Published in final edited form as:
Bioessays. 2004 April ; 26(4): 374–386.

Anterior eye development and ocular mesenchyme:

New insights from mouse models and human diseases

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Summary

During development of the anterior eye segment, cells that originate from the surface epithelium or the neuroepithelium need to interact with mesenchymal cells, which predominantly originate from the neural crest. Failures of proper interaction result in a complex of developmental disorders such Peters' anomaly, Axenfeld-Rieger's syndrome or *aniridia*. Here we review the role of transcription factors that have been identified to be involved in the coordination of anterior eye development. Among these factors is PAX6, which is active in both epithelial and mesenchymal cells during ocular development, albeit at different doses and times. We propose that PAX6 is a key element that synchronizes the complex interaction of cell types of different origin, which are all needed for proper morphogenesis of the anterior eye. We discuss several molecular mechanisms that might explain the effects of haploinsufficiency of PAX6 and other transcription factors, and the broad variation of the resulting phenotypes.

Introduction

Optimal function of the vertebrate retina, which transduces light into electrical signals that are transferred to the brain, depends on a considerable number of highly differentiated tissues in the anterior eye segment. Cornea and lens provide both transparency and refraction. The iris protects the retina from excess of light, while the ciliary body secretes aqueous humor, a clear fluid that is needed for nutrition of the lens and cornea, as both are avascular for optimal transparency. Upon secretion, aqueous humor enters the posterior chamber between iris and lens, and flows subsequently through the pupil into the anterior chamber between the cornea and iris. Aqueous humor leaves the eye by passing through the trabecular meshwork into Schlemm's canal and from there into the venous system. The trabecular meshwork is a porous tissue located in the chamber angle between the iris root and cornea. This structure creates resistance to the flow of aqueous humor. In response to this resistance, an intraocular pressure is generated, which is somewhat higher than the pressure in the episcleral veins outside the eye. Intraocular pressure is needed to stabilize the shape of the eye and to keep constant distances between the retina and refractive surfaces of the cornea and lens.

During development of all these different tissues in the anterior eye segment, cells that originate from the surface epithelium or the neuroepithelium need to interact with mesenchymal cells, which predominately originate from the neural crest. This interaction is under control of a broad range of transcription factors that are active in epithelial or mesenchymal cells, or both. Failure

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of proper interaction results in anterior eye segment dysgenesis, which comprises a complex of developmental disorders that may critically reduce visual function.

Anterior eye development and the role of ocular mesenchyme

The retina and its auxiliary tissues in the front of the eye are formed by rather different developmental processes.(1) In a first critical step during vertebrate eye development, the optic vesicles appear as lateral outgrowths of the prosencephalon (forebrain). The optic vesicles come into contact with the overlying surface ectoderm that responds by formation of a local thickening, the lens placode. In a next step, the distal part of the optic vesicle is invaginated into its more proximal part, thereby converting into a double-layered optic cup. The inner layer of the optic cup will form the neural retina; the outer layer will differentiate into the retinal pigmented epithelium. In parallel to the development of the optic cup, the lens placode enlarges and sinks below the level of the surrounding ectoderm to form the lens pit. Subsequently, it forms the lens vesicle, which at first remains connected to the surface ectoderm by the lens stalk. Finally, the lens vesicle detaches from the surface ectoderm and invaginates into the optic cup. The optic cup is incomplete inferiorly at the so-called embryonic (choroidal) fissure, which is used by the hyaloid artery to pass into the optic cup. This artery supplies nutrients to the inner layer of the cup and the lens vesicle during ocular development.

Shortly after the lens vesicle has become detached from the surface ectoderm, mesenchymal cells start to migrate into the space between the anterior epithelium of the lens vesicle and the surface ectoderm (Fig. 1A). In the mouse eye, four to seven layers of mesenchymal cells are present at embryonic day (E) 12. The cells form long cytoplasmic processes and have a stellate, star-shaped phenotype.(2) As the number of cells between the lens and surface ectoderm continuously increases, the cells condense more and more to form several layers of flat mesenchymal cells that are separated from each other by a loose fibrillar extracellular matrix (Fig. 1B). In parallel, the cavity of the lens vesicle becomes completely closed as it is filled by the primary lens fibers. During the next days (E14.5-E15.5) of mouse anterior eye development, the posterior mesenchyme cells closest to the lens flatten and extend to form apicolateral contacts with adjacent cells.(3-7) Finally, the cells become connected to each other through continuous bands of junctional complexes and an endothelial monolayer is formed (Fig. 2A). At the end of this process, all layers of the future cornea have been defined (Fig. 2A). The endothelial monolayer that has been formed from posterior mesenchyme cells will become the corneal endothelium, the surface ectoderm that covers the anterior side of the mesenchyme will become the corneal epithelium. Mesenchyme cells between the corneal epithelium and endothelium start to differentiate into corneal stroma fibroblasts or keratocytes, which is responsible for the synthesis of the highly specialized extracellular matrix of the corneal stroma. As a result, the cornea finally becomes transparent.

While the early development of the mammalian cornea has been primarily studied in the mouse eye, the available data on corneal development in other mammalian species (cat, cow, pig, hamster) suggest essentially similar mechanisms.(4) In human embryos, migrating mesenchyme cells are seen during the 6th week of development, which form several layers of loosely aggregated cells filling the space between surface epithelium and lens epithelium;(1, 8) this is similar to the situation in the mouse eye at E12. The next step appears to differ somewhat from mouse development, as most of the mesenchyme cells condense to a dense layer that will give rise to the future corneal endothelium.(1,4,8) Some cells remain in the stromal space between surface ectoderm and future corneal endothelium, which thickens again while mesenchyme cells proliferate and/or continue to migrate to the future cornea. A comparable scenario has been observed for corneal development in monkey eyes.(9)

It is of interest to note that corneal development in the mammalian eye appears to differ from that of the avian eye.(10) In birds, an acellular primary corneal stroma consisting of about 30 orthogonally arranged strata of collagen fibrils is deposited between the surface epithelium and the lens. Mesenchymal cells migrate along the inner surface of the primary stroma to form the corneal endothelium. Subsequently, the primary corneal stroma swells and is invaded by secondary mesenchymal cells destined to become keratocytes. A primary corneal stroma comparable to that in birds is not formed during mammalian eye development.(1,3,4)

During differentiation of the corneal endothelium, the lens detaches from the future cornea and a fluid-filled cavity is generated between both structures (Fig. 2A). The peripheral edge of the optic cup grows into this cavity along the anterior lens surface to give rise to the iris and ciliary body (Fig. 2B). The formation of the iris separates the cavity between lens and cornea into the anterior and posterior chamber. Proper differentiation of the corneal endothelium appears to be an essential requirement for the separation of the lens from the cornea, and subsequent anterior chamber formation. In several strains of mutated mice, the corneal endothelium fails to develop and the lens remains attached to the posterior side of the cornea.(5-7)

While the corneal endothelium differentiates, and the lens and future cornea become separated, a new group of mesenchyme cells migrates to the anterior eye in a second larger wave of migration. These cells appear first at the angle between the future cornea and the anterior edge of the optic cup (Fig. 2A). When the anterior edges of the optic cup extend to form the iris and ciliary body, the mesenchyme cells migrate along the epithelial layers of both structures and finally differentiate into the stroma of the iris and ciliary body (Fig. 2B).

The last structures that become differentiated during anterior eye development are the tissues involved in the outflow of the aqueous humor, namely trabecular meshwork and Schlemm's canal (Fig. 3), which both develop in the iridocorneal or chamber angle.(11-13) Shortly after the beginning of iris elongation (E 17-19 in the mouse eye, 15th to 20th week in the human eye), the chamber angle is occupied by a dense mass of mesenchymal cells (Fig. 3A). These cells elongate, flatten, and become separated from each other by small open spaces that are partially filled with extracellular fibers. At that time, vessels appear in the immediately adjacent sclera (Fig. 3B). Subsequently, the extracellular fibers in the chamber angle organize themselves into lamellae or beams that become covered by flat, endothelial-like cells. Thus the trabecular meshwork is formed, which consists of trabecular beams separated by intertrabecular spaces through which the aqueous humor percolates (Fig. 3C). The scleral vessels next to the chamber angle coalesce in human and mouse eyes to a circumferential Schlemm's canal that contacts the outer side of the trabecular meshwork. The major morphogenesis of the trabecular meshwork is complete by postnatal day (P) 21 in the mouse eye, in humans around birth. Some mesenchymal cells with a stellate phenotype remain between the trabecular beams and the endothelial lining of Schlemm's canal. These cells develop cell-cell contacts with the endothelial cells of Schlemm's canal on one side and the cells covering the trabecular beams on the other side, and form the juxtacanalicular or cribriform layer of the trabecular meshwork, where most of the resistance to aqueous humor outflow is located.

Origin and early differentiation of ocular mesenchyme

There has been some debate as to the origin of the ocular mesenchyme. Early studies suggested that these cells are derived from the paraxial mesenchyme. However, fate-mapping studies using quail-chick chimeras showed an extensive cranial neural crest contribution to the formation of the avian trabecular meshwork as well as to the stroma of the iris, ciliary body and cornea, and to the corneal endothelium.(14) More recent cell grafting and cell labelling experiments of craniofacial morphogenesis in the mouse confirmed neural crest derivation of

the same tissues in mammalian development, but additionally provided evidence for a minor contribution of cranial paraxial mesoderm to the ocular mesenchyme.(15) The molecular mechanisms that control migration and/or differentiation of ocular neural crest and/or mesoderm-derived cells are far from clear.

Data from classical transplantation experiments in avian embryos suggest that the differentiation of mesenchymal cells in the cornea and the formation of an anterior chamber depends on inductive signals from the lens.(16,17) Apparently, such inductive lenticular signals are also important during mammalian eye development, as primary defects in lens development are usually associated with malformation of mesenchyme-derived anterior eye segment tissues. Good examples for this are the phenotypes that result from mutations in the transcription factors *MAF, FOXE3* and *PITX3* (Fig. 4). *MAF* encodes a basic region leucine zipper (bZIP) transcription factor that is expressed in the lens placode, vesicle and, later, the primary lens fibers. Affected human patients with mutations in *MAF* suffer from developmental abnormalities in the lens, but also in iris and cornea leading to congenital cataracts, iris coloboma, opaque corneas and Peters' anomaly (see below).(18) In homozygous Maf-deficient mutant mice, the lens fibers do not elongate and the lens vesicle remains open.(19) *FOXE3* encodes a forkhead transcription factor that is expressed in the lens placode and anterior lens epithelium. Mutations in *FOXE3* cause Peters' anomaly, posterior embryotoxon (see below) and cataracts in humans.(20,21) In homozygous mice with mutations in *Foxe3*, the lens vesicle does not close and the lens epithelium remains connected to the corneal epithelium.(22,23) Heterozygous animals show a central corneal opacity and adhesions between corneal stroma and lens, similar to Peters' anomaly in human and often develop polar cataract.(21) *PITX3* is a homeobox-containing gene expressed in the lens.(24) Mice with homozygous mutations in *Pitx3* (*aphakia, ak/ak*) show a severely malformed lens with a persisting lens stalk during early eye development, and an absence of lens structures and anterior chamber at later stages. (24-26) In humans, mutations in *PITX3* are associated with autosomal-dominant congenital cataracts, central opacity of the cornea and adhesions between iris and cornea.(27)

There is a common theme for all of these disorders: a delayed or incomplete separation of the lens vesicle from the surface ectoderm or an incomplete closure of the lens vesicle by failure of lens fiber elongation almost invariably interferes with the signals that are required for early differentiation of the corneal mesenchyme. The resulting phenotype has been termed Peters' anomaly in humans and consists of central corneal opacities (leukoma) with abnormalities of the deepest corneal stromal layers and local absence of the corneal endothelium.(28) The lens may adhere to the back of the corneal opacity and show signs of an anterior polar cataract. Peters' anomaly is usually associated with iridocorneal adhesions that arise from the pupillary region, and with iris hypoplasia, and corectopia (distorted or displaced pupils). Most cases of Peters' anomaly are sporadic. 50-70% of cases have abnormally high intraocular pressure and develop glaucoma, very likely due to dysgenesis of the aqueous humor outflow tissues in the iridocorneal angle.

Differentiation of ocular mesenchyme and Axenfeld-Rieger's syndrome

Differentiation of anterior ocular mesenchyme is not only under the influence of inductive lens-derived factors, but also controlled by transcription factors that are specifically expressed in the mesenchymal cells themselves. Among these factors are the bicoid-like homeobox gene, *PITX2*, and the forkhead/winged-helix transcription factor gene, *FOXC1* (Fig. 4). In the mouse eye, *Pitx2* is expressed in periocular mesenchyme, presumptive cornea, eyelids and extraocular muscle,(29,30) and *Foxc1* in periocular mesenchyme, presumptive cornea and trabecular meshwork.(5,31,32) Neither of the factors is expressed in retina nor lens. In humans, mutations in *PITX2* or *FOXC1* result in a broad spectrum of abnormalities during anterior eye development with different specific clinical phenotypes.(32-37)

Most of these phenotypes belong to the broad spectrum of clinical disorders, which are part of Axenfeld-Rieger's syndrome.(38) Subtypes of Axenfeld-Rieger's syndrome include Rieger's anomaly or syndrome, Axenfeld's anomaly and iridogoniodysgenesis, all of which are commonly inherited in an autosomal-dominant fashion.(39) In Rieger's anomaly, midpheripheral adhesions from the iris to cornea are seen. In addition, there is marked iris hypoplasia and structural defects such as polycoria (extra holes in the iris) and corectopia. When the ocular findings of Rieger's anomaly are associated with characteristic systemic developmental defects such as dental or facial abnormalities, the term Rieger's syndrome is used. Axenfeld's anomaly is characterized by iris strands that attach to a structure called posterior embryotoxon, which is a ring of collagenous fibers at the peripheral end of Descemet's membrane, the basement membrane of the corneal endothelium. It is clinically recognized as a ring-shaped opacity in the peripheral cornea. Patients with iridogoniodysgenesis have an iris with hypoplastic stroma, abnormal chamber angle tissue, and glaucoma. In general, patients with Axenfeld-Rieger's syndrome develop glaucoma in about 50% of cases.

Some patients with mutations in *PITX2* or *FOXC1* have been reported that exhibit the phenotype of Peters' anomaly.(33,34,40) The reasons for this wide spectrum of phenotypes caused by mutations in *PITX2* and *FOXC1* are not clear and have been discussed recently. (41) Mutant proteins may retain partial functions resulting in milder phenotypes. However, individuals with the same mutation may have different phenotypes, even within the same family.(33,40) There is the distinct possibility that different phenotypes result from modifying genes that interact with mutant genes. Indeed, *Foxc1*$^{+/-}$ mice exhibit phenotypes comparable to those of human patients with mutations in *FOXC1* depending on strain, and therefore genetic background.(42) Still, even between mice from the same inbred strain (with essentially the same background), or between right and left eye of the same animal, the severity of the phenotype varies. It has been suggested that stochastic developmental events and/or the local environment during development influence the outcome for each individual eye.(41) Such events may lead to the presence of a more or less active gene product at a given critical point during development. Data from mouse mutants suggest that dosage is an important factor. Heterozygous mutants show phenotypes that resemble those in humans with Axenfeld-Rieger's syndrome,(42) whereas homozygous mutant mice, which die shortly after birth, show corneolenticular adhesions and failure of anterior chamber development similar to the condition in Peters' anomaly.(5) Homozygous *Pitx2*$^{-/-}$ deficient mice are also not viable after birth, and embryonic data suggest a phenotype comparable to that in homozygous *Foxc1*$^{-/-}$ mutant mice with a persistence of corneolenticular adhesions and lens stalk.(43)

Ocular mesenchyme and Pax6

Another gene that is critically required for the morphogenesis of mesenchyme-derived tissues in the anterior eye is *PAX6*, which codes for a paired domain and paired-like homeodomain transcription factor (Fig. 4). Pax6 is a key regulator of eye development that is both essential for eye formation in different organisms as well as capable of inducing ectopic eyes in flies and frogs upon misexpression.(44-46) Humans with heterozygote mutations in *PAX6* exhibit the phenotype *aniridia*, a panocular disease that is associated with iris hypoplasia, corneal opacification, cataract and foveal dysplasia.(47-49) About 50-75% of patients with *aniridia* develop glaucoma, because of abnormal differentiation of the trabecular meshwork and/or complete absence of Schlemm's canal.(50,51) Mutations in *PAX6* have also been found in patients with Peters' anomaly, autosomal dominant keratitis and isolated foveal hypoplasia. (52) Heterozygous *Small eye* (*Sey*) mice or heterozygous *Pax6*$^{lacZ/+}$ mutant mice, which both have null alleles of *Pax6*, show a reduction in eye size and cataracts.(53,54) The iris of the animals remains hypoplastic and corneal abnormalities are present, which include an irregular lamellar alignment, cellular infiltrates and vascularization of the corneal stroma.(53,55)

Defects of the corneal epithelium contribute to the corneal abnormalities.(55,56) In a third of *Pax6*$^{lacZ/+}$ mutant mice, the separation of the cornea from the lens is incomplete, the epithelial layers of lens and cornea are continuous and iridocorneal adhesions are present, all hallmarks of Peters' anomaly.(53) Persistence of the lens stalk has also been observed in *Small eye* (*Sey*) mice.(55) In addition, the trabecular meshwork of *Pax6*$^{lacZ/+}$ mutant mice remains undifferentiated and Schlemm's canal is absent.(53)

Overall, the phenotype of Pax6 haploinsufficiency in mice and humans indicates that Pax6 is critically required for the differentiation of those tissues of the anterior eye segment that are of mesenchymal origin. Pax6 could indirectly act on the morphogenesis of ocular mesenchyme as a strong Pax6 expression has been described in cells that derive from the neuroectoderm of the optic cup or from the anterior surface ectoderm.(57-60) This strong expression is seen during ocular development and is maintained in adulthood. To clarify, if Pax6 acts also directly on mesenchymal differentiation in the developing eye, we recently studied Pax6 expression by β-galactosidase staining of *Pax6*$^{lacZ/+}$ heterozygous mice, and by immunostaining of wild-type littermates and cultured murine trabecular meshwork cells.(53) Positive signals were observed in cells of mesenchymal origin, but the intensity was weaker than in cells of surface ectodermal or neuroepithelial origin and only observed in mid-fetal stages, but not in adult animals. A similar staining pattern was observed by Collinson and coworkers using different antibodies.(61) In addition, these authors analysed gene expression and distribution of Pax6$^{-/-}$ cells in Pax6$^{+/+}$ ↔ Pax6$^{-/-}$ chimeras to find that Pax6 is autonomously required for cells to contribute to corneal stroma and corneal endothelium.(61)

The high and continuous expression of Pax6 in cells that derive from surface ectoderm and optic cup (lens, corneal epithelium, iris, and ciliary epithelium) appears to be required for the expression of transcription factors (Six3, c-Maf, MafA/L-Maf and Prox1,(62,64) structural genes (crystallins and cell adhesion molecules),(65-69) and signaling molecules, which are critical for the morphogenesis of those tissues, and subsequently for the migration of neural crest cells into the eye by inductive processes(53) (Fig. 5). In addition, a low and transient expression of Pax6 plays cell autonomous roles in the differentiation of trabecular meshwork, and in the formation of corneal endothelium and keratocytes(53,61) (Fig. 5).

The role of signaling molecules

It appears reasonable to assume that the various transcription factors that are involved in the control of the morphogenesis of the anterior eye coordinate their signals by modulating the expression of secreted signaling molecules. Studies on mutant mouse models indicate that bone morphogenetic protein 4 (BMP4) and/or transforming growth factor-β2 (TGF-β2) are directly involved in the processes that control mesenchyme morphogenesis in the anterior eye. BMP4 is expressed in the iris, ciliary body and retinal pigment epithelium of embryonic and adult mouse eyes.(70) Mice heterozygous for a null allele of *Bmp4* (*Bmp4*^{tmBLh}) show a variety of ocular segment abnormalities involving the iris (irregular-shaped pupils, anterior synechiae), cornea (opacity at the periphery, diffuse haze), and chamber angle (small or absent Schlemm's canal, hypoplastic or absent trabecular meshwork).(70) The extend of the structural changes in the chamber angle varies along the circumference of individual eyes, and animals with severe structural abnormalities show an increase in intraocular pressure. Similar to the structural changes in *Foxc1* heterozygote mice, the severity of the changes depends on the genetic background, and there is phenotypic variability between eyes of genetically uniform mice.

Similar to BMP4, TGF-β2 is expressed during ocular development and in the adult anterior eye. Homozygous TGF-β2 knockout mice, which die that at birth because of cardial defects, show the phenotype of Peters' anomaly. The cornea is markedly thinner than normal and an anterior chamber is not present.(71) The lens and iris are in contact with the corneal stoma and

the corneal endothelium is completely absent.(72) Transgenic overexpression of TGF-β1 (which is closely related to TGF-β2 and utilizes the same receptors) in the anterior eye causes the formation of a thick and opaque cornea, and prevents the formation of the anterior chamber and that of the tissues in the chamber angle.(7) In addition, the corneal endothelium, iris and ciliary body are absent, emphasizing that the dose of TGF-βs must be critically maintained during development to avoid severe malformations in anterior eye development. It is interesting to note that an interaction between TGF-β signaling and *FOXC1* expression has been observed in cell culture studies. Treatment with TGF-β1 upregulates the expression of *FOXC1* in several human cancer cell lines.(73) Mesenchyme cells from mouse embryos show a characteristic response to added TGF-β, which is not observed in cells from *Foxc1* null mouse embryos. (31)

Molecular mechanisms of Pax6 haploinsufficiency

It is tempting to speculate that Pax6, which is active in both epithelial and mesenchymal cells during ocular development, is a key element to synchronize the complex interaction of cell types of different origin that is needed for proper morphogenesis of the anterior eye. The widespread expression of Pax6 during mammalian eye development, and the broad and quite variable spectrum of the morphological changes that are observed in the anterior eye segment of humans with *aniridia* and heterozygous *Sey* mice, suggest that the mechanisms by which a nonfunctional or only partially functional Pax6 allele causes anterior ocular abnormalities are complex. Any model that seeks to explain these mechanisms will have to address the following particular issues. (1) Why is a single intact copy of Pax6 not sufficient for proper anterior eye formation, and why are some cell types in the anterior eye more severely affected by Pax6 haploinsufficieny than others? (2) Do abnormal Pax6 proteins produced by the mutated allele contribute to the structural alterations that are seen in aniridia or Sey phenotypes and, if so, what biological role(s) do these proteins play? (3) How many and specifically which genes are directly regulated by Pax6, and which of them do directly contribute to the structural changes in Pax6 haploinsufficiency?

A very-likely answer to the first question would be that mutated Pax6 protein does not have any transcriptional activity and that the remaining single normal copy of Pax6 is simply not enough to produce a sufficiently high amount of biologically active Pax6 protein. In support of this, there is clear evidence from in vitro studies that a critical dose of Pax6 is required to initiate the transcription of its target genes.(65,74) Cell types with a constitutively low expression of Pax6 such as the ocular mesenchyme might be more vulnerable, if the amount of available Pax6 is further reduced. A lack or a significant decrease in target gene transcription would contribute to the Pax6 dose effect, if a product of the target gene itself is required to enhance the activation of Pax6. Again, such target genes might be more active in some cell types of the anterior eye than in others. Possible candidates are the transcription factors c-Maf, MafA/L-Maf and Six3. There is evidence that the expression of c-Maf(62) and Six3(75) is regulated by Pax6 and that Pax6 is able to form specific complexes with both factors.(76,77) Moreover, Pax6 itself may be such a target gene, as there are autoregulative mechanisms of *Pax6* gene transcription, which are mediated by Pax6-binding sites in the Pax6 promoter(78) and/or the distal tissue-preferred 3′-enhancer of the *Pax6* gene(75,79) The fact that Pax6 is able to form complexes with other homeodomain-containing proteins such as Six3(77) raises the distinct possibility that Pax6 might form similar complexes with itself. Such Pax6-Pax6 complexes might be critically required for activating the transcription of some selected target genes, but might be less available or difficult to assemble under conditions of haploinsufficiency. Taken together, there is the distinct possibility that the effects of a specific loss of one *Pax6* allele are amplified at the levels of both transcriptional regulation and autoregulation, and that a reduced formation of complexes, which contain both Pax6 and products of its target genes, contributes to these effects. Ultimately, all these processes will

contribute to a reduced availability of Pax6, lead to the reduced expression of one or more critical genes, especially of those that containing “low-affinity” Pax6-binding sites (Fig. 6A), and result in abnormal anterior eye development.

The answer to the second question, if *aniridia* or *Sey* phenotypes are caused by abnormal Pax6 proteins produced by the mutated allele, depends on the type of mutation in the respective abnormal *PAX6* allele. Some missense mutations that result in the translation of full-length Pax6 proteins might clearly have the potential to impair the proper folding of the Pax6 protein and to prevent the formation of its native three-dimensional structure.(80) Indeed, based on the structure of Pax6, it has been generally thought that the naturally occurring missense mutations should compromise the three-dimensional structure and function of Pax6. However, molecular studies on some representative missense mutations in recombinant Pax6 and Pax6 (5a) (a splice variant of Pax6 shown in Fig. 4) proteins showed that such mutated proteins could still interact with a number of different Pax6-binding sites.(81) Moreover the mutated proteins were able to evoke transcriptional responses, which were surprisingly entirely different. In different cell types, transcription decreased, increased or remained unchanged. There is the distinct possibility that a single wild-type *PAX6* allele is actually quite sufficient most of the time except during the activation of a selected number of specific dosage-sensitive target genes in a limited number of ocular cells and tissues.

Nonsense mutations in *PAX6* generate truncated proteins. Clearly, if the premature protein termination produces such short proteins, it is quite likely that the short proteins do not possess any biological activity and are unstable. The resulting “net” effect of the formation of unstable proteins would be similar to that of the “loss-of-allele” mutations described above. However, there are some specific nonsense mutations, e.g. R317X and S353X, which have been shown to result in the formation of more stable truncated Pax6 proteins that still have an intact paired domain (PD) and homeodomain (HD).(82-84) The molecular mechanism behind the structural changes that are caused by this family of missense mutations appears to be a “dominant-negative” effect (see Fig. 6B,C). This dominant-negative effect could be caused by competition for DNA binding between truncated (and partially functional) Pax6 proteins and wild-type Pax6 proteins (Fig. 6B). Dominant-negative effects could also become important if a specific Pax6-mediated transcriptional activation requires the direct interaction with hypothetical auxiliary proteins (termed “P6X” in Fig. 6C) that co-regulate the function of Pax6. Some candidates for P6X have been identified, as Pax6 has been shown to interact with Six3,(77) c-Maf(76) and pRb.(85) Again, truncated Pax6 proteins could compete with wild-type Pax6 proteins for binding of P6X auxiliary proteins and critically reduce the amount of available P6X. Another scenario could come into play, if the function of transcription factors that act downstream of *PAX6*, like *PITX3*, also require binding of the same hypothetical P6X proteins. Missfolded or truncated Pax6 proteins that aberrantly interact with P6X could significantly reduce the amount that is necessary for the proper function of such downstream genes. Such a mechanism could also explain why additional copies of *PAX6*, when introduced into the mouse genome, generate phenotypes similar to Sey/+.(86) Ectopic additional Pax6 might squelch the hypothetical P6X (Fig. 6D) and disrupt the equilibrium between Pax6-P6X and Pitx3-P6X complexes.

Developmental abnormalities in mouse *Sey/+* and human *aniridia* may originate from haploinsufficient expression of a small number of Pax6 target genes

Studies on gene expression during formation of the lens pre-placode and placode in early eye development provide evidence that Pax6 positively regulates the expression of at least two other transcription factors, Six3(75) and c-Maf.(62) Still, the first embryonic process during

eye development that is critically dependent on the correct *Pax6* gene dosage appears to be the formation of the lens placode.(58,66) In contrast, the formation of the pre-placode appears to be unaffected in Pax6-deficient heterozygous mouse strains. Haploinsufficiency for Pax6 does not prevent, but rather delays the induction of lens formation by 12 to 24 hours, suggesting that a prolonged accumulation of Pax6 proteins can overcome the gene dosage effect. The price is a 50% reduction in the number of cells in the lens vesicle, and the frequent failure of the lens vesicle to detach completely from the surface ectoderm.(66) This model would provide an explanation for the reduced size of the lens in the mouse, and the persistence of corneal-lenticular stalks (Peters' anomaly described above) that are observed both in Pax6-deficient heterozygous mice and humans.(28,50,53,66,87) Apparently, lens formation includes multiple steps, each being characterized by its own set of direct Pax6 target genes.(66) N-cadherin, a calcium-binding cell adhesion molecule that is associated with the separation and sealing of cell layers in morphogenesis, might play a critical role for the formation of the lens vesicle.(66) The expression pattern of N-cadherin differs markedly between wild-type and Pax6 heterozygous lens vesicles.

In humans, aniridic lenses are not obviously reduced in size as in the *Sey*/+ mouse, but may be dislocated at a frequency of up to 56%.(50,51) It is unclear why the lens size differs in similar abnormal conditions between mice and humans. It is possible that the human lens size is not affected by changes in *PAX6* gene dosage due to a different sensitivity of the critical target genes and/or because of other species-specific differences. Such differences might have evolved because the relative size of the human lens, when compared to that of the eye, is much smaller than that of mouse lens. Still, the coincidence of cataracts with *aniridia* that has been reported to occur in 50 to 85% of affected cases(50,51) clearly indicates that Pax6 is required for proper gene expression in the human lens. These cataracts evolve from small anterior or posterior lens opacities that are already found at birth.

While the lens size in the *Sey*/+ mouse is systematically reduced, animals with persistent corneal-lenticular stalks or Peters' anomaly are less frequently observed. In the $Pax6^{lacZ/+}$ mouse model with a null allele of Pax6, we observed Peters' anomaly in about one third of the analyzed animals.(53) We speculate that the shift toward Peters' anomaly from *Sey* only might be caused by the action of modifying genes, and/or environmental and stochastic factors. In human patients, Peters' anomaly has been found to be associated with specific PAX6 mutations such as R26G(87) and G18W.(88) In addition, in humans, other factors appear to contribute to the specific phenotype of Peters' anomaly, as the G18W mutation is displayed as cataract or Peters' anomaly in different members of the same family.(88) A comparable familial variability has been observed in humans with the R26G mutation.(87)

It is interesting to note that the original Pax6 null alleles in *Sey/Sey* do not allow the initiation of lens induction (i.e. thickening of the surface ectoderm and its subsequent envagination), while two recently described point mutations, $Pax6^{4Neu}$ and $Pax6^{10Neu}$, allow the envagination of the ectoderm.(89) $Pax6^{4Neu}$ contains the missense mutation S273P in the HD, and $Pax6^{10Neu}$ contains a nucleotide substitution in the Pax6 Kozak sequence, which affects translation.(89) It appears that the Pax6 proteins, which are generated from the 4Neu and 10Neu alleles can execute a certain fraction of the lens induction program, most likely because their initial target gene(s) still cooperate.(89) There is the distinct possibility that the relatively moderate ocular phenotypes, which are seen in about half of *PAX6* missense mutations in humans,(52) are due to the fact that the respective mutated proteins retain their ability to activate a large spectrum of target genes. Hence, a reduction in Pax6 gene dosage may affect only a relatively small number of critical genes, e.g. N- and R-cadherins(66,90) in each of the affected tissues. Even if the overall number of genes that are directly or indirectly affected by Pax6 haploinsufficiency may be in the hundreds or thousands,(68,91) only a dozen of them may be responsible for the generation of developmental abnormalities.

The hallmark of *aniridia* is the absence or hypoplasia of the iris. The molecular and developmental mechanisms of this defect are not well understood. As noted earlier, the two main tissues of the iris, the stroma and the epithelial layers, are of different developmental origin. The epithelia (including the epithelium-derived iris muscles) derive from the optic cup, while the stroma is formed by migrating neural crest cells. An abnormal migration of neural crest cells has been observed in Pax6 homozygous rat embryos.(92) We and others have recently shown that Pax6 is expressed in the ocular mesenchyme that originates from neural crest cells; however, the expression appears to be low and transitory.(53,61) Absence or hypoplasia of the iris in *aniridia* could easily be caused by a combination of defects during the differentiation of the epithelial layers of the iris and a compromised ability of migrating neural crest cells to find their destination in the iris stroma.

It is interesting to note that an iris hypoplasia can be experimentally induced in mice by maternal vitamin A deficiency.(50,51) This correlates with the finding that retinoic acid signaling is altered in mouse *Sey*/+ embryos.(93) Retinoic acid has recently been shown as an environmental modifier of velo-cardio-facial (DiGeorge) syndrome, which is caused by defects in neural crest cell migration,(94) raising the intriguing possibility that the variability of the *PAX6* gene dosage effect might be modified via retinoids. Similar to lens formation, the candidate genes that are under direct or indirect influence of Pax6 in neural crest cells might include cell adhesion molecules,(66-69) although direct molecular evidence for this is still lacking. Since the expression of Pax6 is weak in neural crest cells, many Pax6 target genes, even those with high-affinity Pax6-binding sites (see Fig. 6A), would be negatively affected in Pax6-null heterozygote individuals. It is also possible, that the smaller (in mouse) or abnormal (in human) lens cannot produce adequate amounts of signaling molecules that regulate neural crest cell migration during the formation of the anterior eye segment (Fig. 5). These possibilities are not mutually exclusive.

Conclusions and perspectives

So far, a relatively small group of genes encoding transcription factors has been shown to be critical for the development of the anterior eye. In humans, mutations in these genes cause a spectrum of partially and/or completely overlapping congenital disorders, which all compromise vision to a variable degree. Ocular tissues are of neuroectodermal and neural crest origin, and transcription factors need to coordinate the complex interaction and communication between those two types of cell populations during morphogenesis of the anterior eye. Pax6, which is expressed in all cell types that contribute to anterior eye development, although at different times and with different intensity, appears to be the critical factor that synchronizes the critical events during formation of the anterior eye. In addition, numerous other genes including *PITX2*, *PITX3*, *FOXE3*, *FOXC1* and *c-MAF* appear to regulate similar and/or identical processes as *PAX6*, however, in a limited number of ocular cells and tissues. The challenge for the future will be to clarify the mechanisms and hierarchies that are needed for proper interaction of all these transcription factors. This will involve questioning why these factors have rather specific and important functions in the eye, although they are expressed in numerous tissues outside the eye. An equally important task will be to learn about those genes that are directly regulated by the action of the transcription factors and the elucidation of their specific function during the development of the anterior eye. Such an identification will require high throughput techniques, such as cDNA microarrays, chromatin immunoprecipitations and, maybe equally important, educated guesses. Finally, it is more than likely that more regulatory genes are involved during anterior eye segment formation, which still need to be discovered.

Acknowledgments

We greatly appreciate the assistance of Jörg Pekarsky in generating the drawings of mouse eye development. We also thank Dr. Michael Wegner and Dr. Ruth Ashery-Padan for critically reading this manuscript.

Funding agencies: National Eye Institute EY12200 and 14237 (AC), Research to Prevent Blindness, Inc. (AC), Ernst R. Tamm, Deutsche Forschungsgemeinschaft SFB 539 (ERT); American Health Assistance Foundation (ERT), and Ales Cvekl was a recipient of a Research to Prevent Blindness Inc. (RPB) Career Development Award.

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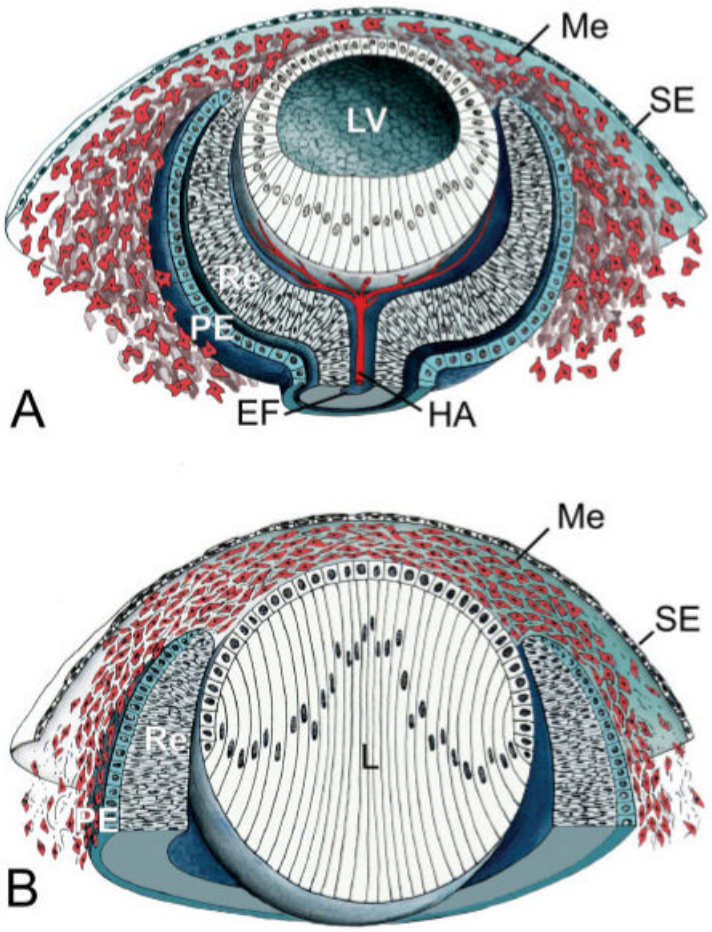

Figure 1.

Schematic diagram of ocular mesenchyme development in the mouse eye between embryonic days (E) 12.5-14.5. **A:** At E 12.5-13.5, the lens vesicle (LV) has detached from the surface epithelium (SE) and has become invaginated into the optic cup. Mesenchymal cells (ME) start to migrate into the space between the anterior epithelium of the lens vesicle and the surface ectoderm. The inner layer of the optic cup forms the neural retina (Re), the outer layer the retinal pigmented epithelium (PE). The optic cup is incompletely inferior at the so-called embryonic (choroidal) fissure (EF), where the hyaloid artery (HA) enters the optic cup. **B:** At E 13.5-14.5, the mesenchyme cells condense to form several flat layers that are separated from each other by a loose fibrillar extracellular matrix. In the lens (Le), the primary lens fibers elongate to close the lumen of the lens vesicle.

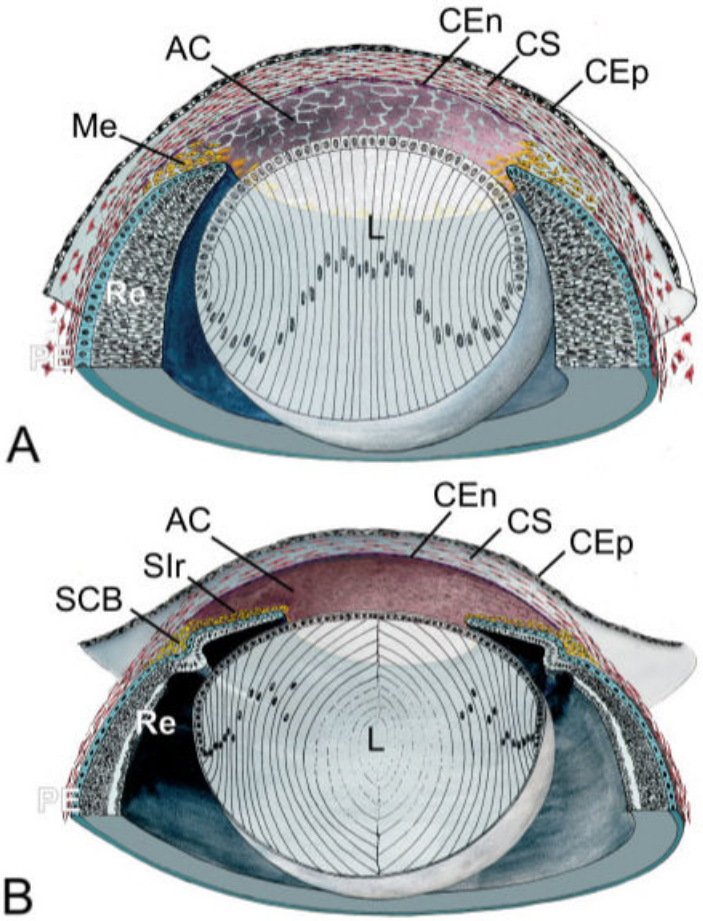

Figure 2.

Schematic diagram of ocular mesenchyme development in the mouse eye between embryonic days (E) 14.5 and 19.5. **A:** At E14.5-15.5, the posterior mesenchyme cells closest to the lens flatten, become connected by apicolateral contacts and form an endothelial monolayer. At the end of this process, all layers of the future cornea have been defined. The endothelial monolayer that has been formed from posterior mesenchyme cells will become the corneal endothelium (CEn), the surface ectoderm that covers the anterior side of the mesenchyme will become the corneal epithelium (CEp). Mesenchyme cells between the corneal epithelium and endothelium differentiate into keratocytes, the specific cell type of the corneal stroma. During differentiation of the corneal endothelium, the lens (L) detaches from the future cornea and a fluid-filled cavity, the anterior chamber (AC), is generated between both structures. In parallel, a new group of mesenchyme cells (Me) arrives at the angle between the future cornea and the anterior edge of the optic cup. **B:** Beginning at approx. E 15.5, the anterior edge of the optic cup enlarges to form the iris and ciliary body. Mesenchyme cells migrate along the epithelial layers of both structures and finally differentiate into the stroma of the iris (SIr) and ciliary body (SCB). Re: Medina, PE: pigmented epithelium.

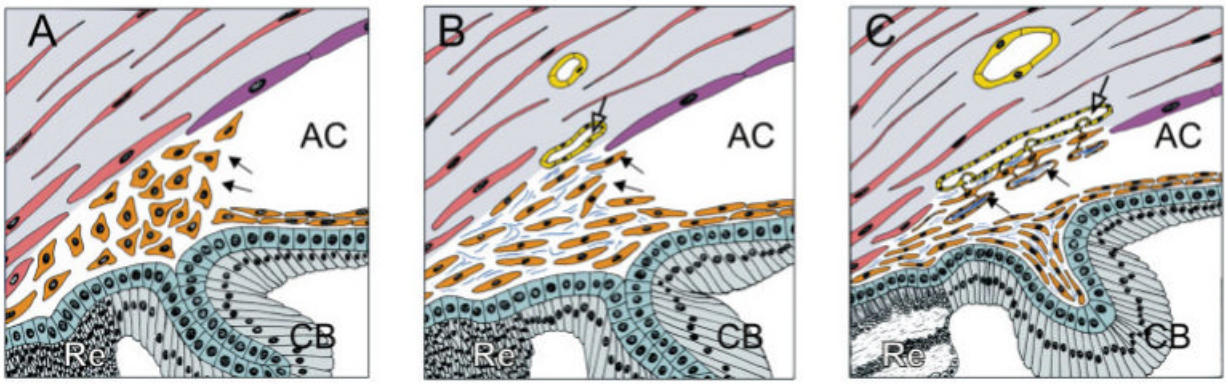

Figure 3.

Schematic diagram of the development of chamber angle and trabecular meshwork in the mouse eye between postnatal days (P) 1 and 14. **A:** From P1-P4, the chamber angle is occupied by a dense mass of mesenchymal cells (arrows). **B:** From P4-P10, chamber angle cells (solid arrows) become separated from each other by small open spaces that are partially filled with extracellular fibers, while vessels appear in the immediate adjacent sclera (open arrows). During this period, the chamber angle is level with the anterior border of the future trabecular meshwork. **C:** From P11-P14, the extracellular fibers in the chamber angle organize themselves into trabecular beams that become covered by trabecular meshwork cells, while the scleral vessels next to the chamber angle coalesce to Schlemm's canal. In parallel, the peripheral margin of the anterior chamber moves posteriorly and the inner surface of the trabecular meshwork becomes exposed to the anterior chamber (AC). Re: Medina, CB: ciliary body.

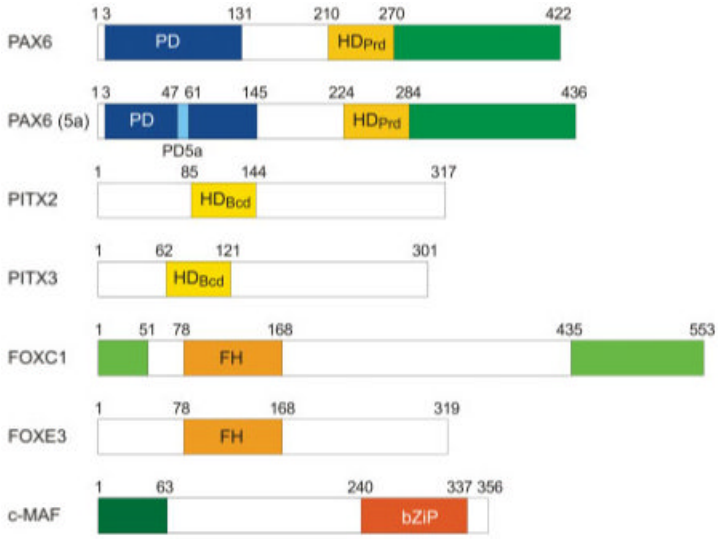

Figure 4.

Schematic drawing of various transcription factors that are involved during anterior eye development. Important functional domains are boxed in colors. DNA-binding domains: PD, paired domain; PD5a, alternatively spliced paired domain; HD_{Bcd}, bicoid type homeodomain; HD_{Prd}, paired type homeodomain; FH, forkhead domain; bZIP, basic leucine zipper domain. Transcriptional activation domains are shown as green boxes.

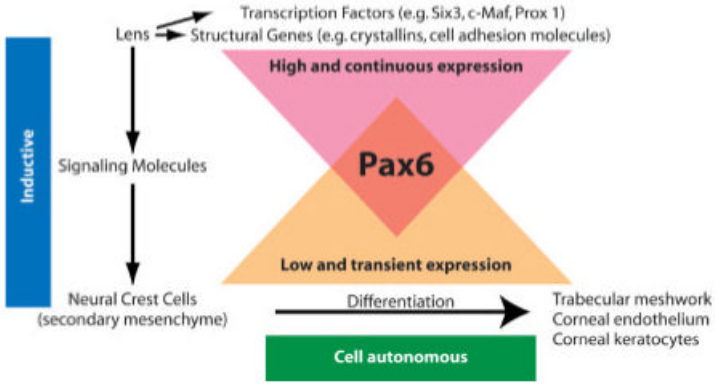

Figure 5.

Different Pax6 roles in ocular cell differentiation. A high and continuous expression of Pax6 in cells of ectodermal origin (lens, corneal epithelium, iris, and ciliary epithelium) is required for expression of transcription factors (Six3, c-Maf and Prox1),(62,63) structural genes (crystallins and cell adhesion molecules),(65-69) and signaling molecules affecting the migration of neural crest cells into the eye by inductive processes.(61) In addition, a low and transient expression of Pax6 plays cell autonomous roles in the differentiation of trabecular meshwork, and in the formation of corneal endothelium and keratocytes.(53)

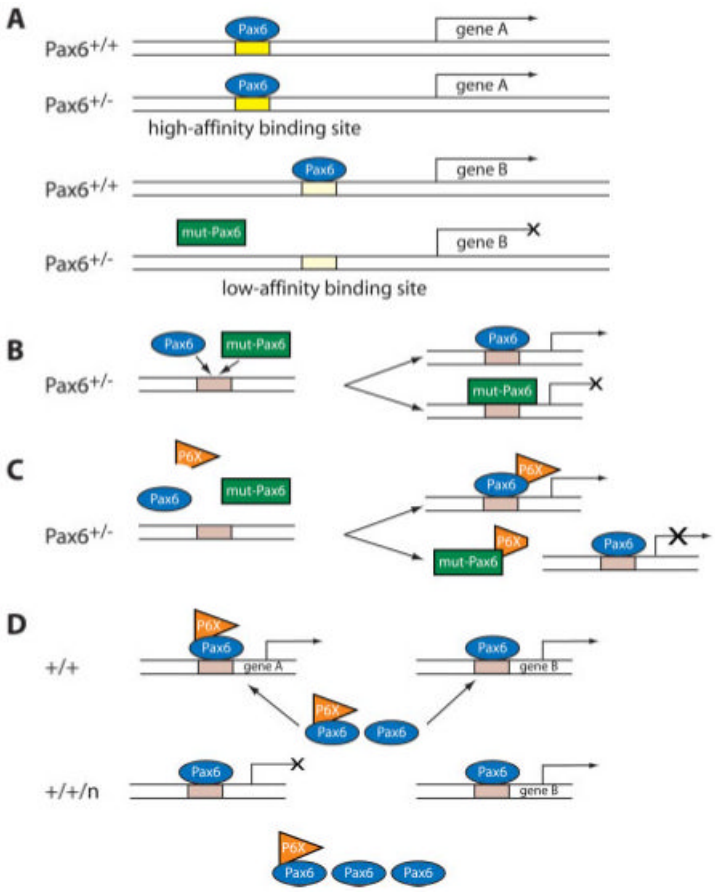

Figure 6.

Molecular models explaining Pax6 haploinsufficiency and overexpression. **A:** Interaction of Pax6 and mutated Pax6 (mut-Pax6) with target genes. A "high-affinity"Pax6-binding site in gene A is occupied in $Pax6^{+/+}$ and $Pax6^{+/-}$ cells. A "low-affinity" Pax6-binding site in gene B is only occupied in $Pax6^{+/+}$ cells. If a mutated Pax6 allele encodes for a nonfunctional protein or no protein at all, the low-affinity site(s) remain(s) free. **B:** Truncated proteins capable of binding to DNA can compete with the normal Pax6 protein as a classical dominant-negative mutation.(83) **C:** Some mutated Pax6 proteins may sequester a hypothetical auxiliary factor (P6X) from Pax6, thereby blocking its function. **D:** Overexpression of Pax6 (+/+/n, n=1-5) generates enough of Pax6 protein to saturate low-affinity binding sites, but may disrupt the equilibrium between Pax6 and the hypothetical P6X. P6X in panels C and D may be the same or different hypothetical protein. Proteins modulating Pax6 activity include pRb, c-Maf, Six3, Sox2, Mitf, and others.