

Methods to generate and evaluate zebrafish models of human kidney diseases

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ABSTRACT Kidney-related disorders affect millions of people worldwide. A survey of chronic kidney disease (CKD) patients showed that the burden of kidney diseases is increasing every year. The global burden of disease (GBD) study 2017 ranked CKD as the 12th leading cause of deaths worldwide. Hence, identification of the causes of kidney diseases, development of accurate diagnostic methods and novel therapeutics is highly relevant. Model organisms that faithfully recapitulate human diseases play important roles in understanding the disease process and provide valuable ground to find their cure. Zebrafish is an excellent model to study the development, pathophysiology and molecular aspects of human kidney diseases. In this review, we summarize various genetic and experimental manipulations that can be carried out in zebrafish to better understand the pathophysiology of human kidney diseases. We suggest that these methods will be helpful in the development of potential therapies to treat kidney diseases.

KEY WORDS: *CKD, AKI, zebrafish model of human kidney diseases, pronephros, renal pathology*

Introduction

Kidney is one of the vital organs in vertebrates that removes waste products and maintains pH, ion and metabolite concentration of blood within physiological range. Kidney secretes erythropoietin that regulates red blood cell production and activates vitamin D which helps bones to absorb calcium. There are about a million nephrons in each human kidney, which are the structural and functional unit of this organ. Consequently, defects in nephrons affect kidney structure and functions. Dysfunction of kidney can occur because of genetic mutations, infections, injury, medicines or exposure to toxic compounds in the environment. Chronic diseases like diabetes, cardiovascular diseases and hypertension are major contributors to kidney disease burden (Levey *et al.*, 2010). Impaired kidney function increases the risk of complications in other organ systems as well (Thomas *et al.*, 2008). The common types of kidney diseases and their causes are outlined in Table 1.

Kidney diseases

Kidney diseases contribute to a significant fraction of the disease burden globally. Around 750 million people worldwide are affected with kidney related disorders (Crews *et al.*, 2019). CKD is most common among them with global prevalence of 13.4% (Hill *et al.*,

2016). The GBD study 2010 ranked CKD as the 18th leading cause of death worldwide, which had jumped to 12th by 2017 for causing maximum number of deaths globally (Jha *et al.*, 2013; Carney, 2020). According to the GBD study 2017, about 697.5 million cases of CKD were reported worldwide, among which 1.2 million people had died. The number of deaths due to CKD is estimated to rise to 4 million by 2040 in the worst-case scenario (Foreman *et al.*, 2018). One third of CKD patients live in two countries, China and India (Bikbov *et al.*, 2020). The status of CKD among Indian population is unclear due to the lack of accurate data collection systems. About 115 million cases of CKD were reported in India in 2017 (Bikbov *et al.*, 2020). The most common diseases in Indian population are diabetes and hypertension (Geldsetzer *et al.*, 2018). As per the Indian Council of Medical Research (ICMR) report, the prevalence of diabetes and hypertension among the urban adult population is 28% and 21.4% respectively (Varma, 2015). Between 40% to 60% of CKD cases occur because of these complications and their number is increasing rapidly (Rajapurkar *et al.*, 2012). The International Society of Nephrology's kidney disease data center reported a 16.8% prevalence of CDK among Indian population (Ene-lordache *et al.*, 2016). The causes of CKD vary throughout India. Andhra Pradesh, Odisha and Goa reported high levels of CKD of an unknown etiology known as chronic interstitial nephropathy (Varughese and Abraham, 2018). As the number of CKD patients

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TABLE 1

DIFFERENT TYPES OF KIDNEY DISEASES AND THEIR CAUSES

Kidney diseases	Causes
Acute kidney diseases	Shock, surgery, medications, infections or impaired cardiac function which causes injury to nephron epithelial cells.
Chronic kidney diseases	Complications like diabetes, high blood pressure, anemia, glomerulonephritis (inflammation in glomerulus), lupus and other autoimmune diseases, urinary tract infections, polycystic kidney diseases, kidney stones or renal tumors which disrupt kidney functions.
Renal stones (Nephrolithiasis)	Impaired kidney functions increase the levels of cystine, oxalate, uric acid or calcium in the urine, which form clumps.
Nephrotic syndrome	Genetic mutations, medications or other disease complications can damage the blood vessels present in the glomerulus and cause loss of proteins through the urine.
Urinary tract infection	An infection in any part of the urinary system, which comprises the glomerulus, bladder, ureters and urethra.
Congenital kidney diseases: Diureter, horseshoe kidney, renal dysplasia, ciliopathies, ADPKD, PKD, MCK, etc.	Genetic mutations.

is increasing worldwide at an alarming rate, there is an urgent need to carry out a thorough analysis of the root causes of common kidney diseases in order to find better ways for their prevention and cure. Model organisms which can mimic human physiology and diseased conditions can provide great avenues in addressing the above problems. Zebrafish has become a useful model to study development and diseases, and provides promising ways to identify new therapeutic targets and drugs. In this review, we have discussed various methods to recapitulate human kidney diseases in zebrafish and how these models can be used to understand disease pathogenicity and its underlying molecular mechanisms. This may result in the generation of novel therapeutic approaches for management and cure of kidney diseases.

Similarities between human and zebrafish kidney

The kidney development in mammals is unique as it takes place through three distinct structures during embryogenesis: pronephros, mesonephros and metanephros. The first two structures are transient in mammals and only the metanephros persists throughout the life (Smyth *et al.*, 2017; Jain, 2014). Pronephros is the functional kidney during embryonic development in lower vertebrates like amphibians and fishes, which is replaced by mesonephros as the functional kidney at later stages (Tahara *et al.*, 1993; Diep *et al.*, 2015). Table 2 describes the time duration of different forms

of kidney in human, mice, *Xenopus* and zebrafish.

Although the overall complexity of kidney increases as we move towards the higher forms, nephrons are the structural and functional units of all types of kidneys. Each nephron has three parts: renal corpuscle to filter blood, tubule to absorb and secrete solutes, and collecting duct to collect unwanted wastes for excretion. The tubular epithelium is patterned into different segments to carry out specific functions. Nephrons from all forms of kidney possess a similar segmentation pattern (Desgrange and Cereghini, 2015). The segmental organization of nephron of zebrafish pronephros and mesonephros is similar to mammalian metanephros, as shown in Fig. 1. The zebrafish pronephric tubule is divided into proximal convoluted tubule (PCT), proximal straight tubule (PST), and distal early (DE) and distal late (DL) tubules, which are analogous to the segmentation pattern of mammalian metanephric nephrons. Zebrafish has an endocrine gland named corpuscles of Stannius (CS), which maintains calcium homeostasis (Krishnamurthy, 1976). The CS is formed by transdifferentiation of renal epithelial cells in between DE and DL segments, which gradually separate from the tubule and form the paired CS gland that is positioned retroperitoneally at the surface of the kidney in adult fish (Roberts and Ellis, 2012; Naylor *et al.*, 2018). One of the major difference between zebrafish and mammalian nephron is the lack of loop of Henle in zebrafish, which acts as countercurrent multiplier to generate medullary osmotic gradient for water conservation. This segment does not have any utility in zebrafish as it is a fresh water fish (Elmonem *et al.*, 2018). Each segment possesses distinct cell types and segment specific gene expressions, which are conserved among vertebrates (Verlander, 1998; Desgrange and Cereghini, 2015). All kidney types follow similar pathways of development. The following four stages are successively involved in nephron development; A: induction of intermediate mesoderm to form renal primordium, B: epithelialization and growth of nephric duct, C: patterning of nephron into specialized segments, and D: vascularization of nephron for blood filtration (Drummond, 2003).

Methods to generate zebrafish models of human kidney diseases

Zebrafish possess one pair of pronephros which start to form after 12 hours post fertilization (hpf) and becomes fully functional by 48 hpf. Availability of whole genome sequence, 71% human genes having at least one zebrafish orthologue, ease of handling,

TABLE 2

THE DIFFERENT FORMS OF KIDNEY AND THEIR TIME DURATION IN HUMAN, MICE, XENOPUS AND ZEBRAFISH

Different form of kidney	Human	Mice	Xenopus	Zebrafish
Pronephros	Forms by the 22nd day and disappears by the 4th week of gestation 6 to 10 pairs of tubules	Begins to form at E8 3 pairs of tubules	Begins to form from stage 12.5, becomes functional by stage 36 and disappears by stage 64 3 pairs of tubules	Development starts at 12 hpf and becomes functional by 48 hpf Single pair of tubules
Mesonephros	Begins to form around the 25th day, remains present up to the 8th week of gestation and gradually degenerates thereafter 30-40 mesonephric tubules	Begins to form from E9, degeneration starts at E14.5 and all tubules disappear within 24 hr 18 pairs of tubules	Begins to form at stage 39, takes 5 days to become functional, 39 extra days to reach its full size, and remains functional throughout life ≈1000 pairs of tubules	Begins to form from 10 dpf, becomes functional by ≈14 dpf and continues to mature in later stages of development ≈300 tubules
Metanephros	Starts developing from the 5th week and nephrogenesis is complete by the 36th week of gestation ≈300,000 - 1 million nephrons	Starts developing from E10.5, and nephrogenesis continues after birth for two weeks ≈12000 nephrons	Not formed	Not formed

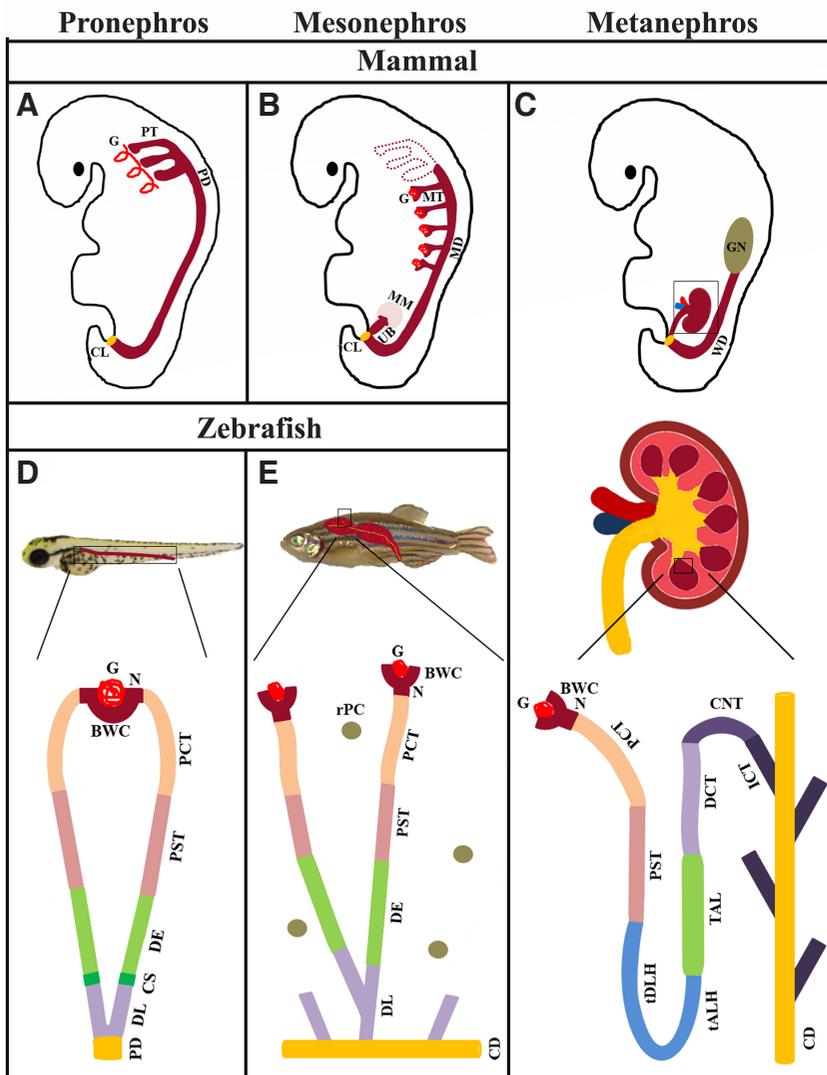


Fig. 1. Schematic representation of different forms of kidney present in mammals and zebrafish, and segmentation of the nephrons. (A) mammalian pronephros (B) mammalian mesonephros (C) mammalian metanephros (D) zebrafish pronephros and (E) zebrafish mesonephros. Pronephros and mesonephros are transient structures in mammals. A comparison of mammalian metanephric nephron segmentation (C) with zebrafish pronephros and mesonephric nephron (D & E) shows a conserved segmentation pattern (the same colour represents equivalent segments), although loop of Henle is absent in zebrafish. BWC - Bowman's capsule, CD - collecting duct, CL - cloaca, CNT - connecting tubule, CS - corpuscles of Stannius, DCT - distal convoluted tubule, DE - distal early, DL - distal late, G - glomerulus, GN - gonad, ICT - initial collecting tubule, MD - mesonephric duct, MM - metanephric mesenchyme, MT - mesonephric tubule, N - neck, PCT - proximal convoluted tubule, PD - pronephric duct, PST - proximal straight tubule, PT - pronephric tubule, rPC - renal progenitor cell, tALH - thin ascending limb of the loop of Henle, TAL - thick ascending limb, tDLH - thin descending limb of the loop of Henle, UB - uretic bud, WD - Wolffian duct.

males are treated with chemical mutagens such as ethyl methane sulphonate (EMS), ethyl nitrosourea (ENU) or physical mutagens like gamma radiation to generate a number of mutations in germ cells (Varshney and Burgess, 2014). The fish carrying mutations are then crossed with wild-type females to generate F1 fishes which contain unique alleles of generated mutation. F1 fishes are out-crossed to generate F2 carriers that are then in-crossed to obtain their homozygous mutants in F3 generation. The offspring having the desired phenotypes are then isolated and used for genetic mapping and sequencing to identify the mutated gene (Patton and Zon, 2001). Other variations of this screen, such as using haploid and homozygous diploid, can help to reduce the time and effort needed to identify the

transparent embryos, short generation time, efficient gene manipulation techniques, methods for transgenic fish generation and its rapid screening make zebrafish the most suitable model organism to study kidney abnormalities (Howe *et al.*, 2013; Pouretezadi and Wingert, 2016). One of the crucial features of adult zebrafish is its ability to regenerate new nephrons by the process of neonephrogenesis in response to renal injury (Chambers and Wingert, 2016). Table 3 describes the comparisons between zebrafish and other common model organisms like mice and *Xenopus*. Genetic manipulations in zebrafish have shown that the novel genetic components of kidney development and function can be identified by using this model organism (Pouretezadi and Wingert, 2016). There are several methods to generate zebrafish models of human kidney diseases.

Forward genetics

The forward genetics screen is used to identify genes that are associated with phenotypes of interest (Lawson and Wolfe, 2011). Both chemical and insertional mutagenesis are used in forward genetics screens (Patton and Zon, 2001). Chemical mutagenesis is a three generation-based screening in which adult

phenotype and the causative genetic mutation (discussed in detail by Patton and Zon, 2001). Transgenic reporter lines expressing fluorescent proteins in the organ of interest can be combined with conventional forward genetic screening to easily screen mutants of interest. An ENU based mutagenesis screen led to the identification of *lightbulb (lib)* mutant zebrafish which show morphological and nephron segmentation defects similar to retinoic acid (RA) deficient zebrafish embryos. It was found that *lib* mutants have C to A transition at nucleotide 174 of *aldh1a2*, which is predicted to synthesize a truncated protein of 58 amino acid length, thus abrogating *aldh1a2* function and affecting synthesis of RA from retinaldehyde. Analysis of *lib*, *neckless (nls)* mutant having point mutation in catalytic domain of *aldh1a2* and DEAB (retinoic acid synthesis inhibitor) treated embryos revealed that the levels of RA modulate nephron segmentation by changing the spatial expression of segments specific transcription factors (Wingert and Davidson, 2011; Begemann *et al.*, 2001; Mullins *et al.*, 1994). Another mutant called *zeppelin* helped to identify *brac2* as a regulator of podocyte development (Kroeger *et al.*, 2017).

Insertional mutagenesis is a transposon or lenti-virus based method that is used to insert DNA randomly at different genomic loci, and this foreign DNA then helps to identify the mutated gene

TABLE 3

A COMPARISON BETWEEN COMMONLY USED MODEL ORGANISMS: MICE, XENOPUS AND ZEBRAFISH.

	Mice	Xenopus	Zebrafish
Human genes that have at least one ortholog	80%	—	71%
Human disease associated genes that have at least one ortholog	90%	79%	84%
Maintenance	High cost	Cost effective	Cost effective
Development	In-utero	Ex-utero	Ex-utero
Generation time	6-8 weeks of age	4 months for <i>X. tropicalis</i> and 12 months for <i>X. laevis</i>	3 months
Brood size	6-12 pups/month	2-3 thousand, 3 to 4 times in a year	≈200 embryos/week
Time duration to generate homozygous mutants/ transgenic lines	5 months to 1 year	1-2 years	9-10 months
Mutant screening techniques	Not easy, some phenotypes take time to develop	Easy phenotype-based screening, embryos are opaque, in vivo imaging not at deep tissue level	Easy phenotype-based screening, embryos are transparent, in vivo imaging
Drug screening	Truly recapitulates human phenotype, low throughput and expensive	High throughput and cost effective	High throughput and cost effective
Difference with human metanephric nephron	Similar	Glomus project in the body cavity not directly connected with tubules	Loop of Henle is absent

(Amsterdam *et al.*, 1999). The Hopkins' Laboratory at MIT performed an insertional mutagenesis screen in zebrafish embryos using a retroviral vector. They identified 12 genes which were associated with defective kidney phenotypes showing cysts in the glomerular-tubular region, among which four were linked with cilia formation (Sun *et al.*, 2004). The drawbacks of the forward genetics approach are that the screening process is cumbersome and time-consuming. However, the advances in new sequencing technology have made the forward genetics approach much more efficient.

Gene knock-out using programmable nucleases

One of the major advantages of using zebrafish as a model organism is the availability of efficient genome editing techniques such as CRISPR/Cas, TALEN and ZFN (Sertori *et al.*, 2016). CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat)/Cas9 is the most versatile and commonly used genome editing technology in zebrafish. A chimera of CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA) is designed as a single guide RNA (sgRNA), which along with Cas9 protein/mRNA are injected into zebrafish embryos at one cell stage (Varshney *et al.*, 2016). The sgRNA binds to the 20bp target DNA sequence adjacent to a protospacer adjacent motif (PAM) NGG sequence in the genome, Cas9 then generates a double strand cut in the target DNA sequence. This is then preferably repaired by non-homologous end joining, which being an error-prone repair mechanism results in the generation of random insertion/deletion (indel) leading to changes in the reading frame of coding sequence, and consequently abrogates the functions of the gene of interest. Advance methodologies have been developed in recent years to increase the efficacy of the CRISPR based genome editing technique (Liu *et al.*, 2019). It is possible to delete a large fragment of DNA using two or more sgRNAs at a time (Kim and Zhang, 2020). It is also possible to simultaneously target multiple genes efficiently in a single zebrafish embryo (Shah *et al.*, 2016). Many studies have been conducted to generate zebrafish mutants in order to recapitulate human kidney diseases by using CRISPR/Cas9 technology. CRISPR mediated knockout of ciliary membrane protein Arl13b resulted in generation of mutants which mimic the "Joubert syndrome" (Cantagrel *et al.*, 2008). It is reported that mutations in human *ELMO1* gene contribute to diabetic nephropathy. *elmo1* mutant zebrafish exhibit

phenotypes seen in hyperglycaemic embryos generated by knock-down of *pdx1* gene such as larger glomerulus, defective podocyte, shorter neck and hyperfiltration, thus highlighting the conserved function of this gene (Sharma *et al.*, 2016). Mutant *magi2a* zebrafish exhibit steroid-resistant nephrotic syndrome which is also observed in people with mutations in *MAGI2* (Jobst-Schwan *et al.*, 2019). These observations highlight the suitability of zebrafish as an organism to model human kidney diseases.

Another method to generate zebrafish mutants is Transcription Activator-Like Effector Nucleases (TALEN) based genome editing. In this technique, the DNA binding domain TALE is made up of monomers, each monomer consisting of tandem repeats of 34 amino acid residues that bind to a particular nucleotide in the DNA sequence. These TALE sequences are derived from pathogenic bacteria *Xanthomonas* that alters the transcription of genes in host cells (Boch and Bonas, 2010). The TALEN targeting construct consists of a nuclear localization signal, the DNA binding domain and the FokI nuclease domain at their carboxyl termini. TALEN work in pairs with their binding sites located on opposite strands of DNA separated by a 12 to 25 bp spacer sequence. The constructs bind to the target site in the nucleus and generate a double strand break, which is then repaired by a non-homologous end joining (Liu *et al.*, 2014). The mutant zebrafish generated by CRISPR or TALEN can activate genes that compensate for the loss-of-gene of interest, thus making it difficult to uncover the gene function (Rossi *et al.*, 2015). However, it is possible to identify these compensatory genes by transcriptome analysis using next-generation sequencing (NGS) methods, which can help to identify the function of the gene of interest. Identification of genes and molecular pathways that compensate for the loss-of-function of a gene can be helpful in finding novel treatment methods for gene mutations that cause serious diseases (El-Brollosy and Stainier, 2017).

Gene knock-down using morpholino antisense oligos

Morpholinos are non-anionic oligonucleotides that are relatively stable as compared to its DNA or RNA oligomers (Summerton, 1999). The morpholino antisense oligo nucleotides bind to the mRNA of a target protein at its translation initiation site and block its translation. Another type of morpholino antisense oligos can target splicing junctions and block pre-mRNA splicing (Summerton,

1999). Morpholino-antisense oligo effect is transient and can be used to study the early developmental role of the genes of interest. Injection of morpholino-antisense oligo is done at the 1-4 cell stage of zebrafish embryos and its effect can be seen up to 3-5 days post fertilization (dpf) (Bill *et al.*, 2009). Morpholino antisense oligo nucleotides often display off-target effects (Robu *et al.*, 2007). Control experiments such as a rescue experiment should be performed by coinjecting morpholino along with mRNA that cannot be targeted by this antisense oligo to verify its specificity (Eisen and Smith, 2008). A photo activated morpholino has been developed, whose activity can be controlled in spatial and temporal manner by UV exposure (Tallafuss *et al.*, 2012). Numerous studies have been carried out using morpholino antisense oligo mediated gene knock-down to understand human kidney diseases. For example, knock-down of *nephrocystin-3* gene led to the formation of cysts and hydrocephaly in zebrafish embryos in a similar way to nephrophthisis type-3 disease (Zhou *et al.*, 2010).

Drug induced kidney damage model

Aminoglycoside antibiotics generally used to treat many life-threatening infections are known for their nephrotoxic and ototoxic effect (Mingeot-Leclercq and Tulkens, 1999). Gentamicin is a commonly used antibiotic that can induce acute kidney injury (AKI) in zebrafish. Gentamicin causes flattening of the brush border epithelium, loss of tubular epithelium, deformation of glomerulus structure, lysosomal phospholipidosis and accumulation of leukocytes or cell debris in the tubular lumen, which mimics gentamicin overdose in humans (Cianciolo Cosentino *et al.*, 2010). Zebrafish has the ability to regenerate and replace damaged nephrons. Gentamicin insult can be used to study renal regeneration in zebrafish (Kamei *et al.*, 2015). Gentamicin injury induces a regeneration response which triggers kidney stem cells to undergo the stages of specification, proliferation and differentiation to generate new nephrons. It takes around 14 to 21 days in adult fish to regenerate nephrons (Diep *et al.*, 2011; McCampbell *et al.*, 2015). Cisplatin, which is used as a chemotherapeutics drug to treat tumors, also has nephrotoxic effects, as observed in zebrafish (Hentschel *et al.*, 2005). Etimicin is another aminoglycoside that can be used while mimicking low nephrotoxicity and ototoxicity in zebrafish embryos (Shao *et al.*, 2020). Thus, AKI and renal regeneration can be studied in zebrafish with the help of these antibiotics.

Mechanical injury to kidney

The zebrafish pronephros can be physically injured using resection, stabbing or cryoinjury. Surgical injury can be performed by using fine tweezers to stab the desired area of the pronephros in order to create an AKI model. This technique was used to damage the pronephric duct close to the cloaca, which impaired fluid flow and led to cyst formation within 30 minutes (Kramer-Zucker, 2005). It was found that the reduction in fluid flow rate in pronephros generates back pressure at the fluid entry site, causing tubule luminal expansion and cyst formation. Another group of researchers made obstruction in the pronephric tubule at 50 hpf or in mesonephric tubules of 12-month-old zebrafish by using tweezers to pinch off the area near the distal collecting tubules (Hellman *et al.*, 2010). They discovered that the tubule scarring leads to an increase in cilia beating rate and an upregulation of the *foxj1a* transcription

factor that regulates ciliogenic gene expression. This suggests that injury to pronephric tubules generates a cilia based mechanosensory signal to maintain nephron homeostasis.

Laser induced kidney injury

Laser mediated cell ablation is used as a tool to study renal injury and mimic AKI in zebrafish. Johnson *et al.*, (2011) have described a method for laser mediated ablation followed by tracking of regenerating nephron. They injected 40 kDa dextran-FITC in the trunk somites of zebrafish embryos at 48-55 hpf to label the proximal tubule epithelial cells, which were then targeted for laser mediated ablation at 72 hpf. The ablated embryos were then reinjected with rhodamine dextran to trace the proximal tubule epithelial cells. They found that a fully developed proximal tubule was formed at the 7th day following laser mediated ablation (Johnson *et al.*, 2011). Another alternative is to use a kidney specific transgenic line to identify cells for laser mediated ablation and monitor behaviors of neighboring cells. A violet laser light of 405 nm wavelength was used to target pronephric tubule of *Tg(atp1a1a.4:GFP)* zebrafish (Palmyre *et al.*, 2014). As GFP excitation spectra lies in the range of blue to violet light, it absorbed 405 nm laser light, which acts as energy sink to potentially induce injury at the target epithelial cells. This experiment led to the discovery that cell migration is the primary response of injured epithelia. Collective cell migration caused mechanical stretch that provided stimuli for cell proliferations to repair the injured tubule. In order to determine the time when the pronephric tubule of zebrafish acquire the ability of regeneration, Yakulov *et al.*, (2018) used a 2 photon laser to ablate a small part of pronephric tubules in *Tg(cldn2b:lyn-GFP)* embryos at different time points and followed the regeneration process. They found that the ablation of pronephric tubules of 2-day old embryos was rapidly repaired by migratory responses, whereas 1 day old embryos did not have this ability. They carried out gene expression profiling of injured zebrafish embryos and found that *cxcr4b* and *myca* are involved in this repair process (Yakulov *et al.*, 2018).

Chemical genetics

Small bioactive molecules can be used to interfere with protein function and understand their biological role. A large number of chemical libraries are commercially available or can be custom-made for probing protein function. These small molecule libraries include kinase inhibitors, protease inhibitors, nuclear receptors and ligands that can be used to identify the role of signaling pathways involved in organ development and function (Kawasumi and Nghiem, 2007). In the last few decades, zebrafish has emerged as a powerful vertebrate model organism for high-throughput chemical screening and phenotypic scoring (Kaufman *et al.*, 2009). Cao *et al.*, (2009) used the chemical screen approach to identify compounds that can reverse phenotypes caused by mutations in *pkd2* (causal gene of PKD) and *ift172* (a gene responsible for cilia formation). They uncovered that a pan-histone deacetylase (pan-HDAC) inhibitor trichostatin A (TSA) and a class-I specific HDAC inhibitor valproic acid (VPA) can suppress kidney cyst formation in *pkd2* knock-out model (Cao *et al.*, 2009). Thus, chemical genetics can be a useful tool to identify new drug candidates that can either reverse or suppress disease conditions.

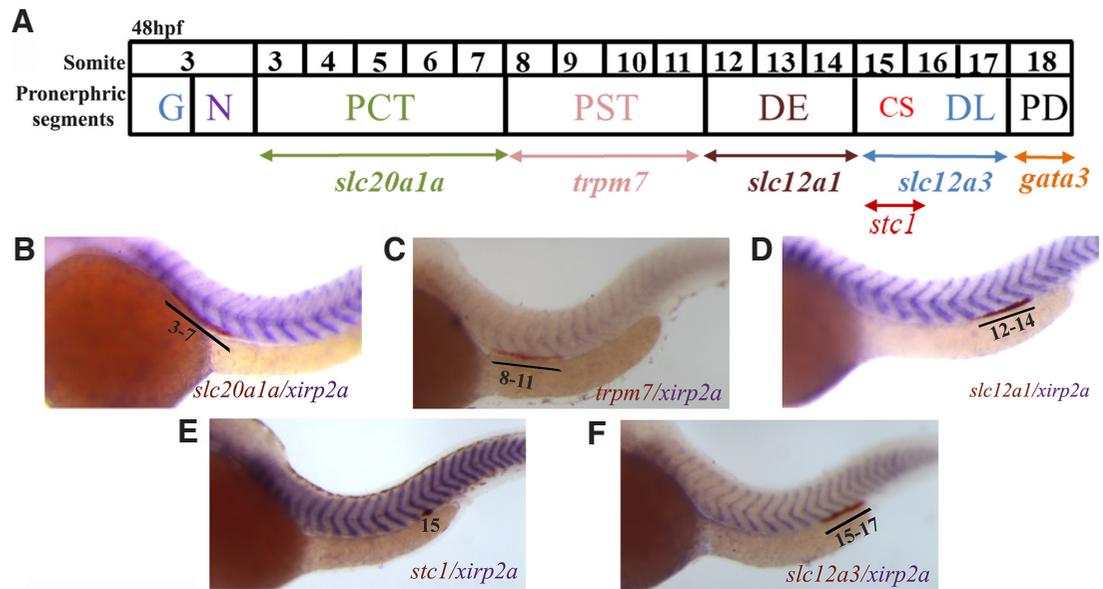


Fig. 2. Identification of segments of zebrafish pronephros using whole mount double *in situ* hybridization at 48 hpf.

(A) Schematic representation of pronephros segmentation with respect to somite and the genes expressed in specific segments (B) PCT markers *slc20a1a* (C) PST marker *trpm7* (D) distal early marker *slc12a1* (E) CS marker *stc1* and (F) distal late marker *slc12a3* in magenta and somite marker *xirp2a* (purple) are shown. The numbers denote the position of each segment with respect to the somite position.

Genetically inducible kidney injury models

A genetically inducible tissue ablation model can be used in zebrafish in which bacterial nitroreductase (NTR) is expressed under the control of a promoter of choice that drives expression of NTR in a particular segment of pronephros. NTR converts metronidazole into a cytotoxic metabolite that can cause death to NTR expressing cells (Curado *et al.*, 2008). Zhou and Hildebrandt (2012) used this technique to induce injury of podocyte by expressing NTR under the control of *podocin* promoter in *Tg(pod:NTR-mCherry)* zebrafish line. They also developed a double transgenic line of VDBP-GFP (Vitamin D binding protein tagged with GFP) as a tracer of proteinuria along with *Tg(pod:NTR-mCherry)* line. Treatment with metronidazole caused injury of podocyte resulting in whole body edema and accumulation of VDBP-GFP in proximal tubules, mimicking the phenotype of the human nephrotic syndrome (Zhou and Hildebrandt, 2012).

Methods to evaluate pronephros development and function in zebrafish

We have described the methods to generate human kidney disease models by using zebrafish. The creation of a disease model using another organism is the initial step, which needs to be evaluated for its ability to faithfully recapitulate various aspects of a disease seen in humans. Zebrafish offers many advantages that can be used to quickly evaluate its ability to serve as a surrogate to understand human kidney diseases as discussed below.

Morphology based screening

Most often mutant zebrafish embryos show morphological differences, compared with the wild-type that can be easily judged by observations under the microscope. The transparency of zebrafish embryos and their ability to survive up to 5 days even with severe developmental defects is a major advantage in morphology-based screens. Common morphological changes observed in zebrafish embryos with defective pronephros include pericardial edema,

pronephric cysts, curved body axis and hydrocephalus (Pouret-ezadi and Wingert, 2016; Outtandy *et al.*, 2019). Edema is one of the common signs of a defective kidney which is also seen in other organ deficiencies such as heart development (Hanke *et al.*, 2013). Mutants with kidney defects may develop cysts because of over-proliferation of epithelial cells, as can be easily observed under a microscope (Zhao and Malicki, 2007; Yamaguchi *et al.*, 2006). Curved body axis is often seen in mutants having pronephric cilia defects. Mutant zebrafish such as *locke (lok)*, *shen yan^{tt214} (shy)*, *garbus^{tm304} (grb)* and *zator^{Ag238a} (zar)* are some of the examples showing curved body axis (Zhao and Malicki, 2007). Zebrafish mutants with kidney defects show multiple morphological defects in the same embryo. The knock-down of two polycystin genes *pkd1a* and *pkd1b* led to dorsally curved body axis, hydrocephaly, cartilage and craniofacial defects with low frequency of pronephric cysts (Mangos *et al.*, 2010). The mutants of intraflagellar transport proteins *ift57*, *ift88* and *ift172*, where cilia were defective, had ventrally curved body axis (Lunt *et al.*, 2009). Thus, the zebrafish mutants with defective kidney exhibit many morphological features that can be easily identified.

Histological analysis

The mutants may not always show sufficiently informative morphological changes. Histological analysis of these embryos or organs of the adults may be necessary to determine the difference between the mutant and wild-type animals. Histological analysis methods for both larvae and adult zebrafish are well established and can be performed in a high throughput manner (Sabaliauskas *et al.*, 2006). Zebrafish embryos or adult tissue can be embedded in paraffin or JB-4 resin followed by microtome sectioning to study tissue architecture (Sullivan-Brown *et al.*, 2011; Copper *et al.*, 2018). Cryo-sectioning can also be performed with zebrafish embryos (Ferguson and Shive, 2019). These tissue sections are then used for immunofluorescence staining, immunohistochemical studies or H&E staining. H&E staining of adult kidney sections showed that the apical side of proximal tubule was stained dark pink and had a wide lumen, while the distal tubule had light pink

stain with narrow lumen, thus clearly marking the differential staining pattern between the segments (McC Campbell *et al.*, 2015). The periodic acid-Schiff (PAS) staining technique which detects polysaccharides in tissues has affinity for brush border epithelium of proximal tubule (McC Campbell *et al.*, 2015; McKee and Wingert, 2015). Methenamine silver stains the basement membranes and can be used for nephric tubules and glomeruli basement membrane staining (McC Campbell *et al.*, 2015). An AKI model of zebrafish by gentamicin insult showed flattening of epithelium, loss of apical brush border, tubular distention and accumulation of debris in lumen, thus highlighting the usefulness of histology in analyzing zebrafish disease models (Cianciolo Cosentino *et al.*, 2013).

Identification of pronephros segmentation defects

The pronephros is patterned into different segments that perform distinct functions. The mechanism behind this segmentation is not clearly understood, although many transcription factors have been identified as regulators of segmentation. The differences in segmental pattern can easily be identified by WISH analysis with riboprobes that specifically mark different segments of the pronephros. The exact position of the pronephros segments can be marked by implementing double *in situ* hybridization of segment specific markers and an antisense riboprobe that marks the somite (such as *smyc1* and *xirp2a*). Most common segment specific markers are *slc20a1a* for PCT, *trpm7* for PST, *slc12a1* for DE, *stc1* for CS and *slc12a3* for DL (Fig. 2). Mutations in human HNF1 β are linked with renal abnormalities like renal dysplasia, glomerulocystic kidney, oligomeganephronia and solitary functioning kidney (Lindner, 1999; Bingham *et al.*, 2002; Bohn *et al.*, 2003). Naylor *et al.*, (2013) analysed pronephros segmentation by WISH in *hnf1b* knock-out zebrafish embryos using segment specific marker genes and found that proximal and distal tubule markers were absent in the mutants. Using similar experiments, it was found that transcription factor empty spiracles homeobox gene 1 (*emx1*) promotes distal late fate and inhibits distal early fate during nephrogenesis (Morales *et al.*, 2018). Wingert *et al.*, (2007) carried out WISH analysis of RA and DEAB treated embryos and found that DEAB treatment resulted in a loss of the proximal segments and an expansion of the distal segments, while exogenous RA treatment reversed this phenotype. They also established a link between caudal transcription factor (*cdx*) and RA in regulating nephron position and segmentation (Wingert *et al.*, 2007). We have shown that the EF-hand domain containing 2 (*efhc2*) knockdown results in expansion of distal early segments and reduction in CS and distal late segment. The expression of *odf3*, which marks multiciliated cells of pronephric tubules, was also reduced in *efhc2* morphants (Barrodia *et al.*, 2018).

Pronephric cilia staining and imaging

Cilia are microtubule-based organelles that are either motile or non-motile. Human disorders caused due to defects in cilia structure and function are called ciliopathies. Defects in cilia present in zebrafish pronephros often lead to body curling, cyst formation and tubule dilation (Sullivan-Brown *et al.*, 2008). Multiciliated cells present in zebrafish pronephros can be visualized by WISH or fluorescence *in situ* hybridization (FISH) using antisense *odf3b* or *rfx2* riboprobes (Liu *et al.*, 2007; Barrodia *et al.*, 2018). Cilia in zebrafish embryos

can be stained using α -acetylated tubulin and γ -tubulin can be used to mark the basal bodies (Jaffe *et al.*, 2010; Zaghoul and Katsanis 2011). Movement of motile cilia can be recorded using a microscope with a high speed camera by employing transgenic zebrafish such as *Tg(Foxj1a:GFP)* (Tavares *et al.*, 2017). A combined technique of FISH and immune fluorescence assay was developed to mark multiciliated cells, cilia and basal bodies (Marra *et al.*, 2017). Different zebrafish mutants with cilia defects such as *locke*, *swt* and *kurly* were examined in detail and it was found that they showed a range of cilia movement defects (Sullivan-Brown *et al.*, 2008). The ciliary motion was reduced in *locke* mutant and cilia were immotile in *swt*, whereas cilia movements in *kurly* ranged from motionless to irregular shifts. Immunostaining with α -acetylated tubulin showed that the length of cilia was normal in *swt* and *kurly* whereas *locke* displayed shorter cilia (Sullivan-Brown *et al.*, 2008). The methods described here have been used extensively to identify cilia defects in kidney diseases involving cilia.

Evaluation of glomerulus function

The main functions of the kidney is to filter blood and remove wastes and excess fluids from the body while preventing the loss of macromolecules into the urine. The glomerulus can filter out molecules of 5 kDa but do not allow excretion of larger molecules such as serum albumin (Chang *et al.*, 1976). Diagnostic methods commonly used to evaluate kidney dysfunction in humans cannot be applied to zebrafish because of its small size. However, fluorescent dyes of different molecular weights mimicking the molecules commonly encountered by the human kidney can be injected into zebrafish, and the assessment of their clearance or retention can be used as a surrogate to determine kidney function (Christou-Savina *et al.*, 2015). It has been proven that the injection of 10 kDa fluorescent dextran into the pericardial cavity of zebrafish embryos results in a loss of about 85% of dye through secretion from the kidney within 24 hours post-injection (hpi) (Christou-Savina *et al.*, 2015). Dyes of higher molecular weight such as 70 kDa or above need injection into vasculature and are retained within in wild-type embryos. However, 70 kDa dextran could be detected in the proximal tubule wall when injected into the vasculature of *cystinosis* (*ctns*) mutant zebrafish, indicating that the integrity of glomerulus filter slits is compromised in *ctns*^{-/-} larvae (Elmonem *et al.*, 2017). Kramer-Zucker *et al.*, (2005) injected 500 kDa FITC-dextran into the cardinal vein of 84 hpf wild-type and *nephrin* and *podocin* morphant zebrafish embryos, and detected the dye in the pronephros indicating dysfunction of nephrons in these morphants.

Evaluation of reabsorption of metabolites

Transmembrane endocytic receptor megalin/LRP2, its adaptor disabled2 (*dab2*) and coreceptor cubilin play a central role in endocytosis mediated clearance of metabolites from glomerular filtrate (Anzenberger, 2006). The injection of 70 kDa fluorescently labelled dextran or fluorescently conjugated receptor-associated protein (RAP), a protein that physically associates with megalin/LRP2 in bloodstream of zebrafish embryos, leads to uptake of these molecules for reabsorption. This serves as a convenient method for evaluating the metabolite reabsorption function of the kidney. In agreement with their central role in the reabsorption of metabolites, the knock-down of either megalin/LRP2 or *dab2*

leads to a complete failure of receptor mediated endocytic uptake of tracers in morphants (Anzenberger, 2006).

Assessment of tubule dilation

The pronephric tubule is lined by a single layer of polarized epithelial cells. The morphology of pronephric tubule and its patterning into distinct segments are controlled by the proliferation of differentiated epithelial cells near the distal end and their migration towards the glomerulus. These events are in turn governed by the fluid flowing in the pronephros, thus providing a correlation between organ morphology and function (Vasilyev *et al.*, 2009). The cells at the proximal end are convoluted and more columnar in shape, whereas the cells at the distal end are cuboidal (Vasilyev *et al.*, 2009). A decrease in glomerular filtration rate, obstruction in tubule or defects in cilia development and motility inhibit this collective cell migration from posterior to anterior direction. However, cells at the distal end continue to proliferate, causing the dilation of pronephric tubules (Naylor and Davidson, 2017). Tubule dilation can be assessed either by directly observing whole embryos under microscope or histological analysis. DIC optics can be used to image and calculate the diameter of pronephric tubule of zebrafish embryos. Sullivan-Brown *et al.*, (2008) compared the tubule dilation in wild-type and *kurly* mutants having defects in cilia, and found that in the wild-type the medial tubule had larger diameter compared with the posterior tubule, and that the diameter of medial tubules decreased over time. In *kurly* mutants the diameter of the medial and posterior tubules was similar to the wild-type at 26-30 hpf, but a constant increase in medial tubule diameter was observed in these mutants at 48 hpf onwards. It was further observed that the number of cells surrounding the medial tubule also increased in mutant embryos (Sullivan-Brown *et al.*, 2008). Mutations in the human *MNX1* (motor neuron and pancreas homeobox 1) gene cause Currarino syndrome, a rare congenital disease characterized by sacral agenesis and urogenital and renal abnormalities such as horseshoe kidney, single kidney, hydronephrosis and anorectal stenosis (Currarino *et al.*, 1981; Lee *et al.*, 2018; Dworschak *et al.*, 2021). Ott *et al.*, (2016) generated *mnx2b* morphants in a *Tg(-8cldnb.1:lynEGFP)^{zf108}* background to image epithelia cells in developing pronephros, and found that the morphants showed enlarged proximal tubule diameters as compared with wild-type controls at 4 dpf. Further analysis revealed that these morphants had altered kidney functions, disorganized pronephric cilia and deformed apical microvilli (Ott *et al.*, 2016). Such analysis using zebrafish would undoubtedly help us to understand the underlying mechanism of human diseases.

Assessment of epithelial cells polarity

The epithelial cells polarity of the pronephric tubule is maintained by protein complexes that segregate the cellular membrane into apical and basolateral domains, and organize membrane subdomains for specific functions like secretion, filtration, absorption and sensory stimulation (Pieczynski and Margolis, 2011). The dislocation of a number of receptors, transporters and channels has been identified in many disease conditions like Na⁺/K⁺-ATPase, Na⁺/K⁺/2Cl⁻ cotransporter and EGFR in PKD and H⁺-ATPase in Dent's disease (Wilson, 2011). The polarity of epithelial cells can be checked by immunofluorescence staining of whole embryos

using antibody against Na⁺/K⁺-ATPase, tight junction marker ZO-1 or alkaline phosphatase (AP) to identify the defects in polarization of tubule epithelia in mutants compared with wild-type embryos. Na⁺/K⁺-ATPase is one of the most abundant proteins in tubular epithelial cells which maintains sodium-potassium homeostasis and regulates the functions of other transporters present in epithelial cells (Fernández and Malnic, 1998). It is localized to the basolateral plasma membrane and is important for epithelial cell polarization and the formation and maintenance of tight junctions (Rajasekaran *et al.*, 2001). ZO-1 and AP are used to mark the apical surfaces of the pronephric epithelial cells. Drummond *et al.*, (1998) analyzed a group of mutants having mild to severe defect in pronephros. They checked the polarity of epithelial cells in 2.5 dpf embryos by immunofluorescence staining with anti-Na⁺/K⁺-ATPase alpha subunit monoclonal antibody (α 6F) followed by tissue sectioning. This analysis showed that Na⁺/K⁺-ATPase localization was altered in most of the mutant lines compared to its normal basolateral expression. In *double bubble (dbb)* and *fleer (flr)* mutants, the Na⁺/K⁺-ATPase was expressed in the apical surface while basolateral surface showed reduced staining. Other mutants had more lateral staining, with unstained apical and basolateral surfaces (Drummond *et al.*, 1998).

Kidney stone detection

Kidney stones are crystals of deposited salts, among which calcium stones are the most common (Evan, 2010). These are composed of calcium oxalate (CaOx) and calcium phosphate (CaP) in different ratios. Calcium stones can be expected in zebrafish mutants having altered calcium homeostasis. Vital dyes such as Alizarin red (red fluorescent) and Calcein (green fluorescent) can be used to detect calcium-containing tissues and kidney stones in zebrafish larvae. Elizondo *et al.*, (2010) showed that 57 - 97% of *trpm7* homozygous mutant embryos developed kidney stones at 5 dpf, whereas only 0-1.4% of wild-type siblings developed such stones. Imaging of alizarin red-stained *trpm7* homozygous mutant embryos at different time points showed that 2-4 dpf embryos had no stones, and the stones were observed at 5 dpf in the lumen and not in the epithelium of pronephric tubule (Elizondo *et al.*, 2010).

Conclusions and future prospects

The incidence of kidney diseases is rising at an alarming rate worldwide. There is an urgent need to identify the causes of these diseases and develop novel methods for their diagnosis and cure. The mammalian metanephric kidney is complex, making it difficult to understand the kidney disease pathology. The pronephros in zebrafish larvae is functional and has only two nephrons on either side of notochord with a shared glomerulus at the anterior and a cloaca at the posterior end. In this review, we have discussed various methods that can be used to generate zebrafish models of human kidney diseases and how to analyse the phenotype of these disease models at morphological, cellular and molecular level. Painstaking research by many groups have established these methods of disease model generation and analysis over the years. These efforts have now established that the zebrafish embryos and adults can be used as human kidney disease models that can faithfully recapitulate various aspects of kidney dysfunction seen in humans. These efforts have also generated many useful tools

and resources, including mutant and transgenic lines. This offers an opportunity not only to understand kidney disease mechanisms using zebrafish, but to use them to discover new drugs for treating kidney diseases. Diabetes is a major contributor to kidney related complications in humans. Zebrafish offers an opportunity where the diabetes-related kidney dysfunction can also be studied (Jörgens *et al.*, 2012). Thus, zebrafish has an excellent foundation as a disease model and offers enormous potential to find novel solutions to human diseases.

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Author contribution

SF conceived and wrote the first manuscript. UN and RKS discussed and modified the manuscript.

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