Neural damage and neuroprotection with glaucoma development in aniridia

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Introduction

Aniridia is a rare congenital disorder distinguished by the complete or partial absence of the iris. This panocular condition presents with a range of eye abnormalities, each to differing extents, such as cataract formation, keratopathy, and foveal and/or optic nerve hypoplasia [1,2]. Common symptoms such as cataracts may be clinically addressed by topical medication or surgical intervention [2]. However, there are no treatments available to reverse neural loss, which is most critical for vision [1-3]. Of particular importance are retinal ganglion cells (RGCs), neurons that project axons to form the optic nerve, and transmit visual information from the eye to central brain structures [4]. About 90% of aniridia patients were found to have underdeveloped fovea, the portion of the retina responsible for acute central vision, and recent data also suggests nerve fiber reduction throughout the retina [5,6]. Similarly, in a rodent model of aniridia, flat-mounted retinas show one-third fewer Brn3a-expressing RGCs than wildtype controls [7]. Optic nerve hypoplasia and associated retinal nerve fiber layer thinning have also been reported in approximately 10% of aniridia patients and may occur independently of foveal hypoplasia [8,9]. In both instances, patients suffer from varying degrees of vision loss [10].

Approximately 90% of aniridia patients have an underlying heterozygous mutation of the *PAX6* gene located on chromosome 11p13 [1,11]. PAX6 is a highly conserved transcriptional regulator crucial for ocular, endocrine, and central nervous system development [1,12]. Two-thirds of aniridia patients inherit an autosomal dominant mutation for the disorder while the disease in the remaining third stems from sporadic mutations. About fifty percent of these *PAX6* haploinsufficiencies are due to premature termination codons (PTCs) introduced by in-frame nonsense mutations [13]. In mice, homozygous loss of *Pax6* gene is neonatally lethal, whereas heterozygotes are viable but show high phenotypic variability due to individual differences in expression levels [12]. Despite variation, *Pax6* haploinsufficient *Small eye* (*Sey*) mouse models have similar mutations and exhibit phenotypes like those seen in human patients, making them ideal systems to study the mechanisms of retinal malformation in aniridia [12] *(Figure 1A)*.

Aniridia's wide range of deleterious symptoms often result in severe vision loss and anterior eye occlusion. Although the neural damage is irreversible, there are several promising treatments being studied to aid proper retinal development in the context of *PAX6*-based aniridia [12,14,15]. For example, PD0325901, a potent small molecule MEK inhibitor, significantly increases the expression of *Pax6*, which in turn limits the development and progression of the congenital defects in *Pax6* deficient aniridia mice (*Pax6^{Sey-Neu/+}*) [15]. Another example is the use of nonsense suppression therapy in the weeks following eye-opening which has presented encouraging evidence for the amelioration of visual deficits and neural health [12,14].

The combination of anterior eye defects and existing retinal damage from ocular hypoplasia contributes to increased RGC loss due to elevated intraocular pressure (IOP). IOP elevation is one of the most potent and damaging risk factors for glaucoma development [16,17]. Commonly identified by visual field tests and optic disc deformation, glaucoma is prevalent in around 50% of aniridia patients [18-20]. However, diagnosis of glaucoma does not typically occur until a mean age of 13.6 years, more than a decade after the initial diagnosis of aniridia (mean 21 months), again emphasizing the importance of early detection and mitigation of neural damage [18].

Elevated IOP is a significant contributor to aniridia phenotypes

Glaucoma is a collection of eye diseases characterized by damage to the optic nerve and subsequent loss of retinal ganglion cells, which manifests as gradually worsening vision. One of the most common indicators of ongoing glaucomatous damage is the increase of intraocular pressure (IOP), resulting in neuronal loss [17,18,21,22]. Intraocular pressure is established by the balance of aqueous humor production and outflow. Aqueous humor is produced by the ciliary body and is needed to maintain transparency of the lens and to provide nutrients to the avascular cornea and lens [23]. The balance between aqueous humor formation in the ciliary body (inflow) and drainage through the iridocorneal (I-C) angle (outflow) is crucial for preservation of a stable and normal IOP. This balance is maintained by drainage tissues such as the trabecular meshwork (TM) and Schlemm's
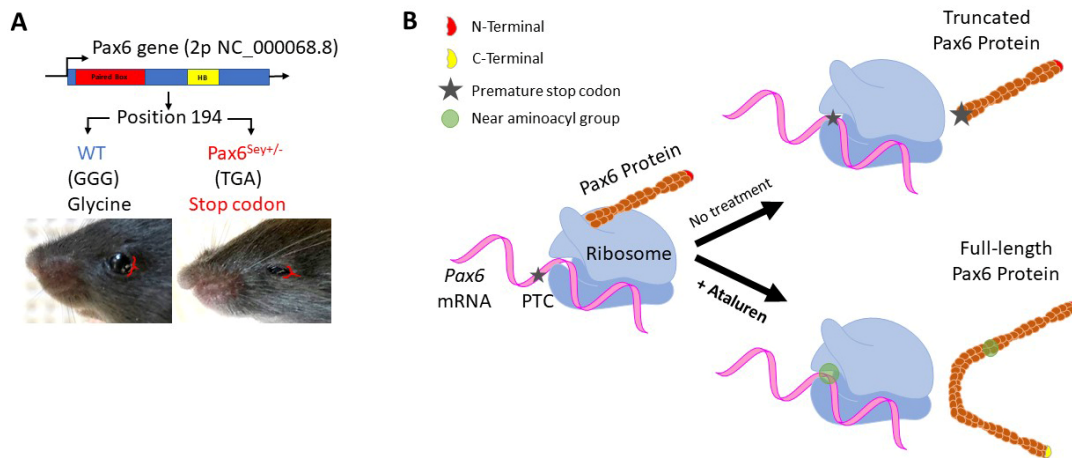
Figure 1. Nonsense mutation in Pax6 produces small-eye aniridia phenotype. (A) Diagram of Pax6 gene in mice (located 2p NC_000068.8). The Pax6^Sey+/- mouse model features a Gly →STOP nonsense mutation, which results in a truncated protein product. Without proper Pax6 dosage, heterozygous mutants are born with a small-eye phenotype. (B) Illustration of the proposed nonsense suppression mechanism of ataluren. When mutant Pax6 mRNA is undergoing translation, ataluren helps to insert a near aminoacyl group in place of the premature stop codon (PTC), allowing full-length translation of the protein.

canal (SC) in the I-C angle. Should these become occluded due to developmental malformation in undifferentiated mesenchyme, glaucomatous symptoms are likely to arise and worsen without medical intervention [17,18,24,25].

Monitoring IOP is the most common clinical method to assess glaucoma. In many experimental and clinical settings, researchers have long relied on rebound tonometry to assess IOP. This technique uses a calibrated probe which, when extended to contact the corneal center of a subject's eye, will then rebound and the relative physical resistance can then be used to extrapolate IOP (typically in mmHg). Furthermore, the versatility of rebound tonometry allows IOP to be measured at regular intervals, and its minimal invasiveness makes measurements quick and easy [20].

However, monitoring IOP in aniridia patients can be unreliable due to considerable variations in ocular axial length, curvature, rigidity, and corneal thickness which leads to incorrectly calculated IOP measurements [26]. Zakrzewska et al. found that even slight increases in central corneal thickness (> 600μm) and curvature could lead to an elevated measurement as high as 1.95 mmHg [27]. The well-documented severity of anterior eye defects in aniridia patients, most notably corneal opacification//thickening (~691.8 ± 75.4μm) [28] may lead to a high incidence of erroneous IOP measurements derived by rebound tonometry. Likewise, the reduced eye growth and pronounced nystagmus typical of aniridia-afflicted mice further complicate IOP measurement, as the corneal center (the intended target of the tonometer's probe) is far smaller and in constant motion [29]. Therefore, it might be more straightforward to apply non-invasive imaging techniques to examine and monitor the neural damage directly.

Neural damage in aniridia

Although anterior eye defects are the most obvious and well-studied component of aniridia, recent evidence reveals that aniridia's developmental defects extend to the retina. Gregory-Evans and colleagues observed thinning or thickening of the inner and outer plexiform layers of the retina and found severe retinal folding [29,30]. These oxbow bends and distended pockets are indicators of lasting neural damage, which surely contribute to permanent visual deficits. This damage, however, is highly variable and a great deal remains unclear about how retinas are directly affected by aniridia [30].

It has been well established in prior literature that classical aniridia can induce severe vision loss [1,9,10,13,31,32]. Existing studies demonstrate that this may be due, in part, to the elevated pressure arising from anterior defects, and thus preexisting retinal damage may be exacerbated. The continued IOP elevation might explain why vision gradually declines with age [18,22]. As previously mentioned, this vision loss likely stems from a combination of the disruption of nerve fiber integrity and an estimated 33% loss of RGCs [7]. However, due to the variability observed in individuals, the average loss of RGCs might be misleading due to disregarding regional and type-specific loss of RGCs.

One problematic factor in aniridia studies is the wide phenotypic diversity among *Pax6* mutants. *Pax6^Sey+/-* mice, a C57BL/6 line with a nonsense mutation in the *Pax6* gene, was found to suffer from a broad range of symptoms [14,30]. Experiments using this line found that eye size, IOP, visual acuity, and ocular deformity ranged from near-normal to extreme levels in individual mice. This range in penetrance is seen in other lines including the Pax129^Sey mice described by Hickmott *et al.* [33] and extends to human subjects as well. Case studies of afflicted families have shown that while the presence of certain symptoms seems consistent among subjects with similar genetic background, the severity of these symptoms still varies greatly between individuals [13,18,34-36]. Such variability is likely due to a combination of individual genetics and environmental factors during early development that influence gene expression.

Secondly, studies, including ours, showed that different types of RGCs respond to the glaucomatous insult differently [37-39]. It is even less known whether and how different subtypes of RGCs are affected with the development of aniridia.

The variability of phenotype contributes to the difficulty of conducting aniridia studies. The efficacy of treatments and the impact of experimental models can be masked by symptom variability. Longitudinal *in vivo* studies of individual subjects are one approach to account for the specific severity of aniridia symptoms. By tracking IOP, visual acuity, eye size, functional retinal output (ERG), and assessing changes in retinal health as a subject ages, one can accurately evaluate developmental changes without the confounding effects of individual variation.

Functional consequences of aniridia phenotypes

Disruption of retinal layers impedes the transmission and processing of visual information, so techniques like optical coherence tomography (OCT) have been employed to visualize layers of the retina *in vivo*. Aniridia patients display an increase in central foveal thickness of around 82μm whereas surrounding parafoveal layers were significantly diminished, supporting observations of disrupted foveal development and hypoplasia [40]. Electroretinogram tests in mice with diminished *Pax6* impairment of retinal function with scotopic responses dropping from ~600μV to nearly 0μV and photopic responses dropping from ~80μV to ~2μV [30]. The degree to which ERG responses decrease varies between individual patients. Variations also exist when comparing mice and humans [10,22,20,32,33,40]. When normalized, studies in humans have shown responses with amplitude reductions of only 14% in the scotopic state and 5% in photopic conditions for patients with PTC mutations [10]. This range of variation further emphasizes the importance of pursuing increased translational studies of *Pax6* mutant phenotypes.

The role of PAX6 as a critical transcription factor supports the finding that much of this neuronal dysfunction in patients with aniridia extends beyond the retina to visual processing centers in the brain [41,42]. Hypoplasia of the pineal gland and disruption of naturally oscillating genes in the hypothalamus indicate lack of synchrony with day/night patterns critical for circadian rhythms [43,44]. Using region-of-interest based estimates, insufficient cell proliferation has been linked to a reduction of cells in the occipital lobe with a mean area loss of 1619 mm^2 in patients severely affected by *PAX6* haploinsufficiency [45]. Increased functional connectivity in the primary visual network of aniridia patients is observed as a compensatory mechanism for the attenuation of regions like the occipital lobe [45,46]. Cortical disruptions are also associated with various intellectual disabilities as well as additional sensory deficits including reduced olfaction and hearing impairment [42].

At the same time, as retinal ganglion cells project to the higher visual centers, miswiring and disrupted connection may affect the structure and function of postsynaptic neurons in the brain, particularly in instances where axon guidance and fasciculation are impaired [47]. Anterograde transsynaptic degeneration has been well established in patients with glaucoma. Lateral geniculate nuclei of glaucoma patients presented with an approximate 55% reduction in laminar volume after eight weeks of elevated intraocular pressure [48]. This volumetric loss incorporates reduction of soma size, soma density, and dendritic branching [48]. Loss of RGC innervation also produces neurons in the superior colliculus with diminished responses to visual stimulation and abnormal receptive field properties [38]. Continued research is needed to further investigate how retinal damage and the brain interact and affect each other in aniridia.

Ataluren and Pax6 mutation suppression

Theoretically, treatments for aniridia that permit ribosomal read-through of the premature termination codon (PTC) should promote a full-length Pax6 protein [49]. Here, we will focus on the translational readthrough-inducing drug (TRIDs) which work by suppressing PTC recognition by the ribosome during the translation process. Aminoglycoside antibiotics, 3-[5-(2-fluorophenyl)-1,2,4-oxadiazol-3-yl] benzoic acid (ataluren), and ataluren oxadiazole analogues are three different classes of drug which act as TRIDs [50]. Aminoglycosides have been used for decades to treat infection by gram negative bacteria by interfering with the bacterial ribosome [51]. There are several subtypes of aminoglycosides, but gentamicin has shown the best efficacy in bypassing the PTC [52]. However, this class of drug has many side effects such as ototoxicity and neurotoxicity and has a low specificity for the premature termination codon, occasionally reading through the correct termination codon. In contrast, ataluren, a nonsense mutation suppressant originally designed to treat Duchenne muscular dystrophy, has shown promising effects in treating aniridia [14,29,53] *(Figure 1B)*. Ataluren (trade name Translarna®) is a member of the oxadiazole drug family, whose members contain aromatic heterocyclic rings, composed of one oxygen and two nitrogen atoms. The arrangement of these atoms in the central five-member ring increases specificity for the PTC [54]. Despite its efficacy in a laboratory setting, the precise mechanism of ataluren remains unclear. Likewise, limitations in drug transport demonstrate some minor application issues [30]. Ataluren is typically taken orally, but this route of administration has shown negligible localization of the drug to posterior eye regions. Similar setbacks have been seen in injection and topical applications [14,30].

Nevertheless, Gregory-Evans et al. have shown near-wildtype levels of rescue in treated *Pax6^(Sey+/−)* mice [14,30]. In their 2014 study, two daily doses of their novel topical START formulation (0.9% sodium chloride, 1% Tween 80, 1% powdered ataluren, 1% carboxymethyl-cellulose) were applied daily from P14 to P60. They later discovered that this time frame was critical, as it represents a time when significant *Pax6* expression is still needed for healthy ocular development. Later studies with adjusted concentration and start time of their treatment, found

that ataluren was most efficacious following eye-opening (P14) until ~P30. Delaying treatment showed reduced rescue effects, and in some cases, induced lens hyperplasia (14). In their initial study, they observed a functional recovery of both scotopic (~600μV, WT: ~620μV) and photopic (~75μV, WT: ~80μV) ERG responses. Likewise, anterior eye defects and retinal layers appear near normal (30). Likewise, it was observed that post-treatment, these rescued phenotypes remained healthy, further highlighting the importance of early intervention. However, due to their reliance on *in vitro* techniques and comparisons of retinal sections, it remains unclear whether these differences in retinal morphology are due to successful treatment or initial symptomatic variance. Likewise, it remains unclear the degree of morphological rescue, as there is not continuous imaging of individual eyes, but rather comparisons between different, and possibly highly varying, samples.

Despite some gaps in our knowledge of its mechanism, ataluren has been approved for the treatment of patients afflicted with Duchenne muscular dystrophy [55]. The clinical trial for ataluren's application in cystic fibrosis was discontinued due to "disagreement between criteria-based CF pulmonary exacerbation definitions and clinical assessments provided by study investigators" [56]. Since 2016 there has been a phase 2 clinical trial, "Study of Ataluren in Participants With Nonsense Mutation Aniridia (STAR)", which is ongoing and awaiting publication [57].

Conclusion

The current rodent models of aniridia, e.g. *Pax6$^{Sey+/-}$*, provide valuable information about the etiology, progression, and drastic phenotypic changes induced by classical aniridia. Their genotypic and phenotypic resemblance to the most common forms of human aniridia make them ideal for studies that seek to understand the underlying relationship between *PAX6*, glaucoma, and subsequent vision loss. However, much more work is needed to account for variable penetrance between subjects and neural damage progression with age. Detailed longitudinal *in vivo* assessments of aniridia phenotypes must be performed regularly throughout early postnatal development to better characterize how early damage can be compounded by other existing conditions such as glaucoma. Likewise, potential drug therapies, such as ataluren, must be tested in a similar long-term *in vivo* context to establish their most efficacious methods of administration.

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Conflict of interest

None.

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