CRISPR/Cas9–Mediated Mutation of αA-Crystallin Gene Induces Congenital Cataracts in Rabbits

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PURPOSE. The present study aimed to investigate the role of the αA-crystallin gene in inducing congenital cataracts in rabbits and to construct a novel animal model for characterization and pathologic analysis of congenital cataracts for future research.

METHODS. We generated αA-crystallin gene knockout rabbits with congenital cataracts by coinjection of Cas9 mRNA and sgRNA into zygotes. Cataract phenotypes were investigated in a repeated study of 19 F0-generation and 11 F1-generation rabbits with αA-crystallin gene mutations. Heritability was analyzed by PCR, sequencing, slim lamp, hematoxylin eosin staining, immunohistochemistry, and Western blot.

RESULTS. We found αA-crystallin gene mutations in all 19 F0-generation pups (100%) with indel mutations in the αA-crystallin gene ranging from 3 to 52 bp. Off-target assay revealed that none of the potential off-target sites exhibited mutations, demonstrating that off-target mutagenesis was not induced by cytoplasmic microinjection of in vitro–transcribed Cas9 mRNA. Slim lamp assay revealed that 15 of 19 live pups (78.9%) exhibited typical phenotypes, including congenital cataracts, microphthalmia, obscurity, and early atrophy of the lens, and failed differentiation of lens fibers. Histologic hematoxylin and eosin staining showed that αA-crystallin gene knockout rabbits exhibited smaller lenses. Production of the αA-crystallin protein was determined to be dramatically reduced in αA-crystallin gene knockout rabbits. We induced αA-crystallin gene mutations and phenotypes in F1-generation rabbits.

CONCLUSIONS. Our data suggest that CRISPR/Cas9–mediated mutation of the αA-crystallin gene in rabbits recapitulates phenotypes of congenital cataracts, microphthalmia, obscurity, and early atrophy of the lens, and failed differentiation of lens fibers. These findings suggest the possibility of a new animal model of congenital cataracts, which should be used to further investigate the association between mutations in αA-crystallin gene and congenital cataracts in humans.

Keywords: rabbits, αA-crystallin, congenital cataracts, CRISPR/Cas9, animal model

Congenital cataracts are characterized by increased opacity and decreased transparency in the lens, and they are the leading cause of blindness in newborn babies.1 Previous studies have reported that nearly a third of congenital cataracts are caused by mutations in the crystalline family genes, αA-crystallin gene (CRYAA) and αB-crystallin gene (CRYAB), which have been shown to be associated with autosomal dominant or recessive congenital cataracts.2–4 The gene CRYAA is expressed at a high level in the lens,5 and is involved in maintaining its structure and transparency.6 In clinical practice, mutations in the human CRYAA have been found to be associated with congenital anterior polar cataracts.5,7,8

Mouse models are widely used to recapitulate human diseases, owing to certain physiologic characteristics and relatively low costs.9 However, mouse models cannot accurately replicate the physiologic and pathologic characteristics of all human diseases due to differences in physiology and gene expression between mouse and human.10 Instead, based on the similar sizes and physiologic structures of human and rabbit eyes, rabbits may represent a more optimal animal model for eye disease studies.

The present study aimed to provide further insight into the relationship between CRYAA mutations and congenital cataracts and to create a new animal model for recapitulating congenital cataracts for in vivo drug screening and assessment of the safety of artificial lenses. We generated CRYAA knockout (KO) rabbits via coinjection of Cas9 mRNA and sgRNA into pronuclear-stage zygotes. We also evaluated the mutational efficiency of the CRISPR/Cas9 system, the resulting congenital cataract phenotypes in the KO rabbits, and the heritability of the CRYAA KO mutations in this animal model.

METHODS

Ethical Statement

We purchased 6-month-old New Zealand rabbits from the Laboratory Animal Centre of Jilin University (Changchun, China). All experiments about rabbits were carried out under...
the supervision of and in accordance with the guidelines on animal care of the Animal Care Center and Use Committee of Jilin University. All experimental protocols were approved by the Ethics Committee of Jilin University, and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Science.

Vector Construction and In Vitro Transcription

Vector construction and in vitro transcription were carried out as described previously by Song et al. 11 Two complementary DNA oligonucleotides were annealed at 95°C for 5 minutes to generate double-strand DNA, which was then cloned into a BbsI-digested pUC57-simple vector expressing Cas9 (Addgene ID 51307). In vitro transcription was performed using commercial kits (mMessagem Machine SP6 and MAXI script T7; Ambion, Austin, TX, USA). Transcripts were then purified with an RNA extraction kit (RNeasy Mini Kit; Qiagen, Hilden, Germany).

Embryo Collection, Microinjection, and Transfer

The protocol for microinjection of pronuclear-stage zygotes has been described previously by Song et al. 11 A mixture of Cas9 mRNA (200 ng/µL) and CRYAA-sgRNA (50 ng/µL) was coinjected into the cytoplasm of pronuclear-stage zygotes. These zygotes were then immediately transferred into the oviducts of surrogate rabbits.

Mutation Detection by PCR and T7E1 Assay

The protocol for mutation detection has been described previously by Lv et al. 13 Genomic (g)DNA was extracted from microinjected blastocysts and CRYAA KO rabbits. We listed PCR primers for CRYAA as follows: F, 5′ AACAGCCAGTCAGCCTTAAC 3′, and R, 5′ ACCGTGTCTCTGGTTGTGGT 3′. At least 30 positive plasmid clones were sequenced by Comate Bioscience Company Limited (Changchun, China) through Sanger sequencing and analyzed using commercial software (DNAman; Lynnon Corp., San Ramon, CA, USA, and Basic Local Alignment Search Tool; National Center for Biotechnology Information, Bethesda, MD, USA).

Off-Target Analysis

To test whether off-target mutations occurred in the CRYAA KO rabbits, seven potential off-target sites (POTS) with top ranking sequence similarity to CRYAA were utilized to perform the T7E1 assay as described previously by Shen et al. 14 Digested samples were analyzed by 12% polyacrylamide TAE gel.

TABLE 1. Summary of Mutation Efficiency and Embryo Cleavage Rate After Injection With the CRISPR/Cas9 System

<table>
<thead>
<tr>
<th>Zygotes, n</th>
<th>2-Cell, n (%)</th>
<th>Morula, n (%)</th>
<th>Blastocyst, n (%)</th>
<th>Blastocysts With CRYAA Mutation, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noninjection</td>
<td>30</td>
<td>29 (96.7)</td>
<td>28 (93.3)</td>
<td>26 (86.7)</td>
</tr>
<tr>
<td>CRISPR injection</td>
<td>30</td>
<td>29 (93.3)</td>
<td>28 (96.7)</td>
<td>26 (86.7)</td>
</tr>
<tr>
<td>30</td>
<td>29 (96.7)</td>
<td>29 (96.7)</td>
<td>27 (90)</td>
<td>27 (100)</td>
</tr>
<tr>
<td>30</td>
<td>29 (96.7)</td>
<td>28 (93.3)</td>
<td>27 (90)</td>
<td>27 (100)</td>
</tr>
<tr>
<td>Total</td>
<td>90</td>
<td>86 (95.5)</td>
<td>84 (93.5)</td>
<td>80 (88.9)</td>
</tr>
</tbody>
</table>

FIGURE 1. CRISPR/Cas9–mediated gene targeting of CRYAA in zygotes. E1-E10 represent various blastocysts used in this study. (A) Schematic diagram of sgRNA targeting the rabbit CRYAA loci. The yellow rectangle represents the protein-coding region of CRYAA. The blue rectangle represents the untranslated regions of CRYAA. Two sgRNA sequences, sgRNA1 and sgRNA2, are highlighted in red, and the protospacer adjacent motif (PAM) sequences are presented in green. Primers F and R were used for mutation detection in blastocysts and pups. ATG, start codon; TGA, termination codon. (B) CRYAA mutation detection in blastocysts by T- cloning and Sanger sequencing. The wild-type sequence is shown at the top. Sequences of sgRNAs are highlighted in red, insertions are highlighted in blue, and deletions are designated by dashes. (C) T7E1 cleavage assay for mutation detection in blastocysts. M, DL2000. Bands of PCR products: WT (368 bp), an additional band of a smaller size was cleaved band in mutants (322 bp). Bands of T7E1: WT band (368 bp), mutant band (322 bp), and T7E1 cleaved band (268 bp). (D) Cytoplasmic injection of zygotes using CRISPR/Cas9 system.
scores for each sgRNA were predicted using an online design tool (http://crispr.mit.edu, in the public domain). Products of POTS PCR were amplified and assessed by Sanger sequencing and T7E1 assay14.

Slit Lamp Examination
The primary diagnosis of congenital cataracts was determined by slit lamp biomicroscopy. At 13 days of age, the rabbits were dilated and examined. In a normal lens, the reflection of the slit lamp from the surface of the cornea is transparency in all panels, while lenses with congenital cataracts result in a dense opacity.

Hematoxylin and Eosin (HE) Staining and Immunohistochemistry (IHC)
We performed HE and immunofluorescence staining as previously described by Xia et al.15 The eyes of CRYAA KO and WT rabbits were fixed in 4% paraformaldehyde for 48 hours at 4°C before being dehydrated, embedded in paraffin medium, and slide-sectioned. Primary anti-CRYAA antibody (1:1000; ab5595; Abcam plc, Cambridge, UK) and secondary anti-rabbit polyclonal antibodies (1:600; A0280; Beyotime Institute of Biotechnology, Shanghai, China) were used in this study. We used 3,3’-diaminobenzidine tetrahydrochloride (DAB) (AR1022; Boster Biological Technology, Wuhan, China) for IHC staining, and sections were imaged with a fluorescence microscope (Eclipse TS100; Nikon Corp., Tokyo, Japan).

Western Blotting
Western blot analysis of CRYAA was conducted as previously described by Lv et al.13 Briefly, primary antibodies for CRYAA (1:10,000; ab5595) and β-actin (1:1000; 60008-1-lg; Proteintech Group) were incubated at 4°C overnight. β-actin was used as an internal control. HRP-conjugated secondary antibodies (1:600; A0280) were used for enhanced chemiluminometry (ECL) detection. The membrane was exposed on an Azure Biosystems (C600) imaging system (Azure Biosystems, Dublin, CA, United States), according to the manufacturer’s instructions.

RESULTS
Mutation of CRYAA in Zygotes Using CRISPR/Cas9 System
To induce KO mutations in CRYAA in rabbits, two sgRNAs targeting exon 2 of CRYAA were designed using an online tool (http://crispr.mit.edu, in the public domain; Fig. 1A). To examine the mutational efficiency of the CRISPR/Cas9 system, we used 3,3’-diaminobenzidine tetrahydrochloride (DAB) (AR1022; Boster Biological Technology, Wuhan, China) for IHC staining, and sections were imaged with a fluorescence microscope (Eclipse TS100; Nikon Corp., Tokyo, Japan).

TABLE 2. Summary of the Generation of CRYAA KO Rabbit Via CRISPR/Cas9 System

<table>
<thead>
<tr>
<th>Recipients</th>
<th>gRNA/Cas9, ng/µL</th>
<th>Embryos, n</th>
<th>Pups Obtained, n</th>
<th>Pups With Mutation, n (%)</th>
<th>Pups With Cataract, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40/180</td>
<td>45</td>
<td>10</td>
<td>10 (100)</td>
<td>8 (80.0)</td>
</tr>
<tr>
<td>2</td>
<td>40/180</td>
<td>40</td>
<td>9</td>
<td>9 (100)</td>
<td>7 (77.8)</td>
</tr>
</tbody>
</table>

FIGURE 2. Generation of CRYAA KO rabbits by CRISPR/Cas9 system. (A) T-cloning and Sanger sequencing of 19 pups (F0-1 to F0-19). F0-1 to F0-19 represent the founder pups (F0). Sequences of sgRNAs are highlighted in red, insertions are shown in blue, and deletions are designated by dashes. (B) T7E1 cleavage assay for CRYAA mutation detection in F0-1 to F0-19 pups. M, DL2000. F0-1 to F0-19 represent various F0 pups used in this study. Bands of PCR products: WT (368 bp), an additional band of a smaller size was cleaved band in mutants (322 bp). Bands of T7E1: WT band (368 bp), mutant band (322 bp), and T7E1 cleaved band (268 bp).
FIGURE 3. Determination of off-target effects in F0 CRYAA KO rabbits. Chromatogram sequence analysis of 14 POTS for (A) sgRNA1 and (B) sgRNA2 in F0 CRYAA KO rabbits showing no double peaks in any sequencing diagrams. Blue area represents sequencing of the POTS. T7E1 cleavage analysis of POTS for (C) sgRNA1 and (D) sgRNA2. M, DL2000; 1–7, seven POTS. (E) Comparison of intended CRISPR DSB sites and POTS showing the number of sequence mismatches. CRYAA-S1, CRYAA-sgRNA1; CRYAA-S2, CRYAA-sgRNA2. Double-strand break sites are highlighted in green, and PAM sequences in blue. Sequence mismatches are highlighted in red.
in zygotes, a mixture of in vitro–transcribed Cas9 mRNA and the two sgRNAs were coinjected into the cytoplasms of rabbit zygotes. Then, the injected zygotes were cultured until the blastocyst stage, and CRYAA mutations were detected in all tested blastocysts (100%; Table 1). This result was confirmed by T-vector and T7E1 assay (Figs. 1B, 1C). These results suggest that mutations in CRYAA were induced by cytoplasmic microinjection of Cas9 mRNA and two sgRNAs in zygotes.

**Generation of CRYAA KO Rabbits**

In order to generate CRYAA KO rabbits, 85 injected zygotes were transferred to two surrogate rabbits. After full-term gestation, the two recipient mothers successfully gave birth to 19 live pups (Table 2). Genomic DNA samples from F0-generation pups were extracted and used to detect of CRYAA mutations. As shown in Fig. 2A, CRYAA mutations were found in all 19 pups (100%). Indel mutations in CRYAA ranged from 3 to 52 bp and were confirmed by T7E1 assay (Figs. 2A, 2B).

To test whether off-target mutations occurred in these CRYAA KO rabbits, PCR products from seven POTS were sequenced and assessed with T7E1 assay (Figs. 3A–D). The number of sequence mismatches between each POTS and the CRISPR double-strand break (DSB) sites is listed in Figure 3E. The results revealed that none of the POTS exhibited mutations, demonstrating that off-target mutagenesis was reduced by cytoplasmic microinjection of in vitro–transcribed Cas9 mRNA.

**Phenotype Assessment of CRYAA KO Rabbits**

Next, we investigated whether the CRYAA mutations caused phenotypes including cataracts. Slit lamp observation revealed that 15 of 19 live pups (78.9%) exhibited congenital cataracts and microphthalmia (Table 2). Opacity was observed in the nuclear regions of the lenses of CRYAA KO rabbits, while wild-type (WT) litter mates exhibited transparent lenses (Fig. 4A). In addition, CRYAA KO rabbits exhibited significantly reduced levels of CRYAA protein (Fig. 4B).

Histologic HE staining showed that CRYAA KO rabbits exhibited smaller lenses (Fig. 4C, a versus e), as well as more severe vacuole-like degeneration in the equatorial regions (Fig. 4C, b versus f) and anterior capsules (Fig. 4C, d versus h) of their lenses compared with those of WT rabbits. In addition, primary fibers were disordered and had migrated to the organelle-free zone (OFZ) in the nuclei of CRYAA KO rabbit lenses, while those of WT rabbits were well ordered (Fig. 4C, c versus g). These findings indicate that phenotypes associated
with congenital cataracts and disrupted organization of the lens were exhibited in CRYAA KO rabbits.

Heritability of CRYAA Mutations and Phenotype Assessment of F1 Rabbits

In order to determine the heritability of the cataract phenotypes, two female founders exhibiting cataract phenotypes (F0-8 and F0-12) were mated with two male founders (F0-4 and F0-6). We genotyped F1 CRYAA KO rabbits by PCR and T-cloning. The results revealed that mutations in CRYAA were detected in 10 of the 11 F1 rabbit pups (Figs. 5A, 5B). We further examined whether these CRYAA mutations led to the observed cataract phenotypes in the F1 generation. As shown in Figures 5C and 5D, F1-8 and F1-3 rabbits presented concentrated opacities in the nuclear regions of the lenses (cataract phenotypes). Microphthalmia, smaller lenses, and early lens atrophy were also found in F1-3 CRYAA KO rabbits. Histologic analysis confirmed that smaller lenses (Fig. 6A, a versus e), as well as anterior capsules (Fig. 6A, d versus h) and more severe vacuole-like degeneration in the equatorial regions (Fig. 6A, b versus f) of lenses were observed in F1 CRYAA KO rabbits. In addition, nuclei were deeply stained pink (Fig. 6A, d versus h) in the anterior capsules, suggesting that lens organization was disrupted in the CRYAA KO rabbits. Furthermore, a dramatic reduction in the production of CRYAA protein was observed via IHC (Fig. 6B) and Western blotting (Fig. 6C) in CRYAA KO rabbits compared with that in WT rabbits. Thus, our data indicate a strong genotype phenotype correlation between CRYAA mutations and congenital cataracts.

DISCUSSION

Previous studies have demonstrated that CRYAA plays key roles in suppressing stress-induced protein aggregation and main-
taining the membrane structures of lens epithelial and fiber cells.\textsuperscript{16,17} This has been confirmed in CRYAA\textsuperscript{KO} mice, which develop small lenses with opacities initially confined to the nucleus but progressing to total cataracts.\textsuperscript{18} In our study, congenital microphthalmia, as well as degradation of the membrane structures in lens fibers were found in CRYAA\textsuperscript{KO} rabbits. In addition, failed maturation of epithelial cells was observed in the OFZ of CRYAA\textsuperscript{KO} rabbits. The OFZ is involved in the synthesis and packaging of free-organelles (nucleus, endoplasmic reticulum, Golgi apparatus, etc.) in the cytoplasm of terminally differentiated lens fiber cells, and it also functions in maintaining the transparency of the lens.\textsuperscript{19,20} Therefore, we speculate that the breakdown of nuclei in the secondary lens fiber cells was due to incomplete organelle degradation in the OFZ, which has been demonstrated in previous studies.\textsuperscript{21,22}

Although cataract phenotypes and microphthalmia have been observed in mice and humans with CRYAA mutations,\textsuperscript{7,18} the mechanisms underlying microphthalmia in these cases have not been investigated. Of note, dramatically smaller lenses that experience early atrophy were observed in CRYAA KO rabbits in this study. It has been shown that CRYAA not only acts as a chaperone but also functions to prevent lens cells from apoptosis both in vitro and in vivo.\textsuperscript{6} Therefore, we suspect that CRYAA mutations may induce apoptosis in lens epithelial cells, resulting in microphthalmia and early atrophy of lenses in CRYAA KO rabbits.

Previous studies reported that mutations in gap junction protein alpha 8 (GJA8) in various species recapitulate congenital cataracts.\textsuperscript{12,23,24} The mechanism leading to cataracts in our previous study\textsuperscript{12} seems to involve the abnormal distribution and expression of membrane protein GJA8, which functions in the formation of connexin and maintains the gap junction channel structure.\textsuperscript{23,25} As for CRYAA mutations, the mechanism underlying the induction of congenital cataracts is the abnormal aggregation of insoluble \(\alpha\)-A-crystallin protein in the lens.\textsuperscript{26} Although phenotypes such as cataracts, lens opacity, microphthalmia, and small lens size were observed in both GJA8 and CRYAA KO rabbits, primary fibers were found to migrate to the OFZ in the nuclei of CRYAA KO rabbit lenses, while smaller gap junctions were observed in the cortical fibers of GJA8 KO rabbits. Based on these differences, the novel CRYAA KO rabbits produced in this study provide a broader understanding of how CRYAA functions as a chaperone, structural, and regulatory protein in lens transparency.

In summary, the CRISPR/Cas9–mediated mutation of CRYAA in rabbits recapitulates phenotypes of congenital cataracts, microphthalmia, early atrophy of the lens, and failed differentiation of lens fibers. These cataract phenotypes can be induced by abnormal aggregation of lens proteins and the failure of OFZ formation in the lenses of CRYAA KO rabbits. This novel and stable animal model can be used in the future to...
study the role of crystallin and to conduct drug screening for cataract prevention in clinical practice.

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