Birth Defects Research (Part C) 93:141–156 (2011)

REVIEW

Zebrafish Kidney Development: Basic Science to Translational Research

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The zebrafish has become a significant model system for studying renal organogenesis and disease, as well as for the quest for new therapeutics, because of the structural and functional simplicity of the embryonic kidney. Inroads to the nature and disease states of kidney-related ciliopathies and acute kidney injury (AKI) have been advanced by zebrafish studies. This model organism has been instrumental in the analysis of mutant gene function for human disease with respect to ciliopathies. Additionally, in the AKI field, recent work in the zebrafish has identified a bona fide adult zebrafish renal progenitor (stem) cell that is required for neo-nephrogenesis, both during the normal lifespan and in response to renal injury. Taken together, these studies solidify the zebrafish as a successful model system for studying the broad spectrum of ciliopathies and AKI that affect millions of humans worldwide, and point to a very promising future of zebrafish drug discovery. The emphasis of this review will be on the role of the zebrafish as a model for human kidney-related ciliopathies and AKI, and how our understanding of these complex pathologies is being furthered by this tiny teleost. **Birth Defects Research (Part C) 93:141–156, 2011.** © **2011 Wiley-Liss, Inc.**

Key words: kidney; zebrafish; ciliopathy; acute kidney injury

INTRODUCTION

The zebrafish (*Danio rerio*) is a freshwater tropical fish that spawns via external fertilization. The *ex utero* developing embryos progress rapidly through embryogenesis and are characterized by optical transparency. These features aid *in vitro* analysis of gene expression via *in situ* hybridization and immunohistochemistry, as well as *in vivo* studies using transgenic reporter lines. Adult fish are small, allowing large numbers of animals to be maintained in a minimal amount of space. Additionally, they breed frequently, yield large numbers of progeny, and have a generation time of three months. Perhaps the greatest reason the zebrafish has become a widely studied vertebrate model is that it is amenable to genetic screens. Several large-scale mutant screens have identified new genes involved in many aspects of organogenesis, including kidney development (Driever et al., 1996; Haffter and Nusslein-Volhard, 1996; Drummond et al., 1998; Amsterdam et al., 1999).

In the 30 years since George Streisinger first broke new ground using zebrafish as a genetically amenable research organism at the University of Oregon (Streisinger et al., 1981; Chakrabarti et al., 1983; Walker and Streisinger, 1983), it has developed from a pet store novelty to a model for studying embryogenesis and human disease pathologies. During this progression from basic to translational research, the zebrafish has been employed in a plethora of studies modeling human disease, including those for congenital defects such as Fraser syndrome (Carney et al., 2010), Waardenburgh syndrome (Dutton et al., 2009), and muscular dystrophy (Thornhill et al., 2008); numerous cancers like melanoma (Patton et al., 2005; Ceol et al., 2011; White et al., 2011), epithelial tumors (Shepard et al., 2007), neuroendocrine carcinoma (Yang et al., 2004), and leukemia (Langenau et al., 2003); as well as neurodegenerative disorders

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Grant sponsor: The NIDDK/NIH; Grant numbers: R01DK069403; RC4DK090770

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View this article online at wileyonlinelibrary.com. DOI: 10.1002/bdrc.20209

including tauopathies (Bai et al., 2007; Paquet et al., 2010), Parkinson's disease (Flinn et al., 2009), and Huntington's disease (Williams et al., 2008). Additionally, several zebrafish models of regeneration have been utilized to understand the repair potential of the limb (fin) (White et al., 1994; Akimenko et al., 1995), heart (Poss et al., 2002; Jopling et al., 2010; Kikuchi et al., 2010), retina (Cameron and Carney, 2000; Vihtelic and Hyde, 2000), lateral line hair cells (Harris et al., 2003; Lopez-Schier and Hudspeth, 2006), and kidney (Reimschuessel 2001; Hentschel et al., 2005; Zhou et al., 2010a; Diep et al., 2011).

This review will focus on the zebrafish kidney and will illustrate how kidney organogenesis studies have evolved into translational-based research. The zebrafish has become an important model system for studying renal disease, thanks to the anatomical simplicity of the embryonic kidney (Drummond, 2005). Although functionally similar, three types of kidneys have evolved in vertebrates: the pronephric, mesonephric, and metanephric kidneys. The metanephros, the most complex kidney, is only present in birds and mammals, but develops from the two simpler, pronephric and mesonephric, kidneys. Zebrafish have pronephric (embryonic) and mesonephric (adult) kidneys. All three types of kidney utilize a common functional substructure called the nephron. Generally, kidney nephrogenesis can be divided into four stages: (1) specification of intermediate mesoderm as nephrogenic mesenchyme, (2) growth and epithelialization of the anlagen, (3) induction and patterning of the nephron, and (4) formation of the glomerular capillary tuft from invading endothelial cells (Drummond, 2003). In the zebrafish embryonic kidney, these stages occur once during organ formation to create two bilaterally paired nephrons, whereas, in the mammalian kidney, they are reiterated many times to create a million nephrons in humans. Significantly, there is a striking similarity in the molecular and segmental organization of the mammalian and zebrafish nephrons (Wingert et al., 2007; Wingert and Davidson, 2008).

The simple nature of the zebrafish pronephric kidney makes it a suitable system to study the early developmental events that lay the foundation for genesis of more complex kidneys. Even though the pronephros in mammalian and avian embryos is not functional, it is important for initiation of the molecular events of kidney development (Vize et al., 2003). Microsurgical inhibition of pronephros development in chick embryos and loss of the pronephros in mouse knockouts results in a lack of mesonephros and metanephros development (Shawlot and Behringer, 1995; Mauch et al., 2000; Bouchard et al., 2002; James and Schultheiss, 2003). Chick embryological experiments also revealed that initial patterning of the intermediate mesoderm is dependent upon signals from the axial/paraxial mesoderm (Mauch et al., 2000; James and Schultheiss, 2003). However, the mechanism of how the specified intermediate mesoderm differentiates into nephric mesenchyme is not completely understood. Several studies have defined a set of genes that are expressed within the intermediate mesoderm of all vertebrate species, which later become restricted to the nephrogenic mesenchyme, and have therefore been implicated in kidney formation, including *lhx1*, *pax8*, *pax2*, *c-ret*, *hnf-1β*, and components of the *Notch* pathway (Barnes et al., 1994; Toyama and Dawid, 1997; Pfeffer et al., 1998; Mauch et al., 2000; Bouchard et al., 2002; Drummond, 2002; Kopan et al., 2007; Drews et al., 2011). The emphasis of this review will be on the role of the zebrafish as a model for human kidney-related ciliopathies and acute kidney injury.

CILIOPATHIES

Cilia are microtubule-based organelles that are anchored and extend from the apical surface of most eukaryotic cells. They can generally be classified as either motile or immotile. The importance of motile cilia has been documented for some time in a number of organisms. For example, the cilia that line the trachea or are found on the ependymal cells of brain ventricles are critical for the movement of mucus and cerebrospinal fluid, respectively (Yoder, 2007; Vincensini et al., 2011). However, the immotile or primary cilium has only recently gained respect within the scientific community. Evidence suggests that the primary cilium can act as a mechanosensor and transducer of various signaling pathways which are critical for normal development and when disrupted, for disease progression (Yoder, 2007; Lancaster and Gleeson, 2009; Goetz and Anderson, 2010).

The assembly of the primary cilium is a dynamic process that is tightly linked to the cell cycle, such that cilia are built during G_1 and retracted as cells enter mitosis (Pedersen et al., 2008; Santos and Reiter, 2008). Derived from the mother centriole, the basal body serves as the foundation of the cilium and is composed of nine triplet microtubules that form a barrel-like structure (Yoder, 2007; Pedersen et al., 2008). The core of the cilium, the axoneme, extends from this basal body and consists of nine microtubule doublets. The presence of a central pair of microtubules (9+2 configuration) has been linked to motile cilia, while this pair is normally absent in primary cilia (9+0 configuration), although exceptions to this rule are found (Yoder, 2007; Vincensini et al., 2011). Construction of the ciliary axoneme requires intraflagellar transport (IFT), a bidirectional motility system that localizes between the axoneme and the membrane of the cilium (Pedersen et al., 2008). IFT particles are transported to the distal tip of the growing axoneme (anterograde) by kinesin-2 motors and return to the base of the cilium (retrograde) with the assistance of cytoplasmic dynein-2. These IFT particles carry a variety of different cargo, including

axonemal precursors and components of important developmental signaling pathways (Goetz and Anderson, 2010).

Deciphering the roles that primary cilia play during development and adult homeostasis has been the driving force behind the work from many laboratories. While it has been appreciated for some time that motile cilia participate in fluid flow, the function of primary cilia was always less clear. However, studies from a number of groups have suggested that primary cilia function as environmental sensors, detecting changes in flow and light (Vincensini et al., 2011). They also appear to function as signaling centers since a number of important developmental pathways, including hedgehog and Wnt, converge on this organelle (Veland et al., 2009; Goetz and Anderson, 2010). The number of proteins that localize to the cilium, basal body, or centrosome (centrioles with associated pericentriolar material) has increased dramatically in recent years. There are currently over 2600 entries in the cilia proteome database (www.ciliaproteome.org), highlighting the importance of this organelle in a variety of cell types and cellular processes. As a result of these localization studies, connections have been made between cilia dysfunction and human diseases/syndromes, collectively termed ciliopathies. Disruption of motile cilia often leads to early embryonic death due to respiratory dysfunction, hydrocephalus, and/or the failure of embryonic turning (Badano et al., 2006b; Cardenas-Rodriguez and Badano, 2009; Tobin and Beales, 2009). In less severe cases, defects in left-right asymmetry are apparent, resulting in the randomization of visceral organs or complete *situs inversus*. Compromising the function of primary cilia results in a range of syndromes, with the development of kidney cysts being a common denominator. Other common phenotypes include liver disease, polydactyly, retinal degeneration, mental retardation, and neural tube defects.

Model organisms have been instrumental in the analysis of gene function, and have been used extensively in the study of human disease, particularly with respect to ciliopathies. While researchers often look to the mouse for answers, the zebrafish is emerging as an excellent system in which to analyze ciliary function. In the zebrafish, apical cilia are present in Kupffer's vesicle (KV) (the equivalent of the mouse node), the spinal cord, and the pronephric kidney (Kramer-Zucker et al., 2005). The cilia in all of these organs are motile but have a slightly different architecture, namely, the cilia in the KV and kidney are 9+2 while those in the central canal are 9+0. Interestingly, knockdown of the zebrafish *IFT88* ortholog (*polaris/oval*) results in phenotypes similar to those observed when *IFT88* function is compromised in the mouse (Pazour et al., 2000; Kramer-Zucker et al., 2005). Not only does this data suggest that the function of this IFT component is conserved within vertebrates but also that studies in the fish can be used to tease out the intricacies within the field of cilia biology.

Polycystic Kidney Disease

Polycystic kidney disease (PKD), which can be subdivided into autosomal dominant (ADPKD) and autosomal recessive (ARPKD), is characterized primarily by the formation of renal cysts (Torres and Harris, 2009). ADPKD is one of the most common monogenic disorders, affecting between 1/400 and 1/1000 individuals, and has been linked to mutations in two genes (Table 1), *PKD1* (polycystin-1) and *PKD2* (polycystin-2). Polycystin-1 contains a large extracellular N-terminus, 11 transmembrane domains, and a shorter cytoplasmic C-terminus, while polycystin-2 is a much smaller protein, spanning the membrane only 6 times with intracellular N- and C- termini (Chapin and Caplan, 2010). Data suggest that the polycystins form a complex that regulates the level of intracellular Ca^{2+}, and this interaction is facilitated by their colocalization, especially to the primary cilium.

Particularly, the zebrafish has served as an excellent model system for analyzing the role of *pkd2* during development. Initial studies showed that *pkd2* was expressed in multiple ciliated-tissues, including KV, the pronephric duct, floorplate, and brain (Bisgrove et al., 2005). Knockdown of *pkd2* gene function resulted in axis curvature, kidney cysts, pericardial edema, and hydrocephalus as well as defects in left-right asymmetry, consistent with phenotypes observed in *Pkd2*$^{-/-}$ mice (Wu et al., 1998, 2000; Pennekamp et al., 2002; Sun et al., 2004; Bisgrove et al., 2005). It was later shown that the polycystin-2 protein localizes to cilia and the basolateral surface of epithelial cells in the anterior pronephros (Obara et al., 2006), and this localization is required for proper function of polycystin-2 within the kidney (Obara et al., 2006; Streets et al., 2006; Fu et al., 2008).

As both polycystin-1 and polycystin-2 localize to the cilium, it is reasonable to suggest that proper function of this organelle is required to prevent cyst formation. In support of this idea, two independent screens in the zebrafish identified a large number of genes that, when mutated, gave rise to cystic kidneys (Drummond et al., 1998; Sun et al., 2004). Two such genes, *curly* and *moe*, were identified as the zebrafish orthologs of *ift57* and *ift172*, respectively (Sun et al., 2004; Cao et al., 2010). Analysis of pronephric cilia in *ift57*^{hi3417} and *ift172*^{hi2211} mutants demonstrated that a subset of ciliated cells was affected. There are both single-ciliated (SCC) and multiciliated (MCC) cells present in the zebrafish kidney at 48 hr postfertilization (hpf) (Liu et al., 2007; Ma and Jiang, 2007). While the SSCs were unaffected, there was a disorganization of the MCCs due to disorganized basal bodies in the tubule epithelial cells (Cao et al., 2010). This phenotype was also observed upon knockdown of *prickle1 (pk1)*, a core component

TABLE 1. Causative Loci in Multiple Ciliopathies and Their Zebrafish Orthologs

Causative Loci in Humans	Zebrafish Ortholog	Phenotype	References
Polycystic Kidney Disease (PKD)			
Pkd1 (Polycystin-1)	*pkd1a/pkd1b*	Dorsal axis curvature, pronephric kidney cysts, hydrocephalus, jaw defects	(Mangos et al., 2010)
Pkd2 (Polycystin-2)	*pkd2/curly up*	Dorsal axis curvature, pronephric kidney cysts, pericardial edema, hydrocephalus, laterality defects	(Bisgrove et al., 2005; Obara et al., 2006; Schottenfeld et al., 2007; Fu et al., 2008; Sullivan-Brown et al., 2008)
Nephronopthisis (NPHP)			
NPHP1	N.D.		
NPHP2/inversin	*inversin*	Ventral axis curvature, pronephric kidney cysts, pericardial edema, laterality defects	(Otto et al., 2003; Simons et al., 2005)
NPHP3	*nphp3*	Ventral axis curvature, pronephric kidney cysts, hydrocephalus, laterality defects, CE defects, fewer and shorter cilia in KV	(Zhou et al., 2010b)
NPHP4	N.D.		
NPHP5	*nphp5/iqcb1*	Axis curvature, pronephric kidney cysts, hydrocephalus	(Schafer et al., 2008)
NPHP6/CEP290	*cep290*	Ventral axis curvature, pronephric kidney cysts, hydrocephalus, CE defects, deficient retinal tissue	(Sayer et al., 2006; Leitch et al., 2008; Schafer et al., 2008)
NPHP7/GLIS2	N.D.		
NPHP8/RPGRIP1L	*rpgrip1l*	Dorsal axis curvature, CE defects	(Khanna et al., 2009)
NPHP9/NEK8	*nek8*	Kidney cysts	(Liu et al., 2002)
NPHP11/MKS3	*mks3*	CE defects	(Leitch et al., 2008)
Joubert Syndrome (JBTS)			
JBTS1/INPP5E	*inpp5e*	N.D.	
JBTS2/TMEM216	*tmem216*	Ventral axis curvature, pericardial edema, hydrocephaly, CE defects	(Valente et al., 2010)
JBTS3/AHI1	*ahi1*	N.D.	
JBTS4/NPHP1	*See NPHP1*		
JBTS5/CEP290	*See NPHP6*		
JBTS6/TMEM67/MKS3	*tmem67*	CE defects	(Leitch et al., 2008; Valente et al., 2010)
JBTS7/RPGRIP1L	*See NPHP8*		
JBTS8/ARL13B	*arl13b/scorpion*	Ventral axis curvature; pronephric kidney cysts; laterality defects, CE defects, reduced number of cilia in pronephros and KV	(Sun et al., 2004; Cantagrel et al., 2008; Duldulao et al., 2009)
JBTS9/CC2D2A	*cc2d2a/sentinal*	Dorsal axis curvature, pronephric kidney cysts	(Gorden et al., 2008)
JBTS10/OFD1	*ofd1*	Axis curvature, pericardial edema, hydrocephalus, laterality defects, CE defects	(Ferrante et al., 2009)
Bardet-Biedl Syndrome (BBS)			
BBS1	*bbs1*	CE defects, KV defects, delay in melasome transport, expansion of Shh expression in fin bud	(Gerdes et al., 2007; Tayeh et al., 2008; Zaghloul et al., 2010)
BBS2	*bbs2*	KV defect, degenerate cila in KV, delay in melanosome transport	(Yen et al., 2006)

TABLE 1. Continued

Causative Loci in Humans	Zebrafish Ortholog	Phenotype	References
BBS3	*arl6/bbs3*	KV defects, laterality defects, delay in melanosome transport	(Tayeh et al., 2008)
BBS4	*bbs4*	CE defects, KV defect, degenerate cilia in KV, laterality defects, delay in melanosome transport	(Yen et al., 2006; Gerdes et al., 2007; Zaghloul et al., 2010)
BBS5	*bbs5*	KV defects, laterality defects, delay in melanosome transport	(Yen et al., 2006)
BBS6/MKKS	*mkks/bbs6*	Pronephric kidney cysts, CE defects, KV defect, degenerate cilia in KV, laterality defects, delay in melanosome transport	(Yen et al., 2006; Gerdes et al., 2007; Zaghloul et al., 2010)
BBS7	*bbs7*	KV defect, degenerate cila in KV, laterality defects, delay in melanosome transport, expansion of Shh expression in fin bud	(Yen et al., 2006; Tayeh et al., 2008)
BBS8	*ttc8/bbs8*	Ventral axis curvature, pronephric kidney cysts, KV defect; laterality defects, delay in melanosome transport	(Yen et al., 2006; Tobin and Beales, 2008)
BBS9	*bbs9*	CE defects	(Zaghloul et al., 2010)
BBS10	*bbs10*	CE defects	(Zaghloul et al., 2010)
BBS11	*trim32/bbs11*	KV defect, delay in melanosome transport	(Chiang et al., 2006)
BBS12	*bbs12*	CE defects	(Stoetzel et al., 2007; Zaghloul et al., 2010)
BBS13/MKS1	*mks1*	Ventral axis curvature, pronephric kidney cysts, CE defects	(Leitch et al., 2008; Tobin and Beales, 2008)
BBS14/CEP290	*See NPHP6*		

Abbreviations: N.D. (Not Determined); CE (convergent extension); KV (Kupffer's Vesicle).

of the planar cell polarity (PCP) pathway. Interestingly, both *ift57* and *ift172* genetically interact with *pk1*, suggesting that alterations in the PCP pathway are responsible for pronephric cyst formation in these *ift* mutants.

Understanding the mechanism of cyst formation is crucial for designing possible treatment options for PKD. Consequently, studies in zebrafish have focused on characterizing this process within the pronephros. Using mutations in *switch hitter* (*swt*), the zebrafish ortholog of *LRRC50*, researchers have found that defects in the motility of pronephric cilia contribute to cyst formation (Sullivan-Brown et al., 2008; van Rooijen et al., 2008). Interestingly, these defects were identified as early as 26 hpf and suggest that disruption of normal ciliary function is the initial cause of pronephric cysts in this mutant. By 48 hpf, cysts within the proximal tubule segments (PCT and PST; Wingert et al., 2007) develop, followed shortly by glomerular dilation. There is also an increase in proliferation and a mislocalization of Na^+/K^+ATPase around and within the tubules, respectively (Sullivan-Brown et al., 2008; van Rooijen et al., 2008). However, these are only thought to contribute to cyst formation because they occur after the identification of cysts (Sullivan-Brown et al., 2008). These phenotypes differ slightly from those observed in *pkd2* morphants (Bisgrove et al., 2005; Obara et al., 2006; Schottenfeld et al., 2007; Sullivan-Brown et al., 2008). While pronephric cysts develop, they occur without defects in ciliary motility and appear localized to the glomerulus. These studies highlight the heterogeneity that exists within cystic kidney disease, as well as the importance for normal ciliary function in the prevention of kidney cyst formation.

Nephronopthisis

While nephronopthisis (NPHP) is a cystic kidney disease, the kidney does not become enlarged due to cyst formation throughout the organ (Hildebrandt and Zhou, 2007; Hildebrandt et al., 2009). Rather, the cysts that develop are localized to the corticomedullary region, resulting in little change in the overall size of the kidney. This disease is also characterized by tubular basement membrane disruption and tubulointerstitial fibrosis. NPHP is an autosomal recessive disorder and represents the most common genetic cause of end-stage renal disease during the first 3 decades of life. To date, mutations in ten genes have been shown to cause NPHP (Hildebrandt et al., 2009) (Table 1). Since these proteins generally localize to primary cilia, basal bodies, and centrosomes, a role for the cilium in the pathogenesis of NPHP has been suggested.

Similar to phenotypes seen in humans and mice (Gagnadoux et al., 1989; Mochizuki et al., 1998; Morgan et al., 1998), knockdown of *inversin (invs)* (*nphp2*) in zebrafish results in ventral axis curvature, severe pronephric cyst development, and randomization of heart looping (Otto et al., 2003). Interestingly, both the axis curvature and cyst development can be rescued by injection of the wildtype mouse *Invs* mRNA, suggesting a conserved function for NPHP2/inversin within the vertebrate lineage. This model of cystogenesis, generated by knockdown of *invs* in zebrafish, was utilized to test the role of canonical Wnt signaling in renal development (Simons et al., 2005). Injection of diversin, a molecule structurally related to inversin and capable of inhibiting the canonical Wnt pathway, prevented renal cyst formation in *invs* morphants, suggesting that proper regulation of Wnt signaling is important for renal development. Knockdown of other NPHP genes in zebrafish using morpholinos produced similar phenotypes to those observed in *invs* morphants (Schafer et al., 2008; Zhou et al., 2010b). Both *nphp3* and *nphp5* morphants displayed body axis curvature, pronephric cyst development, and hydrocephaly. In addition to these phenotypes, left-right patterning defects were also seen upon knockdown of *nphp3* (Schafer et al., 2008; Zhou et al., 2010b). These phenotypes suggest a conserved role for both proteins in proper ciliary function.

In individuals with NPHP, there is often involvement of multiple organs outside of the kidney. When NPHP is combined with cerebellar vermis aplasia, patients are diagnosed with a separate, rare autosomal recessive disorder called Joubert syndrome (JBTS) (Hildebrandt and Zhou, 2007). JBTS occurs in 1 out of 80,000 −100,000 live births and is primarily diagnosed by the presence of the ''molar tooth sign'', a midbrain-hindbrain malformation visible on brain imaging (Brancati et al., 2010). Abnormal eye movements, breathing difficulties, hypotonia, and developmental delay/mental retardation also characterize this syndrome (Harris, 2007; Brancati et al., 2010). Currently, there are 10 causative genes that have been identified and they may explain the pleiotropy associated with this syndrome (Brancati et al., 2010) (Table 1).

Interestingly, the *NPHP6* gene has been implicated in both NPHP and JBTS. Morpholino knockdown of *cep290* (*nphp6*) results in pronephric cyst development, ectopic tissue in the fourth ventricle, and a reduction in retinal tissue (Sayer et al., 2006). These phenotypes are reminiscent of the meningoencephalocele and retinal degeneration seen in some patients with JBTS. Further characterization of the molecular function of NPHP6 has also been performed in the zebrafish. *Nphp6* was shown to genetically interact with *nphp5* (Schafer et al., 2008) as well as with *cc2d2a/JBTS9*, another causative gene linked to JBTS (Gorden et al., 2008). Injection of the *cep290* morpholino into *sentinal (snl)* (*cc2d2a*) mutants resulted in larger and more prevalent pronephric cysts, suggesting a functional synergism between these two genes (Gorden et al., 2008).

Similar to *CEP290*, *RPGRIP1L* has also been linked to both NPHP and JBTS. Retinal degeneration is a common but not universal phenotype in many ciliopathies. Resequencing of known genes involved in ciliopathies identified a mutation in *RPGRIP1L* (A229T) that was enriched in individuals with retinal defects (Khanna et al., 2009). To test the functional significance of this mutation, a splice-blocking morpholino was designed to target the zebrafish ortholog, *rpgrip1l*. Knockdown of this gene resulted in gastrulation defects as well as tail curvature. These phenotypes could be rescued by the wildtype human mRNA but could not be rescued to the same degree by a human mRNA encoding the mutant Thr229. Thus, studies in zebrafish have enabled researchers to identify genes that can serve as phenotypic modifiers, and this may explain, at least in the case of *CEP290* and *RPGRIP1L*, why they have been implicated in multiple ciliopathies.

Many of the studies examined so far have utilized zebrafish to test the functionality of a gene once it has been linked to an established disease. However, one of the strengths of zebrafish as a model organism is the ability to conduct forward genetic screens. As the result of one such screen, the first analysis of a gene associated with JBTS was performed. *Scorpion (sco)/arl13b* mutants displayed a curved body axis as well as kidney cysts (Sun et al., 2004). Because of the phenotypic similarity to other mutants identified in the screen, later determined to be zebrafish orthologs of IFT components, cilia formation was analyzed in *sco* mutants. Based on the absence of acetylated tubulin staining in the kidney (pronephros), it was concluded that *sco* was required for cilia formation. Further analysis showed that *arl13b* was expressed in ciliated tissues and was localized to the cilium in the pronephric duct, neural tube, olfactory placode, and KV (Duldulao et al., 2009).

Genome mapping and sequencing of families affected by Joubert syndrome and related disorders (JSRD) later identified *ARL13B* (JBTS8), a member of the Ras GTPase family, as a causative gene (Cantagrel et al., 2008). Interestingly, this group utilized the zebrafish to test the functionality of the human protein containing the mutations that were identified in individuals with JSRD. Injection of the wildtype human *ARL13B* mRNA was able to rescue the curved axis and kidney cysts observed in zebrafish *sco* mutants, suggesting a conservation of function across vertebrate lineages for this protein. However, two separate human mRNAs containing the missense mutations identified in JSRD families could not show the same degree of rescue. Taken together, the above studies suggest that Joubert syndrome should be considered a ciliopathy. To provide further evidence for this classification, the cilia in *sco* mutants and morphants were analyzed in more detail (Duldulao et al., 2009). In sco^{hi459} mutants, closer examination of cilia in the pronephric duct demonstrated that the number of cilia was significantly reduced and their motility was compromised. Deletion analysis suggested that both the small GTPase domain, as well as the coiled-coil domain, are required for localization of Arl13b to the cilium, and that this localization is required for function. These studies not only demonstrate the conserved function of ARL13B in vertebrates, but also highlight the utility of zebrafish in determining the functionality of a protein associated with human disease.

Bardet-Biedl Syndrome

It has been suggested that Bardet-Biedl syndrome (BBS) is a model ciliopathy because the characteristic features of BBS are shared with other disorders (Zaghloul and Katsanis, 2009). This overlap can be seen at the level of phenotype, involvement of common signaling pathways, and protein co-localization. While BBS is normally inherited in an autosomal recessive fashion, the genetic heterogeneity and presence of second-site modifiers makes this syndrome a representative of the ciliopathy disease spectrum (Zaghloul et al., 2010). Currently, mutations in 14 loci have been shown to cause BBS (Table 1), which is characterized by retinal degeneration, obesity, hypogonadism, polydactyly, renal dysfunction, and mental retardation (Zaghloul and Katsanis, 2009). Through the use of the zebrafish, significant progress has been made with respect to understanding not only the *in vivo* functions of the BBS genes, but also the variants that contribute to this and related syndromes.

Although the proteins encoded by the BBS genes have all been shown to localize to the basal body, centrosome, or cilium (Cardenas-Rodriguez and Badano, 2009), their structures do not place them in similar functional categories (Tayeh et al., 2008). To determine the roles of these genes during development, a number of groups have utilized morpholinos to knockdown the BBS genes individually or in combination. Individual knockdown of *bbs1-8* resulted in defects in KV formation (Yen et al., 2006; Tayeh et al., 2008). Particularly, cilia in the KV appeared to degenerate following injection of morpholinos targeting *bbs2*, *bbs4*, *bbs6*, and *bbs7*, which lead to defects in both heart and gut looping (Yen et al., 2006). It was also shown that knockdown of *bbs1-8* lead to defects in retrograde melanosome transport, a process that requires the minus-end directed motor dynein (Yen et al., 2006; Tayeh et al., 2008). These results suggest that BBS genes are important for ciliary maintenance and intracellular transport.

Data also suggest that BBS proteins play an important role in modulating the noncanonical Wnt pathway and PCP. Disruption of PCP in zebrafish results in convergent extension defects, which include a shortened and widened body axis (Keller, 2002). This phenotype was observed along with widening of the somites and a kinked notochord when *bbs4* or *bbs6* were knocked down (Badano et al., 2006a). Both of these genes genetically interact with *vangl2*, a core PCP gene (Ross et al., 2005), as well as Wnt5a and Wnt11 (Gerdes et al., 2007). Interestingly, while noncanonical Wnt signaling is compromised in *bbs4* and *bbs6* morphants, the level of canonical Wnt signaling is increased, suggesting that BBS proteins and the cilium are important for regulating the balance between these two signaling pathways (Gerdes et al., 2007).

The PCP phenotype that was first observed with knockdown of either *bbs4* or *bbs6* has since been used to identify modifiers of BBS and to characterize the spectrum of this disease. Meckel syndrome (MKS), a lethal autosomal recessive disorder, is characterized by cystic kidneys, liver fibrosis, polydactyly, and encephalocoele (Tobin and Beales, 2009). Since there is significant phenotypic overlap between MKS and BBS, with the primary exception of encephalocoele, it is possible that MKS represents a more severe form of BBS. To this end, mutations in MKS genes could contribute to or modify BBS mutations. The *MKS1* gene was sequenced from over 150 families affected by BBS and a number of mutations were identified (Leitch et al., 2008). Knockdown of *mks1* in zebrafish phenocopied *bbs* morphants and could be rescued with the wildtype *MKS1* human mRNA. These morphants, however, could not be rescued to the same degree by constructs containing the identified human mutations. Depending on the degree of rescue, these mutations were classified as benign, hypomorphic, or null. *Mks1* was also shown to genetically interact with *bbs1*, *bbs2*, and *bbs4*, supporting the hypothesis that MKS genes can modify BBS phenotypes, and that the penetrance of BBS is dependent on the number of mutations affecting overall ciliary function.

This method of analysis was extended to all of the known BBS

genes to test the functionality of 125 identified alleles (Zaghloul et al., 2010). Morpholinos were designed against *bbs1-12* and knockdown resulted in the typical PCP phenotypes previously observed, namely a shortened body axis, broadened somites, and a widened/kinked notochord. Based on the ability of human constructs harboring the identified mutations to rescue the morphant phenotypes, an allelic series was created. A number of dominant negative alleles were isolated and at first, seemed inconsistent with a disease that is inherited in a recessive fashion. However, these alleles were mapped to epistatic loci (rather than primary) and function as dominant modifiers. Taken together, these elegant studies in the zebrafish have begun to unravel some of the complexity associated with this syndrome.

ACUTE KIDNEY INJURY

Acute kidney injury (AKI) is a multifactorial disorder associated with significantly high mortality rates that have remained unchanged for the last twenty years. AKI commonly results from ischemic injury, the use of nephrotoxic agents, and/or sepsis. It occurs in ~7% of in-patient hospital admissions (Lameire et al., 2006), and is an independent predictor of in-hospital mortality (Yalavarthy and Edelstein, 2008). Severe AKI requiring renal replacement therapy occurs in 4% of critically ill patients and has a 50% in-patient mortality (Goldberg and Dennen, 2008). Despite the high mortality rates, at present, renal replacement and dialysis are the only therapies that have proven to be beneficial in humans. Thus, there is an increasing interest in the development of treatments able to prevent or limit kidney injury, and/or enhance regeneration of normal kidney epithelium following injury. Recently, regeneration in the zebrafish kidney has received considerable attention, given the potential for the development of new therapeutic strategies.

Mammalian AKI

The vertebrate kidney possesses a remarkable potential to regenerate after an ischemic or nephrotoxic insult (Duffield et al., 2005; Romagnani and Kalluri, 2009). Mammalian renal epithelium has the capacity to self-renew by symmetric division of differentiated cells that are indistinguishable from their progeny (Vogetseder et al., 2007; Vogetseder et al., 2008). However, the generation of new nephrons has never been observed in mammals, and repair mechanisms often lead to the formation of fibrotic, non-functional tissue (Romagnani and Kalluri, 2009). Following AKI, the injured cells undergo a transient process of dedifferentiation, characterized by cytoskeletal disruption that results in changes in cell polarity, redistribution of the basolateral Na^+/K^+ATPase pump to the apical membrane, loss of brush border, and re-expression of mesenchymal markers and markers typical of early nephron development (Sharfuddin and Molitoris, 2011). This damage results in epithelial cell loss and denudation of areas of basement membrane. In experimental models of toxic and ischemia reperfusion (IR) kidney injury in rodents, epithelial cell death is largely restricted to the S3 segment of the proximal tubule and occurs within the first 12-24 hours of injury. Over this time period, there is loss of cell-cell junctions between surviving renal tubular epithelial cells along with cellular flattening and expression of mesenchymal markers, such as vimentin (Witzgall et al., 1994; Belleri et al., 2005; Verghese et al., 2008). Depending on the severity of the insult, regeneration of injured renal tubular epithelial cells occurs over a one to three week period as rapidly dividing, vimentin-positive cells undergo a mesenchymal-to-epithelial transition and, once again, form functional, polarized epithelia. Recent lineage tracing studies indicate that the bulk of regenerating renal tubular epithelial cells is derived from intrinsic renal epithelium following IR injury (Humphreys et al., 2008). This process of dedifferentiation is believed to promote cell migration into the area where cell death occurred, and the proliferation of these cells reconstitutes the tubular epithelium (Bonventre, 2003; Hader et al., 2010). The identification of renal stem cells and mesenchymal stem cells in the repair process has received considerable attention, given the potential for identifying new therapeutic strategies for treatment of kidney disease (Little, 2006). However, the contribution of adult stem cells, either of intrarenal origin or from the bone marrow, during the repair phase after AKI is still controversial (Cantley, 2005; Benigni et al., 2010).

In addition to the expression of mesenchymal markers, there is evidence that regenerating renal tubular epithelial cells express genetic markers normally associated with the early embryonic renal epithelia (in the pretubular aggregates and renal vesicles), including *Pax2*, *Wnt4*, *Lhx1*, and components of the *Notch* signaling pathway (Terada et al., 2003; Lin et al., 2005; Villanueva et al., 2006; Kobayashi et al., 2008). These markers appear within the first 24 hours following injury and are lost as the cells undergo epithelial differentiation. Since these embryonic markers are required for specification of diverse tubular epithelial cell types during embryonic development of the kidney, it has been proposed that expression of these markers is required to specify a number of different cell types within the regenerating renal tubular epithelium (Kopan et al., 2007). However, the mechanisms regulating embryonic gene expression following AKI are unknown. Interestingly, recent studies have shown that renal ischemia is associated with a reversible reduction in histone deacetylase (HDAC) activity and increased histone acetylation (K9 acetyl-histone H3) in renal tubular epithelial cells (Marumo et al., 2008). As epigenetic regulation of gene expression during development is regulated, in part, by histone acetylation (Haberland et al., 2009), these findings sug-

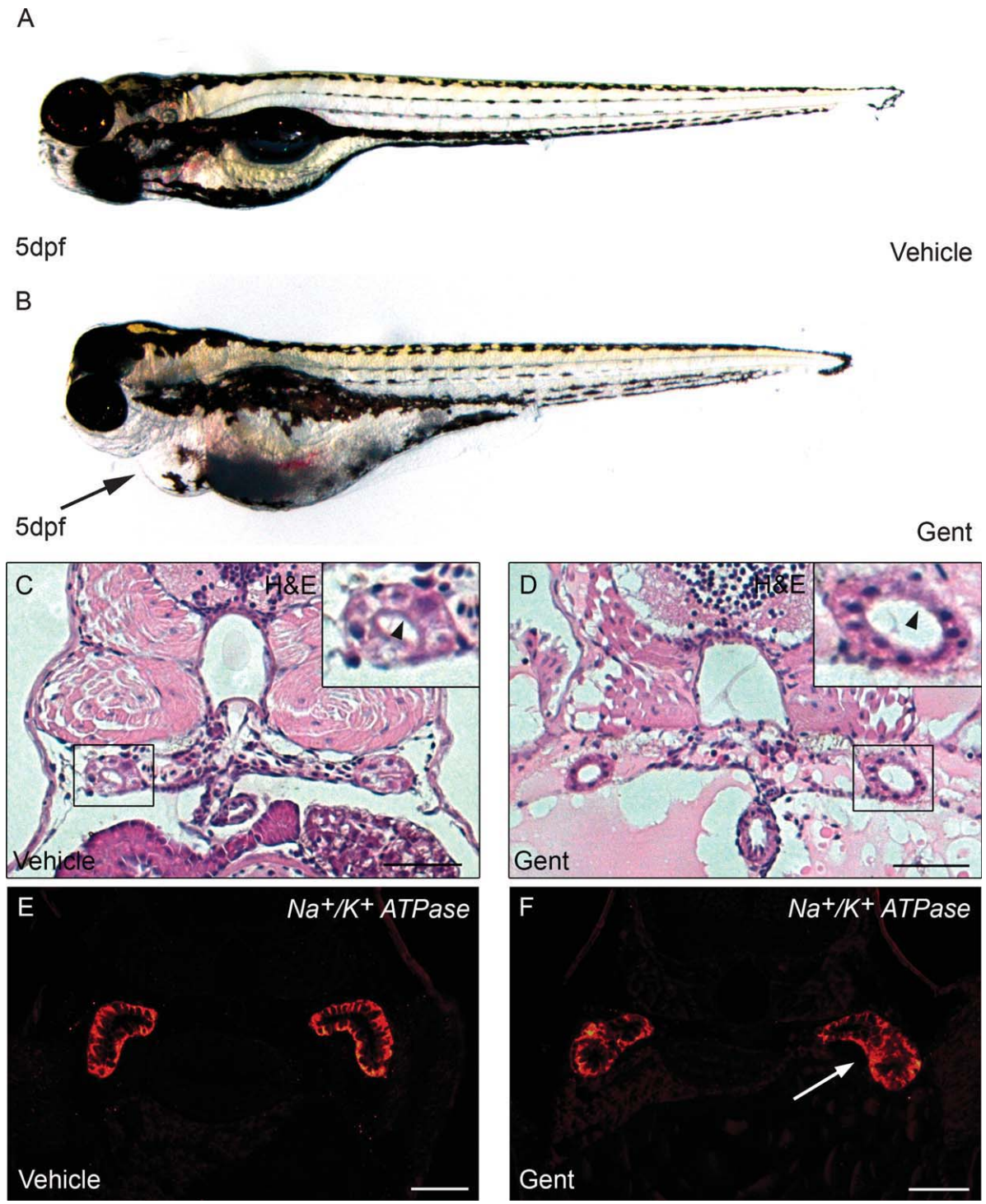

Figure 1. Gentamicin-mediated AKI in zebrafish. (**A,B**) Zebrafish larvae at 5 days post fertilization (dpf) injected with (**A**) vehicle or (**B**) 7.5 ng gentamicin. Arrow indicates pericardial edema reflecting kidney damage. (**C-D**) Haematoxylin and eosin (H&E) staining of kidney sections 72 hours post-treatment in (**C**) vehicle and (**D**) gentamicin-injected larvae. Inset shows the pronephric tubules, with tubular distension and loss of brush border (compare D with C, arrowheads). (**E-F**) Immunofluorescence for Na^+/K^+ATPase 48 hours post-treatment in (**E**) vehicle and (**F**) gentamicin-injected larvae. White arrow (**F**) indicates loss of basolateral polarity in proximal tubules. Scale bar is 20 μm.

gest that alterations in HDAC activity may mediate epigenetic reprogramming of regenerating renal tubular epithelial cells to a more primitive embryonic epithelial state, and that this may be required for the kidney to undergo complete cellular and functional recovery following injury.

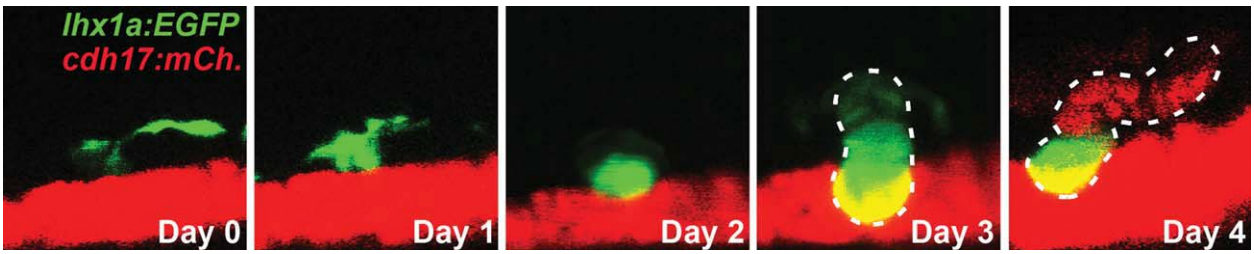

Figure 2. Nascent nephrons develop from *lhx1a:EGFP*-positive cells. Time course of a *Tg(lhx1a:EGFP;cdh17:mCherry)* larva demonstrating that multiple *lhx1a:EGFP*-positive cells coalesce to form a progenitor aggregate, which then differentiates into a nascent nephron.

Zebrafish AKI

The zebrafish pronephric kidney has been established as a valid vertebrate model system for AKI studies (Hentschel et al., 2005; Zhou et al., 2010a; Diep et al., 2011). Using gentamicin and cisplatin as nephrotoxicants, Hentschel et al. (2005) demonstrated nephrotoxic effects in zebrafish larvae similar to the aminoglycoside toxicity observed in mammalian AKI models. Particularly, gentamicin-mediated damage resulted in pronephric tubule flattening of the brush border, tubular and glomerular distension, lysosomal phospholipidosis, formation of debris in the tubular lumen, and accumulation of leukocytes. Similarly, cisplatin treatment caused cellular vacuolization, flattening and loss of tubular brush borders, and distension of the tubular lumen. These changes resulted in a decline in renal function, due to an inability to maintain water balance, and were associated with the development of pericardial edema. Similar results for a gentamicin-mediated AKI model were described in a detailed methodology for inducing AKI in zebrafish larvae (Cianciolo Cosentino et al., 2010). In addition, the loss of tubular cell polarity, one of the first hallmarks of sub-lethal injury in AKI, was clearly demonstrated by the redistribution of the basolateral Na^+/K^+ATPase pump to the apical membrane (Fig. 1).

As opposed to mammals, in which development of new nephrons stops before birth (Kuure et al., 2000), fish can generate new nephrons throughout their lifespan (Reimschuessel and Williams, 1995; Reimschuessel, 2001). This, perhaps, serves as the largest advantage in using zebrafish as an AKI model. Interestingly, neo-nephrogenesis in fish is greatly enhanced after kidney injury. This was first demonstrated in the goldfish, *Carassius auratus*, following exposure to hexachlorobutadiene (Reimschuessel et al., 1990). This same process of neo-nephrogenesis has subsequently been found in other fish species with different nephrotoxicants (Reimschuessel, 2001). The new nephrons appear as basophilic clusters of cells, called nephrogenic bodies, that are either spread throughout the renal mesenchymal tissue (Watanabe et al., 2009; Diep et al., 2011) or, like in the skate, *Leucoraja erinacea,* are restricted to a specific nephrogenic zone, containing mesenchymal cells and immature nephrons which resemble the embryonic kidney (Elger et al., 2003). Interestingly, following partial nephrectomy in skates, the formation of new nephrons, and the rate of proliferation in the nephrogenic zone were greatly enhanced in both the operated and the intact contralateral kidney.

The signals triggering neo-nephrogenesis following injury are unknown, but may be linked to the accumulation of metabolic waste or a salt imbalance (Davidson, 2011). In higher vertebrates, it is still unclear why new nephrons cannot be generated. An intriguing hypothesis suggests that the evolution of higher blood pressures has allowed mammals to respond to an increase in renal demand through incremental increases in glomerular filtration pressure. However, in organisms with low blood pressure, such as the fish, the ability to generate new nephrons has been maintained so that the capacity of the kidney can be expanded (Davidson, 2011).

Recently, two groups, using a gentamicin-mediated AKI model, published *in vivo* studies of neo-nephrogenesis using transgenic zebrafish lines (Zhou et al., 2010a; Diep et al., 2011) Zhou et al. (2010a) followed zebrafish neo-nephogenesis using two transgenic zebrafish lines: *wt1b:GFP*, expressed in the glomeruli and proximal tubules, and *pod:NTR-mCherry*, expressed in the glomeruli. Similar to other fish species, zebrafish continued to generate nephrons throughout adulthood. This was demonstrated by an increase in *wt1b:GFP*-positive cell aggregates, which constituted the new nephrons. Interestingly, this response was enhanced following renal injury. A similar observation was made in medaka where expression of *wt1* was seen in basophilic aggregates following kidney injury (Watanabe et al., 2009).

A more detailed analysis of zebrafish neo-nephrogenesis was conducted, following gentamicin-mediated AKI, and the formation of nephrogenic aggregates in post-AKI animals was observed (Diep et al., 2011). The newly developing nephrons recapitulated mesonephric neo-nephrogenesis, which consisted of small aggregates of *lhx1a* positive cells (Fig. 2) that would later enlarge and express additional renal genes,

such as *wt1b*, *pax2*, *pax8*, and *cdh17*. In an elegant series of transplantation experiments, it was demonstrated that the *lhx1a*-positive aggregates possessed the ability to generate new nephrons. Interestingly, the ability to form new nephrons was maintained after serial transplantation assays, suggesting that this aggregate of cells, termed renal progenitor cells, possessed stem cell-like properties. Based on these results, it was proposed that *lhx1a* represents the earliest marker of renal progenitor cells. This is a significant result, as it is the first indication that a cell-based therapy could be used to treat AKI in the future. It also establishes the zebrafish as the first and only model where adult renal progenitor cells can be studied undergoing successful engraftment into a functioning kidney.

THE ZEBRAFISH KIDNEY AND CHEMICAL GENETICS

The work described in this review supports the use of zebrafish for modeling human ciliopathies and to study the pathophysiology of AKI. However, the success of any animal model also lies in the ability to use that model for the identification of new therapies for a given disease. One promising area for the continued study of ciliopathies and AKI resides in the possibility of identifying small molecules with therapeutic potential to improve the etiology of human kidney diseases.

Small molecule screens represent a novel approach to identify water-soluble chemicals that can alter a biological process and can be a complement to genetic approaches (Stein, 2003; Ding and Schultz, 2004). The pharmaceutical industry has focused on such screens with assays designed to uncover therapeutics with the potential to alleviate disease. However, in academic centers, historically a lack of robotics and automated equipment prevented the exploitation of the newest developments in combinatorial chemistry and rapid target validation. The zebrafish embryo has become an attractive tool for small molecule screens because it is a vertebrate that is small in size (1-2 mm), optically transparent, and can be kept alive in 96-well plates for multiple days without the need for additional nutrients (Peterson et al., 2000). Many research groups have used zebrafish to investigate the effects of small molecules (Stern and Zon, 2003; Peterson et al., 2004; Burns et al., 2005; Murphey et al., 2006; Sachidanandan et al., 2008; Yu et al., 2008; Molina et al., 2009; de Groh et al., 2010), and a wide variety of assay tools have been successfully developed for analysis of zebrafish screens (Pichler et al., 2003; Tran et al., 2007; Vogt et al., 2009).

Fluorescent transgenic reporter zebrafish lines make it possible to discover specific molecules that can alter differentiation events during organogenesis (Molina et al., 2007). For example, Chan et al. (2002) identified molecules that could alter blood vessel formation in the zebrafish embryo, further elucidating the signaling pathways that are employed for vessel formation and regeneration (Bayliss et al., 2006; Chan et al. 2002). Not only did these studies yield insights into vessel formation and the process of angiogenesis in zebrafish, but they may also contribute to the development of assays to screen for anti-angiogenic molecules. In another study, Peterson et al. (2004) identified two structurally similar molecules that could rescue the zebrafish genetic mutant, *gridlock*, which results in the disruption of aortic blood flow and exhibits similar physiological features to aortic coarctation found in humans (Peterson 2004). The implication of these results is that there is now a basis from which to develop molecules that can alleviate aortic coarctation, based on the structures of these two molecules.

Two groups have taken advantage of the zebrafish models for PKD and other ciliopathies to test the ability of several drugs or compounds to ameliorate the presence of kidney cysts. Knockdown of *bbs4*, *bbs6*, *bbs8*, *ift80*, *nphp2*, *nphp5*, *nphp6*, *mks1*, *mks3*, or *ofd1* results in the formation of cystic kidneys along with edema (Tobin and Beales, 2008). Treatment of each morphant with rapamycin, an inhibitor of the mTOR pathway, significantly reduces the edema seen within the kidney. This effect was partially phenocopied in *ift80* morphants by treatment with roscovitine, a cyclin-dependent kinase inhibitor. These data suggest that the cysts forming in the zebrafish models of cystic kidney disease result from a mechanism similar to that which occurs in humans. Thus the identification of new compounds that suppress cyst formation in the zebrafish should translate to an improvement in kidney function in other vertebrates. To this end, a chemical modifier screen was performed, using a custom library of well-defined compounds, to identify those that modify the body axis curvature and/or laterality defects that are present in $pkd2^{hi4166}$ and $ift172^{hi2211}$ mutants (Cao et al., 2009). There were a total of six compounds that modified the axis phenotype and fourteen that affected laterality, with an overlap of only three compounds. One of these compounds, trichostatin A (TSA), was particularly interesting because of its potent ability to inhibit the activity of histone deacetylases (HDACs). Using *pkd2* morphants, it was shown that TSA could effectively reduce kidney cyst formation and this was phenocopied by a second HDAC inhibitor, valproic acid (VPA). Interestingly, a similar effect was observed in $Pkd1^{flox/flox}$;*Pkhd1-Cre* mouse kidneys following injection of VPA. The overall size of the kidney, as well as the cystic area, was reduced with a concomitant improvement in kidney function.

Since innate renal tubular regeneration occurs after tissue injury, there has been considerable interest in developing treatments that enhance the regenerative capacity of the kidney when administered post-injury. This would be a signifi-

cant advance in the field since most therapies for AKI, which have shown potential when administered prior to the onset of renal injury in experimental models, have failed to show therapeutic benefit in humans. Therefore, the identification of pluripotent renal progenitor cells in zebrafish could be important in identifying the latent regenerative pathways that exist in the human kidney (Romagnani, 2009). Likewise, studying self-renewing nephron progenitor cells in zebrafish will allow a better understanding of how to increase the capacity to generate new nephrons. In one zebrafish AKI study, a renal protective effect of the intracellular β-amino acid taurine on gentamicin nephrotoxicity and of Ucf-101, an inhibitor of the serine protease Omi/HtrA2, on cysplatin nephrotoxicity was shown (Hentschel et al., 2005). In addition, since both zebrafish and mammalian AKI models show reactivation of markers of kidney organogenesis, screens that increase the expression of the embryonic kidney genes could potentially enhance the repair process. Toward this end, a chemical screen in zebrafish identified a new HDAC inhibitor, 4-(phenylthio)butanoic acid (PTBA), that was able to expand the pool of *lhx1a*-positive embryonic renal progenitor cells (de Groh et al., 2010). Importantly, another HDAC inhibitor, TSA, has been shown to attenuate or partially reverse nephritic serum nephritis (Imai et al., 2007) and decrease the onset of fibrosis in mice (Pang et al., 2009). Testing these compounds in AKI models thus represents a promising strategy to augment kidney regeneration. Taken together, these studies solidify the zebrafish as a successful model system for studying the broad spectrum of ciliopathies and AKI that affect millions of humans worldwide, and point to a very promising future of zebrafish drug discovery.

ACKNOWLEDGMENTS

The authors thank Maria Cecilia Cirio for critical reading of the manuscript. The authors apologize to those whose work they were not able to include in this review due to space restrictions.

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