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PAX genes in development and disease: the role of *PAX2* in urogenital tract development

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ABSTRACT *PAX* genes play an important role in fetal development. Moreover, heterozygous mutations in several *PAX* genes cause human disease. Here we review the role of *PAX2* in kidney development, focusing on the morphological effects of *PAX2* mutations. We discuss the role of *PAX2* in the context of an inhibitory field model of kidney branching morphogenesis and summarize recent progress in this area.

KEY WORDS: *PAX genes, kidney development, Renal-Coloboma Syndrome*

Introduction

During development many thousands of genes are expressed to control patterning of the developing embryo. In the early phase of development, very rapid cell proliferation and differentiation occurs, but this rapid growth is under the control of a host of developmental genes. Relatively little is yet known about the processes that control patterning and how the precisely regulated number of cells required to form the complete organism is determined. In this review we describe several experimental approaches designed to improve our understanding of these fundamental problems. This review is based on the work of the Developmental Genetics Laboratory, University of Otago, which is focused on the role of *PAX* genes in development and cancer. The reason we have chosen to study the *PAX* family of genes is because individual members of the family control patterning of specific organs, and regulate the decision-making process of cell life and death during development. By understanding the molecular mechanisms of these fundamental processes we hope to gain a better understanding of development and disease, including congenital developmental abnormalities and neoplasia.

PAX Genes

Several gene families have roles in regulating developmental programmes. One such family, called *PAX* genes (Dahl *et al.,* 1997), derives its name from a conserved DNA sequence motif called the paired box, a conserved 128 amino acid domain in the amino-terminal portion of the protein (Treisman *et al.,* 1991). The *PAX* genes are a relatively small family of developmental genes that are grouped into four classes on the basis of their structural similarity, depending on sequence homology, the presence or absence of an octapeptide domain, and either a homeodomain or partial homeodomain (Fig. 1) (Dahl *et al.,* 1997). The paired box and homeodomains encode DNA binding domains within the PAX proteins, so each protein is able to act as a transcription factor regulating the expression of a range of downstream genes (Mansouri *et al.,* 1996). An additional domain in the *PAX* genes is the transactivation domain within the carboxyl terminus of each PAX protein, which is a serine- and threonine-rich domain responsible for transcriptional activation of target genes (Fig. 1) (Ward *et al.,* 1994).

PAX genes have been identified in a wide variety of species, including jellyfish (Sun 1997), the worm *C. elegans* (Chamberlin *et al.,* 1997), insects such as *Drosophila* (Quiring *et al.,* 1994), zebrafish (Krauss *et al.,* 1991), chickens (Nohno *et al.,* 1993), and several species of mammal (Noll 1993). Nine *PAX* genes, *PAX1-PAX9*, have been described in humans and mice (Dahl *et al.,* 1997). The expression of *PAX* genes in many tissues during embryogenesis is associated with critical roles for these genes during development (Dahl *et al.,* 1997). In particular, mutations in four of the *PAX* genes, *PAX2*, *PAX3*, *PAX6* and *PAX8*, have been shown to cause human developmental disorders (Table 1) (Dahl *et al.,* 1997).

In mice, null mutations are associated with developmental abnormalities in all nine *PAX* genes (Table 1). Indeed, mutations in *PAX* genes are often associated with developmental defects in analogous organ systems in disparate species. For example, Gehring and colleagues showed that a *Drosophila PAX* gene,

Abbreviations used in this paper: C. elegans, Caenorhabditis elegans; CNS, central nervous system; GFP, green fluorescent protein; 2-D, two dimensional; Pax, paired box; Wt1, Wilms tumor gene 1.

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termed *eyeless*, which is equivalent to *PAX6* in mice and humans, is involved in eye development (Quiring *et al.,* 1994). Moreover, the same group in 1995 showed *eyeless* functions as a master regulatory control gene in *Drosophila*. When *eyeless* was artificially expressed in *Drosophila* out of the context of the normal eye anlagen, such as in wing or leg tissues, it was able to induce the formation of compound eyes on those structures (Gehring and Ikeo 1999, Halder *et al.,* 1995). These observations suggest that fundamentally important functions for *PAX* genes may have been conserved during evolution.

As mentioned above, the *PAX* genes have been divided into several subgroups based on sequence homology, and the absence or presence of various sub-domains in the proteins (Fig. 1). The *PAX2*, *PAX5* and *PAX8* genes are organized together in group II, as referred to by (Dahl *et al.,* 1997), because their primary sequence is very similar, and each encodes a paired domain, an octapeptide and a partial homeodomain (Ward *et al.,* 1994).

A number of *PAX* genes are also expressed during adulthood, although the distribution of expression is different from that in embryogenesis. For example *PAX5* is expressed during haematopoiesis in adult tissues (Adams *et al.,* 1992). *PAX* genes are also expressed in the adult lens tissue of the eye (Zhang *et al.,* 2001), thymus (Wallin *et al.,* 1996), thyroid (Mansouri *et al.,* 1998), pancreas (Ritz-Laser *et al.,* 2000, Sosa-Pineda *et al.,* 1997, St-Onge *et al.,* 1997), oviduct (Fickenscher *et al.,* 1993), vas deferens, epididymis (Fickenscher *et al.,* 1993, Oefelein *et al.,* 1996), and in myogenic precursors of muscle tissue (Tsukamoto *et al.,* 1994).

PAX2 *in Development and Disease*

The *PAX2* gene is expressed during eye, ear, central nervous system (CNS) and urogenital tract development (Dressler *et al.,* 1990, Eccles *et al.,* 1992, Nornes *et al.,* 1990). A critical role for *PAX2* in eye, ear, mid/hindbrain and kidney development has been established through the analysis of *Pax2* knockout mice (Torres *et al.,* 1995, Torres *et al.,* 1996). Expression of *Pax2* in the Wolffian and Mullerian ducts, as well as the branching ureteric bud and induced nephrogenic mesenchyme is consistent with the observation that *Pax2* plays a role at an early stage in the patterning of the metanephros (Lechner and Dressler 1997). *Pax2* is also expressed in the mesonephros, an earlier transient kidney that is functional for a short time in the fetus. In the developing metanephros, the expression of *Pax2* and the related *Pax* gene, *Pax8*,

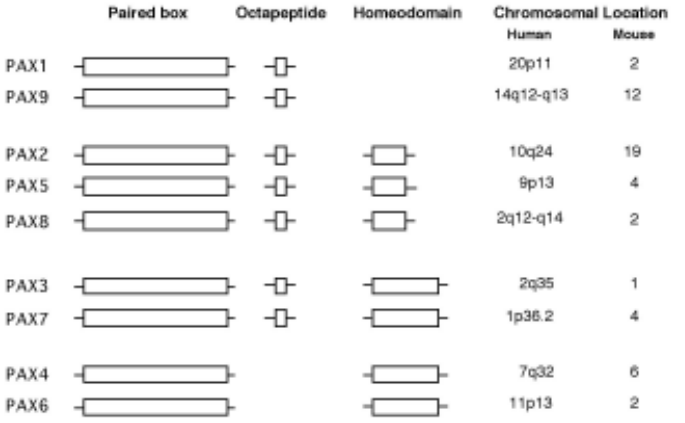

Fig. 1. Schematic representation of human and mouse *PAX* genes. *Shown on the left are the nine human* PAX *genes, which are arranged in groups based on the similarity of the overall structure of the genes. Each of the* PAX *genes in mice has the same structure as the human* PAX *gene. Depicted are the paired box domain contained in each* PAX *gene, as well two other domains. The octapeptide domain (Oct) and the homeobox domain are contained in only some of the* PAX *genes. On the right are shown the chromosomal locations of the human and mouse* PAX *genes. Reproduced with permission from Fig. 1 in Clin. Genet. 56: 1-9 (Eccles and Schimmenti, 1999).*

TABLE 1

ABNORMALITIES IN PATIENTS WITH HETEROZYGOUS *PAX* GENE MUTATIONS, AND MICE WITH HOMOZYGOUS OR HETEROZYGOUS *PAX* GENE MUTATIONS

Gene	Human syndrome	abnormalities	Mouse mutation	Abnormalities
PAX1	-	-	*Undulated* (+/-)	Kinked tail
			Pax1 knockout(-/-)	Skeletal abnormalities in vertebral column and sternum
PAX2	Renal-coloboma syndrome	Optic nerve colobomas or hypoplasia, renal hypoplasia, renal failure, vesicoureteric reflux, sensorineural hearing loss	*Pax2*^{1Neu}, *Krd*, (+/-)	Optic nerve hypolasia, renal hypoplasia
			Pax2 knockout (-/-), *Pax2*^{1Neu} (-/-)	Urogenital tract agenesis, mid/hindbrain deficiency, optic nerve abnormalities, absent chiasma, absent cochlea
PAX3	Waardenburg syndrome	Dystophia canthorum, hypopigmentation sensorineural hearing loss	*Splotch* (+/-)	Hypopigmentation patches, hearing loss
			Splotch (-/-)	Deficient muscle and neuromuscular development, hypopigmentation
PAX4	-	-	*Pax4* knockout (+/-)	No abnormalities
			Pax4 knockout (-/-)	Absence of alpha cells of the pancreas
PAX5	-	-	*Pax5* knockout (+/-)	No abnormalities
			Pax5 knockout (-/-)	Absence of B lymphocyte development
PAX6	Aniridia	Absence of iris, cataracts, Peters' anomaly, foveal hypoplasia, autosomal dominant keratitis	*Small eye* (+/-)	Small eyes
			Small eye (-/-)	Deficient cortical development of brain, absent eyes, absent nasal structures, absent Beta cells of the pancreas
PAX7	-	-	*Pax7* knockout (+/-)	No abnormalities
			Pax7 knockout (-/-)	Absence of myogenic satellite cells
PAX8	Congenital hypothyroidism	Hypothyroidism, thyroid dysgenesis, thyroid hypoplasia	*Pax8* knockout (+/-)	No abnormalities
			Pax8 knockout(-/-)	Absent follicular cells of thyroid
PAX9	Oligodontia	Oligodontia and tooth agenesis	*Pax9* knockout (+/-)	No abnormalities
			Pax9 knockout (-/-)	Absent thymus, parathyroid glands, ultimobranchial bodies, and teeth, supernumary digits, absent hindlimb toe, disturbed craniofacial and visceral skeletogenesis

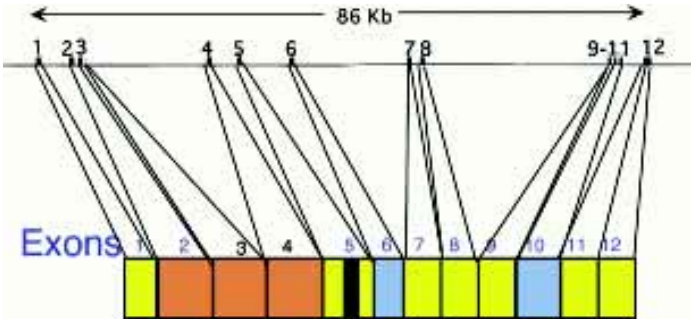

Fig. 2. Genomic organization of the human *PAX2* gene. *The 12 exons are shown, spanning 86 Kb. The cDNA is shown below the line; orange boxes represent the Paired box domain; the alternatively spliced exon 6 and exon 10 are shown as light blue boxes and the octapeptide domain is shown as a black box.*

attenuates in the S-body before the nephron is fully formed (Dressler *et al.,* 1990, Dressler and Douglass 1992, Eccles *et al.,* 1992, Eccles *et al.,* 1995, Plachov *et al.,* 1990). However, in juvenile and adult human kidney, transcription of *PAX2* and *PAX8* is much lower than that in fetal kidney (Dressler and Douglass 1992, Eccles *et al.,* 1992, Poleev *et al.,* 1992).

Genes homologous to *PAX2* have been identified in *Xenopus* (*XPax2*) (Heller and Brandli 1999), zebra-fish (*Pax2.1* and *Pax2.*2, also called *PAX[zf-b]* and *Pax[zf-a],* respectively) (Fjose 1994, Lun and Brand 1998, Majumdar *et al.,* 2000), *C. elegans* (*Egl-38*) (Chamberlin *et al.,* 1997), sea urchin (Czerny *et al.,* 1997), and *Drosophila* (*Sparkling* and *Shaven*) (Fu *et al.,* 1998). In zebrafish, *Pax2* is expressed during development of the excretory structures of the pronephros and mesonephros, as well as in the mid/hindbrain region, and eye. Mutations in zebrafish *Pax2* result in deletion of the mid/hindbrain region (Fjose 1994), as well as kidney abnormalities (Majumdar *et al.,* 2000). *Sparkling* is a mutation in an enhancer of the *Pax2* gene that affects the developing insect compound eye (Fu and Noll 1997), while *Shaven* is a mutation in a separate enhancer that affects mechano-sensory bristle formation (Fu *et al.,* 1998). The *C. elegans* gene, *egl-38*, is involved in development of the male tail and hermaphrodite egg-laying system (Chamberlin *et al.,* 1997).

The *PAX2* gene resides close to the boundary of bands q24 and q25 on human chromosome 10 (10q24-25) (Narahara *et al.,* 1997), and consists of 12 exons as depicted in Fig. 2 (Sanyanusin *et al.,* 1996). The first four exons encode the paired box domain (Sanyanusin *et al.,* 1995b), while the fifth exon contains another highly conserved motif called the octapeptide sequence (Sanyanusin *et al.,* 1996, Sanyanusin *et al.,* 1995b). Three exons in *PAX2* are alternatively spliced in humans and mice; exons 6, 10, and an alternative acceptor splice site located within exon 12 (Dressler *et al.,* 1990, Sanyanusin *et al.,* 1996, Tavassoli *et al.,* 1997, Ward *et al.,* 1994). *Xenopus Pax2* has several more alternatively spliced exons than those identified in mammals (Heller and Brändli 1997), although in *Xenopus*, *Pax2* appears to have functions that are fulfilled by *Pax8* in mammals, and *vice versa* (Heller and Brandli 1999). The exact function of the alternatively spliced exons in *PAX2* is not clear. The carboxy-terminal portion of the PAX2 protein, encoded by exons 7-12, is required for transcriptional activation of target genes by PAX2 protein (Lechner and Dressler 1996), and has strong activating and inhibitory domains (Dorfler and Busslinger 1996, Lechner and Dressler 1996).

The chromosomal location of *PAX2* in humans did not suggest the possible involvement of *PAX2* in a mapped human genetic disease (Eccles *et al.,* 1992). To identify a syndrome caused by *PAX2* mutations we searched for a disease in humans that would look similar to the disease in *Krd* mice (Keller *et al.,* 1994). We identified a *PAX2* mutation in a father and three sons, whose anomalies included optic nerve defects (called optic nerve colobomas), renal anomalies, and vesicoureteric reflux (Fig. 3) (Sanyanusin *et al.,* 1995b). This syndrome is renal-coloboma syndrome, also called papillo-renal syndrome, which is a poorly characterised, recently identified syndrome (Eccles and Schimmenti 1999, Parsa *et al.,* 2001), which has only been reported in 10 families to date. Thirty patients with mutations in various parts of the *PAX2* gene have been identified (Fig. 3). Some of these patients have, in addition to eye and kidney anomalies, high frequency hearing loss and CNS defects (Amiel *et al.,* 2000, Cunliffe *et al.,* 1998, Dureau *et al.,* 2001, Ford *et al.,* 2001, Narahara *et al.,* 1997, Porteous *et al.,* 2000, Sanyanusin *et al.,* 1995a, Schimmenti *et al.,* 1997, Schimmenti *et al.,* 1999). The incidence of renal-coloboma syndrome in the population is unknown but includes as part of the spectrum, primary renal hypoplasia, which is a relatively frequent congenital abnormality. Primary renal hypoplasia, a sub-type of renal hypoplasia, called oligomeganephronia, and renal-coloboma syndrome, have been shown to be caused by *PAX2* mutations (Nishimoto *et al.,* 2001, Salomon *et al.,* 2001). These conditions are associated with potentially life-threatening illness due to the possibility of progression to end-stage renal failure.

Mouse Pax2 *Mutants*

There are many similarities between renal-coloboma syndrome in humans and the phenotype in heterozygous *Pax2* mutant mice. In humans a frequent mutational site was identified in exon 2 (Eccles 1998, Eccles and Schimmenti 1999, Sanyanusin *et al.,* 1995a). An identical mutation has been identified in the mouse *Pax2* gene (Favor *et al.,* 1996). In heterozygous mutant animals the disease is very similar to renal-coloboma syndrome in humans, and so may be an exact animal model in which to study the effects of the *Pax2* mutation in urinary tract development. *Pax2* null mutant mice lack any functional Pax2 protein, and die perinatally, lacking kidneys, ureters, oviducts, vas deferens, and epididymis, and have anomalies of the cochlea as well as defects of the optic nerves and CNS (Favor *et al.,* 1996, Torres *et al.,* 1995, Torres *et al.,* 1996).

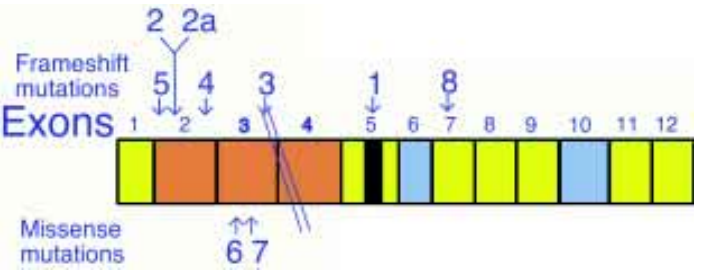

Fig. 3. Mutations in the human *PAX2* gene. *Shown are eight mutations in the various exons of* PAX2. *The exons are the same as in Fig. 2. Reproduced with permission from Fig. 2 in Clin. Genet. 56: 1-9 (Eccles and Schimmenti, 1999).*

Major Questions in the Field

We are presently interested in several questions about *PAX* genes and their role in development and disease:
- What is the molecular basis of haploinsufficiency in *PAX* mutants?
- How does increased or decreased (mutated) *PAX* gene expression cause disease, and what is the function of PAX proteins?
- What pathways and genes do PAX proteins regulate?
- How do PAX proteins fit into the transcriptional control networks in cells?

The following sections outline several experimental approaches that we are using to address the above questions.

What is the Molecular Basis of Haploinsufficiency?

In humans, heterozygous mutations in *PAX2*, *PAX3* and *PAX6* cause developmental abnormalities associated with specific syndromes (Dahl *et al.,* 1997). Heterozygous *PAX2* mutations cause renal-coloboma syndrome, as mentioned above. Heterozygous *PAX3* mutations in humans cause Waardenburg syndrome, an autosomal dominant syndrome characterized by pigmentary disturbances and sensorineural hearing loss (Tassabehji *et al.,* 1992). Heterozygous mutations in the *PAX6* gene cause aniridia, a condition characterised by severe hypoplasia of the iris, accompanied by cataracts and hypoplasia of the ciliary body and retina (Glaser *et al.,* 1992, Jordan *et al.,* 1992). Homologous genetic loci in mice are also associated with naturally occurring *PAX* gene mutations *Pax2*^{1Neu} (*Pax2*) (Favor *et al.,* 1996), *Splotch* (*Pax3*) (Epstein *et al.,* 1991), and *Small eye* (*Pax6*) mutations (Hill *et al.,* 1991). These mice have characteristic abnormalities of the urogenital tract, CNS, coat colouration, nasal structures, eyes, or ears. In mice, as in humans, heterozygous mutations in *Pax2*, *Pax3* and *Pax6* cause developmental abnormalities. Little is known about dosage sensitivity with regard to *PAX* genes.

Dosage sensitivity associated with the *PAX* genes is of clinical relevance, as syndromes caused by *PAX2*, *PAX3* and *PAX6* mutations are inherited in an autosomal dominant fashion (Glaser *et al.,* 1992, Jordan *et al.,* 1992, Sanyanusin *et al.,* 1995b, Tassabehji *et al.,* 1992). However, these syndromes are not truly dominant, and it is better to think of them as semi-dominant. This is because homozygous mutant individuals are more severely affected (often embryonic lethal), than heterozygous mutant individuals (Epstein *et al.,* 1991, Favor *et al.,* 1996, Hill *et al.,* 1991). Phenotypically, this phenomenon is referred to as haploinsufficiency. As with mice, homozygous mutations in *PAX3* or *PAX6* in humans cause more severe phenotypes. Marriages between people who each carry a heterozygous mutation for the same *PAX* gene may result in severely affected offspring (Glaser *et al.,* 1994, Zlotogora *et al.,* 1995).

Haploinsufficiency is consistent with the notion that the level of PAX protein required for the full development of certain tissues is important. Another explanation is that there is stochastic allelic inactivation of *PAX* gene expression in certain tissues. This has been observed for the *PAX5* gene, but not for *PAX2* (Nutt *et al.,* 1999, Porteous *et al.,* 2000).

Analysis of PAX2 Protein in Kidney and Oviduct Tissues in PAX2 *Mutant Mice*

The expression of PAX2 protein is detectable by immunohistochemistry in specific cells of the neural tube. Later PAX2 is expressed in the spinal cord, mid/hindbrain, ear, eye, and urogenital system, which includes the Wolffian duct, ureteric bud, collecting duct epithelia, early nephrons, uterus, oviducts, vas deferens, and epididymis. Our recent studies indicate a strong relationship between kidney development and PAX2 expression (Porteous *et al.,* 2000). Therefore, we are interested to determine whether gene dosage, in normal and *PAX2* mutant individuals, is mirrored by changes in the level of PAX2 protein expression. Relatively little is known about the PAX2 protein. For instance, is the PAX2 protein post-translationally modified by phosphorylation, and is it of equivalent size and sequence in different tissues where PAX2 is expressed? In human and mouse fetal kidney the PAX2 protein is 48-50 kDa in size (Dressler and Douglass 1992), but it is not known if PAX2 protein is of a similar size in other tissues.

It is not known whether the level of PAX2 protein is precisely regulated in some tissues and not in others? Recent studies suggest that a decreased PAX2 protein level as a result of *PAX2* mutation could cause loss or paucity in ureteric bud growth with an excessive amount of apoptosis, especially during a critical window in kidney development (Ostrom *et al.,* 2000, Porteous *et al.,* 2000, Torban *et al.,* 2000). Clinically, renal-coloboma syndrome in humans is associated with small kidneys containing fewer nephrons, and predisposition to renal failure. Heterozygous *Pax2*^{1Neu} mutant mice show a similar phenotypic spectrum to humans with renal-coloboma syndrome, including defects in kidney and eye tissue. Interestingly, abnormal development occurs in some tissues (the ureteric buds and collecting ducts, optic nerve, and cochlea) in heterozygous *Pax2* mutants, whereas in other tissues (uterus, oviduct, vas deferens and epididymis) developmental abnormalities occur only in the presence of a homozygous *Pax2* mutation. In heterozygous mutant mice the uterus, oviduct, vas deferens and epididymis are unaffected (Table 1).

One possibility is that the PAX2 protein plays different roles during development in different tissues (Table 1). During embryogenesis the kidney develops from the mutual inductive interaction between the metanephric blastema and the ureteric bud, which gives rise to the nephrons, stroma, and collecting system of the kidney. The Wolffian and Mullerian ducts precede development of these structures, and also precede development of the male and female genital tracts. These two ducts are adjacent to each other during embryogenesis, and exhibit strong PAX2 protein expression. We hypothesize that differences in the sensitivity of the oviduct to *PAX2* dosage, as compared to kidney tissue, may be due to differences in the PAX2 protein expression in the tissues. Such differences in protein expression may include alternative splicing, post-translational modifications, or differences in sensitivity of the tissue to PAX2 protein level.

To explore whether PAX2 protein is differently expressed in the kidney as compared to oviduct, we have analyzed the protein complement from these tissues by 2-D polyacrylamide protein gel electrophoresis (PAGE). This method resolves more than 1000 proteins and is sensitive to both single changes in single amino acids in proteins, as well as the more subtle changes in charge caused by changes in post-translational modification by glycosylation and phosphorylation. In oviduct and kidney tissues from adult wild type and *Pax2* mutant mice (*Krd* and *Pax2*^{1Neu} mutants), the protein maps from 2-D gels were grossly equivalent. To further resolve Pax2 protein expression using a proteomics approach we are currently using enhanced direct protein staining techniques such as silver staning as well as immunolocalisation

of Pax2 protein with anti-Pax2 antibody. Ultimately we will undertake comparative analysis of Pax2 protein expression using micro-sequencing techniques and mass spectrometry.

How do *Pax* Gene Mutations cause their Respective Syndromes?

As discussed above, mutations in several members of the *PAX* gene family result in developmental abnormalities. In contrast, mutations in highly homologous *PAX* genes, expressed in the same cell types, do not necessarily cause developmental abnormalities. For example *Pax3* and *Pax7*, which are highly homologous, are co-expressed in developing somites, myogenic precursor cells, and neural crest-derived cells. Heterozygous *Pax3* mutations affect neural crest cell migration, and homozygous mutations affect development of the somites, yet *Pax7* mutations do not (Borycki *et al.,* 1999, Epstein *et al.,* 1991). What is the basis of this difference?

Another relevant example is that of *PAX2* and *PAX8*, which encode almost identical proteins (Ward *et al.,* 1994). These proteins are co-expressed in several of the same cell types in the developing kidney (Eccles *et al.,* 1995). *PAX2* is required for kidney development, while *PAX8* is not (Mansouri *et al.,* 1998). Why does one gene result in disease when it is mutated and not the other? There is also a difference between the *Pax2* and *Pax8* genes in their dosage sensitivity, as *Pax8* is expressed in the developing thyroid gland, and homozygous, but not heterozygous, mutations in murine *Pax8* cause thyroid gland abnormalities (Mansouri *et al.,* 1998). Kidneys are normal in homozygous *Pax8* mutants, yet heterozygous *Pax2* mutations cause kidney abnormalities.

Exactly how *PAX2* is involved in development of the urogenital tract is unknown. What causes the kidneys of patients with *PAX2* mutations to be smaller than normal, and why are these patients predisposed to renal failure? Recently we found that apoptosis in the collecting ducts of *PAX2* mutants is greatly increased (Porteous *et al.,* 2000, Torban *et al.,* 2000). However, the precise events orchestrated by *PAX2* during fetal growth of the kidneys are not well characterised.

The Role of PAX2 in Ureteric Bud Branching and Development of the Renal Collecting Duct System

Analysis of Kidney Development in *PAX2* Mutant GFP Transgenic Mice. Since the discovery of the cause-effect relationship between mutations in *PAX2* and renal-coloboma syndrome, we have pursued research to further characterize the functional role of *PAX2* and to understand this developmental syndrome. To date, the majority of this research has been in the form of retrospective studies and the data that has been generated has mostly been 2-dimensional, while the process of development itself is 3-dimensional.

Our current research aims to better understand the role of *PAX2* in the development of the collecting duct system. We are breeding and crossing several strains of mice, including *Pax2*^{1Neu} mice and Hoxb7/GFP transgenic mice to study branching morphogenesis in the collecting duct system. The latter transgenic mice have been shown to express green fluorescent protein (GFP) during development under the control of the Hoxb7 promoter (Srinivas *et al.,* 1999). Of significance is the GFP expression in the urogenital system, where GFP has been localized in the epithelia of the Wolffian duct, the ureteric bud, and the collecting ducts. This fluorescence can be captured using confocal microscopy, following excitation of the chromophore with a 488 nm laser. This technique allows serial sections to be taken through the fluorescent tissue, and reconstructed into a 3-dimensional image.

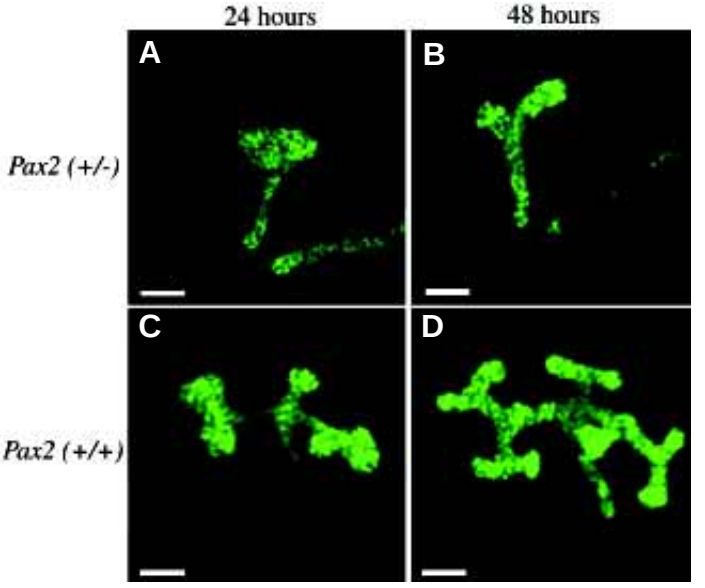

Fig. 4. Green Fluorescent Protein expression in *Pax2*^{1Neu} mutant kidneys. (A,B) *Expression of Green Fluorescent Protein in HoxB7-GFP/* Pax2^{1Neu} *mutant (*Pax2*+/-) kidneys.* **(C,D)** *Expression of Green Fluorescent Protein in HoxB7-GFP/wild type (*Pax2*+/+) kidneys. The kidneys in A and C were cultured for 24 hrs, while the kidneys in B and D were cultured for 48 hrs. Fewer ureteric buds and less development is seen in the* Pax2^{1Neu} *mutant kidneys. The bar represents 200 μM.*

We have characterized the development of the metanephric kidney in wildtype and *Pax2* mutant mice at different stages of embryological development: E12, E14, E16 and E18. Between E14 and E18 we are using kidney explant organ culture to compare metanephric development between littermates. Using confocal microscopy, it is possible to take time-lapsed images of these cultures, and to analyze metanephric development in wildtype kidneys compared to *Pax2* mutant kidneys (Fig. 4). The key question that we are addressing by this procedure is how *Pax2*^{1Neu} mutant fetal mice and wild type littermates differ in the induction of the metanephric mesenchyme by the ureteric bud. A related question to this is how do these mice differ in the relative rates of proliferation of cells in the collecting ducts, and of apoptosis during development?

Analysis of Kidney Development in *PAX2* Mutant Aggregation Chimaera Mice. To investigate the role of *Pax2* in kidney development we have generated aggregation chimeras using *Pax2*^{1Neu} and *Krd* compound heterozygous mutant embryos. The Krd mouse, has a chromosomal deletion of the *Pax2* gene and 400cM of flanking DNA (Keller *et al.,* 1994). Unlike *Pax2*^{1Neu} mice, homozygous *Krd* mutants are preimplantation lethal (Keller *et al.,* 1994). However, heterozygotes of both strains of mice provide good animal models of renal-coloboma syndrome.

The double-mutant embryos are fused to wild type embryos at the 8-cell stage (Fig. 5A). The fused embryos are then cultured to

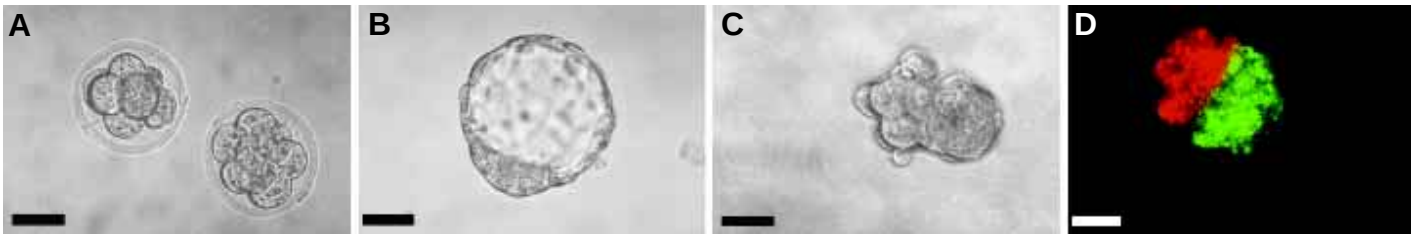

Fig. 5. Aggregation chimaeras of *Pax2*^{1Neu}/*Krd* double mutant embryos fused with wild type embryos. (A) *Eight cell stage embryos.* **(B,C)** *Blastocysts comprised of a fusion of* Pax2^{1Neu}/Krd *double mutant embryos. One of the embryos in C is developing more slowly, leading to a deformity in the blastocyst in C.* **(D)** *Morula prior to transfer, in which the two embryos have been labeled with red and green dyes. Some mixing of the cells is already evident. The bar represents 60 μM.*

blastocysts (see Fig. 5B) before being transferred to pseudopregant hosts. The chimeras formed by the fusion of the two embryos therefore represent an aggregation of cells of two different genotypes; *Pax2*^{1Neu}/*Krd* double mutants and wildtype (Fig. 5C). The embryos are then harvested at various times after implantation and the contribution of wild type and mutant cells in kidney development is analyzed.

The generation of aggregation (fusion) chimaera *Pax2* mutant mice in this way allows us to determine the mode by which the kidneys fail to develop in homozygous *Pax2* mutants. Although *Pax2* is clearly a critically required developmental gene, the role of *Pax2* in initiating kidney development is difficult to determine because of failed organogenesis and perinatal lethality. Aggregation chimaera mice have mosaicism in all tissues. The percentage of mosaicism for *Pax2* double-mutant cells and wild-type normal cells varies from mouse to mouse, but is relatively constant throughout different tissues.

By making aggregation chimaeras we can determine whether *Pax2* specifies an early survival function that is required for subsequent kidney development, or whether the missing signal in homozygous *Pax2* mutant mice is able to be complemented by the presence of wild type cells. If *Pax2* is required for the autonomous survival of kidney epithelial cells, then *Pax2* mutant cells would make up a very low percentage of the total complement of cells in the chimaeric kidneys because the mutant cells would die. The kidney would of course be mosaic for mutant and wild type cells in the chimaeric mice, but there would be a much lower percentage of mutant cells in the kidneys than in the other tissues of the fetus which do not depend on *Pax2* expression for cell survival. On the other hand, if *Pax2* induces a signal(s) during kidney development, and if wild type cells can complement this signal in the chimaeras, then *Pax2* mutant and wild type cells would together make up an equal percentage of cells. Wild type and mutant cells would then contribute equally to the mosaicism of the chimaeric fetal kidneys, and the percentage mosaicism in the kidney would then not be significantly different than in the other tissues of the chimaeric fetal mice.

Conceivably the kidney phenotype of the homozygous mutants might be completely rescued by the presence of wild type cells, and that the percentage of normal cells in the kidneys of the chimaeras would be enough to support normal growth. In addition, we might find that there is a critical time period for the expression of *Pax2*, after which kidney development is not able to proceed normally.

How are PAX *Genes involved in Cell Survival and Renal Failure?*

Is *PAX2* required for kidney cell survival, or is apoptosis simply a default mechanism to eliminate cells that develop abnormally as a result of the *PAX2* mutation? We have addressed this question using mIMCD-3 cells. These cells are murine adult kidney collecting duct cells that constitutively express high levels of *Pax2*. Inhibition of *Pax2* expression in mIMCD-3 cells using an antisense *PAX2* expression construct causes reduced cell survival. Conversely, enhanced expression of *PAX2* in HEK293 human kidney epithelial cells, that do not express *PAX2*, leads to enhanced cell survival (Torban *et al.,* 2000). We interpret these data to mean that *PAX2* is involved in cell survival in mIMCD-3 cells. The mechanism of survival conferred by *PAX2* is unknown, but may involve gene regulation.

Identification of PAX2 *Modulated Genes using Microarray and Real Time PCR*

In previous studies we showed that three promoter-luciferase expression constructs, N-*myc*, *p53*, and Wilms tumour 1 (*WT1*) were regulated by *PAX2* in transient co-transfection assays (McConnell *et al.,* 1997). We also identified important regions of the *WT1* promoter that were regulated by the PAX2 protein. In addition, others have shown that the *p53* and *WT1* promoters are regulated by PAX2 (Stuart *et al.,* 1995; Dehbi *et al.,* 1996).

To confirm the above findings, and to identify further downstream targets of PAX2, we have undertaken cDNA micro-array experiments using several different experimental systems. Our approach has been to hybridize human or mouse cDNA microarrays, containing 6,347 cDNAs and ESTs (purchased from Affymetrix) with probes generated from RNA isolated from human or mouse fetal kidney cells, respectively. HEK293 human fetal kidney cells were stably transfected with a tetracycline regulated *PAX2* expression construct. This construct enabled expression of *PAX2* in the absence of tetracycline, and repression of *PAX2* expression when tetracycline was present. HEK293 cells do not normally express *PAX2*. RNA populations were isolated before and after tetracycline treatment and used to make probes to hybridize to human cDNA microarrays. The results of this experiment are in progress.

In a different microarray experiment we have isolated RNA from embryonic day 16 (E16) *Pax2*^{1Neu}/+ heterozygous mutant and wild type kidneys, and generated probes for microarray hybridization. One cDNA microarray was hybridized with the probe made from the *Pax2*^{1Neu} +/- mutant fetal kidney RNA, while another cDNA microarray was hybridized with the probe made from wild type fetal kidney RNA. *Pax2* gene dosage was halved in these experiments by the heterozygous *Pax2* mutation, and therefore not all genes regulated by Pax2 would be likely to be affected. However, because the mutant phenotype shows decreased kidney size and fewer poorly developed nephrons, we expected that at least some

changes in gene expression would be observed, and that some of these changes would be related to the *Pax2*^{1Neu} mutant phenotype. To maximize the chances of observing changes in gene expression, kidneys were sorted into small mutant, large mutant, and wild type kidneys by weight and genotype. Kidneys were isolated from fetal mice at E16 and E19.

Genes showing differences of 2-fold or more were identified and the changes in gene expression were validated on the same RNA samples using quantitative Real-Time PCR. The level of expression of each gene was normalized to a simultaneously amplified house-keeping gene, Beta 2 Microglobulin (B2M). Interestingly, several genes in the insulin-like growth factor signaling pathway were changed in expression in the mutant kidneys. There is already some evidence that insulin-like growth factors influence branching morphogenesis and cell survival in the kidney (Feld and Hirschberg 1996, Werner *et al.,* 1994).

Transcription Factor Networks during Kidney Development: Wt1 and Pax2

The interactions between genes that control growth and differentiation of the embryo, and that may act to limit proliferation by specifying terminal differentiation, are fundamental to understanding the molecular mechanisms of development and oncogenesis. A variety of transcription factors have been identified that are required for development of the kidney (Table 2). However, little is known about the interactions between developmentally regulated transcription factors and the networks of gene regulation that exist to control kidney development. One transcription factor of great interest in urogenital tract development is WT1, encoded by the Wilms tumour suppressor gene locus (Call *et al.,* 1990).

WT1 harbours mutations and deletions in approximately 10-15% of Wilms tumours (Call *et al.,* 1990, Huff *et al.,* 1991, Pelletier *et al.,* 1991). *WT1* is expressed in the nephrogenic mesenchyme during normal kidney development in a developmentally regulated pattern, suggesting that it is involved in kidney development (Pritchard-Jones *et al.,* 1990). *WT1* null mutant mice fail to develop kidneys (Kreidberg *et al.,* 1993). *WT1* also plays an important role in male sexual differentiation and in the control of key factors involved in androgen synthesis and metabolism (Kim *et al.,* 1999, Shimamura *et al.,* 1997).

The WT1 protein contains four zinc finger domains, and has greatest similarity to the zinc finger transcription factor, EGR-1 (Rauscher *et al.,* 1990). As the *WT1* gene has a major role in the developing urinary tract, it is clearly of significance to determine what regulates this gene, and whether these factors may also be important in the development of the urinary tract.

The *Pax2* gene is normally expressed in the mesonephric duct, the ureteric bud, the condensing metanephric mesenchyme and in early epithelial stuctures derived from the mesenchyme, and is quickly down regulated as the tubular epithelium matures (Dressler *et al.,* 1990, Eccles *et al.,* 1992). As the down-regulation of *Pax2* in these structures proceeds, WT1 protein expression increases (Fig. 6). Normally in the induced mesenchyme, PAX2 and low levels of WT1 are co-expressed at early stages of renal development (Eccles *et al.,* 1995). WT1 is also expressed in the differentiating epithelium, with maximum levels in the podocyte cells of the glomerular epithelium (Grubb *et al.,* 1994). Data from co-transfection studies suggest that WT1 is capable of transcriptionally repressing the *PAX2* promoter and could be responsible for the attenuation of *PAX2* expression during kidney morphogenesis (Ryan *et al.,* 1995, Stayner *et al.,* 1998). On the other hand, *PAX2* is able to transcriptionally activate the *WT1* promoter (Dehbi *et al.,* 1996, McConnell *et al.,* 1997). Therefore, the possibility exists that there is cross-transcriptional control between these two genes, such that PAX2 initially modulates the transcriptional activity of *WT1*, and at a later stage when a threshold level of WT1 protein is reached the WT1 protein level in cells may then repress *PAX2* transcription (Dressler 1995).

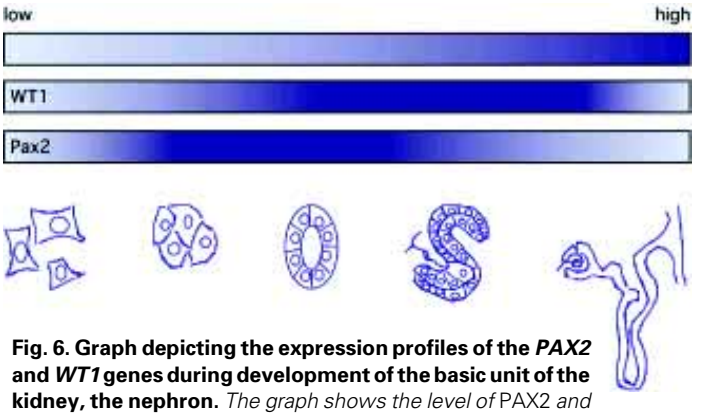

Fig. 6. Graph depicting the expression profiles of the *PAX2* and *WT1* genes during development of the basic unit of the kidney, the nephron. *The graph shows the level of* PAX2 *and* WT1 *expression as a variable strength band at various stages of kidney development (depicted below the bands). Darker coloring represents stronger expression. Along the horizontal axis are various structures that are observed in fetal kidneys; from left to right; diffuse mesenchyme, condensed mesenchyme, comma-body, S-body, mature nephron. WT1 expression continues in mature nephrons after birth.*

In the mouse, complete loss of *WT1* function results in multiple developmental abnormalities including renal agenesis (Kreidberg *et al.,* 1993). There is no expression of *Pax2* in the mutant metanephric blastema of *WT1*-/- mice, but *Pax2* expression is observed in the Wolffian duct and in the mesonephros of *WT1*-/- mice (Kreidberg *et al.,* 1993). In the epithelial components of human Wilms' tumors, *PAX2* expression persists (Dressler and Douglass 1992, Eccles *et al.,* 1992), suggesting that negative regulators of *PAX2* are inactive.

Identifying the molecular mechanism of *PAX2* down-regulation during normal nephrogenesis and understanding how these mechanisms are lost during the initiation and progression of renal tumors, as well as how these genes function, may illuminate the genetic basis of renal oncogenesis. It would also be of interest to identify auxiliary proteins that interact with PAX2 protein and that would therefore have an important role in urinary tract development by being part of a PAX2 transcription factor complex. WT1

TABLE 2

TRANSCRIPTION FACTOR PROTEINS THAT ARE EXPRESSED DURING URINARY TRACT DEVELOPMENT

Transcription factor(s)	Type of protein
Hox A.3 - Hox A.6, Hox B.4, Hox B.5, Hox B.7, Hox C.6, Hox C.8, Hox C.9, Mox-1, LFB1, LFB3, HNF-1	Homeobox domain
PAX2, PAX8	PAX (paired box domain)
KDN-1, HNF-1,	Pou domain
Evi-1, Kid-1, WT1, SP1, Kox-1, ZNF141, KRK-1	Zinc finger domain
c-myc, L-myc, N-myc, MFH-1, Id (limb deformity)	basic helix-loop-helix domain
p53, E2F	–

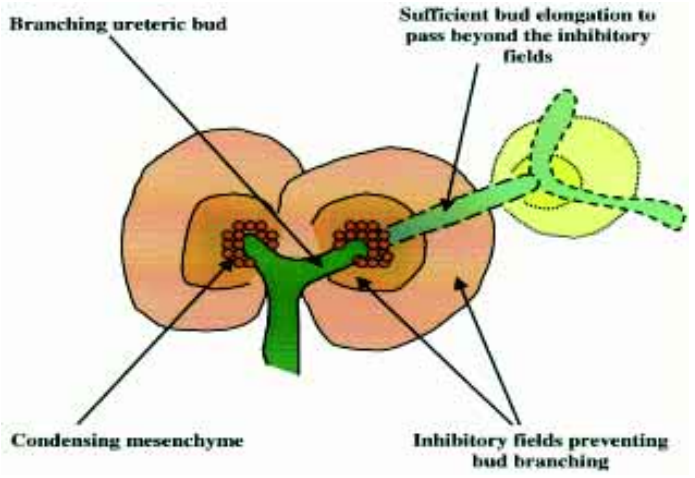

Fig. 7. The Inhibitory Field Model. *The ureteric bud (green) grows progressively until it has reached a critical threshold (inhibitory field), beyond which there is no further inhibition of ureteric bud branching.*

is thought to regulate a number of other genes such as the insulin-like growth factor II gene *(IGFII),* platelet derived growth factor receptor *(PDGFR),* and epidermal growth factor receptor *(EGFR)* among others (Little *et al.,* 1999).

An Inhibitory Field Model of Kidney Branching Morphogenesis

To explain why apoptosis might reduce branching morphogenesis, and consequently affect the ultimate size of the mature kidney, we have developed an inhibitory field model, which links the control of cell survival to branching morphogenesis (Torban *et al.,* 2000). We postulate that the growing uretric bud must escape an inhibitory field established by condensing mesenchyme before the next generation of nephrons is produced (Fig. 7). Excessive apoptosis deletes cells from the growing ureteric bud, delaying the escape of the ureteric bud from the field (Fig. 8). *PAX2* may function at the interface of proliferation and differentiation, decoding the signals destined for proliferation, differentiation, and cell survival, while at the same time promoting interactions between cells required for tissue patterning. The 'Inhibitory Field' model attempts to link the consequences of increased apoptosis in the collecting duct, and reduced branching morphogenesis.

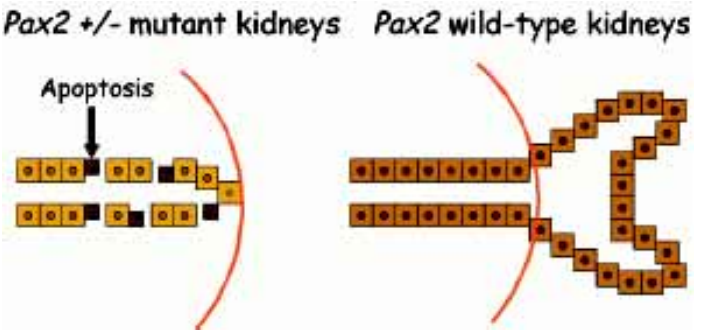

Fig. 8. Apoptosis Model. *The growth of the ureteric bud in* $Pax2^{1Neu}/+$ *mutant mice is impeded by the high rate of apoptosis (shown). Growth of the ureteric bud beyond the inhibitory field (red line) is prevented by apoptosis.*

Summary

As we begin to understand the role of *PAX2* and pathways in kidney development, we may learn ways to manipulate the growth of kidneys during embryogenesis, and perhaps even prevent disabling or dysmorphic abnormalities that occur in individuals who carry mutant genes that affect kidney growth and neoplasia. A future focus of the Developmental Genetics Laboratory will be to investigate these possibilities.

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