cre-cryaa:EGFP)* can constitutively express Cre recombinase in lens tissue (Figure 3) and that EGFP is a primary selective marker for transgenic zebrafish.

To evaluate Cre activity in the zTg*(cryaa:Cre-cryaa:EGFP)* lens, we constructed and administered two plasmids (Figure 5): p*cryaa:RFP-cmlc:EGFP* and p*cryaa:Flox-RFP-Flox-cmlc:EGFP.* A total of 98.4% of the zTg*(cryaa:Cre-cryaa:EGFP)* zebrafish exhibited Cre activity in the lens (Table 3). These results demonstrate that zTg*(cryaa:Cre-cryaa:EGFP)* expresses an active Cre recombinase in lens tissues. Cre-transgenic mouse lines are a common tool for studying lens development or cataracts by manipulating specific gene expression in the lens. There are transgenic *Le-Cre*, *MLR10*, *MLR39*, *Nes-Cre*, *P0-P3.9GFPCre*, and *LR-Cre* mice [11]. Among them, MLR39 Cre-transgenic mice use the αA-crystallin promoter to drive Cre expression in lens fiber cells. αA-crystallin promoter sequences in zebrafish are conserved with those in mice [27]. The results presented in Figure 2 and Figure 3 indicate that the *cryaa* gene promoter could drive Cre expression in lens fibers in the zTg*(cryaa:Cre-cryaa:EGFP)* zebrafish model. Like transgenic *MLR39* mice, our zTg*(cryaa:Cre-cryaa:EGFP)* zebrafish is an alternative tool to edit gene expression in the lens fibers. By breeding this Tg-zebrafish with the zebrafish carrying the Flox-targeted genes, the researchers can narrow down the genes' specific regulation toward the lens, rather than another organ—for example, the *pax6* [40], *six3* [41], *or sox1* [42] genes.

Additionally, using this zTg*(cryaa:Cre-cryaa:EGFP)* zebrafish model, we also ensured the regulation of *cryaa* mRNA expression during zebrafish embryo development. We found that maternal *cryaa* mRNA was distributed in early zebrafish embryos (Figure 2). The embryos started to express their own *cryaa* mRNA at the 16–18 hpf stage, and following the embryos' growth, this expression occurred predominantly in lens fiber tissue (Figure 2C,D and Figure 3A–C). This expression pattern of *cryaa* in zebrafish is consistent with previous reports that *cryaa* mRNA was detectable at 10 somites (14 hpf) as a ubiquitous expression pattern [43] and that *cryaa* mRNA was detectable with qRT-PCR at 0.5 dpf (12hf) even at a low level [27].

In conclusion, we established a zTg(*cryaa*:Cre-*cryaa*:EGFP) zebrafish line with lens-specific expression of an active Cre recombinase. The Cre recombinase in zTg*(cryaa:Cre-cryaa:EGFP)* could efficiently recognize and cleave *loxP*-flanked RFP (Figure 5 and Table 2), and the ectopic expression of Cre and EGFP did not influence lens development or transparency (Figure 4). Accordingly, this zTg*(cryaa:Cre-cryaa:EGFP)* zebrafish is a useful tool for specifically manipulating gene expression in zebrafish lenses when the fish are crossed with another transgenic zebrafish line in which the target's particular sequences are flanked by the *LoxP* site.

ACKNOWLEDGMENTS

REFERENCES

1. Cvekl A, Ashery-Padan R. The cellular and molecular mechanisms of vertebrate lens development. Development 2014; 141:4432-47. [PMID: 25406393].

2. Shiels A, Hejtmancik JF. Inherited cataracts: Genetic mechanisms and pathways new and old. Exp Eye Res 2021; 209:108662[PMID: 34126080].

3. Li J, Chen X, Yan Y, Yao K. Molecular genetics of congenital cataracts. Exp Eye Res 2020; 191:107872[PMID: 31770519].

4. Wu X, Long E, Lin H, Liu Y. Prevalence and epidemiological characteristics of congenital cataract: a systematic review and meta-analysis. Sci Rep 2016; 6:28564-[PMID: 27334676].

5. Shiels A, Bennett TM, Hejtmancik JF. Cat-Map: putting cataract on the map. Mol Vis 2010; 16:2007-15. [PMID: 21042563].

6. Duncan MK, Kozmik Z, Cveklova K, Piatigorsky J, Cvekl A. Overexpression of PAX6(5a) in lens fiber cells results in cataract and upregulation of (alpha)5(beta)1 integrin expression. J Cell Sci 2000; 113:3173-85. [PMID: 10954416].

7. Krall M, Htun S, Anand D, Hart D, Lachke SA, Slavotinek AM. A zebrafish model of foxe3 deficiency demonstrates lens and eye defects with dysregulation of key genes involved in cataract formation in humans. Hum Genet 2018; 137:315-28. [PMID: 29713869].

8. Cui X, Feng R, Wang J, Du C, Pi X, Chen D, Li J, Li H, Zhang J, Zhang J, Mu H, Zhang F, Liu M, Hu Y. Heat shock factor 4 regulates lysosome activity by modulating the αB-crystallin-ATP6V1A-mTOR complex in ocular lens. Biochim Biophys Acta Gen Subj 2020; 1864:129496[PMID: 31786107].

9. Wu SY, Zou P, Mishra S, Mchaourab HS. Transgenic zebrafish models reveal distinct molecular mechanisms for cataract-linked αA-crystallin mutants. PLoS One 2018; 13:e0207540[PMID: 30475834].

10. Jia ZK, Fu CX, Wang AL, Yao K, Chen XJ. Cataract-causing allele in CRYAA (Y118D) proceeds through endoplasmic reticulum stress in mouse model. Zool Res 2021; 42:300-9. [PMID: 33929105].

11. Yang Y, Zhao H, Rizo CM, Robinson ML. (2002). Influences on the Expression Pattern of Cre Recombinase Driven by the −290/+43 alpha A-Crystallin Promoter. Arvo Annual Meeting Abstract Search & Program Planner Abstract.

12. Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, Collins JE, Humphray S, McLaren K, Matthews L, McLaren S, Sealy I, Caccamo M, Churcher C, Scott C, Barrett JC, Koch R, Rauch GJ, White S, Chow W, Kilian B, Quintais LT, Guerra-Assunção JA, Zhou Y, Gu Y, Yen J, Vogel JH, Eyre T, Redmond S, Banerjee R, Chi J, Fu B, Langley E, Maguire SF, Laird GK, Lloyd D, Kenyon E, Donaldson S, Sehra H, Almeida-King J, Loveland J, Trevanion S, Jones M, Quail M, Willey D, Hunt A, Burton J, Sims S, McLay K, Plumb B, Davis J, Clee C, Oliver K, Clark R, Riddle C, Elliot D, Threadgold G, Harden G, Ware D, Begum S, Mortimore B, Kerry G, Heath P, Phillimore B, Tracey A, Corby N, Dunn M, Johnson C, Wood J, Clark S, Pelan S, Griffiths G, Smith M, Glithero R, Howden P, Barker N, Lloyd C, Stevens C, Harley J, Holt K, Panagiotidis G, Lovell J, Beasley H, Henderson C, Gordon D, Auger K, Wright D, Collins J, Raisen C, Dyer L, Leung K, Robertson L, Ambridge K, Leongamornlert D, McGuire S, Gilderthorp R, Griffiths C, Manthravadi D, Nichol S, Barker G, Whitehead S, Kay M, Brown J, Murnane C, Gray E, Humphries M, Sycamore N, Barker D, Saunders D, Wallis J, Babbage A, Hammond S, Mashreghi-Mohammadi M, Barr L, Martin S, Wray P, Ellington A, Matthews N, Ellwood M, Woodmansey R, Clark G, Cooper J, Tromans A, Grafham D, Skuce C, Pandian R, Andrews R, Harrison E, Kimberley A, Garnett J, Fosker N, Hall R, Garner P, Kelly D, Bird C, Palmer S, Gehring I, Berger A, Dooley CM, Ersan-Ürün Z, Eser C, Geiger H, Geisler M, Karotki L, Kirn A, Konantz J, Konantz M, Oberländer M, Rudolph-Geiger S, Teucke M, Lanz C, Raddatz G, Osoegawa K, Zhu B, Rapp A, Widaa S, Langford C, Yang F, Schuster SC, Carter NP, Harrow J, Ning Z, Herrero J, Searle SM, Enright A, Geisler R, Plasterk RH, Lee C, Westerfield M, de Jong PJ, Zon LI, Postlethwait JH, Nüsslein-Volhard C, Hubbard TJ, Roest Crollius H, Rogers J, Stemple DL. The zebrafish reference genome sequence and its relationship to the human genome. Nature 2013; 496:498-503. [PMID: 23594743].

13. Bournele D, Beis D. Zebrafish models of cardiovascular disease. Heart Fail Rev 2016; 21:803-13. [PMID: 27503203].

14. Chhetri J, Jacobson G, Gueven N. Zebrafish–on the move towards ophthalmological research. Eye (Lond) 2014; 28:367-80. [PMID: 24503724].

15. Takamiya M, Weger BD, Schindler S, Beil T, Yang L, Armant O, Ferg M, Schlunck G, Reinhard T, Dickmeis T, Rastegar S, Strähle U. Molecular description of eye defects in the zebrafish Pax6b mutant, sunrise, reveals a Pax6b-dependent genetic network in the developing anterior chamber. PLoS One 2015; 10:e0117645[PMID: 25692557].

16. Zou P, Wu SY, Koteiche HA, Mishra S, Levic DS, Knapik E, Chen W, Mchaourab HS. A conserved role of αA-crystallin in the development of the zebrafish embryonic lens. Exp Eye Res 2015; 138:104-13. [PMID: 26149094].

17. Greiling TMS, Houck SA, Clark JI. The zebrafish lens proteome during development and aging. Mol Vis 2009; 15:2313-25. [PMID: 19936306].

18. Alpha-crystallin HJ. Exp Eye Res 2003; 76:145-53. [PMID: 12565801].

19. Reddy GB, Kumar PA, Kumar MS. Chaperone-like activity and hydrophobicity of alpha-crystallin. IUBMB Life 2006; 58:632-41. [PMID: 17085382].

20. Koteiche HA, Claxton DP, Mishra S, Stein RA, McDonald ET, Mchaourab HS. Species-Specific Structural and Functional Divergence of α-Crystallins: Zebrafish αBa- and Rodent αA(ins)-Crystallin Encode Activated Chaperones. Biochemistry 2015; 54:5949-58. [PMID: 26378715].

21. Zhao H, Yang Y, Rizo CM, Overbeek PA, Robinson ML. Insertion of a Pax6 consensus binding site into the alphaA-crystallin promoter acts as a lens epithelial cell enhancer in

transgenic mice. Invest Ophthalmol Vis Sci 2004; 45:1930-9. [PMID: 15161860].

22. Smith AA, Wyatt K, Vacha J, Vihtelic TS, Zigler JS Jr, Wistow GJ, Posner M. Gene duplication and separation of functions in alphaB-crystallin from zebrafish (Danio rerio). FEBS J 2006; 273:481-90. [PMID: 16420472].

23. Farnsworth DR, Posner M, Miller AC. Single cell transcriptomics of the developing zebrafish lens and identification of putative controllers of lens development. Exp Eye Res 2021; 206:108535[PMID: 33705730].

24. Runkle S, Hill J, Kantorow M, Horwitz J, Posner M. Sequence and spatial expression of zebrafish (Danio rerio) alphaA-crystallin. Mol Vis 2002; 8:45-50. [PMID: 11925526].

25. Posner M, Kantorow M, Horwitz J. Cloning, sequencing and differential expression of alphaB-crystallin in the zebrafish, Danio rerio. Biochim Biophys Acta 1999; 1447:271-7. [PMID: 10542326].

26. Wu S-Y, Zou P, Mishra S, Mchaourab HS. Transgenic zebrafish models reveal distinct molecular mechanisms for cataract-linked αA-crystallin mutants. PLoS One 2018; 13:e0207540[PMID: 30475834].

27. Posner M, Murray KL, McDonald MS, Eighinger H, Andrew B, Drossman A, Haley Z, Nussbaum J, David LL, Lampi KJ. The zebrafish as a model system for analyzing mammalian and native α-crystallin promoter function. PeerJ 2017; 5:e4093[PMID: 29201567].

28. Kos CH. Cre/loxP system for generating tissue-specific knockout mouse models. Nutr Rev 2004; 62:243-6. [PMID: 15291397].

29. Schwenk F, Baron U, Rajewsky K. A cre-transgenic mouse strain for the ubiquitous deletion of loxP-flanked gene segments including deletion in germ cells. Nucleic Acids Res 1995; 23:5080-1. [PMID: 8559668].

30. Maddala R, Chauhan BK, Walker C, Zheng Y, Robinson ML, Lang RA, Rao PV. Rac1 GTPase-deficient mouse lens exhibits defects in shape, suture formation, fiber cell migration and survival. Dev Biol 2011; 360:30-43. [PMID: 21945075].

31. Ni TT, Lu J, Zhu M, Maddison LA, Boyd KL, Huskey L, Ju B, Hesselson D, Zhong TP, Page-McCaw PS, Stainier DY, Chen W. Conditional control of gene function by an invertible gene trap in zebrafish. Proc Natl Acad Sci U S A 2012; 109:15389-94. [PMID: 22908272].

32. Sugimoto K, Hui SP, Sheng DZ, Kikuchi K. Dissection of zebrafish *shha* function using site-specific targeting with

a Cre-dependent genetic switch. Elife 2017; 6:6-[PMID: 28513431].

33. Hoshijima K, Jurynec MJ, Grunwald DJ. Precise Editing of the Zebrafish Genome Made Simple and Efficient. Dev Cell 2016; 36:654-67. [PMID: 27003937].

34. Liu F, Kambakam S, Almeida MP, Ming Z, Welker JM, Wierson WA, Schultz-Rogers LE, Ekker SC, Clark KJ, Essner JJ, McGrail M. Cre/*lox* regulated conditional rescue and inactivation with zebrafish UFlip alleles generated by CRISPR-Cas9 targeted integration. Elife 2022; 11:11-[PMID: 35713402].

35. Mukherjee K, Liao EC. Generation and characterization of a zebrafish muscle specific inducible Cre line. Transgenic Res 2018; 27:559-69. [PMID: 30353407].

36. Westerfield M. The Zebrafish Book. A Guide for The Laboratory Use of Zebrafish (Danio rerio). zebrafish book a guide for the laboratory use of zebrafish. 2000.

37. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. Dev Dyn 2010; 203-[PMID: 8589427].

38. Li X, He J, Hu W, Yin Z. The essential role of endogenous ghrelin in growth hormone expression during zebrafish adenohypophysis development. Endocrinology 2009; 150:2767-74. [PMID: 19264876].

39. Casadei R, Pelleri MC, Vitale L, Facchin F, Lenzi L, Canaider S, Strippoli P, Frabetti F. Identification of housekeeping genes suitable for gene expression analysis in the zebrafish. Gene Expr Patterns 2011; 11:271-6. [PMID: 21281742].

40. Kannan RR, Vincent SG. ELISA based quantification of Pax6 expression in the developing Zebrafish embryos. Ann Neurosci 2015; 22:171-5. [PMID: 26130925].

41. Samuel A, Rubinstein AM, Azar TT, Ben-Moshe Livne Z, Kim S-H, Inbal A. Six3 regulates optic nerve development via multiple mechanisms. Sci Rep 2016; 6:20267-[PMID: 26822689].

42. Nishiguchi S, Wood H, Kondoh H, Lovell-Badge R, Episkopou V. Sox1 directly regulates the gamma-crystallin genes and is essential for lens development in mice. Genes Dev 1998; 12:776-81. [PMID: 9512512].

43. Marvin M, O'Rourke D, Kurihara T, Juliano CE, Harrison KL, Hutson LD. Developmental expression patterns of the zebrafish small heat shock proteins. Dev Dyn 2008; 237:454-63. [PMID: 18161059].