PAX6 in sensory development

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PAX6 function was first identified through aniridia-associated null mutations. Since then, this transcription factor, with a paired domain and a homeodomain, has become a paradigm, illustrating functional conservation in developmental pathways. The Small eye mouse and Drosophila eyeless have served as major model systems in defining the multistage roles for Pax6 in eye and olfactory system development throughout evolution. The overt phenotypic consequences of heterozygous human and mouse Pax6 mutations were initially confined to the eye, with some interesting genotype–phenotype correlations being noted. Recently, structural and functional abnormalities in the olfactory system have been identified. Alterations in brain structure have also been documented, in line with the wider forebrain and cerebellar expression of Pax6. The broad PAX6 expression pattern is controlled by a number of long-range control elements, and is reflected in the severe homozygote phenotype. Upstream regulators and a multitude of downstream targets of PAX6 have been identified, and its varied tissue-specific functions are emerging.

The PAX6 gene was originally defined by homology to Drosophila paired as a member of the vertebrate PAX family (1) (reviewed in 2) and immediately suggested by positional cloning as a candidate gene for the developmental eye anomaly aniridia (3). The role of PAX6 in human eye disease has been confirmed through the identification of a large number of independent mutations (4). Considerable insight into PAX6 function has been gained from detailed genotype-phenotype comparison. PAX6 orthologues, highly conserved in terms of both sequence and function, have been isolated from a wide spectrum of invertebrates and lower chordates as well as other vertebrate classes (5,6). Pax6 mutations, associated with eye phenotypes, have been identified in mouse (7–9), rat (10) and Drosophila (11–13) and as the cause of sensory abnormalities in Caenorhabditis elegans (14,15). These impressive findings highlight the appropriateness of using a wide spectrum of organisms as models for the dissection of Pax6 function, providing experimental systems not feasible in humans. The mouse, with several independent Pax6 Small eye mutations (7–9), conditional knockouts (16,17) and transgenic studies (18–23), has proved to be a particularly useful model for developmental analysis. Strong contributions to our understanding of gene function and interactions have also come from studies of the Drosophila Pax6 homologue eyeless (ey) (11–13,24–27). Here we attempt to summarize and integrate the findings from human disease analysis and the model systems, with particular emphasis on recent data in both areas.

The PAX6/Pax6 gene: structure and evolution

PAX6, on chromosome 11p13, encodes a 422-amino-acid transcriptional regulator, occupying 14 exons in a 22 kb genomic region, with two DNA-binding domains: a bipartite paired domain (PRD) and a paired-type homeodomain (HD) (28). The N-terminal PRD, including an inframe 14-amino-acid alternatively spliced exon (5a), is separated from the HD by a linker region. There is a C-terminal proline, serine and threonine-rich trans-regulatory domain. Throughout the whole coding region, human and rodent proteins are 100% identical, with chick (96%) and zebrafish (93%) showing strong similarity; PRD and HD maintain 80–90% identity from mammals to Drosophila and C. elegans (6), suggesting that the DNA-binding targets may also be highly conserved across this evolutionary timespan.

Duplication in an ancestral insect lineage led to Drosophila having a second Pax6-related gene, twin of eyeless (toy), with close similarity to ey and also implicated in eye development, acting upstream of ey (25). Another gene with an eyeless mutant phenotype and some similarity to ey is eyeone (eyg), with a Pax6-like HD, and a PRD showing little sequence similarity, although some structural homology to the 5a form of the vertebrate Pax6 protein (29). Partial duplication in a branch of the vertebrate lineage has led to two somewhat diverged and differentially regulated Pax6 genes in zebrafish (30).

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LESSONS FROM THE STUDY OF ANIRIDIA; COMPARISONS WITH THE Small eye MOUSE MODEL

The panocular features of aniridia reflect the wide expression of PAX6 in the developing eye, in both the neur ectoderm and the surface ectoderm, and in their derivatives (reviewed in 31). Expression continues in adult retina, lens and cornea (32,33). The fact that aniridia is a progressive condition may reflect the maintenance functions of PAX6 in the adult eye. The primary defects in aniridia include iris and foveal hypoplasia, often accompanied by nystagmus (rapid uncontrolled eye movements), cataracts, corneal abnormalities and glaucoma. Small eye mice heterozygous for Pax6-truncating mutations provide a good model for aniridia, including its variability, which encompasses multiple phenotypes including the anterior segment disease Peters anomaly, with central corneal opacification and adhesions between the cornea and the lens or iris. Sensory tissue expression extends to the olfactory system, from the earliest nasal placode to the mature olfactory bulb and the olfactory epithelium. Pax6 is further expressed in the developing telencephalon, thalamus, pituitary, pineal, cerebellum, spinal cord and pancreas.

Heterozygous intragenic mutations in PAX6 have been identified in the majority (80–90% - our unpublished data) of classical aniridia patients tested (4), suggesting that PAX6 is the sole gene implicated in this autosomal dominant phenotype. The majority of classical aniridia cases are due to PAX6 haploinsufficiency, resulting from heterozygous null mutations. The homozygous null phenotype in mice (7,34) and a very rare human case (35) reflect more closely the broad Pax6 expression pattern, revealing perinatal lethality associated with absence of eyes, nasal structures and pancreas, and with severe brain defects. Overexpression of Pax6 is also deleterious, leading to reduced eye size in mice transgenic for five copies of a human PAX6-bearing YAC (36), suggesting that correct Pax6 dosage is critical for eye development.

GENOTYPE–PHENOTYPE CORRELATIONS

While most classical aniridia cases are associated with deletions or premature protein truncations, a high proportion of ‘variant’ aniridia cases carry missense mutations clustering in the highly conserved residues of the PRD (Fig. 1) (37). Recently, as more independent missense changes are identified, there is a suggestion that some may be recurrently associated with specific sub-phenotypes. Thus an R128C mutation, first reported from Japan (38) in a case with isolated foveal hypoplasia, has just turned up again in an independent European case with the same phenotype (our unpublished data). The same amino acid change in the mouse is associated with the regular Small eye phenotype (9), but there is no foveal region in the mouse retina. G18W was first reported in a family with Peters anomaly (39), while recently we identified a G18R change in another family with bilateral Peters anomaly in an affected member. Although Peters anomaly is genetically heterogeneous, the previously reported PAX6-associated case also carried a mutation in the N-terminal region of the PRD (R26G) (40).

Figure 1. Domain structure of PAX6 with the paired domain structure shown in detail to illustrate the paired domain missense mutations identified in human and mouse. The protein consists of a paired domain (PRD) including the checkered alternatively spliced exon 5a; the homeodomain (HD), separated from the PRD by a linker region; and the proline, serine and threonine-rich transactivation domain (PST). The first three exons and most of the fourth exon constitute the 5’UTR; the 3’UTR is about 1 kb. The expanded paired domain includes α1–α3, denoting the N-terminal subdomain alpha helices, and α4–α6, C-terminal subdomain alpha helices. The amino acid sequence is shown, with residues in red contacting the DNA backbone and those in blue contacting the minor groove or the major groove (underlined). Human missense mutations are highlighted in pink, mouse mutations in green. Numbering for the alternatively spliced exon (checkered box) is from the start of that segment only. Mutations are referenced in the text, at www.hgu.mrc.ac.uk/Softdata/PAX6/ and in (9); the two mutations in bold are our unpublished data.
Four, apparently independent, identical mutations have been reported (41) in the seventh codon (V7D) of the 14-amino-acid alternatively spliced exon (4). The phenotypes included Peters anomaly and microphthalmia. In vitro analysis of the PRD + 5a protein domain revealed a reduced DNA target-binding capacity, however, it is not clear whether in vivo these mutations may lead to aberrant splicing control and thus to altered + 5a/− 5a ratios (42). The role of PAX6 alternatively spliced forms is not yet fully explored. Familial cataract has been associated with one reported mutation affecting alternative splicing (43).

Very few mutations have been found in the HD. R208W, in the linker region just preceding the HD, was first seen in an aniridia family, now lost to follow-up (44). R208Q was reported by Gronskov et al. (45) in a compound heterozygote with mild aniridia, with two inherited PAX6 mutations, where the unaffected parent carried the same mutation. More recently, we described a child with unilateral iris coloboma and normal visual acuity and with an unaffected mother carrying the same R242T change (46). It is surprising to find such mild, incompletely penetrant, phenotypic changes in two well-documented cases with HD mutations, particularly when the altered amino acids are highly conserved across phyla, and the HD in its entirety is the most highly conserved region of the PAX6 gene (6). Further analysis of this phenomenon may be possible with the recent identification of two HD missense mutations in mice: V270E (9) and S273P (8) (human equivalent residues V256 and S259). V270E is indistinguishable from loss-of-function Small eye mutants, while S273P is clearly hypomorphic with a milder phenotype. It is interesting to note that in Drosophila, the HD was recently shown to be redundant for correction of the eyeless phenotype or for eliciting the development of ectopic eyes (47). In C. elegans, the alternative Pax6 isoform mab18 with no PRD but an intact HD, is required for peripheral nervous system development (15). There is a surprising dearth of information on the separate and combined roles of the PRD and HD. Most in vitro binding studies use each domain separately.

Other than premature truncations, few mutations have been identified in the trans-regulatory domain among the eye phenotype cohort. A missense mutation, Q422R, in the last coding exon has been reported to be associated with uveal ectropion (48) and also with classical aniridia (49). In vitro binding studies show reduced HD binding in the presence of the altered C-terminal glutamine residue (49). An interesting subset of cases has emerged with predicted C-terminal protein extensions, associated, perhaps, with a distinct, more progressive retinal phenotype (A.T. Moore, personal communication). Several carry a heterozygous X423L (49–51) or X423Y (52) mutation, abolishing the stop codon and leading to predicted elongation of the PAX6 protein. Others are associated with late frameshift mutations in the trans-regulatory domain (53 and our unpublished data). In addition to the eye phenotype, behavioural abnormalities are described in the family reported by Heyman et al. (53). In two families with X423L mutations (51 and unpublished), three affected individuals have epilepsy. Two further epilepsy cases are associated with different PAX6 mutations (51).

CONTROL OF PAX6 EXPRESSION BY cis REGULATION

A wide array of upstream enhancers, promoters and intronic regulatory elements have been defined for PAX6 over the years (19,54–56). These have been judiciously used to dissect PAX6 function (see below). A whole series of additional downstream

Figure 2. The genomic organization of the PAX6 gene with its long-range control elements. The 14 exons of the PAX6 gene are shown in red, with the alternatively spliced exon 5a in yellow. The neighbour gene, originally named PAXNEB, is now known to be the ELP4 subunit of a transcriptional elongation factor complex (83). Only the last three exons of ELP4 are shown (in blue). Direction of transcription is indicated by arrows. The table below the diagram details the regulatory elements shown in the diagram and discussed in the text.
regulators of PAX6 are now being assessed functionally (21,23). Their existence was deduced originally from aniridia-associated chromosomal rearrangements well outside the PAX6 transcription unit. Initial analysis was by DNaseI hypersensitivity and transgenic analysis, but with the growing availability of multiple genomic sequences, sequence conservation has revealed all the previously assessed sites and identified additional ones, which have subsequently been confirmed functionally. A map of some functionally tested PAX6 regulatory elements is shown in Figure 2. Many human disease-associated genes, particularly genes implicated in complex developmental regulation, have been shown to be under long-range control (57). In most cases, distant chromosomal breaks elicit loss-of-function phenotypes, as demonstrated for PAX6 (58), but we need to be aware that positive control changes, leading to aberrant gene expression, may also be caused by translocations, insertions, deletions and point mutations in distant control elements. In such cases, very specific phenotypes, distinct from the null phenotype, may be observed. (59,60).

NEW PHENOTYPES SUGGESTED BY EXPRESSION PATTERN

In view of the broad expression pattern of Pax6, it has always been surprising that the haploinsufficient phenotypic effects are confined to the eye. Sisodiya et al. (51) carried out structural MRI and olfactory function tests in 14 adult aniridia subjects (including familial and sporadic cases), each carrying one of nine independently arising, identified PAX6 mutations. Of the 14 cases, 12 were found to have absent or hypoplastic anterior commissures, without obvious functional consequences. Similarly, 12 of the 14 cases were revealed to have functional hyposmia or anosmia, in keeping with one previous report of aniridia-associated anosmia (61), and reduced olfactory bulb size described in heterozygous Small eye mice (62). Several less consistent additional structural brain changes were noted in these aniridia cases, but none of these alterations were seen in 150 controls. Intriguingly, development and function of the Drosophila olfactory associative learning centres, the mushroom bodies, is disrupted in flies with new null mutants of ey (12,13).

Recent assessment of subjects with aniridia for pancreatic function has suggested that glucose intolerance and diabetes may be associated with the eye disorder and PAX6 mutations (63).

‘Missing’ PAX6 mutations – for example other HD missense changes – may be associated with neurological or cognitive deficits. Tissue maintenance in adult cells that continue to express PAX6 may be another unexplored role for this gene, for example in age-related macular degeneration. The advent of faster, cheaper sequence analysis techniques may be the required trigger for identifying these.

PAX6 INTERACTIONS AND FUNCTIONAL MECHANISMS

A great deal of work in this area is beginning to yield results now, and we are in a position to draw up fairly reliable lists of interacting genes. We have summarized in Figure 3 some of the upstream controlling loci and downstream targets – direct and indirect. Only a few recent examples of these analyses are mentioned here specifically.

The interactive roles of hedgehog (hh) and decapentaplegic (dpp) signalling in Drosophila eye development is well established (64), but evidence for a comparable reiterative morphogenetic wave function for vertebrate sonic hedgehog (Shh) has only recently emerged (65). The role of vertebrate homologues of dpp, the TGFB family members Bmp4 and Bmp7, in lens induction, has been unfolding (66,67), and recently interaction with FGF signalling in regulation of Pax6 (as well as Sox2 and Foxe3) expression has been reported (68).

Complex developmental regulators such as Pax6 often play distinct, but overlapping and recurrent, roles in spatiotemporally different sites. In the eye, this has been elegantly
demonstrated by the Gruss lab, using a Pax6 conditional knockout carefully engineered to turn on the Lox-rearranging enzyme Cre only after earlier critical stages of Pax6 function had been accomplished. Loss of Pax6 function in lens development (with Cre driven by the ectodermal enhancer EE; Fig. 2), after the early role in placode formation had been completed, led to disruption not only of lens but also retinal development (16), emphasising the developmental interdependence of these two structures in the vertebrate eye. These experiments also identify several downstream targets of Pax6: Sox2, Six3 and Prox1. Ablation of Pax6 in the retina, under the control of the NRE element (Fig. 2), following completion of early precursor cell fate determination, abrogated the differentiation of all but amacrine cells, through failure, in the absence of Pax6, to turn on the different bHLH genes (Atoh7/Math5, Atoh4/Ngn2 and Hes1; Fig. 3) that control retinal cell fates (17).

Targeted deletion of the EE element alone led to relatively mild developmental anomalies of the lens (22), perhaps because EE is not the only element controlling Pax6 lens expression (21,69). The deletion of EE leads to reduced Pax6 and loss of Foxe3 expression, as well as delayed lens fibre differentiation.

Crystallins were the first clear-cut targets of Pax6 regulation. The regulation of several members of this family has been described, and yet another new crystallin gene target has very recently been identified through microarray analysis comparing gene expression in heterozygous Pax6-mutant mice with wild type (70). This approach also uncovered a number of new targets such as Pitx3 and paralemmin.

Expression screening by high-throughput in situ hybridization in mutant and wild-type mice revealed the calcium-binding protein Necab (Necab2) as a downstream target of Pax6 (71). Interestingly, ectopic expression of Necab leads to Chx10 expression, confirming it as at least an indirectly regulated target of Pax6.

An additional complexity of many of the gene interactions described reveals both autoregulation, for example by Pax6, and mutual regulation of two or more genes. The latter is illustrated by the Pax6/Six3 interactions (16), and also by the modulation of Pax6 and Pax2 (20): expression is refined from largely overlapping in early neurectoderm and retina, to mutually exclusive functions in later retinal differentiation (Pax6) and in the optic stalk (Pax2).

Some genes interact both at the DNA and at the protein level. Sox2 and Pax6 proteins form a co-DNA-binding complex with a key role in the initiation of lens development (72), as well as acting to control Sox2 expression through binding to its promoter. Similarly, Pax6 and Maf proteins interact in regulating glucagon expression in the pancreas (73) and perhaps at other targets too (74). DNA-level regulation of Maf by Pax6 has also been demonstrated (75).

ROLE OF Pax6 IN DEVELOPMENTAL INDUCTION, PROLIFERATION AND DIFFERENTIATION

Pax6 plays multiple complex parts in all expressing tissues. Its pivotal role in eye induction was highlighted by the original report of ectopic Pax6 expression triggering eye formation at ectopic sites in Drosophila (76). Subsequently, a more restricted inductive capacity was also demonstrated in vertebrates (77). However, several members of the complex interwoven pathways regulating eye development, the ‘eye specification genes’ (78), can fulfill a similar role, alone or in collaboration (6).

Detailed examination of Pax6 cellular functions in the eye suggests involvement in cell proliferation, differentiation and migration/adhesion. Pax6-null and heterozygous cells are excluded in chimeric mice from contributing to the lens (79,80), and mutant cells are sequestered together in the retina (79). Expression of Pax6 in the optic vesicle is required for the persistent surface ectoderm contact necessary for lens placode induction. Reduced proliferative capacity in the developing lens has been demonstrated for heterozygous mutant cells (81). A role in cell proliferation is also suggested by long-term Pax6 expression in independently derived virally transformed adult lens cells (21 and our unpublished data) that nevertheless fail to express the protein markers of mature lens cells. Limbal stem cell deficiency has been documented in aniridia (82), and Pax6 expression has been observed when wounded cornea regenerates (33).

In the developing retina, Pax6 expression is maintained in all proliferating retinal progenitor cells, but downregulated following the differentiation of most neuronal cell types, except in amacrine and bipolar cells (31). In the olfactory system, Pax6-expressing neurons migrate along the rostral migration stream, constantly throughout life. Most of these cells are tyrosine hydroxylase-positive dopaminergic cells (61), and may serve as a source of neural stem cells.

We have addressed here predominantly the role of Pax6/Pax6 in sensory development and disease. A large body of work on Pax6 function in development of the central nervous system remains unexplored here.


