Kidney Development and Disease in the Zebrafish

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Unraveling the molecular pathogenesis of human disease presents many experimental challenges, not the least of which is that experiments on humans are generally frowned upon. Model organisms, including the zebrafish, allow for experimental analysis of gene function and the detailed characterization of disease processes. Zebrafish have matured as a vertebrate model organism now that genetic tools for targeted “knockdowns” and unbiased mutagenesis approaches are in hand. The fish larval pronephros is a relevant kidney in which to pursue many aspects of human kidney development and disease. This short review outlines recent progress in applying the zebrafish pronephros to issues of human health and development.

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tudies of kidney function in lower vertebrates have had a significant impact on our understanding of basic kidney physiology. The first demonstration of tubular epithelial secretion was performed by Marshall (1) in the agglomerular kidneys of the goose fish and toadfish. Our current understanding of fluid secretion mechanisms were originally worked out using the shark rectal gland (2). Renal micropuncture experiments that first revealed the composition of the glomerular filtrate were performed in the frog (3). Smith (4) studied the kidney in many species of fish, taking advantage of their broad range of habitats to explore important aspects of renal physiology as well as vertebrate evolution. Exploring kidney development and disease in the fish is a natural extension of these early studies. Studies of kidney development and disease aim to uncover the origins of complexity in the kidney and molecular pathogenesis underlying tissue dysfunction (5): How are specialized cells and structures of the kidney derived from undifferentiated embryonic tissues? Can developmental processes be recapitulated in the context of organ injury and repair? How can organogenesis in the fish be exploited to learn about human disease? This short review illustrates how genetic analysis of zebrafish pronephric development is helping to answer these questions.

Zebrafish as a System for Functional Genomics

The maturation of the zebrafish as a model of vertebrate organogenesis owes to (1) its relative simplicity in terms of organ size and cell number, (2) the feasibility of applying both forward and reverse genetic tools to define gene function, and (3) its accessibility to observation. Zebrafish embryos develop in freshwater outside the mother and are transparent; observation and manipulation of organ development are relatively straightforward (6). Development occurs rapidly, with embryos progressing from fertilized egg to free-swimming larvae in 2.5 d; all stages of organ development can be observed in this short time window. Their high fecundity, their relatively short generation time, and the ability to observe internal organ development have made large-scale, unbiased mutagenesis screens possible in a vertebrate (7). Hundreds of mutations that affect individual organ systems have been isolated, with various defects including cardiac arrhythmias (8), gut and hepatic degeneration (9), failed blood cell development (10), and cystic disease of the kidney (11). Sequencing the zebrafish genome is predicted to be complete in 2005 (12), and the existing sequence data are already useful for finding gene homologs in zebrafish using bioinformatic methods. Full-length cDNA projects are also under way, and microarrays that represent approximately 15,000 genes are available for screening. Gene targeting with antisense morpholino oligos is highly efficient for gene “knockdowns” and is effective for at least the first 50 h of development, well within the time period of organogenesis (13). With these methods for defining gene function coupled with the feasibility for high-throughput screening of bath-applied small molecules and drugs for effects on development and disease phenotypes, the zebrafish is a viable system for modeling many human diseases and offers an in vivo assay system for drug development (14).

Structure and Function of the Zebrafish Pronephros

In zebrafish and other teleosts, the functional larval pronephros consists of only two nephrons with glomeruli fused at the embryo midline just ventral to the dorsal aorta (Figure 1) (11,15–17). Although simple in form, the pronephric glomerulus is composed of cell types that are typical of higher vertebrate kidneys, including fenestrated capillary endothelial cells, podocytes, and polarized tubular epithelial cells (Figure 1, B and C) (11). Two pronephric tubules connect the glomerulus to the pronephric ducts, which run caudally and fuse just before their contact with the outside world at the cloaca. The zebrafish pronephric nephrons form a closed system of blood filtration,
tubular resorption, and fluid excretion. The primary function of the fish pronephros is for osmoregulation. Without a functional kidney, larvae die of gross edema because they have salty blood and yet live in a very dilute environment.

The pronephric kidney forms in a stepwise progression that, in general terms, follows the sequence observed in mammalian kidney development (18). First, the pronephric duct, the future collecting system, is formed by the conversion of loose fibroblastic cells into a polarized epithelial tube. The nephrons then form and connect to the pronephric duct at its anterior tips, and, finally, blood vessels sprout and invade the podocytes to form the glomerulus. This process occurs only once in the zebrafish embryo during the first 2 d of development. Thus, all of the relevant cellular interactions and the genes that control kidney development can be studied over a relatively short period in a simple and accessible kidney.

**Zebrafish Pronephros as a Model of Human Disease**

**Defects in Glomerulus Formation and Nephrotic Syndromes**

Proper functioning of the kidney requires a structural integration of glomerular podocytes and blood vessels. In zebrafish, evidence that podocytes act to organize vessel in-growth can be seen in (1) the expression patterns of genes that are known to play an important role in angiogenesis and (2) the recruitment of endothelial cells to clusters of podocytes in mutant embryos that lack the dorsal aorta, the normal blood supply for the pronephric glomerulus. Zebrafish pronephric podocytes express two known mediators of angiogenesis: Vascular endothelial growth factor (VEGF) and angiopoietin 2 (19,20). In a complementary manner, capillary-forming endothelial cells express flk1, a VEGF receptor and an early marker of the endothelial differentiation program (21). In normal zebrafish embryos at 40 hpf, flk-1-positive endothelial cells invade the glomerular epithelium and form the capillary loop. In *floating head* mutant embryos, the dorsal aorta is absent (22), and so the nascent glomeruli are deprived of their normal source of vasculature. Nonetheless, podocytes continue to express *wt1* and *vegf* and seem to recruit flk-1-positive endothelial cells from nearby veins and go on to form a reasonably functional glomerulus (19). These results support the idea that podocytes, by expressing *vegf*, play a primary role in attracting and assembling the glomerular capillary tuft.

Surprisingly, zebrafish mutants that lack blood flow as a result of defects in cardiac function (23) fail to form a proper glomerular capillary tuft (Figure 2, A and B). This suggests that vascular shear force *per se* is required to drive capillary formation (24). Although vascular cells seem normal, they fail to express matrix metalloproteinase-2. Inhibition of matrix metalloproteinase activity by tissue inhibitor of metalloproteinase-1 injections results in a similar failure to form the glomerulus (24), indicating that degradation and remodeling of the glomerular basement membrane is a key step in capillary tuft formation.

Filtration of blood by the pronephric glomerulus can be detected by injections of fluorescence compounds into the general circulation and then monitoring the appearance of fluorescent endosomes in the apical cytoplasm of pronephric duct cells (Figure 2C) (11,19). From these data, it can be inferred that the fluorescence tracer has passed the glomerular basement membrane and entered the lumen of the pronephric tubules and ducts, where it is actively endocytosed. Using this assay, we have established that blood filtration by the zebrafish pronephros begins at approximately 40 hpf (11).

A major feature of the mammalian glomerular blood filter is the podocyte slit diaphragm, a specialized adherens junction that forms between the podocyte foot processes (25). Failure of the slit diaphragm to form results in proteinuria or leakage of high molecular weight proteins into the filtrate. Proteinuria is the cardinal feature of several human congenital nephropathies and also a common complication of diabetes (26). Several disease genes that are known to function in the slit diaphragm have been cloned. Nephrin is a transmembrane protein present in the slit diaphragm and is thought to contribute to the zipper-like extracellular structure between foot processes (27). Podocin is a podocyte junction-associated protein (28) that resembles stomatin proteins, which play a role in regulating mechanosensitive ion channels (29). Electron microscopy of the zebrafish pronephric glomerulus reveals that like mammalian
podocytes, zebrafish podocytes form slit diaphragms between their foot processes (Figure 2D). Zebrafish homologs of podocin and nephrin are specifically expressed in podocyte precursor cells as early as 24 hpf (Figure 2, E and F). These functional similarities between mammalian and zebrafish podocytes, coupled with assays for glomerular remodeling and morphogenesis. (C) Measuring renal clearance: Rhodamine-dextran filtration and uptake by pronephric epithelial cells. 10KD lysine-fixable rhodamine dextran injected into the general circulation can be seen as red fluorescence in glomerular capillaries (gl), and filtered dye is seen in apical endosomes of pronephric duct cells (arrowheads). Counterstain: FITC wheat germ agglutinin. (D) Electron micrograph of the glomerular basement membrane (gbm) region in the glomerulus. Individual profiles of podocyte foot processes resting on the glomerular basement membrane (gbm) are connected by slit diaphragms (arrowheads at top). Cl, capillary lumen; bs, Bowman’s space. Whole-mount in situ hybridization shows expression of zebrafish podocin (E) and nephrin (F), genes associated with human nephrotic syndromes, specifically in the forming podocyte precursor cells.

Figure 2. The zebrafish as a model of glomerular structure and function. (A) An electron micrograph of the forming glomerulus at 2.5 dpf with invading endothelial cells from the dorsal aorta shaded in red and podocytes shaded in blue (image false-colored in Adobe Photoshop). (B) A similar stage glomerulus in the mutant island beat, which lacks blood flow as a result of a mutation in an L-type cardiac-specific calcium channel. The endothelial cells and podocytes are present, but there is no sign of glomerular remodeling and morphogenesis. (C) Measuring renal clearance: Rhodamine-dextran filtration and uptake by pronephric epithelial cells. 10KD lysine-fixable rhodamine dextran injected into the general circulation can be seen as red fluorescence in glomerular capillaries (gl), and filtered dye is seen in apical endosomes of pronephric duct cells (arrowheads). Counterstain: FITC wheat germ agglutinin. (D) Electron micrograph of the glomerular basement membrane (gbm) region in the glomerulus. Individual profiles of podocyte foot processes resting on the glomerular basement membrane (gbm) are connected by slit diaphragms (arrowheads at top). Cl, capillary lumen; bs, Bowman’s space. Whole-mount in situ hybridization shows expression of zebrafish podocin (E) and nephrin (F), genes associated with human nephrotic syndromes, specifically in the forming podocyte precursor cells.

Defects in Tubules and Models of Cystic Disease

One of the most common human genetic diseases is polycystic kidney disease, which affects 1 in 1000 individuals (30). Kidney cysts are the result of grossly expanded kidney tubule lumens and, when present in sufficient size and number, lead to kidney fibrosis and end-stage renal failure. Our work has identified a relatively large set of genetic loci associated with cystic pronephroi in zebrafish (11) (Figure 3). Recently, the results of a large-scale retroviral insertional mutagenesis screen have identified 10 zebrafish genes that when mutated cause pronephric cysts (31). The requirement for a relatively large number of genes underlying maintenance of tubule structure is consistent with the idea that maintenance of lumen size and epithelial cell shape is a complex process that is controlled by many cellular proteins or signaling pathways.

A surprising convergence of findings from studies of cystic disease, left-right asymmetry, retinal degeneration, and flagella formation in the simple eukaryote Chlamydomonas have led to the idea that defects in the formation or function of cilia may underlie the pathology observed in all of these conditions. Cloning the gene that is responsible for the oakridge polycystic kidney (orpk) mouse was the first link between cilia and kidney cystic disease. The mutant gene, polaris, is a homolog of a Chlamydomonas gene, IFT88, that is required for intraflagellar

Figure 3. The zebrafish as a model of polycystic kidney disease. Three-day-old larvae showing wild-type (A) and the mutant double bubble (dbb; B) with a grossly distended pronephric tubule that appears as a bubble (arrow) just behind the pectoral fin. (C) Three-day-old wild-type kidney structure showing the pronephric duct (pnd; cross-section), pronephric tubule (pt), and glomerulus (gl). (D) A section of a dbb mutant pronephros shows the cystic distended lumen of the pronephric tubule (*) and distended glomerulus at the midline. (E) Whole-mount confocal immunofluorescent image of a 3-d-old embryo stained with anti-acetylated tubulin to reveal cilia in the pronephric ducts (arrowhead). Dorsal structures are the neural tube and motor neurons. (F) Close-up view of acetylated tubulin–positive pronephric cilia. (G) Electron micrograph of a single apical cilium (arrowhead) on a pronephric duct cell.
transport, an essential process in flagellum formation (32,33). Human and mouse kidney cells are not flagellated but have a single, nonmotile apical cilium. Orpk mutant mouse kidney epithelial cells have short, malformed apical cilia (32–34), suggesting a functional link between cilia and maintenance of epithelial tubule lumen diameter. Subsequent studies revealed that most known cystic mutant genes, including polycystin 1, polycystin 2, cystin (opk mouse), polaris, inversin, and the Caenorhabditis elegans polycystin homologs lov-1 and pkd2, were, at least in part, localized to cilia (35–39). The results of a large-scale insertional mutagenesis screen in zebrafish lend further support to the link between cilia and cystic disease. Of 10 genes reported in this work, three were IFT genes associated with ciliogenesis (31). Recent studies of polycystin 1 and polycystin 2, the genes that are responsible for autosomal dominant polycystic kidney disease, indicate that they act together to mediate calcium entry into cells upon flow-induced cilium deflection (40,41). The current model of cilia function is that the cilium acts as a sensor of tubule lumen mechanics and flow, providing a feedback signal that limits lumen diameter or cell proliferation. Our recent observations in zebrafish indicate that cilia in the pronephros are motile and have a “9 + 2” microtubule doublet organization that is typical of motile cilia and flagella (Kramer-Zucker et al., submitted). Because motile cilia are often associated with fluid flow, this leads to an alternative hypothesis that cilia may act as a fluid pump in the zebrafish pronephros. It seems that cystic kidney tubules could arise by multiple different mechanisms related to cilia function.

Several other genes that can account for tubule cyst formation have been identified in zebrafish. Nek8 is a member of the NIMA family of serine/threonine kinases and is mutated in the juvenile cystic kidney (jck) mouse (42). Disrupting the function of zebrafish Nek8 causes severe cystic distension of the pronephric tubules. Other Nek kinases have links to cytoskeletal functions: Nek2 is localized to centrioles and acts to promote splitting of duplicated centrioles during the cell cycle (43). Further studies are needed to test whether Nek8 may have a similar role in cilia or centrosome/basal body function (44–47).

Disruption of the zebrafish homolog of the human cystic disease gene polycystin 2 also causes pronephric cyst formation (31; Obara et al., unpublished results, 2004). We have found that co-injected human polycystin 2 mRNA can rescue this phenotype, indicating that the function of polycystin 2 has been highly conserved between fish and human. This kind of result opens to the door for functional analysis of variant forms of the human PKD2 gene in an easily manipulated, in vivo model of human disease.

Disruption of the zebrafish inversin gene results in kidney cysts. The human condition nephronophthisis type 2 (NPHP2) is associated with mutations in the human inversin gene (13). Both inversin and the mammalian NPHP1 gene nephrocystin are found in basal bodies and cilia and have been shown to interact biochemically. Deletion of the putative nephrocystin-binding domain in zebrafish inversin results in severe cyst formation, supporting the idea that NPHP proteins act as a multiprotein complex to regulate the function of basal bodies and/or cilia (13).

The transcription factor hepatocyte nuclear factor 1β (HNF-1β) is required for normal zebrafish pronephric tubule development (48) and, when mutated, results in glomerular cysts. HNF-1β has been shown to regulate the expression of several other cyst-associated genes in the mouse (49). Mutations in HNF-1β in humans are associated with glomerulocystic disease and maturity onset diabetes of the young, type V (50).

Conclusions

Despite some differences in organ morphology between the mammalian and teleost kidneys, many parallels exist at the cellular and molecular levels that can be exploited to further our understanding of kidney development and disease. The same genes and cell types are used in the development and function of all vertebrate kidneys. Genes that are mutated in human disease are also essential for the formation and function of the zebrafish pronephros. The zebrafish thus presents a useful and relevant model for studies of kidney development and disease. Both gene-targeted and unbiased mutagenesis approaches to genetic manipulation in the zebrafish will no doubt continue to reveal the function of genes and cell–cell interactions that underlie the development of all kidney forms.

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References

4. Smith HW: From Fish to Philosopher, Boston, Little Brown, 1953


45. Potter EL: Normal and Abnormal Development of the Kidney, Chicago, Year Book Medical Publishers, 1972