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Molecular and developmental mechanisms of anterior segment dysgenesis

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Abstract

Anterior segment dysgenesis (ASD) is a failure of the normal development of the tissues of the anterior segment of the eye. It leads to anomalies in the structure of the mature anterior segment, associated with an increased risk of glaucoma and corneal opacity. Several different gene mutations have been identified underlying these anomalies with the majority of ASD genes encoding transcriptional regulators. In this review, the role of the ASD genes, *PITX2* and *FOXC1*, is considered in relation to the embryology of the anterior segment, the biochemical function of these proteins, and their role in development and disease aetiology. The emerging view is that these genes act in concert to specify a population of mesenchymal progenitor cells, mainly of neural crest origin, as they migrate anteriorly around the embryonic optic cup. These same genes then regulate mesenchymal cell differentiation to give rise to distinct anterior segment tissues. Development appears critically sensitive to gene dosage, and variation in the normal level of transcription factor activity causes a range of anterior segment anomalies. Interplay between *PITX2* and *FOXC1* in the development of different anterior segment tissues may partly explain the phenotypic variability and the genetic heterogeneity characteristic of ASD.
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Introduction

The anterior segment of the eye comprises all the structures lying in front of the anterior vitreous face and includes the ciliary body, lens, iris, and cornea. An anterior segment dysgenesis (ASD) is a developmental abnormality of the tissues of the anterior segment. The aqueous filled space lying behind the cornea and in front of the lens is separated into two chambers by the iris (Figure 1c). The two-layered epithelium covering the finger-like ciliary processes in the posterior chamber produces the aqueous that flows through the pupil into the anterior chamber. At the margin of the anterior chamber in the angle between the cornea and the iris, the structures of the outflow system, the trabecular meshwork and Schlemm's canal drain the majority of the aqueous from the anterior chamber. Continual regulation of the production of aqueous and its drainage is needed to maintain an optimal intraocular pressure. Typically, ASDs include combinations of congenital abnormalities affecting the iris and cornea, such as iris hypoplasia or rupture (corectopia) or ectopic pupils (polycoria), corneal opacity (leukoma), tissue strands or adhesions between the iris and the cornea (peripheral anterior synechiae), malformation of the irido-corneal angle drainage structures, or adhesion between the cornea and the lens or the iridic-pupillary border (Peters anomaly). In addition to the psychological effect of cosmetic changes to the anterior segment, vision may be at risk from reduced corneal transparency or high-tension glaucoma. Over the last decade, molecular and developmental genetic research has transformed our understanding of the molecular basis of ASD and the developmental mechanisms underlying these conditions. Here,

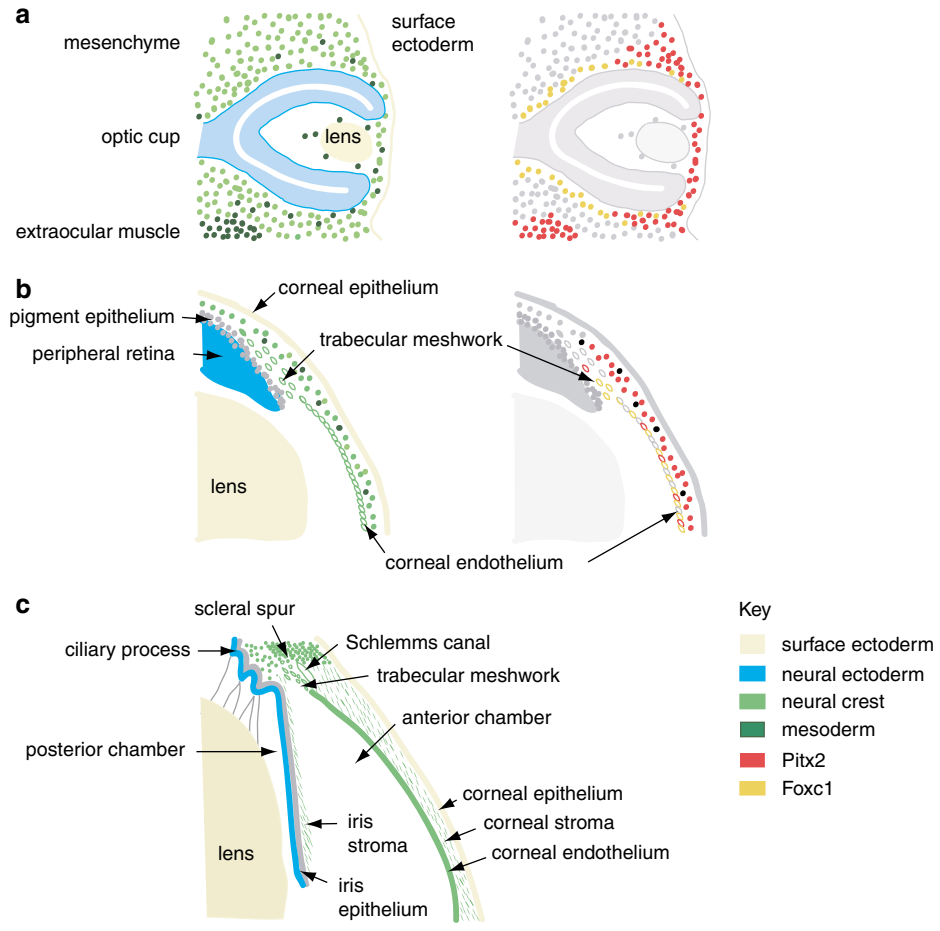

Figure 1 The embryonic and fetal development of the anterior segment of the eye. (a) Optic cup stage, embryonic day 10.5 in the mouse equivalent to week 5 in human development. (b) Formation of anterior chamber, embryonic day 15.5 in the mouse equivalent to the 5th month of human gestation. (c) Mature anterior segment depicting the lens, iris, irido-corneal angle, and the cornea. Key shows the colour coding used to represent the embryonic origin of the anterior segment tissues in the right-hand plates, and the pattern of expression of the *Foxc1* and *Pitx2* genes in the left-hand plates, based on published expression data.[40,42]

a current model of the disease aetiology is provided by considering findings from basic laboratory-based studies in combination with clinical and genetic analysis in patients.

Embryology of the anterior segment

To understand the aetiology of this group of conditions an overview of the complex embryonic origin of the anterior segment tissues is essential. By the 6th week of human development, morphogenetic movements have formed the bi-layered embryonic optic cup from the forebrain neuroectoderm, and the lens vesicle has invaginated and separated from the overlying surface ectoderm. At this stage, the rudimentary eye is loosely surrounded by mesenchymal progenitor cells, mainly of neural crest origin, that are migrating anteriorly (Figure 1a). By processes of tissue morphogenesis and differentiation, these cells, together with cells of the anterior-most peripheral region of the optic cup and the overlying surface ectoderm, give rise to the cornea, iris, and drainage structures of the iridocorneal angle. Correct specification and differentiation of mesenchymal progenitor cells in early embryogenesis is critically required for normal anterior segment development. First, a primitive endothelium forms for the cornea and future trabecular meshwork, lying posterior to the surface ectoderm (the future corneal epithelium) (Figure 1b). Mesenchymal cells migrate anterior to the lens and differentiate to form the fibroblasts and melanocytes of the anterior iris stroma; the two layers of cells at the periphery of the optic cup proliferate and extend inwards between the lens and the iris stroma to form the iris epithelium. Between the corneal endothelium and corneal epithelium, migrating cells then form the corneal stroma, and synthesise collagen in a lamellar arrangement. By the 5th month of gestation, development of the iris, and cornea is advanced and the

Table 1 Clinical features of ASD, and the gene mutations causing each condition

	Axenfeld rieger syndrome	*Peters anomaly*	*Iris hypoplasia*	*Primary congenital glaucoma*	*Aniridia*
ASD clinical features					
Corneal opacity		■			■
Corneal opacity with iris/lens adhesions		■			
Absent iris					■
Iris hypoplasia			■		
Pupil-polycoria corectopia	■	■			
Abnormal angle iris strands to trabecular meshwork/cornea-peripheral anterior synechiae	■	■	■	■	
ASD genes					
PITX2 4q25	■	■	■		
FOXC1 6p25	■		■		
PAX6 11p13	■	■			■
FOXE3 1p23		■			
CYP1B1 2p22		■		■	

Abbreviation: ASD, anterior segment dysgenesis.
See http://www.ncbi.nlm.nih.gov/omim/ for identified ASD gene mutations. MIM number for *PITX2* (601542), *FOXC1* (601090), *PAX6* (607108), *FOXE3* (601094), *CYP1B1* (601771). Note that the same gene causes more than one condition and one condition is not always caused by the same gene.

anterior chamber is well defined (Figure 1b). Subsequent maturation of the angle tissue involves further tissue movement and differentiation; as the scleral spur develops it separates the ciliary body and iris root from the developing trabecular meshwork. By birth, the trabecular meshwork is located anterior to the iris root and is exposed to the aqueous (Figure 1c). Figure 1 shows the embryonic development of the anterior segment and is colour coded to show the contribution that four types of embryonic tissue, the neuroectoderm, the surface ectoderm, the neural crest cells, and mesoderm-derived cells make to the mature anterior segment structures.

Classification of ASD

The ASDs are complex and affect multiple structures, which have made their clinical classification and description difficult.[1] It is generally advisable to characterise the features of the malformation affecting each anterior segment structure as this avoids the use of different clinical diagnoses to describe the same condition.[2] Table 1 shows the overlap in clinical features between typical ASD. Several clinical features are found in more than a single condition. For example, abnormalities of the angle may be found in patients with a diagnosis of Axenfeld–Rieger syndrome, Peters anomaly, and iris hypoplasia. These conditions are all associated with raised intraocular pressure and an increased incidence of glaucoma with around 50% of patients developing glaucoma. Identification of the genetic changes underlying ASD has gradually led to the recognition that these conditions are part of a disease spectrum.[3]

As long ago as 1925 a German ophthalmologist, Karl Axenfeld described congenital angle anomalies with iris strands, and by 1935 an Austrian ophthalmologist, Herwigh Rieger characterised Rieger syndrome as a dominantly inherited condition involving these eye anomalies together with dental anomalies (OMIM 180500). Since then, the description of similar ASD in different families lead to a series of clinical classifications describing similar conditions, for example, Rieger syndrome, iridogoniodysgenesis, iris hypoplasia, Rieger anomaly, Axenfeld anomaly, glaucoma iridogoniodysplasia, and Axenfeld–Rieger anomaly. About 80 years after it was first described, Elena Semina *et al* characterised the first gene mutations causing dominantly inherited Rieger syndrome in the *PITX2* gene on chromosome 4q25.[4] However, mutations in the *PITX2* gene are not the sole cause of ASD; at least four other gene loci have been identified on 6p25, 13q14 and 16q24, and 11p13.[5–7] The genes *FOXC1* and *PAX6* at 6p25, and 11p13 respectively have been identified,[8,9] but the others remain elusive. Nonetheless, these and other studies have firmly established the fact that is ASD genetically heterogeneous (Table 1); more than one gene causes the

same clinical condition. For example, mutations in *FOXC1* and *PITX2* lead to ocular malformations that are indistinguishable from one another. Conversely, each gene causes what had previously been described as more than a single condition. Therefore, the umbrella term Axenfeld–Rieger syndrome (ARS) is best applied to the range of conditions with overlapping clinical features[3] most commonly associated with *PITX2* and *FOXC1* mutations.

One of the best examples of a single ocular gene causing a range of clinical conditions is the *PAX6* gene, first identified as the gene for aniridia (lack of iris),[10] but now known to underlie a range of other ocular conditions[11] including Peters anomaly and a rare case of ARS.[7,12,13] As well as causing ARS, *PITX2*, and *FOXC1* mutations have been demonstrated in cases of Peters anomaly and primary congenital glaucoma (PCG).[9,14,15] Peters anomaly has also been associated with mutations in two other genes, the *CYP1B1* gene, commonly mutated in PCG and the FOXC1 related gene, *FOXE3*.[16] These findings indicate that PCG may also be considered as part of the ASD spectrum affecting common and interacting genetic pathways.

It is also common for a single gene mutation within an affected family to show variable expression between different individuals.[15] For example, in a family that we recently studied, of three individuals with the same *PITX2* mutation, the phenotype ranged from normal pressure and iris hypoplasia, to polycoria, corneal opacity, and glaucoma.[17] While it is not always possible to predict confidently the type of genetic change underlying a specific patient phenotype seen in the clinic, sets of likely contenders are emerging for each condition. In the future, ophthalmologists may find the non-ocular disease features in the ASD patient, or in other family members, increasingly useful for genetic diagnosis. The association between ASD and dental hypoplasia, first described by Rieger has most commonly been found with *PITX2* gene mutations although the dental anomalies have not been systematically described. Rarely, *FOXC1* mutation has been found in patients with dental malformations.[14] In our recent study, we classified the tooth abnormalities in patients with *PITX2* mutation, and compared these with other reported cases in order to catalogue the most frequently lost teeth and common malformations associated with ARS[17]; see also www.phenodent.org for diagnosing dental defects.

Expression of ASD genes

Analysis of patterns of gene expression in animal models, particularly the mouse, as well as the limited number of studies possible on human tissue, are important for indicating the role of ASD disease genes in development of specific tissues. In the mouse, *Pitx2* gene-specific expression in the developing face and the dentition,[18] correlates with the observed dental anomalies seen in patients. In embryogenesis, the *Pitx2* gene has a range of prominent functions notably a role in left right asymmetry and its absence causes anomalies in heart, lung, and pituitary development.[19,20] At least three different Pitx2 protein isoforms exist, but only the Pitx2c transcript is expressed asymmetrically and has been demonstrated to be involved in the generation of laterality.[21] The Pitx2a isoform is most prevalent in the developing eye and to date variations in gene expression have not been related to the frequently observed asymmetric nature of ASD. One intriguing disease feature is that patients with *PITX2* mutation often present with abnormal belly buttons, due to a failure of involution of the pre-umbilical skin post-natally.[4,15] In mouse models that completely lack *Pitx2* function there is failure to close the ventral body wall of the embryo leaving the heart and abdominal organs exposed,[19] and this seems to represent the severe end of a malformation spectrum ranging to the mild umbilical phenotype seen in patients with heterozygous mutations.

One of the other interesting sites of *Pitx2* expression is the developing brain,[22] although its function in the brain is not well studied due to the embryonic lethality of homozygous *Pitx2* mutations;. However, analysis of the embryonic brain of mice lacking Pitx2 function suggests that *Pitx2* regulates neuronal differentiation in the developing ventrolateral thalamus and superior colliculus of the midbrain.[22] We recently examined a family with a heterozygous *PITX2* mutation and found brain abnormalities including an enlarged cisterna magna together with an executive skill deficit, which supports the idea that this gene may be important for brain development.[17] The mutation in the homeobox was predicted to abolish DNA binding activity and act in the same way as many other reported mutations. Learning difficulties have also previously been reported in association with a *PITX2* mutation.[15] It therefore seems likely that a brain pathology may be a feature associated with *PITX2* mutations. Hence, it may be important to note associated dental and umbilical features with ARS syndrome as these patients are most likely to carry *PITX2* mutation and may also have impaired cognitive ability and need special referral.

Clinical reports of ARS describe a range of associated features, including anal stenosis, hypospadias, growth hormone deficiency, congenital heart defects, hearing defects, hydrocephaly, and psychomotor retardation (OMIM 602482, 109120, 601499, 180500). The genetic basis of ARS in these reports is unknown and more work needs to be done to establish whether these associated disease features are caused by specific genes. With future research, a patient's genetic diagnosis

may provide a useful indicator of the risk of other clinical complications.

Molecular mechanisms: How *PITX2* and *FOXC1* mutations affect protein function?

Novel insights have been gained over the last decade through the investigation of the function of the key proteins encoded by the ASD disease genes. Specifically, exploration of the normal function of these proteins during development and analysis of how mutations perturb the normal developmental processes has provided a focus for understanding the aetiology of ASD.

The prevalent class of ASD gene encode developmental transcription factors. These genes are expressed in precisely regulated spatial and temporal patterns during embryonic development and act via DNA–protein interactions to control the transcription of other downstream target genes and thereby orchestrate programs of cell migration and differentiation. The genes most commonly associated with ASD are *PITX2* and *FOXC1* and these have been extensively studied.[23] FOXC1 is a member of the fork head transcription factor family[24] and PITX2 is a *bicoid*-like homeodomain protein. The crystal structures of the DNA-binding domains in these two types of transcription factor have been characterised; last year the first structure of a PITX2–DNA complex was elucidated, showing how this protein recognises and binds to specific DNA sequences.[25] The effect of disease-causing mutations on protein structure and function can be modelled. In addition, the function of mutant protein can be compared with normal protein in biochemical assays of transcription factor function.[23,26] *In vitro* assays typically test two functions, the ability of a transcription factor protein to bind to DNA and the ability to *trans*-activate the transcription of a reporter gene by binding to a cognate DNA sequence. Intriguingly mutations have been found, which both decrease and increase *trans*-activation activity *in vitro*.[27,28] These studies indicate that more than enough activation of downstream target genes can be as detrimental as a lack of regulation of downstream target genes.

A second disease-causing mechanism acts to cause alterations in the level of functional protein. Chromosomal deletions including the *PITX2* and *FOXC1* genes leading to haploinsufficiency have been documented as causes of ARS.[29,30] In addition, several studies have demonstrated that duplication of *FOXC1* can equally cause an ARS phenotype.[31,32] Overexpression of *Pitx2* in animal models also causes ARS type phenotypes.[33] The important question of how common a disease causing mechanism this is, remains to be established.

The problem of why both these genes cause the same condition, was partially answered in an elegant study that showed that the PITX2 and FOXC1 proteins interact with each other.[34] *In vitro* assays demonstrated that PITX2 binds to FOXC1, and can act to repress activation of putative FOXC1 target genes. Loss of PITX2 function in such cells would lead to activation of repressed FOXC1 targets. Intriguingly, this would perhaps be synonymous to a *FOXC1* gene duplication causing increased levels of FOXC1 protein in a single cell that may also act to overcome PITX2 repression. Identification of the downstream target genes regulated by the PITX2 and FOXC1 transcription factors will provide deeper understanding of the ASD tissue defects and the interplay between these two regulators. To date, *in vitro* studies have suggested that genes expressed in the cornea and encoding enzymes responsible for hydroxylysing lysines in collagens (procollagen lysyl hydroxylase PLOD1 and 2) may be regulated by PITX2.[35] In the periocular mesenchyme and the cornea, the secreted signalling molecule FGF19, a member of the fibroblast growth factor family seems to be regulated by FOXC1 and reduced fgf19 activity in zebrafish gives rise to an ASD phenotype.[36]

In summary, two distinct molecular genetic mechanisms have emerged that interfere with the level of transcription factor activity during anterior segment development, altered gene dosage and gain or loss of function mutations. If the level of transcription factor activity falls below, or increases above a critical level, then normal development is disrupted. As the extent of perturbation varies between individuals, other interacting factors must also influence these critical developmental processes.

Developmental mechanisms of anterior segment dysgenesis

The study of *Pitx2* and *Foxc1* in mouse models using modern genetic techniques to generate conditional deletions of gene function is elucidating the genetic pathways disrupted in ASD. In light of new research, it is timely to reassess the commonly cited model proposed by Shields[37] 'that a developmental arrest, in the third trimester of gestation, of tissues derived from the neural crest cells accounts for the ocular and most of the non-ocular abnormalities in this group of disorders'. Analysis of human histopathology specimens indicated retention of a primordial-like endothelium on the iris and in the iridocorneal angle, and showed compacted TM tissue[37].

The view that the ASDs are neural crestopathies is based largely on classical embryology studies, in which quail neural crest cells were grafted into the chick

embryo and their contribution to anterior segment structures monitored.[38,39] These lead to the model that much of the anterior segment derives from neural crest cells and developmental arrest of these cells causes the ASD pathology in humans. Similar fate mapping studies had not been carried out in the mouse or in any mammalian model until more recently.[40,41] In fact, it seems that there are significant differences in the contribution of cells to the anterior segment structures between mouse and chicks, and the human situation is probably closer to the mouse. In mouse, but not in chick, mesoderm-derived progenitors contribute to the anterior segment, particularly in the corneal stroma[40] (No. 30). The identity of these cells and their role has not been established, but the fact that they express *Pitx2* raises the possibility that non-neural crest cells may contribute to the primary anterior segment pathology, in addition to neural crest-derived cells.

In addition to similarities, there are also differences in the expression patterns of *Pitx2* and *Foxc1* during eye development.[40,42] It is striking that both genes are expressed very early in embryogenesis, equivalent to the first trimester of human development (Figure 1a). Both genes are not expressed in neural crest cells until they adopt a periocular location, hence they do not serve any function in regulating the migration of neural crest cells from the crest of the closing neural tube to the eye primordia. At E10.5 in the mouse (equivalent to week 5 of human gestation) *Pitx2* is activated in the anterior-most cells, whereas *Foxc1* is most strongly expressed in posterior periocular cells around the optic stalk.[40] Expression of both genes subsequently spreads around the periocular mesenchyme. Importantly, *Pitx2* and to a lesser extent *Foxc1* is also expressed in progenitors of mesodermal origin in the presumptive anterior segment. *Pitx2*, but not *Foxc1* is expressed in the developing extra ocular muscles, which fail to develop in the homozygous $Pitx2^{-/-}$ mice,[43] although this phenotype has not yet been associated with *PITX2* mutation in patients. There are however clinical reports of absence of ocular muscles.[1,44,45] Interestingly in heterozygous $Pitx2^{+/-}$ mice, superior, and inferior oblique muscles are affected, but the rectus muscles are less affected showing a differential effect of the gene dose.[46] Later expression differences include the fact that *Pitx2*, but not *Foxc1* is expressed in the corneal stroma; whereas both genes are expressed in the trabecular meshwork[42] (Figure 1b).

Activation of *Foxc1* and *Pitx2* in the presumptive anterior segment is a critical event in specifying the mesenchymal progenitor population that will give rise to the different angle tissues. Inductive signalling from the anterior lens epithelium has been shown to be important in anterior segment development. Anterior lens epithelial cells grafted in chick embryos induced adjacent mesenchyme cells to differentiate into corneal endothelium.[47] One of the factors implicated in activating the anterior segment transcription factors is the secreted signalling molecule TGF-β expressed in the early lens vesicle. Loss of TGF-β receptor activity only in the neural crest cells (by a process of genetic engineering referred to as conditional gene ablation) caused loss of activation of *Foxc1* and *Pitx2* in the presumptive anterior segment tissue,[42] suggesting that these progenitor cells are dependent on signalling from the lens. Loss of TGF-β receptor activity as well as loss of another member of the TGF-β growth factor family, Bmp4 in animal models, both cause anterior segment malformations similar to ARS[42,48] indicating these factors are essential for induction of anterior segment development. Significantly, mutations in several lens-specific genes, for example *MAF* and *PITX3* cause ASD in combination with cataract formation.[49,50] In such cases, the anterior segment anomalies likely arise via failure of normal induction of anterior segment tissue via lens signalling factors. *PITX3* and *PITX2* are highly related homeobox genes, which arose via ancient gene duplication events; their divergent expression in lens *vs* anterior ocular mesenchymal tissue, and sequential activity of anterior segment induction followed by specification of anterior segment differentiation provides an intriguing example of co-ordinated divergence of gene function during evolution.

Analysis of the function of ASD genes in animal models can provide a comprehensive understanding of the embryonic origin of the malformations. In fact, a mouse model of ASD caused by *Foxc1* mutation, called *congenital hydrocephalus* was first described by the pioneering mouse embryologist Hans Grunenberg.[51] But only after characterisation of the first *FOXC1* human gene mutation was the mouse model studied to understand the disease. Homozygous loss of function of both *Pitx2* and *Foxc1* cause embryonic lethality in mice and the same is likely to be true for humans. Study of embryos completely lacking either *Foxc1* or *Pitx2* show profound failure in the normal process of differentiation of the anterior segment structures.[43,52,53] Although the optic cup and lens vesicle form, the anterior progenitor cells fail to organise and differentiate to produce a corneal stroma and endothelium. Extrusion of lens tissue into the primitive anterior tissue or adhesion between the lens and the posterior cornea are found, reminiscent of Peters anomaly. These primitive arrested cells extend to the angle between the cornea and the developing iris, and are likely the origin of adhesions between cornea and iris that arise in the heterozygous state, as seen in ARS. At the angle, progenitors also

fail to differentiate to produce the drainage structures, the trabecular meshwork and Schlemms canal. Without formation of the corneal endothelium, a separate anterior chamber between the cornea and the lens fails to form. Since such studies show the absolute requirement of these genes for normal differentiation of the angle progenitor cells; in humans lacking normal levels of functional *PITX2* or *FOXC1* transcription factor a degree of failure of these same processes probably occurs.

One of the intriguing features of the phenotypes of *FOXC1/PITX2* mutations is the variability in phenotype. Clear evidence of the contribution of other modifying genetic factors was provided by analysis of the ASD in mice heterozygous for *Foxc1* in different genetic backgrounds.[54] Iris malformation ranged from normal to severe corectopia and polycoria. Similarly, the effect on the trabecular meshwork was variable, ranging from normal to severely hypoplastic. The direct role of these genes in development of the trabecular meshwork and the variable expression of mutations suggest that these genes may be critical for the development of normal drainage structures and maintenance of healthy intraocular pressures and hence that variations in the function of these genes could predispose to raised pressures and glaucoma susceptibility. Combining mutations of *Foxc1* with mutations of the tyrosinase gene (*Tyr*) (an enzyme essential for pigment production) caused a more severe ASD, and similarly lack of *Tyr* and the congenital glaucoma gene *Cyp1b1* caused a worsening of the angle phenotype.[55] Such studies are beginning to identify the network of modifying factors that are essential for normal angle development and when perturbed cause ASD and developmental glaucoma.

Summary

Study of ASD is revealing the genetic pathways concerned with embryonic development of the anterior segment. The most common genetic causes of ARS have been identified as mutations affecting the function of the *PITX2* and *FOXC1* transcription factors, although additional chromosomal loci are also implicated. ARS is now generally accepted to be a single disease spectrum with genetic heterogeneity and variable expression. *Pitx2* and *Foxc1* genes are activated in periocular mesenchymal cells early in development, at stages equivalent to the first trimester of pregnancy, via secreted signalling from the lens. Precise levels of PITX2/FOXC1 transcription factor activity are critically required in the mesenchymal progenitor cells that give rise to the angle drainage structures, and the corneal stroma and endothelium. Further study of the downstream targets regulated by these transcription factors particularly in the cornea and trabecular meshwork may aid in understanding and treatment of the serious sight threatening conditions of glaucoma and corneal opacities associated with ASD.

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