



# HHS Public Access

Author manuscript

*Curr Pathobiol Rep.* Author manuscript; available in PMC 2017 July 05.

Published in final edited form as:

*Curr Pathobiol Rep.* 2015 June ; 3(2): 163–170. doi:10.1007/s40139-015-0080-4.

## Zebrafish Models of Kidney Damage and Repair

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### Abstract

The vertebrate kidney possesses the capacity to repair damaged nephrons, and this potential is conserved regardless of the complexity of species-specific kidneys. However, many aquatic vertebrates possess the ability to not only repair existing nephrons, but also generate new nephrons after injury. Adult zebrafish have the ability to recover from acute renal injury not only by replacing lost injured epithelial cells of endogenous nephrons, but by also generating de novo nephrons. This strong regeneration potential, along with other unique characteristics such as the high degree of genetic conservation with humans, the ease of harvesting externally fertilized, transparent embryos, the accessibility to larval and adult kidneys, and the ability to perform whole organism phenotypic small molecule screens, has positioned zebrafish as a unique vertebrate model to study kidney injury. In this review, we provide an overview of the contribution of zebrafish larvae/adult studies to the understanding of renal regeneration, diseases, and therapeutic discovery.

### Keywords

Acute kidney injury; Chronic kidney disease; Polycystic kidney disease; Regeneration; Small molecule screening; Zebrafish

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This article is part of the Topical Collection on *Zebrafish as a Model for Pathobiology*.

Compliance with Ethics and Guidelines

**Conflict of Interest:** Maria Cecilia Cirio and Neil Hukriede have no conflicts of interest. Mark de Caestecker is a consultant for Nephrogenix.

**Human and Animal Rights and Informed Consent:** This article does not contain any studies with human or animal subjects performed by any of the authors.

## Introduction

For more than a decade, zebrafish researchers have been developing larval and adult models of acute kidney injury (AKI). AKI is defined as a sudden loss of renal function resulting in an increase in circulating waste products [1]. AKI results from largely multifactorial insults, including but not limited to decreased renal blood flow, urinary obstruction, exposure to toxic agents, and as a consequence of sepsis, with US healthcare costs for AKI estimated at \$10 billion annually [2, 3]. Each year in the United States, AKI incidents requiring dialysis affect more than 90,000 patients, while non-dialysis-dependent AKI affects more than 1.5 million patients [4–6]. AKI is also thought to be one of the risk factors for chronic kidney diseases (CKD), and there is an increased risk of end-stage renal disease in patients with severe AKI [7–12]. Despite this, no established therapies are proven to significantly prevent renal injury, accelerate the rate of renal recovery, or prevent post-injury fibrosis and chronic renal insufficiency after AKI [13]. Using the anatomically simple zebrafish kidney, researchers hope to provide insights into kidney regeneration mechanisms with the promise of developing future renal therapeutics.

The vertebrate kidney has the ability to repair damaged nephrons, which is conserved across species with varying regenerative potential. While a thorough understanding of the molecular mechanisms that govern kidney repair are still incompletely understood [14], there is a well-defined sequence of cellular events that drives functional organ recovery [15, 16]. The mammalian kidney does not likely harbor a stem cell population, but instead, the repair process is driven by dedifferentiation of surviving injured renal tubule epithelium followed by proliferation and repopulation of the denuded tubules [17–20]. This process is thought to be phenotypically and mechanistically conserved between human [20], mouse [15, 17], and larval zebrafish kidneys [21•], allowing for reagents and pathways discovered in zebrafish to be utilized for mammalian regeneration studies. In contrast to this, in adult zebrafish, an adult “stem cell” population has been identified that drives neo-nephrogenesis during both organism growth and injury events [22•]. While this mechanism does not occur in the mammalian kidney, findings from post-injury neo-nephrogenesis in zebrafish may also provide insight into pathways that promote whole nephron regeneration in the mammalian kidney after injury.

Zebrafish larvae contain a functional pronephros composed of two nephrons whose glomeruli fuse at the trunk midline. The zebrafish larval pronephric kidney is anatomically simple, while conserving the cellular and molecular complexity of higher vertebrates. Its accessibility for visualization and manipulation represents a great advantage for the study of the mechanisms involved in the pathophysiology of AKI. As with mammalian nephrons, the zebrafish pronephric tubules are segmented into different specialized regions: a neck, proximal convoluted tubule, proximal straight tubule, distal early tubule, Corpuscle of Stannius, distal late tubule, and duct [23]. The zebrafish pronephros is thought to possess a lumen by 24 h post-fertilization (hpf) and shows initial functionality by 48 hpf [24]. Development of the mesonephros, the juvenile and adult kidney, begins at 12 days post-fertilization (dpf) and remains functional the entire adult life [25]. In adult fish, the nephrogenic capacity of cells within the mesonephric field is maintained throughout life (termed neo-nephrogenesis) [22•, 25, 26], whereas in mammals the potential to generate

nephrons *de novo* ends a few days after birth. Zebrafish mesonephric kidney nephrogenesis proceeds in an anterior to posterior manner and the kidney is divided into four morphologically distinct regions with different densities of nephrons: the anterior nephron-dense region, the medial nephron-sparse region, the medial nephron-dense region, and the posterior nephron-sparse region. Interestingly, the number of nephrons in the two medial regions is directly proportional to the body mass of the fish, indicating the existence of a mechanism that coordinates neo-nephrogenesis and renal capacity required by the fish [22•, 25]. This capacity of the zebrafish mesonephros to generate a neo-nephrogenic response provides a unique model for studying both developing nephrons and renal regeneration in an adult vertebrate organism. In this review, we provide an overview of zebrafish larval/adult models of AKI, genetic models of chronic kidney disease (CKD), and discovery screens for compounds that enhance recovery from kidney damage.

## Zebrafish Models of Acute Kidney Injury

In this section, we will review both larval and adult models of AKI. We have made a distinction between larval and adult models, since larvae do not have the capacity to generate *de novo* nephrons through neo-nephrogenesis. The models generated to date have focused either on translating mammalian models to zebrafish (i.e., nephrotoxins) or focused on the unique utility of the zebrafish (i.e., laser ablation, inducible transgene) (Fig. 1).

### Larval Model of Gentamicin-Induced Injury

Perhaps the best-characterized model of AKI used in zebrafish is a nephrotoxic model using the aminoglycoside gentamicin. At high doses, gentamicin causes AKI as a result of proximal tubular cell damage [27, 28]. Injection of gentamicin in the circulation of 48–72 hpf zebrafish larvae results in histological changes characteristic of mammalian AKI such as flattening of the apical brush border, loss of cell polarity, and enlargement of the tubular lumen with accumulation of debris in the tubular lumen [21•, 29, 30]. The tubular obstruction as a consequence of the gentamicin-induced tubular injury results in a reduction of the pronephros' ability to filter and eliminate fluid and subsequent development of edema [30]. Together, these findings demonstrate the promise of the zebrafish larval gentamicin AKI model as a simple model to augment mammalian AKI studies [31].

### Adult Model of Gentamicin-Induced Injury

While larval zebrafish AKI models have considerable advantages such as the simplicity of the kidney, and ease to manipulate and visualize the organism, the adult zebrafish mesonephric kidney, with its potent regenerative capacity, represents a relevant model to understanding differing repair potentials when compared to the limited regeneration potential of mammalian metanephric kidneys [19, 22•, 25, 26, 32]. Using gentamicin-induced injury, the zebrafish mesonephros has been shown to undergo *de novo* formation of nephrons (neo-nephrogenesis) [22•, 25]. Utilizing this model, two independent groups used the transgenic zebrafish lines, *lhx1a:EGFP* [22•] and *wt1b:GFP* [25], to follow GFP expression in cellular aggregates within the nephrogenic field and characterize the regenerative response following gentamicin-induced injury. Using the *lhx1a:EGFP* line, one

study found populations of  $lhx1a^+$  single cells, cellular aggregates, and renal vesicle-like bodies, which are believed to be nephron progenitors and early stage nephrons [22•]. In agreement with the *lhx1a:EGFP* studies, another group using the *wt1b:GFP* line identified *wt1b*<sup>+</sup> cellular aggregates form 72 to 96 h following gentamicin treatment that could give rise to new nephrons [25]. In serial transplantation assays, using purified *lhx1a*<sup>+</sup> tubular aggregates of 10–30 cells, it was discovered that these cells can form new functional nephrons in recipient adult zebrafish, and able to maintain a self-renewal capacity [22•]. Further characterization of this renal “stem-cell” population in an adult vertebrate, including defining the cellular niche, will provide a solid foundation for understanding the differences between potent renal regeneration (zebrafish) and a limited renal regeneration potential (mammals).

## Laser Ablation-Induced Injury

In an attempt to overcome some of the limitations of gentamicin-induced damage, mainly bilateral (complete) damage at undefined time points after delivery, researchers have developed a laser ablation model in the larval zebrafish kidney [33]. Injury is driven by the induction of cell death in a small area of the renal field with a low degree of larval lethality, thus allowing for a longer timeframe to study the injury than allowed by gentamicin-driven injury [33]. The increased spatial and temporal specificity of the damage and the fact that varying regions of the kidney can be targeted allows for the study of repair processes in different segments of the tubule. Using controlled laser ablation to target the distal tubule in a larval transgenic zebrafish expressing GFP in the kidney tubule, it was demonstrated that the repair process is driven by cell migration and this process is independent of cell proliferation [34]. Results from this work showed that after unilateral epithelial injury proliferation is confined in the injured kidney to cells adjacent to the wound, supporting the idea that the initial signals inducing repair are intrinsic to the injured tubule itself. The main limitations of this model are the uncertain relevance of the physiological characteristics of laser-driven damage to mammalian AKI and the relatively small field of damage induced. However, this model still represents a useful tool to answer questions that require a highly controlled degree of injury.

## Aristolochic Acid-Induced Injury

Aristolochic acid (AA) is a nephrotoxic compound found in the birthwort (Aristolochiaceae) plant family and was used in the past for treatment of arthritis and wound healing [35]. To date, in zebrafish, the effect of AA-induced nephrotoxicity has only been evaluated in developing embryos by soaking of the embryos at 24 hpf in 10 ppm of AA for 5–7 h, and found to induce tubular and glomerular damage [36]. Although tubular and interstitial damage is associated with AA treatment in mammals, glomerular damage has not been reported in mice and humans with AA nephropathy [37, 38]. While this model has a high appeal because zebrafish larvae could simply be soaked in a chemical toxin, more work needs to be done to determine if this approach actually leads to classic AKI hallmarks.

## Puromycin Aminonucleoside (PAN)-Induced Injury

The glomeruli are responsible for the filtration of low-molecular-weight plasma waste products into the urine, while retaining large macromolecules. The glomerular filtration barrier (GFB) is a complex structure containing endothelial, mesangial, and podocyte cells. Perturbation of the GFB integrity, and in particular damage to the podocytes, results in leakage of blood plasma proteins into the urine, a hallmark of most glomerular diseases termed proteinuria [39, 40]. Progressive proteinuria frequently leads to permanent glomerular damage and renal failure. Therefore, there is a need to develop models that can serve to understand GFB function under normal and damage conditions. The glomerular toxin Puromycin aminonucleoside (PAN) has been used to study podocyte injury in zebrafish embryos. When injected into the circulation of 2.5 dpf larvae, PAN toxicity leads to effacement of podocyte foot processes and development of edema [41]. Similar changes in glomerular function have also been observed in rats treated with PAN, validating the use of the zebrafish pronephros to study glomerular injury [42].

## Inducible Transgenic Lines for AKI

Generation of transgenic lines is a widely used method in the zebrafish field for understanding the function of genes involved in kidney development and regeneration. Given the density of the metanephric kidney, *in vivo* imaging of glomerular structures in mammalian systems is technically difficult. Taking advantage of the transparency of zebrafish embryos and larvae allows for imaging of podocytes in the zebrafish glomerulus. For example, a transgenic zebrafish line expressing GFP under the control of the *podocin* promoter was generated to visualize podocytes for dissection and electron microscopy analysis [43]. Using this transgenic line in a *crb2b* morphant with compromised glomerular function that leads to pericardial edema and formation of pronephric cysts, there was a loss of *podocin:GFP* expression, validating the potential use of this transgenic line for the *in vivo*, real-time study of glomerular injuries [43].

A limitation of disrupting glomerular function in zebrafish embryonic kidneys is that it leads to death within a few days. In order to model human diseases that lead to disruption of the glomerular filtration barrier, inducible transgenic zebrafish lines have been generated [25, 44, 45]. These lines were generated using the bacterial nitroreductase (NTR) gene under the control of the *podocin* promoter, such that NTR expression is limited to the podocytes of both pronephric and mesonephric kidneys. Glomerular injury can be induced by treatment in either larvae or adult zebrafish with the pro-drug metronidazole (Mtz), as this is converted by NTR into a DNA cross-linking agent that induces cell death [46, 47]. Using *podocin:NTR-GFP* transgenic larvae subjected to Mtz treatment, pericardial edema and podocyte depletion as suggested by reduction in *GFP* expression were observed [44]. Close examination of the glomerulus in Mtz-treated zebrafish also revealed the presence of podocyte foot process effacement, a phenotype resembling nephrotic syndrome in humans. Interestingly, glomerular barrier functionality and foot process structures were recovered within a week after washout of Mtz. A group of proliferating cells in the glomeruli was detected during the recovery period suggesting the presence of a resident progenitor cell population [44]. A complementary study used a similar transgenic line *podocin:NTR-*

*mCherry* to investigate the mechanism of podocyte damage and regeneration in adult zebrafish [45•]. Similar to the observations in larvae, Mtz treatment of adult zebrafish leads to severe edema and decreased fluorescent reporter intensity, as well as an impaired GFB and podocyte foot process effacement [45•]. Interestingly, employing the *wt1b:GFP* transgenic line that was previously used as a marker for kidney regeneration studies, it was demonstrated that *wt1b* expression is reinitiated in cells on the wall of Bowman's capsule after Mtz-mediated podocyte injury [25, 45•]. The recent description in a mammalian system of a parietal epithelial cell population in Bowman's capsule that can act as podocyte progenitors positions zebrafish as a great alternative model to investigate the identity of these cells and their mechanism to repair glomerular damage [48, 49].

## Fluorescent Conjugates to Delineate Injury

Small dextran conjugates delivered to the kidney via the circulation can be endocytosed by the proximal tubule, enabling specific labeling of the renal epithelial tubular cells in zebrafish [24, 30]. However, using such dextran conjugates in high-throughput small molecule or genetic screens is not feasible, since individual injection of the conjugates into larval zebrafish is required. To overcome this limitation, researchers have developed a small fluorescent molecule as an alternative tool for visualization of the proximal tubule after injury [50]. The compound, named PT-Yellow, specifically labels the proximal tubule of zebrafish embryos soaked for 20 min. It is absorbed into the blood, filtered by the glomerulus, and endocytosed by the proximal tubular epithelial cells. Additionally, injection of PT-Yellow into adult zebrafish results in labeling of the mesonephric proximal tubules and a significant loss of fluorescence follows gentamicin-induced injury [50]. These observations indicate that PT-Yellow may be useful for studying normal and altered proximal tubule function in the zebrafish.

## Genetic Models of Kidney Disease

Since zebrafish are amenable to genetic modification, strength is to identify mutants that model the etiology of different types of kidney disease. As part of a large-scale mutagenesis screen, numerous recessive mutations affecting the development of the zebrafish pronephric kidney were identified and characterized [24, 51]. Since the screens were performed to uncover mutants that compromised kidney function based upon renal cyst development at 2 dpf, these mutants were found to be more akin to CKD, and affected architecture and function of glomeruli and terminal differentiation of tubular epithelial cells [24]. A second screen, using insertional mutagenesis with a pseudo-typed retrovirus as the mutagen, also assessed cystic kidneys. This study recovered twelve different mutant loci and two of the genes identified, *vhnf1* and *pkd2*, were previously shown to be responsible for cystic kidney diseases in humans [52–54]. Six of the loci were found to encode components of intraflagellar transport complex B, a multi-protein complex essential for cilia function, biogenesis, maintenance, and signaling [54–56], findings consistent with a role of cilia in polycystic kidney disease (PKD) pathogenesis [57]. PKD, a common CKD, is characterized by the formation of multiple epithelium-lined renal cysts, renal enlargement, and abnormal tubule development [58]. Given the identification of multiple homologs of human PKD genes and the observation of characteristic phenotypes associated with the formation of

pronephric kidney cyst, the kidney of the zebrafish larvae serves as a good model for the discovery and study of PKD-associated genes [54, 59–61]. There is now evidence in mammalian systems pointing to primary cilia defects in the pathogenesis of PKD [62–66]. The role of cilia function in response to injury was investigated in a model of pronephric and mesonephric obstruction in zebrafish embryos and adult, respectively. The findings from this study suggest that expression of *foxj1a*, a transcription factor required for cilia formation, is a primary response to injury and is needed to maintain enhanced cilia function in injured epithelial cells. Interestingly, *foxj1a* expression is also increased in uninjured zebrafish cystic mutants representing a putative novel therapeutic target for PKD [60].

## Small Molecule Screening Assays

Phenotypic in vivo high-throughput screening (HTS) has become a widely used drug discovery method [67–70]. Since traditional HTS use has not increased the number of newly marketed drugs [71], it has been proposed that HTS screens which better recapitulate in vivo events are needed to improve the discovery of new drug candidates [70, 72, 73]. To comprehend pathophysiological processes, the interactions between compounds, pathways, cells, tissues, and organs must be understood; whole-animal models have been shown to be more informative in this regard than in vitro and ex vivo models [74]. The concept is supported by a review of FDA-approved new molecular entities over a decade, which showed that the majority of first-in-class agents were discovered in phenotypic, not target-based screens [75]. The zebrafish is uniquely positioned for chemical screens. Due to its small size and high fecundity, it is compatible with multi-well plate formats used in large-scale, phenotype-based screens. Because small molecule examination occurs in a physiologically relevant context, zebrafish screens select for compounds with favorable physiochemical properties. Despite using limited numbers of compounds, zebrafish screens have yielded valuable compounds for cardiac [76–79], vascular [80], cancer [81], and CNS biology [82, 83], among others. The best example, from a therapeutic standpoint, is a prostaglandin E2 analog, which accelerates hematopoietic stem cell engraftment in the bone marrow and has been moved into Phase 1b clinical trials within 2 years of the initial screen of 2500 compounds [84].

As discussed in the genetic screen section, compromised kidney function in the zebrafish embryo and larvae is associated with pericardial edema, axis curvature, and, in the case of cystic kidney diseases, also a defective left–right asymmetry of the body plan [24, 54]. Together, these phenotypic characteristics have enabled the use of the zebrafish embryo for a chemical screen of compounds that ameliorate cysts in a model of PKD. The chemical modifier screen was done using *pkd2* mutant zebrafish larvae that develop cystic kidneys by treating with a collection of compounds and using a computer algorithm to quantify the body axis curvature [59]. This research identified a role of the HDAC inhibitor, VPA, for the reduction of axis curvature and kidney cyst formation in zebrafish and lessening the progression of cyst formation in a mouse model of PKD [59]. Another study performed screening for compounds capable of increasing the size of the kidney field through proliferation-based mechanisms [85]. The screen identified a compound, PTBA, which was able to induce an increased proliferation of renal progenitor cells with persistent expansion of the kidney field [85]. Follow-up studies demonstrated that in a zebrafish larvae AKI

model, a PTBA analog (m4PTB) enhanced functional renal recovery [21•]. In addition, m4PTB enhances renal recovery and reduces renal fibrosis after IR-AKI in mice by promoting cell cycle progression of the surviving regenerating renal tubular epithelial cells [21•]. Further investigation of m4PTB revealed that the compound also had activity in the aristolochic acid-induced mouse model [86]. Importantly, the cellular mechanism by which m4PTB ameliorates recovery from AKI, namely increased proliferation of dedifferentiated progenitor cells, is preserved among the two species and models [21•, 86], and mirrors that seen in human clinical specimens [20]. Validating the use of the zebrafish system as a novel, innovative model to identify agents that ameliorate recovery from kidney damage.

## Conclusion

The insights gained over the past decade into the applicability of zebrafish models of kidney injury and disease to mammalian models of renal damage have provided valuable groundwork for utilizing the zebrafish as a therapeutic discovery model. While several valid models of AKI and CKD have been developed in zebrafish, and potential therapeutic compounds have been identified in zebrafish screens that have been successfully translated to mammalian models, more work needs to be done to determine if these hits will be effective in the clinic. An important first step is to elucidate whether the molecular mechanisms found during zebrafish AKI are ultimately conserved in human injury and disease events. Such studies would help to provide an early evaluation step in the discovery process to predict whether a compound should be moved forward for development.

## Acknowledgments

The laboratory of Neil Hukriede was supported by the National Institutes of Health Grants 2R01 DK069403, 1RC4 DK090770, 2R01 HD053287, and 1P30DK079307. The laboratory of Maria Cecilia Cirio was supported by the Agencia Nacional de Promoción Científica y Tecnológica de Argentina, FONCyT, PICT 2013. The laboratory of Mark de Caestecker was supported by National Institutes of Health Grants 1R01 HL093057 and 1RC4DK090770.

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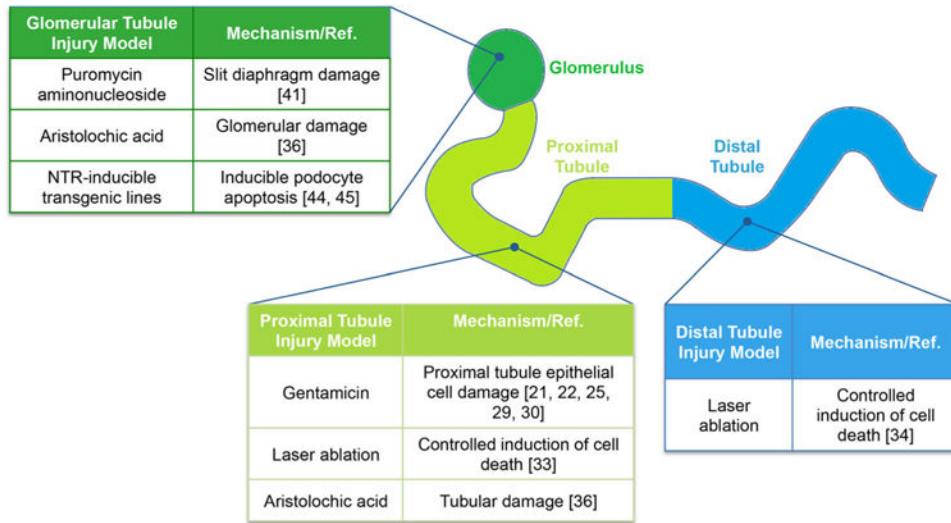


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**Fig. 1.** Summary of zebrafish renal injury models. The studies highlighted in the review, as indicated by reference number, are organized by location of damage in the nephron and the type of injury model