Germline CRISPR/Cas9-Mediated Gene Editing Prevents Vision Loss in a Novel Mouse Model of Aniridia

Seyyedeh Zeinab Mirjalili Mohanna,1,2 Jack W. Hickmott,1,2 Siu Ling Lam,1 Nina Y. Chiu,1,2 Tess C. Lengyell,1 Beatrice M. Tam,3 Orson L. Moritz,3 and Elizabeth M. Simpson1,2

1Centre for Molecular Medicine and Therapeutics at British Columbia Children’s Hospital, The University of British Columbia, Vancouver, BC V5Z 4H4, Canada; 2Department of Medical Genetics, The University of British Columbia, Vancouver, BC, Canada; 3Department of Ophthalmology and Visual Sciences and Centre for Macular Research, The University of British Columbia, Vancouver, BC, Canada

Aniridia is a rare eye disorder, which is caused by mutations in the paired box 6 (PAX6) gene and results in vision loss due to the lack of a long-term vision-saving therapy. One potential approach to treating aniridia is targeted CRISPR-based genome editing. To enable the Pax6 small eye (Sey) mouse model of aniridia, which carries the same mutation found in patients, for preclinical testing of CRISPR-based therapeutic approaches, we endogenously tagged the Sey allele, allowing for the differential detection of protein from each allele. We optimized a correction strategy in vitro then tested it in vivo in the germline of our new mouse to validate the causality of the Sey mutation. The genomic manipulations were analyzed by PCR, as well as by Sanger and next-generation sequencing. The mice were studied by slit lamp imaging, immunohistochemistry, and western blot analyses. We successfully achieved both in vitro and in vivo germline correction of the Sey mutation, with the former resulting in an average 34.8% ± 4.6% SD correction, and the latter in restoration of 3xFLAG-tagged PAX6 expression and normal eyes. Hence, in this study we have created a novel mouse model for aniridia, demonstrated that germline correction of the Sey mutation alone rescues the mutant phenotype, and developed an allele-distinguishing CRISPR-based strategy for aniridia.

INTRODUCTION
The paired box 6 (PAX6) gene encodes a transcription factor that controls many aspects of early development of the central nervous system, eye, and some non-neuronal tissues such as the pituitary and pancreas.1 This transcription factor is conserved in evolution and has complex regulatory regions that span beyond 200 kb.2 Heterozygous loss-of-function mutations of PAX6 result in a rare congenital eye disorder known as aniridia.3 Patients with aniridia are typically born with low vision, mainly due to fovea hypoplasia, and become legally blind in young adulthood due to progressive sight-threatening complications, including glaucoma, cataract, and corneal opacification.4 Currently, there are no long-term vision-saving therapies or cures for aniridia; however, some of the secondary ocular abnormalities can be partially managed through medical and surgical interventions with limited success, as surgeries on aniridic eyes can expedite aniridia-related keratopathy and worsen vision.5,6 Therefore, new treatment strategies are needed for aniridia.

One possible approach to treating aniridia is gene therapy, where genetic material is delivered into cells to make the required protein (augmentation) or correct the defective gene (gene editing).7 However, similar to haploinsufficiency, PAX6 overexpression has been shown to negatively affect normal eye development.8–11 Thus, very precise regulation of PAX6 would be necessary to achieve therapeutic success with augmentation gene therapy.12 In contrast, gene editing, for example by clustered regularly interspaced short palindromic repeats (CRISPR), can be used to revert a mutation to the correct sequence in the genome, thereby allowing for the normal regulation of expression levels and timing in appropriate cell types. Unfortunately, CRISPR-mediated gene editing can also create small insertions and deletions (indels), or even large deletions and complex rearrangements, which are important considerations when taking this approach.13–19

Studies on cellular and animal models are often the first steps toward developing a gene therapy. In the case of aniridia, this approach is supported by the fact that the human PAX6 gene is homologous to the mouse Pax6 gene, and the protein products from these two genes have identical amino acid sequences.17 The small eye (Sey) mouse was first described in 1996 and is a widely accepted model for aniridia, which carries a spontaneous point mutation causing a premature stop codon in exon 8 of the Pax6 gene.16–18 Mice that are homozygous for the Sey allele are anophthalmic and die shortly after birth, whereas mice that are heterozygous for Sey are viable and exhibit iris hypoplasia, lens abnormalities, and corneal clouding.17–19 In both cases, the
phenotypes are comparable in humans, with the latter modeling aniridia.19,20 Furthermore, the mutation present in Sey mice (G194X) has been reported in a patient with aniridia,21 making Sey an exciting model for proof-of-principle gene therapy studies.

Given the role of PAX6 in stem/progenitor cells,22 and the overall plasticity of the eye,23,24 we think a postnatal CRISPR-based gene therapy is a feasible option for treating aniridia. Supporting this is evidence of successful delivery of CRISPR/Cas9 components via viral vectors to rodent and non-human pri-

mulate eyes,25–30 as well as the ability of the aniridic mouse eye to improve after augmenting PAX6 levels postnatally.23,24 Therefore, we hypothesized that increasing the expression of PAX6 through a CRISPR-based gene-editing strategy would improve the structure and function of the eye, and ultimately rescue the mutant phenotype in a mouse model of aniridia.

For in vivo CRISPR strategy development, it would be advantageous to have a mouse model that allows us to distinguish both the gene and protein of wild-type (WT) PAX6 from CRISPR-corrected mutant PAX6, for quantification of the targeted genome editing efficacy (Figure 1). Thus, using CRISPR for endogenous-gene tagging, we have created a new model for preclinical CRISPR-based therapy development for aniridia. Furthermore, since the Sey mutation was first identified more than half a century ago, there was a possibility that the Sey locus might have acquired additional genetic mutations over time.16 Hence, as an important step, we validated the causality of this mutation by germline correction. Lastly, we developed a CRISPR allele-distinguish ing strategy that successfully corrected the Sey mutation both in vitro and in vivo in the mouse germline.

RESULTS

PAX6 and 3xFLAG/PAX6 Constructs Induced Ectopic Eyes in Xenopus laevis Tadpoles

Sey mice produce WT PAX6 protein, so to distinguish the CRISPR-corrected PAX6 protein from WT, a protein tag was necessary. To tag PAX6, FLAG tag was selected, as it is small,31 can be stained with high-affinity monoclonal antibodies,32 and has been previously used for endogenous Pax6 tagging in vivo.33 Triple FLAG (3xFLAG) tag was selected over single FLAG tag, as it is easier to detect with
antibodies. PAX6 is highly conserved between diverse species, and it is a concern that endogenous gene tagging may cause a functional deficit. However, evidence showing successful use of N-terminally tagged PAX6 in in vitro experiments supported the possibility of tagging this protein. Overexpression of Pax6 induces the formation of ectopic eyes in Xenopus laevis (X. laevis). Therefore, to determine whether the 3xFLAG-tagged PAX6 remains functional, N- and C-terminally tagged human PAX6 cDNA constructs were tested in a rapid ectopic eye formation assay. X. laevis embryos were injected with mRNA encoding emerald GFP (EmGFP), PAX6, N-terminally 3xFLAG-tagged PAX6 (3xFLAG/PAX6), or C-terminally 3xFLAG-tagged PAX6 (PAX6/3xFLAG). The resulting tadpoles were examined 14 days later for the formation of ectopic eye structures (Figure 2). PAX6, 3xFLAG/PAX6, and PAX6/3xFLAG constructs, but not EmGFP or water, induced ectopic eyes, confirming the functionality of all three constructs. The N-terminally tagged PAX6 was selected for future use, as it produced the greatest number of ectopic eyes.

An Optimized CRISPR Strategy Successfully Inserted 3xFLAG Tag in Mouse Embryonic Stem Cells

Guide RNA (gRNA) and single-stranded oligodeoxynucleotide (ssODN) template designs can tip the balance toward more successful gene editing, and therefore optimizing CRISPR/Cas9 system components prior to in vivo studies is an important step. The N-terminal 3xFLAG-tag location was based on our results with the ectopic eye formation assay in X. laevis tadpoles. To improve the gene editing efficiency, and to readily use the optimized CRISPR strategy in the micro-injection, we used the Cas9 ribonucleoprotein (RNP) complex. The Cas9 RNP complexes with their corresponding ssODNs were electro-porated into mouse embryonic stem cells (ESCs), and the outcome of genomic manipulation was quantified by site-specific next-generation sequencing (NGS). Figure 3A shows the schematic of our best CRISPR strategy for addition of the 3xFLAG tag at the Pax6 start codon (ATG) that was able to successfully insert the intact 3xFLAG tag in 1,007 out of 19,573 NGS reads (5.14%) (Figure 3B). In 7,986 out of 19,573 reads (40.8%), Cas9-induced double-stranded breaks were repaired by the non-homologous end joining (NHEJ) pathway, creating relatively small indels in close vicinity to the cut site.

Endogenous Sey Tagging Created a Novel Mouse Model for Aniridia without Compromising the Protein Function in the Control Mice

We used an earlier in vitro-optimized CRISPR strategy (Figure 3A) to insert a 3xFLAG tag to the Sey allele in mouse zygotes in a rapid manner, creating the 3xFLAG-tagged Sey mouse strain (C57BL/6J [B6]-Pax6[Sey-em1(3xFLAG)Ems]), called Fey hereafter. Since the Sey mutation causes a premature stop codon and no detectable protein, we were unable to directly characterize the function of the Sey allele. Thus, we created the matched WT control strain (B6-Pax6em3[Ems]), called Fax hereafter. Both strains were backcrossed to WT B6 mice to remove any incidental mutations. As expected, the slit lamp imaging of adult Fey mouse eyes revealed abnormalities comparable to those seen in Sey mice. However, even when required for the entire eye development in a complex and sophisticated mammalian system, the addition of the 3xFLAG tag to the conserved PAX6 in Fax mice resulted in no eye phenotypes (Figure 4A). Moreover, we scored eye size and corneal cloudiness by visual inspection in 106 homozygous Fax and 76 WT eyes. We found no significant difference in the ocular phenotype between...
Sey Mutation Was Efficiently Corrected in Mouse ESCs

Optimization of CRISPR/Cas9 components in cells enabled us to ensure on-target cleavage activity and specificity prior to in vivo studies. One recent approach that has been quite successful in distinguishing alleles uses Cas proteins with unique protospacer adjacent motifs (PAMs) such that correcting the mutation removes the PAM.40–42 However, this approach is restricted by the PAM sequences available, and it is currently not applicable to the Sey mutation. We worked with mouse Pax6Sey/Sey and Pax6+/+ ESCs, as homologyosity made it easier to interpret modifications from sequencing chromatograms. The on-target cleavage activity was determined by Sanger sequencing of edited whole-cell lysates normalized against unedited controls (defined as either Pax6Sey/Sey or Pax6+/+ ESCs electroporated with Cas9 and an ssODN template in the absence of gRNA). According to the results, our best CRISPR strategy corrected the Sey mutation with a 42.9% success rate and modified the PAM with a rate of 41.4% to avoid repeated targeting of edited alleles (Figure 5A).

Upon repeating the in vitro gene editing in triplicate, the average correction rate for the Sey mutation was 34.8% ± 4.6% (mean ± standard deviation), while the average PAM change rate was 42.9% ± 3.2%. In a head-to-head experiment, our best CRISPR strategy was then tested in both Pax6Sey/Sey and Pax6+/+ ESCs to determine its specificity. Quantification of the PAM change in these cells revealed a 2-fold enrichment of gRNA interaction with the mutant allele.

CRISPR/Cas9-mediated gene editing is known to lead to sequence heterogeneity among cells from the same sample.43 Hence, to look at the samples at a single molecule level, we sent Pax6Sey/Sey and Pax6+/+ whole-cell lysates edited with our best CRISPR strategy and the unedited controls for site-specific NGS, which enabled us to observe the various on-target mutations that CRISPR created. As expected, in the unedited Pax6Sey/Sey sample, most of the NGS reads (11,404/11,458; 99.5%) aligned completely to the reference sequence. The remainder of the reads in the unedited sample had single nucleotide substitutions, which could be explained by the error rate associated with this sequencing technique.44 Among the 18,291 aligned reads in the edited Pax6Sey/Sey sample, 3,644 reads (19.9%) had both knock-in variants with no further changes in PAM sequence available, and it is currently not applicable to the Sey mutation. The indel profile analysis revealed various sizes of NHEJ-induced indels around the Cas9 cut site (Figure 5B). Consistent with the earlier Sanger sequencing results, the indel frequency analysis of edited Pax6Sey/Sey and Pax6+/+ samples showed a 2-fold enrichment of gRNA interaction with the mutant allele.

In Vivo Gene Editing Corrected the Sey Mutation with a 25% Success Rate

Rescuing the mutant phenotype by correcting the Sey mutation in the mouse germline proves causality and serves as a proof-of-principle experiment for a future somatic gene therapy. The corrected mice were generated by microinjecting the in vitro-optimized CRISPR

---

**Figure 3. Site-Specific Next-Generation Sequencing Revealed the Correct Insertion of 3xFLAG Tag in 5.14% of Sequences**

Cas9 ribonucleoprotein complexes were assembled in vitro and electroporated into mouse embryonic stem cells. Site-specific next-generation sequencing was used to quantify the knock-in. (A) Schematic of the best guide RNA (gRNA) and single-stranded oligodeoxynucleotide (ssODN) for CRISPR/Cas9-mediated insertion of a 3xFLAG tag at Pax6 ATG (yellow). In addition to the tag sequence (green), the ssODN contained 60-bp homology arms flanking the Cas9-induced double-stranded break. Blue indicates Pax6 exons; black indicates Pax6 introns. The diagram is not to scale. (B) The top panel shows mutation positions relative to the Cas9 cut site. 1,007 out of 19,573 reads (5.14%) showed the correct 3xFLAG insertion.

---

**Figure 4B.** Immunohistological analysis was performed to look at the co-localization and expression of PAX6 and 3xFLAG-tagged PAX6 proteins. Sections from Sey, Fey, WT, and Fox retinas showed similar anti-PAX6 staining patterns in the ganglion cell layer (GCL) and inner nuclear layer (INL). Additionally, for Fox retinas, co-labeling of PAX6 and FLAG was detected in both the GCL and INL.
Figure 4. Fey Is a Novel Mouse Model of Aniridia and, Importantly, the Fax Control Mice Showed No Phenotype

An optimized Cas9 ribonucleoprotein complex and a single-stranded oligodeoxynucleotide containing the 3xFLAG sequence were microinjected into one-cell mouse zygotes to generate transgenic animals in one step. (A) Slit lamp analysis of the transgenic mouse eyes. As expected, images of Fey showed small eyes and corneal opacity, not significantly different from the Sey (Pax6 small eye) mouse model (two-tailed test, $p > 0.999$). Slit lamp images of Fax (FLAG-tagged Pax6) mouse eyes showed normal iris and clear cornea, which were not significantly different from WT eyes (one-tailed t test, $p > 0.999$). Fax, FLAG-tagged Pax6; Fey, FLAG tagged Sey; Sey, Pax6 small eye; WT, wild-type. (B) Histological analysis of mouse retinas. Adult mouse eyes were directly fixed and stained with FLAG and PAX6 antibodies. While FLAG staining was absent in the negative controls (Fey and WT retinas), Fax mouse retinas showed positive co-labeling of FLAG and PAX6. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer. Blue shows Hoechst staining. All images were taken at the same magnification. Scale bars, 34 μm.
gene editing strategy into Pax6Fey/+ and Pax6+/+ zygotes. Fifteen potential founder mice were born. A summary of the phenotyping, genotyping, and sequencing results of these mice is presented in Table 1. The proportion of animals with eye abnormalities observed by visual inspection was 46.7% (7/15), which was not significantly different from the expected 50:50 phenotypic ratio of Sey to WT. Moreover, the FLAG allele was present at 26.7% (8/30), which again was not significantly different from the expected 25%. Mice 1-9 and 1-12 were positive for 3xFLAG, which was genetically linked to the Sey allele, but had a normal eye phenotype. Sequencing results of ear notches from these two mice confirmed the correction of Sey mutation and presence of the silent base change at the original PAM site. On the allelic level, we achieved a correction rate of 25% (2/8) for the Fey allele. Sequencing results also revealed that the WT allele was hit at a rate of 22.7% (5/22), while only 13.6% (3/22) of this rate represents a deleterious hit. All 8 FLAG alleles were hit by our CRISPR strategy, whereas 77.3% (17/22) of WT alleles remained unmodified.

Germline Correction of the Sey Mutation Restored 3xFLAG-Tagged PAX6 Expression and Normal Eyes

Upon breeding, founders 1-9 and 1-12 were shown to stably transmit the transgene to their progeny. A first pedigree mouse was chosen for each founder, resulting in establishment of two new transgenic strains, B6-Pax6Fey-em4(c.[580TG;591GA])Ems and B6-Pax6Fey-em5(c.[580TG;591GA])Ems, which are called hereafter Fey correction em4 (FeyCor-em4) and Fey correction em5 (FeyCor-em5), respectively. Both strains were backcrossed to WT B6 mice to remove any incidental mutations. Excitingly, unlike Fey mice, FeyCor-em4 and FeyCor-em5 mouse eyes were not significantly different from WT by slit lamp examination (one-tailed t test, p > 0.999) (Figure 6A). Additionally, FLAG and PAX6 co-labeling in the immunohistological analysis of mouse retinas from both FeyCor strains confirmed the expression of 3xFLAG-tagged PAX6 in the GCL and INL (Figure 6B).

PAX6 Level Was Increased in the Brain of Germline-Corrected Mice

Being homozygous for a Pax6 or PAX6 null mutation causes anophthalmia and is neonatal lethal in both mice and humans, respectively. Therefore, to examine PAX6 levels in an expressing tissue, we decided to perform western blot analysis on brain samples isolated from fetuses at embryonic day 18.5 (E18.5). PAX6 level influenced the phenotype of fetuses as expected, with Pax6Sey/Sey and Pax6Sey/Fey fetuses showing microphthalmia and anophthalmia, respectively, while Pax6Sey/Fey and Pax6 control fetuses had normal eyes. The germline correction of Sey mutation led to Pax6SeyFeyCor/FeyCor fetuses having normal eyes that were not

---

Figure 5. In Vitro CRISPR-Mediated Gene Editing Corrects the Sey Mutation in Pax6Sey/Sey Embryonic Stem Cells

Sey is a point mutation where the wild-type G nucleotide is mutated to a T, resulting in a premature stop codon. Various CRISPR-mediated editing strategies to correct the Sey mutation were tested in mouse embryonic stem cells, and the editing efficiencies were quantified by Sanger sequencing and site-specific next-generation sequencing (NGS). (A) Sanger sequencing chromatogram of the best CRISPR strategy. Sanger sequencing revealed a correction rate from T to G of 42.9% (red box). To prevent repeat targeting of previously edited alleles, the single-stranded oligodeoxynucleotide (ssODN) also included a silent base change in the protospacer adjacent motif (PAM) sequence, giving an alteration rate from G to A of 41.4% (green box). RFU, relative fluorescent units. (B) Site-specific NGS histograms of the best CRISPR strategy. 11,404 out of 11,458 (99.5%) reads from the unedited cells (negative control) matched the reference sequence. For the cells edited with the best strategy, 3,644 out of 18,291 reads (19.9%) had both the correction and the PAM change with no other changes in Pax6 exon 8. 7,855 out of 18,291 reads (42.9%) were unmodified.
significantly different from WT (one-tailed t test, p > 0.999) (Figure S2). Next, we looked at PAX6 levels using western blotting. Immunoblots of E18.5 brains showed co-staining of 3xFLAG-tagged PAX6 and PAX6 proteins for the FeyCor genotype, suggesting that the rescue of 3xFLAG-tagged PAX6 expression in the brain of these fetuses (Figure 7A). PAX6 protein levels were determined from two technical replicates with an understanding of the semiquantitative nature of these results. For each immunoblot, PAX6 protein levels were normalized against a non-specific protein revealed by the secondary antibody (Figure 7B). See Table S2 for relative PAX6 levels in each sample. Statistical analysis revealed a significant decrease in PAX6 levels in the brains of heterozygous Fey fetuses compared to homozygous FeyCor fetuses (one-tailed t test, p = 0.017), while no significant difference was observed in the mean PAX6 levels between homozygous FeyCor and WT genotypes (two-tailed t test, p = 0.999). As expected, statistical analysis also revealed similar PAX6 levels in the brains of homozygous Fax and WT fetuses (two-tailed t test, p = 0.937), which was significantly more than brains from heterozygous Fey fetuses (one-tailed t test, p = 0.016), confirming the observation that the PAX6 genotype, but not the endogenous tagging, influences PAX6 protein levels.

**DISCUSSION**

Aniridia was first described in 1818. Despite considerable research efforts during the past 200 years, there remains a therapeutic gap for aniridia patients around the world. Therapy development for rare diseases such as aniridia relies heavily on efficacy and safety studies performed on animal models. In this study, we developed a new mouse model of aniridia, Fey, with the addition of 3xFLAG to the Sey allele, that can be used to explore gene-editing therapies for this debilitating disease.

Prior to making Fey, we tested the impact of the 3xFLAG tag on WT Pax6, naming this allele Fax. We were surprised that the heterozygous, and even homozygous, addition of 3xFLAG to Pax6 in vivo did not result in any observed undesired phenotypes in the "control" Fax mice, suggesting that our endogenous gene tagging did not influence the function of PAX6. This is critical since it shows that when the Fey mouse is "corrected", the new allele will be fully functional. However, note that we focused our characterization on the ocular phenotype, since aniridia is primarily an eye-related disorder, and thus we cannot rule out the possibility of other subtle phenotypes in non-ocular Pax6-expressing tissues.

**Sey is an old mutation, and thus this non-functional allele could have undergone random genetic drift during the at least 52 years of continuous breeding since discovery. Such additional mutations could be difficult to detect even by sequencing if located in non-coding Pax6 regulatory elements.** The success of our mutation-dependent

---

**Table 1. The Sey Phenotype Was Rescued In Two Mice By Germline Correction**

| Mouse ID | Eye Phenotype | 3xFLAG Genotype | Fey Allele Corrected and PAM Change | Fey Allele Unedited | WT Allele Change | WT Allele Indel | WT Allele Unedited |
|----------|---------------|-----------------|-----------------------------------|-----------------|-----------------|-----------------|-------------------|
| 1-1      | abnormal      | F/+             | NA                                | NA              | NA              | NA              | NA                |
| 1-2      | abnormal      | F/+             | NA                                | NA              | NA              | NA              | NA                |
| 1-3      | normal        | +/+             | NA                                | NA              | NA              | NA              | NA                |
| 1-5      | abnormal      | F/+             | NA                                | NA              | NA              | NA              | NA                |
| 1-6      | abnormal      | F/+             | NA                                | NA              | NA              | NA              | NA                |
| 1-7      | abnormal      | F/+             | NA                                | NA              | NA              | NA              | NA                |
| 1-8      | normal        | +/+             | NA                                | NA              | NA              | NA              | NA                |
| 1-9      | normal        | F/+             | NA                                | NA              | NA              | NA              | NA                |
| 1-10     | normal        | +/+             | NA                                | NA              | NA              | NA              | NA                |
| 1-11     | normal        | +/+             | NA                                | NA              | NA              | NA              | NA                |
| 1-12     | normal        | +/+             | NA                                | NA              | NA              | NA              | NA                |
| 1-13     | normal        | +/+             | NA                                | NA              | NA              | NA              | NA                |
| 1-14     | normal        | F/+             | NA                                | NA              | NA              | NA              | NA                |
| 1-15     | normal        | +/+             | NA                                | NA              | NA              | NA              | NA                |
|          | 7/15 (46.7%)  | 8/30 (26.7%)    | 2/8 (25.0%)                       | 6/8 (75.0%)     | 0/8 (0%)        | 2/22 (9.10%)    | 17/22 (77.3%)     |

F, 3xFLAG-tagged Pax6 allele; +, wild-type Pax6 allele; ID, identifier; Indel, insertion or deletion; WT, wild-type Pax6; present in one allele; present in both alleles; NA, not applicable.

*aAllele of interest/total alleles.

*bAbnormal/total ratio.

*cAllele of interest/total WT alleles.

**This refers to either a PAM change or point mutations with no effect on the phenotype.
CRISPR strategy relied on the causality of the Sey mutation. Therefore, as an important step, we validated the causality of the Sey mutation in the Fey mouse through a CRISPR-based germline correction experiment and demonstrated that correction of just this 1 bp fully restored the ocular phenotype to WT. These results further validate Fey as a novel mouse model for the development of CRISPR-based mutation correcting therapies.

The Fey mouse will also be valuable for the development and quantification of efficacy for numerous other therapeutic approaches. For example, nonsense suppression therapies using readthrough compounds such as G418, amlexanox, and ataluren to overcome the presence of nonsense mutations in aniridia. G418 has shown readthrough activity both in vitro and in vivo, and amlexanox has shown activity in vitro. Ataluren has shown activity in mice, and has been in clinical trial for aniridia (ClinicalTrials.gov: NCT02647359). Fey mice can be also used to design and explore CRISPR/Cas13 strategies that target Pax6 mutations at the RNA level without permanently altering the genome. Additionally, mutation-independent strategies such as the intronic insertion of the Pax6 cDNA sequence immediately downstream of the AUG, as well as use of the spliceosome-mediated RNA trans-splicing (SMaRT) technique, will benefit from having the new protein FLAG tagged. The former and the latter therapeutic approaches have been shown to be effective in treating mouse models of hemophilia and retinitis pigmentosa, respectively.

We successfully achieved both in vitro and in vivo germline correction of the Sey and Fey mutations, respectively, restoring the mutant protein expression. In vitro delivery by electroporation into ESCs resulted in an average 34.8% ± 4.6% SD correction, while in vivo delivery by microinjection into zygotes resulted in 25% correction. Furthermore, the bias toward the mutant allele was 2-fold and 4-fold higher in our in vitro and in vivo experiments, respectively. Thus, although both were successful, our allele-distinguishing CRISPR strategy performed quite differently depending on the delivery method and recipient cell. Therefore, we now hypothesize that the effectiveness of this strategy when delivered by viral or non-viral delivery vectors to somatic cells.
will need empirical testing. PAX6 plays a critical role in maintaining the corneal limbal stem cell (LSC) niche in both human and mouse eyes. LSC deficiency is the underlying mechanism responsible for cornea opacification and ultimately blindness in aniridia.\(^{57-60}\) Hence, in future studies, we aim to deliver our CRISPR strategy to LSCs of post-weaned Fey mice to model gene therapy in recently diagnosed children with aniridia.

Taken together, we have created a novel mouse model for testing CRISPR-based genome editing, as well as other therapeutic approaches for preclinical assay development for aniridia. We demonstrated that germline correction of the Sey mutation alone rescues the mutant phenotype. Finally, we have developed an allele-distinguishing CRISPR strategy that corrected the Sey mutation in both cells and the mouse germline.

MATERIALS AND METHODS

Cloning

To generate plasmids for \textit{in vitro} transcription, EmGFP, PAX6, 3xFLAG/PAX6, and PAX6/3xFLAG open reading frames (ORFs) were cloned into pBluescript as previously described.\(^{12,61}\) Briefly, EmGFP, PAX6, 3xFLAG/PAX6, and PAX6/3xFLAG ORFs, flanked by NotI restriction sites, were commercially synthesized (DNA2.0, Menlo Park, CA, USA). pBluescript and DNA2.0 plasmids containing EmGFP, PAX6, 3xFLAG/PAX6, and PAX6/3xFLAG were electroporated into 50 μL of OneShot TOP 10 Electrocomp \textit{Escherichia coli} (C404052; Thermo Fisher Scientific, Waltham, MA, USA) as directed, and cultured on Luria-Bertani (LB) plates. Resulting colonies were picked and cultured, and the DNA was prepped (27104; QIAGEN, Germantown, MD, USA). The resulting plasmids were digested with NotI (R0189; New England Biolabs, Ipswich, MA, USA), size separated, and gel extracted, and the ORFs were ligated into pBluescript using T4 DNA ligase (M0202; New England Biolabs). Ligation products were electroporated into TOP 10 cells, cultured, and DNA was prepared as described above. Correct ligation was confirmed by restriction digest with NcoI (R0193; New England Biolabs) and XhoI (R0146; New England Biolabs), as well as Sanger sequencing that was executed at the Centre for Molecular Medicine and Therapeutics (CMMT) DNA Sequencing Core Facility.

mRNA Production and Injection into \textit{X. laevis}

To produce mRNA for injection into \textit{X. laevis} embryos, EmGFP, PAX6, 3xFLAG/PAX6, and PAX6/3xFLAG ORFs were \textit{in vitro} transcribed, polyadenylated, and 5' capped. Ligation products were electroporated into TOP 10 cells, cultured, and DNA was prepared as described above. Correct ligation was confirmed by restriction digest with NotI (R0193; New England Biolabs) and XhoI (R0146; New England Biolabs), as well as Sanger sequencing that was executed at the Centre for Molecular Medicine and Therapeutics (CMMT) DNA Sequencing Core Facility.

Cell Culture

\textit{Pax6}^{Sey/Sey} and \textit{Pax6}^{Sey/+} ESCs were produced by setting up timed pregnancies with B6-\textit{Pax6}^{Sey/+} dam mice and 129-\textit{Pax6}^{Sey/+} stud...
males and B6-\textit{Pax6}\textsuperscript{+/+} dam mice and 129-\textit{Pax6}\textsuperscript{+/+} stud males, respectively. The resulting embryos were cultured in high-glucose Dulbecco’s modified Eagle’s medium (DMEM) (LS11960044; Thermo Fisher Scientific) supplemented with 1,000 UmL ESGRO recombinant mouse leukemia inhibitory factor (ESGI1107; Chemicom/MilliporeSigma, Burlington, MA, USA), 16% fetal bovine serum (080-150; Wisent Bioproducts, Canada), 0.1 mM MEM non-essential amino acids solution (11140050; Thermo Fisher Scientific), 2 mM l-glutamine (25030081; Thermo Fisher Scientific), and 0.01% 2-mercaptoethanol (M7522; Sigma-Aldrich, St. Louis, MO). ESC lines were genotyped for \textit{Pax6} using a WT-specific polymerase chain reaction (PCR) assay with primer pair oEMS6076 (5’-AACACCAAATCTCCATGTTTCAATG-3’) and oEMS6075 (5’-GCAAAATATAAGCATCCCCAGTGCAT-3’), as well as a Sey-specific PCR assay with primer pair oEMS6071 (5’-TCACCTATTTTCCAAACACAGCC-3’) and oEMS6073 (5’-CTGAGCTTCAATCCGGACTTTCTTTTA-3’). The selected ESC lines were expanded on mouse embryonic fibroblast cells until passage 8. Subsequently, the cells were maintained on tissue culture plates coated with 0.1% gelatin (G7-500; Thermo Fisher Scientific) in sterile distilled water for a minimum of five passages prior to use in \textit{in vitro} gene editing experiments.

\textbf{In Vitro Gene Editing}

The online tool Benchling (https://benchling.com/crispr) was used to design gRNAs for the \textit{Streptococcus pyogenes} Cas9 nucleases in the region of interest. The top three gRNAs were selected based on their predicted on-target and off-target effects, and their proximity to either the \textit{Pax6} start codon for 3xFLAG insertion, or the Sey mutation for the correction strategy. The commercially synthesized CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA) (GenScript, Piscataway, NJ, USA) were annealed at a 1:1 ratio by incubating at 95°C for 5 min to generate gRNA. Next, ssODN templates with 60- to 80-bp homology on each side of a Cas9 cleavage site were ordered (Integrated DNA Technologies, Coralville, IA, USA). When required, a silent mutation was introduced in PAM to prevent Cas9 nuclease (Integrated DNA Technologies, Coralville, IA, USA) to be assayed for gene-editing frequency by site-specific NGS with self-designed primers.

\textbf{Mouse Husbandry}

All mice were bred and maintained in the pathogen-free mouse core facility of the CMMT at the University of British Columbia (UBC). Animal work was performed in accordance with the guidelines set by the Canadian Council on Animal Care and adhered to the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research. All work was done following protocols approved by the UBC Animal Care Committee (protocols A17-0204 and A17-0205). For characterizing the strains, WT B6 (The Jackson Laboratory [JAX] strain code 006664) dams were bred with heterozygous transgenic sires (backcross N2 or higher). Pups were weaned and ear notched at postnatal day 21. Collected ear notches were digested in THB with protein kinase and genotyped by PCR using primer pair oEMS6145 (5’-GATTCTCACTGTCGGACCC-3’) and oEMS6146 (5’-CTACTAGTTGTGC CCGGATAGA-3’) and primer pair oEMS6077 (5’-CGTATATCC CAGTTCAGTGCAGTCCGGTTG-3’) and oEMS6144 (5’-CTTGTACATCGT CATCCTTTGT-3’), allowing for detection of 3xFLAG insertion based on size difference and sequence specificity, respectively. Primer pairs used for WT and Sey-specific PCR assays were as mentioned earlier. For phenotyping the \textit{FAX} strain, WT and homozygous \textit{FAX} mice littermates (N2F2 or higher) obtained from intercrosses were used to set up WT or \textit{FAX} incrosses. The progeny eyes were scored for abnormal phenotypes by visual inspection at 4 weeks, and representative eyes were imaged by slit lamp at a minimum of 3 months of age.

\textbf{In Vivo Gene Editing}

For the creation of the \textit{Fey} and \textit{FAX} strains, zygotes from day 0.5 postcoitus (PC) plugs were obtained by the superovulation of B6 females mated with B6-\textit{Pax6}\textsuperscript{+/+} and B6 males, respectively. For the \textit{FeyCor} strain, superovulated \textit{Pax6}\textsuperscript{+/+} females were set up with B6 males. The day of injection, all females were plug checked, and only those with a PC plug present were harvested. Zygotes were harvested into an EmbryoMax M2 medium (MR-015-D; MilliporeSigma) or Research Vitro Wash (K-RVWA-50; Cook Medical, Bloomington, IN, USA), then treated with a 300 \textmu M solution of hyaluronidase (H272; Sigma-Aldrich) to remove cumulus cells. Zygotes were then placed

\textbf{Sanger and Site-Specific NGS}

The region around Sey mutation was PCR amplified using a Q5 high-fidelity PCR kit (E05555; New England Biolabs), with primer pair oEMS6299 (5’-AGGGCTTTCCGATTTC-3’) and oEMS6171 (5’-GCTGTCGATTTCTTTG-3’). Following gel electrophoresis, DNA bands were excised and purified using a QIAquick gel purification kit (29706; QIAGEN). Cleaned DNA samples were then sent to the CMMT DNA Sequencing Core Facility for Sanger sequencing using the same primers as above.

Whole-cell lysates from electroporated cells were sent to Desktop Genetics (London, UK) to be assayed for gene-editing frequency by site-specific NGS with self-designed primers.
into cultured KSOM (potassium simplex optimization medium) (MR-121-D; MilliporeSigma) or Research Vitro Cleave (K-RVCL-50; Cook Medical) medium until the time of injection. The CRISPR RNP complex was formed immediately prior to microinjection by mixing 2.5 µL of 1 µg/µL Cas9 (CP01-20; PNA Bio) with 3.8 µL of 0.3 µg/µL crRNA/tracrRNA (GenScript) and incubating the mixture on ice for 10 min. For 3xFLAG insertion, 4.3 µL of 1.2 µg/µL ssODN (Integrated DNA Technologies) was added to the corresponding complexed RNP and the mixture was topped up to 50 µL using an embryo-grade Tris-EDTA (TE) buffer (CytoSpring, Mountain View, CA, USA). For the cyttoplasm of one-cell mouse zygotes using the XenoWorks digital microinjection system (Sutter Instrument, Novato, CA, USA). Post-microinjection zygotes were placed back into KSOM and incubated until transferred into day 0.5 PC pseudopregnant CD-1 females

E18.5 Fetus Generation

A previously described, timed pregnancy protocol was followed. Heterozygous dams and sires were bred, producing homozygous, heterozygous, and WT offspring for each strain. On E18.5, pregnant dams were sacrificed by cervical dislocation and their uteruses were removed. Fetuses were isolated by removing the yolk sac. Fetuses were imaged at ×0.8 magnification using a Leica MZ125 microscope with a CoolSnap-Pro CF camera (Leica Microsystems). Fetuses were then decapitated and brains were collected. Brains were divided into hemispheres by directly cutting down the longitudinal fissure. Each hemisphere was flash-frozen in liquid nitrogen and stored at −80°C. Lastly, tail tip samples were taken to be used in PCR-based genotyping of the E18.5 fetuses.

Western Blot Analysis

Protein extraction, quantification, and western blotting were performed according to a previously described protocol. Briefly, E18.5 brain hemispheres were homogenized in lysis buffer. The extracted proteins were quantified and 34 µg of each protein sample was run on the gel before being transferred to the polyvinylidene fluoride (PVDF) membrane. The blots were blocked and incubated with primary antibodies against PAX6 (1:1,000; 901301, BioLegend) and FLAG (1:500; F3165, Sigma-Aldrich). Blots were washed and incubated with anti-rabbit (1:5,000; A11037; Thermo Fisher Scientific) and anti-mouse (1:10,000; A11029; Thermo Fisher Scientific) secondary antibodies. After the final wash, the fluorescence signals were detected using the LI-COR Odyssey imaging system (LI-COR Biosciences, Lincoln, NE, USA).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2020.03.002.

AUTHOR CONTRIBUTIONS

Conceptualization, E.M.S.; Methodology, S.Z.M.M., J.W.H., and S.L.L.; Investigation, S.Z.M.M., J.W.H., S.L.L., N.Y.C., T.C.L., and B.M.T.; Writing – Original Draft, S.Z.M.M.; Writing – Review & Editing, E.M.S.; Visualization, S.Z.M.M., J.W.H., and B.M.T.; Supervision, O.L.M. and E.M.S.; Funding Acquisition, O.L.M. and E.M.S.

CONFLICTS OF INTEREST

The authors declare no competing interests.
ACKNOWLEDGMENTS

We thank Tom W. Johnson, of the Mouse Animal Production Service (MAPS), for making the Fey mouse. We also thank Dr. Jingsong Wang at the British Columbia Children’s Hospital Research (BCCHR) Institute Imaging Core Facility for access to the confocal microscope and Dr. Elizabeth Hui at the BCCHR Institute Sequencing and Bioanalyzer Core for Sanger sequencing support. This work was funded by a Canadian Institutes of Health Research (CIHR) grant (2OR77895) awarded to E.M.S. The Micron IV retinal imaging microscope and SonomSuite were funded by the Canada Foundation for Innovation (CFI) (grant 2OR76139) and the BCCHR Institute (grant CRG74760), respectively. Additionally, salary support was provided to S.Z.M.M. by the Brain Canada Foundation through the Canada Brain Research Fund, with the financial support of Health Canada and the BCCHR Institute. The views expressed herein do not necessarily represent the views of the Minister of Health or the Government of Canada. We also acknowledge salary support to J.W.H. from the University of British Columbia Four Year Doctoral Fellowship and Graduate Student Initiatives, and the CIHR Canadian Graduate Scholarship.

REFERENCES

1. Simpson, T.I., and Price, D.J. (2002). Pax6: a pleiotropic player in development. BioEssays 24, 1041–1051.
2. Tyas, D.A., Simpson, T.I., Carr, C.B., Kleinjan, D.A., van Heyningen, V., Mason, J.O., and Price, D.J. (2006). Functional conservation of Pax6 regulatory elements in humans and mice demonstrated with a novel transgenic reporter mouse. BMC Dev. Biol. 6, 21.
3. Vincent, M.C., Pujo, A.L., Olivier, D., and Calvès, P. (2003). Screening for Pax6 gene mutations is consistent with haploinsufficiency as the main mechanism leading to various ocular defects. Eur. J. Hum. Genet. 11, 163–169.
4. Calvão-Pires, P., Santos-Silva, R., Falcão-Reis, F., and Rocha-Sousa, A. (2014). Congenital aniridia: clinic, genetics, therapeutics, and prognosis. Int. Sch. Res. Notices 2014, 305350.
5. Tsai, J.H., Freeman, J.M., Chan, C.C., Schwartz, G.S., Derby, E.A., Petersen, M.R., and Holland, E.J. (2005). A progressive anterior fibrovascular syndrome in patients with post-surgical congenital aniridia. Am. J. Ophthalmol. 140, 1075–1079.
6. Edén, U., Riese, R., and Tornqvist, K. (2010). Corneal involvement in congenital aniridia. Cornea 29, 1096–1102.
7. Naldini, I. (2015). Gene therapy returns to centres stage. Nature 526, 351–360.
8. Scheld, A., Ross, A., Lee, M., Engelkamp, D., Rashbass, P., van Heyningen, V., and Hastie, N.D. (1996). Influence of Pax6 gene dosage on development: overexpression causes severe eye abnormalities. Cell 86, 71–82.
9. Ouyang, J., Shen, Y.C., Yeh, L.K., Li, W., Coyer, B.M., Liu, C.Y., and Fini, M.E. (2006). Pax6 overexpression suppresses cell proliferation and retards the cell cycle in corneal epithelial cells. Invest. Ophthalmol. Vis. Sci. 47, 2397–2407.
10. Manuel, M., Pratt, T., Liu, M., Jeffery, G., and Price, D.J. (2008). Overexpression of Pax6 results in microphthalmia, retinal dysplasia and defective retinal ganglion cell axon guidance. BMC Dev. Biol. 8, 59.
11. Davis, J., and Piatigorsky, J. (2011). Overexpression of Pax6 in mouse cornea directly alters corneal epithelial cells: changes in immune function, vascularization, and differentiation. Invest. Ophthalmol. Vis. Sci. 52, 4158–4168.
12. Hickmott, J.W., Chen, C.Y., Arenillas, D.I., Korecki, A.J., Lam, S.L., Molday, I.L., Bonaguro, R.J., Zhou, M., Chou, A.Y., Mathelier, A., et al. (2016). Pax6 MiniPromoters drive restricted expression from rAAV in the adult mouse retina. Mol. Ther. Methods Clin. Dev. 3, 16051.
13. Ran, F.A., Hsu, P.D., Wright, J., Agarwala, V., Scott, D.A., and Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. Nat. Protoc. 8, 2281–2308.
14. Kosicki, M., Tomberg, K., and Bradley, A. (2018). Repair of double-strand breaks induced by CRISPR-Cas9 leads to large deletions and complex rearrangements. Nat. Biotechnol. 36, 765–771.
15. Gehring, W.J. (1996). The master control gene for morphogenesis and evolution of the eye. Genes Cells 1, 11–15.
16. Roberts, R.C. (1967). Small-eyes, a new dominant mutant in the mouse. Genet. Res. 9, 121–122.
17. Hogan, B.L., Horsburgh, G., Cohen, J., Hetherington, C.M., Fisher, G., and Lyon, M.F. (1986). Small eyes (Sey): a homologous lethal mutation on chromosome 2 which affects the differentiation of both lens and nasal placodes in the mouse. J. Embryol. Exp. Morphol. 97, 95–110.
18. Hill, R.E., Favor, J., Hogan, B.L., Ton, C.C., Saunders, G.F., Hanson, I.M., Prosser, J., Jordan, T., Hastie, N.D., and van Heyningen, V. (1991). Mouse small eye results from mutations in a paired-like homeobox-containing gene. Nature 354, 522–525.
19. Ramesh, T., Collins, J.M., Ramaesh, K., Kaufman, M.H., West, J.D., and Dhillon, B. (2003). Corneal abnormalities in Pax6fl/fl small eye mice mimic human aniridia-related keratopathy. Invest. Ophthalmol. Vis. Sci. 44, 1871–1878.
20. Yoko, T., Nishina, S., Fukami, M., Ogata, T., Hosono, K., Hotta, Y., and Azuma, N. (2016). Genotype-phenotype correlation of Pax6 gene mutations in aniridia. Hum. Genome Var. 3, 15052.
21. Hever, A.M., Williamson, K.A., and van Heyningen, V. (2006). Developmental malformations of the eye: the role of Pax6, SOX2 and OTX2. Clin. Genet. 69, 459–470.
22. Sansom, S.N., Griffiths, D.S., Faedo, A., Kleinjan, D.J., Ruan, Y., Smith, J., van Heyningen, V., Rubenstein, J.L., and Livesey, F.J. (2009). The level of the transcription factor Pax6 is essential for controlling the balance between neural stem cell self-renewal and neurogenesis. PLoS Genet. 5, e1000511.
23. Gregory-Evans, C.Y., Wang, X., Wasan, K.M., Zhao, J., Metcalfe, A.L., and Gregory-Evans, K. (2014). Postnatal manipulation of Pax6 dosage reverses congenital tissue malformation defects. J. Clin. Invest. 124, 111–116.
24. Wang, X., Gregory-Evans, K., Wasan, K.M., Sivak, O., Shan, X., and Gregory-Evans, C.Y. (2017). Efficacy of postnatal in vivo nonsense suppression therapy in a Pax6 mouse model of aniridia. Mol. Ther. Nucleic Acids 7, 417–428.
25. Hung, S.S., Chrysostomou, V., Li, F., Lim, J.K., Wang, J.H., Powell, J.E., Tu, L., Daniszewski, M., Lo, C., Wong, R.C., et al. (2016). AAV-mediated CRISPR/Cas9 editing of retinal cells in vivo. Invest. Ophthalmol. Vis. Sci. 57, 3470–3476.
26. Ruan, G.X., Barry, E., Yu, D., Lukason, M., Cheng, S.H., and Scarl, A. (2017). CRISPR/Cas9-mediated genome editing as a therapeutic approach for Leber congenital amaurosis 10. Mol. Ther. 25, 331–341.
27. Yu, W., Mookherjee, S., Chaitanya, V., Hirayama, S., Kim, J.W., Brooks, M., Atzarenannai, Y., Sun, X., Dong, L., Li, T., et al. (2017). NF knockdown by AAV-delivered CRISPR/Cas9 prevents retinal degeneration in mice. Nat. Commun. 8, 14716.
28. McCullough, K.T., Boye, S.L., Fajardo, D., Calabro, K., Peterson, J.J., Strang, C.E., Chakraborty, D., Gliskowski, S., Haskett, S., and Samuelsson, S. (2019). Somatic gene editing of GUCY2D by AAV-CRISPR/Cas9 alters retinal structure and function in mouse and macaque. Gene. Hum. Ther. 30, 571–589.
29. Giannelli, S.G., Luoni, M., Castoldi, V., Massimo, L., Cabassi, T., Angeloni, D., Demontis, G.C., Leocani, L., Andreazelli, M., and Broccoli, V. (2018). Cas9/sgRNA selective targeting of the P23H Rhodopsin mutant allele for treating retinitis pigmentosa by intravitreal AAV9.PHP.B-based delivery. Hum. Mol. Genet. 27, 761–779.
30. Jo, D.H., Song, D.W., Cho, C.S., Kim, U.G., Lee, K.J., Lee, K., Park, S.W., Kim, D., Kim, J.H., and Kim, J.S. (2019). CRISPR-Cas9-mediated therapeutic editing of Rpe65 ameliorates the disease phenotypes in a mouse model of Leber congenital amaurosis. Sci. Adv. 5, eaax1210.
31. Hopp, T., Prickett, K., Price, V., Libby, R., March, C., Cerretti, D., Urdal, D.L., and Conlon, P.J. (2008). Short polypeptide marker sequence useful for recombinant protein identification and purification. Nat. Biotechnol. 6, 1204–1210.
32. Terke, P. (2003). Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. Appl. Microbiol. Biotechnol. 60, 523–533.
33. Chow, R.L., Altmann, C.R., Lang, R.A., and Hemmati-Brivanlou, A. (1999). Pax6 induces ectopic eyes in a vertebrate. Development 126, 4213–4222.
34. Karmakar, U.K., Ishikawa, N., Toume, K., Arai, M.A., Sadhu, S.K., Ahmed, F., and Ishibashi, M. (2015). Sesquiterpenes with TRAIL-resistance overcoming activity from Xanthium strumarium. Bioorg. Med. Chem. 23, 4746–4754.

35. Wawersik, S., and Maas, R.L. (2000). Vertebrate eye development as modeled in Drosophila. Hum. Mol. Genet. 9, 917–925.

36. Aota, S., Nakajima, N., Sakamoto, R., Watanabe, S., Ibaraki, N., and Okazaki, K. (2003). Pax6 autoregulation mediated by direct interaction of Pax6 protein with the head surface ectoderm-specific enhancer of the mouse Pax6 gene. Dev. Biol. 257, 1–13.

37. Gan, Q., Lee, A., Suzuki, R., Yamagami, T., Stokes, A., Nguyen, B.C., Pleasure, D., Wang, J., Chen, H.W., and Zhou, C.J. (2014). Pax6 mediates β-catenin signaling for self-renewal and neurogenesis by neocortical radial glial stem cells. Stem Cells 32, 45–58.

38. Jang, E.S., and Goldman, J.E. (2011). Amlexanox enhances premature termination codon read-through in COX7A1 and expression of full length type VII collagen: potential therapy for recessive dystrophic epidermolysis bullosa. J. Invest. Dermatol. 137, 1842–1849.

39. Heier, C.R., and DiDonato, C.J. (2009). Translational readthrough by the aminoglycoside geneticin (G418) modulates SMN stability in vitro and improves motor function in SMA mice in vivo. Hum. Mol. Genet. 18, 1310–1322.

40. Brasell, E.J., Chu, L., El Kares, R., See, J.H., Loesch, R., Iglesias, D.M., and Goodyer, P. (2019). The aminoglycoside geneticin permits translational readthrough of the CTNS W138X nonsense mutation in fibroblasts from patients with nephropathic cystinosis. Pediatr. Nephrol. 34, 873–881.

41. Gonzalez-Hilarion, S., Béglyn, T., Jia, J., Debrecven, N., Berte, G., Mamchaouki, O., Mouly, V., Gruneri, D.C., Déprez, B., and Lejeune, F. (2012). Rescue of nonsense mutations by amlexanox in human cells. Orphanet J. Rare Dis. 7, 58.

42. Atanasova, V.S., Jiang, Q., Prisco, M., Gruber, C., Pihtón Houbauer, J., Chen, M., Has, C., Bruckner-Tuderman, L., McGrath, J.A., Uitto, J., and South, A.P. (2017). Amlexanox enhances premature termination codon read-through in COX7A1 and expression of full length type VII collagen: potential therapy for recessive dystrophic epidermolysis bullosa. J. Invest. Dermatol. 137, 1842–1849.

43. Sahel, J.A., and Marazova, K. (2014). Toward postnatal reversal of ocular congenital malformations. J. Clin. Invest. 124, 81–84.