Aniridia is a congenital and progressive panocular condition with poor visual prognosis that is associated with brain, olfactory, and pancreatic abnormalities. Development of aniridia is linked with nonsense mutations that result in paired box 6 (PAX6) haploinsufficiency. Here, we used a mouse model of aniridia to test the hypothesis that manipulation of Pax6 dosage through a mutation-independent nonsense mutation suppression strategy would limit progressive, postnatal damage in the eye. We focused on the nonsense suppression drugs 3-[5-(2-fluorophenyl)-1,2,4-oxadiazol-3-yl]benzoic acid (ataluren) and gentamicin. Remarkably, we demonstrated that nonsense suppression not only inhibited disease progression but also stably reversed corneal, lens, and retinal malformation defects and restored electrical and behavioral responses of the retina. The most successful results were achieved through topical application of the drug formulation START (0.9% sodium chloride, 1% Tween 80, 1% powdered ataluren, 1% carboxymethylcellulose), which was designed to enhance particle dispersion and to increase suspension viscosity. These observations suggest that the eye retains marked developmental plasticity into the postnatal period and remains sensitive to molecular remodeling. Furthermore, these data indicate that other neurological developmental anomalies associated with dosage-sensitive genetic mutations may be reversible through nonsense suppression therapeutics.

Results and Discussion

Postnatal systemic nonsense suppression. As we had previously tested aminoglycosides in rat and zebrafish nonsense mutations models (8, 9), we chose gentamicin for preliminary tests in vivo. We also selected 3-[5-(2-Fluorophenyl)-1,2,4-oxadiazol-3-yl]benzoic acid (ataluren) for nonsense suppression, because it is known to be the least toxic drug in humans (12). For in vivo studies, we used the semidominant small eye (Pax6<sup>Sey</sup>+/–) model of aniridia, which has a naturally occurring Gly194X stop codon mutation (UGA) in the mouse Pax6 gene (13). Our initial therapeutic paradigm was daily subcutaneous injections of 30 μg/g ataluren or 6.25 μg/g gentamicin from P4 to P14 in Pax6<sup>Sey</sup>+/– mice. In mutant eyes, prior to treatment at P4, the cornea was thickened, there was a lenticular stalk in which the underdeveloped lens was attached to the cornea, and the retina was thickened and abnormally infolded at the ciliary margin (Figure 1A). Without treatment, at P14 the Pax6<sup>Sey</sup>+/– globe was further distorted, progressive infolding was seen in the mutant retina, and the lens was abnormally small. However, ataluren treatment resulted in correction of retinal infolding and also resulted in an increase in lens size to 70% of that observed in wild-type eyes. Similarly, treatment with gentamicin, starting either prenatally at E12.5 or postnatally at P4 and continuing until P21, resulted in remarkable normalization of the eye malformation defects (Supplemental Figure 2; supplemental material available online with this article; doi:10.1172/JCI70462DS1). This demonstrated that tissue development controlled by Pax6 expression is still sensitive to manipulation after birth. However, observations in the anterior segment of the eye showed that the corneal stroma in treated animals was abnormally thickened (Figure 2A). Detailed analysis showed that at P14 the thickness of the corneal epithelium in untreated eyes was 9 ± 2 μm (n = 6), in treated eyes was 10 ± 3 μm (n = 6), and in wild-type eyes measured 38 ± 2 μm (Figure 2B). Continued treatment with ataluren to P60 did not significantly

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improve this abnormal corneal epithelial thinning (14 ± 3 μm, n = 6). This suggested that limited drug from systemic delivery had reached the cornea. It should be noted, however, that development of the anterior segment of the eye is in part dependent upon non-cell autonomous PAX6 signaling from the lens and from migrating neural crest cells (14) and may have contributed to the corneal defects. More detailed assessment of the retina showed that there was also a significantly higher packing density of photoreceptor nuclei in ataluren-treated Pax6Sey+/– retinas (376 ± 17 nuclei per 200 μm retinal width; P < 0.001, n = 5) compared with that in wild-type Pax6+/– retinas (263 ± 12 nuclei per 200 μm retinal width, n = 5) and that photoreceptors had shorter inner and outer segments (Figure 2E). This is consistent with a less mature retina compared to age-matched wild-type animals. The untreated retina had no outer segments, and all other retinal layers were thinner that the wild-type or treated eyes. The packing density of photoreceptor nuclei in the untreated retina was 172 ± 11 nuclei per 200 μm width (n = 5).

Systemic nonsense suppression partially rescues functional deficits of the retina. We next determined whether ataluren had a discernible effect on the functional deficits of the retina using electroretinography (ERG) (9). The retinas of untreated Pax6Sey+/– mice at P60 had an undetectable ERG response (Figure 3A, trace 2). However, substantial ERG responses were recorded in mice treated up to P60 (Figure 3A, trace 3). Both scotopic and photopic b-wave amplitudes (Table 1) were significantly smaller (P ≤ 0.01, P ≤ 0.05, respectively, n = 6) than those in wild-type mice (Figure 3B), suggesting incomplete rescue. Importantly, in withdrawal experiments in which nonsense suppression was stopped at P21 and mice were tested at P60, we did not observe any reduction in the ERG (Table 1), suggesting that the benefits of treatment were stable.

Postnatal topical nonsense suppression reverses pathology. Since our observations suggested that the postnatal eye was still responsive to Pax6 dosage but that systemic therapeutic benefit was limited in the cornea, we hypothesized that a better response might be achieved using topical application to the eye. This
would increase drug concentration in the eye while further limiting any systemic toxicity (12). A 1% aqueous ataluren suspension was delivered topically to the eye (50–100 μg per eye) twice a day from P14 (when the eyes open) to P60. This resulted in only partial histological rescue of the retina and lens defects (Figure 1B). Also, we noted that the ataluren suspension caused marked ocular irritation in treated animals immediately upon drug administration. We therefore tested a number of reformulations of the drug suspension to improve particle dispersion properties (Supplemental Figure 1) and to increase the suspension viscosity to improve particle dispersion properties (13). For comparison, a mouse model with a Pax6 splice-site mutation (Pax6<sup>Sp<sub>Ey</sub></sup>–<sup>Neu</sup> mice) (13) did not respond to START therapy, indicating specificity for nonsense mutations.

**Topical nonsense suppression improves functional deficits of the retina.** ERG testing showed that both topical formulations resulted in improved responses to light stimuli (Figure 3A, traces 4 and 5). However, the scotopic and photopic b-wave responses for topical ataluren in H<sub>2</sub>O were smaller than those in wild-type animals (35% and 29%, respectively, <i>n</i> = 5), which were similar to systemic treatment responses (Figure 3B). One-way ANOVA with Tukey post-hoc comparisons between treatment groups showed no statistically significant difference in the responses between systemic and topical ataluren in H<sub>2</sub>O (<i>P</i> = 0.994). However, the responses for START therapy showed a significant benefit over other treatments (<i>P</i> < 0.01). Additionally, the scotopic b-wave amplitudes in START-treated animals were not significantly different from those of wild-type controls (<i>P</i> = 0.78).

To further evaluate the functional effects of treatment, we assessed the optokinetic tracking response, a behavioral response mediated through retina-brain circuitry (15) that approximates a measurement of visual acuity. Under optokinetic tracking test conditions, Pax6<sup>Sp<sub>Ey</sub></sup>–<sup>Neu</sup> mutants demonstrated limited tracking responses (0.04 cycles/degree [c/d]) (Figure 1D) when compared with those of wild-type mice (0.42 c/d). Mice treated topically with aqueous ataluren had an improved spatial frequency threshold (0.3 c/d). However, mice treated with the
START formulation showed higher spatial frequency thresholds (0.42 c/d), and these were not significantly different from responses seen in wild-type animals ($P = 0.42$).

Here, we demonstrated that, in the haploinsufficient postnatal mouse eye, manipulation of $Pax6$ dosage stably reversed the congenital ocular malformations and, importantly, that this was best achieved through topical administration using the START formulation. Based on our observations, we propose that abnormally developed ocular tissue is still remarkably sensitive to molecular remodeling postnatally. In mammals, although most tissue morphogenesis is completed by birth, it has already been found that this is not the case in the CNS. Both the brain and eye continue to adapt after birth, with significant remodeling of new synaptic connections (16). Our data suggest that this postnatal plasticity might be sufficiently versatile to also allow for reversal of abnormal tissue modeling if the underlying molecular defect is corrected. The mechanism by which this remodeling occurs warrants further investigation.

Another key finding in this study was that extensive remodeling could be achieved in vivo in the retina and corneal epithelium with START therapy that correlated directly with increasing $PAX6$ protein expression to approximately 90% of wild-type levels. Based on previous studies (11, 12), this suggests that ataluren was able to suppress the nonsense mutation, allowing full-length $PAX6$ protein to be synthesized. Recently, the effectiveness of ataluren as a nonsense suppression agent has been questioned (17). Despite this, several in vivo studies (12, 18), including this one, show beneficial efficacy of ataluren as a treatment strategy. Further research is needed to fully define the exact mechanism of action of this drug.

The effects of varying $Pax6$ gene dosage have been investigated in haploinsufficient and overexpression models of ocular malformation (19). However, the minimum $Pax6$ dosage that is required for normal tissue development has yet to be determined, but it is likely to vary in different tissues and at different times. Other genes associated with abnormal ocular development, such as $Pitx2$, $Foxc1$, $Foxc2$, and $Foxe3$, are also dosage-sensitive (20–22). Manipulation of these genes after birth may also lead to corrective tissue remodeling, suggesting a common therapeutic paradigm that could be applicable to many congenital eye defects. In addition, nonsense suppression therapy is also emerging as a therapy outside the eye. For example, in cystic fibrosis (7) and Duchenne muscular dystrophy mouse models (18), ataluren treatment lead to an approximately 25% increase in protein production, and this correlated with a similar improvement in functional studies. Thus, a moderate increase in gene expression is able to have a beneficial effect, but presumably the levels would need to be closer to 50% in the affected tissues for full rescue.

In conclusion, we have uncovered a viable therapeutic option for children with $PAX6$ nonsense mutations that reverses a debilitating eye malformation defect. In addition, it is also advantageous that the most benefit appears linked to topical application,
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since this will improve patient compliance and reduce the risk of systemic adverse effects to a minimum. Based on this study in a directly relevant animal model, we would advocate the initiation of clinical trials for a nonsense suppression paradigm in aniridia.

Methods

Animals and drug administration. Both Pax6 mouse alleles (Pax6<sup>Sey+/–</sup>; a gift of Veronica van Heyningen, MRC Human Genetics Unit, Edinburgh, United Kingdom, and Pax6<sup>Sey–1Neu</sup>; a gift of Elizabeth Simpson, University of British Columbia) were maintained on the C57BL/6 background. Pax6 mutant offspring were identified by genotyping (details in Supplemental Methods). Daily subcutaneous injections of 30 μg/g body weight of an aqueous suspension of ataluren (Selleckchem) started at P4 and continued until P14. For gentamicin studies, subcutaneous injections (6.25 μg/g) aqueous suspension of ataluren (Selleckchem) started at P4 and continued until P14. For gentamicin studies, subcutaneous injections (6.25 μg/g; Sigma-Aldrich) were carried out from P4 to P21 or began in time-mated pregnant mice from E12.5 to P21. For topical administration, drops were instilled into the eyes twice daily beginning at P14 until analysis at P60 using 2 different formulations: a 1% ataluren aqueous suspension or the systemic therapy –210.0 ± 18.9 400.8 ± 15.3 58.1 ± 3.1 30.6 ± 3.3

Pax6<sup>Sey+/–</sup> mice with topical 1% ataluren in H2O to P60 (n = 6) –161.7 ± 27.8 374.5 ± 57.4 55.9 ± 5.2 32.1 ± 3.7

Pax6<sup>Sey+/–</sup> mice with topical START formulation to P60 (n = 6) –215.8 ± 20.3 638.0 ± 26.9 72.4 ± 5.8 38.1 ± 2.1

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