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Brad A. Amendt

The Molecular Mechanisms of Axenfeld-Rieger Syndrome

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Brad A. Amendt, Ph.D.
The Texas A&M University System
Health Science Center
Institute of Biosciences and Technology
Center for Environmental and Genetic Medicine
Houston, Texas, U.S.A.

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Dedication

We are indebted to the families and individuals who generously provided material for the studies described in this book and to the many geneticists for identifying and counseling patients with Axenfeld-Rieger syndrome. Finally to the members of my laboratory, both past and present, for their excellent work on the molecular basis of Axenfeld-Rieger syndrome.

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EDITOR

Brad A. Amendt, Ph.D.
The Texas A&M University System
Health Science Center
Institute of Biosciences and Technology
Center for Environmental and Genetic Medicine
Houston, Texas, U.S.A.
Chapters 4, 8, 10

CONTRIBUTORS

Sally A. Camper
Department of Human Genetics
University of Michigan Medical School
Ann Arbor, Michigan, U.S.A.
Chapter 5

Michael A. Charles
Department of Human Genetics
University of Michigan Medical School
Ann Arbor, Michigan, U.S.A.
Chapter 5

YiPing Chen
Department of Cell and Molecular Biology
Tulane University
New Orleans, Louisiana, U.S.A.
Chapter 6

Philip J. Gage
Department of Human Genetics
University of Michigan Medical School
Ann Arbor, Michigan, U.S.A.
Chapter 5

Donna M. Martin
Department of Human Genetics
University of Michigan Medical School
Ann Arbor, Michigan, U.S.A.
Chapter 5

James F. Martin
Albert B Alkek Institute of Biosciences and Technology
Texas A&M University
Center for Cancer Biology
Houston, Texas, U.S.A.
Chapter 7

Igor O. Nasonkin
Department of Human Genetics
University of Michigan Medical School
Ann Arbor, Michigan, U.S.A.
Chapter 5

Darryl Y. Nishimura
Department of Pediatrics
University of Iowa
Iowa City, Iowa, U.S.A.
Chapter 2

Ruth Riise
Department of Medical Genetics
National Hospital
University of Oslo
Oslo, Norway
Chapter 3

Elena V. Semina
Department of Pediatrics
Human and Molecular Genetics Center
Medical College of Wisconsin
Milwaukee, Wisconsin, U.S.A.
Chapters 1, 9

Hoonkyo Suh
Department of Human Genetics
University of Michigan Medical School
Ann Arbor, Michigan, U.S.A.
Chapter 5

Ruth E. Swiderski
Department of Pediatrics
University of Iowa
Iowa City, Iowa, U.S.A.
Chapter 2

Shusheng Wang
Department of Cell and Molecular Biology
Tulane University
New Orleans, Louisiana, U.S.A.
Chapter 6

Xueyan Yu
Department of Cell and Molecular Biology
Tulane University
New Orleans, Louisiana, U.S.A.
Chapter 6

PREFACE

We are excited to bring together recent research on the molecular biology of Axenfeld-Rieger syndrome (ARS) disorders. In the following chapters we will review and provide direct evidence for the molecular basis of this group of heterogeneous disorders, which include Rieger syndrome and Rieger anomaly. While ARS patients were initially diagnosed in the early 1930s the genetic basis for ARS was unknown until the recent identification of chromosomal loci associated with this genetic disorder. In the mid-1990s Drs. Jeffrey C. Murray and Elena V. Semina identified *PITX2* through positional cloning techniques as a gene associated with ARS. These researchers were able to identify point mutations in *PITX2* that were linked with ARS patients. ARS patients presented clinically with several developmental anomalies that further provided clues about the function of the *PITX2* homeobox transcription factor. The phenotypic variability of ARS patients indicates that PITX2 can participate with many other factors to control normal development processes. The hallmarks of ARS developmental anomalies are eye, tooth and umbilical defects. However, abnormal pituitary, heart, and craniofacial development are also detected. Thus, ARS patients provided the first link of *PITX2* involvement in the development of these organs and structures. Some of these anomalies are recapitulated in epigenetic and genetic mouse, chick, zebrafish and frog studies which will be reviewed in the following chapters. Clearly one of the most astounding features identified through genetic studies in mice, chicks, zebrafish and frogs is the laterality defects seen with overexpression or homozygous deletions of the *Pitx2* gene. We will discuss these findings in several chapters and relate them to defects associated with ARS patients. Several laboratories have provided direct evidence for the molecular basis of *PITX2* mutations using PITX2 target gene promoters in transfected cells. Furthermore, studies on the molecular/biochemical role of PITX2 provide important details as to its function.

Interestingly, other chromosomal loci have also been linked to ARS and these include chromosome 6,11, and 13. This is not surprising since approximately 40% of ARS patients do not harbor mutations in *PITX2*. The *FOXC1* gene (formally known as "*FREAC3*" and "*FKHL7*") has been directly linked to ARS; this gene is located on human chromosome 6. The implications of this gene in ARS and its associated mutations will be addressed in several chapters. Recently a gene located on human chromosome 11 was identified which harbors a deletion of the *PAX6* gene. The case history of this patient and the molecular findings will be reviewed. Linkage studies have identified chromosomal abnormalities associated with chromosome 13; however, the affected gene has not been identified. Thus, while *PITX2* mutations can account for the majority of ARS cases, clearly other genes can elicit similar defects. However, all three genes identified to date en-

code transcription factors that are expressed very early during embryogenesis and in cells that give rise to the affected organs and tissues. The following chapters will review the expression patterns and activities of these genes and their involvement in development processes.

Clearly, many researchers have contributed to the wealth of knowledge concerning ARS and the functional studies of the genes associated with this family of human genetic disorders. While it was impossible to have each laboratory or researcher write a chapter on their findings we have made every attempt to include their important discoveries. We apologize to those researchers who have provided critical data for the importance of genes involved in ARS that were not selected to write a chapter. However, our intention was to include their work and reference these researchers in this report on the molecular mechanisms of ARS. I would like to mention the contribution of Dr. Michael A. Walter's laboratory (University of Alberta, Edmonton, Alberta, Canada) to our current understanding of ARS. Another researcher who must be identified is Dr. Andrew F. Russo (Department of Physiology and Biophysics, The University of Iowa, Iowa City, IA) in whose laboratory the initial studies were performed on the molecular/biochemical basis of ARS. Dr. Russo is a valued colleague who has worked with me to research the transcriptional properties of PITX2.

We acknowledge Dr. Jeffrey C. Murray (Department of Pediatrics, The University of Iowa, Iowa City, IA) and his exceptional work on finding genes responsible for ARS. Dr. Murray started researching genetic linkages to ARS patients in the 1980s and has spent a huge effort on finding the genetic basis for this disorder. His work with Dr. Michael Solursh at The University of Iowa provided the initial discoveries of genes associated with ARS. He initially named the gene cloned by him and Dr. Elena Semina, *SOLURSHIN* in recognition of the support from Dr. Solursh. It was subsequently termed *RIEG* and now is referred to as *PITX2* (pituitary homeobox transcription factor 2). I want to personally thank Dr. Murray for his help, advice, support, wisdom and for graciously allowing me to work with him and his group at The University of Iowa on the molecular mechanisms of PITX2 transcriptional activity. This book would not have been written without his scientific contributions to understanding the genetic basis of ARS.

Sincerely,
Brad A. Amendt
Editor

CHAPTER 1

Identification of the Gene Involved in 4q25-Linked Axenfeld-Rieger Syndrome, *PITX2*

Elena V. Semina

Axenfeld-Rieger syndrome (ARS) is a rare autosomal dominant disorder. ARS is considered to be fully penetrant, but variable expressivity was reported in families. The three cardinal features of ARS include specific ocular anomalies of the anterior segment, dental anomalies and redundant periumbilical skin. A variety of other abnormalities have been reported in ARS patients such as pituitary, hearing, heart and limb defects that may represent coincidental findings in some cases and be associated with specific mutations in others. Identification of genes and chromosomal regions associated with ARS demonstrated extreme genetic heterogeneity of this condition and allowed genotype-phenotype correlation studies. In this chapter, we describe identification of a gene, *PITX2*, which to-date represents a major gene for Axenfeld-Rieger syndrome accounting for approximately 40% of mutations in classic ARS patients. The *PITX2* gene was discovered by positional cloning approach and is located at 4q25 in humans. The *PITX2* gene appears to play an important role in development of many different systems and its involvement in human disorders needs to be further elucidated.

Introduction

Axenfeld-Rieger anomalies were first reported by Vossius[1] and Darwin[2] at the end of 19th century and defined as a genetic disorder by Axenfeld[3] and Rieger.[4] Axenfeld-Rieger syndrome (ARS) is characterized on the basis of specific ocular anomalies associated with other, generally dental and umbilical defects. Ocular anomalies include a prominent annular white line near the limbus at the level of Descement membrane (posterior embryotoxon), hypoplastic iris, irido-corneal adhesions, and glaucoma.[5,6] Childhood glaucoma is considered to be the most debilitating feature of this condition and affects approximately 50% of patients. Dental anomalies vary from misshapen teeth to complete anodontia with missing lateral incisors being the most common manifestation. Abdominal defects include redundant periumbilical skin and omphalocele in more severe cases (Fig. 1). A number of occasional features have also been associated with ARS suggesting that some causative genes could influence cardiac, limb, pituitary and brain development as well.[7]

Axenfeld-Rieger syndrome is an autosomal-dominant disorder characterized by complete penetrance but variable expressivity of the phenotype. A genetic heterogeneity has always been suspected due to a variety of associated features and cytogenetic anomalies reported in ARS patients. This has recently been confirmed by identification of three genes and at least two

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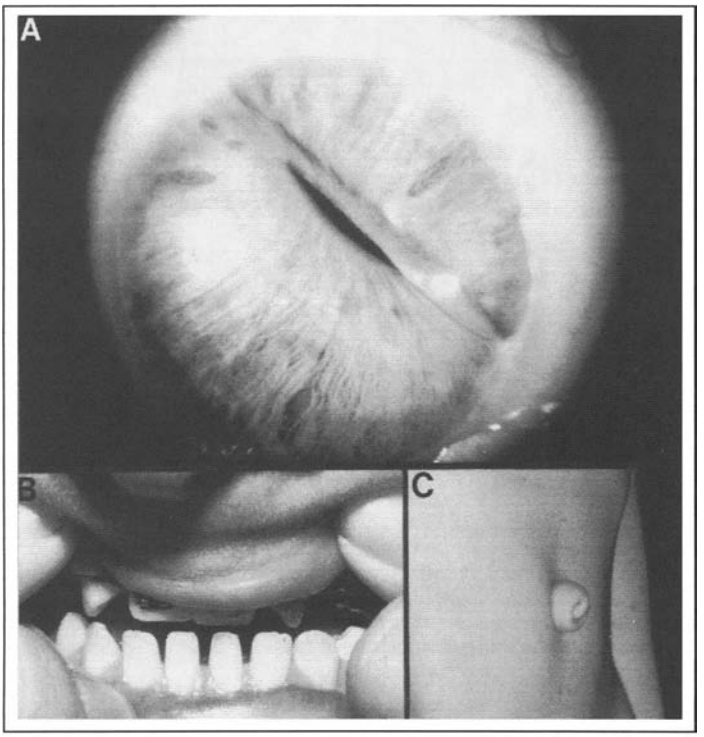

Figure 1. Features of Axenfeld-Rieger syndrome in one patient. On the top, A) the hypoplastic iris allows the pupillary sphincter muscle to be seen as a band around the pupil. The pupil is distorted (corectopia). The distinct white line in the periphery of the cornea is termed "posterior embryotoxon" (see text). On the bottom left, B) this patient is an example of both oligodontia, as evident by the missing maxillary lateral incisors. On the bottom right, C) a photograph of the abdominal area of an ARS patient showing characteristic umbilical abnormalities due to failure of the periumbilical skin to involute. (Published with permission from Nature Genetics.)

additional chromosomal regions associated with this syndrome (see other chapters in this book) (for a review see refs. 6, 8, 9). The most frequent cytogenetic anomalies in ARS patients included chromosomes 4, 6, 13 and sometimes 16, 20, and others. Linkage of Axenfeld-Rieger syndrome to chromosome 4q25 was reported by Murray and associates in 1992 and marked an identification of a first genetic locus for this condition.[10]

Cloning of the *PITX2* Gene and Identification of Mutations in Axenfeld-Rieger Syndrome Patients

Successful positional cloning efforts often rely on several independent and complementary approaches. The first approach that we used, once the genetic linkage of ARS to 4q25 was determined, was the candidate gene approach. Genes at 4q25 were examined for their candidacy for Axenfeld-Rieger syndrome and two genes, epidermal growth factor (EGF) and basic fibroblast growth factor (FGF2), were selected and screened for mutations in ARS patients.[11] No disease-causing mutations were identified in these genes. In parallel with this, we also identified two patients who had balanced translocations with nearly identical cytogenetic breakpoints on the long arm of chromosome 4 and features of Axenfeld-Rieger syndrome. We

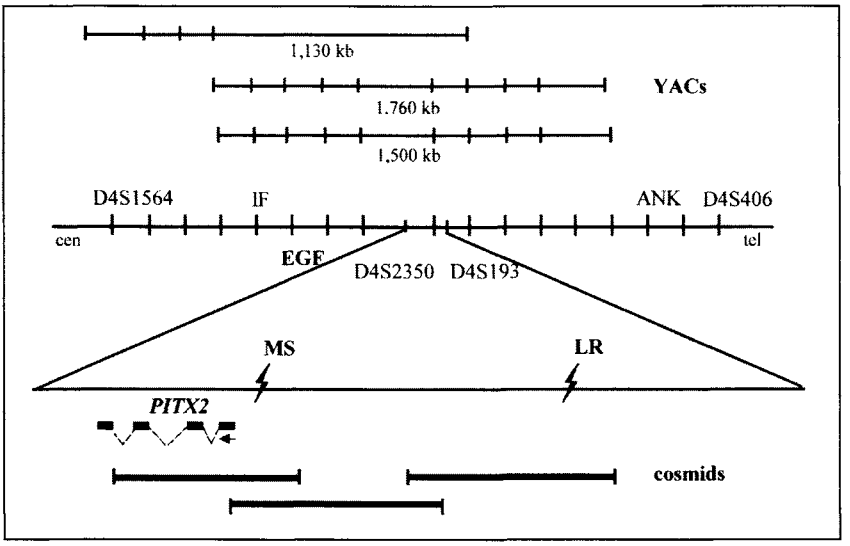

Figure 2. YAC and cosmid contig across the Axenfeld-Rieger syndrome critical region. The positions of the patient MS's and LR's breakpoints are shown with a schematic lightning bolt. *PITX2* exons are shown as gray boxes. The DNA inserts of the YACs' and cosmids' clones are shown at the top and at the bottom of the drawing, correspondingly.

hypothesized that these breakpoints interrupted ARS gene sequence and therefore next used these two translocations to isolate and characterize the Axenfeld-Rieger syndrome critical region at 4q25.

Somatic cell hybrids were created using lymphoblastoid cells established from ARS patients carrying translocations and hamster cells. Hybrid clones that retained the human der(4) and lost all other chromosome 4 material were isolated and used for fine mapping of the breakpoints. Preliminary mapping of the breakpoints was performed by PCR screening of the DNA extracted from these hybrid cell lines for the presence/absence (+/-) of different chromosome 4 specific markers/regions. This resulted in an identification of the markers, D4S1571 and D4S193, flanking the breakpoints and located at the distance of 3cM from each other according to the human linkage map (Fig. 2). These markers were then used to isolate genomic clones containing large DNA pieces (YACs, BACs) that were then fragmented into smaller pieces and subcloned into cosmids. The cosmid clones were next arranged into a contig using cosmid fingerprinting technique and the ends of overlapping cosmid inserts were sequenced to obtain additional chromosome 4 specific markers. These new markers/ regions were analyzed for their position in respect to the breakpoints using the same +/- PCR screen and the hybrid cell lines containing translocated der(4) chromosomes. The smallest region encompassing the both breakpoints was identified and it consisted of three overlapping cosmids comprising approximately 120 kb of genomic sequence; the breakpoints were localized at the distance of about 50 kb from each other (Fig. 2).[12,13]

In order to isolate genes from the ARS critical region several approaches were used: identification of conserved regions using zooblot analysis, isolation of CpG islands by restriction enzyme analysis and exon trapping. Several CG-rich regions were identified and one showed significant conservation with nonhuman genomic sequences on zooblot suggesting that this sequence may be a part of a gene. Screening of a human craniofacial cDNA library with this fragment resulted in an identification of several matching transcripts. These cDNA clones were

isolated, sequenced and sequences were arranged into a cDNA contig. The composite cDNA was 2,125 bp in length and contained an 813-nt open reading frame predicted to encode a protein of 271 amino acids. The overall cDNA sequence represented a new gene that was initially called *RIEG1* and was later renamed by nomenclature committee into *PITX2* gene.[12]

Analysis of the nucleotide and of the predicted amino-acid sequence of *PITX2* indicated that this gene encodes a homeodomain (HD) protein. Homeodomain proteins are characterized by the presence of the 60-amino-acids motif HD, which is encoded by the 180-nucleotides region (homeobox) of the gene. The homeodomain proteins are transcription factors that are most active during embryonic development governing expression of many other genes. The HD is the domain that binds to specific DNA sequences in the regulatory regions of downstream genes to manipulate their expression. On the basis of their HD sequence, these proteins are divided into groups. The PITX2 protein belongs to *paired* group (by overall similarity) and *bicoid* subgroup (because of lysine at position 50 of PITX2 HD) and therefore was predicted to bind to DNA sequences similar to those that were identified for other proteins from these groups. In agreement with this prediction, the PITX2 protein was shown to specifically interact with the *bicoid* DNA binding site, 5'-TAATCC-3'.[8,14-16] Other interesting regions of Pitx2 protein included 14-amino-acids motif located in the C-terminus that was conserved in several *paired*-like HD-containing genes and a 270-nucleotides region in the 3'UTR that was shown to be 97% identical between the human and mouse genes.[12] Functional roles of these conserved elements are yet to be determined. The 14-amino-acids motif is likely to be involved in interactions with accessory proteins, which are important for DNA binding specificity of these transcription factors (for a review see ref. 17). The 3'UTR sequences were shown to play a role in mRNA turnover, regulation of translation and other post-transcriptional regulatory pathways (for a review see refs. 18, 19).

Identification of the mouse *Pitx2* gene facilitated studies of its expression during embryonic development. The *Pitx2* transcripts were detected in the mesenchyme surrounding the developing eye, maxillary and mandibular epithelia, at the base of limbs, in the midbrain and at the site of umbilicus (Fig. 3).[12] Therefore, the *Pitx2* expression sites corresponded well with the regions that are affected in Axenfeld-Rieger syndrome patients.

The *PITX2* gene represented an excellent candidate for Axenfeld-Rieger syndrome because of its location and expression during embryonic development. In order to perform a mutation screening of *PITX2* in ARS patients, the genomic structure of *PITX2* was determined and primer sets were developed to amplify all of the exon sequences and to include the adjacent intron sequences. DNA samples from ten unrelated ARS patients were amplified with these *PITX2*-specific primers and PCR products were analyzed on single-strand-conformational-variant (SSCV) gel to detect bands with abnormal mobility (Fig. 4). Six mutations were identified: three resulted in different amino acid changes in the PITX2 homeodomain, two nucleotide changes were found at exon-intron splice junctions and are likely to affect normal splicing of *PITX2* transcript, and one was a stop codon mutation predicted to cause a premature termination of PITX2 protein (Fig. 4).[12] All of the mutations were found to cosegregate with the affected phenotype in families and were not present in more than 200 normal control chromosomes.

The translocation breakpoints in ARS patients, which were originally used to identify the critical interval and to subsequently isolate the *PITX2* gene, were positioned in respect to *PITX2* using the constructed cosmid contig (Fig. 2). None of the breakpoints was found to interrupt any *PITX2* gene sequences. In fact, the breakpoints were estimated to be at the distance of approximately 5-15 kb (MS patient) and 50-70 kb (LR patient) from the most 5' end of the gene. Similar cases associated with chromosomal rearrangement outside the transcription and promoter regions have been identified for a variety of human disorders and are categorized as position effects (for a review see ref. 20). The general assumption is that the regulation of gene expression has been compromised in these cases and a number of different mechanisms have

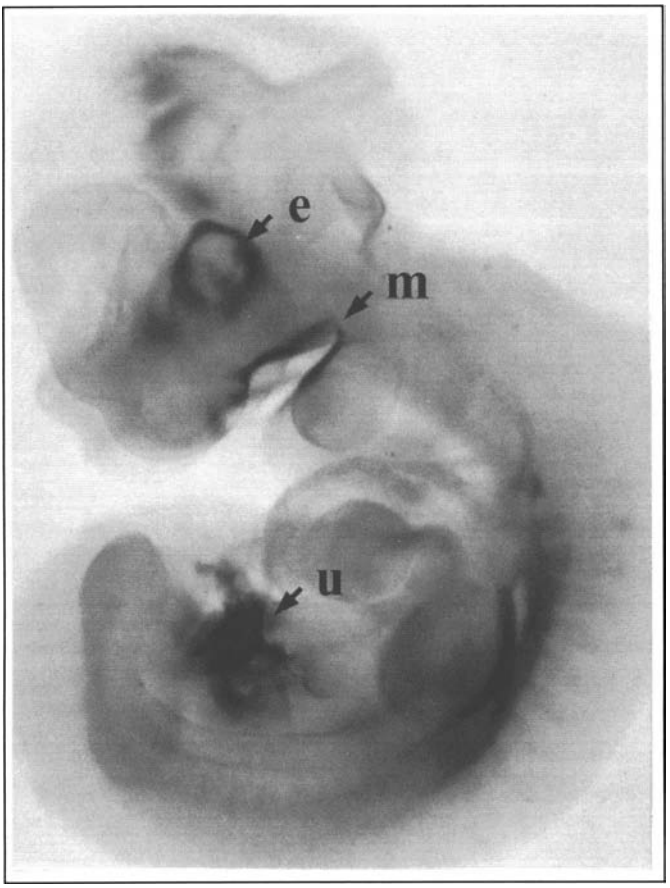

Figure 3. *Pitx2* expression in the mouse embryo. Whole-mount in situ hybridization on mouse day-11 embryos with an anti-sense digoxigenin-labelled riboprobes derived from the 3'UTR of the mouse *Pitx2* cDNA. The expression is seen in the mesenchyme surrounding the developing eye (e), maxillary and mandibular epithelia (m), umbilical cord (u) and also in the midbrain and at the base of forelimbs.

been proposed: removal of a specific enhancer element(s) or a chromatin organizing region that is normally present on intact chromosome, or an introduction of heterochromatin or regulatory elements from rearranged allele that are normally absent on intact chromosome and now can influence gene expression.

Collectively, these facts strongly suggested that the *PITX2* is the gene responsible for 4q25-linked cases of Axenfeld-Rieger syndrome. First, it maps to the 4q25 region that is known to contain a gene for this disorder; second it lies in close proximity to two ARS breakpoints; third, six out of ten ARS patients in the original study were found to have etiologic mutations in *PITX2* gene and finally, expression of the mouse *Pitx2* gene is consistent with the human disease phenotype.

Since the original description of the *PITX2* gene, many additional mutations in this gene have been identified in several laboratories.[21-26] In summary, *PITX2* mutations are most commonly associated with the complete Axenfeld-Rieger syndrome phenotype that includes ocular, dental and umbilical features. Approximately forty percent of these classic

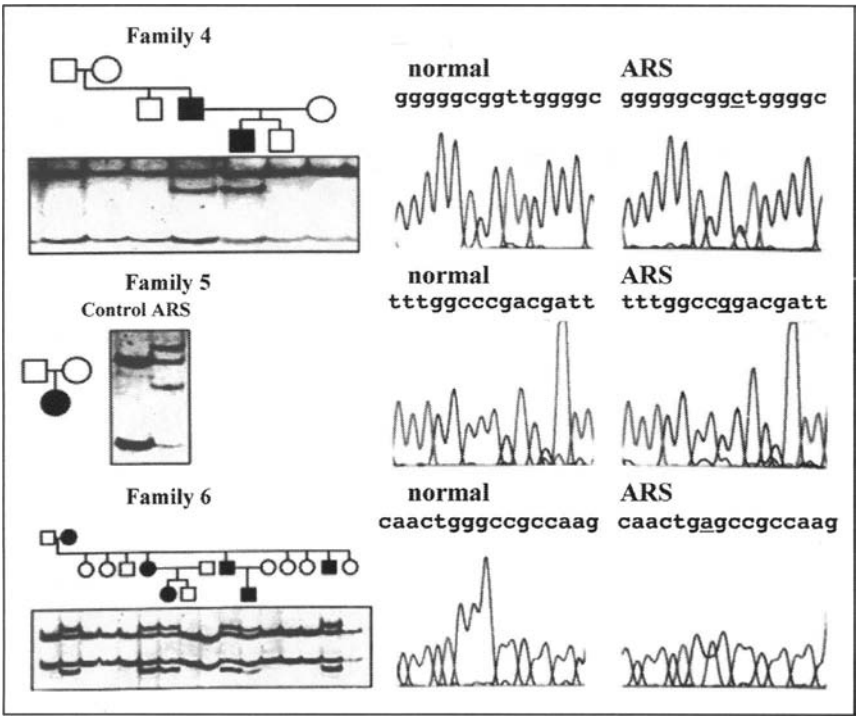

Figure 4. Mutations in the *PITX2* gene in Axenfeld-Rieger syndrome patients. The pedigrees and single-strand-conformational-variant (SSCV) gel pictures are shown on the left: the mutated allele cosegregates with the affected phenotype in families 4 and 6, in family 5 a de novo mutation has been identified. Nucleotide sequence analysis of DNA from affected individuals from families 4-6 is shown on the right: mutated nucleotides are underlined. (Published with permission from Nature Genetics.)

ARS cases demonstrate *PITX2* mutations. The remaining cases could be attributed to mutations in other regions of *PITX2* gene that are yet to be identified and mutations in other genes. In some cases mutations in *PITX2* gene resulted in isolated ocular phenotypes[21,25] suggesting that the ocular development might be particularly sensitive to mutations in the *PITX2* gene.

Haploinsufficiency, caused by a removal of one normal allele of *PITX2* due to deletions,[27] translocations[12,27] or mutations [12,22-25] appears to be a dominant etiologic mechanism of ARS associated with *PITX2* mutations. In addition to this, the ocular phenotype of ARS was suggested to be dosage-dependent because some *PITX2* mutations that produced mutant proteins that retained some residual function resulted in a less severe ocular phenotype.[23] In agreement with this, a dominant-negative *PITX2* mutation that was shown to be nonactive and, in addition, affecting activity of the normal *PITX2* allele, was found to be associated with a particularly severe ocular phenotype, partial aniridia, in one patient.[16] Studies performed by Drs. Amendt and Espinoza demonstrated that different promoters activated by PITX2 have different sensitivities to *PITX2* mutations and suggested that this phenomenon may be responsible for the fact that some *PITX2* mutations are associated with isolated ocular conditions as, for example, iris hypoplasia mutation.[21,28]

Mouse mutants with alterations in the *Pitx2* gene have been created in several laboratories and demonstrated a variety of severe developmental defects.[29-32] In addition to arrest of the development of the anterior segment of the eye, arrest of teeth development and abdominal wall defects that all are consistent with *Pitx2*'s role in Axenfeld-Rieger syndrome, the *Pitx2-/-* mice also exhibited other multiple congenital anomalies such as cardiac, pituitary, brain and lung defects leading to embryonic death of these animals (see following chapters). Although anomalies in these systems have been occasionally reported in association with ARS, humans with *PITX2* mutations are usually presented with the ARS phenotype without any distinct other associated features.[12,23-25] Human *PITX2* mutations affecting development of other systems might be embryonically lethal and therefore are removed from human population or to be associated with a different phenotype that is yet to be discovered. Several human syndromes demonstrate a combination of anomalies in systems affected in *Pitx2*-deficient mice. Screening of *PITX2* gene in CHARGE patients excluded this gene as a major cause of this condition.[33] Studies of other human conditions are underway.

Different isoforms of *PITX2* gene have been discovered: four in humans,[34,35] three in mice[36,37] and two in *Xenopus* and other species.[38,39] The isoforms differ in their N-terminal regions but all share the same homeodomain and the C-terminal region. The *Pitx2* isoforms were shown to have different expression pattern and protein activities.[34,38,39] No isoform-specific mutations have been identified in humans as all of the known mutations cluster in the homeodomain and C-terminal regions and therefore affect all the isoforms. It is possible that isoform-specific mutations may result in a different ARS phenotype that is yet to be discovered.

Conclusion

Identification of the *PITX2* gene marked the discovery of the first gene associated with Axenfeld-Rieger syndrome and developmental glaucoma. Since then several other genes involved in ARS and related conditions have been reported and many more still need to be discovered. There is a clear variability of the Axenfeld-Rieger phenotype, which is likely to be due to gene-gene and gene-environment interactions. Identification and characterization of the other genes involved in Axenfeld-Rieger syndrome and, particularly, studies of animal models should help to determine these interactions and lead to a better prediction and treatment of these conditions. At the same time, the *PITX2* gene plays a role in development in many other organs in addition to ones affected in ARS patients and therefore is a candidate for a wide range of human genetic defects. All of the known mutations associated with ARS cluster in the homeodomain and C-terminal region of the gene suggesting that mutations in other domains might be responsible for alternate phenotypes. Future studies of *PITX2* should reveal its complex interactions with other factors and multiple developmental roles.

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CHAPTER 2

Winged Helix/Forkhead Transcription Factors and Rieger Syndrome

Darryl Y. Nishimura and Ruth E. Swiderski

A second locus for Rieger syndrome (RS) was identified from chromosomal abnormalities involving chromosome 6p25. Study of the breakpoint revealed mutations in the forkhead transcription factor (*FOXC1*) gene. Initially, this defect was identified in two patients with RS and glaucoma and later FOXC1 mutations were associated with RS patients. Several studies have shown linkage of anterior segment abnormalities to the same region of chromosome 6. These abnormalities include Axenfeld anomaly, Rieger anomaly, iridogoniodysgenesis anomaly and familial glaucoma iridogoniodysplasia. Axenfeld-Rieger syndrome (ARS) is a heterogeneous condition as noted by the identification of different chromosomal aberrations and phenotypes of ARS patients. This chapter will focus on the identification of *FOXC1* and its association with the group of disorders that comprise Axenfeld-Rieger syndrome.

Introduction

The winged helix/forkhead family of transcription factors plays key roles in many aspects of development. One member of this family, *FOXC1*, has been extensively studied recently because of its involvement in causing defects of the anterior chamber of the eye and glaucoma. *FOXC1* has been implicated as a major cause of Rieger syndrome, an autosomal dominant syndromic form of inherited glaucoma. This chapter covers the studies that led to the identification of *FOXC1* as the gene responsible for these anterior chamber defects, as well as more recent studies on *FOXC1* gene expression and function. In addition to *FOXC1*, the mouse gene *Foxc2* as well as the human *FOXE3* and mouse *Foxe3* genes appear to play a role in the normal development of the eye. This raises the possibility that other members of this transcription factor family may also be involved in eye development.

Genetic Characterization of 6p25 Glaucoma Phenotypes

In December of 1996, Mears et al[1] described the genetic localization of iridogoniodysgenesis anomaly (IGDA), an autosomal dominant disorder with variable expressivity that is characterized by the presence of iris hypoplasia, goniodysgenesis and juvenile glaucoma. A genome-wide linkage analysis was performed with greater than 300 microsatellite markers on two large IGDA families before significant linkage was detected on the short arm of chromosome 6. A gene for IGDA was localized to an 8.3-cM region distal to the marker D6S477 at 6p25. Recombination events in two unaffected individuals allowed the interval to be narrowed to a 6.4-cM region flanked by the markers D6S1600 and D6S1617 (Fig. 1). This region of 6p25 was also identified

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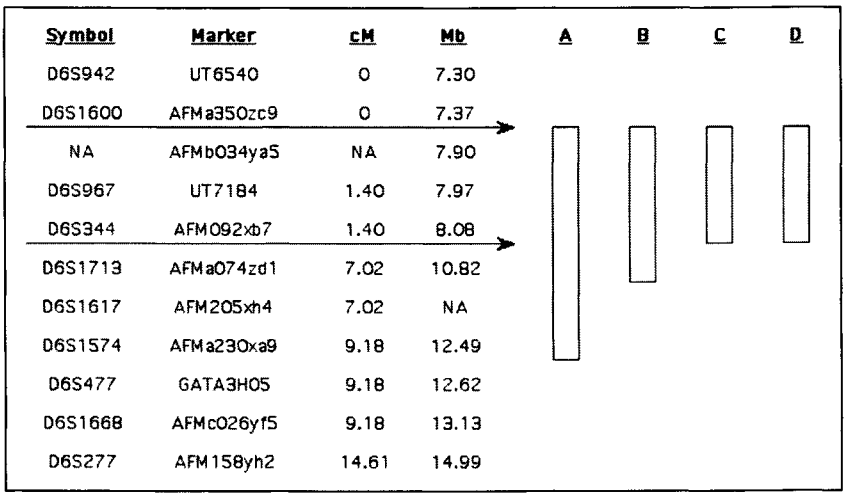

Figure 1. Comparison of 6p25 genetic intervals. A comparison of the published candidate intervals for the IRID1 locus at 6p25. The boxes indicate the regions of 6p25 to which the IRID1 locus was localized in various genetic mapping studies. Intervals for the following studies are shown: A—Mears et al,[1] B—Gould et al,[3] C—Jordan et al,[4] D—Graff et al.[6]

as containing the IGDA locus by genome-mismatch scanning, a different method of linkage analysis that quickly isolates regions of identity between two genomes.[2]

In September of 1997, Gould et al[3] reported on the mapping of a locus for Axenfeld-Rieger anomaly (ARA) to 6p25. ARA is an autosomal dominant disorder that can include the presence of posterior embryotoxon (a prominent, anteriorly displaced Schwalbe's line), iris hypoplasia, displaced pupils (corectopia), iridocorneal adhesions and juvenile glaucoma. ARA has similar ocular manifestations as those found in Rieger syndrome (RS), but none of the nonocular findings of RS. A single large family with seven individuals affected with ARA was used to identify linkage to 6p25. A recombination event in one of the affected individuals from this family was consistent with the proximal boundary (D6S1617) defined by the results from an unaffected individual from the IGDA family (Fig. 1).

In October of 1997, Jordan et al[4] presented the mapping of familial glaucoma iridogoniodysplasia (FGI) to 6p25. FGI is an autosomal dominant form of juvenile open-angle glaucoma that is characterized by defects of the iris and iridocorneal angle. Affected individuals present with distinctive irises and may also have pale tissue, possibly of mesenchymal origin, covering the trabecular meshwork. Linkage analysis of a single large family suggested linkage to a 6.4-cM region at 6p25 flanked by the markers D6S1600 and D6S1713 (Fig. 1). In the same month, Morissette et al[5] described the mapping of a gene for developmental and familial open-angle glaucoma to the D6S1600/D6S1713 region in a single large family with 30 affected individuals. Finally, in December 1997, Graff et al[6] confirmed the candidate interval for IGDA to a roughly 6 cM segment in 6p25 flanked by the markers D6S1600 and D6S1713 (Fig. 1).

The clinical disorders described in the various linkage studies likely represent a broad spectrum of phenotypic expression that is consistent with Rieger syndrome (RS).[7] The combined data from the various studies strongly supports the hypothesis of a RS locus at 6p25 and suggests that a RS gene is contained within a roughly 3.5 Mb interval flanked by the markers D6S1600 and D6S1713. A number of potential candidate genes were identified based on their known location within this interval, including the winged helix/forkhead transcription factor FOXC1. The 6p25 locus has been assigned the standard nomenclature of *IRID1* (MIM: 601631).

Positional Cloning of *FOXC1*

In 1998, two groups simultaneously and independently used positional cloning techniques to identify the gene responsible for the *IRID1* locus at 6p25.[1,8] One group used a focusing cytogenetic rearrangement to narrow the search, while the second group refined the genetic localization of the *IRID1* locus in five *IRID1* families. Both groups were able to demonstrate mutations in the transcription factor *FOXC1* in a number of patients with RS. Considerable variation was observed in the expression of the RS phenotype, both within families and between families. The large variation in the expressivity of the phenotype between patients makes it difficult to subcategorize the phenotype in families in which a small number of affected individuals have been clinically evaluated. Therefore, the terminology Rieger syndrome (RS) will be used as a general descriptor for the spectrum of anterior chamber anomalies associated with the *IRID1* locus.

Nishimura et al[8] used a positional cloning strategy that focused on the identification of the chromosomal breakpoints in a congenital glaucoma (CG) patient who was found to harbor a balanced translocation between 6p25 and 13q22. The cloning and identification of the 6p25 breakpoint from this patient led to the identification of two candidate genes based on their proximity to the breakpoint. One gene, GDP-mannose 4,6-dehydratase (*GMDS*), was found to contain the 6p25 breakpoint within an intron upstream of the penultimate exon. A second gene, the winged helix/forkhead transcription factor *FOXC1*, was found to be located 16.2 kb from the 6p25 breakpoint and was translocated to the derivative chromosome 13. A similar finding has been observed for *RIEG1* in which the breakpoint in an affected patient has been demonstrated to be roughly 90 kb from the *PITX2* gene.[9] Northern blot analysis of the expression pattern of each gene in the mouse demonstrated that *Foxc1* was abundantly expressed during embryogenesis and at high levels in the kidney and eye, while *Gmds* was expressed at basal levels during embryogenesis as well as in most of the adult tissues. Based on the expression pattern and putative function, *FOXC1* was considered an attractive candidate. A mutation screen of 19 probands (6 CG and 13 RS) found no mutations in *GMDS*, but detected mutations in four of the probands for *FOXC1*.

Mears et al[10] used a strategy that focused on the refinement of the genetic mapping of the *IRID1* locus. Five of the families used to map the *IRID1* locus were genotyped for known polymorphic markers at 6p25 as well as for several additional new markers that were developed for the study. Most of the data generated was consistent with a localization of *IRID1* between *D6S1600* and a polymorphism in the *NQO2* gene. As this interval contained the *FOXC1* gene, it was screened in five of the I*RID1* families used to map the locus, as well as in 16 sporadic patients with a variety of defects of the anterior segment of the eye. Three novel mutations were detected (one *IRID1* family and two sporadic patients). However, complete sequencing of the coding region of *FOXC1* in the remaining four *IRID1* families did not yield any evidence for additional mutations. Furthermore, an insertion polymorphism within the *FOXC1* gene excluded it from being the causative gene in one of the *IRID1* families. This recombination event when considered in conjunction with a second recombination event at *D6S344* in an unaffected individual from another *IRID1* family prompted the authors to suggest that a second *IRID1* locus might be localized between *D6S1600* and *D6S344*.

Winged Helix/Forkhead Gene Family

The winged helix/forkhead family of transcription factors contains a roughly 100 amino acid, monomeric DNA binding domain that was first identified in *Drosophila melanogaster* and the rat.[11-13] Mutations in the *Drosophila* forkhead gene result in a homeotic transformation of gut structures into elements of the head. There have been well over 100 members of this family identified in a variety of species.[14] Figure 2 shows a phylogenetic analysis of many of the winged

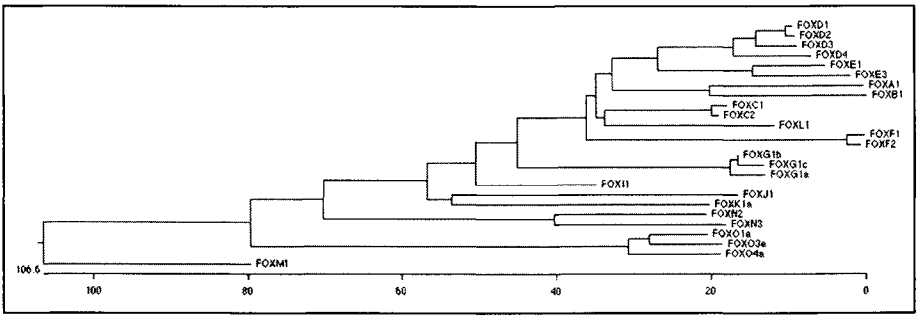

Figure 2. Phylogenetic analysis of winged helix/forkhead genes. Phylogenenetic analysis of human winged helix/forkhead (Fox) genes using the Clustal algorithm of the LaserGene software suite. The conserved forkhead domain of each gene was used for the analysis.

helix/forkhead proteins that have been identified in humans. The rapid identification of such genes has led to a great deal of variation in naming and classification. To counteract this problem, a standardized nomenclature has been proposed which assigns all chordate winged helix/fokhead transcription factors the symbol Fox (Forkhead box). Fox proteins are assigned to subclasses based on phylogenetic analysis and numbered according to the following schema, "Fox, subclass, member".[11] Fox genes have been shown to play key roles in various aspects of development.[14]

FOXC1 is a member of the winged helix/forkhead transcription factor family and is encoded by a single exon. The human and mouse proteins both contain 553 amino acids and are highly homologous, demonstrating 91% identity across the entire coding region. The forkhead domain demonstrates 100% conservation between the human and mouse at the protein level. The DNA binding specificity of four of the Fox genes has been examined by the selection of binding sites from random sequence oligonucleotides and all four genes were found to share a core binding sequence of RTAAAYA, but differed in positions flanking this core sequence.[15] In addition to the human and mouse, *FOXC1* orthologues have also been identified in *Xenopus*, chicken and zebrafish.[16-18] Interestingly, in the zebrafish two *FOXC1* homologues (*Foxc1a* and *Foxc1b*) were identified.

FOXC1 Mutations Cause Anterior Segment Anomalies

Since the identification of *FOXC1* as the 6p25 gene responsible for RS phenotypes at the *IRID1* locus, more than 20 different mutations have been described as are summarized in Table 1. In addition to the two patients with CG and chromosomal translocations, Nishimura et al[8] described three missense mutations (F112S, I126M and S131L) and one frameshift mutation (A51fsX73) that were detected in a screen of 19 unrelated probands (13 RS, 9 CG). Mears et al[10] performed a mutation screen of *FOXC1* in five *IRID1* families and 16 isolated probands with RS. One missense mutation was detected in an *IRID1* family (S82T). Among the 16 probands, one missense mutation (I87M) and one frameshift mutation (A31fsX41) were found. Two other DNA sequence variations were detected (insertion of an extra GGC triplet into GGC repeats) within the *FOXC1* coding region which were determined to be polymorphisms due to the detection of the variants in both patient and control samples.

In September of 1999, Swiderski et al[19] described a frameshift mutation (Q70fsX77) in a RS family in which the proband was also found to have a congenital heart defect (atrial septal defect). In addition, a second RS family (F112S mutation) that had been published previously[8] was found to harbor three RS individuals with mitral valve defects. In January of 1998,

Table 1. Mutations in Axenfeld-Rieger syndrome

Nucleotide	Protein	Phenotype	Reference
g.26-47ins	S9fsX89	Axenfeld anomaly	25, 27
g.67C>T	Q23X	Rieger syndrome	24
g.93-102del	A31fsX41	Axenfeld anomaly	10
g.99-108del	G33fsX41	Axenfeld anomaly	25
g.116-122del	A39fsX42	Rieger anolmaly	25
g.153-162del	A51fsX73	Axenfeld anomaly	8
g.210delG	Q70fsX77	Rieger anomaly	19
g.235C>A	P79T	Rieger syndrome	26
g.236C>T	P79L	Rieger anomaly	25
g.245G>C	S82T	IRID1	10
g.256C>T	L86F	Rieger syndrome	This Study
g.261C>G	I87M	Axenfeld anomaly	10
g.262-265del	T88fsX100	Axenfeld anomaly	25
g.272T>G	I91S	Axenfeld anomaly	27
g.287insG	D96fsX305	Axenfeld anomaly	27
g.335T>C	F112S	Rieger anomaly	8
g.378C>G	I126M	Axenfeld anomaly	8
g.380G>A	R127H	Axenfeld anomaly	27
g.388C>T	L130F	Axenfeld anomaly	This Study
g.392C>T	S131L	Axenfeld anomaly	8
g.392C>T	S131L	Rieger anomaly	25
g.1512delC	F504fsX518	Axenfeld anomaly	25

Cunningham et al[20] described a small family in which atrial septal defect and sensorineural hearing loss was found in conjunction with RS. Several additional reports also suggest that cardiac anomalies may be an occasional finding of RS.[21-23] In January of 2000, Mirzayans et al[24] identified a nonsense mutation (Q23X) in a three-generation RS family. Currently, this is the only report of a nonsense mutation in the *FOXC1* gene.

In February of 2001, Nishimura et al[25] described a variety of mutations found in the screening of 70 RS probands in which *PITX2* mutations had not been detected. The mutations included two missense mutations (P79L and S131L) and five frameshift mutations (S9fsX89, G33fsX41, A39fsX42, T88fsX100 and F504fsX518). The S131L mutation represents the second instance this particular change has been identified in an RS patient. It is not known if the two families with S131L *FOXC1* mutations are related. One of the frameshift mutations (F504fsX518) is interesting as it represents the most distal mutation detected in the *FOXC1* gene to date. The mutation would be expected to cause premature termination of translation and thus the loss of 36 amino acids from the FOXC1 protein. It is therefore possible that this region of FOXC1 contains an element that is important for the function of the protein.

In October of 2001, Suzuki et al[26] identified a second missense mutation (P79T) at position 79 of the FOXC1 protein in a three-generation family of Japanese ancestry. Later in 2001, Kawase et al[27] described four additional mutations that were detected in a screen of six Japanese RS families. One of the mutations (S9fsX89) is the same mutation that was reported by Nishimura et al previously.[25] The three other mutations include two missense mutations (I91S and R127H) and a frameshift mutation (D96fsX305) within the conserved forkhead domain. In February of 2002, Borges et al[28] published the results of a screen of five RS families from Brazil. Although no mutations were detected in the *FOXC1* gene, two mutations were found in

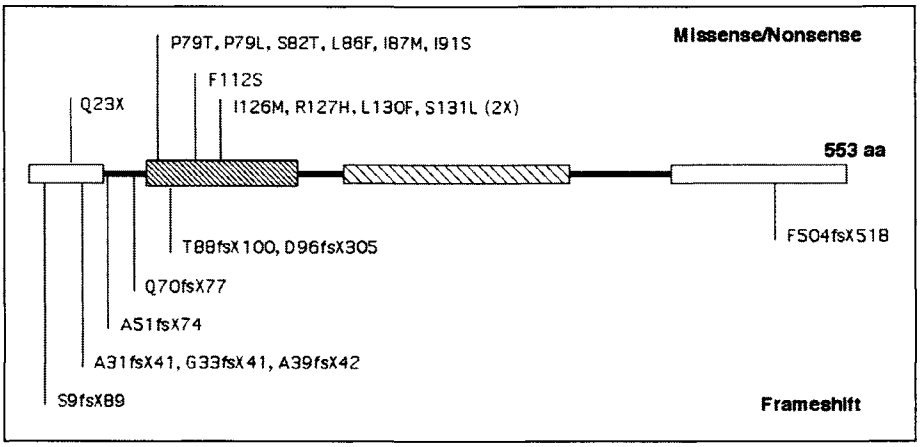

Figure 3. Summary of mutations in *FOXC1*. The locations of the various mutations are depicted within the graphical representation of the *FOXC1* gene. Missense and nonsense mutations are shown above the representation of the *FOXC1* gene while the frameshift mutations are shown below. The box with the diagonal lines represents the winged helix/forkhead domain. The open boxes represent the two transcriptional activation domains, while the box with the sparse diagonal lines represents the transcription inhibitory element.[31]

the *PITX2* gene at 4q25. Recently, we have been able to identify two additional missense mutations (L86F and L130F) in the *FOXC1* gene in RS probands.

The distribution of mutations within the *FOXC1* gene is shown in Figure 3. To date, 12 missense mutations (11 novel) have been reported. It is of interest that all of the missense mutations in *FOXC1* have been observed within the conserved winged helix/forkhead domain. Figure 4 illustrates the location of the missense mutations within the FOXC1 winged helix/forkhead domain. All of the mutations disrupt highly conserved residues within this important functional region of FOXC1. The single nonsense mutation as well as eight of the nine frameshift mutations has been found to cluster at the beginning of the protein. These mutations result in truncated FOXC1 proteins in which the winged helix/forkhead domain is either altered or missing. If such proteins are stable, they might be expected to demonstrate defects in DNA recognition and binding as well as to exhibit drastically altered regulatory capabilities. A single frameshift mutation has been observed that alters only the final 36 amino acids of the FOXC1 protein. As was mentioned previously, it is possible that these 36 amino acids may play an important role in the function of FOXC1.

In November of 2000, Lehmann et al[29] described a large family with an autosomal dominant form of iris hypoplasia and glaucoma. Significant linkage (maximum two-point lod score of 6.20 at recombination fraction of 0) was found for the 6p25 marker *D6S967*. Although no mutations were detected in *FOXC1* by direct sequence analysis, a partial chromosomal duplication of the 6p25 region was detected by genotyping with microsatellite repeat markers and confirmed by FISH. The authors note that four other pedigrees with similar phenotypes have been mapped to 6p25, but sequencing of *FOXC1* has not found any evidence for mutations.[10] Although it was proposed that a second glaucoma gene might be present at 6p25, the authors note that alterations in gene dosage may be another explanation for the failure to detect mutations in the four pedigrees. The authors speculate that altered *FOXC1* gene dosage may result in an altered expression pattern of *FOXC1* target genes and that increased *FOXC1* dosage may result in a more severe phenotype than reduced dosage.

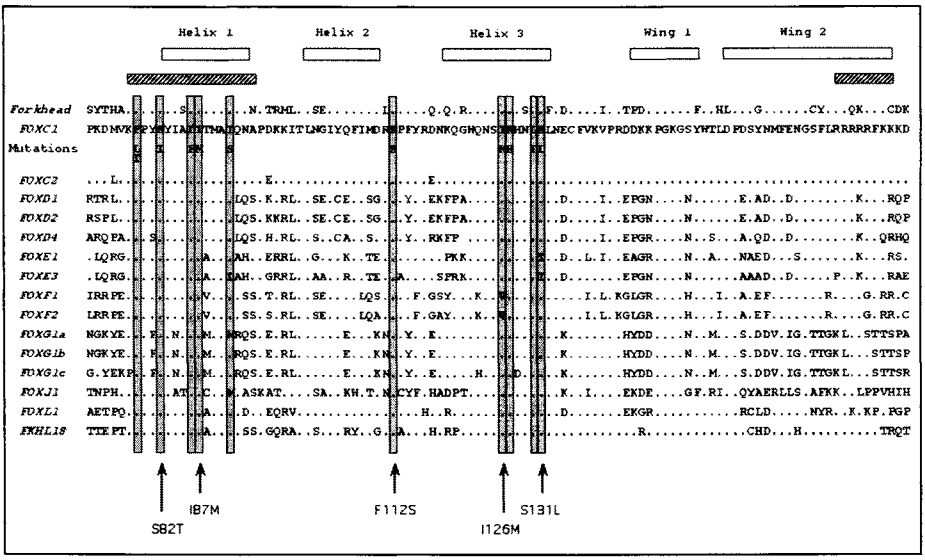

Figure 4. Missense mutations within the *FOXC1* forkhead domain. Locations of the *FOXC1* missense mutations within a comparison of forkhead domains from various members of the winged helix/forkhead transcription factor family. The sequence for the *Drosophila* fork head gene is shown above that for *FOXC1*. The missense mutations are depicted below the *FOXC1* sequence. The shaded boxes show the corresponding position for each mutant in the various family members. The missense mutations that were used for functional studies are shown at the bottom of the figure.[30] The open boxes illustrate the location of the three alpha helices and the two wing domains.[49] The boxes with the diagonal lines show the location of the two nuclear localization signals.[31]

In February of 2001, Nishimura et al[25] described a partial chromosomal duplication of 6p25 in two families. The first family was a nuclear family in which the affected proband was found to have Peters' anomaly in addition to other nonocular findings. Cytogenetic analysis detected extra material at 6p25 that proved to be derived from chromosome 6 upon further cytogenetic analysis. The second family consisted of four individuals diagnosed with iris hypoplasia and glaucoma. The presence of the 6p25 duplication in this family was initially detected with the genetic marker *AFMb034ya5*. These initial results were confirmed by the analysis of additional genetic makers. Mapping of the duplicated region in each family suggested that both regions encompassed the *FOXC1* gene leading to increased dosage for *FOXC1* as well as other genes within the duplicated region.

In June of 2002, Lehmann et al[29] presented a second family with iris hypoplasia and glaucoma that was found to have a partial 6p25 chromosomal duplication. This pedigree represented one of two recombination events upon which a proposal for a second 6p25 glaucoma locus, IRID1b had been based.[10] In addition, screening of 21 RS probands for 6p25 cytogenetic anomalies detected a proband with an interstitial deletion of a portion of 6p25. The presence of the deletion was confirmed in two other affected family members. Seven other indviduals with telomeric cytogenetic abnormalities were also studied. The three individuals that were trisomic for 6p25 were found to have micrococornea, while the four individuals that were monosomic for 6p25 were each diagnosed with RS. The authors speculate that the balance in gene dosage of a gene or genes in the interstitial duplicated regions versus that of the larger (telomeric) regions may influence the dimensions of the anterior chamber.

Functional Characterization of FOXC1

Saleem et al[30] investigated the effects of five missense mutations (S82T, I87M, F112S, I126M and S131L) on the structure and function of the *FOXC1* protein. Molecular modeling of the *FOXC1* forkhead domain indicated that the five missense mutations would not change the structure of the FOXC1 protein. The I87M mutation was found to drastically reduce the level of FOXC1 protein that was produced as compared to wild type FOXC1 (wtFOXC1) and the other four mutants. All five of the mutants were localized to the nuclei similar to what was observed for wtFOXC1. Due to the instability of the I87M mutation, further experiments with this particular mutant were not carried out. DNA-binding experiments showed that both the S82T and S131L mutations demonstrated decreased levels of DNA binding, while the binding levels of the F112S and I126M mutants were unchanged. In addition, the I126M mutant also demonstrated altered DNA binding specificity. Although both the F112S and I126M mutants were able to bind to FOXC1 binding sites at near wild type levels, both mutants exhibited defects in transactivation. The net effect of all five missense mutations was a reduction in FOXC1 transactivation activity though the I126M mutant did also display altered DNA binding specificity.

In March of 2002, Berry et al[31] identified and characterized important functional elements of the *FOXC1* gene. Two regions within the FOXC1 winged helix/forkhead domain were identified as being necessary for nuclear localization. The first region was observed to be rich in basic amino acids while the second region was found to be highly conserved among the various winged helix/forkhead proteins (Fig. 4). Of the two regions, only the basic region proved to be sufficient for nuclear localization. Two distinct transcriptional activation domains were identified at the extreme N- and C-terminal regions of the FOXC1 protein, while a transcription inhibitory element was found to be located in the central portion of the gene. These functional elements are graphically depicted as part of (Fig. 3). The transcription inhibitory domain was predicted to contain a number of amino acids that could be phosphorylated. Removal of the domain results in increased transactivation activity by FOXC1 and a reduction of phosphorylation.

FOXC1 Expression

The forkhead/winged helix family of DNA-binding transcription factors play important roles in embryogenesis, tissue-specific gene expression and tumorigenesis.[14] Widespread *Foxc1* gene expression during mouse embryogenesis, as well as in a variety of adult mouse and human tissues, has been reported by several laboratories.[8,10,15] Kume et al have shown that the classical mouse mutant *congenital hydrocephalus* is a result of a nonsense mutation (Q123X) in the mouse *Foxc1* gene.[32] Recent studies using mutant heterozygous *Foxc1*$^{+/-}$ mice revealed anterior segment abnormalities of the eye similar to those seen in Axenfeld Rieger syndrome patients including iris hypoplasia, displaced pupils, displaced Schwalbe's line, aberrantly developed trabecular meshwork, a small or absent Schlemm's canal and corneal opacity.[32-35] The combined data from patients with *FOXC1* mutations, as well as information from mouse models with *Foxc1* modifications suggest that both *FOXC1* haploinsufficiency and increased gene dosage can cause anterior chamber defects in the eye, and that precise regulation of *FOXC1* levels is critical for proper eye, kidney, urinary tract, heart, cartilage and bone development.[8,10,19,25,32-39]

Until recently, the majority of *FOXC1* studies have focused on the morphological aspects of ocular anterior segment dysgenesis using primarily knockout animal models. However, a study of *FOXC1* gene expression in isolated normal, nondiseased human ocular tissues using quantitative RT-PCR analysis demonstrated that *FOXC1* was expressed in discrete regions of the adult eye.[40] *FOXC1* expression was observed in the trabecular meshwork, optic nerve head, choroid/RPE, and ciliary body. The cornea, iris and optic nerve demonstrated lower levels of

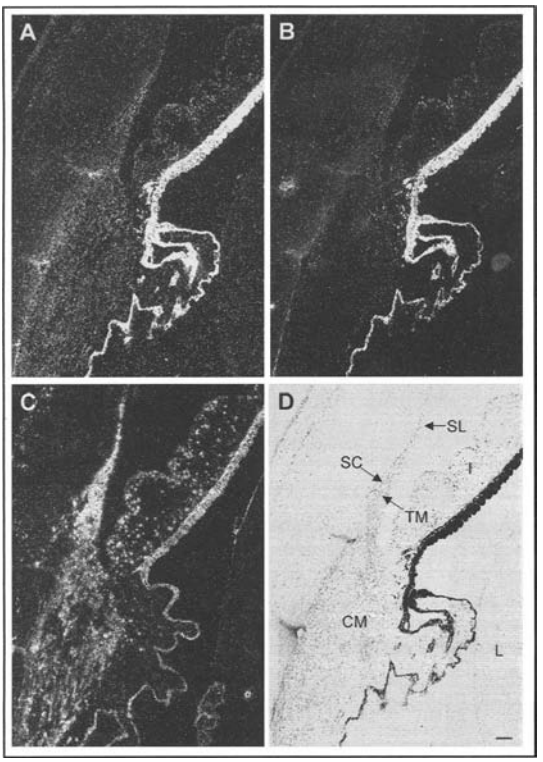

Figure 5. Expression of *FOXC1* in the normal human eye. Detection of *FOXC1* transcripts in longitudinal sections of normal human eye anterior segments by in situ hybridization. A) Antisense *FOXC1* riboprobe was localized in the trabecular meshwork, Schlemm's canal, scleral spur, ciliary muscle and iris. This expression pattern was observed consistently in all five donor eyes. B) Sense (control) *FOXC1* riboprobe showed no hybridization. C) Antisense *MYOC* (myocilin) riboprobe was localized in adjacent sections in the trabecular meshwork, Schwalbe's line, scleral spur, Schlemm's canal, ciliary muscle, and iris. D) Bright-field optics illustrate the morphology of the anterior segment. The autofluorescence observed in the iris posterior layer and ciliary epithelium in (A) and (C) is due to the presence of pigment when viewed using dark-field optics and is not a postitive hybridization signal. SL, Schwalbe's line, SC, Schlemm's canal, TM, trabecular meshwork, I, iris, CM, ciliary muscle, L, lens. Scale bar equals 100 microns. Autoradiography time *FOXC1*, 3 wks; *MYOC* 1 wk.

expression, and *FOXC1* transcripts were barely detectable in the retina and lens. It was noteworthy that elevated levels of expression were observed in the trabecular meshwork and optic nerve head; two tissues that are associated with glaucoma pathogenesis. The trabecular meshwork is involved with the development of elevated intraocular pressure, a key risk factor for the development of glaucomatous optic neuropathy. Cupping of the optic nerve head, optic nerve atrophy, and retinal ganglion cell death are hallmarks of all the glaucomas. The *FOXC1* expression profiling study was significant since it was the first report of *FOXC1* expression in either adult animal or human ocular tissues.

To further refine the analysis, in situ hybridization was used to localize *FOXC1* transcripts in the intact normal human eye and optic nerve. *FOXC1* expression was seen throughout the trabecular meshwork, as well as in the anterior-most nonfiltering region of the trabecular meshwork (Fig. 5A). In the ciliary body, *FOXC1* transcripts were observed in the ciliary muscle,

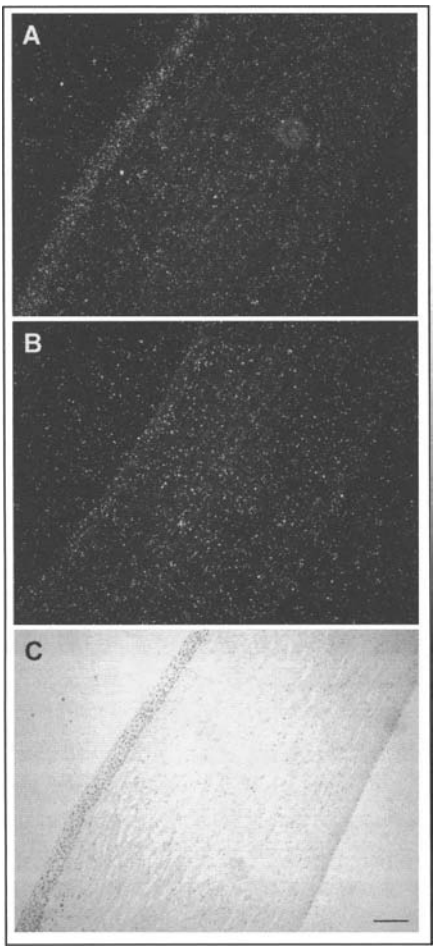

Figure 6. Expression of *FOXC1* in the human cornea. Detection of *FOXC1* transcripts in the cornea using in situ hybridization. A) *FOXC1* expression was noted in the epithelial cell layer of the cornea. B) No signal was detected using the *FOXC1* sense (control) riboprobe. C) Bright-field optics illustrate tissue morphology. Scale bar equals 50 microns. Autoradiography time: 3 wks.

which regulates aqueous humor outflow through the trabecular and uveoscleral pathways (Fig. 5A). Expression was noted in the contractile cells of the scleral spur; the region of the sclera between the ciliary body and Schlemm's canal. As seen in Figure 5A after 3 weeks of autoradiography, the level of *FOXC1* expression in the anterior segment was considerably lower than that which was observed after only 1 week of autoradiography in an adjacent section hybridized to the *MYOC* (myocilin) gene. *MYOC* is a gene that is associated with primary open angle glaucoma[41] and is abundantly expressed in the anterior chamber, particularly in the trabecular meshwork, ciliary muscle and iris (Fig. 5C).[42] *FOXC1* expression was also observed in a punctate manner in the iris (Fig. 5A), throughout the corneal epithelium (Fig. 6), and in the endothelium and media surrounding scleral blood vessels (Fig. 7). No detectable expression was noted in the retina, choroid, lens, or sclera (data not shown). In all tissues, no signal was detected using *FOXC1* or *MYOC* control sense strand riboprobes. The autofluorescence

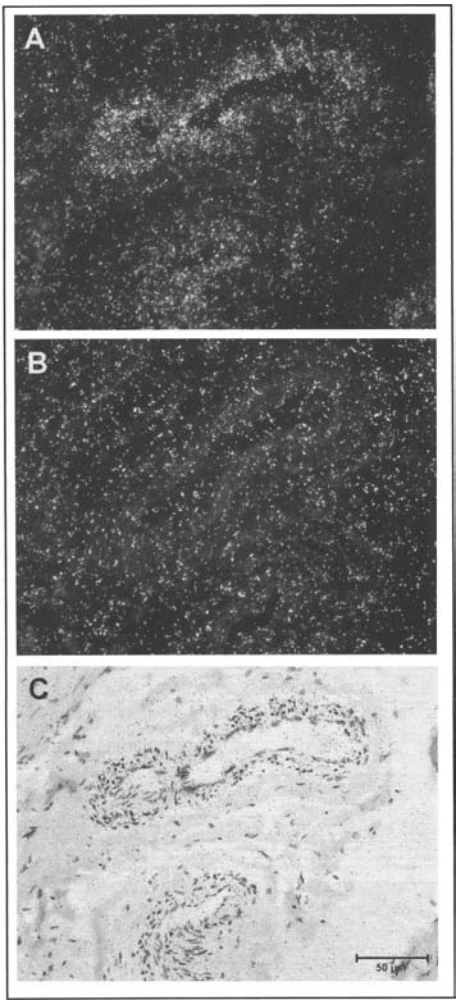

Figure 7. Expression of *FOXC1* in the human scleral blood vessels. Detection of *FOXC1* transcripts in scleral blood vessels using in situ hybridization. A) *FOXC1* expression was noted in the endothelium and media surrounding scleral vessels. B) No signal was detected using the sense (control) riboprobe. C) Bright-field optics illustrate tissue morphology. Scale bar equals 50 microns. Autoradiography time: 3 wks.

observed in the iris posterior layer and ciliary epithelium using both sense and antisense *FOXC1* and *MYOC* riboprobes is due to the presence of pigment when viewed using dark-field optics and is not a positive hybridization signal.

The highest level of *FOXC1* expression was seen in the meningeal sheath that surrounds the optic nerve at the level of the optic nerve head as well as in the retrolaminar region of the optic nerve. Following three weeks of autoradiography, an abundance of *FOXC1* transcripts were present in the dura mater, the outermost layer of the meningeal sheath that is composed of dense bundles of elastic tissue and collagen which surrounds and protects the intraorbital optic nerve (Fig. 8A). Expression was also detected in the arachnoid, or intermediate meningeal layer, which is made up of delicate connective tissue trabeculae lined by meningothelial cells.

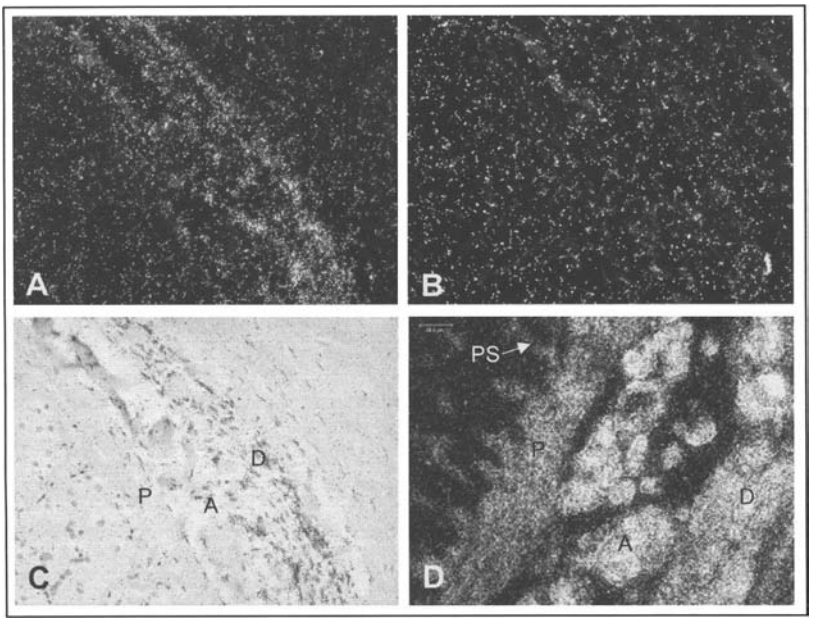

Figure 8. Expression of *FOXC1* in optic nerve. Detection of *FOXC1* transcripts in the meninges of the optic nerve using in situ hybridization. A) *FOXC1* expression was noted in the (A) arachnoid and (D) dura mater of the retrolaminar region of the meningeal sheath surrounding the optic nerve in sections cut 200 microns distal to the optic nerve head. B) No signal was seen using the *FOXC1* sense (control) riboprobe. C) Bright field optics illustrate tissue morphology in panels A and B. D) Localization of *MYOC* (myocilin) transcripts in the dura mater, arachnoid, pia mater and pial septa of retrolaminar sections of the same donor optic nerve cut 500 microns distal to the optic nerve head. P, pia mater. Scale bar equals 50 microns. Autoradiography time : *FOXC1*, 3 wks; *MYOC*, 2 wks.

Expression was not detected in the pia mater, the innermost meningeal layer comprised of fibrous tissue with multiple small blood vessels. Likewise, *FOXC1* transcripts were not detectable in the pial septa that divide and support the optic nerve fiber bundles. As seen previously in the anterior segment, the level of *FOXC1* expression in the meninges of the optic nerve head and in the retrolaminar region of the optic nerve is considerably lower than that seen for the *MYOC* gene (Fig. 8D). Although both *FOXC1* and *MYOC* transcripts were localized in the dura mater and arachnoid, only *MYOC* transcripts were observed in the pia mater and pial septa. Adjacent sections of the retrolaminar region of the optic nerve were stained with luxol fast blue to verify myelination of the optic nerve axons. A very low level of *FOXC1* expression was barely detectable above background in the substance of the axons at the level of the optic nerve head (data not shown).

Inability to detect *FOXC1* expression in the choroid/RPE may have been due to the abundance of pigment in these layers of the retina and their artifactual autofluorescence using dark-field optics that might have obscured a bona fide hybridization signal. *FOXC1* expression was barely detectable in the axons of the optic nerve head, yet expression in the dura mater and arachnoid layers of the surrounding meninges was readily observed using in situ hybridization. It is possible that the donor eyes may have experienced a degree of post-mortem decay unlike those used by Wang et al[40] that were deposited directly into the preservative RNA*later*. It is also possible that *FOXC1* expression in the substance of the optic nerve head can be detected only by the more sensitive technique of quantitative RT-PCR.

It appears that *FOXC1* expression in adult ocular tissues may perpetuate early embryonic expression patterns. *Foxc1* transcripts have been observed in the periocular mesenchyme, cornea, iris, hyoid blood vessels, presumptive trabecular meshwork, and in the ectoderm of the future inner eyelids of the developing mouse eye.[10,32,34] *Foxc1* is also responsible for the differentiation of the arachnoid cells in the meninges of the embryonic mouse brain.[32] One possibility is that *FOXC1* gene expression in the adult human trabecular meshwork and ciliary muscle regulate downstream target genes that influence the structure and function of these tissues and thereby influence intraocular pressure. Approximately 50% of RS patients develop glaucoma, the severity of which has been correlated with the level at which the iris is inserted into the iridocorneal angle.[43] As a consequence, the outflow of aqueous humor through the drainage structures located in the iridocorneal angle of the anterior segment can be impeded, leading to the elevated intraocular pressure and the progressive optic nerve destruction that is typical of glaucoma. Perhaps adult expression of aberrant FOXC1 protein in the developmentally malformed trabecular meshwork and ciliary body exacerbates the impeded aqueous humor outflow observed in RS patients with *FOXC1* mutations.

Other Forkhead Genes and Anterior Eye Chamber Defects

Smith et al[35] examined *Foxc1*$^{+/-}$ mice and found that these mice exhibit defects similar to those found in human RS patients with penetrance of observable phenotype in the mouse being dependant on the genetic background. However, despite the anterior chamber anomalies, the intraocular pressure (IOP) was found to be normal in all of the mice examined. *Foxc1* and *Foxc2* exhibit a very high degree of homology between their winged helix/forkhead domains (97% identity). Similar anterior segment anomalies were observed in both *Foxc1*$^{+/-}$ and *Foxc2*$^{+/-}$ mice suggesting that human FOXC2 might be involved in RS. However, screening of 32 RS patients failed to yield any viable mutation candidates. Thus, it appears that *FOXC2* mutations are unlikely to be a common cause of RS in humans. However, mutations in *FOXC2* have been shown to cause lymphedema-distichiasis syndrome, an autosomal dominant disorder that manifests as lymphedema of the limbs with variable age of onset and double rows of eyelashes.[44] Although a Y;16 translocation was used to narrow the candidate region within 16q24.3, the breakpoint was found to lie roughly 120kb distal to the *FOXC2* gene.

Dysgenetic lens (*dyl*) is an autosomal recessive mouse phenotype that is characterized by anterior segment defects such as lens and iris hypoplasia, fusion of the lens, iris and cornea, corneal dysplasia as well as severe cataract.[35] Defects in the mouse *Foxe3* gene have been demonstrated to cause dyl.[45,46] Saleem et al[30] noted that the F98S *dyl* mutant in Foxe3 corresponds to the F112S mutant in *FOXC1* and that in each case phenylalanine is replaced by a serine. It was speculated that like F112S, the F98S *Foxe3* mutant might result in a decrease in Foxe3 transactivation activity. Semina et al[47] screened 161 patients with a variety of anterior segment anomalies and detected a frameshift mutation 15 nucleotides upstream of the termination codon in a single family with anterior ocular dysgenesis and cataracts. The mutation was found in two affected individuals from the family, but was not seen in 180 control chromosomes. Ormestad et al[48] have proposed that *Foxe3* haploinsufficiency in the mouse may be a model for the human Peters' anomaly phenotype.

Future Directions

Although little is known about the downstream targets of *FOXC1* in the embryonic and adult eye, it will be important to determine if there are overlaps in downstream gene targets and whether continued *FOXC1* expression is a requirement for proper adult ocular tissue structure or function. It is noteworthy that *Foxc1* and *Foxc2*, a closely related winged-helix transcription factor, have recently been reported to interact with the Notch signaling pathway during

murine somitogenesis, thus providing the first evidence of *Foxc1* interaction in a known signaling pathway.[37] Continued study of *FOXC1* ocular target genes and the signaling pathways in which they function may lead to a better understanding of their malfunction in the development of RS. Additional functional studies of *FOXC1* as well as the creation of animal models to explore *FOXC1* gene dosage are promising areas of research. Also, as three members of the Fox family of transcription factors have been demonstrated to cause anterior segment defects, it is possible that other family members may also play a role in the normal development of the eye.

Acknowledgments

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CHAPTER 3

Rieger Syndrome and PAX6 Deletion

Ruth Riise

Rieger syndrome is a rare autosomal dominant inherited disorder mainly characterized by congenital anomalies of the anterior segment of the eye, the teeth and the skin around the umbilicus.[1] The anomaly of the eye is an iridogoniodysgenesis, Rieger anomaly including iris stromal hypoplasia with corectopia and/or polycoria. In the anterior chamber angle strands of iris tissue cross the angle (Rieger-Axenfeld anomaly) where Schwalbe's line can be anteriorly displaced (posterior embryotoxon). It has been known for a long time that this anterior segment anomaly involves a risk of glaucoma[2] stipulated at 50% by the age of 20 years.[3] The features of the teeth include hypodontia (missing teeth) especially the upper incisors, and peg-shaped teeth.[4] There is regularly a failure of the periumbilical skin to involute.[5] Some patients have cranial abnormalities with hypoplasia of the midface[6] and an underdeveloped premaxilla.[7] Empty cella and growth hormone deficiency may be found.[1] Several other abnormalities have been reported: cardiovascular disease,[8] hypospadias,[5] anal stenosis[9] and psychomotor retardation.[7]

In 1883 Vossius described the typical anterior chamber anomalies in combination with hypodontia and peg shaped teeth.[10] Rieger described the ocular features as a heritable disorder in 1935[11] and later also included the dental anomalies.[12] His name has since been connected to the syndrome, but not without some controversy.[13]

The phenotype of Rieger syndrome can vary.[13,14] Especially interesting are the asymmetric findings by Perveen and coworkers, who in a material of Rieger syndrome patients found various anterior segment anomalies; for example Rieger anomaly in one eye and Peters' anomaly in the other. Anterior segment anomalies as isolated findings can be caused by mutations in various genes including the *PAX6* gene[15] and the *PITX3* gene.[16]

The complete Rieger syndrome phenotype with anterior segment dysgenesis and dental and umbilical anomalies has predominantly been associated with mutations in the *PITX2* gene on chromosome 4q25,[16] the *FOXC1* gene on chromosome 6p25[17] and an unidentified gene on chromosome 13q14.[18] However, the syndrome has also been associated with partial monosomi 21q22.2.[19] In addition, recent studies have shown that the complete Rieger syndrome phenotype is also associated with a deletion in the *PAX6* gene,[20] a gene in which mutations had previously only been associated with anterior segment dysgenesis in isolated forms.[15] Riise and coworkers[20] described an 8-year old girl, the only child of healthy nonrelated parents. Her height and weight for age measurements were always within normal limits: height: 127 cm and weight 24.2 kg at seven years of age. She had been treated with mydriatics in the right eye because of corectopia. The patient had bilateral iris atrophy with a visible sphincter muscle (Figs. 1 and 2). In addition an excentric pupil and polycoria were found in the right eye, where the anterior chamber angle was extremely narrow. In the left eye strands of iris tissue traversed

Molecular Mechanisms of Axenfeld-Rieger Syndrome, edited by Brad A. Amendt.

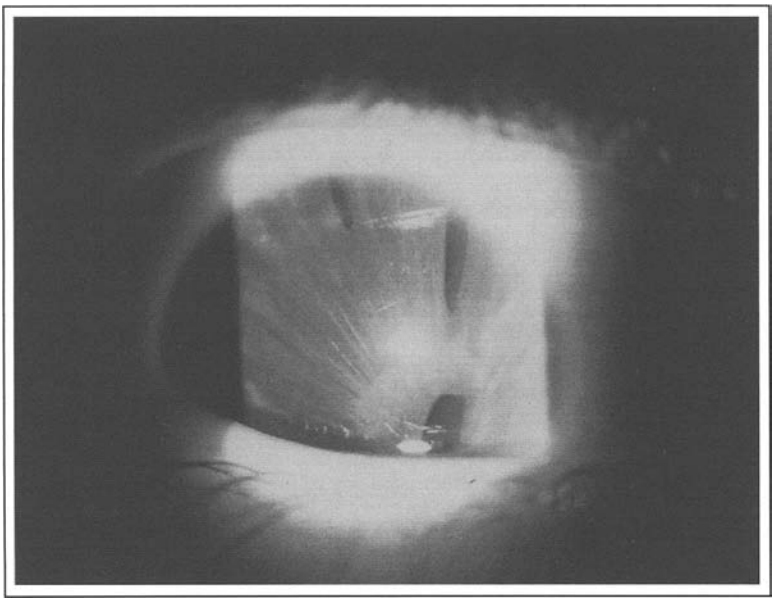

Figure 1. Eye anomalies. Iris atrophy and corectopia downward nasally with two iris defects upward in the right eye in an eight year-old girl with Rieger syndrome.[20]

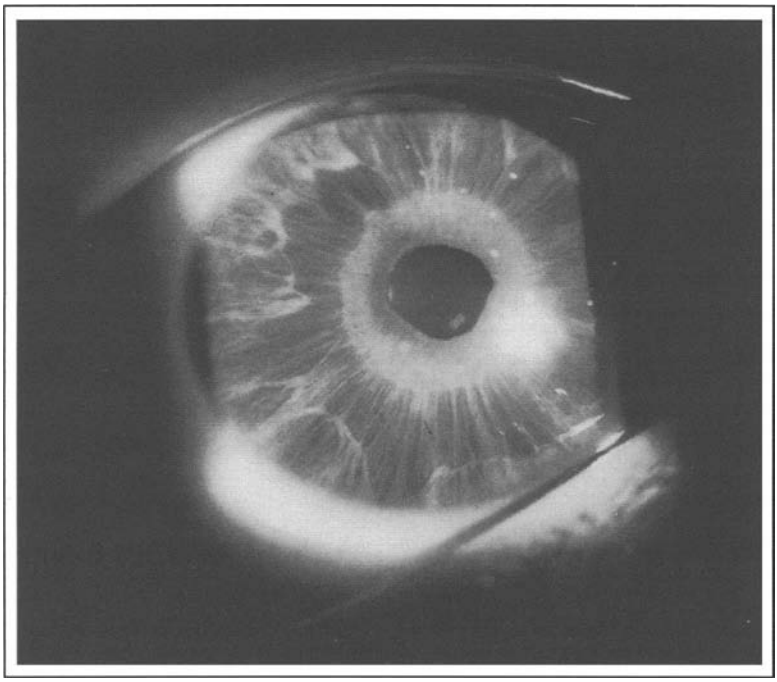

Figure 2. Eye anomalies. Iris atrophy in the left eye in an eight year-old girl with Rieger syndrome.[20]

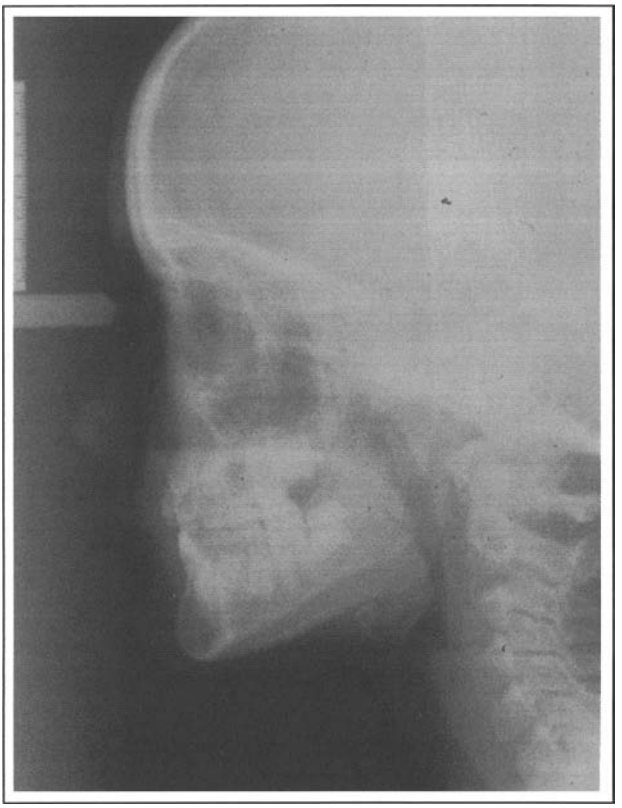

Figure 3. Craniofacial anomalies. A flat midface and an underdeveloped maxilla in an eight year-old girl with Rieger syndrome.[20]

the trabecular meshwork. Both eyes were microphthalmic with an axial length of 19.5 mm in the right eye and 19.8 mm in the left eye. The corneal diameter was reduced to 9 mm in the right and 11 mm in the left eye. The refraction was moderately hypermetropic with a spherical equivalent of +3.75 on the right and +3.0 in the left eye. Corrected visual acuity was 0.5 in the right and 1.0 (Snellen) in the left eye. Intraocular pressure was 7 mm Hg in the right eye and 9 mm in the left eye. Her inner canthal distance was 34 mm which is just beneath upper normal limit (age-specific normal range 23-34 mm).[21] Her midface was flat with hypoplasia of the premaxilla (Fig. 3). She had dental anomalies with small teeth and peg-shaped incisors in the mandible. The following teeth were missing 15, 13, 12, 11, 21, 23, 25, 51 and 61 (Fig. 4). There was failure of involution of the periumbilical skin (Fig. 5).

Chromosome Analysis

Conventional cytogenetic chromosome analysis showed a normal female karyotype 46XX. Fish (fluorescent in-situ hybridization) analysis with a probe for the *PAX6* gene showed a deletion encompassing the *PAX6* gene on one homologue of the *PAX6* gene on chromosome 11 (Fig. 6). The deletion did not include the Wilm's tumor gene. Neither parent had deletion of the *PAX6* gene. This patient matched clinically with a typical Rieger syndrome, with the ocular, dental and umbilical anomalies. In addition, microphthalmos was documented by ultrasound

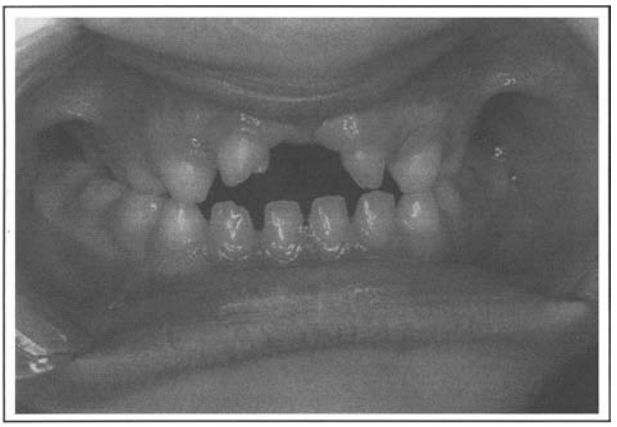

Figure 4. Dental hypoplasia. Congenital absence of teeth in the maxilla and peg-shaped front teeth in the mandible in an eight year-old girl with Rieger syndrome.[20]

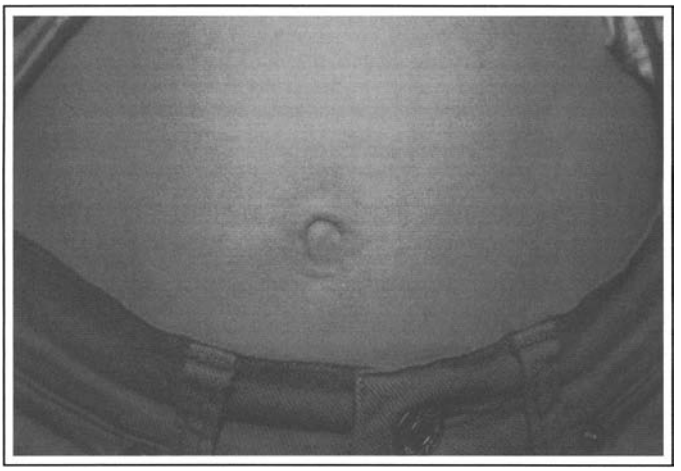

Figure 5. Umbilical anomalies. Failure of involution of the periumbilical skin in an eight year-old girl with Rieger syndrome.[20]

in our patient. This is an anomaly that has not been described in other cases of the Rieger syndrome unless the Rieger syndrome case with microcornea published by Frandsen[6] in reality represented a microphthalmic eye since ultrasound examination was not available. Microphthalmos has been found together with anterior segment dysgenesis in isolated forms.[22]

It is not surprising that a mutation in the *PAX6* gene, which is central for eye morphogenesis and evolution, could involve microphthalmos. *PAX6* mutations and deletions may also result in for example Peters' anomaly[15] and aniridia.[23]

Pax6 Expression

Major sites of *PAX6* expression include the neural tube, the brain and the developing eye. *Pax6* is strongly expressed in the ectoderm- and neuroectoderm-derived tissues of the eye,

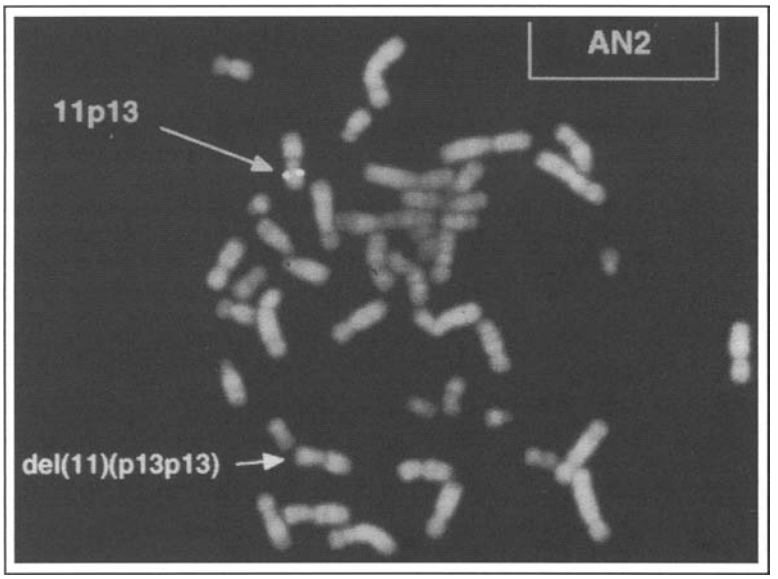

Figure 6. Abnormal chromosome 11. Deletion on one homologue of the chromosome 11 shown by FISH (fluorescent in-situ hybridization) analysis with probes for the PAX6 gene.[20]

including the lens, the corneal epithelium and the rim of the optic cup.[24,25] Pax6 is a key transcription factor in lens development. Pax6 was originally identified as a member of the Pax protein family having paired DNA-binding domains. Pax6 contains a paired domain and a second DNA-binding domain, the paired-type homeobox at the carboxy-terminal end.[26] In addition to eye development, Pax6 is also necessary for normal development of the nose, pancreas and the central nervous system.[27-30] *Pax6* is first expressed in a broad region of the head ectoderm and later becomes restricted to the presumptive lens placode.[31]

Summary

The anomalies in most of the involved tissues in Rieger syndrome (anterior segment of the eye, cranial bones, teeth, periumbilical skin) are derived from the neural crest. If *PAX6* expression in the neuroectoderm includes the neural crest, most of the Rieger-anomalies could be explained by a *PAX6* deletion. However, little is known about the behavior of cells within the human anterior neural tube and the molecular action of PAX6 has not been determined.

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CHAPTER 4

The Molecular and Biochemical Basis of Axenfeld-Rieger Syndrome

Brad A. Amendt

Mutations in the *PITX2* homeobox gene are associated with Axenfeld-Rieger syndrome (ARS) and provided the first link of this transcription factor to tooth, eye, heart, and pituitary development. We are investigating the molecular basis of developmental anomalies associated with human *PITX2* mutations. PITX2 mutant proteins exhibit a variety of transcriptional defects including, instability, decreased DNA binding activity, DNA binding without transcriptional activation, phosphorylation defects, increased transcriptional and dominant negative activities. ARS is a haploinsufficiency disorder and because PITX2 proteins can dimerize the effects of the *PITX2* mutations can cause heterogeneous developmental anomalies in these patients. *FOXC1* is a member of the Forkhead Box transcription factor family that play key roles in development, including morphogenesis and cell fate specification. *FOXC1* mutations are associated with ARS and Axenfeld-Rieger Anomaly (ARA) that result in a spectrum of glaucoma phenotypes in humans. Missence mutations in the FOXC1 forkhead domain result in impaired DNA binding and reduced transactivation of target genes. *Pax6* is a *paired*-like homeobox gene and recently a mutation in this transcription factor has been linked to Rieger syndrome. This report will summarize the molecular/biochemical mechanism of these developmental transcription factors and how they correlate with the clinical manifestations of ARS and ARA.

Introduction

The human *PITX2* gene (MIM 601542) is a member of the *paired*-like homeobox transcription factor family.[1,2] The homeobox gene family members play fundamental roles in the genetic control of development, including pattern formation and determination of cell fate (for a review see refs. 3-5). The homeodomain of PITX2 has a high degree of homology to other paired-like homeodomain proteins, P-OTX/Ptx1/Pitx1,[6,7] Pitx3,[8] and to a lesser extent to unc-30, Otx-1, Otx-2, otd and goosecoid.[1] The homeobox proteins contain a 60 amino acid homeodomain that binds DNA. PITX2 contains a lysine at position 50 in the third helix of the homeodomain that is characteristic of the Bicoid-related proteins.[9-11] This lysine residue selectively recognizes the 3'CC dinucleotide adjacent to the TAAT core.[4,12] We have shown that PITX2 binds to the DNA sequence 5'TAATCC3',[13] which is also recognized by Bicoid protein.[14] PITX2 also contains a highly conserved 14 amino acid C-terminal domain that is found in the *paired* class of homeodomain genes *Otp, aristaless and Rx*[1,15] and we term this the OAR (otp, aristaless and Rx) domain.

The human *FOXC1* gene (MIM 601090) is a member of the winged-helix family of transcription factors.[16] The forkhead/winged-helix family of transcription factors are required

Molecular Mechanisms of Axenfeld-Rieger Syndrome, edited by Brad A. Amendt.

for a variety of developmental processes including embryogenesis and cell/tissue differentiation. These transcription factors are characterized by a 110 amino acid DNA binding domain.[17] The DNA binding domain consists of three α helices and two large loops that form "wing" structures, this conformation is termed a "winged-helix". *FOXC1* (formerly *FREAC3* and *FKHL7*) mutations are associated with ARS and more commonly seen as affecting anterior eye-segment defects associated with ARA, mapping to chromosome 6p25.[18-21] Recently, it has been reported that missense mutations of the *FOXC1* transcription factor produce mutant proteins with altered functions.[22]

Pax genes encode a family of transcription factors that contain paired box DNA binding domains and function in developmental control. The *Pax* genes have diverse tissue-specific expression patterns and homozygous mutations in the majority of them result in specific developmental defects.[23] *Pax 6* gene dosage has been shown to correlate with specific developmental defects.[24] Heterozygous mutations in *Pax6* are responsible for the Small eye (Sey) phenotype in the mouse, aniridia and Peters' anomaly in humans.[25-27] Homozygous *Pax6* mutants fail to form a lens placode.[28] It has been shown that reduced levels of Pax6 resulting from a heterozygous condition caused a delay in lens placode formation.[24] Researchers have now demonstrated that ARS is associated with specific mutations in *PAX6*.[29] However, the mechanism of PAX6 mutant proteins in causing the ARS developmental defects has not yet been determined.

This review will focus on the molecular/biochemical mechanisms of the PITX2 and FOXC1 developmentally regulated transcription factors. The structure and function of wildtype proteins will be compared to the effects of specific mutations associated with ARS, Rieger syndrome, Axenfeld anomaly and Rieger anomaly.

Molecular/Biochemical Analysis of PITX2 Transcriptional Activities

The molecular and biochemical properties of the human homeodomain transcription factor, PITX2 are being studied by several laboratories.[1,13,15,30-35] Three major PITX2 isoforms have been identified which result from alternative splicing and alternative promoter mechanisms (Fig. 1).[1,15,36-38] *PITX2A* and *PITX2B* are generated by alternative splicing mechanisms and *PITX2C* uses an alternative promoter located upstream of exon 4 (Fig. 1). All isoforms contain dissimilar N-terminal domains while the homeodomain and C-terminal domains are identical.

One mechanism for the regulation of gene expression is by alternatively spliced transcription factors. Alternative splicing of transcription factors provides a mechanism for the fine-tuning of gene expression during development. The three major *Pitx2* isoforms have been shown to differentially regulate organogenesis. However, the molecular mechanism for this development preference of the PITX2 isoforms is unknown. The transcriptional mechanisms of these PITX2 isoforms are beginning to be understood. Our laboratory has been studying the mechanism of PITX2 transcriptional regulation and has identified several genes regulated by PITX2.[39-41] Our studies reveal a promoter and cell dependent activation by the three major PITX2 isoforms. The PITX2 isoforms can interact by forming homodimers or heterodimers to synergistically activate or repress gene expression.[34]

Recent research has shown that PITX2 isoforms can interact with other transcription factors to regulate their activity. A recurring theme among homeodomain proteins is the important role of protein-protein interactions in modulating activity. For PITX2, the C-terminal region of the protein has been identified as a site for protein-protein interactions.[34,39,41] A mechanism for regulating the transcriptional actions of PITX2 is its interaction with other transcription factors. PITX2 can directly bind at least one other homeodomain protein, Pit-1, via the C-terminal domain of PITX2.[39] Pit-1 is a POU homeodomain protein that regulates pituitary cell differentiation and expression of pituitary hormones, including prolactin.[42] At least one manifestation of this interaction is increased PITX2 DNA binding in

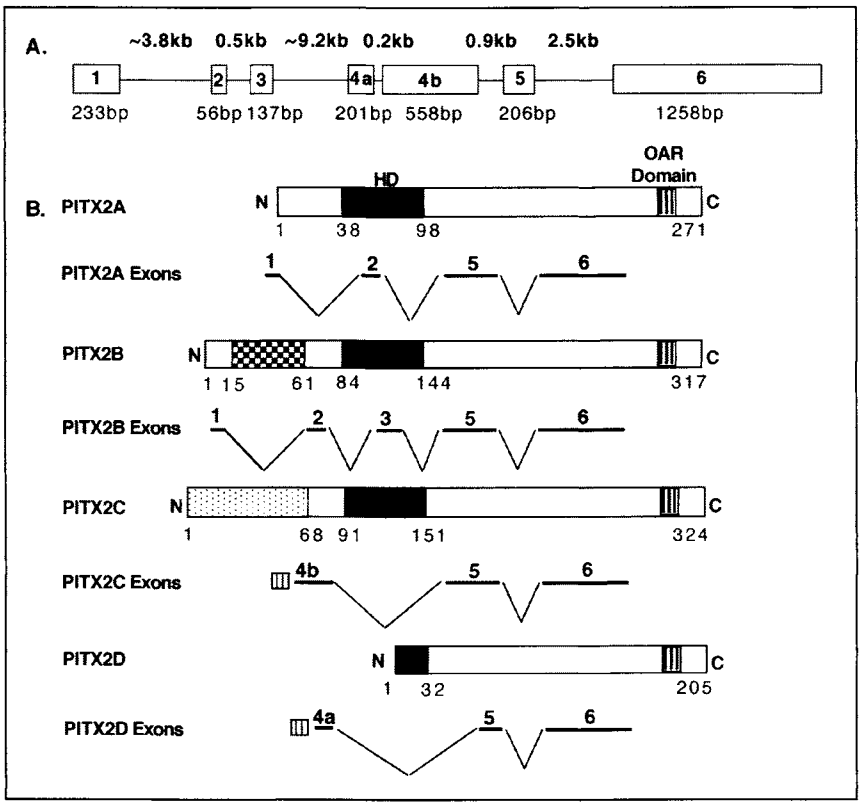


Figure 1. PITX2 major isoforms found in humans. A) Genomic organization of the *PITX2* gene, intron sizes are shown on the top and exon sizes at the bottom; exons are numbered. B) The protein structure is shown with the location of the homeodomain (HD) and 14 amino acid conserved OAR domain. Checkered and stippled boxes denote the differences in the N-terminal region of the isoforms. The exons that code for the respective proteins are shown below each isoform. PITX2C and PITX2D RNA's are transcribed using an internal promoter shown as a striped box flanking exon 4.

vitro. Furthermore, Pit-1 and PITX2 synergistically interact to activate the *prolactin* promoter.[13,39] All of the major PITX2 isoforms can interact with Pit-1 to synergistically activate the *prolactin* promoter, (unpublished observations).[43] New insights into pituitary development were revealed by demonstrating that the three major PITX2 isoforms interact to significantly increase *prolactin* expression.[34] Thus, the levels and combinations of *PITX2* isoform expression would contribute to the dosage-response model proposed for pituitary and other organ development.[44, 45] Because the three major PITX2 isoforms all activate the *prolactin* promoter at similar levels this may explain why pituitary development is mostly unaffected in ARS patients.

Other pituitary-specific PITX2 target genes have been described.[43] Three genes outside of the pituitary have been identified that are specifically regulated by PITX2. PITX2 regulates procollagen lysyl hydroxylase (*PLOD1*) and *Dlx2* gene expression.[41,46] The *PLOD1* gene encodes an enzyme responsible for hydrolyzing lysines in collagens, which plays a role in specifying the extracellular matrix and provides a foundation for the morphogenesis of tissues and organs. The *Dlx2* gene encodes a transcription factor expressed in the mesenchymal and epithelial cells of the mandibular and maxillary regions and expressed in the diencephalon. *Dlx2*, a member of the distal-less gene family, has been established as a regulator of branchial arch

development.[47,48] Homozygous mutants of *Dlx2* have abnormal development of forebrain cells and craniofacial abnormalities in developing neural tissue, *Dlx* genes exhibit both sequential and overlapping expression, implying that the temporal-spatial regulation of *Dlx* genes are tightly regulated.[49] *Pitx2* and *Dlx2* genes are expressed in the same tissues early during development with *Pitx2* expression occurring earlier than *Dlx2* in the craniofacial region. Interestingly, PITX2B is unable to activate the *PLOD1* and *Dlx2* genes, however it synergistically activates these two promoters in combination with PITX2A or PITX2C.[34]

A PITX2 target gene was identified that is specifically involved in heart development. The 3.0 Kb *atrial natriuretic factor* (*ANF*) promoter contains multiple PITX2 binding sites and is positively regulated by PITX2. *Pitx2* and *ANF* have overlapping expression patterns in the heart during development. *ANF* expression is differentially activated by the three major PITX2 isoforms however, only PITX2C in combination with Nkx2.5 can synergistically activate the *ANF* promoter. Interestingly, Nkx2.5 represses PITX2A activation of the *ANF* promoter in the C3H10T1/2 embryonic cell line. Pitx2 and Nkx2.5 are two transcription factors that represent early markers in heart development and both play major roles in vertebrate cardiogenesis.[44,50-54] Nkx2.5 is required for early cardiogenesis through its role of specifying early cardiac progenitors. Nkx2.5 is essential for cardiomyogenesis, homeostasis and survival of cardiac myocytes in the adult heart.[55,56] Furthermore, mutations in *Nkx2.5* have been shown to cause congenital heart disease.[57-59] The identification of cardiogenic target genes for these transcription factors presents a major challenge for those studying their functional activities. We demonstrate that PITX2 regulates *ANF* expression and furthermore that *PLOD1* is a target gene for Nkx2.5. More importantly these data place PITX2 in the class of myocardial transcription factors required for commitment of heart development. These data provide a molecular basis for PITX2 function in heart development and for heart defects in ARS patients. These data corroborates genetic and epigenetic studies, which demonstrate that the *Pitx2c* isoform is the major effector of heart development. Furthermore, a negative or repressive effect was identified that regulates PITX2A transcriptional activation of the *ANF* promoter. In support of these findings it has been suggested that a negative regulatory mechanism may be acting on Pitx2 to regulate looping morphogenesis (see Chapters 6 and 7). A majority of heart defects in ARS patients involve the septum and atria, which coincide with *Pitx2* and *ANF* expression.

The identification of a fourth minor *PITX2* isoform expressed in humans adds another level of regulation to the transcriptional activity of PITX2.[34] A new *PITX2* isoform was identified from a human craniofacial library. It is made by alternative splicing of a transcript produced from the internal promoter located in intron 3 which also produces the *PITX2C* isoform (Fig. 1). *PITX2D* results from splicing of exon 4a to a cryptic 3' splice site in exon 5, which produces a truncated homeodomain and complete C-terminal tail. This isoform does not bind to DNA as expected since it does not contain a functional homeodomain.[34] PITX2D has no transcriptional activity when transfected with promoters in transient transfection assays. However, when cotransfected with PITX2A, PITX2D caused a 3-fold reduction in PITX2A transcriptional activity in CHO cells.[34] When cotransfected with PITX2C it caused a 2-fold repression of PITX2C transcriptional activity in CHO cells. These data reveal that PITX2D can negatively regulate the transcriptional activities of PITX2A and PITX2C isoforms.[34]

A possible explanation for these results could involve a mechanism where the *PITX2D* RNA inhibits the translation of the other PITX2 isoforms in transfected cells. However, PITX2A and PITX2C protein expression was unaffected by coexpression of PITX2D.[34] These results would then suggest that factors specific for CHO cells might be interacting with PITX2D to facilitate its repression of PITX2A and 2C transcriptional activity. However, the repressive effect by PITX2D does not appear to be due to specific factors associated with a specific cell line. The repressive effects by PITX2D were also observed with other promoter constructs including the *PLOD1* promoter demonstrating that this effect is not restricted to a specific promoter. Yet

another explanation for suppression of PITX2A and PITX2C transcriptional activity could be due to PITX2D inhibiting the DNA binding activities of the other PITX2 isoforms.

PITX2D *Physically Interacts with* PITX2A *and* PITX2C *Isoforms*

The PITX2D isoform can physically interact with the other PITX2 isoforms.[34] These experiments corroborate previous experiments demonstrating that PITX2 isoforms interact through their C-terminal tails.[41] Since all PITX2 isoforms contain identical C-terminal tails, then each isoform has the capability to interact with other isoforms. One explanation for the suppression of PITX2A and PITX2C activity by PITX2D might be due to the inability of a PITX2A/2D or PITX2C/2D complex to bind DNA. Electrophoretic mobility shift assays (EMSA's) were performed using PITX2A and PITX2C mixed with PITX2D and found neither a loss of binding or increased PITX2A or PITX2C binding activity. Clearly, the easiest explanation is that PITX2D is binding factors essential for PITX2 activity, thereby sequestering that factor(s) from interacting with PITX2 isoforms. While this is a possibility, we speculate that PITX2D directly binds to PITX2A and PITX2C to inhibit their transcriptional activity. This mechanism is analogous to a previous report demonstrating that the C-terminal 39 amino acid peptide can also inhibit the transcriptional activity of PITX2A (Fig. 2).[39]

We propose that intramolecular folding of the full length PITX2 protein brings the C-terminal tail in direct contact with the N-terminal domain (Fig. 2A). This folding would interfere with DNA binding by the homeodomain. However, after PITX2 binds DNA this disrupts the C-terminal tail interaction with the N terminus. Expression of the PITX2 C39 peptide (last 39 amino acids of the PITX2 C-terminal tail) inhibits transactivation by wildtype PITX2.[39] The C39 peptide appears to interact with PITX2 through the N-terminal 16-38 residues (Fig. 2C). Thus, in the absence of a cofactor, the C-terminal 39 residues interact with a domain in the N-terminus of PITX2 to modulate the DNA binding and transcriptional activities of PITX2. When a specific cofactor such as Pit-1 binds to the C terminus it relieves this inhibition. Pit-1 binding to PITX2 may cause a conformational change in the C-terminal tail that unmasks the homeodomain and a potential transactivation domain (Fig. 2B). The model predicts that PITX2 may not be fully activated until expression of the appropriate cofactors. The PITX2D isoform can interact with the other PITX2 isoforms to inhibit their activity presumably through the same mechanism as the PITX2 C39 C-terminal peptide.[34] Because PITX2D can interact with the C-terminal tail of the other PITX2 isoforms we predict that PITX2D binding inhibits the transcriptional activity of the other isoforms by masking transactivation domains. The conformational structure of the dimer complex apparently inactivates the transcriptional activity of the dimer complex (Fig. 2D). Analogous to the inhibition of PITX2 activity by the C39 peptide and PITX2D, it was shown that a dominant negative PITX2 K88E ARS mutation can form dimers and inhibit wildtype activity (Fig. 2E).[31]

In another ARS PITX2 mutation, a C-terminal truncation at amino acid 133 causes several developmental defects. This mutation demonstrates the importance of the PITX2 C-terminal tail. Thus, in addition to its expression in the pituitary, PITX2 is also required for eye and tooth development suggesting that PITX2 is regulated in multiple tissues by a combination of interacting factors.[1] The ability of PITX2 to be activated during development could be a function of factors interacting with its C terminus to increase DNA binding and transcriptional activity. The C-terminal tail contains a 14 amino acid stretch that is conserved among the PITX family members and several other homeodomain proteins (Fig. 1).[1] Many of these proteins, prx1, 2, Cart1, aristaless, chx10, otp, and Pitx1, are expressed at high levels in the craniofacial region, suggesting an important role for this multifunctional C-terminal regulatory mechanism in craniofacial development.

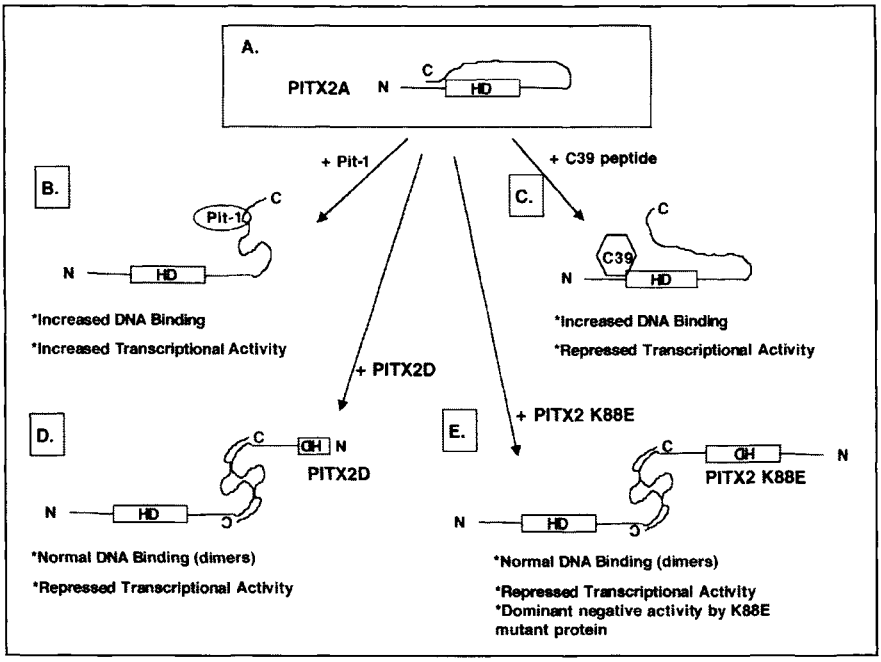


Figure 2. Model for the multifunctional role of the PITX2 C-terminal tail. A) The PITX2A protein is shown as an intramolecular folded species. The folding interferes with DNA binding of PITX2A. B) Pit-1 binds to the C-terminal tail of PITX2A and disrupts the inhibitory function of the C terminus. This allows for a more efficient homeodomain interaction with the target DNA and transactivation. C) PITX2 C39 peptide interaction with the N terminus of PITX2A displaces the C-terminal tail and increases its binding activity. However, the C39 peptide masks an N-terminal transactivation domain that results in repressed transcriptional transactivation. D) PITX2D isoform interacts with the C-terminal tail of PITX2A, B and C isoforms and also displaces the C-terminal tail but does not affect DNA binding. However, the PITX2D isoform acts to inhibit the C-terminal transactivation domain of the other PITX2 isoforms, which results in repressed transcriptional activation. E) The PITX2A K88E ARS mutant protein does not bind DNA by itself but instead interacts with the C-terminal tail of PITX2 isoforms to inhibit their transcriptional activity. This represents a dominant negative effect by an ARS mutant protein. N = N-terminal end; C = C-terminal end; HD = homeodomain.

Phosphorylation of PITX2 *by Protein Kinase C*

Another mechanism for regulating the activity of transcription factors is through phosphorylation. Analysis of the PITX2 protein revealed 10 consensus protein kinase C (PKC) sites (S/T X K/R, K/RXXS/T, K/RXS/T), located throughout the PITX2 protein (Fig. 3A).[60] PKC selectively phosphorylates serine and threonine residues in specific amino acid sequences. There also appears to be several casein kinase II (CKII) sites (S/T XX EX) located within the PITX2 protein. We have observed specific phosphorylation of PITX2 with PKC and no phosphorylation by CKII or protein kinase A (PKA), other known serine/threonine kinases.[35] PKC site specific mutagenesis has demonstrated that all 10 PKC sites within PITX2 are phosphorylated by PKC. Phosphorylation of the C-terminal tail increases PITX2 transcriptional activty while phosphorylation of the N terminus is inhibitory for transcriptional activity.

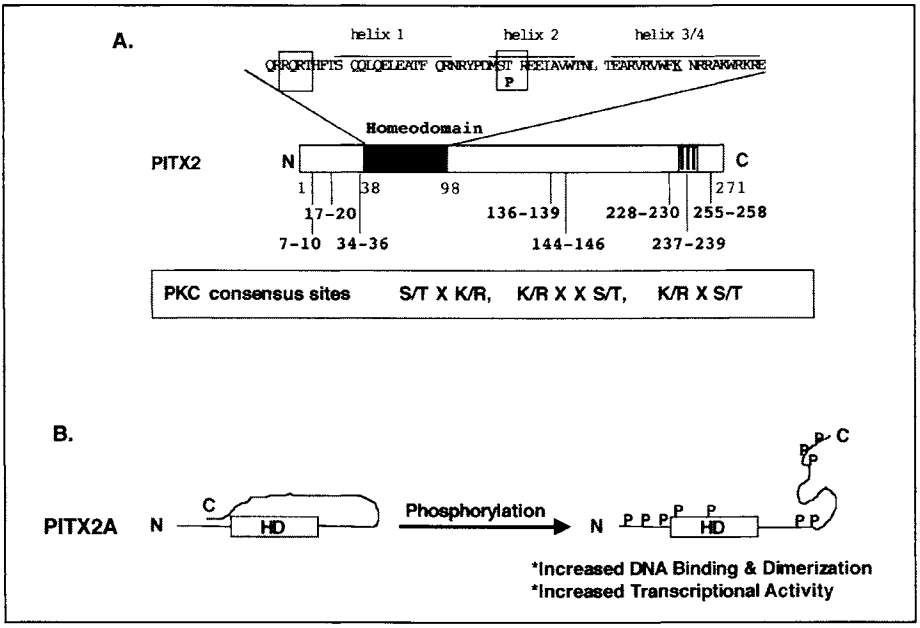


Figure 3. PITX2 phosphorylation by protein kinase C. A) Schematic of the consensus PKC sites within the PITX2A homeodomain protein. Two sites are located in the homeodomain and are boxed out; one site contains the PITX2A T68P ARS mutation. The PKC consensus sites are shown within the protein by the amino acid numbers in bold. One site in the C terminus is located within the OAR domain (stripped box). The PITX2A T68P mutation is shown with a bold letter under the wildtype sequence. B) A model for the displacement of the C-terminal tail by phosphorylation of serine/threonine residues in the N terminus, homeodomain and C terminus. PITX2A is highly phosphorylated by PKC, which leads to increased DNA binding, formation of dimers and transcriptional activity.

Phosphorylation of PITX2 *Acts to Increase Its DNA Binding Activity*

PITX2 is phosphorylated by PKC and it has been reported that phosphorylation of homeodomain proteins can either stimulate DNA binding or inhibit DNA binding activity.[61] To examine this possibility EMSA's were performed with phosphorylated PITX2 proteins to determine if phosphorylation affected DNA binding. A 2-fold increase in DNA binding was observed for PITX2 phosphorylated proteins.[35] Interestingly, phosphorylation acts to facilitate dimer formation of PITX2 (Fig. 3B).

PITX2 *Transcriptional Activity Is Stimulated by Phorbol 12-Myristate 13-Acetate (PMA) an Activator of PKC Activity*

Addition of PMA to *PITX2* transfected CHO cells increased the activation of the *Dlx2* promoter from 30-fold to 65-fold activation.[35] These data suggest that PITX2 phosphorylation facilitates protein interactions (Fig. 3B).

Biochemical Analysis of *PITX2* Mutations Associated with ARS

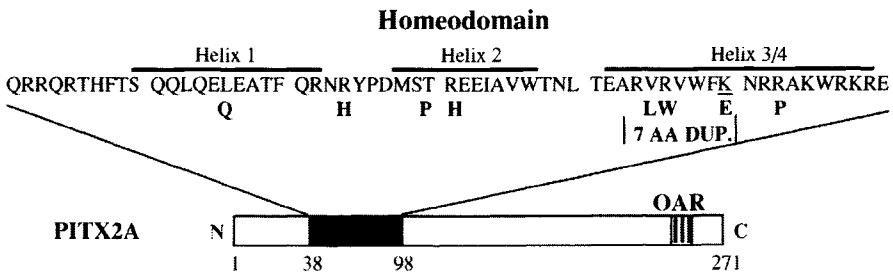
In patients with ARS, eleven mutations have been identified in *PITX2* (Table 1). Seven of these mutations are single amino acid changes and three others cause premature termination due to altered splicing or a stop mutation. One splice site mutation produces an unstable protein and both splice site mutations alter the reading frame of PITX2. Interestingly, one

mutation is a 7 amino acid duplication of residues 43 to 49 at position 82 to 88 of the homeodomain (Table 1). Two PITX2A mutations result in mild forms of ARS. The R69H mutation is associated with a patient presenting clinically with iridogoniodysgenesis, and the R84W mutation is seen in a patient with iris hypoplasia or Rieger anomaly (Table 1). The importance of the C-terminal region is best highlighted by the identification of at least two human mutations in this region (Table 1). One of these mutations causes a premature termination that predicts a protein lacking most of the C-terminal region. These patients have Axenfeld-Rieger syndrome. The second mutation is a missense mutation in the C-terminal domain. This second mutation is fairly rare and has only been found in a patient that has a partially penetrant Rieger phenotype with sclerocornea. This latter mutation raises the possibility that a partially functional protein is generated.

PITX2 binds to the *bicoid* DNA element (5'TAATCC3') with a high specificity and affinity.[13,39] PITX2 can also bind to *bicoid*-like DNA elements such as TTATCC, GAATCC, TATTCC, CAATCC however, PITX2 binds to these elements less efficiently than the consensus *bicoid* element (unpublished data).[13,39,41] The 3' CC dinucleotide appears to be essential

Table 1. Analysis of PITX2 mutations associated with Axenfeld-Rieger syndrome

A. PITX2 protein and homeodomain location of specific ARS mutations



B. Human PITX2 mutations

PITX2 Mutation	Region	Frequency	Diagnosis
L54Q	homeodomain	1/30	ARS
R62H	homeodomain	1/30	ARS
T68P	homeodomain	1/30	ARS
R69H	homeodomain	1/30	Iridogoniodysgenesis
T82 to K88	homeodomain	ND	ARS
V83L	homeodomain	ND	ARS
R84W	homeodomain	1/30	Iris Hypoplasia
g(+6)c	5' ss, intron3/homeo	1/30	ARS
a(-11)g	3' ss, intron3/homeo	1/30	ARS
K88E	homeodomain	1/30	ARS
R91P	homeodomain	1/15	ARS
W94 STOP	homeodomain	1/30	ARS
W133 STOP	C-terminal	1/30	ARS
A135T	C-terminal	ND	Sclerocornea

for PITX2 binding activity with the exception of the *paired* class P3 element (5'TAAT CTG ATTA3') which can bind PITX2 albeit at lower levels than the *bicoid* element (unpublished data).[39] The initial studies on the binding characteristics of PITX2 were performed using artificial probes containing known DNA binding elements. However, when PITX2 DNA binding was tested using native promoter DNA sequences, PITX2 binding to these elements resulted in increased DNA binding and the formation of PITX2 homodimers.[41] The formation of PITX2 homodimers was observed with both bacteria purified PITX2 and endogenously expressed PITX2 in nuclear extracts.[41] It is clear that PITX2 binds to DNA as a dimer, which has reciprocal effects on gene expression by ARS mutant proteins and PITX2 isoform proteins. Thus, the type of *bicoid* element and sequences flanking the element greatly influence binding of the PITX2 protein.

The binding specificity of homeodomains is dictated mostly by residues in the recognition helix and the N-terminal arm.[9,12,62-64] The side chains of amino acids at positions 25, 28 and 31 of the Antp homeodomain contact phosphate groups of the 5'TAAT3' DNA binding element.[4] The amino acids located in positions 6 and 10 in helix 3 of the *Drosophila* Bicoid homeodomain have been shown to recognize the TAAT core sequence.[65] It has been demonstrated that the amino acid at position 50 of the Bicoid homeodomain is critical for recognizing the 3'-CC dinucleotide of the DNA binding sequence.[4,12]

Several PITX2A mutant proteins do not bind the *bicoid* DNA or at severely reduced levels. These include PITX2A L54Q, g(+6)c, a(-11)g, R91P, K88E and the T82-K88 duplication mutants (Table 2A). In the case of PITX2A L54Q, g(+6)c and a(-11)g mutants these proteins are unstable in both bacteria and mammalian cell lysates (unpublished data).[13] The PITX2A K88E mutant protein is stable but does not bind the *bicoid* DNA probe as expected since this mutation occurs at the lysine residue required for recognizing the CC dinucleotide.[31] The PITX2A R91P and T82-K88 mutants are also stably expressed but bind the *bicoid* element at severely reduced levels compared to wildtype.[13,66] These data indicate that certain mutations in the homeodomain of PITX2A can greatly affect the DNA binding properties of the protein. Loss of DNA binding activity would not allow specific gene expression required during embryogenesis of the affected morphological structures.

Other PITX2A mutant proteins bind the *bicoid* element at moderately to slightly reduced levels compared to wildtype protein (Table 2A). The PITX2A T68P, R69H, V83L and R84W mutants bind DNA at reduced levels and all are stably expressed in mammalian cells.[13,32,35,66] However, specific mutations affect binding to the *bicoid* probe they could also change the DNA binding specificity. Thus, the PITX2A mutant proteins could bind efficiently to other DNA elements. These results are the easiest explanation for the phenotypic anomalies observed in ARS patients. Since a lack of specificity of the mutant protein would lead to defective gene expression. A mechanism for the anomalies seen in ARS patients could be due to inappropriate gene expression caused by the mutant PITX2 proteins binding to other DNA elements contained in their promoters. Two of these mutant proteins, PITX2A T68P and R84W do not have altered DNA binding specificity's.[13,35] Research indicates that at least in the case of PITX2A T68P and R84W that the *PITX2A* mutations do not change the DNA binding specificity of the ARS mutants.

There are several *PITX2* gene mutations that result from chromosomal breakpoints and deletions (Table 2B). These either change expression of *PITX2* or do not produce a protein product. These *PITX2* mutations will not be described in this report.

Transcriptional Activities of PITX2A *Mutations*

The PITX2A L54Q, T68P, K88E, R91P and T82-K88 mutants are transactivation defective (Table 2A). They are unable to activate promoters containing *bicoid* elements when assayed in several cell lines.[13,31,32,66] As discussed above these mutant proteins are either unstable

Table 2A. Summary of PITX2 mutations

Mutation	Diagnosis	Exon	Gene Region	Effect on Protein Function	Reference
Leu54Gln	Axenfeld-Rieger syndrome	5	homeobox	Unstable protein	Semina et al, 1996 Amendt et al,1998
Arg62His	Axenfeld-Rieger syndrome	5	homeobox	Not determined	Semina et al, unpublished
Thr68Pro	Axenfeld-Rieger syndrome	5	homeobox	Reduced DNA binding Transactivation defective Enhanced phosphorylation	Semina et al, 1996 Amendt et al, 1998
Arg69His	Iridogoniodysgenesis syndrome	5	homeobox	Reduced DNA binding Reduced transactivation	Kulak et al, 1998 Kozlowski et.al, 2000
Val83Leu	Axenfeld-Rieger syndrome	5	homeobox	Reduced DNA binding Increased transactivation	Priston et al, 2001
Arg84Trp	Iris hypoplasia	5	homeobox	Reduced DNA binding Reduced transactivation	Alward et al, 1997 Espinoza et.al, 2002
G to C	1. Axenfeld-Rieger syndrome	5	position +5, ss	Frameshift, Semina et al, unpublished	Semina et al, 1996
	2. Rieger anomaly	-//-	-//-	Unstable protein	Amendt et al, unpublished
A to G	Axenfeld-Rieger syndrome	6	Position -11, ss	Frameshift, Unstable protein	Semina et al, 1996 Amendt et al, unpublished
Lys88Glu	Axenfeld-Rieger syndrome	6	homeobox	Defective DNA binding, transactivation defective, dominant neg. mutant	Saadi et al, 2001
Arg91Pro	1. Axenfeld-Rieger syndrome	6	homeobox	Defective DNA binding	Semina et al, 1996
	2. Axenfeld-Rieger syndrome	-//-	-//-		Amendt et al, 1998
Trp94 STOP	Axenfeld-Rieger syndrome	6	homeobox	Truncates protein	Semina et al, unpublished
Trp133 STOP	Axenfeld-Rieger syndrome	6	C-terminal	Truncates protein	Semina et al, 1996
21 bp dup. at 713-733 Thr82 to Lys88	Axenfeld-Rieger syndrome	5	homeobox	Reduced DNA binding, transactivation defective	Priston et al, 2001

Table 2B. Summary of* PITX2 *breakpoints and deletions

Mutation	Diagnosis	Exon	Gene Region	Effect on Protein Function	Reference
Break point t(4;16)	Axenfeld-Rieger syndrome	none	5'region	Changes expression	Semina et al, 1996
Break point t(4;11)	Axenfeld -Rieger syndrome, polydactyly, developmental delay	none	5'region	Changes expression	Semina et al, 1996
Break point t(4;12)	Axenfeld-Rieger syndrome	none	5'region	Changes expression	Flomen et al, 1998
Deletion del(4) (q24q26)	Axenfeld-Rieger syndrome, multiple congenital anomalies	all	Whole gene	No protein	Schinzel et al, 1997
Deletion del(4) (q25q27)	Rieger syndrome, dyscrania, dysplastic ears	all	Whole gene	No protein	Flomen et al, 1998

or unable to bind to the *bicoid* element, with the exception of the PITX2A T68P mutant. The PITX2A T68P mutant binds DNA at slightly reduced levels but can not activate reporter constructs containing *bicoid* elements.[13,31] This lack of transcriptional activity is puzzling since we and others have reported the existence of a transactivation domain in the C terminus of PITX2.[39,43,53] Our data also suggests the presence of a transactivating domain in the N terminus of PITX2A.[39] Thus, how would a mutation in the homeodomain of PITX2A affect transactivation? Several mechanisms could explain why PITX2A T68P transactivation is defective. The easiest explanation is that a mutation imparts a conformational change in the protein that while not adversely affecting DNA binding would render the protein transactivation deficient. Several modeling experiments have shown how *PITX2A* mutations affect DNA binding and these conformation changes could be extended to affect transactivation properties of the mutant proteins.[31,67] PITX2A can form homodimers through interactions with the homeodomain as well as the C-terminal tail.[39] Therefore, a mutation in the homeodomain that inhibits PITX2A dimerization could have deleterious effects on its ability to activate transcription. Because PITX2A binds to the consensus *bicoid* site as a homodimer the inhibition of dimer formation could account for the loss of transcriptional activity. Another possibility would be that the conformational change imparted on the protein by the mutation would not allow it to interact with other factors required for transcriptional activation. This effect was reported for the K88E mutant in that while this protein is unable to bind DNA when it was cotransfected with wildtype PITX2A it inhibited synergism with the Pit-1 POU homeodomain protein (Fig. 2E).[31] Because PITX2A can form dimers, presumably the heterodimer formed between PITX2A wildtype and PITX2A K88E resulted in a structure that did not allow Pit-1 to interact with PITX2. Thus, the interaction of the PITX2A K88E mutant probably disrupts this interaction and inhibits Pit-1 from interacting with PITX2A. This results in a dominant negative mechanism by this mutant PITX2A protein. Interestingly, when the PITX2A T68P mutant protein was cotransfected with wildtype PITX2A no suppression of Pit-1 synergism was observed. The PITX2A R91P ARS mutation also results in a dominant negative form of PITX2.[68] This mutation was also shown to prevent the PITX2A/Pit-1 synergistic activation of the *prolactin*

promoter.[68] These data would support a model where the different PITX2A mutations associated with ARS can form conformations that have different effects on transcriptional activation. Because ARS is due to haploinsufficiency, one normal copy of *PITX2A* combined with a defective gene product could contribute to the phenotypic variation seen in these patients.

The PITX2A V83L mutant protein has reduced DNA binding activity but surprisingly results in increased transactivation activity.[66] This mutation causes >200% increase in transcriptional activity of an artificial promoter containing several PITX1 binding sites (Table 2A).[66] This is the first *PITX2A* mutation described that results in an increase in its transcriptional activity and provides evidence that increased PITX2A activity can cause ARS. This is similar to the overexpression of MSX2 in causing Boston-type craniosynostosis.[69] Thus, increased activity of one *PITX2* allele may be as physiologically disruptive as a mutation that nullifies a *PITX2* allele, with either condition resulting in ARS.

Two *PITX2A* mutations are associated with milder forms of the group of anterior segment disorders of which ARS is the most severe and include Iridogoniodysgenesis (IGDS) and Iris hypoplasia (IH). A nice study using several *PITX2A* mutations demonstrated differences in PITX2A mutant activities.[32] In this report the differences in residual PITX2A mutant activities were correlated to the phenotypic differences observed in the patients that presented with the mutations.[32] Using artificial promoter constructs it was demonstrated that the PITX2A R69H mutant associated with IGDS and the PITX2A R84W mutant associated with IH retained more transcriptional activity than other ARS mutant proteins. Thus, the mutant PITX2A proteins that retain partial function result in milder anterior segment aberrations (Table 2A).[32] These observations were extended using natural PITX2 target promoters and the PITX2A R84W mutation. Because *Pitx2* and *Dlx2* are expressed in the dental epithelium and *Pitx2* is the first transcriptional marker of tooth development it was shown that PITX2 could transactivate the *Dlx2* promoter.[34,41] The patient with the *PITX2A R84W* mutation presents clinically with only IH and has normal tooth development. Therefore, if the PITX2A R84W mutant protein could transactivate the *Dlx2* promoter, this would provide evidence for the normal tooth development in this patient. The PITX2A R84W mutant did activate the *Dlx2* promoter a low levels compared to wildtype PITX2A but higher than ARS mutations that result in abnormal tooth development.[35] Thus, we speculate that the PITX2A R84W mutant protein provided enough residual activity along with a normal *PITX2* allele to allow normal tooth development in that patient (see Chapter 8). However, the PITX2A T68P mutant, which was unable to transactivate the *Dlx2* promoter was identified in an ARS patient with abnormal tooth development.[35]

Phosphorylation of PITX2A *Mutant Proteins*

The PITX2A T68P mutant protein is hyperphosphorylated by PKC.[35] In contrast to wildtype PITX2A protein, phosphorylation of PITX2A T68P did not increase its DNA binding activity or transcriptional activity. Thus, the proline substitution in helix 2 of the homeodomain while it does not abolish DNA binding activity it completely inhibits transcriptional activity and increased phosphorylation has no effect on its activity.[35] We observed increased DNA binding for both the PITX2A and PITX2A R84W phosphorylated proteins. Interestingly, phosphorylation acts to facilitate dimer formation of these proteins. Quantitation of the EMSA gels demonstrated a 2-fold increase in binding by both PITX2A and PITX2A R84W proteins. In contrast, the phosphorylated PITX2A T68P mutant protein demonstrated only a slight increase in binding to the *bicoid* probe. The PITX2A T68P mutant protein has an overall reduced DNA binding activity for the *Dlx2 bicoid* labeled oligonucleotide. Therefore, the *PITX2A R84W* mutation causes a less severe phenotype because the protein can bind DNA similar to wildtype and is activated by PKC phosphorylation and retains some transcriptional

activity.[35] However, the PITX2A T68P mutation produces a protein with increased phosphorylation activity but reduced DNA binding activity and defective transcriptional activity. Addition of PMA to PITX2A R84W transfected CHO cells increased the activation of the Dlx2 promoter from 6-fold to 13-fold while PITX2A T68P transcriptional activity was not affected. Thus, activation of PKC by PMA increased PITX2A and PITX2A R84W transcriptional activity but not PITX2A T68P.[35]

All of the *PITX2* mutations discussed in this report were constructed in the *PITX2A* isoform. The three major *PITX2* isoforms (A,B,C) have been shown to differentially regulate organogenesis. *Pitx2a* and *Pitx2c* are the major isoforms expressed in the heart, lung and gut. In the brain, craniofacial region and pituitary all three *Pitx2* major isoforms are expressed and provide a mechanism to tightly regulate gene expression through interactions of the PITX2 isoforms. PITX2 isoforms can differentially regulate gene expression through both cell-dependent and promoter-dependent mechanisms.[34] Thus, the levels and combinations of *PITX2* isoform expression contribute to the dosage-response model proposed for pituitary and other organ development.[44,45] We are currently studying the effect of *PITX2* ARS mutations in the other isoforms. The ARS mutations will be carried in all isoforms since PITX2A, PITX2B and PITX2C all contain identical homeodomains and C-terminal tails. Because the isoforms have different transcriptional properties the mutations may manifest themselves differently in the specific isoforms. It will be of interest to determine if the other PITX2 isoforms contribute different activities and correlate these activities to the phenotypic and heterogeneous spectrum of the group of disorders associated with ARS.

Mechanism of *FOXC1* Mutations Associated with ARS

The genetic analysis of *FOXC1* mutations seen in Axenfeld-Rieger syndrome and related disorders has been describe elsewhere in this book. I will describe the limited research that has been performed using FOXC1 mutant proteins and detail their activities.

FOXC1 is a member of the winged-helix family of transcription factors and binds the core DNA sequence (5'GTAAATAAA3').[70] Because the natural target genes of FOXC1 have not been reported a synthetic reporter construct was made to assess the transcriptional activity of FOXC1 and FOXC1 mutant proteins.[22] Six *FOXC1* binding elements were cloned upstream of the herpes simplex-virus *thymidine kinase* (TK) promoter and activation of this promoter was compared to the activity of the *TK* promoter construct without *FOXC1* binding elements. While wildtype FOXC1 activated the minimal promoter the addition of the six *FOXC1* binding sites to this promoter resulted in a two-fold activation above the minimal promoter.[22] Thus, it appears that FOXC1 can transactivate an artificial promoter containing *FOXC1* DNA binding elements.

While many mutations have been reported in *FOXC1* only the DNA binding and transcriptional activities of five missense mutations have been described (Table 3).[22] The *FOXC1 I87M* mutation produces a protein that is unstable in transfected cells and was not detected by Western blot analysis. This isoleucine is highly conserved in all FOX proteins (see Chapter 2) in the first helix of the forkhead domain and substitution with a methionine appears to produce a proteolytic sensitive region. The *FOXC1 S82T* mutation gene product demonstrates reduced DNA binding and reduced transactivation. The serine at this position is highly conserved however substitution with a threonine would not appear to have much affect since both residues are not bulky and have uncharged polar groups. The *FOXC1 S82T* mutation occurs in a putative nuclear-localization signal of the forkhead domain, but it was shown that this mutant protein correctly localizes to the nucleus.[22] These data demonstrate that replacing a highly conserved amino acid with another like amino acid can have deleterious effects on protein function. However, this *FOXC1* mutation is associated with iridogoniodysgenesis syndrome

Table 3. Summary of the FOXC1 forkhead domain missense mutations

Mutation	Diagnosis	Gene Region	Effect on Protein Function	Reference
Ser82Thr	Axenfeld-Rieger Anomaly	Forkhead domain	Reduced DNA binding Reduced transactivation	Saleem et al, 2001
Ile87Met	Axenfeld-Rieger Anomaly	Forkhead domain	Unstable protein	Saleem et al, 2001
Phe112Ser	Axenfeld-Rieger Anomaly	Forkhead domain	Normal DNA binding Reduced transactivation	Saleem et al, 2001
Ile126Met	Axenfeld-Rieger Anomaly	Forkhead domain	Normal DNA binding Reduced transactivation	Saleem et al, 2001
Ser131Leu	Axenfeld-Rieger Anomaly	Forkhead domain	Reduced DNA binding Reduced transactivation	Saleem et al, 2001

(IGDS) the less severe form of the anterior segment disorders. Therefore, it is interesting to speculate that the replacement of a residue with another that is similar in charge and size produces a less severe phenotype. The *FOXC1 S131L* mutation occurs in a highly conserved region of helix 3 and results in a protein with reduced DNA binding and transactivation. Two mutations (F112S and I126M) produce proteins that have normal DNA binding activity however, transcriptionally they have low activation potential. It was speculated that these two mutations may affect protein-protein interactions and transcription initiation, respectively.[22] Overall the *FOXC1* mutations parallel the *PITX2* mutations in their functional deficiencies which include defective DNA binding and transcriptional activity (Table 3).

Discussion

A recurring theme among homeodomain proteins is the important role of protein-protein interactions in modulating activity. Some of these interactions can stimulate[71-74] while others act to inhibit transcriptional activity.[75-79] A mechanism for regulating the transcriptional actions of PITX2 is its interaction with other transcription factors. The C-terminal region of PITX2 can directly bind the POU homeodomain protein, Pit-1. Pit-1 is well known to regulate pituitary cell differentiation and expression of pituitary hormones, including prolactin.[42] At least one manifestation of this interaction is increased PITX2 DNA binding in vitro. There is precedence for protein interactions yielding increased DNA binding activity.[77,80,81] For example, Pbx can increase the DNA binding of Hox proteins and the engrailed homeodomain protein.[82,83] Likewise, Prospero (Pros) has been shown to increase the DNA binding of Deformed (Dfd) and Hoxa-5.[76] Interestingly, Pros is not part of the Dfd-DNA complex,[76] similar to Pit-1 and PITX2.[13] Pit-1 binding to PITX2 also results in a synergistic activation of the *prolactin* promoter. Thus, the C-terminal protein-protein interaction domain of PITX2 regulates DNA binding and transcriptional activities in response to specific factors, such as Pit-1 (Fig. 2B). Our model predicts that PITX2 may not be fully activated until expression of the appropriate cofactors.

Our laboratory has cloned several genes that interact with the PITX2 C-terminal tail using the yeast two-hybrid assay. One gene has homology to High Mobility Group (HMG) genes, which are involved in chromatin remodeling. A major question that those of us studying developmental gene regulation seek to answer is, how does a transcription factor find the appropriate gene target during embryogenesis? Furthermore, how is the gene activated when in the condensed form of chromatin? We may have an answer in that our new gene may bind

chromatin and recruit PITX2 to the active chromatin sites. This would allow PITX2 to complex with the transcription machinery and specifically activate genes during embryogenesis. We are currently working on chromatin remodeling using PITX2 and our new gene.

The Role of *PITX2* Phosphorylation

PITX2A is phosphorylated by PKC and phosphorylation increases DNA binding activity. ARS mutant proteins are phosphorylated by PKC and the PITX2A T68P mutation acts to increase phosphorylation at that site. Since phosphorylation of wildtype protein increased its activity we expected that hyperphosphorylation of PITX2A T68P to produce a gain-of-function mutation. However, increased PITX2A T68P phosphorylation only had a minimal effect on DNA binding. Thus, the proline mutation probably imparts a conformational change in the protein that generally inhibits its DNA binding activity. Interestingly, the PITX2A T68P mutation suggests that the serine residue is phosphorylated by PKC, since removal of the threonine residue does not inhibit phosphorylation. Additional mutation analysis has shown that all 10 PITX2A PKC sites are phosphorylated through mostly serine residues. Furthermore, phosphorylation of both PITX2A and PITX2A R84W acts to increase dimer formation. PITX2 phosphorylation may also act to facilitate protein-protein interactions. Protein interactions occur through the C-terminal tail of PITX2 and phosphorylation may regulate these interactions.

A Molecular Basis for Phenotypic Variations in ARS Patients

We have demonstrated a molecular basis for two ARS phenotypes, one patient that has normal tooth development and dental anomalies in another. In ARS the *PITX2A T68P* point mutation lies in helix 2 at position 30 of the homeodomain, whereas the *R84W* point mutation is in helix 3 at position 46 of the homeodomain. No other proteins have been identified with a proline at position 30 and it appears that this position can accommodate some changes in amino acid identity without a complete loss of DNA binding activity. Interestingly, a previous report using the PITX2A T68P mutant protein revealed only a 2-fold reduction in DNA binding activity using an artificial *bicoid* oligonucleotide.[13] However using the naturally occurring *Dlx2* consensus *bicoid* element and flanking sequences binds poorly to the natural element. In the PITX2A R84W mutant the R residue at position 84 may be similar to the K residue of Engrailed, which contacts the sugar residue of the DNA backbone. However, our data indicates that this position can accommodate amino acid changes without adverse effects.

The molecular basis of ARS appears to result from reduced activation of PITX2 arising from the various missense mutations found in the homeodomain and C-terminal tail. However, a dominant negative effect from coexpression of a *PITX2A K88E* mutation with the wildtype gene has been observed. These proteins may interact to abolish the interaction of wildtype with other PITX2A interacting proteins such as Pit-1.[31] These results were recently reported with the PITX2A R91P mutant.[68] Furthermore, a new study has identified two PITX2 mutations associated with Axenfeld-Rieger syndrome.[66] Interestingly, one mutation appears to yield a mutant PITX2A protein with reduced DNA binding activity compared to wildtype but results in an increase in transcriptional activation. Thus, it appears that a combination of instability, phosphorylation, transcription inactivity, dominant negative and dominant positive mutations will all contribute to the variable ARS phenotypes.

The Role of PITX2 Isoforms

The location of *PITX2* isoform expression in regions of the developing embryo could have important effects on PITX2 target gene expression such as *Dlx2*. During embryogenesis the levels of PITX2 isoform expression will greatly influence *Dlx2* gene expression. This type of

regulation has been reported by using different cell lines, which reveal differences in PITX2 isoform activities.[34] PITX2 isoform transcriptional activity may be similarly different in tissues of the developing embryo. Since the major PITX2 isoforms differ only in their N termini, the N-terminus must play a role in the differential transcriptional activities of these isoforms. However, because each isoform binds DNA similarly, the N-terminus must be interacting with tissue/cell specific factors to regulate their activities. We observe transcriptional activation differences in several cell lines, indicating that cell specific factors regulate PITX2 isoform activities.

To address the functional properties of the different N-termini, each N-terminal specific peptide was cotransfected with each PITX2 isoform. The rationale was that if the N termini were binding specific factors then expression of the N-terminal peptides would sequester cellular factors by binding them and thus allow for differential regulation by the wildtype isoforms. Interestingly, cotransfection of the PITX2A, PITX2B or PITX2C isoforms with the PITX2A and PITX2B N-terminal peptides resulted in a 2-fold increase in their transcriptional activity. Thus, in these experiments the N-terminal peptides could be "soaking up" factors that regulate the activities of the PITX2 isoforms. These data could suggest that factors binding to the PITX2 N-terminal residues negatively regulate PITX2 transcriptional activity. Our speculations are composed from the idea that the N-terminal peptides are binding or sequestering cellular factors and when they can not bind to the full-length isoforms their transcriptional activities are enhanced. However, cotransfection of the PITX2C N-terminal peptide had no effect on PITX2A, PITX2B or PITX2C activity. While these results are difficult to interpret they do provide clues that the N-terminal residues of each PITX2 isoform maybe binding cell specific factors that regulate their activities.

Furthermore, all isoforms can form heterodimers to both positively and negatively regulate their activities. This provides a unique mechanism to regulate PITX2 activity in a cell/tissue dependent manner. There is a PITX2C isoform preference for *ANF* expression through the interaction with Nkx2.5. PITX2C and Nkx2.5 synergistically regulate *ANF* gene expression however, PITX2A and PITX2B are unable to synergistically activate the *ANF* promoter in the presence of Nkx2.5. Surprisingly, Nkx2.5 can act to repress the transcriptional activity of the PITX2A isoform using the *ANF* promoter in a cell dependent manner. Researchers are beginning to demonstrate differences in PITX2 isoform activation through interactions with other factors. These types of factor interactions would be expected to spatially and temporally regulate PITX2 transcriptional activity during development.

PITX2D Inhibits the Transcriptional Activity of the Other PITX2 Isoforms

The mechanism of this suppressive effect is currently unknown and we are investigating its action. But interestingly there is a transactivation domain in the C-terminus of PITX2.[39,43,53] Thus, if the PITX2D protein is forming a heterodimer with the other isoforms it should be able to activate the promoters in transfection assays since it contains the C-terminal transactivation domain. Since mixing PITX2D with each PITX2 isoform does not inhibit their DNA binding activity then suppression of PITX2A and PITX2C transcriptional activity is not due to a loss of DNA binding activity. One explanation for the suppressive effect would involve PITX2D binding cellular factors required for PITX2 activity. Thus, PITX2D may act to suppress the activity of the other PITX2 isoforms by "soaking up" factors that normally bind to PITX2. We have titrated PITX2D in our transfection experiments and do not observe a corresponding decrease in PITX2A activation upon increased PITX2D DNA concentrations. We are currently investigating the mechanism of this novel PITX2 isoform. Developmentally, it does provide an interesting mechanism for the regulation and fine-tuning of PITX2 transcriptional activity, which appears to be required for the normal morphogenesis of several organs.

In summary, these studies provide evidence that PITX2 isoforms differentially activate genes involved in development. These data provide a molecular basis for organ/tissue development by PITX2 isoforms, where the expression of PITX2 isoforms can greatly influence gene expression. Furthermore, they provide evidence for the regulation of PITX2 isoform transcriptional activity in a cell dependent manner and through the action of a novel PITX2 isoform.

PITX2 Mutations

The PITX2A T68P point mutation lies in helix 2 at position 30 of the homeodomain, while the PITX2 L54Q point mutation is in helix 1 at position 16 of the homeodomain. Furthermore, the PITX2A R84W point mutation is in helix 3 at position 46 of the homeodomain. To our knowledge there are no reports of amino acids in these positions affecting DNA binding specificity in homeodomain proteins. Comparison of the amino acid sequence of over 300 homeobox proteins reveals that position 30 of the homeodomain is not conserved and several amino acids can be located at this position.[84] While no other proteins have been identified with a proline at position 30 it appears that this position can accommodate changes in amino acid identity without affecting DNA binding activity. In contrast, the amino acid residues at position 16 and 46 of the homeodomain are highly conserved.[84] In the approximately 300 homeobox proteins analyzed, the residue at position 16 is a leucine, except for EgHbx4, ap, and LH-2 which contain a methionine and Lmx-1 which has a phenylalanine.[84] This strong conservation suggests that the leucine residue plays a fundamental role in the homeodomain. Our results support this prediction by demonstrating that a mutation of the leucine to a glutamine (L54Q) is detrimental for PITX2 activity. Since this mutant protein could not be detected in transfected mammalian cells, this suggests that the leucine at position 16 is important for stability of the homeodomain. The strong conservation of the argininine residue at position 46 of the homeodomain (R84W) would also suggest that this residue plays a fundamental role in homeodomain activity. However, this mutation is seen clinically as being less severe and does bind DNA slightly less than wildtype levels thus, a change to a tryptophan residue does not appear to adversely affect its DNA binding activity.

The binding specificity of homeodomains is dictated mostly by residues in the recognition helix and the N-terminal arm.[9,12,62-64] While we have shown that the PITX2 T68P mutation binds DNA and is transactivation deficient the mechanism of this mutation is not yet known. The results with PITX2 deletion constructs suggest that PITX2 transactivation domains lie outside of the homeodomain. Thus, how a mutation in the homeodomain affects transactivation is not entirely clear. In unpublished data we have confirmed that this mutation is correctly localized to the nucleus demonstrated by immunoflourescence. A summary of *PITX2* mutations associated with Axenfeld-Rieger syndrome and their molecular and biochemical activities are shown in Table 3.

The molecular basis of Boston-type craniosynostosis was determined to involve a point mutation in the N-terminal arm of the MSX2 homeodomain.[69] Similar to the PITX2A mutants T68P and R84W, a mutation in the MSX2 homeodomain did not abolish DNA binding as one might expect. Overexpression of the wildtype *Msx2* gene can also produce craniosynostosis; therefore enhanced binding by the MSX2 mutant is implicated as the cause for this disorder. PITX2A T68P binds DNA however, this mutation results in reduced DNA binding specificity and capacity. This reduction in binding specificity might account for the loss of PITX2 transactivation activity. However, while we can not rule out this possibility, it seems unlikely that these changes are sufficient to yield no detectable transacting activity especially in the presence of Pit-1.

In addition to its expression in the pituitary, PITX2 is also required for eye, heart, and tooth development suggesting that *PITX2* is regulated in multiple tissues by a combination of

interacting factors.[1] The ability of *PITX2* to be activated during development could be a function of factors interacting with its C terminus to increase DNA binding and transcriptional activity.

FOXC1 Mutations

The penetrance of FOXC1 defects within the eye are high and similar to PITX2 it has variable expressivity. Therefore, the level of *FOXC1* expression is critical for normal development through the regulation of developmentally important downstream target genes. It has been shown that mutant FOXC1 proteins are associated with ARS and ARA and related disorders, however it was reported that duplication of the *FOXC1* gene can result in anterior eye-segment defects.[85] Thus, increased FOXC1 activity can also result in defects and suggests a critical role in the regulation of FOXC1 transcriptional activity during development. In contrast to *PITX2* mutations a genotype-phenotype relationship has been established for *FOXC1* mutations and ARS. These observations could indicate that *FOXC1* mutations and *PITX2* mutations use different pathways or mechanisms to cause the developmental defects seen in patients with these specific mutations. Studies using mice deficient for *Foxc1* expression have demonstrated a role for Foxc1 in cell migration and differentiation.[86,87] Foxc1 appears to be involved in a dose-dependent mechanism during gastrulation for cardiogenesis and somitogenesis.[87] The dysgenesis of the anterior chamber of the eye in affected ARA patients resembles that of heterozygous *Foxc1* mice. These data suggest that both *FOXC1* haplo-insufficiency and increased gene dosage can cause anterior chamber defects of the eye.[21]

PAX6 Mutations

Fluorescent in-situ hybridization analysis using probes for the PAX6 gene showed a small deletion for the PAX6 gene on one homologue of chromosome 11.[29] This patient had the three cardinal features of Axenfeld-Rieger syndrome and is described in Chapter 3. However, this report describes that in addition to *PITX2* and *FOXC1* mutations ARS is also associated with mutations in *PAX6*.

Future Directions

We are just beginning to determine the transcriptional mechanisms used by PITX2 to regulate developmental gene expression. The biochemical mechanism of the PITX2D isoform will provide new insights into how protein isoforms can regulate the transcriptional activities of the other isoforms. This unique type of regulation may play a major role in the spatial temporal expression of PITX2 target genes. Clearly the differential expression patterns of the major PITX2 isoforms must play a role in specifying tissues and organ development. Furthermore, do ARS mutations act differently in the other PITX2 isoforms? The transcriptional activities of these ARS mutations in the different isoforms may yield clues to the phenotypic heterogeneity observed in patients and the effects on different organs and tissue. Of course a major problem for those of us working with transcription factors is to identify their downstream targets. More PITX2 target genes will need to be identified to fully understand the developmental defects associated with ARS. The same problems occur with both the FOXC1 and PAX6 transcriptional analysis. We are working to identify new genes that either directly interact with PITX2 or by themselves are associated with ARS. Because PITX2 is the first transcriptional markers observed for several tissues and it is developmentally regulated we are pursuing its role in chromatin remodeling and gene activation. We have cloned a gene that directly interacts with the PITX2 C-terminal tail and appears to bind chromatin. We speculate that this new gene product binds chromatin, is involved in chromatin remodeling and recruits PITX2 to active sites of transcription during embryogenesis. There are many aspects of PITX2 structure and function to be researched and this manuscript outlined a few areas of interest.

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CHAPTER 5

Role of PITX2 in the Pituitary Gland

Hoonkyo Suh, Donna M. Martin, Michael A. Charles, Igor O. Nasonkin, Philip J. Gage and Sally A. Camper

The pituitary gland is a neuroendocrine organ composed of specialized peptide hormone-producing cells that control many bodily functions. Pituitary development depends on the combined activity of extrinsic signaling molecules and intrinsic transcription factors. One of the earliest acting transcription factors in pituitary development is PITX2, a homeobox transcription factor required for expansion of the pituitary primordium, Rathke's pouch. Analysis of an allelic series in mice revealed that pituitary gland size and the specification of individual pituitary cell types are also dependent upon *Pitx2*, and this dependence is sensitive to *Pitx2* gene dosage. Mechanistically this pituitary phenotype results from the inability of low levels of PITX2 to activate gene expression of several lineage specific transcription factors, such as *Gata2*, *Sf1* (*Nr5a1*), *Egr1* and *Pit1*. Our understanding of *Pitx2* gene dosage effects on pituitary development suggests a basis for dosage sensitive defects in other organs of Rieger syndrome patients. In addition, analysis of the allelic series in mice raises the possibility of gonadotropin and PIT1 lineage defects in individuals with loss of PITX2 function.

Introduction to Pituitary Development and Function

The pituitary gland is a master neuroendocrine organ that regulates production and secretion of peptide hormones important for both development and function of many target organs, including the thyroid, adrenal glands, gonads, mammary glands and liver. Acting as an intermediary between the brain and peripheral organs, the pituitary gland receives processed inputs from the brain, mainly from the hypothalamus, and produces and releases six distinct peptide hormones that subsequently act on endocrine systems for control of growth, lactation, reproduction and stress responses. Pituitary hormone target organs have compensatory mechanisms that regulate hypothalamic input and pituitary function. This allows the pituitary to monitor and respond to changing needs of the body and to maintain homeostasis.

The rodent pituitary gland is comprised of anterior, intermediate, and posterior lobes. Within the anterior pituitary gland, five pituitary hormone producing cell types are primary sites for endocrine regulation (Table 1). They are somatotropes, thyrotropes, lactotropes, corticotropes and gonadotropes, producing and secreting growth hormone (GH), thyroid stimulating hormone (TSH), prolactin (PRL), adrenocorticotropic hormone (ACTH), and the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), respectively. The glycoprotein hormone α-subunit (αGSU) is a common partner for the unique β-subunits that give specificity to thyrotropin and the gonadotropins. The production and secretion of all six hormones is controlled by factors synthesized and released from the axonal terminals of hypothalamic neurons. Normal somatic growth requires adequate production of both GH and TSH.

Molecular Mechanisms of Axenfeld-Rieger Syndrome, edited by Brad A. Amendt.

Table 1. Anterior pituitary cell types, hormone products, and regulation

Cell Type	Hypothalamic Signals	Hormone Products	Onset of Hormone [7] Transcription	Target Organs
Corticotropes	CRH	ACTH	e12.5	Adrenal Gland
Somatotropes	GHRH, Somatostatin	GH	e15.5	Liver and Other Tissues
Lactotropes	Dopamine	PRL	e15.5	Mammary Glands
Thyrotropes	TRH	TSH	e16.5	Thyroid
Gonadotropes	GnRH	LH	e16.5	Gonads
		FSH	e17.5	

Abbreviations: ACTH, adrenocorticotropic hormone; GH, growth hormone; PRL, prolactin; TSH, thyroid stimulating hormone; LH, luteinizing hormone; FSH, follicle stimulating hormone; CRH, corticotropin releasing hormone; GHRH, growth hormone releasing hormone; TRH, thyrotropin releasing hormone; GnRH, gonadotropin releasing hormone.

Two different types of embryonic ectoderm contribute to the formation of the mature pituitary gland. While the posterior lobe, a neural ectoderm derivative, arises from the ventral diencephalon, the anterior and intermediate lobes originate from the oral ectoderm.[1,2] Pituitary development undergoes several successive, but highly regulated steps: thickening of the oral ectoderm, invagination and separation of the pituitary primordium from the roof of the mouth, cell proliferation in the luminal area of Rathke's pouch, and differentiation of each cell type.[3] Thickening of oral ectoderm begins at embryonic day (e) 8.0 in the mouse. This thickening, or placode, invaginates, making direct contact with ventral diencephalon at e9.5. The contact between invaginating oral ectoderm and evaginating ventral diencephalon is required for reciprocal induction of development.[4,5] This invaginating structure is referred to as a rudimentary pouch or pituitary primordium. The pouch separates from the remaining oral ectoderm and forms a definitive Rathke's pouch by e12.5 (Fig. 1). The anterior-ventral portion of Rathke's pouch expands and becomes a glandular structure that produces and releases pituitary specific hormones by e16.5.[6] The posterior - dorsal aspect of the pouch develops into the intermediate lobe, which produces melanocyte stimulating hormone and endorphins.

The differentiation of each of five pituitary hormone-producing cell types appears in a spatially and temporally regulated manner during pituitary organogenesis, despite the nearly complete mixing of the specialized cells in the adult organ.[7,8] Cells positive for αGSU are the first type to appear, at approximately e10.5 in the mouse. These cells are thought to become the thyrotropes located at the rostral tip of the anterior lobe at e18.5 (Fig. 1). ACTH-producing corticotropes appear dorsal to the αGSU-positive cells at e12.5, and become dispersed throughout the caudo-medial part of the anterior lobe as it expands. The caudo-medial area also contains somatotropes and lactotropes, producing and secreting GH and PRL, respectively, and an independent thyrotrope population. GH and PRL transcripts are detected at e15.5, and *Tshb* transcripts appear in the caudo-medial region at e16.5. *Lhb* and *Fshb* transcripts are detected at e16.5 and e17.5, respectively, in the most ventral aspect of the pituitary gland. Each pituitary hormone producing cell type appears to be specified as pituitary organogenesis progresses. This suggests that there might be a direct correspondence between the cell birth dates of each lineage and the initial position of the cell population. If this hypothesis were true, the last cells to appear (i.e., the gonadotropes) would be most sensitive to developmental delay and/or depletion of stem cells or precursor pools.

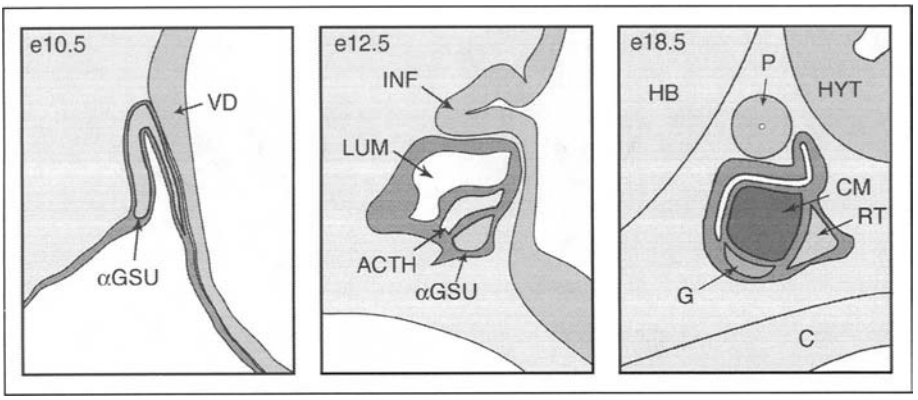

Figure 1. Pituitary gland development and spatial specification of anterior cell types. Mid-sagittal sections of e10.5, e12.5 and e18.5 mouse pituitary glands are illustrated. They represent the formation of a rudimentary pouch (e10.5), a definitive pouch (e12.5), and a mature glandular structure (e18.5), respectively. Abbreviations: VD, ventral diencephalon; INF, infundibulum; LUM, lumen; HB, hindbrain; HYT, hypothalamus; CM, caudomedial region; G, gonadotropes; RT, rostral tip region; C, cartilage. Adapted from a review by Camper and colleagues.[69]

Identification of *Pitx2*

Several genes encoding homeobox transcription factors are important for normal pituitary development, cell specification and differentiation, and hormone production (Table 2).[9-11] Lesions in these genes cause similar defects in pituitary development and function in mouse and man. Because some cases of combined pituitary hormone deficiency (CPHD) do not have mutations in known genes, we hypothesized the existence of additional genes whose functions are essential for normal pituitary development. In an effort to identify such genes, we undertook the identification and characterization of novel homeobox genes expressed in the pituitary primordium during cell specification. Reverse transcriptase (RT) polymerase chain reaction (PCR) was performed using degenerate oligonucleotide primers to amplify transcripts from expressed homeobox transcription factor genes in an adult pituitary cDNA library, and this approach identified two mRNA isoforms of the homeobox gene *Pitx2* that arise by alternative splicing.[12] Subsequently, a third isoform was discovered that uses an alternative promoter and produces a protein with a unique amino terminus.[13] The PITX2 homeodomain bears considerable homology to the paired class of homeodomain transcription factors. PITX2 contains an OAR domain (otp, *aristaless,* Rx) in the C-terminus that is exclusively found in the paired class of homeodomain proteins.[12,14] The OAR domain has transactivating properties. The 50th amino acid position of the PITX2 homeodomain contains a lysine (K) instead of a glutamine (Q), placing *Pitx2* in the K50 subclass of *paired*-like genes, which includes *goosecoid* and *Otx*. The K50 residue is one characteristic of the bicoid class of proteins, and electrophoretic mobility shift assays suggest that PITX2 binds to bicoid-like sites, although a PITX2 consensus binding study has not been done.[15]

Pitx2 is expressed in embryonic and adult pituitary gland, and other organs.[13,16,17] *Pitx2* is transcribed throughout the oral ectoderm and invaginating rudimentary pouch and constitutes one of the very earliest markers of the pituitary. It is ubiquitously expressed throughout Rathke's pouch, including the prospective intermediate and anterior lobes, but not in the infundibulum.[18] *Pitx2* expression is low in the perinatal period, but immunoreactivity is readily detectable in adult pituitaries (unpublished data). The temporal expression pattern of *Pitx2* is consistent with roles in many steps of pituitary development and function, including initial

Table 2. Mutations in homeobox genes cause pituitary defects in mouse and man

Gene Name	Human Disease	Mouse Model	Mouse Pituitary
Pitx1 (*Ptx1*)	none known, limb deformity and cleft palate likely	knockout[35,36]	altered size of specialized cell populations
Pitx2 (*Ptx2*)	Rieger syndrome,[56] GH deficiency[66]	knockout[13] hypomorph[18]	developmental arrest e10.5-12.5 deficiency gonadotropes & PIT1 lineage
Lhx3 (*Lim3, pLim*)	CPHD with rigid cervical spine[40]	knockout[3]	developmental arrest after corticotrope specification
Lhx4	CPHD with skull abnormalities[41]	knockout[38]	hypoplasia due to few differentiated cells of each type
Hesx1 (*Rpx*)	Septo-optic dysplasia with CPHD[28] or IGHD[29]	knockout[28]	highly variable, can include ectopic anterior lobe, cell differentiation failure
Prop1	CPHD[70]	Spontaneous mutant[71]: Ames dwarf = Ser83Pro, gene knockout*	hypoplasia with deficiency of PIT1 lineage,[72] lowered gonadotropin production.[73]

Abbreviations: Combined pituitary hormone deficiency, CPHD; Isolated growth hormone deficiency, IGHD. * Nasonkin and Camper, unpublished.

cell specification, differentiation, and regulated hormone production. Another pituitary transcription factor with multiple roles is *Pit1*, which is critical for specification, expansion, and function of three cell types: somatotropes, lactotropes and thyrotropes.[19,20] *Pit1* is necessary for these three cell types to develop and for transcriptional regulation of the hormone genes *Gh*, *Prl* and *Tshb*.[21,22,23,24]

Allelic Series of *PITX2* Mutations in Mice

To understand the role of *Pitx2* in pituitary organogenesis, we generated and analyzed a series of *Pitx2* alleles with varied activity (Fig. 2). A *Pitx2* hypomorphic or reduced function allele, termed $Pitx2^{neo}$, was generated using homologous recombination in embryonic stem cells, and a *Pitx2* null allele was derived from it using the *cre* loxP site-specific recombination system. Intermediate levels of PITX2 were obtained by crossing mice carrying the null or hypomorphic alleles to produce compound heterozygotes.[13,18]

Homozygotes for the *Pitx2* null allele ($Pitx2^{-/-}$) exhibit abnormal formation of the anterior-posterior body axis and multiple defects in eye, heart, and pituitary development consistent with *Pitx2* expression in those organs.[13,25,26,27] Morphologically, the pituitary rudiment is nearly normal in *Pitx2* null homozygotes at e10.5, but development is completely arrested at e11.5-e12.5. Transcription of the paired-like homeobox transcription factor gene *Hesx1*, also known as *Rpx*, is not detectable in $Pitx2^{-/-}$ pituitaries. *Hesx1* is important for the development of craniofacial midline structures including the pituitary.[28] The pituitaries of *Hesx1* deficient mice vary considerably. They are often ectopically placed and dysmorphic with evidence of developmental arrest and degeneration. Humans with mutations in *HESX1* have septo-optic

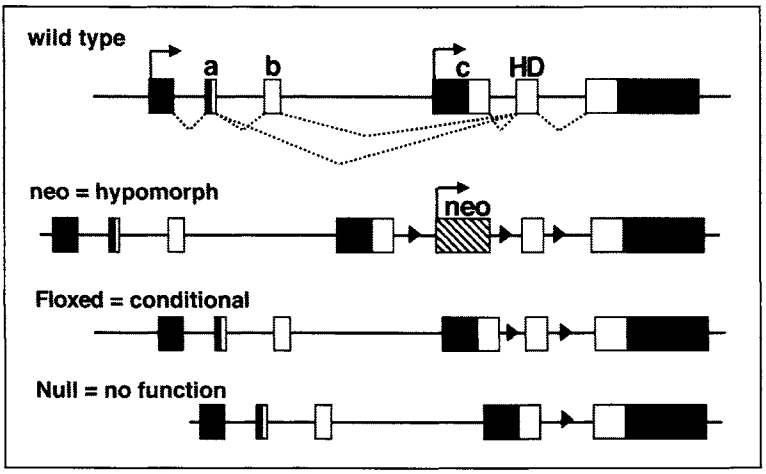

Figure 2. *Pitx2* gene structure and modified alleles. The coding regions (open) and noncoding regions (black) of the mouse *Pitx2* gene are illustrated. Transcription can initiate at two different places (arrows). Alternative splicing and promoter usage produce three PITX2 protein isoforms, PITX2A, PITX2B, and PITX2C, which differ only in their amino termini. A hypomorphic allele was generated by inserting a neomycin resistance cassette (neo) in the intron just upstream of the penultimate exon, which encodes the homeodomain (HD). The number of mature *Pitx2* transcripts is reduced in mice carrying the neo allele because a high percentage of the transcripts undergo cryptic splicing and polyadenylation within the neo cassette. The site specific recombinase *cre* was used to generate a null allele and a "floxed" allele, which consists of a functional Pitx2 gene in which the critical HD containing exon is flanked by lox P sites, (triangles), the recognition sites for *cre* recombinase.[13]

dysplasia (SOD), with defects in development of forebrain, midline craniofacial structures and pituitary gland, and sometimes isolated growth hormone deficiency.[28,29] Analysis of the *Pitx2* null allele proved that *Pitx2* is absolutely required for Rathke's pouch expansion and for pituitary expression of the transcription factor *Hesx1*.

Anterior pituitary size depends on *Pitx2* gene dosage.[18] The size of the gland is only marginally reduced in *Pitx2* hypomorphic homozygotes relative to wild type, and the *Pitx2* null homozygotes have severe pituitary hypoplasia. The pituitaries of *Pitx2* compound heterozygotes ($Pitx2^{neo/-}$) are intermediate in size relative to the other *Pitx2* mutants, indicating that the size of the organ is reduced proportional to declining levels of PITX2. The specification of several pituitary hormone producing cell types is also affected by reduced levels of PITX2. Gonadotropes are essentially not detectable in *Pitx2* hypomorphic homozygotes, and the number of somatotropes and thyrotropes is reduced. *Gata2*, *Sf1* (officially *Nr5a1*), and *Egr1* encode transcription factors involved in gonadotrope lineage specification, and expression of these transcription factors is nearly absent when *Pitx2* expression is reduced.[18,30,31] The reduction in somatotropes and thyrotropes in animals with lower levels of PITX2 is attributable to the reduced level of *Pit1* transcripts, as the POU-homeodomain transcription factor *Pit1* is critical for somatotrope, thyrotrope and lactotrope cell fates, and *Pitx2* has been shown to activate the *Pit1* promoter in cell culture.[15,20,32] Collectively these data reveal that *Pitx2* has an important role for pituitary cell lineage specification at the later stages of gestation, and that a threshold level of *Pitx2* is critical for activation of downstream lineage determining transcription factors in the anterior pituitary.

The similiarity in PITX1 and PITX2 protein sequences and completely overlapping expression patterns in early pituitary gland development suggests that the genes have overlapping

functions. The PITX1 and PITX2 homeodomains are 97% identical, and the carboxy terminal regions exhibit about 70% identity. Both *Pitx1* and *Pitx2* encode several isoforms with differing amino termini, but there is no similarity between any of the amino termini.[33] In addition to the coincident patterns of *Pitx1* and *Pitx2* expression in the pituitary primordium, both genes are expressed in the adult pituitary gland.[18] PITX1 is expressed in all cell types of the adult rat pituitary, including the folliculo-stellate cells, but the penetrance of expression varies by cell type.[34] No studies have been published on pituitary expression of *Pitx2* in adult mice, but preliminary data suggest that PITX2 is not uniformly expressed in the five hormone producing cells (unpublished data). Low levels of PITX2 in homozygotes for the hypomorphic allele lead to reduction in the number of thyrotropes and somatotropes, and complete absence of gonadotropes.[18] This pituitary phenotype is similar to that of *Pitx1*$^{-/-}$ mice which have nearly normal pituitary gland size and shape at birth, but gonadotropes and thyrotropes are reduced in number, somatotropes are unchanged, and corticotropes are increased.[85,36] Either the allocation of precursor cells to five different pituitary cell fates is abnormal in *Pitx1* and *Pitx2* mutants, or differentiation of some cells is incomplete. Regardless of the mechanism, the similarities in the phenotypes support the idea that PITX1 and PITX2 have overlapping functions in regulation of cell fate and/or differentiation.

To test for functional overlap between *Pitx1* and *Pitx2* during early pituitary morphogenesis, we generated double mutants with the *Pitx1* null and *Pitx2* neo alleles.[18] The size of the pituitary gland is similar in wild type mice and *Pitx1*$^{-/-}$ mice, or *Pitx2*$^{neo/neo}$ mice, but the pituitary primordia of double mutants (*Pitx1*$^{-/-}$, *Pitx2*$^{neo/neo}$) is as hypoplastic as that of *Pitx2*$^{-/-}$ mutants. This indicates that the combined dosage of *Pitx1* and *Pitx2* is critical for establishing a normally sized pituitary primordium.

We hypothesize that *Pitx2* resides at the top of the genetic hierarchy controlling pituitary development (Fig. 3). Together with *Pitx1*, *Pitx2* is required for activation of the LIM homeobox genes *Lhx3* and *Lhx4* (unpublished data). *Lhx3* and *Lhx4* are expressed after the primitive pouch develops from the oral ectoderm.[8,37] The LIM domain in LHX3 facilitates physical interaction with another pituitary specific paired-like transcription factor, PIT1,

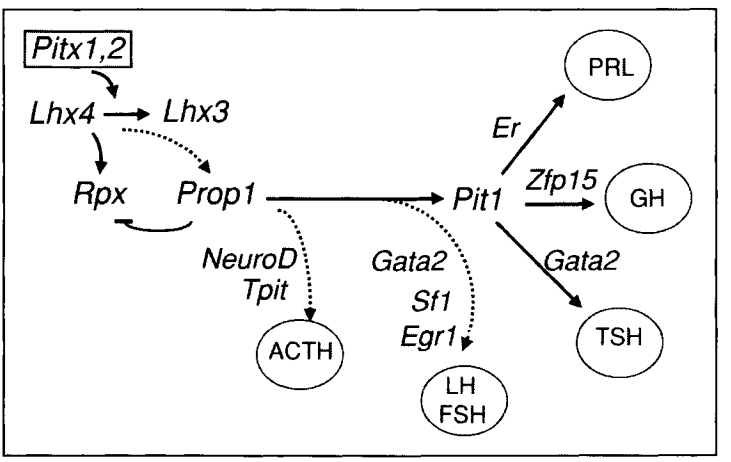

Figure 3. *Pitx2* acts early in the genetic hierarchy of pituitary transcription factors. *Pitx1* and *Pitx2* have overlapping functions that are required for expansion of the pouch and activation of the LIM homeobox genes *Lhx3* and *Lhx4*. The LIM genes are necessary for sustaining *Rpx* (*Hesx1*) expression. *Hesx1* has repressor functions and is antagonized by *Prop1*. *Prop1* is not necessary for specification of gonadotropes, but it is essential for normal gonadotropin production and for development of the *Pit1* lineage.

and this interaction is required for the synergistic induction of the downstream gene, *αGSU*.[37] Targeted inactivation of mouse *Lhx3* results in failure of Rathke's pouch to expand and failure of all but a few corticotrope cells to differentiate.[38] In the absence of *Lhx4*, initiation of *Lhx3* expression is delayed and pituitary hypoplasia results.[3,39] In addition, humans with CPHD have mutations in *LHX3* and *LHX4*. Patients with LHX3 mutations have a complete deficit of serum pituitary hormones except ACTH,[40] while patients with LHX4 mutations have low GH, corticosterone and thyroid hormone, but normal prolactin.[41] Just like *Pitx1* and *Pitx2*, *Lhx3* and *Lhx4* have overlapping functions in the development of the pituitary primordium, such that double mutant mice have an earlier and more severe arrest of development than single mutants.[3]

Cell Transfections Illustrate the Role of *Pitx2* in Pituitary Hormone Production

Immortalized cell lines have been used to study transcriptional regulation of pituitary hormone genes. Several cell lines exist that mimic the differentiated pituitary cell types.[42] Northern blot, RT-PCR and Western blot analysis of these cell lines suggests that both PITX1 and PITX2 are expressed in all five hormone producing cell types of the pituitary gland.[13,15,43,44,45,46] Both of these PITX family members are capable of enhancing expression of the hormone genes in transfection assays, and act synergistically with other transcription factors including PIT1, SF1, and EGR1.[15,46,47] Recent data suggest that individual isoforms have different transactivating properties.[48] Taken together, these studies with transfected cell lines suggest roles for PITX1 and PITX2 in regulated hormone production within the specialized cell types, in addition to their roles in formation of the pituitary primordia and activation of gonadotrope lineage specific transcription factors.

PITX2 can form homodimers.[48,49] A dominant negative K88E PITX2 mutant protein does not have DNA binding activity or transactivation properties.[49] Moreover, cotransfection of the K88E mutation with wild type *PITX2* inhibits the synergism between PITX2 and PIT1, suggesting that the K88E mutation interferes with normal dimeric formation of PITX2. It is likely that other *Pitx2* dependent genes are activated by this protein-protein interaction mechanism. Thus, one might expect selected *Pitx2* mutations to cause different phenotypes by eliminating only a subset of the protein-protein interaction domain functions.[15]

Mutations in *PITX2* Are One Cause of Rieger Syndrome

Mesodermal dysgenesis of the iris and cornea were originally observed by Vossius and Darwin.[50,51] In 1935, Rieger described autosomal dominant inheritance of anterior segment dysgenesis in humans, after which the term Rieger anomaly was coined.[52] Multiple clinical reports followed this original description, many of which emphasized a frequent association of the Rieger anomaly with other congenital abnormalities.[53,54] Among the clinical findings reported with Rieger anomaly, dental dysgenesis and umbilical abnormalities were the most consistent, leading to their ultimate inclusion, along with the Rieger anomaly, into what is now considered a triad of clinical findings for Rieger syndrome (OMIM #180500; http://www.ncbi.nlm.nih.gov/htbin-post/Omim/getmim).

Genes responsible for RGS map to multiple loci including 4q25, 6q25 and 13q14. *PITX2* and *FOXC1* are the responsible genes on 4q25 and 6q25, respectively, but the gene(s) in 13q14 has not been identified yet.[55,56] Abnormal formation of the anterior segment of the eye, malformation of the teeth, and umbilical abnormalities are the three clinical hallmarks of RGS. Occasionally RGS patients also have heart defects. Although RGS phenotypes are highly variable, all patients carrying *PITX2* mutations have congenital defects in the anterior portion of the eye.

Pituitary dysfunction has been associated with Rieger syndrome. Short stature and growth hormone deficiency with Rieger syndrome were first reported in a 7 year-old boy whose other relatives with Rieger syndrome were of normal stature.[57] In a later report, multiple individuals in a family had both growth hormone deficiency and Rieger syndrome, consistent with autosomal dominant inheritance.[56,57,58] Blunted growth hormone response to insulin and mild short stature were also reported in a mother and son with Rieger syndrome.[59] However, these individuals also exhibited lipodystrophy, delayed bone age, and hypotrichosis, suggesting they may represent a separate entity now referred to as SHORT syndrome (S = short stature, H = hyperextensibility of joins or inguinal hernia, O = ocular depression, R = Rieger anomaly, T = teething delay; OMIM #269880; http://www.ncbi.nlm.nih.gov/htbin-post/Omim/getmim).

Empty sella syndrome, a condition where the subarachnoid space extends into the sella turcica, has also been reported in multiple individuals of a family with Rieger syndrome.[60] Like other patients with empty sella syndrome, the individuals in this report had radiographic evidence of enlarged sella turcicae but did not have pituitary endocrine dysfunction. Cryptorchidism and hypospadius have also been associated with Rieger syndrome in some individuals,[54] but there is no evidence for decreased testosterone levels in these patients.

In 1996, mutations in *RIEG1/PITX2* were identified in individuals with Rieger syndrome.[56] All six *RIEG1/PITX2* mutations identified in their original report were missense mutations, thought to result in loss of function. Since their initial study, many more mutations in *RIEG1/PITX2* have been found.[49,61-65] Recently, a *RIEG1/PITX2* deletion was identified in the family with growth hormone deficiency,[66] consistent with the demonstration that PITX2 has an important role in mouse pituitary development.[13,18] The animal studies also suggest that altered levels of PITX2 in humans might result in hypogonadotropic hypogonadism and delayed puberty, in addition to short stature due to GH and/or TSH deficiencies.

The majority of PITX2 mutations in RGS patients are missense or nonsense mutations within or near the homeodomain. There are a few mutations that interfere with exon-intron splicing. Although a dominant negative form of PITX2 has been reported (K88E), biochemical analysis reveals that most mutations result in loss of PITX2 activation of artificial transcription targets, suggesting that RGS is generally a haploinsufficiency disease.[47,49,67,68] Recent functional analysis of novel PITX2 mutations in RGS pedigrees, however, suggests that increased activity of PITX2 can also feature RGS.[65] Specifically, a Val45Leu mutation in the homeodomain causes reduced DNA binding affinity but increased transactivation properties in target gene induction. This gain of function mutation results in phenotypes commonly found in RGS including iris atrophy, corectopia (asymmetrically placed pupils) and craniofacial abnormalities. It is noteworthy that the same phenotypes are observed in the affected organs of patients with gain and loss of function mutations, suggesting that normal development requires a specific concentration of PITX2 rather than achievement of a threshold level. Given this observation, it is possible that elevated levels of PITX2 could also interfere with pituitary development or function.

In summary, the series of mouse *Pitx2* alleles revealed the reliance of pituitary development on the *Pitx2* gene dosage. *Pitx2* is absolutely required for the initial allocation of cells for Rathke's pouch and for its expansion. Together with *Pitx1*, *Pitx2* is essential for the activation of early acting pituitary transcription factors including LHX3 and HESX1, both of which are also required for normal pituitary development and function. Examination of a hypomorphic reduced function allele revealed that *Pitx2* is also required for specification of the gonadotrope and *Pit1* cell lineages. These data give support to the recent observation that lesions in *PITX2* can cause growth insufficiency in humans and suggest that *PITX2* mutations might cause hypogonadotropic hypogonadism or infertility in some individuals.

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CHAPTER 6

Expression and Function of Pitx2 in Chick Heart Looping

Xueyan Yu, Shusheng Wang and YiPing Chen

Rightward looping of the straight heart tube, a vital process for the formation of multi-chambered heart, is the first morphological manifestation of left-right (L-R) asymmetry during vertebrate embryonic development. In the developing chick embryo, sophisticated genetic pathways involving numerous signaling molecules and transcription factors in the regulation of dextral cardiac looping have been recently established. Among these asymmetric molecules is Pitx2, a paired-like transcription factor expressed in the left side lateral plate mesoderm and left half of the cardiac tube. Studies demonstrated that *Pitx2* resides downstream of the Shh/Nodal signaling pathway and functions to execute the L-R developmental program.

Chick Cardiac Looping

The heart is the first functional organ to form in vertebrate embryos. It arises through a complex series of morphogenetic interactions involving cells from several embryonic origins.[1] In the chick, cardiac precursor cells are originally located in the epiblast lateral to the primitive streak, and move through the primitive groove to join the mesoderm during gastrulation.[2] These cardiac precursors continue to migrate in an anterior/lateral direction, forming a pair of cardiac primordia residing in the lateral mesoderm on either side of the primitive streak. Beginning at HH-stage 6,[3] with the formation of head fold and anterior intestinal portal, the lateral regions of anterior embryo begin to approach each other to form the foregut. As part of this process, the two cardiac primordia, now each forms a heart tube, are brought together on the ventral surface of the forming foregut. From HH-stage 9, these two separate cardiac tubes meet at the ventral midline and fuse to form a single straight heart tube. It contains three layers: a thin outer layer of myocardium, a middle layer of extracellular matrix known as cardiac jelly, and an inner layer of endocardium. The cardiac tube then undergoes a dextral looping, a feature which is conserved among all vertebrate species.

The dextral heart looping that breaks the bilateral symmetry of the embryo is vital to normal cardiac morphogenesis and laterality. Failure to establish the proper left-right (L-R) asymmetric development leads to misplacement of internal organs that is classified as isomerism (symmetrical organ situs), heterotaxia (one or more organs develop with reversed L-R polarity), and situs inversus (complete reversion of the L-R axis). Cardiac looping is a complex process rather than a simple rightward bending of the straight heart tube (for a review see ref. 4). In the developing chick embryo, heart looping starts around HH-stage 10, and becomes discernible at HH-stage 11. During this period, the straight heart tube is transformed into a c-shaped loop through the rightward flapping/lateralization of the primitive ventricular bend

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and the right kinking of the primitive conus.[4,5] This process is followed by the transformation of the c-shaped cardiac loop into the s-shaped loop via significant morphogenetic events. The later contains four morphologically distinguishable components: the sinus venosus, the primitive atria, the primitive ventricular bend, and the primitive conus, establishing architecture for the formation of the multichambered heart (for a review see ref. 4).

The mechanisms of heart looping have been studied extensively in the chick. Heart looping appears to be intrinsic to the heart with consistent rightward looping directionality.[6,7] Patten,[8] put forward a model in which the dextral-looping was thought to be driven by compressive axial forces as the heart grows longer within a confined space. However, he could not explain the preference for rightward looping. Several hypotheses were proposed that postulated the involvement of differential cell movement, cell redistribution, or differential rate of cell proliferation and cell death in myocardial walls across the heart tube (for a review see ref. 4).[6,9] Others took account of cardiac jelly pressure or an initial tension in the dorsal mesocardium.[10,11] But none of them was experimentally proven or consistent with all available experimental evidence.

Molecular Cascades Regulating Cardiac Looping

Although the cellular mechanisms that drive cardiac looping remain unclear, significant progress has been made in the past few years regarding the molecular basis for the rightward looping of the heart tube.[12] Classic transplantation experiments suggested that directionality of heart looping is determined during gastrulation and controlled by the lateral plate mesoderm (LPM).[13] It is now known that dextral heart looping is controlled by a set of genes that interact to establish the L-R asymmetric developmental program. The molecules involved in breaking the L-R symmetry and regulating heart looping directionality are mostly derived from the original studies in the chick. The seminal work of Levin et al[14] initiated a wave of molecular studies on L-R patterning. In the chick, the L-R asymmetric gene expression is first seen in Hensen's node and perinodal area. During early gastrulation, asymmetric expression of *Activin-βB* and gene encoding for its type II receptor (*cAct-RIIa*) in the right side of Hensen's node inhibits otherwise symmetrically expressed *Sonic hedgehog* (*Shh*) in the right side of the node, which leads to the restriction of *Shh* expression to the left side around HH-stage 5.[14,15] The left-sided *Shh* expression in Hensen's node is maintained by BMP4 that, together with FGF4 and FGF8, exhibits asymmetric expression in the right side of Hensen's node and repress expression of *Shh* and the Shh-dependent pathway.[16-18] The asymmetric expression of these genes in the node is unique to the chick and not found in other species including mice and *Xenopus* at similar developmental stages. Other genes that exhibit an asymmetric expression pattern in and around Hensen's node and participate in the development of the L-R axis in chick embryos include *Wnt-8c*, *PKI*, and *N-cadherin*.[19-21]

The L-R asymmetric pathway established in Hensen's node is then converted into much broader domains of side-specific gene expression in the LPM. On the left side of the chick embryo, the asymmetric *Shh* expression is responsible for the asymmetric expression of the *Nodal* gene that encodes a member of TGF-β superfamily to the left LPM around HH-stage 6.[14,15,22] This induction of *Nodal* by *Shh* is mediated by the product of the *Cerberus*-related gene *Caronte* (*Car*) that antagonizes the repressive activity of bilaterally expressed BMPs.[23-25] The chick CFC, a member of the EGF-CFC family, appears to maintain the asymmetric *Nodal* expression in the LPM that is crucial for establishing the L-R asymmetric program.[26] Alteration of *Nodal* expression in the LPM randomizes the directionality of heart looping.[15] Genes downstream from the Nodal signaling in the left LPM include the *paired*-type homeobox gene *Pitx2*, the homeobox gene *Nkx3.2*, and the zinc finger gene *SnR*. Nodal functions as an activator for both *Pitx2* and *Nkx3.2* but a repressor for *SnR*.[23,27-29] Other laterality molecules, such as

Lefty1, Flectin, hLAMP, and Fibrillin-2, also display L-R asymmetric expression and may participate in the regulation of heart laterality.[23,24,30,31] Perturbation of expression of these molecules during early chick embryogenesis could result in a laterality defect of the heart. For a complete list of laterality genes and a summary of signaling pathways in L-R determination in several different species, please see a recent review by Mercola and Levin.[32]

Expression and Regulation of Pitx2 in Early Developing Chick Embryo

The chick *Pitx2* gene was cloned and shown to be expressed asymmetrically in the LPM and developing organs in early embryo simultaneously by several laboratories.[28,29,33] Two differentially spliced chick *Pitx2* isoforms have been isolated that correspond to the mouse and human *Pitx2a* and *Pitx2c*.[28,29,33-35] The chick Pitx2a and Pitx2c, while differing only by 77 amino acids at the N-terminal region between themselves, show 100% identity within the homeodomain to their mouse and human correspondents at the amino acid level.

In the developing chick embryo, strong *Pitx2* expression could be detected at early gastrulation stage (HH-stage 4), with its transcripts localizing symmetrically in the hypoblast and area opaca.[33,35] Around HH-stage 7, asymmetric *Pitx2* expression initially appears as a small patch restricted to the left LPM, just lateral and anterior to Hensen's node, while strong and symmetric *Pitx2* expression is also seen in the head mesenchyme (Fig. 1A,D).[33] The asymmetric *Pitx2* expression domain in the left LPM then extends both anteriorly and posteriorly, and

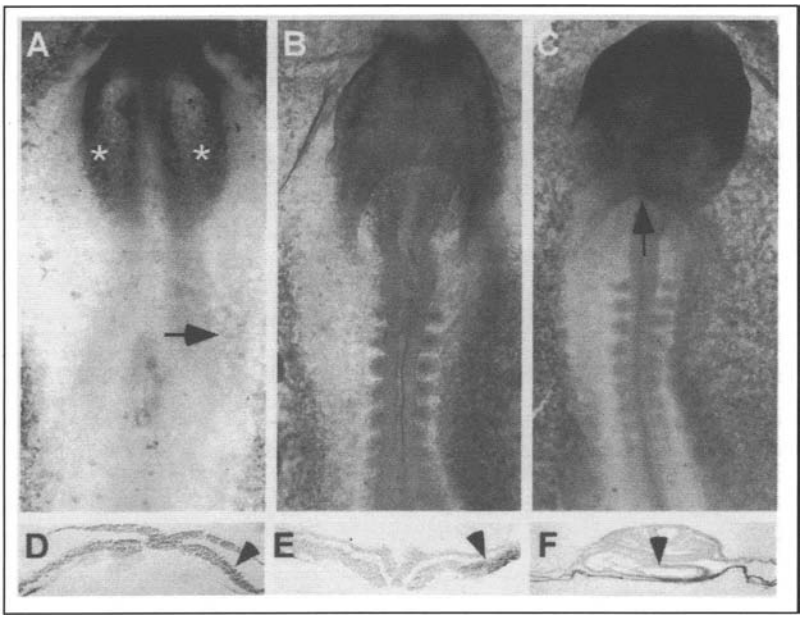

Figure 1. Expression of *Pitx2* in early developing chick embryo. A,D) *Pitx2* expression in the left LPM can be detected as early as HH-stage 7 as a small patch (arrow) (A), as confirmed (arrowhead) by section shown in (D). *Pitx2* expression in the head mesoderm is indicated by stars. B,E) An embryo at HH-stage 9 shows strong *Pitx2* expression in the left LPM (B), and is confirmed (arrowhead) by section as shown in (E). C,F) At straight heart tube stage (HH-stage 10), *Pitx2* is exclusively expressed in the LPM as well as the left half of the straight heart tube. Arrow in (C) and arrowhead in (F) point to the midline of the heart tube. All images of panels A-C are ventral view of embryo.

becomes evident along the entire left LPM by HH-stage 8 (Fig. 1B,E).[28,29,36] This asymmetric *Pitx2* expression in the left LPM appears slightly later than and overlaps with that of *Nodal*.[14,15,22] At the straight heart tube and looping stages (HH-stages 10-11), *Pitx2* expression remains limited to the myocardium of the left sided heart tube and the left vitelline vein (Fig. 1C,F). By stage 15, expression in the heart is only seen in the atrial myocardium. In addition, asymmetric *Pitx2* expression is also detected in the primitive gut and its derivatives.[28,29,33,36] Of the two chick *Pitx2* isoforms, *Pitx2c* is exclusively expressed in the left LPM, left half of the heart tube, and the head mesoderm. In contrast, *Pitx2a* transcripts are present in head mesoderm and extraembryonic mesoderm, but are absent in the LPM.[35] The asymmetric expression of *Pitx2c* in the left LPM is also conserved in mouse and *Xenopus*.[37] In contrast, in Zebrafish, *Pitx2c* only exhibits left-sided expression in the developing diencephalon but *Pitx2a* shows asymmetric expression in the left LPM.[38]

The expression pattern and timing of *Pitx2* in the LPM suggested that it might be a downstream target of the Shh-Nodal signaling pathway. Indeed, ectopic expression of *Shh* to the right LPM led to bilateral *Pitx2* expression. On the other hand, blockade of Shh activity in the left side of Hensen's node by application of anti-Shh antibody before HH-stage 6 repressed *Pitx2* expression in the left LPM (Fig. 2).[28,29,33,36] Furthermore, misexpression of *Nodal* to the right LPM also caused bilateral *Pitx2* expression through the LPM.[28,29,36] *Pitx2* expression is thus regulated through the Shh-Nodal signaling pathway in the developing chick embryo. It

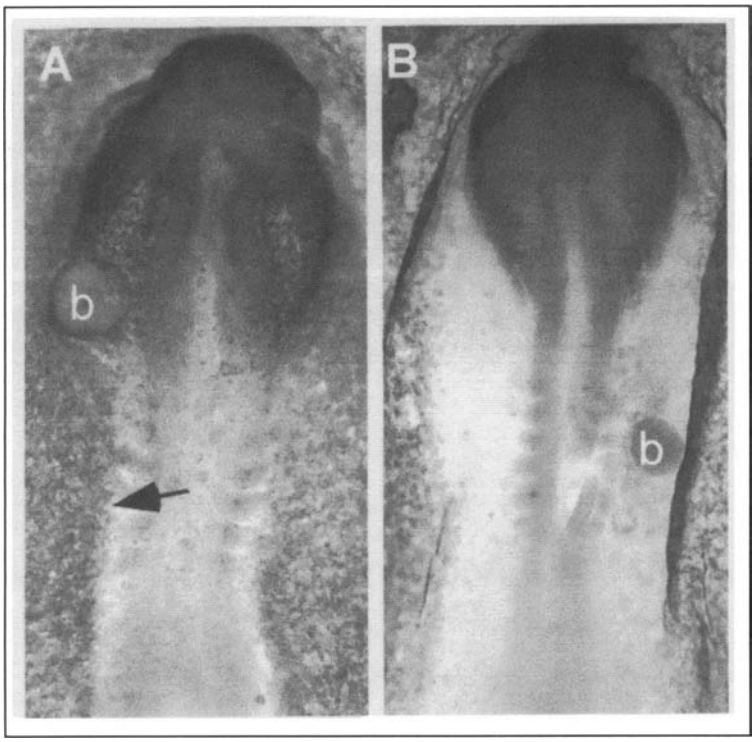

Figure 2. Regulation of *Pitx2* by Shh in chick embryo. A) A HH-stage 9 embryo which received a bead (b) soaked with Shh protein at stage 5 exhibits bilateral *Pitx2* expression in the LPM. Arrow points to the ectopic *Pitx2* expression in the right LPM. B) A HH-stage 9 embryo which recived a bead (b) soaked with antibody against Shh at stage 4 showes repression of *Pitx2* in the left LPM. Both images are ventral view of embryo.

was demonstrated in mice that the asymmetric expression of *Pitx2* is directly induced by Nodal signaling through the action of the transcription factor FAST and maintained by Nkx2.5.[39] In addition to the regulation by Nodal, Activin, another member of TGF-β superfamily, was also able to activate *Pitx2* expression in *Xenopus* embryo.[40] However, in chick embryo, activation of *Pitx2* in the left LPM by Nodal is mediated by the repression of a zinc finger transcriptional repressor SnR.[41] *SnR* is initially expression bilaterally, and then restricted to the right LPM when *Nodal* begins to be expressed in the left LPM.[27] Repression of *SnR* expression by anti-sense oligonucleotides could result in ectopic *Pitx2* in the right LPM.[41]

Retinoic acid (RA) is known to influence cardiac looping in vertebrate embryos by regulating expression of genes involved in L-R patterning.[42-45] RA has been demonstrated to be required for the normal expression of *Pitx2* in the left LPM in developing avian embryos. Either excess or deficiency in the retinoid signal leads to abnormal *Pitx2* expression.[43,45] Down-regulation of *Pitx2* was observed in the retinoid-deficient quail embryo that exhibits randomization of heart looping direction.[45] Similarly, application of RA antagonist to the left side of Hensen's node represses *Pitx2* expression in the LPM, while application of exogenous RA to the right side induces ectopic *Pitx2* in the right LPM.[43] Paralleled with the repression or ectopic expression of *Pitx2* regulated by RA signal is the aberrant expression of *Nodal* and *Lefty-1* but not *Shh*, indicating that the effect of RA on *Pitx2* expression may be mediated through *Lefty-1* and *Nodal* in a pathway downstream or in parallel to Shh.[43]

Normal *Pitx2* expression in the left LPM also depends on the activity of N-cadherin that is distributed asymmetrically in Hensen's node, with restriction to the right side. Blocking of N-cadherin function with anti-N-cadherin antibody at HH-stage 3 to 4 modified *Pitx2* expression to either a bilateral or reversed pattern in the LPM.[19] It was suggested that N-cadherin might mediate a pathway in parallel to that mediated by Nodal, since blocking N-cadherin function did not perturb *Nodal* expression in the developing chick embryo.[19] In contrast, another study demonstrated application of a blocking anti-N-cadherin antibody to the right side of Hensen's node resulted in ectopic expression of *Nodal* to the right LPM.[21] The right sided expression of N-cadherin in the node seems to normally antagonize the Shh-independent Wnt signaling pathway which otherwise induces *Nodal* expression in the right side of the embryo. It appears that Nodal plays a converging role to mediate the regulation of *Pitx2* by several independent signaling pathways.

Function of Pitx2 in the Regulation of Heart Looping Direction

In vitro assays have demonstrated that *Pitx2* encodes a transcription activator, with the transactivation domain mapped to its C-terminus.[35,46,47] As a transcription factor downstream from the Shh-Nodal signaling pathway, *Pitx2* products may interpret and execute the L-R developmental program. The function of *Pitx2* in the regulation of heart looping in developing chick embryo has been examined by both loss-of-function and gain-of-function approaches. Misexpression of *Pitx2* via infection of RCAS-retrovirus carrying *Pitx2* gene to the right side LPM randomized heart looping directions (Fig. 3B).[28,29,35] Interestingly, when ectopically expressed in the right LPM, both *Pitx2a* and *Pitx2c* equally resulted in randomization of the direction of heart looping. This was explained by the fact that an identical C-terminus containing the transactivation domain is present in both isoforms that may activate the same set of downstream genes or execute similar downstream functions.[35] The capability of *Pitx2* to direct the situs of heart looping was verified by misexpressing *Pitx2* to the right LPM while blocking the endogenous *Pitx2* in the left LPM with anti-Shh antibody. Reversion in heart looping direction was seen in such treated embryos.[28] The importance of *Pitx2* in the regulation of heart looping direction in the chick was further corroborated by loss-of-function studies. Elimination of *Pitx2c* in the left LPM by anti-sense oligonucleotide treatment or by misexpression of a dominant negative form of *Pitx2c* to the left LPM randomized the direction of heart looping

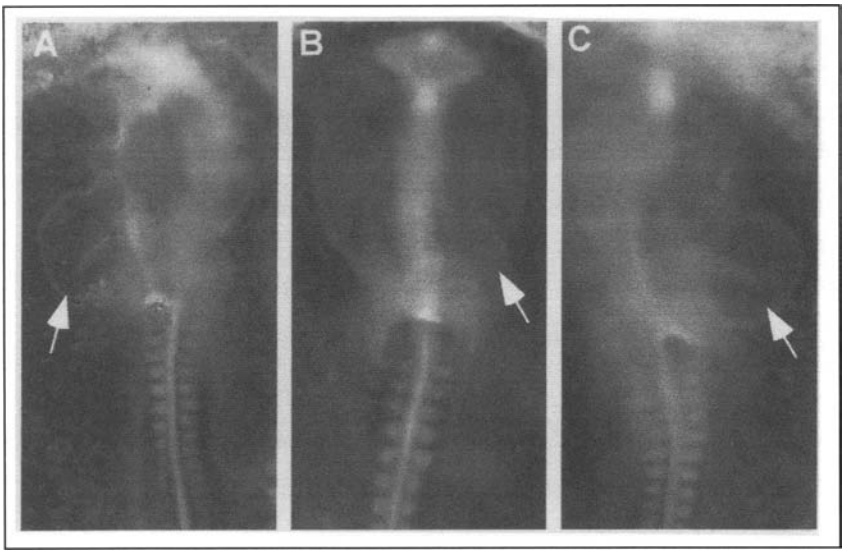

Figure 3. Misexpression of *Pitx2* in chick embryo leads to randomization of heart looping direction. A) A control embryo at HH-stage 11 exhibits left sided looping of the heart (arrow). B) A HH-stage 11 embryo which was infected with RCAS-*Pitx2c* on the right LPM at stage 4 shows rightward heart looping (arrow). C) A HH-stage 11 embryo which was treated with antisense oligonucleotides to *Pitx2c* at stage 6 exhibits rightward heart looping (arrow).

(Fig. 3C).[35] These studies provided unambiguous evidence for Pitx2 as a critical effector downstream from the Shh-Nodal signaling pathway to regulate heart looping directionality in the chick. However, it was also observed that asymmetric *Pitx2* expression is not always coupled with the heart looping direction in chick embryos. Abnormal heart looping still occurred even if *Pitx2* in the left LPM remained unaltered.[19,41] Furthermore, *Pitx2*-deficient mice do not exhibit a looping defect, although severe laterality defects were observed in other organs (see the following chapter for details on the role of *Pitx2* in mouse heart development).[48-52] It was thus suggested that other equally important genes are also involved in asymmetric development of the heart.[53]

Being a transcription activator, Pitx2 may control heart looping by regulating the expression of downstream gene(s). It was speculated that Pitx2 does so through contractile proteins based on the observation of *Pitx2* expression in the muscular layers in the heart and gut and in the sites where muscle differentiation occurs.[28,54] Recently, one putative downstream target of *Pitx2*, procollagen lysyl hydroxylase (Plod)-2 has been identified by chromatin precipitation in mice.[55] Members of the *Plod* gene family encode for enzymes that hydroxylate lysines in collagens. The hydroxylysine residues provide more stable attachment sites for carbohydrate units. *Pitx2* was shown to coexpress with *Plod-1* and *Plod-2* in several embryonic and adult mouse tissues, including the heart, lung, brain, and skeletal muscle.[55] Mutations in *PLOD-1* are often associated with Ehlers-Danlos syndrome, kyphoscoliosis type (EDVI) patients who share several characteristics with the Axenfeld-Rieger syndrome patients.[55] Examination of *Plod/PLOD* expression in *Pitx2* mutant mice or Axenfeld-Rieger syndrome patients would validate *Plod* genes as downstream targets of Pitx2. Evidence derived from studies in chick embryo indicates that Pitx2 may control heart looping by modulating the expression of the downstream

morphoregulatory ECM molecule flectin in the heart.[56] Flectin is an extracellular matrix molecule that shows L-R asymmetric localization during heart looping in mice and chick, with its expression predominantly in the left heart field and heart tube.[31,57] Blocking of flectin activity by a monoclonal antibody against flectin causes cardiac laterality defect which may result from perturbation of mechanical interactions between flectin and other components of the extracellular matrix.[56] In those chick embryos that carried misexpresison of *Pitx2c* to the right LPM or were treated with antisense oligonucleotide to *Pitx2c* and showed leftward heart looping, flectin expression pattern was reversed. These embryos exhibited a predominant flectin expression in the right mesocardial fold and right side of the heart extending from the fold region.[56] However, the regulation of flectin by *Pitx2* is unlikely to be directly because flectin is also normally expressed in the right sided heart field and heart tube where *Pitx2* is absent. Although the specific biochemical interactions or cell signaling mediated by flectin are elusive, flectin apparently functions downstream of *Pitx2* and participates in morphoregulatory pathways involved in coordinating heart looping.

Heart looping is an intricate process involving multiple interactions between numerous factors. Significant progress has been made in the past few years in identifying molecules and in establishing signaling cascades involved in the regulation of heart looping. However, at the present time, there is no model that can integrate the molecular data into the biomechanics of heart looping. The mechanisms of Pitx2 in regulating the cellular or biomechanical processes that drive dextral heart looping remains unknown. Pitx2 is undoubtedly not the only factor, but a central mediator in the regulation of heart looping. Identification of *Pitx2* downstream target genes and understanding of their associated cellular mechanisms including cell adhesion, migration, proliferation, and apoptosis would definitely provide mechanistic insights into this crucial process during cardiac morphogenesis.

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CHAPTER 7

The Multiple Roles of *Pitx2* in Heart Development

James F. Martin

PITX2 is a *paired*-related homeobox gene that has been shown to be the mutated gene in Axenfeld-Rieger syndrome (ARS). The focus of this chapter will be to review recent studies that address the role of *pitx2* in cardiac morphogenesis. Since ARS patients usually manifest ocular, dental and abdominal wall phenotypes, this review will deal primarily with experiments performed in model systems used to study gene function, such as the mouse.

Introduction

PITX2 *in Heart Morphogenesis: Evidence from Human Genetics*

Cardiac anomalies, such as atrial septal defects and valvular defects, have long been recognized to be occasionally associated with ARS suggesting a function for the ARS gene in cardiac development. However, until recently it was unclear if the ARS gene was directly involved with the cardiac phenotypes observed in ARS patients and the suggestion was made that ARS with cardiac manifestations may be a separate entity or contiguous gene syndrome.[1-3] Adding to the difficulty in sorting out these questions was the genetic and phenotypic heterogeneity in ARS patients.[4] The identification of *Pitx2* as the gene mutated in ARS has helped to clarify the role that *Pitx2* plays in cardiac morphogenesis.[5] Importantly, studies performed in chick, mouse, zebrafish and Xenopus embryos made a firm connection between *pitx2* and the development of left right asymmetry (see below). Defects in left right asymmetric morphogenesis have been documented to be closely associated with a range of congenital heart anomalies suggesting that *Pitx2* function may have a direct role in complex cardiac morphogenesis.[6,7] More recent studies performed in model systems have begun to unravel the function of *Pitx2* in the heart.

Pitx2 As a Final Effector in Left Right Asymmetry

Although vertebrates are outwardly symmetrical all internal organs show morphologic differences in the left versus right. An example of this is the number of lobes in the lungs. In the mouse, the left lung has one lobe while the right lung has four. The first left right asymmetric morphologic event is the rightward looping of the heart tube. This is followed closely by the directional rotation of the embryo from a dorsally flexed to the ventrally flexed position.[8,9] These asymmetric events are followed by left right asymmetric morphogenesis of all internal viscera. Importantly for this discussion, it has been recognized from clinical studies that congenital cardiac defects are often associated with other defects in left right asymmetry (LRA).[6] Thus, normal LRA is a critical determinant of cardiac morphogenesis.

Molecular Mechanisms of Axenfeld-Rieger Syndrome, edited by Brad A. Amendt.

Current models divide LRA into three components: breaking of symmetry, transduction of the asymmetric signal, and interpretation of the asymmetric signal by each individual organ.[8-10] Breaking of symmetry has been proposed to result from the directional movement of cilia at the node resulting in a leftward flow of a critical morphogen that initiates a left sided signal transduction cascade.[11-13] This left sided signaling serves to instruct or bias each individual organ to develop asymmetrically.

Analysis of the *pitx2* expression pattern in mice and chick embryos revealed that *pitx2* was expressed on the left side of the anterior precardiac splanchnic mesoderm. This left asymmetric expression persisted into the left heart tube prior to looping morphogenesis. *Pitx2* was also expressed in the left lateral plate and splanchnic mesoderm and the left side of forming organs. This early expression domain of *pitx2* suggested a role for *pitx2* in cardiac looping morphogenesis.[14-16]

The expression of *pitx2* in the *iv* and *inv* mouse mutants that have laterality defects also suggested a fundamental role for *Pitx2* in regulation of asymmetric morphogenesis. In the *iv* mutant mice, that display randomization of organ situs, *Pitx2* expression was observed to be either absent, reversed or correct suggesting a direct correlation of the *iv* mutant phenotypes with *pitx2* expression. Similarly, in the *inv* mutant mouse that has reversal of LRA, *Pitx2* expression in left lateral plate was reversed.[16-18] Consistent with these ideas, gain of function studies in chick embryos demonstrated that *Pitx2,* when overexpressed in right lateral plate mesoderm, resulted in hearts with reversed or ambiguous situs. Moreover, *nodal* misexpression in the right lateral plate of chick embryos also resulted in induction of ectopic *Pitx2* expression on the right.[15,16,19] This data, in combination with earlier experiments showing that *shh* was able to induce expression of *nodal* in lateral plate mesoderm, suggested the existence of a linear signaling cascade with *Pitx2* serving as the final effector within each organ primordium.[9,20]

In addition to a potential role for *Pitx2* in cardiac looping morphogenesis, other expression studies suggested a direct role for *Pitx2* in patterning a broad range of left-sided cardiac and vascular structures. *Pitx2c* was expressed in the left atrium and atrioventricular canal, left outflow tract, right ventricle and interventricular myocardium. Moreover, *Pitx2* was also expressed in the primary and secondary interatrial septum, left atrial appendage, left superior caval vein and pulmonary vein myocardium.[21,22] Thus, the expression analysis pointed to a role for *Pitx2* in cardiac looping morphogenesis and also in the morphogenesis of complex cardiac and vascular structures.

The Role of *Pitx2* Isoforms

The *Pitx2* gene encodes four isoforms *Pitx2a*, *Pitx2b*, *Pitx2c*, and *Pitx2d* that are generated by a combination of alternative splicing and alternative promoter usage.[23] The *Pitx2d* isoform has recently been described in humans and it is presently unknown if this isoform has conserved functions in other vertebrates.[24] Expression studies have shown that only *Pitx2c* is asymmetrically expressed in the developing embryo while the *Pitx2a* and *Pitx2b* isoforms are coexpressed with *Pitx2c* in symmetrical regions of the embryo.[22,25-27] *Pitx2c* is expressed in left lateral plate mesoderm and in the left side of the developing guts, heart and lungs. The three *Pitx2* isoforms in mice are coexpressed in periocular mesenchyme, oral and dental epithelium, as well as anterior body wall. Overexpression of a *Pitx2c engrailed repressor* (*en*^{r}) fusion protein resulted in randomization of cardiac looping morphogenesis.[27] Moreover, a similar experiment performed with the *Pitx2a en*^{r} failed to interfere with endogenous *Pitx2c* function suggesting that *Pitx2* isoforms have distinct target genes. Thus, these studies implicate *Pitx2c* as the important isoform in left right asymmetry and cardiac development primarily on the basis of the appropriate spatiotemporal expression of the *Pitx2c* isoform.

Evidence From Loss of Function Studies in Mice

Inactivation of *Pitx2* in mice has supported the models for *Pitx2* in left right asymmetry and cardiac development with the exception of looping morphogenesis. *Pitx2* null mutant mice had multiple defects in cardiac development but the mutant heart tube looped correctly rightward.[25,28-30] This result contrasts to the phenotypes observed in studies performed in chick embryos that showed randomization of cardiac looping after misexpression of *Pitx2* or a *Pitx2 en*^{r} fusion construct as described above. Explanations for the inconsistencies of these data may be that the function of *Pitx2* in chicks and mice may not be completely conserved. This is plausible since it is now clear that many components of the left right signaling pathways have divergent functions between species.[31] Also genetic redundancy such that another gene can compensate for *Pitx2* may be at play. A related, and more interesting possibility is that the overexpression studies have uncovered the existence of a negative regulatory mechanism to prevent *Pitx2* from regulating looping morphogenesis. Thus, by overexpressing *Pitx2* in the right lateral plate or recruiting a strong transcriptional repressor to *Pitx2* binding sites in the left lateral plate mesoderm, a more severe phenotype was observed.[15,16,19,27] Thus, these experiments suggest the possibility for a mechanism that prevents *Pitx2* from gaining access to all its potential target genes in the left lateral plate mesoderm. Future experiments are needed to clarify these questions.

Pitx2 null embryos had severe defects in AV valve formation with complete AV canal. There were also defects in sinuatrial morphogenesis including failure of outgrowth of the primary interatrial septum and isomerized or reversed atrial appendages.[22,25] Arterio-ventricular connections were abnormal with double outlet right ventricle (DORV) as the most common anomaly observed.[22] Growth of the ventricular myocardium was also defective resulting in right ventricular hypoplasia.[22,25] Thus, analysis of the *Pitx2* null mutant embryos revealed a wide range of defects in mutant hearts revealing many potential functions for *Pitx2* in heart morphogenesis.

Dosage Dependence for *Pitx2* in Heart Development

The haploinsufficiency of ARS suggested a requirement for two functional copies of *PITX2* for normal development. The underlying basis for this dosage requirement has been examined in mouse studies using a *Pitx2* allelic series that encodes different levels of *Pitx2c* activity.[22] These studies revealed that the heart requires only low levels of *Pitx2c* in comparison to other organ systems, such as the lungs, that require relatively high doses of *Pitx2c* for normal morphogenesis.[22]

Moreover, within the forming heart the structure that was most sensitive to small reductions in *Pitx2c* was the left atrial appendage. In mice that expressed intermediate levels of *Pitx2c,* valvular, ventricular and arterioventricular formation were normal while the atrial appendages were still isomerized.[22] Other atrial defects such as atrial septation were also normal in embryos with these intermediate levels of *Pitx2c.* Thus, these experiments identified the atrial appendages as the cardiac tissue that is most sensitive to *Pitx2* levels.

These experiments clearly illustrate the central role of *Pitx2* dose in organ morphogenesis. Moreover, they provide an explanation for the observation that cardiac defects are only rarely found in ARS patients. Models that can be gleaned from these experiments include the idea that transcription of *Pitx2c* levels within each organ primordial may vary. Alternatively, there may be mechanisms within each organ to modulate *Pitx2* activity. For example, phosphorylation has been shown to play a role in regulating *Pitx2* function.[32] Also, there may be tissue specific cofactors that either augment or inhibit the ability of *Pitx2* to regulate target genes. There is precedent for this idea in the pituitary where *Pitx2* has been shown to directly interact with *Pit1* to regulate pituitary target genes.[33]

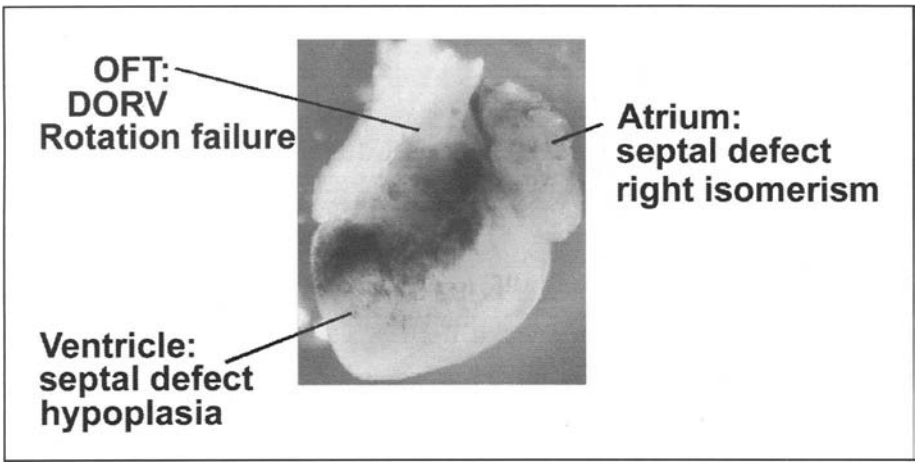

Figure 1. *Pitx2* functions in multiple areas of the developing heart. Whole mount of a *Pitx2 cre recombinase* knock in mutant 16.5 dpc heart crossed to the *rosa 26 reporter* mouse. *Pitx2* daughter cells are visualized by the blue staining. Labeled areas of the heart refer to the primary heart defects that have been identified in the *Pitx2* null mutant embryos. A color version of this figure is available at http://www.Eurekah.com.

Pitx2 in a Prospective Secondary Heart Field That Contributes to Ventricular and Outflow Tract Myocardium

Studies in mice have shown that *Pitx2* null mutants have defective orientation of the OFT and right ventricular hypoplasia.[25,28-30] Expression studies showed that *Pitx2c* was specifically expressed in the OFT myocardium and right ventricle,[34] suggesting a direct role for *Pitx2* in formation of the conotruncal region of the heart. It has become clear that there are multiple fields that contribute to the forming heart. Perhaps the best understood is the cardiac neural crest that derives from the dorsal neural tube and contributes to the conotruncal cushions and valves.[35]

It has also been established that cells derived from the epicardial organ contribute to the coronary vessels.[36] More recently, it has been shown that cells from the branchial arch and splanchnic mesoderm contribute to the OFT and the right ventricular myocardium.[37-39] *Pitx2c* is expressed in these cells as they move out from the splanchnic and branchial arch mesoderm and contribute to the OFT myocardium and right ventricle.[34] Thus, these experiments suggest that *Pitx2c* provides laterality not only to the primary heart field but also to cells that move into the heart after it has looped to the right.

The phenotypes observed in the *Pitx2c* mutant mice that have recently been generated, as well as the previous data from *Pitx2* null embryos, supported a role for *Pitx2c* in the splanchnic and branchial arch mesoderm heart field.[34] The data from the *Pitx2c* mutant mice suggested that the function of *Pitx2* in this secondary heart field was important after cardiac progenitors had arrived within the OFT and right ventricular myocardium. Fate mapping experiments using a *Pitx2 cre recombinase* knock in allele and the *rosa 26* reporter mice revealed that *Pitx2c* daughter cells were capable of migrating to the OFT and right ventricular myocardium.[34] One idea to explain these data would be that *Pitx2c* is required to regulate signaling between the OFT myocardium and the underlying conotruncal mesenchyme that will form the cushions and valves. Alternatively, *Pitx2c* may be required for expansion or proliferation of myocardium. Experiments are currently underway to address these issues.

The fate mapping experiments using the *Pitx2 cre* allele also provided insight into the function of *Pitx2* in AV valve formation.[34] Using this approach to visualize *Pitx2* daughter cells, it was observed that *Pitx2* daughters contributed to the AV cushion mesenchyme in heterozygous embryos but failed to enter the cushion mesenchyme of *Pitx2* mutant embryos. Consistent with this, *Pitx2* daughters were observed in the AV valve but were not detected in the abnormal valves of *Pitx2* mutant embryos.[34] Thus, these experiments raise a number of important questions about the function of *Pitx2c* in valve and cushion morphogenesis. What is the source of *Pitx2* daughters that contribute to the valves and cushions? One idea, that needs to be tested, is that these cells originate in myocardium that surrounds and move into the forming cushions in a process referred to as myocardilization.[40,41] An alternative idea is that *Pitx2* daughters move from the dorsal mesocardium that is in close proximity to the AV cushions and expresses *Pitx2c*.[34] Does the failure of *Pitx2* to move into the AV cushion reflect a direct requirement for *Pitx2c* in cell movement. This notion is supported by the recent observation that *Pitx2a* functions in cell migration through a Rho GTPase regulated pathway.[42] These important questions await future experiments.

Pitx2 Function in Pulmonary and Systemic Vein Development

Studies in mice have demonstrated an important role for *Pitx2* in patterning of the atrial appendages and atrial septation.[22,25] Recent work analyzing *Pitx2c* mutants also revealed a role for *Pitx2c* in morphogenesis of the pulmonary and caval veins.[34] Fate mapping studies revealed a direct role for *Pitx2c* in vein morphogenesis since *Pitx2c* daughters were shown to populate both pulmonary and caval veins. In addition, *Pitx2c* mutant mice had severe defects in vein morphogenesis.[34] In wild type mice, the pulmonary veins drain into a common pulmonary vein that empties into the left atrium. The left superior caval vein normally drains into the coronary sinus and the right superior and inferior caval veins are connected to the right atrium. In *Pitx2c* mutants, this normal venous architecture is lost and all the veins run together into a common sinus. Diminished contribution of *Pitx2* daughters to the *Pitx2c* mutant pulmonary vein suggested a role for *Pitx2c* in cell movement or cell sorting that may be similar to *Pitx2c* function in AV cushion morphogenesis. Alternatively, *Pitx2* may function to regulate proliferation or survival of pulmonary vein and AV cushion progenitors. Experiments are underway to distinguish between these possibilities.

Future Directions

As has been alluded to multiple times in this review, there are many important questions about the function of *Pitx2* in heart development that remain to be answered. Perhaps the largest void in our understanding of *Pitx2* function relates to the paucity of known *Pitx2* target genes in heart development. However, the prospects for this problem are bright as current methodologies to identify target pathways are available and continue to improve. Despite the unknowns, the last few years have seen a dramatic expansion of our understanding of *Pitx2* in development and disease and the pace of discovery will only increase.

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CHAPTER 8

The Role of *PITX2* in Tooth Development

Brad A. Amendt

The transcriptional mechanisms underlying tooth development are only beginning to be understood. Axenfeld-Rieger syndrome (ARS) patients provided the first link of *PITX2* to tooth development. ARS patients present clinically with dental hypoplasia, which includes microdontia, hypodontia and misshapen teeth. *Pitx2* is the earliest known transcription factor that is selectively expressed in the oral ectoderm. Since *Pitx2, Msx2, Lef1* and *Dlx2* are expressed in the dental epithelium we are examining the transcriptional activity of PITX2 in concert with these factors. We demonstrate that Msx2 binds to a variety of DNA elements and may play a more central role in regulating genes in tissues expressing this transcriptional repressor. We have identified the *Dlx2* promoter as a target of PITX2 during tooth development. Msx2 represses the *Dlx2* promoter and coexpression of both PITX2 and Msx2 resulted in transcriptional antagonism of the *Dlx2* promoter. Furthermore, a *PITX2A* mutation associated with ARS (*PITX2A T68P*) is unable to transactivate the *Dlx2* promoter. ARS patients with this point mutation present clinically with missing teeth. In contrast, a patient that presents clinically with only iris hypoplasia and normal tooth development has a *PITX2A* mutation (*PITX2A R84W*) that transactivates the *Dlx2* promoter. These data suggest a molecular mechanism for the dental anomalies associated with Axenfeld-Rieger syndrome. We will review the role of *PITX2* in tooth development and speculate on potential downstream targets of PITX2.

Introduction

Over the past several years there has been an explosion of information on the control of tooth formation. A database on gene expression in the tooth has been constructed and provides a tool for learning and research (http://bite-it.helsinki.fi/).[1] The development of teeth, like all organs, is regulated by inductive interactions between an epithelial layer and mesenchymal layer of cells (for a review see ref. 2). Genes and signaling pathways between and within these cells are rapidly coming to the forefront. Much of this information has come from mouse studies, although insight has also come from mapping of genes in human disorders, including Axenfeld-Rieger syndrome (ARS). PITX2 mutants cause defective transcription in patients with ARS. ARS is an autosomal-dominant human disorder characterized by dental hypoplasia, mild craniofacial dysmorphism, ocular anterior chamber anomalies causing glaucoma, and umbilical stump abnormalities. The dental hypoplasia is manifested as missing, small, and/or malformed teeth.[3] Teeth anomalies occur as abnormally small teeth (microdontia) giving rise to spaces between teeth, misshapen teeth, and missing teeth (hypodontia). The clinical presentations of ARS patients with regard to tooth anomalies are varied and may include all of the aforementioned anomalies or only one. In older ARS patients it has been observed that their teeth become brittle and they suffer from tooth loss. Some of the naturally occurring *PITX2A*

Molecular Mechanisms of Axenfeld-Rieger Syndrome, edited by Brad A. Amendt.

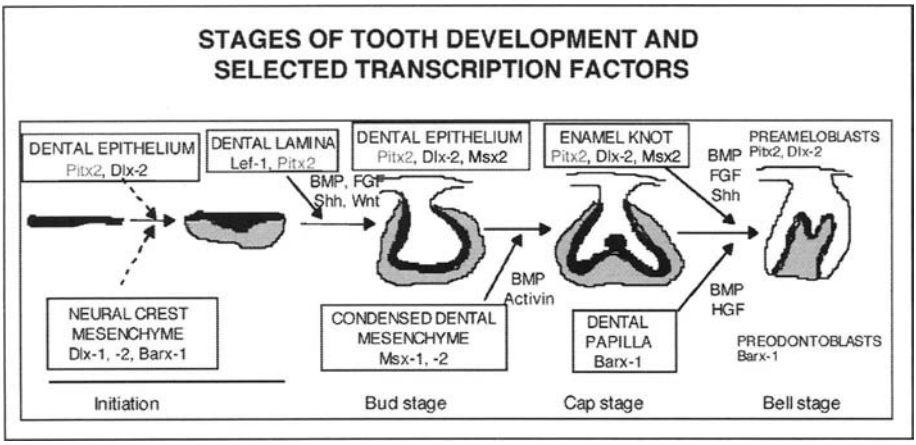

Figure 1. Sequential and reciprocal epithelial-mesenchymal signaling factors and transcription factors in tooth morphogenesis. The stages of tooth development are indicated at the bottom of the figure. The signaling tissues and associated transcription factors are boxed. During the early stages, the tissue source of the signal and the factors are not known hence the dashed lines. *Pitx2* is expressed throughout the process. *Pitx2* expression in the stomatodeal ectoderm, the precursor to the oral epithelium and anterior pituitary, was seen as early as day E8.5. By day E11.5, *Pitx2* expression in the oral epithelium is restricted to the thickenings of the dental placodes. *Pitx2* expression was high in the epithelium derivatives of both incisors and molars and is maintained at high levels through at least postnatal day 4 in the molar epithelial diaphragm. While *Pitx2* is expressed in the preameloblasts, it begins to be down regulated at this stage, suggesting a role in morphogenesis, but not differentiation. Figure is adapted from Thesleff and Sharpe, 1997.

mutations associated with ARS are defective for either DNA binding or transcriptional activation (see Chapter 4). Thus, the molecular basis of tooth anomalies in ARS appears to be the inability of PITX2 to activate genes involved in tooth morphogenesis (for a review see ref. 4). Studies by Mucchielli[5] have shown that *Pitx2* is the earliest known marker for dental initiation in the mouse (day E8.5). While the precise role of PITX2 in this process is not yet known, it is expressed in the appropriate tissues and times to be playing an instructive role in tooth morphogenesis and the dental hypoplasia of ARS patients supports a key role for PITX2.

We are working to establish the regulation of *PITX2* expression and protein interactions that modulate PITX2 function. In the past few years, a number of signaling molecules and transcription factors have been identified and shown to be critical for tooth formation (Fig. 1). In tooth formation, as in all organs, a single gene or even cell type does not act alone, but rather is influenced by other genes and cell types. The developing tooth primordia consists of two basic cell lineage's: a layer of epithelial cells, which arise from the oral ectoderm, and an underlayer of mesenchymal cells from the cranial neural crest (Fig. 1). Explant studies have elegantly revealed a series of reciprocal epithelial-mesenchymal interactions, with signals sent between both layers leading to a progressive differentiation of cells that eventually give rise to the mature tooth.[2,6]

Pitx2 Expression Patterns in the Developing Tooth

As noted above, *Pitx2* is the earliest known transcription factor that is selectively expressed in the oral ectoderm.[5,7] Expression was detected as early as day E8.5 in the mouse stomatodeal ectoderm, but not other ectoderm. Within 1 day, this region has acquired inductive capacity to

induce odontogenic properties in cranial neural crest. The first morphological evidence of tooth formation is at day E10.5, when there is a thickening of the mouse oral epithelium at the sites of odontogenesis. At this point, several transcription factor genes, including *Msx2*,[8] *Dlx2*,[9] and *Lef1*[10] are expressed in this region (Fig. 1). In addition, several secreted signaling molecule genes are also expressed, including bone morphogenetic protein (BMP-4),[11] fibroblast growth factor-8 (FGF-8),[12] and sonic hedgehog (Shh).[13] The expression of *Pitx2* remains specific to the oral epithelium with a progressive restriction to the dental placodes, followed by high level expression in the dental lamina and enamel knot in embryonic tooth primordia (Fig. 1).[5] In another report, *Pitx2* expression was found to be weakly expressed in the enamel knot, high in the inner enamel epithelium and weakly expressed in the outer enamel epithelium.[7] Postnatal expression is still detected in relatively undifferentiated epithelial tissue in the tooth germs, in the later developing second and third molar anlage. *Pitx2* is found in the preameloblasts, although the levels are lower, and it is absent from the fully differentiated ameloblasts.[5] However, *Pitx2* transcripts and protein are present in a cell line (LS-8) derived from neonatal mouse molar epithelium.[14] Pitx2 immunofluorescence studies have shown Pitx2 protein expression in the developing tooth bud.[15] The epithelium of both molars and incisors express Pitx2 protein. Pitx2 protein is also seen in the enamel epithelium of late stage (E18.5) mouse incisors.[15]

Mucchielli and colleagues have also shown that a mesenchymal signal is required to maintain *Pitx2* expression in the epithelium.[5] Using explant recombination experiments, they found that epithelial expression after the cap stage, E13 (mouse embryonic day 13) required a diffusable signal from the dental mesenchyme. This unidentified signal was capable of inducing *Pitx2* expression in nondental epithelium. Peptide growth factors such as BMPs and FGFs act as morphogenetic signals mediating inductive interactions throughout tooth development.[6,16,17] The regulation of transcription factor expression by these morphogenetic factors in the dental mesenchyme has been previously reported however little is known about the regulation of epithelial expressed transcription factors. An elegant study has demonstrated that antagonistic signals between BMP4 and FGF8 define the expression of *Pitx1* and *Pitx2* in developing mice teeth.[7] These researchers have demonstrated that BMP4 and FGF8 regulate the expression of *Pitx1* and *Pitx2* in both mesenchyme and epithelium of the developing mandible. Furthermore, the epithelial-derived signaling molecules not only activate or repress gene expression in the adjacent mesenchyme in a paracrine manner, but also affect gene expression in the epithelium itself in an autocrine manner.[7] Thus, the antagonistic effects of epithelial expressed *Fgf8* and *Bmp4* regulate and restrict both *Pitx1* and *Pitx2* expression in the mesenchyme as well as the epithelium with FGF8 being a positive regulator while BMP4 acts as a negative regulator. Based on *Pitx2* expression patterns and timing it was suggested that *Pitx2* plays a role in cell proliferation but not differentiation.[7] The restricted expression patterns of *Pitx2* could be accomplished by a concert of factors acting both transcriptionally and post-transcriptionally.

Tooth Epithelial Transcription Factors

The transcriptional mechanisms of tooth development are beginning to be defined and several key transcription factors have been shown to be involved in tooth morphogenesis.[1,2] It is known that homeodomain proteins play a role in tooth development.[1,3,5,8,18-21] Several of these homeobox genes have been shown to have an overlapping pattern of expression that correlates with tooth development.[18] Recent evidence has demonstrated that Pitx2, Msx2, and Dlx2, all homeodomain proteins, are involved in tooth morphogenesis.[3,5,7,8,15,18,19,22]

Because *Pitx2* is the first transcriptional marker of tooth development PITX2 may regulate the expression of other transcription factors or interact with the transcription factors expressed in the dental epithelium to regulate its transcriptional activity. Msx proteins are known to act as repressors of transcription[23] and Lef-1 and Dlx2 as transcriptional activators.[10,24-27]

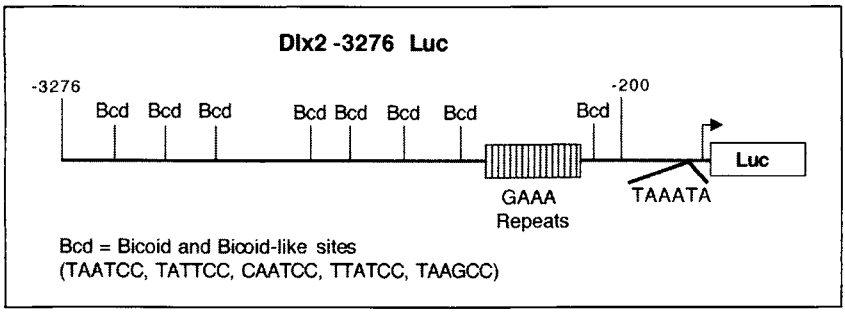

Figure 2. Schematic of the *Dlx2* promoter used in transient transfection assays. The location of *bicoid* and *bicoid*-like DNA elements, Bcd, *bicoid* and *bicoid*-like sequences are shown. The *Dlx2* promoter was linked to the luciferase gene in order to assay for luciferase activity as a measure of *Dlx2* promoter activity in transfected cells.[14,53,58] There are eight *bicoid* elements spaced throughout the 5' flanking region of *Dlx2*.

Furthermore, Msx and Dlx proteins can heterodimerize, which results in a functional antagonism.[27] We speculate that Pitx2 may act alone or complex with other proteins to regulate its own transcription or that of other genes involved in tooth morphogenesis. The *Dlx2* promoter appears to be an excellent candidate as a target gene for PITX2 transactivation. It contains multiple *bicoid* elements and derivatives of the element that can act as sites for PITX2 and PITX1 binding to confer transactivation (Fig. 2).

Dlx2, a member of the distal-less gene family, has been established as a regulator of branchial arch development.[28,29] Homozygous mutants of *Dlx2* have abnormal development of forebrain cells and craniofacial abnormalities in developing neural tissue, *Dlx* genes exhibit both sequential and overlapping expression, implying that temporal spatial regulation of *Dlx* genes are tightly regulated.[30] Within the mandibular and maxillary divisions of the first branchial arch, whose mesenchyme and epithelium eventually form the teeth, *Dlx2* is expressed proximally in the mesenchyme and distally in the epithelium.[29] *Dlx* genes are believed to play a role in tooth morphogenesis because homozygous *Dlx1/Dlx2* mutants are missing maxillary molars.[19] There are no obvious tooth abnormalities in either *Dlx1* or *Dlx2* mutant mice. These mice do however show abnormalities in development of specific facial skeletal elements.[28,31] The *Dlx1* and *Dlx2* double mutant mice show normal development and cyto-differentiation of the incisors and mandibular molars in newborn mice but the maxillary molar teeth were missing.[19] Maxillary molar tooth development was halted at the epithelial thickening stage and no bud- or cap-stage tooth germs were identified.

Tooth development is arrested in *Pitx2*$^{-/-}$ mice.[32,33] *Fgf8* and *Bmp4* expression patterns are disturbed and the enamel knot fails to develop. It was suggested that tooth development proceeds through the initial signaling and determination phases, but that the emergence, migration and expansion of distinct cell types in the developing ectoderm fail to progress past the full bud stage.[33] In the heterozygous *Pitx2*$^{-/+}$ mutant mice the tooth phenotypes are small, misshapen or mal-occluded teeth.[34] *Pitx2*$^{hd-/-}$ mice mandibular teeth arrested as tooth buds and maxillary teeth arrested at the placode stage.[33] Thus, there appears to be differences in the dental phenotypes between the *Pitx2* and *Dlx1/Dlx2* mutant mice. However, it is also clear that humans with *PITX2* mutations present with different phenotypes than mice.

A third homeobox protein, Msx2, is also implicated in the development of the teeth and other craniofacial structures.[8,18,32] Msx2 is a transcriptional repressor that has been shown to bind to the Msx1 binding motif 5' TAATTG 3'.[26] Some evidence indicates that the repressive activity is due to protein-protein interactions rather than direct binding with DNA.[27,35,36] Like other homeodomain proteins, *Msx2* expression is both spatially and temporally regulated

primarily through interactions between epithelial and mesenchymal tissue.[11] In the dental ectoderm, *Msx2* expression overlaps with that of *Dlx2*.[18]

Lymphoid enhancer-binding factor 1 (LEF-1) is a cell type-specific transcription factor expressed in lymphocytes of the adult mouse and in the neural crest, mesencephalon, tooth germs, whisker follicles and other sites during embryogenesis.[10,37-41] LEF-1 is a member of the high mobility group (HMG) family of proteins and activates transcription only in collaboration with other DNA-binding proteins and may promote the assembly of a higher-order nucleoprotein complex by juxtaposing nonadjacent factor binding sites.[24,25,42] Tooth development is initiated in *Lef*$^{-/-}$ mouse embryos however it is arrested before the formation of a mesenchymal dental papilla at E13, after formation of the epithelial tooth bud and mesenchymal condensation but before morphogenesis.[40] From E10 to E12, *Lef1* transcripts are detected initially in the epithelium and subsequently in the mesenchyme, consistent with the change in the developmental dominance of these tissues.[10] The role for *Lef1* in the mesenchyme is unclear since an essential function for *Lef1* expression could be demonstrated only in the dental epithelium between E13 and E14, corresponding to the presence of *Lef1* transcripts in the epithelial tooth bud. Because the epithelial tooth bud and future enamel knot structure has been proposed to function as a signaling center for tooth morphogenesis these results suggest that LEF-1 may regulate this process.[10]

Transcriptional Mechanisms of PITX2 during Tooth Development

We have shown that the *Dlx2* promoter is a target of PITX2.[14] The *Dlx2* promoter, with 3.8 Kb upstream sequence has been shown to contain the regulatory elements directing expression of *Dlx2* in the epithelium, but not the mesenchyme of the first arch.[29] The epithelial specificity of this region is maintained in the late stages of tooth formation.[43] PITX2 binds to the *bicoid* element that is present in numerous copies in the *Dlx2* promoter. In addition, Msx2, also binds to the *bicoid* element and competes with PITX2 for binding. Msx2 is a promiscuous DNA binding protein and may act to regulate a wide variety of genes by binding to a variety of DNA elements. Our results suggest that during tooth development Msx2 functionally antagonizes PITX2 activation of the *Dlx2* promoter.

PITX2 and Msx2 Act to Regulate Promoters Containing Bicoid and Bicoid-Like Elements

Most homeodomain proteins bind to DNA at a site that contains a TAAT core.[44] Homeodomain proteins are known for binding to specific DNA sequences despite the promiscuity of TAAT motifs. The specificity for which protein binds to a promoter is provided by the bases immediately 3' to the TAAT core.[45,46] For example, members of the *Fushi tarazu* class bind to a 5'TAATGG3' motif. Bicoid-like proteins bind to a 5'TAATCC3' motif.[46,47] Binding of a transcription factor to DNA can result in either activation or repression of the promoter.

To our knowledge *Dlx2* is the first reported downstream target of PITX2 identified in tooth morphogenesis. Previous work has shown that Pitx2 binds to the *bicoid* motif (5'TAATCC3').[48] In fact, both Pitx2 and Msx2 protein can bind to *bicoid* elements, competing with each other for binding. This has several implications on the regulation of transcription by Pitx2 in vivo. First, Pitx2 can activate genes that contain *bicoid* elements in their promoters. Second, competition for these binding sites with a repressor such as Msx2 would reduce or disrupt this activation.[14]

Msx2 can functionally antagonize Dlx5, a transcriptional activator, through protein-protein interactions, but not through competitive binding to sites within the promoter.[36] Other evidence demonstrates that Msx and Dlx proteins functionally antagonize each other, and this antagonism may be the result of the dimerization of the proteins preventing DNA binding.[27]

We demonstrate that Msx2 and Pitx2 have differential binding specificities for consensus and nonconsensus *bicoid* sites, allowing DNA interactions to dictate the transcriptional regulation by these proteins. We were unable to demonstrate heterodimerization between these two proteins. These results are similar to another report describing transcriptional antagonism between an activator and a repressor for a shared DNA-binding site.[49] Hmx1 was identified as repressing transcription from a promoter containing 5'CAAGTG3' elements while Nkx2.5 activated this promoter. Hmx1 can antagonize Nkx2.5 activation of this promoter and conversely Nkx2.5 can attenuate Hmx1 repression.[49] The expression levels of these two gene products determine the level of promoter activity. Therefore, during development the activity of the *Dlx2* promoter would be regulated by the dosage of *PITX2* and *Msx2* gene products.

During tooth morphogenesis, as the level of Msx2 rises, it may act to reduce the activation caused by PITX2, which is expressed earlier. In this case, the response of Pitx2 is changed not due to heterodimerization with Msx2, but due to the binding of both factors to the promoter. Interestingly, in Axenfeld-Rieger syndrome, a haploinsufficiency disorder, the variability in defects associated with mutations in *PITX2* has been attributed to *PITX2* tissue-specific dosage-dependence.[34] Since ARS patients present with tooth abnormalities the low level of *PITX2* expression would be counteracted by higher levels of *Msx2* expression and result in the continued repression of the *Dlx2* promoter.

Because *Pitx2* expression occurs before *Dlx2* our results are consistent with PITX2 activating *Dlx2* expression. Furthermore, because *Msx2* expression appears after *Pitx2* and *Dlx2* our model where Msx2 antagonizes PITX2 activation of *Dlx2* expression would tightly control *Dlx2* expression during tooth morphogenesis (Figs. 1,3). In the later stages of tooth morphogenesis *Pitx2*, *Dlx2* and *Msx2* expression is restricted to the inner enamel epithelium, which forms the ameloblast layer.[5,9,50] *Dlx2* expression appears to be reduced in the epithelium during later stages of tooth morphogenesis. Quantitative PCR data demonstrates a decrease in *Dlx2* tanscripts in *Pitx2*$^{-/-}$ mutant mouse face tissue compared to wildtype mouse tissue (unpublished observation).

LS-8 Cells Contain Factors That Regulate PITX2 Transcriptional Activity

We have shown that a cell line derived from mouse enamel organ epithelia,[51] endogenously expresses *Pitx2* isoforms *Pitx2a* and *Pitx2c*.[14] Interestingly, the activity of the full-length *Dlx2* promoter is significantly reduced in LS-8 cells compared to CHO cells transfected with PITX2. LS-8 nuclear extract (NE) contains factors that interact with PITX2. We speculate that factors in the LS-8 cell line interact with PITX2 to attenuate its activity. We propose a model for PITX2 and Msx2 in regulating *Dlx2* expression (Fig. 3). In CHO cells a simple model of mutual exclusion may exist where either PITX2 or Msx2 bind and activate or repress *Dlx2* expression, respectively. However, in the tooth epithelial cell line PITX2 expression has little effect on *Dlx2* promoter activity. In contrast, Msx2 appears to exert more of a repressive effect on the *Dlx2* promoter. This may be due to Msx2 interacting factors that enhance Msx2 activity or as we have shown, factors in the tooth epithelial LS-8 cell line complexing with PITX2 to inhibit its transcriptional activity.[14] These PITX2-protein complexes appear to enhance PITX2 binding to the *bicoid* probe similar to Pit-1 however, PITX2 transcriptional activity is repressed while Pit-1 causes transcriptional synergism. We are cloning genes from an LS-8 cDNA library to determine the nature of this effect, and have identified several genes that interact with PITX2 using the yeast two-hybrid assay. One of these genes I mentioned in chapter 4 and has homology to the high mobility group genes that play a role in chromatin remodeling. Other clones have been identified that may act as transcription factors interacting with PITX2 to regulate tooth development. It will be interesting to see how these factors regulate PITX2 activity and their role in tooth development.

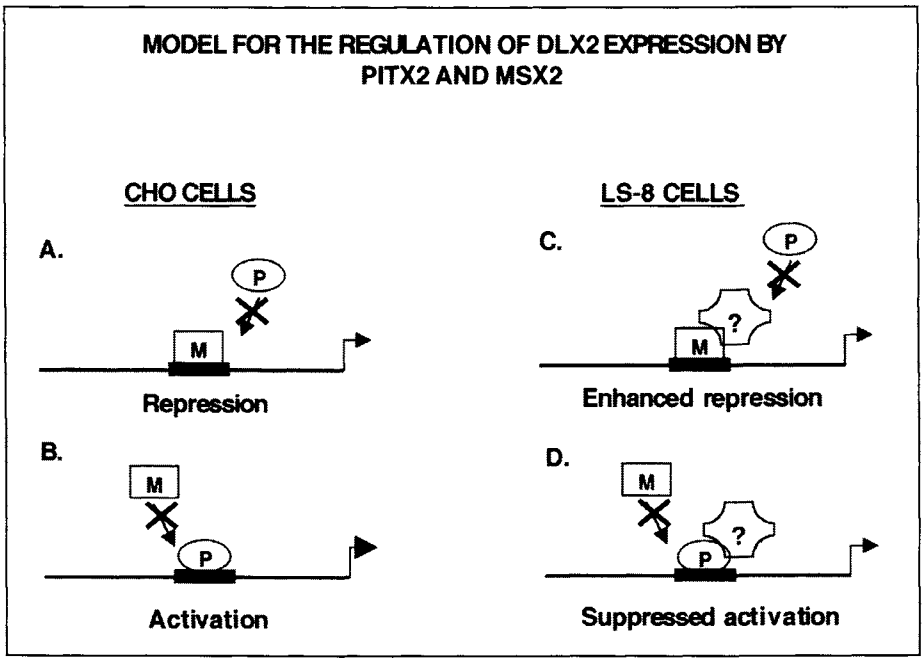

Figure 3. Model for PITX2 and Msx2 activation of the *Dlx2* promoter in a nontooth cell line, (CHO) and the LS-8 tooth epithelial cell line. A and B) In CHO cells a mutually exclusive binding of PITX2 and Msx2 to *bicoid* and *bicoid*-like elements may occur. The binding of one factor excludes the binding of the other factor. The relative concentrations of these proteins during development would dictate either activation or repression of the *Dlx2* promoter. C) In LS-8 cells a factor interacting with Msx2 may enhance its binding to DNA preventing PITX2 from binding to the same element. D) A factor in LS-8 cells interacts with PITX2, which may enhance PITX2 binding while inhibiting its transactivation of the *Dlx2* promoter. M, Msx2 protein; P, PITX2 protein; ?, unidentified protein(s) in LS-8 nuclear extract.

Dlx2 is a target of PITX2 in tooth morphogenesis. While Msx2 can attenuate *Dlx2* expression, factors in the tooth epithelium at later stages may interact with PITX2 to attenuate its transcriptional activity. Based on the expression patterns of *Pitx2*, *Msx2* and *Dlx2* it would seem possible that Msx2 is regulating *Dlx2* to a reduced level in the distal region of the mandible. However, PITX2 may increase *Dlx2* expression specifically at the sites of tooth development. In ARS patients with tooth abnormalities these defects may be linked to PITX2 mutant proteins that cannot transactivate the *Dlx2* gene.

We are currently studying the activity of LEF-1 protein and its interaction with PITX2. Because *Lef1* is expressed after *Pitx2* in the tooth epithelium it is a good candidate to test for interactions with PITX2. As mentioned before, LEF-1 is transcriptionally active only in combinations with other factors and PITX2 may interact with LEF-1 to regulate gene expression in the developing tooth epithelium (unpublished observations). Furthermore, our studies on *Msx2* promoter activation has revealed that PITX2 does not directly regulate *Msx2* expression but may act through other factors to indirectly regulate its expression. Experiments are underway to determine these transcriptional mechanisms involving PITX2 and these factors.

Recently, we have demonstrated that PITX2 activates the *LEF-1* promoter and in combination with LEF-1 protein synergistically activates *LEF-1* expression.

A Molecular Basis for Differential Developmental Anomalies in ARS

We have provided direct evidence using a natural PITX2 target gene for the molecular basis of the phenotypic variation associated with ARS.[53] We focused on two *PITX2A* mutations associated with ARS, which present clinically with very different phenotypes. The *PITX2A T68P* mutation was identified in an individual with Rieger syndrome, the most severe of the ARS disorders and clinically presents with the full-spectrum of developmental abnormalities.[3,48] These defects include ocular anterior chamber anomalies causing glaucoma, dental hypoplasia, mild craniofacial dysmorphism and umbilical stump abnormalities. The *PITX2A R84W* mutation was identified in 15 patients of a five-generation pedigree with IH.[54,55] This mutation represents the least severe phenotype associated with the ARS disorders, affecting mainly eye development. Both of these disorders are characterized by ocular defects and the lack of other anomalies in IH suggests that these mutations have distinct molecular effects during development.

Different DNA Binding and Transcriptional Properties of Two ARS Mutant Proteins

The PITX2A T68P mutant protein has reduced DNA binding activity, loss of binding specificity and defective transcriptional activation.[4,48] The DNA binding and transcriptional activity of *PITX2A T68P* was compared to the phenotypically less severe mutation *PITX2A R84W*. In contrast to the PITX2A T68P mutant the PITX2A R84W mutant retained normal DNA binding and specificity compared to wildtype protein. It was recently reported that the PITX2A R84W mutant caused a reduction in binding activity, but those investigators did not address binding specificity.[56] A possible explanation for the differences may be due to their use of proteins expressed in nuclear extracts with a 4 kDa flag epitope tag and inability to quantitate the amount of PITX2-proteins used in their experiments. However, both of our PITX2A T68P mutant proteins demonstrated reduced DNA binding activity. A possible explanation for the lack of or reduced transcriptional activity by these mutant proteins could be due to a change in their binding specificity. This would allow binding to different DNA elements, which might not be present in the natural targets of PITX2. To address this we asked if these mutant proteins could bind more efficiently to other known homeodomain binding sites. The *PITX2A T68P* mutation produces a protein with altered DNA binding specificity, which is in contrast to the PITX2A R84W mutant protein.

The transcriptional properties between these two mutant proteins are also quite different. PITX2 binds to both consensus *bicoid* elements (5'TAATCC3') within the *Dlx2* promoter and nonconsensus *bicoid* elements such as 5'TATTCC3', 5'TTATCC3', 5'TAAGCC3' and 5'CAATCC3' (Fig. 2). However, PITX2 binds to the consensus *bicoid* element as a dimer, which we speculate facilitates transcriptional activation.[14] We have used the natural *Dlx2* promoter to characterize the transcriptional properties of these two mutant proteins. Our data demonstrates a molecular basis for the lack of dental anomalies associated with the *PITX2A R84W* mutation since this mutant can activate the *Dlx2* promoter approximately 6-fold. PITX2A R84W while able to bind DNA does not form dimers similar to wildtype. Thus, one explanation for the lack of transcriptional activation may be due to its reduced ability to form homodimers. PITX2 homodimers occur naturally in a cell line endogenously expressing PITX2.[14] However, the more phenotypically severe mutation, *PITX2A T68P* is unable to activate the *Dlx2* promoter. We speculate that the 6-fold activation of the *Dlx2* promoter by PITX2A R84W would allow for normal tooth development in patients with this mutation. A dosage response model has been proposed to explain the differential organ development seen in *Pitx2* heterozygous $^{+/-}$ and homozygous $^{-/-}$ mice.[34] Recently, investigators have shown that thresholds of the PITX2C isoform dictates development of asymmetric organ morphogenesis.[57] These

models would explain the normal development of teeth in patients with the *PITX2A R84W* mutation as this protein would allow for some expression of the *Dlx2* gene. The PITX2A R84W mutant activity combined with the levels of *PITX2* expressed from the normal allele would provide an increased Dlx2 dosage response compared to the PITX2A T68P mutant.

The Role of PITX2 Phosphorylation

PITX2 is phosphorylated in vitro and in vivo by PKC and phosphorylation increases DNA binding activity.[53] Two ARS mutant proteins are phosphorylated by PKC and the PITX2A T68P mutation acts to increase phosphorylation at that site. Since phosphorylation of wildtype protein increased its activity we expected that hyperphosphorylation of PITX2A T68P to produce a gain-of-function mutation. However, increased PITX2A T68P phosphorylation only had a minimal effect on DNA binding. In contrast, phosphorylation of wildtype and PITX2R84W facilitates dimerization and increased transcriptional activation. Thus, the proline mutation in *PITX2 T68P* probably imparts a conformational change in the protein that generally inhibits its DNA binding activity.

Summary

The *PITX2* gene provides a tool to study dental development by investigating the mechanisms and mutations that control PITX2 function. The established requirement for PITX2 in normal human tooth development based on the ARS mutations and the early and sustained expression pattern of *Pitx2* in the tooth primordia suggest that PITX2 plays a major role in tooth development. Identification of interacting partners that regulate *PITX2* expression and function will then further our understanding of the genetic and epigenetic factors important in dental development.

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CHAPTER 9

PITX Genes and Ocular Development

Elena V. Semina

The anterior segment of the vertebrate eye is a complex arrangement of interdependent tissues of different embryonic origins. Despite its critical role in normal vision, relatively little is currently known about the development of the anterior segment and its molecular determinants. All three members of the *PITX* homeobox-containing transcription factor gene family were found to be expressed in the anterior segment structures during embryonic development. Two of these genes, *PITX2* and *PITX3*, were shown to be responsible for a spectrum of developmental anterior segment phenotypes associated with glaucoma, corneal opacities and cataracts in humans and mice. These findings demonstrate a requirement of *PITX* genes for normal development of the anterior segment of the eye and provide tools to study the molecular control of development of these structures.

Introduction

PITX genes represent a recently identified family of homeodomain-containing transcription factor genes that were shown to play an important role in development. In humans, the family was found to consist of three genes, *PITX1*,*PITX2* and *PITX3*, cognates of which were discovered in different species.[1] The *Pitx* genes were shown to have distinct but overlapping expression patterns during mouse development (Table 1), (for images of *Pitx2* expression see other chapters in this book).[2-5] In respect to ocular development, products of all three *Pitx* genes are detected in the developing eye: *Pitx2* expression is strong in the periocular mesenchyme and developing cornea, *Pitx3* in the developing lens, and *Pitx1* is weakly expressed at both locations, periocular mesenchyme and the lens, probably representing ancient nondivided expression of the original *Pitx* gene before duplications. The phenotypes caused by mutations in *Pitx* genes correspond well with their expression patterns: inactivation of *Pitx1* gene results in limb and pituitary defects in mice.[4,5] Mutations in the human *PITX2* gene cause Axenfeld-Rieger syndrome,[2] and inactivation of the mouse gene results in embryonic death and abnormal development of heart, lungs, eyes, pituitary, teeth and abdominal wall defects (see other chapters).[6-9] Mutations in the *PITX3* gene result in anterior segment dysgenesis and cataracts in humans and *aphakia* in mice.[3,10,11] Therefore, normal function of *PITX* genes appears to be essential for proper formation of the anterior segment of the eye and studies of these genes should provide critical knowledge about mechanisms of development of these structures that play important role in normal vision.

Molecular Mechanisms of Axenfeld-Rieger Syndrome, edited by Brad A. Amendt.

Ocular Phenotypes Associated with Mutations in *PITX2* and *PITX3* Human and Mouse Genes

PITX2 *Mutations*

PITX2 gene was discovered by positional cloning as a gene involved in Axenfeld-Rieger syndrome (see other chapters in this book).[2] A main characteristic feature of this condition is anomalies of the anterior segment of the eye that most frequently include posterior embryotoxon, irido-corneal adhesions, iris hypoplasia and glaucoma, which is developed by ~50% of affected individuals. Glaucoma associated with Axenfeld-Rieger syndrome is typically difficult to control and therefore often leads to substantial optic nerve damage and visual loss. Surgical intervention such as trabeculotomy is a primary choice of treatment and is aimed to facilitate the aqueous humor outflow in the irido-corneal angle towards the Schlemm's canal and the subconjunctival space.

Several theories about possible mechanisms of this condition have been proposed with the most popular one being presented by Shields in 1983.[12] Shields suggested that Axenfeld-Rieger anomalies result from developmental arrest at late stages of anterior segment development. Anterior segment structures receive a substantial contribution from neural crest cells through three successive migration waves. These mesenchymal cells of neural crest origin produce primordial corneal endothelial layer that then creates a closed cavity to form the anterior chamber, later this layer progressively disappears with some remaining cells establishing the endothelial lining of the trabecular meshwork. Shields suggested that in Axenfeld-Rieger anomaly there is a retention of this endothelial layer over portions of iris and anterior chamber leading to the corectopia (abnormal position of the pupil to one side of the center of the iris) and other iris anomalies as the iris cellular layer contracts. Also, this arrest prevents the normal migration of the iris and ciliary body posteriorly in relation to the angle structures, resulting in an anteriorly inserted iris root and therefore compression of the trabecular meshwork, incomplete formation of the trabecular lamellae and Schlemm's canal. Therefore, obstruction to aqueous humor outflow in patients with glaucoma associated with Axenfeld-Rieger spectrum are due to either compression of trabecular meshwork or incomplete development of trabecular meshwork and Schlemm's canal. Neural crest cells give rise to most of mesenchyme related to forebrain and pituitary gland, bones and cartilage of the upper face, and dental papillae. Therefore, developmental arrest involving neural crest could also explain the anomalies of facial bones, and teeth that are characteristic for this condition.[12,13]

Ocular expression of *Pitx2* is detected in neural crest-derived periocular mesenchyme at early stages of ocular development and at later stages *Pitx2* transcript and protein are found in the developing cornea, iris, trabecular meshwork and thin layer of mesenchymal cells surrounding the developing eye.[2,14] In mutant mice with complete loss of *Pitx2* function, ocular development is arrested at the stage when the lens vesicle separates from corneal ectoderm allowing formation of anterior segment structures in the opening space. Notable defects in these mice include displaced irregular pupil and a lack of anterior chamber, extraocular muscles and differentiated cornea, specifically corneal endothelium. Instead these mice demonstrate a 5- to 10-fold increase in thickening of the mesothelial layer of the cornea between the cuboidal epithelium constituting the anterior part of the lens and corneal ectoderm in 12.5-13.5 days embryos. The combination of extraocular muscle dysgenesis and corneal thickening results in enophthalmos in these *Pitx2*-deficient animals.[6-9] Thus, *Pitx2* was shown to be essential for proper migration and/or differentiation of neural crest-derived periocular mesenchyme in animal models, indicating that this process is likely disturbed in Axenfeld-Rieger patients.

Mechanism of *PITX2* mutations in Axenfeld-Rieger syndrome appears to be haploinsufficiency, which means that mutations abolish function of one *PITX2* allele while the

Table 1. Current genetic, expression and phenotype data for* PITX1, 2 *and* 3 *genes

Gene	Location: Human/ Mouse	Major Developmental Expression	Human Phenotype	Mouse Phenotype
PITX1	5q31/13	Pituitary, hindlimb, mandible, palate, teeth, abdominal region, olfactory system	unknown	*Pitx1-/-*: hindlimb, pituitary anomalies, cleft palate
PITX2	4q25/3	Eye (periocular mesenchyme, anterior segment structures, extraocular muscles), maxilla and mandible, teeth, umbilicus, pituitary, heart, limbs, brain, lung	Axenfeld-Rieger syndrome	*Pitx2-/-*: embryonic death around day 10.5 to 13: arrest in eye, teeth development, abdominal wall defects, hypoplastic pituitary, heart defects, lung isomerism
PITX3	10q25/19	Eye (lens, extraocular muscles), midbrain, tongue, head muscles, mesenchyme around spinal column and sternum	Anterior segment mesenchymal dysgenesis and congenital cataracts	*Aphakia* mice: small eyes with no lens

References are included in the text.

other retains its qualities but is not sufficient to fulfill a required task. Some dominant-negative and one possible gain-of-function mutation have been described that still resulted in Axenfeld-Rieger syndrome suggesting broader importance of *PITX2* dosage in this condition (see other chapters in this book for details).[15-17]

PITX3

The *PITX3* gene was discovered in a search for additional members of the *PITX* family.[3,18] Expression studies revealed a strong presence of the *Pitx3* transcript during lens development starting from expression in the lens placode in day-10.5 embryos throughout all following stages of lens development and at lower levels in the adult lens (Fig. 1). This finding, in combination with localization of this gene to the region implicated in *aphakia* ("no lens") phenotype in mice, suggested that *PITX3* gene is likely to be involved in ocular conditions with altered lens and/or anterior segment development in humans.

Localization of the *PITX3* gene to 10q25 region facilitated linkage studies in related ocular pedigrees that resulted in an identification of a linkage of anterior segment mesenchymal dysgenesis and cataracts (ASMD) in one family to *PITX3* with lod score 4.8 at ∂=0.[3,19] This family was characterized by a highly variable phenotype that included cataracts of varying severity (100%), corneal opacities with or without irido-corneal adhesions (100%) and optic nerve abnormalities (20%) (Fig. 2).

Screening of the *PITX3* coding region in the ASMD family and other families affected with various anterior segment conditions resulted in identification of two mutations. A 17-nt

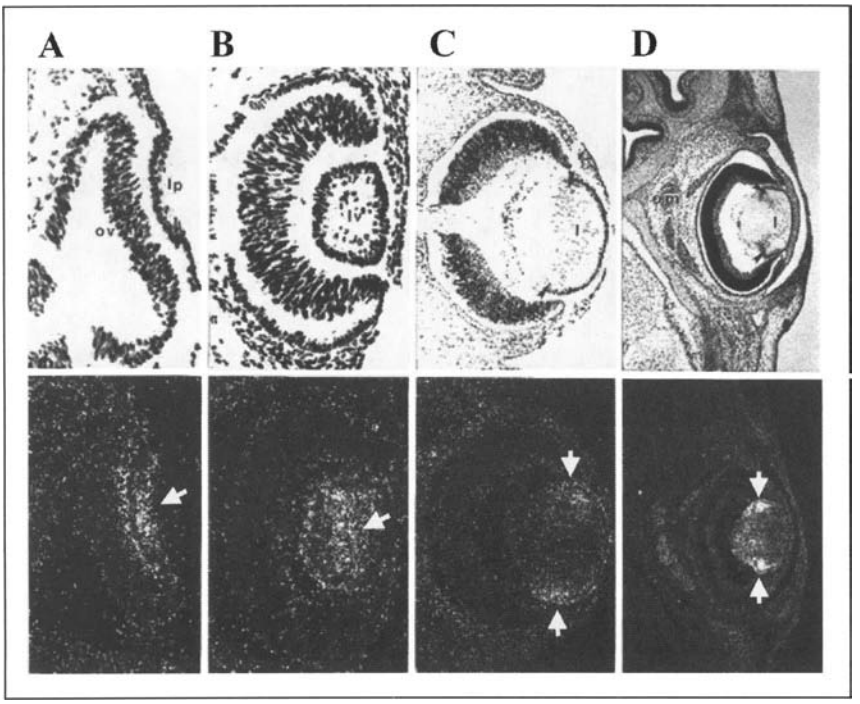

Figure 1. Expression of the mouse *Pitx3* gene during ocular development. Subsequent stages of ocular development are presented staring from late lens placode in day 10.5 embryo (A), lens vesicle in day 11 embryo (B), differentiating lens in day 13.5 embryo (C) and maturing eye in day 15.5 embryo (D). The Pitx3 expression is seen at all stages and is confined to the developing lens at earlier stages and later is also present in the extraocular muscles. lp- lens placode, ov- optic vesicle, lv- lens vesicle, l- lens, om- extraocular muscle.

insertion resulting in a frame shift in the C-terminal region of the protein, altering 82 out of 302 amino acids, in ASMD family; and a G-to-A nucleotide substitution resulting in a serine-to-asparagine substitution in the N-terminal region of the translated protein in a family with congenital cataract and glaucoma but without irido-corneal adhesions or corneal opacities, characteristic for the ASMD family. Neither mutation was detected in 300 normal control individuals and both of them completely cosegregated with the affected phenotypes in these families.[3] Both families had no other systemic features indicating that eye development is the most sensitive to normal *PITX3* function. Other expression domains of the rodent *Pitx3* gene include mesencephalic dopaminergic neurons in the brain,[20] head and trunk muscles, tongue, mesenchyme around spinal column and sternum.[3]

Studies of *aphakia* mice for a mutation in the *Pitx3* gene initially yielded negative result as no mutation was identified in the coding region of the gene.[18] Further analysis of *aphakia* mouse's RNA demonstrated lack of *Pitx3* transcripts in the lens during development that encouraged studies of the promoter and other potential regulatory regions of this gene. Deletions in the promoter region of *Pitx3* have been subsequently discovered in genomic DNA of *aphakia* mice that cosegregated with the phenotype and were not present in other mouse strains.[10,11] These findings strongly suggested that lack of *Pitx3* expression caused by the promoter deletion resulted in the arrest of lens development and *aphakia* phenotype in these animals.

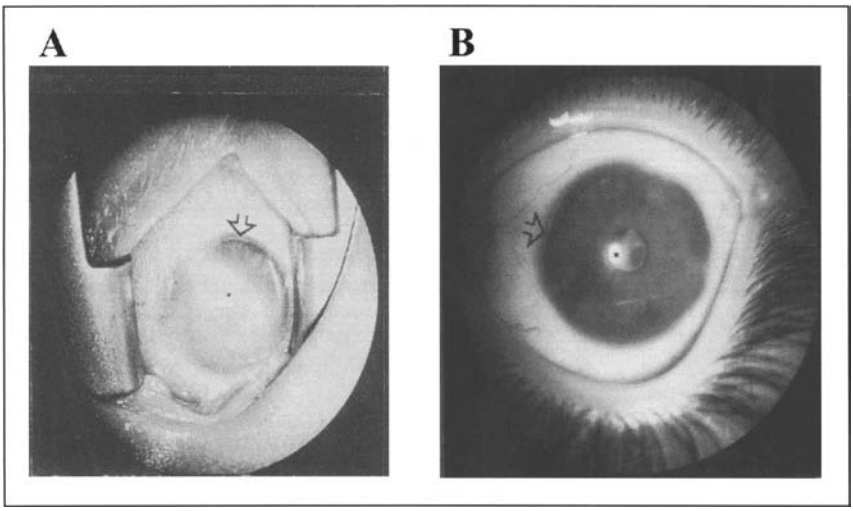

Figure 2. Anterior segment mesenchymal dysgenesis in a family with *PITX3* mutation. A) A dense central corneal leucoma with an iris adhesion obscures a small central cataractous lens. B) A dense central cataractous lens overshadows a translucent central corneal leucoma. Open arrowhead indicates Schwalbe's ring. As seen in Semina et al (1998); published with kind permission from Nature Genetics.

Ocular development in *aphakia* is arrested at the stage when the lens vesicle separates from the corneal ectoderm making space for the anterior segment. Easily observable changes in *aphakia* eyes include a persistent connection between the lens and the corneal ectoderm (lens stalk) and rudiment lens vesicle filled up with undifferentiated cells released from the lens epithelium. There is no differentiation of anterior segment structures or lens fibers, lens vesicle is later dissolved and the space, which is normally occupied by lens, is progressively filled with retinal folds.[21] It has always been suggested that the primary defect in *aphakia* eyes stems from the abnormal lens development as lensless eyes demonstrate the same abnormalities. Zwaan and Kirkland[22] noted that the lens placode and early lens cup of mouse *aphakia* embryos show significantly greater numbers of maloriented mitoses than normal control lenses resulting in the production of daughter cells, which are unequal by their position at the tissue surface. Also Zwaan and Webster[23] demonstrated that extracellular matrix components were observed intercellularly in the presumptive neural retina and lens rudiment of some *aphakia* specimens and suggested that these intercellular deposits may be secreted abnormally due to some disturbed cellular polarity. Defects in cellular adhesion, migration and differentiation of neural crest cells have been suggested to occur in *aphakia* eye development, it is still unclear whether these abnormalities play role in pathogenesis of *aphakia* phenotype or present secondary effects. Identification of a causative gene for this condition, homeodomain-containing transcription factor *Pitx3*, and studies of its developmental pathway(s) should assist in better understanding of this phenotype and also provide an insight into normal processes involved in lens-cornea separation and subsequent differentiation of lens and anterior segment structures.

Disorders of the Anterior Segment of the Eye: A Variety of Conditions and Causative Genes, Possible Interactions with PITX Factors

Developmental defects of the anterior segment represent a continuum of abnormalities characteristic of multiple diseases, each likely to have a unique etiology. A considerable effort

has been made in the last few years to identify genetic loci for anterior segment disorders in both human and mouse genomes. This has facilitated the discovery of many causative genes with a total of fifteen genes currently known to be involved in defective development of the anterior segment structures in humans, seven of which have been associated with glaucoma (for a review see refs. 24-35). Variable expressivity of many of these phenotypes suggested modification by other, genetic, environmental and random, factors that can now be investigated.[36]

Majority of the identified causative genes represent transcription factors of different types. Research into possible interactions of some of these genes with PITX factors has been initiated. Studies of mutual interactions of these factors, their developmental functions and downstream targets will lead to better understanding of the processes involved in patterning of the complex diversity of the anterior segment structures.

Conclusions

Anomalies in the formation of the anterior segment and lens lead to a variety of debilitating ocular disorders. At the same time, the development of these structures is still not fully understood. One approach to reveal these developmental processes could be based on studies of its defective forms. *PITX* family of homeodomain-containing transcription factor genes was demonstrated to be involved in a variety of ocular conditions resulting from abnormal development of the anterior segment of the eye. Humans with mutations in *PITX2* and *PITX3* genes exhibit corneal opacities, glaucoma and cataracts that often result in poor or no vision. Mice with *Pitx2-* or *Pitx3-* deficiencies demonstrate arrest of ocular development and lack of the anterior segment structures. Therefore, the *PITX* family represents an important component of the network of genes that control normal development of the anterior segment and lens in mammals. Studies of these genes will improve our knowledge about mechanisms of ocular development and reveal its novel determinants. This will lead to superior diagnosis, treatment and prevention of these conditions in the future.

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CHAPTER 10

An Overview of Axenfeld-Rieger Syndrome and the Anterior Segment Developmental Disorders

Brad A. Amendt

The preceding chapters have described the current research on the genetic, molecular and biochemical basis for Axenfeld-Rieger syndrome (ARS). They also provided information on anterior segment disorders by *PITX* and *FOXC1* genes characterized by maldevelopment of the anterior segment of the eye. In chapter 1, Dr. Elena Semina described their initial studies that identified the human *PITX2* gene on chromosome 4q25 using a positional cloning strategy. This gene was cloned from mouse at the same time by a group from the University of Michigan,[1] and several other groups and has been assigned various names (*Rieg, Ptx2, Otlx2, Brx1, ARP1*).[2-4] The three cardinal features of ARS include specific ocular anomalies of the anterior segment, dental anomalies and redundant periumbilical skin. However, other anomalies were reported in ARS patients such as pituitary, hearing, heart and limb defects that may be associated with other gene defects. Dr. Semina noted that approximately 40% of classic ARS patients have mutations in the *PITX2* gene. This indicated that other genes are involved in the etiology of this disorder. Dr. Semina and Dr. Murray as well as others have identified many *PITX2* mutations, which are mainly clustered in the homeodomain, however recently two missense mutations were found downstream of the homeodomain in ARS patients.[5] The phenotypic presentations of the ARS patients provided the first clues to the role of *PITX2* in embryogenesis and development of the affected tissues.

Drs. Nishimura and Swiderski described their work in chapter 2 on a second locus for ARS, which was identified from chromosomal abnormalities on chromosome 6p25. Their research has identified mutations in the *FOXC1* gene from patients with Axenfeld anomaly, Rieger anomaly and iris hypoplasia. Subsequently, *FOXC1* mutations were associated with ARS patients from several laboratories. *FOXC1* has been linked to the group of anterior segment disorders including ARS. The authors describe the clinical presentations of patients with *FOXC1* mutations. The molecular/biochemical studies on FOXC1 are evolving and initial work demonstrates that FOXC1 mutant proteins are transactivation defective.

Dr. Riise has extended the chromosomal loci for ARS by reporting that a small deletion on chromosome 11 was associated with a patient presenting with Axenfeld-Rieger syndrome. She described a new ARS patient associated with a *PAX6* mutation. The affected individual has the three cardinal features of ARS, and FISH analysis using probes for the *PAX6* gene showed a small deletion in the *PAX6* gene on one homologue of chromosome 11. The molecular/

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biochemical basis for this *PAX6* mutation is unknown. A fourth locus for ARS has been associated with abnormalities at chromosome 13q14, however the gene has not been identified.

Functional studies on the molecular basis of ARS by the PITX2 protein are being performed in several laboratories and are discussed in Chapter 4. Studies in the editor's laboratory have shown that PITX2 mutant proteins are either defective for DNA binding, transcriptional activation, phosphorylation or are unstable and degraded in the cell. Other researchers have demonstrated that PITX2 mutations can produce a dominant negative effect or an increase in transcriptional activity. Because PITX2 isoforms have different transcriptional activities and they can dimerize we hypothesize that these activities cause the phenotypic differences observed in ARS patients. Furthermore, because ARS is a haploinsufficiency disorder the heterodimers formed between mutant and normal PITX2 proteins would directly affect gene expression is a dose dependent manner. It is not clear how ARS mutations in PITX2 isoforms other than PITX2A affect the transcriptional properties of this homeodomain transcription factor. Several PITX2 target genes have been identified and the activities of the PITX2 isoforms reveal unique mechanisms for the regulation of gene expression. A molecular basis for organ development by PITX2 isoforms has been suggested through transcriptional analysis.

Drs. Camper and Gage were one of the first groups to clone mouse *Pitx2* and her group has provided a wealth of knowledge on the role of *Pitx2* in pituitary and other organ development. They have shown that *Pitx2* is required for the expansion of the pituitary primordium or Rathke's pouch. Using an allelic series of mice they elegantly demonstrate that pituitary gland development and cell specification is sensitive to *Pitx2* gene dosage. Moreover, their demonstration of *Pitx2* gene dosage effects on pituitary development provides a basis for dosage sensitive effects in other organs of ARS patients. Interestingly, the heterozygous *Pitx2*$^{-/+}$ mice produced in their laboratory present with similar defects seen in ARS patients.

One of the most interesting findings from genetic and epigenetic animal studies of the *Pitx2* gene is the effect on left-right asymmetry. Because congenital heart anomalies are sometimes associated with ARS patients we wanted to bring together the current research on heart development by two outstanding researchers. Dr. Chen has described his studies on the expression and function of *Pitx2* in the chick heart. He discusses the complex genetic pathways involving signaling molecules and transcription factors in the regulation of dextral cardiac looping. His work and others have shown that *Pitx2* is downstream of the Shh/Nodel signaling pathway and functions in establishing the left-right developmental program. Interestingly, heart looping is affected in chick *Pitx*$^{-/-}$ embryos while it is unaffected in *Pitx2*$^{-/-}$ mice. Dr. Martin reviews the role of *Pitx2* in heart development referencing some of the mouse studies performed in his laboratory. He correlates the atrial and septum defects associated with ARS patients and the role of *Pitx2* in heart morphogenesis. His studies on the *Pitx2* isoforms have provided a nice model for gene dosage in regulating heart development and other organs and tissues. He defines specific roles for *Pitx2* in specifying tissues of the heart. Together the work of these two researchers has demonstrated the importance of *Pitx2* in heart morphogenesis.

Because ARS patients present with dental anomalies and *Pitx2* has been shown to be the first transcriptional marker of tooth development my laboratory is studying the molecular/biochemical role of PITX2 in regulating tooth morphogenesis. Drs. Chen, Sharpe, Mucchielli and colleagues and others have provided essential information on the function of *Pitx2* through their animal studies. Their work is reviewed in this chapter along with our own studies on the functional properties of the PITX2 protein. *Dlx2* is a downstream target of PITX2 during tooth development and we discuss the activities of PITX2, Dlx2 and other transcription factors in the tooth epithelium. Little is known how these transcription factors coordinately regulate tooth morphogenesis through activation of target genes. There are several of us (including Dr. Michael Rosenfeld) who are working to establish a model for the action of these factors.

Chapter 9 reviews the current research on the role of *PITX* genes and ocular development. Dr. Semina has performed some initial work demonstrating a critical role for all three *PITX* genes in regulating eye development. Two of these genes, *PITX2* and *PITX3* are shown to be responsible for a spectrum of developmental anterior segment phenotypes associated with glaucoma, corneal opacities and cataracts in humans and mice. All three *PITX* family members are expressed in the anterior segment structures during embryonic development. Dr. Semina is using the ARS *PITX2* mutations as a tool to study the molecular control of eye development.

The authors wish to thank their collaborators and all of the researchers who have provided data for these chapters. Many researchers are working on the development and gene regulation of the organs and tissues discussed in this book. Our goal was to provide a current review of only the genes involved in ARS and associated mutations in the development of affected cells, tissues and organs.

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