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Brief Report

Correction of a Genetic Disease in Mouse via Use of CRISPR-Cas9

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SUMMARY

The CRISPR-Cas9 system has been employed to generate mutant alleles in a range of different organisms. However, so far there have not been reports of use of this system for efficient correction of a genetic disease. Here we show that mice with a dominant mutation in *Crygc* gene that causes cataracts could be rescued by coinjection into zygotes of Cas9 mRNA and a single-guide RNA (sgRNA) targeting the mutant allele. Correction occurred via homology-directed repair (HDR) based on an exogenously supplied oligonucleotide or the endogenous WT allele, with only rare evidence of off-target modifications. The resulting mice were fertile and able to transmit the corrected allele to their progeny. Thus, our study provides proof of principle for use of the CRISPR-Cas9 system to correct genetic disease.

The CRISPR-Cas9 system from bacteria has been recently applied to genome editing in different species, including *Drosophila* (Yu et al., 2013), *C. elegans* (Dickinson et al., 2013; Friedland et al., 2013), zebrafish (Chang et al., 2013; Hwang et al., 2013; Jao et al., 2013), mouse (Cho et al., 2013; Cong et al., 2013), rat (Li et al., 2013a, 2013b), and human (Cong et al., 2013; Jinek et al., 2013; Mali et al., 2013). Impressively, by directly injecting Cas9 mRNA and single-guide RNAs (sgRNAs) into zygotes, mice or rats carrying mutations in transgenes or multiple endogenous genes can be generated in one step (Li et al., 2013a, 2013b; Shen et al., 2013; Wang et al., 2013; Yang et al., 2013), indicating that the CRISPR-Cas9 system can be used as an effective tool for genome engineering (Gaj et al., 2013). Nevertheless, successful correction of disease-causing mutations in animal models via the CRISPR-Cas9 system has not yet been demonstrated. In this study, we report the efficient correction of a mutant gene in a mouse disease model by zygote injection of CRISPR-Cas9, and we report subsequent successful transmission of the corrected trait to the next generation.

We chose a mouse model of dominant cataract disorder caused by a defined mutation in the *Crygc* gene (Zhao et al., 2010). These mice carry a 1 bp deletion in exon 3 of *Crygc*. This mutation leads to a stop codon at the 76th amino acid (Figure 1A) and thus the production of truncated γC-crystallin, resulting in nuclear cataracts (Figure 1B) in both homozygous and heterozygous mutant mice at weaning (Zhao et al., 2010). We first tested the feasibility of CRISPR-Cas9-mediated genetic repair at the cellular level. To this end, we designed five sgRNAs targeting different regions in the mutant *Crygc* gene. sgRNA-1, sgRNA-2, and sgRNA-3 were designed to target regions spanning the site of 1 bp deletion in mutant allele (Figure 1C and Supplemental Experimental Procedures available online). sgRNA-4 was designed to target the region immediately downstream of the 1 bp deletion, which we reasoned might be highly specific for the mutant allele due to the fact that the 1 bp deletion in the mutant allele produced a neo-protospacer adjacent motif (neo-PAM) that is not present in the WT allele (Figure 1C and Supplemental Experimental Procedures). sgRNA-5 targeted the normal sequence upstream of the deletion (Figure 1C and Supplemental Experimental Procedures). In preliminary experiments, we investigated the specificity of sgRNAs by separately transfecting plasmids expressing both the mammalian-codon-optimized Cas9 and each of the five sgRNAs into WT E14 embryonic stem cells (ESCs) (Cong et al., 2013; Mali et al., 2013). For each sgRNA, PCR products corresponding to the putative target region were amplified from the resulting ESC clones. The results showed that sgRNA-2 and sgRNA-5 efficiently targeted the WT alleles and led to nonhomologous end joining (NHEJ)-mediated mutations of the *Crygc* gene, while sgRNA-1 and sgRNA-3 did so much less frequently (Table S1). By contrast, no mutation was detected in ESC clones transfected with sgRNA-4 (Table S1). We performed similar experiments in heterozygous mutant ESCs (termed m*Crygc* ESCs) and found that all of the sgRNAs could induce cleavage at the mutant *Crygc* allele (Table S1). Consistent with the results from the E14 ESCs, we found that sgRNA-2 and sgRNA-5 targeted both the WT and mutant alleles in m*Crygc* ESCs, while the other three sgRNAs only targeted the mutant allele. Importantly, while all of the sgRNAs could induce

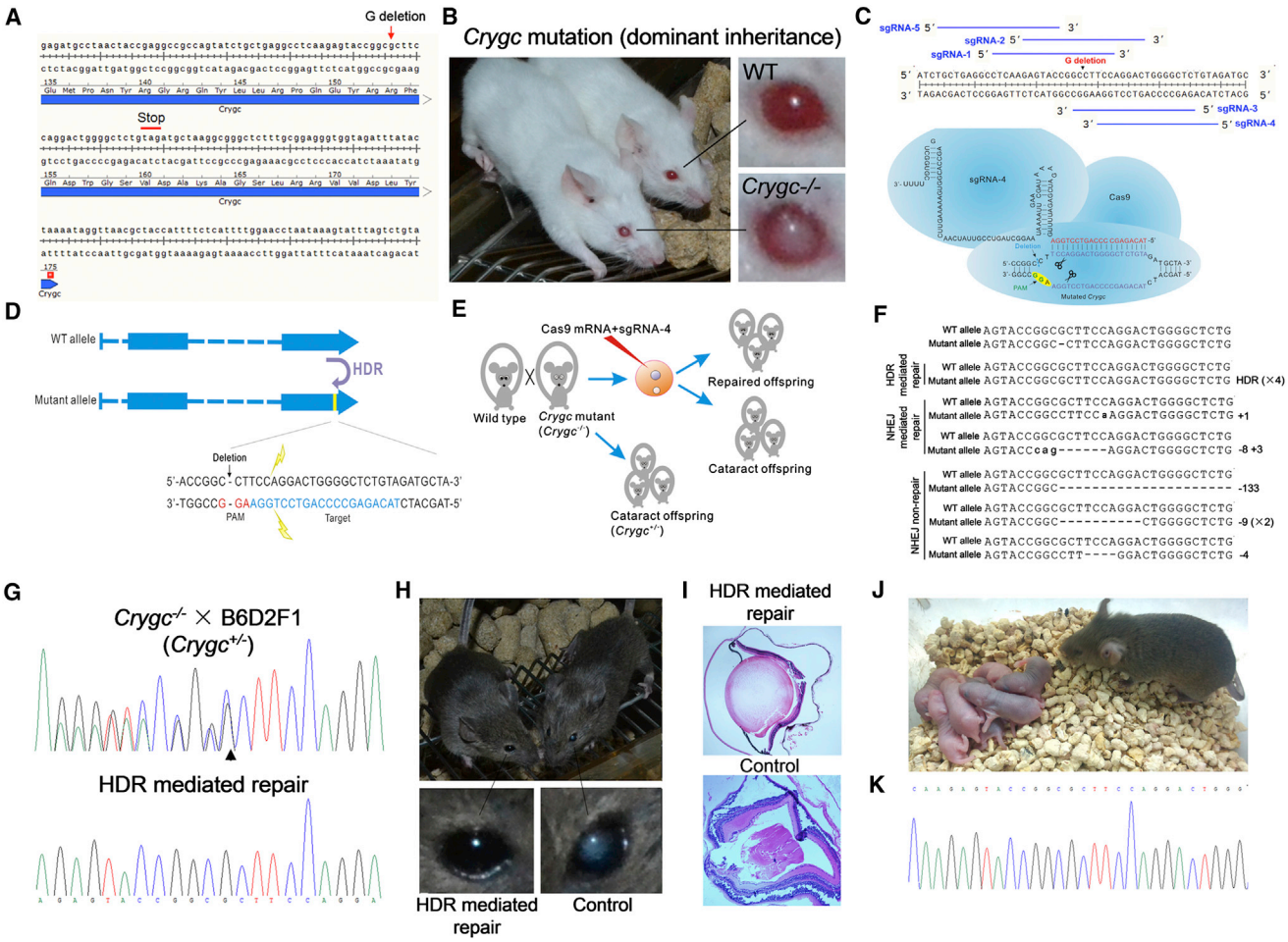

Figure 1. CRISPR-Cas9-Mediated Gene Correction in a Cataract Mouse Model

(A) The sequence of the mutant *Crygc* gene. The cataract phenotype is induced by 1 bp deletion in exon 3 of *Crygc*. This mutation leads to a stop codon at the 76th amino acid and thus the production of the truncated γC-crystallin.

(B) The eyes of *Crygc*$^{-/-}$ mutant mouse display cataract phenotypes compared to normal eyes of BALB/c control mouse.

(C) Schematic of the Cas9-sgRNA-targeting sites in mutant *Crygc* gene. Blue lines label the sgRNA-targeting sequences.

(D) Schematic for a gene correction via homology-directed repair (HDR) induced by the CRISPR-Cas9 system using normal allele on the homologous chromosome as a template. sgRNA-4 targeting site is labeled in blue and the PAM is marked in red.

(E) Outline of one-step correction of a genetic defect in cataract mouse model. The heterogeneous F1 pups delivered from WT female mice (B6D2F1) mated with *Crygc*$^{-/-}$ mice bear cataracts. Upon microinjections of Cas9 mRNA and sgRNA-4 into heterogeneous zygotes carrying mutant *Crygc* allele, some of the embryos develop to term with a normal phenotype.

(F) The sequences of the *Crygc* gene in mice carrying CRISPR-Cas9-induced gene modifications. Insertions are indicated with (+), deletions are indicated with (−). Small letters represent the inserted nucleotides.

(G) DNA sequence of PCR products amplified from the *Crygc* gene of a heterogeneous mutant mouse (*Crygc*$^{+/-}$) and a repaired mouse carrying the corrected allele induced by HDR after zygote-injection of Cas9 mRNA and sgRNA-4. Two peaks can be observed in the sequence of the control heterogeneous mutant (*Crygc*$^{+/-}$) mouse, while the HDR-repaired mouse has only one peak.

(H) A repaired mouse carrying the correct allele induced by HDR and a control heterogeneous mutant (*Crygc*$^{+/-}$) mouse. The repaired mouse after CRISPR-Cas9-mediated gene correction is free of cataracts, as measured by the appearance of black lenses compared to the opacity of the lens in control heterogeneous mutant mouse (*Crygc*$^{+/-}$).

(I) Histological analysis of lenses prepared from repaired mice via HDR (6 weeks old) and control heterogeneous mutant mice (*Crygc*$^{+/-}$) (6 weeks old). While the heterogeneous mutant mouse (*Crygc*$^{+/-}$) shows vacuole-like degeneration in the equatorial region of the entire eye, the cataract-free mouse exhibits normal histological features.

(J) Progeny with a repaired mouse (HDR-based) developed from a zygote after injection of Cas9 mRNA and sgRNA-4.

(K) DNA sequencing analysis of progeny. Note that the sequence of PCR products amplified from the *Crygc* gene shows only one peak in all 13 pups, indicating that the corrected trait of the repaired mice could be successfully transmitted to the next generation.

See also Figures S1 and S2 and Tables S1 and S2.

Table 1. CRISPR-Cas9-Mediated Gene Correction in Cataract Mice

Oligo	Injected Embryos	Blastocysts (Percentage of Injected Embryos)	Transferred Blastocysts	Live-Born Pups	Genetic Modification		NHEJ-Mediated Repair/Nonrepair	HDR-Mediated Repair
					WT allele	mutant allele		
-	172	157 (91%)	135	22	0	10	2/4	4
Oligo-1	245	213 (87%)	178	29	0	14	4/5	5
Oligo-2	221	190 (86%)	159	27	0	12	5/3	4

Cas9 mRNA and sgRNA-4 targeting mutant allele of *Crygc* gene were coinjected into fertilized oocytes with or without exogenous oligonucleotides (Oligo-1 or Oligo-2). The blastocysts derived from the injected embryos were transferred into uteri of pseudopregnant females. Newborn pups were obtained and genotyped. See also Figures 1, S1, and S2.

NHEJ-based deletions and insertions as expected (Table S1), sgRNA-4 could also efficiently promote HDR-based precise gene editing (16 of 36 ESC clones sequenced) (Table S1), indicating that Cas9-induced DNA breaks in the mutant *Crygc* allele could be repaired through HDR using the normal allele on the homologous chromosome as a template (Figure 1D). Taken together, these data suggest that sgRNA-4 specifically targets the mutant allele; this effect is likely due to the neo-PAM contained in the mutant allele. We thus selected sgRNA-4 for our subsequent targeting experiments.

We next asked whether the genetic defect in the cataract mouse model could be corrected at the organism level (defined as the functional correction of the cataract phenotype) by injection of CRISPR-Cas9 into zygotes (Shen et al., 2013; Wang et al., 2013). Cas9 mRNA and sgRNA-4 were coinjected into the cytoplasm of zygotes that were harvested from B6D2F1 females mated with homozygous cataract males (Figure 1E). The injected zygotes developed into blastocysts at a rate of 91% (Table 1), indicating a low toxicity of the injected RNAs. Of the 135 transferred blastocysts that were derived after the injection, a total of 22 live pups were born (Table 1). Upon DNA sequencing analysis, we identified 10 mice carrying genetic modifications of the mutant allele (Figure 1F). We failed to detect any gene editing events in the WT allele of these gene-modified mice, confirming the specificity of sgRNA-4 in targeting the mutant allele. Similar to our previous observations in ESCs, gene-editing events induced by the CRISPR-Cas9 system included NHEJ-mediated insertions and deletions (6 of 10 mice) and HDR-mediated repair (4 of 10 mice) (Figures 1F and 1G). As expected, all four mice that carried the corrected allele induced by HDR were free of cataracts, as measured by the appearance of black lenses compared to the opacity of the lens in control mice developed from heterozygous mutant embryos (20 of 20 mice) (Figure 1H). Interestingly, two of the six mice that carried NHEJ-mediated insertions or deletions also displayed normal lenses (Figure 1F and Figure S1A). An analysis of the nucleotide sequences of the mutant allele in these two mice revealed that the cataract phenotype is likely corrected by the new insertion of one nucleotide in one case or the new net deletion of five nucleotides in another, both resulting in the restoration of the correct open reading frame for the WTγC-crystallin (Figure 1F and Figure S1B).

Next, we asked whether supplying an exogenous WT single-stranded DNA oligo (termed Oligo-1) would lead to increased efficiency of HDR-mediated precise genome repair by coinjecting Cas9 mRNA, sgRNA-4, and Oligo-1 into the cytoplasm of heterozygous cataract mutation-bearing zygotes (Figures S2A and S2B and Supplemental Experimental Procedures). From this set of experiments, a total of 29 live pups were born from 178 transferred blastocysts (Table 1). DNA sequencing results (Figure S2C) showed that 14 mice carried gene modifications of the mutant allele. Of these, nine were free of cataracts (Figure S2D), a frequency similar to the case of injection of Cas9 mRNA and sgRNA-4 (9/29 versus 6/22). Further analysis of DNA sequences showed that five of these nine cataract-free mice carried a corrected *Crygc* gene derived from HDR. The remaining four mice were apparently repaired by NHEJ, with either a new insertion of one nucleotide (two pups) or new deletions of 2+3n nucleotides (two pups) (Figure S2C), restoring the correct open reading frame in all cases. To dissect whether the CRISPR-Cas9-induced HDR utilized information from the normal allele on the homologous chromosome or the exogenous oligo template, we designed a *Crygc* oligo (termed Oligo-2) harboring two synonymous mutations (Supplemental Experimental Procedures); this oligo would allow us to discriminate the origin of the recombination template (Figure S2E). A total of 27 live pups were born from 159 transferred blastocysts; of these, 9 were free of cataracts (Table 1). Of the four mice cured via HDR, we found that three of them carried modified *Crygc* genes with a DNA sequence that was same as the exogenous oligo, and the remaining one had a DNA sequence identical to that of the WT allele (Figures S2F and S2G). Taken together, our data suggest that while supplying exogenous oligos may not be necessary, the exogenous oligonucleotide could serve as an optional template for repairing the mutant gene, especially when the endogenous allele cannot be used as the template (as in homozygous genetic diseases).

In this study, 24 mice were cured of cataracts using the CRISPR-Cas9 system. To further confirm the correction of the disease, we performed histological analysis of lenses prepared from repaired and control cataract mice. As shown in Figure 1I and Figure S2H, control cataract mice showed pathological changes in the equatorial region of their entire eyes, but the cataract-free mice that were cured via HDR or NHEJ-mediated gene editing (two or three mice, respectively) exhibited normal histological features. One major hindrance to the therapeutic application of the CRISPR-Cas9 system is the potential for off-target mutations; we therefore examined off-target effects in the repaired mice. Given the recent findings that a maximum level of mismatch that could be tolerated in the "target" sequence is no more than five nucleotides (Fu et al., 2013; Hsu et al., 2013; Pattanayak et al., 2013), we searched the genome

for sites with a minimum of 14 consecutive nucleotides of shared identity with the authentic target sequence. This led to the identification of a total of 10 potential "off-target" sites (Table S2). DNA sequencing of PCR products amplified from these genomic sites showed no mutations at these loci in the majority of the repaired mice (10 out of 12). For the remaining 2 mice, off-target mutations were detected at 1 of the 10 potential off-target sites (Table S2). These results are consistent with previous observations that injection of CRISPR-Cas9 into mouse and rat embryos results in genetic-modified animals carrying off-target mutations in only rare instances (Li et al., 2013a, 2013b; Wang et al., 2013; Yang et al., 2013). Finally, we analyzed the fertility of repaired mice by mating them with WT mice. Progeny were obtained from four cataract-free mice carrying the *Crygc* gene that was corrected through various mechanisms (two via HDR using information from the endogenous WT allele, one via NHEJ, and one via HDR using information from Olig-2) (Figure 1J and Figures S1C and S2I). DNA sequencing of the progeny showed that the pups carried the repaired *Crygc* allele originated from their parents (Figure 1K and Figures S1D and S2J), indicating that the corrected allele could be successfully transmitted to the next generation through the germline.

In summary, we have demonstrated that the CRISPR-Cas9 system can be used to cure a genetic disease in mouse by directly correcting the genetic defect through NHEJ- or HDR-mediated gene editing. In the future, it would be of interest to investigate whether similar gene-correction strategies could be used for mutation correction in a setting related to human diseases, such as human stem cells.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes Supplemental Experimental Procedures, two figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2013.10.016.

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