Molecular genetics of Axenfeld–Rieger malformations

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Axenfeld–Rieger (AR) malformations are autosomal dominant developmental defects of the anterior segment of the eye, and often result in glaucomatous blindness. AR malformations are associated with mutations in two transcription factor genes (PITX2 and FOXC1) expressed throughout eye ontogeny. Studies of disease-associated mutant proteins have provided insights into the aetiology of AR malformations, while delineating residues and domains important to DNA binding, transactivation and nuclear localization. The availability of mouse models for both PITX2 and FOXC1 has allowed detailed study of their expression and mutant phenotypes. Dissection of the normal functions and domain structures of these factors will aid in future elucidation of how alterations of the developmental program produce the dysgenic phenotypes seen in AR. There are at least two AR loci still awaiting molecular cloning on chromosomes 13q14 and 16q24. Identification of further genes implicated in aberrations of human ocular development will advance our understanding of the mechanisms whereby pattern is established in the eye, and may be of clinical value in treating the glaucoma that is the most serious consequence of AR malformations.

Anterior segment dysgenesis of the eye is a genetically heterogeneous spectrum of clinical disorders (1–8) that result from malformation of neural-crest-derived endothelial tissues (9–12), conferring a high risk of blindness due to glaucoma. The diagnosis of Axenfeld–Rieger (AR) malformation refers to a constellation of dominantly inherited ocular findings, including anomalies of the anterior chamber angle and aqueous drainage structures (iridogoniodysgenesis, IGD), iris hypoplasia (IH), eccentric pupil (corectopia), iris tears (polycoria), and iridocorneal tissue adhesions traversing the anterior chamber (13–16). Iris strands are frequently fused with Schwalbe’s line, which may appear as a prominent, centrally displaced annular ridge (posterior embryotoxon) (17). The corneal endothelium and Descemet’s membrane may also be absent in affected eyes (18). The corneal stroma is sometimes centrally opaque in AR malformation, perhaps due to edema associated with permeability of the endothelial barrier (7,18,19). Syndromic features can also occur in conjunction with these eye anomalies, and are seen with incomplete penetrance and variable expressivity. The systemic AR malformation phenotype variously includes any of small, missing or absent teeth, redundant periumbilical skin, sensory hearing loss, congenital heart defects, and skeletal limb anomalies (Fig. 1) (20–23). The gross changes in eye morphogenesis in AR malformation are highly penetrant and produce an approximately 50% risk of glaucomatous optic nerve damage and visual field loss. Dysgenesis of the trabecular meshwork (TM) likely reduces aqueous outflow facility, increasing intraocular pressure and placing stress on the optic nerve head, leading to glaucoma.

AFFECTED OCULAR STRUCTURES SHARE A NEURAL CREST LINEAGE

It has long been suspected that both the ocular and systemic AR phenotypes result from disruption of migration and differentiation processes specific to the neural crest (NC) lineage (12,24). Cells derived from the embryonic NC produce an uninterrupted endothelium that lines the anterior chamber until the seventh or eighth month of gestation, forming each of the mature connective tissues between the lens and the corneal endothelium (9–11). Differentiation of this layer is intertwined with the normal development of aqueous drainage structures (TM, juxtacanicular tissues and Schlemm’s canal) needed to establish intraocular pressure (IOP) homeostasis.

The development of aqueous outflow facility is a gradual process, coinciding with progressive recession of the reticular, NC-derived angle mesenchyme covering the angle apex and embryonic TM (9). Observed anomalies of this developmental process in AR malformation include high insertion of the iris root over the TM, with sheet-like adhesions over the angle, or presence of excessive ‘wooly’ mesenchyme at the angle (12,16,25–28). Physical occlusion of the angle structures is not prerequisite for elevated IOP and glaucoma in AR malformations.

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malformation, suggesting that functional anomalies of the angle drainage structures themselves might also occur (22,29).

Components of the cornea and iris are also derived from the embryonic NC (11). In the chick, anterior chamber development is prefaced by three successive waves of invasion, during which migrating, NC-derived mesenchymal cells at the optic cup rim insinuate themselves between the lens placode and overlying surface ectoderm. These movements define the prospective corneal endothelium, the corneal stroma and the anterior iris respectively.

**GENES ASSOCIATED WITH AXENFELD–RIEGER SYNDROME**

There are currently two genes encoding transcription factors, in addition to at least two incompletely defined genetic loci, that are associated with the AR malformation phenotype. The following is a brief review of the molecular features of these factors, concluded by a brief discussion of immediate avenues open to future exploration in the study of anterior segment dysgenesis.

**PITX2 (pituitary homeobox transcription factor 2)**

PITX2 (RIEG1) was positionally cloned by Semina and colleagues as the gene underlying a Rieger syndrome locus on chromosome 4q25 (1,3,29–32). PITX2 is a member of the paired-bicoid family of homeodomain transcription factors, defined by the presence of a lysine residue at position 50 of the third helix of the homeodomain (HD). This residue confers specificity for two bases (CC) following a core target sequence (TAAT) common for all homeodomains (HD) (33–35). There are three (a, b and c) alternate transcripts of PITX2, encoding proteins of 271, 317 and 324 amino acids, respectively (36,37). The deduced PITX2 isoforms contain the same homeodomain and C terminus but are encoded by different combinations of the first four N-terminal exons. The largest of these isoforms (c) is transcribed from a distinct promoter (36).

Numerous pathologic mutations in PITX2 have been described, producing a continuum of clinical phenotypes (Fig. 2) including AR malformation, IGD and IH (1,3,5,38–40), as well as rarer cases of a Peter's-like anomaly (7). Null alleles (deletions) of the gene point to PITX2 haploinsufficiency as a model for AR malformation, although chromosomal rearrangements some 90 kb from the gene may also be
pathological (41). Characterized patient PITX2 mutations imply a preliminary genotype–phenotype correlation between the severity of clinical defect(s) and proportion of normal PITX2 transactivation activity remaining in affected individuals (42). DNA-binding and reporter transactivation assays illustrated that IH alleles retained some activity (37% of normal), while alleles causing AR malformations were transcriptionally inactive. Similar functional studies have unearthed a hypermorphic missense allele (V45L in the homeodomain) of the PITX2 recognition helix that shows decreased DNA binding but greatly enhanced transactivation (39). Interestingly, this mutation produces a syndromic AR malformation phenotype, suggesting that increased as well as reduced PITX2 activities are deleterious during development. Localization of PITX2 may also be impaired by homeodomain mutations. A patient R53P mutation partially mislocalizes PITX2 to the cytoplasm, either because of destabilization of the homeodomain or as a result of loss of a potential nuclear localization signal (NLS) within the HD, homologous to the position of the NLS of the distantly related homeodomain gene PDX-1 (42).

These experiments and others (43) have supported the results of in silico threading predictions of patient PITX2 homeodomain mutations (44), in which a number of distinct mechanisms were suggested. It was proposed that substitution of the leucine residue at homeodomain position 16 with any other amino acid may disrupt hydrophobic packing interactions necessary to form a HD, while the arginine residues at positions 21 and 46 might mediate repulsive electrostatic interactions needed to properly position helices two and three for DNA binding. The original modeling analysis of the PITX2 T30P missense mutation found no significant change in the homeodomain threading energies of this mutant (44). However, while the DNA-binding potential of the mutant protein remains controversial (42,43,45), the T30P allele is transactivation-defective (42,43,45,46).

A C-terminal, 14-amino-acid domain of PITX2 has been found to inhibit DNA binding in a manner dependent on the N terminus of the protein. This presumably autoregulatory interaction is relieved by binding of the pituitary homeodomain protein (PIT1) at the C terminus (47). A mutation of the lysine residue (K50) that confers DNA-binding specificity produces a dominant-negative allele of PITX2 that abrogates PIT1 synergism (45). Although PIT1 appears to be expressed exclusively in the anterior pituitary, it seems possible that other relevant transcription factor(s) could interact with PITX2 via a similar mechanism in the eye.

**Pitx2 mouse model**

The mouse Pitx2 and human PITX2 genes are very highly conserved, with identical HD peptide sequences and only two amino acid differences throughout the (α) isoform (1). In situ hybridization and immunofluorescence studies of murine Pitx2 expression have confirmed overlap of the mRNA distribution and the organs affected in AR malformations, with Pitx2 expression being detected in the periorcular mesenchyme,
umbilicus, heart, gut, dental epithelia and limb bud, as well as in Rathke’s pouch of the forming anterior pituitary (1,48,49). The ocular distribution of embryonic mouse Pitx2 protein progresses from the periciliar mesenchyme to include some layers of the cornea and iris, as well as the hyaloid plexus found posterior of the lens, notably excluding the NC-derived optic cup and stalk (49). After E18.5, Pitx2 eye expression is confined to the iris.

Targeted gene disruption approaches have been used to produce both hypomorphic and null alleles of Pitx2, causing a pleiotropic murine phenotype that overlaps with the Pitx2 expression pattern and provides a good model for human AR malformation (50–52). About one in ten heterozygous mice display corectopia and iris tears, a reduced but significant proportion relative to the highly penetrant ocular dysgeneses of human AR malformation (50). Some heterozygotes are small-bodied, perhaps corresponding to rare reports of human pituitary insufficiency in association with AR syndrome (20,53). Homozygosity for null or hypomorphic alleles of Pitx2 is lethal at day 10 of gestation, with features including incomplete neural tube closure, lung isomerism and failed heart septation. Similar septal defects of the heart atria have been observed in conjunction with AR malformation, and while this is a heterozygous phenotype in humans, heart defects may be situated at the severe end of the AR malformation phenotypic spectrum (23,54,55). Pitx2 is an important director of left-right body plan symmetry, and participates in a sonic hedgehog/nodal/lefthanded signaling pathway of abdominal organs, including the heart and viscera (56–61). There is currently no evidence that left-right axis defects are a feature of AR malformation.

PITX2 is also involved in the interpretation of global morphogens during odontogenesis. Very recently, PITX2 was identified as a coexpressed transactivator of procollagen lysyl hydroxylase PLOD, implying that the ocular and dental malformations observed in AR malformation may be related to the Ehlers–Danlos syndrome phenotype of PLOD1 mutations (46). Additionally, PITX2 activates expression of the distal-less homeodomain protein Dlx2, with which it is coexpressed in the dental epithelium during tooth development (62). The regulation of Pitx2 itself in the mandibular epithelium is defined by antagonistic signals mediated by the global regulators Bmp4 (inhibitory) and Fgf8 (activating) during odontogenic site selection (63). The dental and/or cardiac features of syndromic AR are likely entwined with abnormal interpretation of asymmetrically expressed global morphogen signals such as BMP4 and FGF8.

FOXC1 (forkhead box transcription factor C1)

The forkhead family of transcription factors are distinguished by a highly conserved DNA-binding domain (the forkhead domain), initially identified on the basis of homology between the Drosophila forkhead (64) and rat hepatocyte nuclear factor 3 (HNF3) (65) genes. The Forkhead-related factors are directors of morphogenesis that have a plurality of distinct roles in development. Expression of forkhead homologues at different points through development has ramifications on the early establishment of embryonic cell layers, determination of cell fates in blood and other tissues, and the control of organogenesis (66–69).

In 1994, Pierrou et al. (70) reported the cloning of seven human forkhead orthologues from a craniofacial cDNA library. One such gene, FREAC-3 (now called FOXC1), was mapped to a subtelomeric region at 6p25 (71). FOXC1 has been implicated as the gene underlying a cluster of loci for AR malformation, IGD and related disorders, leading to the recognition that FOXC1 mutations produce a spectrum of allelic anterior segment disorders associated with glaucoma (Fig. 2B) (4,6,72–77). Duplications of varying extents on the 6p subtelomere cause IGD, a phenotype similar to the AR malformations that result from FOXC1 frameshift or missense mutations, suggesting an upper threshold on the appropriate level of FOXC1 activity in vivo (75,78). Targeted mutations of the FOXC1 gene in mice indicate that AR-like ocular defects can occur by haploinsufficiency, and manifest with varying penetrance depending on genetic background (19,79), consistent with the AR malformation phenotype of a number of 5′ frameshift mutations abolishing the forkhead domain in humans (75). While extensive genotype–phenotype correlations are lacking at present, missense alleles displaying as much as 56% of normal transactivation in synthetic reporter assays produce AR malformations in heterozygous patients (80). The normal developmental window of FOXC1 activity may therefore be very narrow indeed.

FOXC1 is expressed nearly ubiquitously, primarily as a 3.9 kb transcript that is highly expressed in heart, liver, muscle and kidney (70). FOXC1 is also present during eye organogenesis, with expression in the periciliar and pre-endothelial mesenchyme, as well as in the sclera and all layers of the developing cornea (6,19). Staining in the adult eye is most notable in the TM, consistent with AR angle malformations, and is also found in the conjunctival epithelium (19).

Domain structure of FOXC1: the forkhead domain

Members of the forkhead transcription factor family bear a conserved 110-amino-acid forkhead domain (FHD) that mediates binding to target DNA sequences. The FHD forms a ‘winged helix’ structure consisting of a three-helix bundle packed against two loop regions that reside C-terminal of the third helix (81). The third ‘recognition’ helix orthogonally crosses the DNA helical axis, forming sequence-specific contacts with the major groove of the core target sequence GTATA[T/c]AAA (70,81). Additional protein–DNA contacts are provided by the second wing region, which inserts lengthwise into the minor groove immediately 5′ of the core sequence. The FHD binds DNA as a monomer, and circular permutation DNA binding assays produce observations consistent with kinking of the target DNA when bound to FOXC1 (70). The current understanding of the functional domain structure of FOXC1 is outlined below and shown diagrammatically in Figure 2B.

Patient mutations of the forkhead domain have provided a means of dissociating the DNA-binding, nuclear localization and activation functions of this region of FOXC1, given sensitive in vitro assays to assess each of these functions separately. Notably, two patient missense mutations in the forkhead domain (F112S and I126M) decreased transactivation potential in vitro but retained DNA-binding activity (80). The F112S mutation, which resides in the short loop directly...
N-terminal of the specificity helix, is not likely to alter protein-DNA contacts and may interfere with activating protein interactions. The I126M mutation appears to alter the binding specificuty but not the affinity of helix 3-DNA contacts.

Transcriptional activation and inhibition domains

Recent studies of recombinant FoxC1 deletion constructs have begun to unmask the domains involved in the mediation and regulation of transactivation by this factor (82). Transactivation by FoxC1 requires both a novel N-terminal activation domain and a C-terminal, glutamine/bulky hydrophobe-rich activating region, harbored by residues 1–51 and 435–553, respectively (82). The activity of these domains is attenuated by a phosphorylated inhibitory domain (ID) between residues 215 and 366, which is not intrinsically repressive to transcription but does oppose the function of activation domains in synthetic activator–FoxC1 fusion proteins (82). Phosphorylation of FoxC1 alters its trypsin digest pattern, hinting at the likelihood of regulation via conformational change. Altered conformation may affect the availability of the activating domains of FoxC1 for putative protein–protein contacts, implicating kinase and/or phosphatase involvement in FoxC1 regulation. Identification of such regulatory molecules may aid in the delineation of signaling pathway(s) relevant to the development and function of the eye.

Nuclear localization

FoxC1-green fluorescent protein (FoxC1–GFP) fusion proteins localize entirely to the cell nucleus only if a novel C-terminal FoxC1 NLS (residues 168–176) is included. A second nuclear localization accessory domain in the HD (residues 77–93) is required for full localization of FoxC1 (82). As no patient mutations noted to date appear to interfere exclusively with nuclear localization of FoxC1, the etiologic significance of FoxC1 nuclear import remains to be determined.

FoxC1 mouse model

The mouse orthologue FoxC1 (formerly Mf1) has been disrupted in targeted gene approaches, and a homozygous nonsense mutation of FoxC1 is also responsible for the lethal, congenital hydrocephalus phenotype of the ch mouse (79,83,84). The study of such models has had mixed results. FoxC1 heterozygotes do have ocular findings like those of human AR malformation, including TM abnormalities and SC hypoplasia. However, malformation of these structures is confined to sectors of the murine eye, and does not produce either elevated IOP or glaucoma (79). Nonetheless, a number of other interesting findings in mouse may warrant pursuit in humans. Interestingly, angle malformations of other interesting either elevated IOP or glaucoma (79). Nonetheless, a number of conhuman AR malformation, including TM abnormalities and SCclusion malformations, but instead have been associated with adipocyte metabolism abnormalities producing lymphedema–distichiasis syndrome (OMIM 153400), implying that there may be key differences in the function of human and mouse FoxC2 (88,89).

FoxC1–/– homozygotes have a perinatally lethal skeletal phenotype (83) that includes failed separation of the primordial cornea and iris, leading to absence of the anterior chamber (19). The corneas of such mice are abnormally thick, show disorganized collagen fibers, and do not develop a flattened, differentiated endothelium with proper occluding junctions. Transforming growth factor α (TGF-α) expression can drive the chemotactic invasion of NC-derived cells from the pericircular mesenchyme (90), where FoxC1 is abundantly expressed during development (6,19). In mice ectopically expressing either TGF-α or epidermal growth factor (EGF) in the lens, the corneal endothelium fails to differentiate, leading to an ocular phenotype similar to that of FoxC1–/– mice (91). Such studies suggest that anterior chamber formation requires the prior establishment of the endothelial layer, and signal interactions between the lens ectoderm and NC-derived anterior tissues appear integral to this process. In any case, these observations in the mouse support the view that the corneal edema and opacity observed in AR as well as in Peter’s anomaly may be due to altered growth factor signaling between the primordial anterior lens and posterior cornea.

Other loci: PAX6 and MAF

Prevalence studies of FoxC1 and PITX2 suggest that there are likely other candidates, remaining to be cloned, that contribute to the locus heterogeneity of anterior segment dysgenesis. A mutation of the PAX6 paired-class transcription factor gene on 11p13 has been described in an isolated case of AR malformation (8). Mutations of PAX6 more commonly produce aniridia (92,93), as well as malformations of anterior structures, including the cornea (96,97).

Linkage analysis and chromosomal rearrangements indicate the presence of additional AR malformation loci at 13q14 and 16q24 that remain to be cloned (29,89). The 13q14 locus may comprise a clinically distinct disorder including both AR and sensorineural hearing loss (55). FoxC2 was previously considered a logical candidate for the 16q locus, based on its position, its significant homology to FoxC1 and the phenotype of mouse FoxC1+/–, FoxC2+/– double heterozygotes. However, mutations of FoxC2 appear to produce lymphedema syndromes rather than AR malformations (88,89,100). Recently, a second relevant candidate has been identified at 16q. Mutations of the MAF transcription factor gene on 16q23.2 cause cataract, corneal opacity and microcornea, iris coloboma, and anterior segment dysgenesis (101). MAF is normally expressed in surface ectodermal components of the embryonic eye (i.e. the lens placode and vesicle), suggesting a lens-specific signaling defect blocking anterior chamber formation. Signals generated by the lens epithelium are required for induction of the corneal endothelium – itself a prerequisite for anterior chamber formation in animal models (91,102). Thus, it is possible that defect(s) of lens–cornea signaling might account for some proportion of AR malformation.
Members of the Maf family of leucine zipper proteins (including NRL) are upregulated by and transcriptionally synergize with PAX6 (103–105). Additionally, deletion of a portion of the Pitx3 promoter containing a MAF response element produces absence of the lens and anterior chamber in the aphakia mouse (106). The iris coloboma and anterior segment features produced by MAF mutations grossly resemble the corectopia of anterior segment dysgenesis, consistent with a suggestion that MAF may be a good candidate for an AR-like clinical disorder at 16q. Future developments in this area will therefore be watched with interest.

CONCLUSIONS

Study of the genetic basis of ocular disorders such as AR malformation will inevitably advance our understanding of the genetic pathways of ocular development. Simultaneously, the syndromic features of this disorder may indicate developmental and mechanistic ties between the eye and other organ systems. It seems likely that PITX2 and FOXC1 play equally important roles in the development of the eye, given that mutations of either gene produce virtually identical phenotypes. It will be interesting to determine if this phenotypic resemblance reflects either coordinate regulation or function of these factors. The study of genetic diseases such as AR malformation may further aid in the development of prospective therapies for common ocular diseases such as glaucoma.

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REFERENCES


38. Flomen, R.H., Vatcheva, R., Gorman, P.A., Baptista, P.R., Groet, J., fi


