REVIEW

The Genetics of Childhood Disease and Development: A Series of Review Articles

This review is the ninth in this series. Dr. Hanson describes the remarkable studies that have determined the relationship between the PAX6 gene and eye development. These studies include observations of anomalies in humans and also experimental studies in animals to determine the mechanism of action of the gene. In children with aniridia, the relationship between this determinant of ocular development and the WT1 gene of Wilms Tumor is discussed. The PAX6 gene is a prime example of a specific gene responsible for normal human development.

Alvin Zipursky
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PAX6 and Congenital Eye Malformations

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ABSTRACT

The *PAX6* gene is a paradigm for our understanding of the molecular genetics of mammalian eye development. Twelve years after its identification it is one of the most intensively studied genes, both in terms of its diverse and complex functions during oculogenesis and its role in an ever-increasing variety of human congenital eye malformations. The *PAX6* field has benefited greatly from the continued input of clinicians, human geneticists and developmental biologists. This review summarizes the latest data on the *PAX6* mutation spectrum and recent

insights into *Pax6* function from the mouse. (*Pediatr Res* 54: 791–796, 2003)

Abbreviations

kb, kilobases
MRI, magnetic resonance imaging
PST, proline, serine, and threonine-rich domain
WAGR, Wilms tumor, aniridia, genitourinary malformations, and mental retardation

PAX6 was identified as a candidate aniridia gene during the search for the genes responsible for the WAGR syndrome (Wilms tumor, aniridia, genitourinary malformations, and mental retardation), which is caused by hemizygous deletions of 11p13 (1). Heterozygous intragenic mutations were subsequently described in many nonsyndromic aniridia cases, confirming *PAX6* as the aniridia gene (2–5). At the same time

Pax6 mutations were found in the classical mouse microphthalmia mutant *small eye* (6).

The *PAX6* protein is a transcriptional regulator with two highly conserved DNA binding domains, a paired domain and a homeodomain (1, 3, 7, 8; Fig. 1). The homeodomain is followed by a proline, serine, and threonine-rich trans-activation domain (9). The last 30 amino acids constitute a highly conserved C-terminal peptide that modulates DNA binding by the homeodomain (10).

THE SPECTRUM OF HUMAN PAX6 MUTATIONS

Molecular analyses of WAGR syndrome cases showed that aniridia can be caused by deletion of one copy of *PAX6* (1, 11).

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Figure 1. The *PAX6* cDNA and protein. The cDNA is represented as a horizontal bar (top). Vertical lines indicate exon boundaries (3). *PAX6* has 10 constitutive coding exons, 4–13, and one alternatively spliced exon, 5a. Inclusion of exon 5a results in the insertion of 14 amino acids into the paired domain. PB, paired box (red); LNK, linker region; HB, homeobox (yellow); PST, proline, serine, threonine-rich domain; CT, C-terminal region (green). A graphical representation of the *PAX6* protein (bottom) shows the different domains of the translated protein. The paired domain (red) and the homeodomain (yellow) are shown in contact with DNA. The homeodomain consists of 3 α -helices (parallellograms linked by ribbons) while the paired domain consists of 6 α -helices. The structures of the PST domain and the C-terminal peptide are unknown. N and C indicate the N- and C-termini of the protein, respectively.

This led to the proposal that aniridia results from *PAX6* haploinsufficiency and is caused by loss-of-function of one copy of the gene (1). Once a significant number of intragenic mutations had been reported (2–5) it was clear that the classical aniridia phenotype could be caused by mutations throughout the *PAX6* coding region. The simplest explanation for the observation that deletions and intragenic mutations cause the same phenotype is that both types of mutation are functionally null (4, 5, 12).

Human *PAX6* mutations are archived in the *PAX6* Allelic Variant Database, <http://pax6.hgu.mrc.ac.uk/> (13). The database contains 292 records of which 275 refer to pathologic mutations in the coding region of the gene. Fig. 2 shows a breakdown of these 275 mutations by class.

The most common *PAX6* mutations are the nonsense mutations R240X (21 reports), R317X (15 reports), and R203X (15 reports). These mutations, which involve CpG dinucleotides, account for 50% of all nonsense mutations and 19% of all *PAX6* mutations.

Frameshift, nonsense, and splice mutations, which together make up 71% of *PAX6* mutations, would all be predicted to introduce a premature termination codon into the *PAX6* reading frame. The resulting mutant mRNAs are likely to be detected by RNA surveillance and degraded by nonsense-mediated decay (14–16). RNA surveillance is a powerful mechanism to prevent the accumulation of truncated peptides that could interfere with the function of the normal protein or have toxic effects in the cell.

“Anti-termination” mutations – where the stop codon is changed to a coding codon – have recently emerged as a significant new mutation class (10, 17–20). The consequence

Figure 2. The *PAX6* mutation spectrum. Pie-chart showing the different mutation types among 275 pathologic mutations in the *PAX6* coding region. N, nonsense mutation; FS, frame-shifting insertion or deletion; S, splice mutation; M, missense mutation; AT, anti-termination mutation; IF, in-frame insertion or deletion.

of such a mutation is predicted to be continuation of translation into the 3' untranslated region of the *PAX6* gene. The C-terminus of the *PAX6* protein, including the position of the stop codon, is highly conserved between species and functional studies show that the C-terminal region is important for DNA binding by the homeodomain (10). Addition of a peptide encoded by the 3' untranslated region would be expected to disrupt the function of the C-terminus. Of 11 anti-termination mutations in the database, 8 are associated with aniridia and 3 are associated with milder iris defects (10, 19, 20). From these phenotypes it seems likely that anti-termination mutations generate alleles with partial or complete loss of function.

Missense mutations account for 18% of *PAX6* mutations (Fig. 2). Missense mutations would not be detected by RNA surveillance and are predicted to result in a full-length *PAX6* protein with a single amino acid substitution. Half of all missense mutations are associated with the aniridia phenotype and are therefore likely to have significant loss-of-function (20–23; Fig. 3). However missense mutations can potentially cause partial loss-of-function or gain-of-function. This may explain why the remaining 50% of missense mutations are associated with congenital eye malformations that are distinct from classical aniridia. These include isolated foveal hypoplasia, ectopia pupillae, and Peters' anomaly, a rare form of anterior segment dysgenesis characterized by central corneal opacification (23–28; Fig. 3). Two-thirds of missense mutations are in the paired domain and may affect the ability of the resultant protein to bind some, but not other, *PAX6* targets. Indeed, differences in DNA binding activity and trans-activation activity have been reported for a number of paired domain missense mutant proteins (20, 21, 29).

The spectrum of phenotypes associated with *PAX6* missense mutations was extended recently with the report of missense mutations, including 5 in the PST domain, in a cohort of

Figure 3. Distinct ocular phenotypes associated with *PAX6* missense mutations. (a) Aniridia with iris remnants (arrowheads) and congenital cataract (arrow) caused by the missense mutation A33P (23). (b) Ectopia pupillae caused by the missense mutation V126D (23). (c) Gonioscopic view of Peters anomaly, showing corneal opacification, caused by the missense mutation R26G (24). Panels (a) and (b) reproduced from Hanson *et al.*; 1999 Human Molecular Genetics vol 8 pp162–175, by permission of Oxford University Press. Panel (c) reproduced from Holmstrom *et al.* (1991) British Journal of Ophthalmology vol 75 pp591–597, by permission of the BMJ Publishing Group.

patients with a variety of optic nerve malformations including morning glory disc anomaly and optic nerve hypoplasia (29). The mutant proteins are impaired in their ability to auto-activate *PAX6* and repress *PAX2*, which may affect the formation of the boundary between the optic cup and the optic stalk (see below).

Surprisingly the most highly conserved region of the *PAX6* protein, the homeodomain, has just two reported missense mutations, one in an individual with a phenotype of very mild partial aniridia (30) and one in a patient with iris defects, coloboma of the optic nerve, retina and choroid, and persistent hyperplastic primary vitreous (29). The fact that the *PAX6* homeodomain is very highly conserved throughout evolution argues that most changes in the amino acid sequence are selected against, presumably because they impair the function of the protein and cause a deleterious phenotype. Perhaps homeodomain missense mutations predominantly cause non-ocular phenotypes, thus explaining the low frequency of reported mutations to date.

The *PAX6* gene has one alternatively spliced exon, 5a (Fig. 1). Inclusion of exon 5a results in the insertion of 14 amino

acids into the paired domain and changes the DNA binding specificity of the protein (31). Mutations that affect exon 5a cause congenital eye malformations in humans and mice (27, 31, 32).

ANIRIDIA AS PART OF THE WAGR SYNDROME

It is well known that congenital sporadic aniridia is associated with an elevated risk of later developing Wilms tumor (33). The molecular basis of this association is hemizygous deletions that remove one copy of *PAX6* – thus causing aniridia – and one copy of *WT1* – thus making it much more likely that a “second hit” in the remaining functional copy of *WT1* will cause tumorigenesis (1, 11, 33). The *PAX6* and *WT1* genes lie 650 kb apart in 11p13 (<http://www.ensembl.org/>).

The proportion of sporadic aniridia cases with a deletion of both *PAX6* and *WT1* has been determined in a number of studies (18, 34, 35). Of 51 sporadic aniridia cases, 19 (37%) had a deletion of both genes. Of these 19 patients, 9 developed Wilms tumor. Thus the proportion of all sporadic cases developing Wilms tumor was 9/51 or 18%, while the proportion of deletion cases developing Wilms tumor was 9/19 = 47%. Wilms tumor was not observed in any nondeletion patients.

Familial aniridia patients may also carry deletions of the *PAX6* gene (11) although the frequency is lower than in sporadic patients. The deletions are usually small and tend not to encompass the *WT1* gene; however in one rare case a deletion of *PAX6* and *WT1* was passed from mother to son (36).

PAX6 deletions have been described in association with other eye anomalies. A child with bilateral Peters’ anomaly had a deletion of *WT1* and *PAX6* (24) and a child with congenital bilateral microphthalmia and severe anterior segment dysgenesis who later developed Wilms tumor had a deletion of 11p13–15.1 (37). A child with sporadic Rieger syndrome, including typical dental and maxillary anomalies, had a *PAX6* deletion (38). Since *PAX6* is not expressed in the developing teeth or maxilla, this individual may also have a mutation of *PITX2*, the Rieger syndrome gene, at 14q25 (39).

GENETIC TESTING AND COUNSELING

Most familial aniridia patients have mutations within the *PAX6* gene, of the kind shown in Fig. 2. Aniridia is dominantly inherited with high penetrance. Affected individuals have a 50% chance of passing the mutant allele, and therefore the disease, to each offspring.

As mentioned above, about one-third of sporadic aniridia cases have a deletion of the *WT1* and *PAX6* genes and half of these will develop Wilms tumor (18, 34, 35). This emphasizes the importance of performing chromosomal deletion analysis in a newborn with sporadic aniridia. If *WT1* is deleted, there is a significant risk of Wilms tumor and monitoring should be performed; however if no deletion is found the risk of Wilms tumor is reduced to that of the general population (33).

The remaining two-third of sporadic cases are most likely to have *de novo* mutations within the *PAX6* gene, of the sort shown in Fig. 2. These mutations would be expected to be

transmitted to the next generation in an autosomal dominant fashion.

EXTRA-OCULAR PHENOTYPES

Extra-ocular sites of *PAX6* expression include the developing olfactory system, brain, neural tube, and endocrine pancreas (28, 40). A number of recent studies have highlighted nonocular phenotypes in aniridia patients.

Impaired olfaction is a form of sensory deprivation that is relatively overlooked in humans, but a recent study found that of 14 aniridia patients, all but one had impaired sense of smell, ranging from mild hyposmia to anosmia (19).

MRI scans of the brains of aniridia patients have revealed a range of anomalies including polymicrogyria, absent or hypoplastic anterior commissure and absent or hypoplastic pineal gland (19, 41). The functional consequence of these changes is not yet known. The phenotypes were highly variable even in family members with the same mutation.

Three reports have documented behavioral anomalies in aniridia patients, including cognitive dysfunction (42), aggressive behavior (42, 43), autism (20), and mild mental retardation (43). Two of these cases were familial, with the behavioral phenotype segregating with aniridia (42, 43); however neither pedigree was large enough to prove linkage between the behavioral phenotype and the *PAX6* mutation.

Four aniridia patients with *PAX6* mutations all had glucose intolerance related to impaired insulin secretion (44). This is the first report of a possible link between *PAX6* heterozygosity and impairment of pancreatic function.

LESSONS FROM THE MOUSE

Important insights into *Pax6* function have come from the naturally occurring mouse *Pax6* mutant *small eye* and from experimentally engineered animals. Many recent studies have highlighted the role of *Pax6* in forebrain development including regionalization, cell migration, and axon guidance (40). The role of *Pax6* in eye development has been covered in recent reviews (45, 46). Here I discuss the eye phenotype in heterozygous *small eye* mice and advances in our understanding of the regulation of *Pax6* transcription.

Some *Pax6* heterozygous mice have aniridia-like iris anomalies (2) but others have corneal opacities and lens-corneal adhesions that resemble Peters' anomaly (24, 46, 47; Fig. 3C). Detailed histologic studies of neonatal mice with Peters-like defects revealed that the lens frequently fails to separate from the cornea (48). Trabecular meshwork development was also abnormal in heterozygotes.

CIS-ACTING DNA ELEMENTS

The sequences responsible for regulating the spatially and temporally complex pattern of *Pax6* transcription have come under intense scrutiny. Many of the key regulatory regions were initially identified in quail (49, 50) but have now been more rigorously analyzed in mice. The availability of genome sequence from different organisms has greatly facilitated the identification of potential regulatory elements, since regulatory

sequences often show up as blocks of high nucleotide sequence conservation (50–56). The pattern of expression directed by each putative control element can be determined by reporter gene expression analysis in transgenic mice.

In mice, *Pax6* expression can be initiated at two 5' promoters, P0 and P1, both of which are associated with distinct regulatory elements (52, 53). P0 and P1 transcripts are both abundant in the lens placode but P0 transcripts predominate in the corneal and conjunctival epithelia while P1 transcripts predominate in the optic vesicle and CNS (52). Intron 4 contains a third potential promoter, P α , and regulatory sequences that direct expression to the retina, ciliary body, and iris (52, 53, 57; Fig. 4).

A number of *cis*-acting regulatory elements lie far downstream of the 3' end of the *Pax6* gene. These were identified by studying chromosomal rearrangements in a small number of aniridia patients with breakpoints up to 130kb distal to the *PAX6* coding region (58, 59). The downstream region was shown to be essential for normal eye development in transgenic mice (54, 60). Long-range sequence comparisons revealed several highly conserved noncoding elements in the downstream region that could direct expression to specific regions of the eye, brain, and olfactory system (54, 55; Fig. 4). When the mutant and normal chromosomes were separated in somatic cell hybrids, no *Pax6* transcription could be detected from the allele on the rearranged chromosome, indicating that these downstream elements must be present in *cis* for normal *Pax6*

Figure 4. The *PAX6* locus, showing regulatory elements of the *PAX6* gene and target genes of the *PAX6* protein. (a) The *PAX6* genomic region. The *PAX6* gene is shown as a green rectangle. The last 3 exons of the neighboring *ELP4* gene are shown as red rectangles (54, 61). The *PAX6* protein is represented as a green oval. Examples of genes that are activated (+) or repressed (-) by the *PAX6* protein are shown (63, 66, 68, 69, 72–78). Single arrows represent direct control; double arrows show that direct control has not yet been proven. (b) *PAX6* regulatory elements. Regions of the *PAX6* locus that contain control elements are shown in expanded form. Numbered green rectangles represent individual exons of the *PAX6* gene. Lettered rectangles represent control elements that have been analyzed by reporter studies in transgenic mice. The expression patterns are as follows: A, endocrine pancreas (52, 53); B (the ectodermal element), surface ectoderm, lens, cornea (51, 53); C, telencephalon, hindbrain, spinal cord, photoreceptors (52, 53); D (the α -element), retina, iris, ciliary body, spinal cord (52, 53, 57); E, pretectum, neural retina, olfactory structures (55); F, lens, diencephalon, hindbrain (54); G, neural folds, optic primordia, optic vesicle, retina (54). Hypersensitive sites (HSS) that have been identified within the downstream regulatory region are indicated by arrows (54). Colored ovals represent proteins that directly bind and activate (SIX3, *PAX6*, SOX2, MEIS) or repress (*PAX2*) the regulatory elements (56, 66, 68, 69). Brackets indicate that *PAX6* and SOX2 physically interact (69, 70).

expression (59). Remarkably, the downstream elements are located in an intron of the neighboring *ELP4* gene (61; Fig. 4). *ELP4* encodes a subunit of an RNA polymerase-associated histone acetylase (62). Although the patients with chromosomal rearrangements necessarily lose one functional copy of *ELP4*, this appears to have no phenotypic consequence.

Physically distant elements may combine to bring about the correct expression pattern in any particular structure; lens expression is directed by two elements, one upstream of P0 (51, 53) and another over 150 kb away (54). Deletion of the upstream element reduces but does not abolish *Pax6* expression in the lens placode suggesting that different elements may act in a combinatorial fashion to determine the correct level of transcription (63).

The well-studied β -globin gene cluster has given insights into the mechanism of long-range control of gene expression (64, 65). Recent evidence supports a model in which physically distant sites (such as a long-range enhancer and a promoter) interact directly, with the intervening chromatin looping out. This may create a chromatin domain that is permissive for transcription (64, 65).

Trans-ACTING FACTORS

Progress has also been made in identifying the proteins that bind to these regulatory elements. The *Pax6* α -element (box D, Fig. 4) contains two sites that bind both Pax2 and Pax6 (66). Pax2 represses, while Pax6 activates, reporter expression from this element. Competition between Pax6 and Pax2 binding may help to define the sharp boundary of *Pax6* and *Pax2* expression between the optic cup and the optic stalk (66).

The ectodermal element (box B, Fig. 4) binds the homeoproteins Six3, Meis1, and Meis2, which are expressed in the lens placode (56, 67, 68). Pax6 expression is up regulated in the lenses of transgenic mice that over-express Meis2 (56).

The ectodermal element also binds the $-5a$ isoform of the Pax6 protein (which lacks the additional 14 amino acids in the paired domain), and the Sox2 and Sox3 proteins (69). Both Sox2 and Sox3 can synergistically activate the enhancer when co-expressed with Pax6, and Sox2 and Pax6 physically interact as judged by a co-immunoprecipitation assay with tagged proteins (69). Pax6 and Sox2 also form a co-DNA binding complex on the chick δ -crystallin enhancer (70). *SOX2* gene mutations were recently described in anophthalmia patients (71). Sox2 may be an important co-factor of Pax6 during many key stages of eye development.

The Pax6, Six3, Meis, and Sox binding sites lie within 60 bp of each other in the core of the ectodermal enhancer (53, 56, 68, 69). This high density of sites hints at how *Pax6* transcription can be fine-tuned by specific combinations of *trans*-acting factors in different cell types.

DOWNSTREAM TARGETS OF THE PAX6 PROTEIN

Genes that are directly activated by the Pax6 protein include *Pax6* itself (66, 69), bHLH genes such as *Ngn2* (72) and *Maf* (73), homeobox genes such as *Six3* (68), *crystallin* genes (74, 75) and pancreatic hormone genes such as *glucagon* (76; Fig. 4). Pax6 is a repressor of *Pax2* and β -crystallin (66, 77).

Expression of the calcium-binding protein gene *Necab* and the forkhead gene *Foxe3* are both absent in mutant mice, suggesting that they are downstream of Pax6, but a direct interaction has not yet been demonstrated (63, 78; Fig. 4).

For most developmental genes associated with human disease, a handful of mutations are described before attention turns to functional studies in animal models. In the case of *PAX6* however, large numbers of mutations are still being reported, thus providing a rich molecular pathology backed up by extensive functional work. The challenge ahead is to better understand the phenotypes associated with different mutations in terms of functional information gained from model systems.

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