Review Article

PAX6 disease models for aniridia

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Aniridia is a pan-ocular genetic developmental eye disorder characterized by complete or partial iris and foveal hypoplasia, for which there is no treatment currently. Progressive sight loss can arise from cataracts, glaucoma, and aniridia-related keratopathy, which can be managed conservatively or through surgical intervention. The vast majority of patients harbor heterozygous mutations involving the *PAX6* gene, which is considered the master transcription factor of early eye development. Over the past decades, several disease models have been investigated to gain a better understanding of the molecular pathophysiology, including several mouse and zebrafish strains and, more recently, human-induced pluripotent stem cells (hiPSCs) derived from aniridia patients. The latter provides a more faithful cellular system to study early human eye development. This review outlines the main aniridia-related animal and cellular models used to study aniridia and highlights the key discoveries that are bringing us closer to a therapy for patients.

Key words: Aniridia, hiPSC, LESC, PAX6, primary cells, retinal organoids, sey mouse, zebrafish

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Aniridia is a sight-threatening pan-ocular disorder characterized primarily by partial or complete iris hypoplasia. Other ocular manifestations include foveal and optic nerve hypoplasia, early-onset glaucoma, nystagmus, cataracts, and corneal keratopathy [Fig. 1a–c].[1-5] Aniridia is a rare disease with a prevalence of 1:40,000–100,000 births. The majority of cases are caused by heterozygous mutations resulting in haploinsufficiency of the *PAX6* gene, either inherited in a highly penetrant autosomal dominant manner (70%) or caused by *de novo* sporadic mutations (30%).[1,5] Recent reports have also shown the presence of paternal mosaicism in four aniridia families.[6,7]

PAX6 and Aniridia

Paired box 6, denoted as PAX6 in humans, is a highly evolutionary conserved transcription factor and has a fundamental role in the development and maintenance of eyes, as well as being expressed in regions of the central nervous system, pancreas, gut, and olfactory epithelium.[8-10] *PAX6* is located on chromosome 11p13 with 14 exons, but the first three are noncoding.[11] Heterozygous mutations in the mouse *Pax6* gene cause the small eye (*Sey*) mouse, a strain that phenotypically resembles human aniridia [Fig. 1d].[12-14] Additional *PAX6* homologs have been detected in zebrafish (*pax6a* and *pax6b)* [Fig. 1e], quail, and *Drosophila*.[15-17] Overexpression of the *PAX6* gene induces the formation of ectopic eyes in *Drosophila* and *Xenopus*; for this reason, PAX6 is categorized as the "master regulator" of the eye.[18,19]

It has been demonstrated that *PAX6* is highly regulated and dosage sensitive. For this reason, several elements and promoters both within and upstream the gene are involved in precise regulation of its complex spatial and temporal expression [Fig. 1a].[8,20-22] The PAX6 protein has two DNA-binding sites – the paired domain and the homeodomain, which are adjoined by a linker region. The DNA-binding ability of the homeodomain is regulated by a proline–serine–threonine-rich transactivation domain (PSTD) that is located immediately downstream of the homeodomain. Equivalently, the paired domain comprises an N-terminal subdomain and a C-terminal subdomain, each binding specific motifs and altering the conformation of the paired domain [Fig. 2b].[8] Canonical PAX6 and PAX6 (5a) are the two main protein isoforms that have been identified in humans; the isoforms are thought to have different downstream targets and are expressed at varying ratios throughout development [Fig. 2b].[8,10,23] Furthermore, a third isoform, Pax6ΔPD, has been found in mice and has also recently been detected in human retinal organoids.[24,25]

Over 600 *PAX6* mutations have been observed in aniridia patients, with the most common introducing a premature termination codon (PTC) through nonsense variants or insertion–deletion frameshift variants. In such cases, the PAX6 protein is truncated and likely results in loss of function, or the mutated mRNA transcript is degraded through nonsense-mediated decay preventing translation.[8,26,27] Because of the dosage sensitivity of PAX6, the reduction of protein levels induces haploinsufficiency.[8] A recent longitudinal

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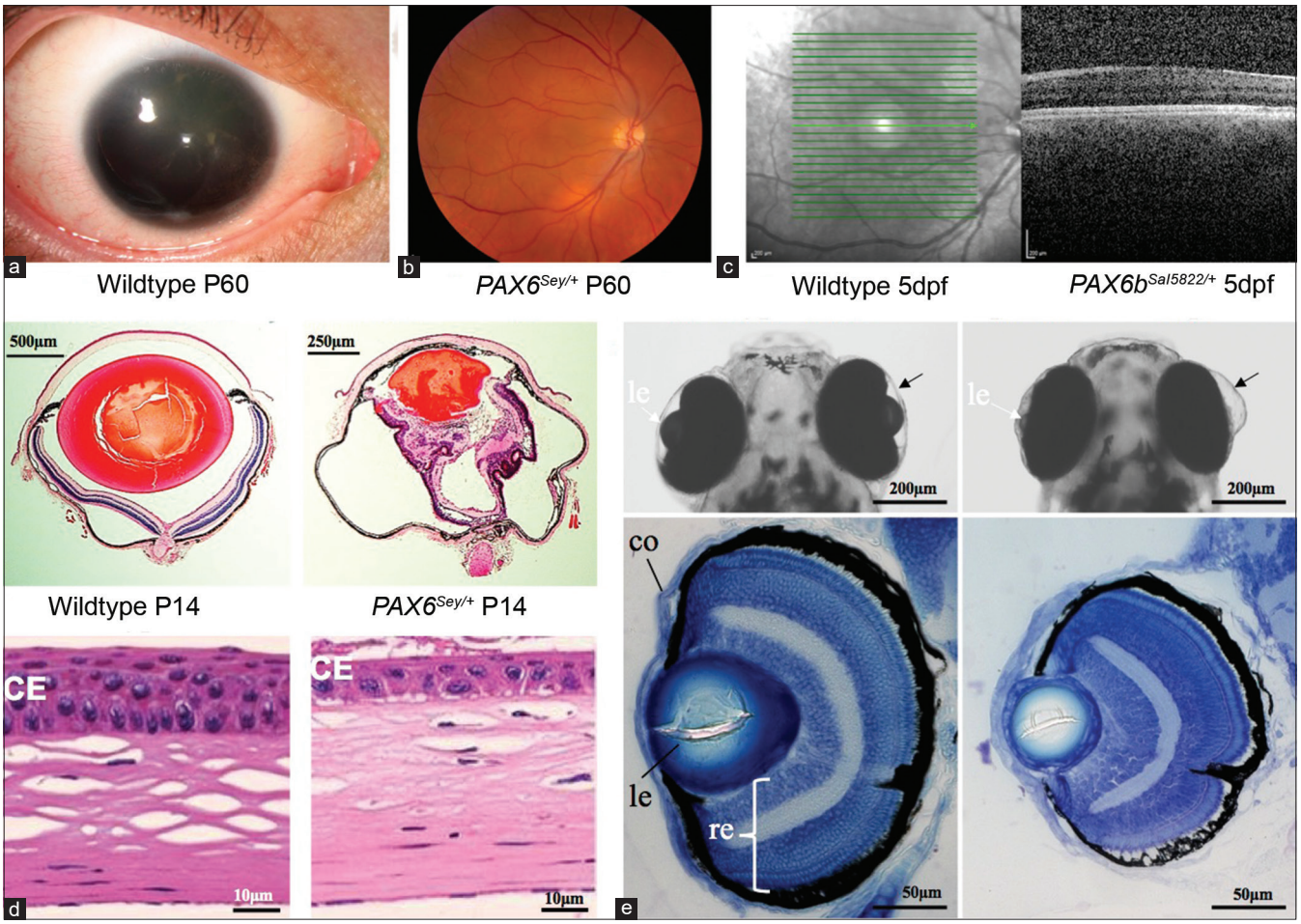

Figure 1: (a) Right anterior segment showing complete iris hypoplasia. (b) Color fundus photograph lacking foveal reflex and (c) Spectral domain optical coherence tomography (SD-OCT) of the right macula showing complete foveal hypoplasia. (d) *Pax6*$^{Sey/+}$ mouse H and E whole eye sections at postnatal (p) day 60 (top) showing a small eye, lens, and retinal dysplasia, and a thinned P14 corneal epithelium (bottom). (e) Brightfield images (top) and toluidine blue-stained sections (bottom) displaying anterior segment dysgenesis (black arrow), small eye and lens (le) (white arrow), and thickened cornea (co) in 5dpf pax6bSa15822 zebrafish mutant. dpf = days post-fertilization, H and E = hematoxylin and eosin, re = retina

study of 86 aniridia patients in the UK supported the previous findings that missense variants are associated with milder phenotypes (milder grades of foveal and iris hypoplasia, cataracts, and aniridia-related keratopathy), with the exception to those that disrupt PAX6 DNA-binding activity, such as the c.372C > A, p.(Asn124Lys) variant, which gives rise to a non-aniridia phenotype of microphthalmia and coloboma.[28] Several studies have also shown that PTC and C-terminal extension (CTE) mutations result in more severe phenotypes with poorer visual outcomes.[29,30] The mean age ± standard deviation (SD) of developing cataracts for patients with nonsense variants was 11.8 ± 11.8 years. It developed at a later age in patients with missense (17.2 ± 9.8 years), intronic (18.7 ± 16.2 years), and frameshift (22.9 ± 11.0 years) variants.[31] The mean age ± SD of glaucoma diagnosis was 25.0 ± 17.3 years, with missense (28.5 ± 26.0) and frameshift (50.7 ± 2.3) variants being diagnosed later. Overall, there was no significant difference observed in the mean age of glaucoma diagnosis between the mutation groups (P = 0.22). However, the prevalence of glaucoma was significantly different (P < 0.001), with a higher prevalence in those with whole gene deletions compared to those with frameshift mutations who showed the lowest prevalence.

Animal Models for Aniridia

Given the limited access to the human eye, animal and cellular disease models have been, and continue to be, crucial in identifying the genetics and pathophysiology underlying aniridia.[14,18,32,33] The function and structure of the mature eye is similar across different vertebrate species.[34] Animal models allow us to consider genotype–phenotype correlations, as well as potentiate the identification of molecular pathways of disease progression.[35]

Ever since the discovery of *PAX6* homology across different species, *Drosophila*, zebrafish, quail, *Xenopus*, and mice have been used to model aniridia, the pathways affected by *PAX6* haploinsufficiency, and potential therapies. This review will focus on mice, zebrafish, and cellular models and their contributions to our knowledge of aniridia.

Mouse Models

Advantages of using mouse models

Genomic conservation of 99% between humans and mice has allowed mouse models to be the most commonly explored animal model for biomedical research.[36,37] Mice are

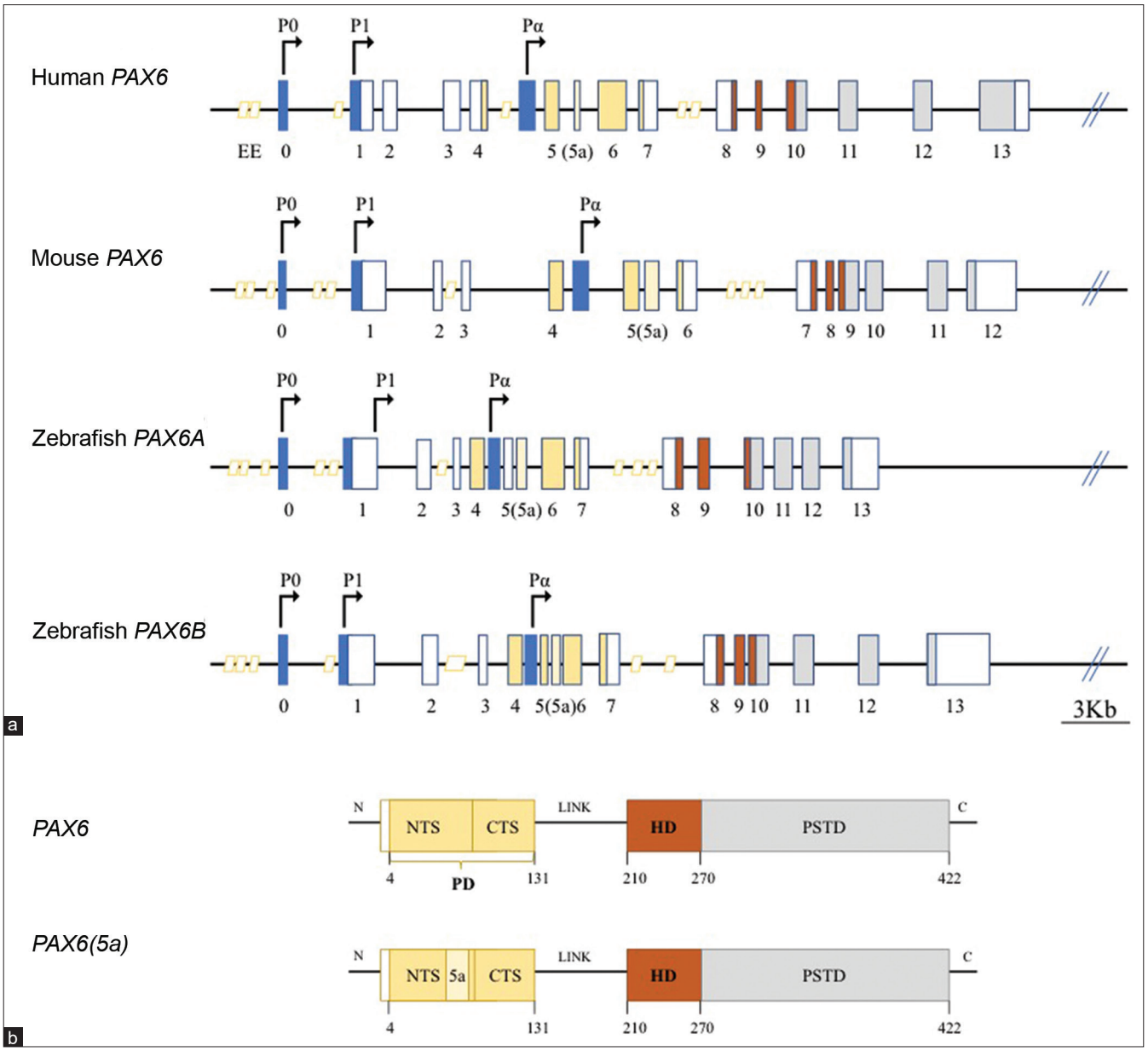

Figure 2: (a) Human, mouse, and zebrafish *PAX6* gene structure (boxes show exons and colors correspond to the respective protein domain as shown in b). (b) The two main PAX6 isoforms in humans. The DNA-binding domains PD and HD are illustrated. CTS = C-terminal subdomain, EE = ectodermal enhancer, HD = homeodomain, LNK = linker region, NTS = N-terminal subdomain, P0, P1, Pα = promoters (blue boxes) and regulatory elements (parallelograms), PD = paired domain, PSTD = proline–serine–threonine domain

suitable for large-scale studies because they are small, have a short generation time, and are relatively cost-effective to maintain.[34,38] The mouse eye is similar to the human eye in terms of physiology, anatomy, and development, with the only major difference in development being the lack of cone-rich macula. Additionally, the size of the mouse eye facilitates morphological analysis.[34] Mice are particularly a good model for congenital aniridia because the human *PAX6* and mouse *Pax6* genes are homologous and translate into the same amino acid sequence [Fig. 2a].[39]

Generation of mouse models and phenotypes

Pax6 mutations in mice are either spontaneous, chemically/radiation-induced or targeted mutations [Table 1]. The "original" *Sey* mouse contains a spontaneous mutation and was characterized by Roberts in 1967.[13] Hill *et al*.,[14] in 1991, demonstrated the link between the *Pax6* gene on chromosome 2 and the small eye phenotype. $Pax6^{Sey}$ carries the point mutation c.622G>T p.(Gly208*) that results in a PTC. Homozygous $Pax6^{Sey/Sey}$ mice exhibit anophthalmia and lack nasal cavities and die shortly after birth.[14] However, the heterozygous $Pax6^{Sey/+}$ mice display iris hypoplasia, abnormal lens morphology, cataracts, corneal opacification, and incomplete separation of lens from the cornea, all of which correspond to patient-related aniridia features.[13,14,32,40] Similar to $Pax6^{Sey}$, $Pax6^{Sey\text{-}Neu}$ and the $Pax6^{Neu}$ series (2Neu–10Neu) contain point mutations (missense or nonsense), with the difference being that the mutation has been chemically induced

Table 1: Animal (mouse and zebrafish) and cellular models of *PAX6* mutant genes

Genotype	Generation of mutation	Mutation details	PAX6 domain	Predominant ocular phenotype	References
		Mouse			
$PAX6^{Sey}$/ $PAX6^{+}$	Spontaneous	Nonsense Exon 7 c. 622G>T p.(Gly208*)	Termination before the HD	Abnormal cornea Abnormal lens Mic	Roberts, 1967[13]
$PAX6^{Leca1}$/ $PAX6^{Leca1}$	Chemically induced	Missense Exon 10 c. 971T>A p.(Val270Glu)	HD	Fused lens and cornea Mic	Thaung *et al.*, 2002[44]
$PAX6^{Leca2}$/ $PAX6^{Leca2}$	Chemically induced	Missense Exon 7 c. 586C>T p.(Arg142Cys)	PD	Fused lens and cornea Mic	
$PAX6^{Leca3}$/ $PAX6^{Leca3}$	Chemically induced	Nonsense Exon 12 c. 1266C>A p.(Tyr369*)	PSTD	Fused lens and cornea Mic	
$PAX6^{Leca4}$/ $PAX6^{Leca4}$	Chemically induced	Missense Exon 6 c. 354C>A p.(Asn64Lys)	PD	Fused lens and cornea Mic	
$PAX6^{SeyDey}$/ $PAX6^{+}$	Spontaneous	Intergenic deletion Large deletion of *PAX6* gene affecting the *Wt1* gene	Whole protein	Coloboma Small lens Cataract Retinal abnormalities Absent anterior chamber Mic	Theiler *et al.*, 1980[45]
$PAX6^{SeyH}$/ $PAX6^{+}$	Radiation induced	Large deletion of *PAX6* gene Likely affects the *Wt1* gene	Likely the whole protein	Coloboma Mic	Hogan *et al.*, 1986[32]
$PAX6^{SeyNeu}$/ $PAX6^{+}$	Chemically induced	Frameshift, G T transversion at the +1 position of a splice donor site, retention of 116 nucleotides of intronic sequence	Lacks 15 amino acids from the C terminus, including the transactivation domain	Abnormal cornea Abnormal lens Anterior chamber defect Mic	Hill *et al.*, 1992[46]
$PAX6^{2Neu}$/ $PAX6^{+}$	Chemically induced	Intron 9, 2T>C 5′ splice after 269	HD	Abnormal iris Abnormal pupil Cataract Mic	Favor *et al.*, 2001[42]
$PAX6^{3Neu}$/ $PAX6^{+}$	Chemically induced	Frameshift Insertion Exon 7 598insAla	Deletion of HD, LR, and PSTD	Abnormal iris Abnormal pupil Corneal opacity Fused cornea and lens Cataract Mic	
$PAX6^{4Neu}$/ $PAX6^{+}$	Chemically induced	Missense Exon 10 c. 979T>C p.(Ser273Pro)	HD	Abnormal iris Abnormal pupil Cataract Mic	
$PAX6^{5Neu}$/ $PAX6^{+}$	Chemically induced	Nonsense Exon 6 c. 517A>T p.(Arg119*)	Deletion of HD, LR, and PSTD	Abnormal iris Abnormal pupil Cataract Mic	

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Table 1: Contd...

Genotype	Generation of mutation	Mutation details	PAX6 domain	Predominant ocular phenotype	References
			Mouse		
PAX6^{6Neu}/ PAX6^{+}	Chemically induced	Nonsense Exon 10 c. 1092C>A p.(Tyr310*)	PSTD	Abnormal iris Abnormal pupil Cataract Mic	
PAX6^{7Neu}/ PAX6^{+}	Chemically induced	Missense Exon 4 c.-3A>T	Kozak consensus sequence	Abnormal iris Abnormal pupil Cataract Mic	
PAX6^{8Neu}/ PAX6^{+}	Chemically/radiation induced	Nonsense Exon 10 c. 1092C>A p.(Tyr310*)	PSTD	Abnormal iris Abnormal pupil Mic	
PAX6^{9Neu}/ PAX6^{+}	Chemically induced	Nonsense, deletion Exon 5 7bp 261-267	PD	Abnormal iris Abnormal pupil Mic	
PAX6^{10Neu}/ PAX6^{+}	Chemically induced	Nonsense Exon 6 c. 469C>T p.(Gln103*)	Deletion of PD, LR, HD, PSTD	Abnormal iris Abnormal pupil Mic	
PAX6^{tm1Pgr}/ PAX6^{+}	Targeted	Insertion of β-galactosidase The start codon in exon 4	HD is replaced with a β-galactosidase gene followed by a neomycin cassette	Abnormal iridocorneal angle Abnormal optic cup Anterior iris synechia Fused cornea and lens Absent anterior chamber	St-Onge *et al*., 1997[47]
PAX6tm^{2Pgr}/ PAX6^{+}	Targeted	Insertion of loxP sequences flanking the initiator ATG and exons 4-6	PD	Abnormal iris Abnormal optic nerve Mic	Ashery-Padan *et al*., 2000[48]
PAX6^{Coop}/ PAX6^{+}	Chemically induced	Nonsense Exon 10 c. 1033C>T p.(Glu329*)	HD and PSTD	Abnormal iris Corneal opacity Mic	Lyon, 2000[49]
PAX6^{Aey11}/ PAX6^{Aey11}	Chemically induced	Nonsense Exon 8 c. 644C>T p.(Glu209*)	Loss of entire HD and PSTD	Small eye Corneal adhesion Corneal and lens opacities	Graw, 2005[50]
PAX6^{Aey18}/ PAX6^{+}	Chemically induced	Splice defect Last base of intron 5a G>A skipping exons 5a and 6	PD	Small eye Corneal adhesion Corneal and lens opacities	
PAX6^{ADD4802}/ PAX6^{+}	Chemically induced	Frameshift due to changed splicing intron 8 G>A	Loss of C-terminal, half of the HD	Small eye Corneal adhesion Corneal and lens opacities	
PAX6^{13214Neu}/ PAX6^{+}	Radiation induced	Missense c. 1099T>A p.(Phe272Ile)	HD	Abnormal cornea Fused cornea and lens Abnormal lens Cataract	Favor *et al*., 2008[51]

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Table 1: Contd...

Genotype	Generation of mutation	Mutation details	PAX6 domain	Predominant ocular phenotype	References
			Mouse		
PAX6$^{132\text{-}14Neu}$/ PAX6$^{132\text{-}14Neu}$				Abnormal cornea Abnormal lens Anterior chamber defect Coloboma Mic	
			Zebrafish		
PAX6b^{tq253a}/ PAX6b^{tq253a} (*sri*)	Chemically induced	Missense Exon 8 c. 770T>C p.(Leu244Pro)	HD	Abnormal cornea Abnormal lens Iris hypoplasia Retinal malformations Shallow anterior chamber Small eye	Heisenberg *et al*., 1996[52]
PAX6a^{ka709}/ PAX6a^{ka709}	CRISPR/Cas9	Small deletion Exon 8-12 3011 bp	HD and PSTD	Marginal anterior segment dysgenesis Small eye and lens	Takamiya *et al*., 2020[53]
PAX6a^{ka709}/ PAX6a^{ka709}; PAX6b^{tq253a}/ PAX6b^{tq253a}	Chemically induced and CRISPR/Cas9	*PAX6b*: Missense Exon 8 c.770T>C p.(Leu244Pro) *PAX6a*: Deletion Exon 8-12 3011 bp	HD and PSTD	Absent lens Absent anterior chamber Corneal endothelium malformation Small eye	
			hiPSCs		
UCLi013-A	Patient-derived skin fibroblasts	Missense Exon 7 c. 372C>A p.(Asn124Lys)	PD	Aniridia Cataracts Optic nerve Coloboma Nystagmus Mic	Harding *et al*., 2021[28]

CRISPR=clustered regularly interspaced short palindromic repeats, HD=homeodomain, hiPSCs=human-induced pluripotent stem cells, LR=linker region, MGI=Mouse Genomic Informatics, mic=microphthalmia, PD=paired domain, PSTD=proline-serine-threonine-rich domain Mouse genotype and phenotype have been taken from MGI database (http://www.informatics.jax.org/). Zebrafish data have been taken from The Zebrafish Information Network (ZFIN) (http://zfin.org/)

by ethyl nitrosourea (ENU).[41,42] Except for *Pax6^{4Neu}* and *Pax6^{7Neu}*, all of the discussed mice represent null mutants [Table 1]. More recently, Mohanna *et al*.[43] have tagged the mutant *Sey* allele with 3xFLAG, generating a model termed *Fey*. By doing this, it is possible to quantify Pax6 protein levels that are translated from the corrected mutant allele following gene editing.

Other commonly used mouse models for aniridia carry *Pax6* deletions. *Pax6$^{Sey\text{-}Dey}$*, *Pax6$^{Sey\text{-}H}$*, and *Pax6^{tm1Pgr}* (Pax6^{lacZ}) are examples of these models.[54,55] The latter two mutant strains contain large chromosomal deletions encompassing the whole *Pax6* gene and culminate in more severe aniridia phenotypes.[9,14] However, in *Pax6^{tm1Pgr}* mice, the entire sequence encoding the paired domain has been removed and replaced with a β-galactosidase gene followed by a neomycin cassette.[9,47] The aforementioned gene replacement reflects the endogenous *Pax6* expression and can be detected in live embryos. This model has recently been utilized by Voskresenskaya *et al*.[55] to compare the lens defects in aniridia, which revealed there may be differences in the *PAX6*/*Pax6*-controlled mechanisms between humans and mice. For example, they found that in humans, unlike mice, *PAX6* mutations do not delay lens placode development or alternatively, it recovers from the delay.

Contribution to our knowledge of aniridia

The *Sey* mouse has been crucial in understanding the pathophysiology of aniridia and testing potential therapeutics. Using mouse models, the dosage sensitivity of the *Pax6* gene has been demonstrated and consequently, therapies that target gene dosage of *Pax6* have been explored.[20] Nonsense suppression drugs have been shown to inhibit disease progression and rescue corneal, lens, and retinal malformations postnatally.[56,57] More recently, the inhibition of mitogen-activated protein kinase kinase (MEK) via small-molecule drugs in *Pax6$^{Sey\text{-}Neu/+}$* mice has illustrated upregulation of Pax6 and alleviation of Pax6 haploinsufficiency-related corneal phenotypes.[58] Identification of downstream *Pax6* targets in the developing iris and ciliary

body and of *Pax6*-dependent gene regulatory network in the lens are among the other discoveries made using mouse models.[59-61]

Limitations

Different mutant mice produce a spectrum of aniridia phenotypes; also, mice that carry genomic deletions could exhibit severe phenotypes that are not observed in patients, making them less appropriate for studying human aniridia.[9] Alternatively, mutations in the mouse could cause a less-severe phenotype than that seen in patients.[55] The small eye mouse, as indicated by the name, develops microphthalmia, a symptom that is not common in human aniridia patients, as one of its main phenotypes. In contrast, foveal hypoplasia, one of the main manifestations in humans, is absent in mice due to the lack of fovea in these animals.[56]

Zebrafish Models

Advantages of using zebrafish models

Zebrafish (*Danio rerio*) have become an increasingly popular organism to study vertebrate development and pathophysiology. Genomic sequencing revealed a significant genetic similarity between humans and zebrafish; 70% of human genes have a zebrafish ortholog and 84% of human disease-causing genes have a zebrafish counterpart.[62] Zebrafish are easy to breed through external fertilization with a large number of eggs (100–200) produced, making them cost-effective to maintain with a short regeneration time of 2–4 months.[63] The transparency of zebrafish embryos enables early visualization of organogenesis. Moreover, zebrafish eye development occurs rapidly with the retinal layers resembling adult-like pattern by 72 h post-fertilization (hpf).[34,63] At this timepoint, a highly organized heterotypical mosaic photoreceptor structure is formed in the retina, which is rich in cone photoreceptors and is remarkably similar to humans.[64] Retinal regeneration is possible in zebrafish, although this does not always occur in the presence of pathogenic variants, resulting in ocular maldevelopment or retinal degeneration.[64] The overall zebrafish corneal structure is evident as early as 5–7 days post-fertilization (dpf) and is similar to that of humans with corneal epithelium, stroma, and endothelium layers.[65]

Generation of aniridia models and phenotypes

About 350 million years ago, zebrafish underwent a whole-genome duplication; consequently, many genes, including *pax6*, have duplicates (*pax6a* and *pax6b*).[66] There has been some division of roles between the two duplicates, with varying ratios of gene expression in different organs and tissues, similar to PAX6 isoforms in humans. This neutral partitioning of role is termed "subfunctionalization."[66] Zebrafish can be easily genetically manipulated; in the 1990s, large-scale chemical mutagenesis using ENU was carried out in zebrafish, resulting in the generation of thousands of mutants bearing developmental defects. One of the identified mutants is *sunrise* (*sri*), a mutant that carries a leucine to proline missense mutation c. 770T > C, p.(Leu244Pro) in the *pax6b* homeodomain gene [Table 1].[52,66-68] The *sunrise* (*sri*) mutant was utilized to study the subfunctionalization of the *pax6* gene and the spatiotemporal manner in which each ortholog is expressed.[66] *Sunrise* is the most widely explored zebrafish model with a *pax6b* mutation and exhibits aniridia-like phenotypes, for example, abnormal lens and corneal structure, thick cornea, iris hypoplasia, retinal malformations, shallower anterior chamber, and a smaller eye.[65,66] The defects in the *sri* mutant are due to reduced DNA binding. Other than chemically induced mutations, *pax6b*-mutant fish have been produced via retroviral insertions [Table 1].[69] Several other mutants have been generated by either exposure to ENU or retroviral insertion; however, these have not been phenotypically characterized.

Limitations

Due to the genomic duplication and the consequent subfunctionalization of the *pax6* gene, the phenotypes are milder in mutant zebrafish than in aniridia patients. The homozygous *sunrise* fish have milder phenotypes and can grow into adulthood and breed, suggesting a compensation by the unaffected *pax6a* gene.[66] Fish that have been injected with both *pax6a* and *pax6b* morpholinos show more severe phenotypes, like microphthalmia and general developmental delay.[66]

Human Cellular Models

As discussed previously, substantial genetic and phenotypic distinctions exist between human and animal models; thereupon, patient-derived cells are important *in vitro* models to investigate human disease mechanisms and test potential therapeutics including drug screening.[70] The use of representative cellular models could reduce or even replace the number of animals used in biomedical preclinical experiments.[71]

Primary cells

Primary cells are generated by isolating the cell type of interest from the patient. As they are not modified, they are useful for studying signaling pathways, pathophysiology, drug efficacy, and toxicity.[34] If a gene of interest is expressed in primary cells, it has the machinery to support protein production, modifications, assembly, and transport, whereas genes that are overexpressed may not have this innate intracellular support. The disadvantage of using primary cells is their senescence as they have limited capacity of growing and dividing *in vitro*.[34]

Aniridia-Related Keratopathy (ARK) is one of the main manifestations of aniridia in patients and is mainly caused by limbal epithelial stem cell (LESC) deficiency.[72] Cultured LESCs previously extracted from aniridia patients showed a reduction in PAX6 protein.[73] This model was further used to identify morphological and molecular alterations, such as impaired migration and ability to differentiate into corneal epithelial cells, as well as novel PAX6 downstream targets, for instance, *ADH7*, *ALDH1A1*, and *SPINK7*.[73-76] MEK/extracellular signal-regulated kinases (ERK) signaling pathway repressor drugs, duloxetine and ritanserin, have been recently tested on mutant LESCs and have shown to rescue *PAX6* haploinsufficiency and restore *PAX6* production.[77,78]

Immortalized cell lines

Continuous (immortalized) cell lines have the indefinite ability to proliferate and generate an unlimited supply of cells. They are generally more robust, easier to maintain, and more cost-effective than primary cells. Immortalized cells are useful for the *in vitro* study of gene function and their pathological role. Theoretically, the function of continuous cell lines should closely resemble that of primary cells, but the genetic manipulation of the cell lines predisposes them to varied phenotype, function, and responsiveness. For example,

ARPE-19 cells lose their characteristic epithelial phenotype, the formation of tight junctions, after a few passages, consequently affecting the other roles of the cells, like fluid homeostasis, that were regulated through the epithelial characteristics.[79] There also exists the risk of further genotypic variation following prolonged passaging and contamination.[80] Roux *et al*.[72] have used clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 to introduce a *PAX6* heterozygous nonsense mutation within an immortalized line of LESCs to establish an aniridia LESC model and, through the use of soluble recombinant PAX6 protein, illustrated a successful rescue of the ARK phenotype by activating the endogenous *PAX6* gene.

Human-induced pluripotent stem cells

Human-induced pluripotent stem cells (hiPSCs) are generated through reprogramming of adult somatic cells and, like embryonic stem cells (ESCs), have the capacity of unlimited self-renewal and differentiation into all adult cell types.[81] hiPSCs circumvent the ethical concerns associated with ESCs, which is mainly the use of human embryos.[82] Patient-derived hiPSC lines are particularly valuable models to study congenital diseases because they carry the same disease-causing mutation (s) as the affected individuals, allowing for the *in vitro* analysis of molecular pathways involved in disease development. Also, by differentiating into cell types of interest, these become important tools for drug screening and personalized medicine.[28,81] However, they can be costly and difficult to culture with variable differentiation efficiencies.[83] Thus far, only a few hiPSC lines carrying *PAX6* mutations have been generated.[28,84] One of these lines has been derived by Zhang *et al*.[84] from a patient who suffered from optic nerve malformations, but not aniridia. Harding *et al*.[28] have most recently generated a heterozygous *PAX6* missense c. 372C>A, p.(Asn124Lys)-mutant hiPSC line from the dermal fibroblasts of a patient with aniridia, microphthalmia, cataracts, optic nerve coloboma, nystagmus, and type 2 diabetes. There are currently no publications available that explore hiPSC-derived aniridia lines; however, this strategy would provide a novel and valuable human model to study the disease.

3D cellular models (organoids)

Although 2D cellular models provide researchers with information about molecular pathophysiology and drug action, they may not replicate the intercellular signaling or the tissue complexity that exists *in vivo*.[34,85] Organoids are generated from hiPSCs or ESCs and closely resemble the temporal and spatial developmental stages of human organs *in vitro*, allowing to study human organogenesis in the early fetal stages.[85,86] Molecular characterization of events taking place in the optic vesicle or early retina is now possible using organoids, hence making it an increasingly popular model for the study of developmental eye diseases.[87-91]

Ocular organoid models are still incapable of fully mimicking the *in vivo* complexity of the human eye. Retinal organoids are prone to degeneration and loss of orientation as they develop. This drawback is often caused by nutritional deficits and inadequate passive diffusion in the inner layers. Retinal pigment epithelium (RPE) is an important ocular structure for the maintenance of the photoreceptors in the outer retina. The RPE in organoid cultures does not mature to a functional layer nor correctly localizes around the photoreceptors, making them susceptible to malfunction and degeneration.[92]

Another drawback of current retinal organoids is the lack of three distinctive nuclear layers, leading to disorganization of the rods and cones photoreceptors compared to the human retina *in vivo*.[93] More importantly, organoids lack vasculature and immune cells, both of which are important components of adult tissues. The variability of the differentiation process and low efficiency in organoid formation are other factors to be considered; different lines vary on differentiation effectiveness and their ability to assemble in 3D structures. To circumvent this issue, methodology improvements as well as isogenic controls are continuously being developed.[86,87]

The use of organoid models has not yet been explored for *PAX6*-related conditions. The application of retinal and, very recently, corneal organoid technology to aniridia research would be paramount to understand the mechanisms behind *PAX6* haploinsufficiency and its effects in the development and homeostasis of these tissues.[94,95]

Management of Aniridia and Potential Clinical Prospects

Management of aniridia is directed at the patient's symptoms and preserving vision. Regular eye examination and correction of refractive error to improve visual acuity are recommended. Patients with photosensitivity secondary to their iris hypoplasia should use tinted lenses or glasses.[1] For those with cataracts, surgery can lead to improvement in vision,[96] although in one study with a mean of 16.3 years of follow-up, no significant improvement in visual acuity was observed.[31] Serious complications like secondary glaucoma may arise after the surgery. Managing ARK can be achieved by topical preservative-free lubricants in mild cases to slow down the progress. Cultured LESCs have been successfully transplanted in more severe cases of ARK and a variety of culture techniques have since been developed to optimize the transplant.[97] The common transplantation procedure is through injection; however, tissue-engineered grafts for transplantation have been produced by combining the cells with a biomaterial, for example, amniotic membrane.[98] Glaucoma is also initially treated with topical antiglaucoma medications and requires surgery (cyclodiode laser, tube surgery, and trabeculectomy) in eyes that are unresponsive to medical treatment; however, surgical intervention for glaucoma was correlated with a worse visual acuity in the study carried out by Kit *et al*.[31]

It was previously discussed that topical application of nonsense suppression drugs was tested in mice postnatally, which showed the upregulation of *Pax6* and stable rescue of the disease phenotype.[56,57] However, the Phase 3 clinical trial using ataluren (NCT02647359) to treat aniridia through oral delivery failed to meet its endpoints. This may have been due to the adoption of incorrect endpoints and the mode of delivery, that is, a topical application with a focus on corneal parameters may have been more optimal. Mouse models, as well as primary cells, have demonstrated that inhibitors of the MEK/ERK signaling pathway, either directly or indirectly, show promising results with rescue of the aniridia phenotype. Therefore, these are other promising pharmacological agents that could potentially upregulate *PAX6* levels and prevent disease progression. The advantage of these drugs would

be their availability and not being selective on the type of mutation.[58,77,78] From a practical clinical perspective, family planning and preimplantation genetic diagnosis should be discussed with prospective parents.

Future Directions

Many aniridia mouse models have been identified and characterized that vary in phenotypic severity. Further work in other species, such as the zebrafish, could provide valuable insights due to their genetic homology to humans and their accessible and rapid eye development for the discovery of molecular pathways and testing potential pharmaceutical agents. Furthermore, generation of aniridia hiPSCs and 3D cellular models allows for more in-depth and specific investigation of the genetic causes and genotype–phenotype correlation, especially in fetal development. The patient-derived nature of these models also allows for drug screening and the generation of personalized medicine, thus providing effective treatment options for aniridia patients.

Conclusion

PAX6 is expressed in several tissues including the adult eye, brain, and pancreas. Therefore, the effect of haploinsufficiency extends beyond the ocular tissue, and aniridia is starting to be recognized as a systemic disease.[6] Animal models allow the exploration of systemic manifestations within the whole organism and have thus far been fundamental in our understanding of aniridia, the role of *PAX6*, and its regulatory mechanisms.[99]

Contributors

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Conflicts of interest

There are no conflicts of interest.

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