New tides: using zebrafish to study renal regeneration

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Over the past several decades, the zebrafish has become one of the major vertebrate model organisms used in biomedical research. In this arena, the zebrafish has emerged as an applicable system for the study of kidney diseases and renal regeneration. The relevance of the zebrafish model for nephrology research has been increasingly appreciated as the understanding of zebrafish kidney structure, ontogeny, and the response to damage has steadily expanded. Recent studies have documented the amazing regenerative characteristics of the zebrafish kidney, which include the ability to replace epithelial populations after acute injury and to grow new renal functional units, termed nephrons. Here we discuss how nephron composition is conserved between zebrafish and mammals, and highlight how recent findings from zebrafish studies utilizing transgenic technologies and chemical genetics can complement traditional murine approaches in the effort to dissect how the kidney responds to acute damage and identify therapeutics that enhance human renal regeneration. (Translational Research 2014;163:109–122)

Abbreviations: AG = aminoglycoside antibiotic; AKI = acute kidney injury; CKD = chronic kidney disease; dpf = days postfertilization; H&E = hematoxylin and eosin; hpf = hours postfertilization; hpi = hours postinjection; IRI = ischemia/reperfusion injury; m4PTB = methyl-4-(phenylthio) butanoate; MSC = mesenchymal stem cell; PCT = proximal convoluted tubule

The kidney plays several functional roles, including the removal of waste metabolites, electrolyte and acid-base balance, water homeostasis, and blood pressure regulation. Humans have a pair of bean-shaped kidneys located at the rear of the abdominal cavity. Each kidney is comprised of nephrons, which are the functional units of the organ, and are found packed in an intricate three-dimensional array (Fig 1, *A*). The nephrons are characterized as specialized epithelial tubes that consist of 3 major parts: (1) the glomerulus, which acts as a blood filter; (2) the tubule, which is comprised of segments that function to secrete and/or reabsorb specific molecules; and (3) the collecting duct, where final changes in solute and water composition occur as the urine is conveyed out of the kidney for excretion (Fig 1, *A*).[1] Overall nephron segment composition is conserved, though differences are found even between closely related mammalian species.[1] The number of nephrons in a normal, healthy human kidney varies, ranging from 800,000 to 1.5 million.[2] During development, vertebrate species possess a series of up to 3 kidney structures that arise sequentially: the pronephros,

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The work of the Wingert laboratory is supported by the following: National Institutes of Health grants K01 DK083512, DP2 OD008470, and R01 DK100237; a March of Dimes Basil O'Connor Starter Scholar grant award #5-FY12-75, start-up funds from the University of Notre Dame College of Science and Department of Biological Sciences, and a generous gift to the University of Notre Dame from Elizabeth and Michael Gallagher on behalf of the Gallagher Family to support adult stem cell research. The authors have read the journal's policy on disclosure of potential conflicts of interests, and have no disclosures to report.

Submitted for publication June 15, 2013; revision submitted September 24, 2013; accepted for publication October 8, 2013.

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1931-5244/$ - see front matter

http://dx.doi.org/10.1016/j.trsl.2013.10.003

the mesonephros, and the metanephros.[3] In these various kidney iterations, the nephron serves as the basic structural and functional unit.[3] The metanephros is the most complicated in terms of the number and arrangement of the nephrons, and becomes the permanent kidney in humans and other mammals after the other structures degenerate in succession during fetal development.[3] Lower vertebrates like amphibians and fish develop a functional embryonic pronephros followed by a more complex mesonephros that serves as the adult organ.[4-7] For example, the zebrafish pronephros is a rather simple kidney comprised of just 2 nephrons, whereas the subsequent mesonephros structure is comprised of several hundred nephrons that are progressively added to the initial pronephros framework.[7]

Kidney disorders and diseases can interfere with normal nephron development or cause nephron impairment, affecting millions of people worldwide. Disruptions in kidney function can arise from acute kidney injury (AKI), in which partial or complete restoration of renal function is possible. Renal diseases also arise from chronic kidney disease (CKD), in which the progressive scarring of the organ is too catastrophic to be repaired. Both AKI and CKD can lead to kidney failure, known as end-stage renal disease, which requires patients to undergo life-long dialysis or an organ transplant. Understanding how nephrons are made and how they regenerate has received increasing attention because of the possible clinical applications—which could be relevant to treating the aforementioned kidney diseases, and a long list of others including renal birth defects and genetic conditions like polycystic kidney disease.[8] Although considerable information has been amassed about how the kidney senses and responds to damage, many questions remain. For example, the identification of adult renal stem cells in the human kidney is a central issue in nephrology, as is the prospect of cell-based regenerative medicine for kidney disease.[9]

In this review, we discuss how the attributes of the zebrafish embryonic and adult kidneys have made these models particularly amenable to studying the mechanisms of renal regeneration associated with AKI, and for translational research to identify AKI therapeutics. Zebrafish nephrons have been shown to possess multiple proximal and distal tubule domains that resemble the overall pattern of mammalian nephron segmentation and share histologic characteristics with mammals (Fig 1, *B* and *C*, and Fig 2). These observations have led to the hypothesis that fundamental mechanisms of nephron development and regeneration are likely to be conserved, even though there are differences as to whether certain segments are present in fish (eg, intermediate tubule segments) and because zebrafish do not form a third, metanephric kidney like humans.[7,10] In fact, zebrafish exhibit a multifactorial regenerative response to AKI that distinguishes them from mammalian species; they restore nephron epithelia and make new nephrons. Understanding these intriguing similarities and differences between zebrafish and humans may proffer powerful novel insights for translational medicine.[11] Here, we focus primarily on recent findings that demonstrate the potential of zebrafish research to discover innovative ways to promote regeneration following AKI. We further define AKI, review the current zebrafish AKI paradigms, and discuss avenues of renal regeneration research afforded by the zebrafish.

AKI PATHOPHYSIOLOGY

AKI is a multifactorial disorder characterized by the abrupt partial or complete loss of kidney functions (Fig 3). AKI leads to life-threatening complications such as pulmonary edema, hyperkalemia, and metabolic acidosis, and is also associated with high mortality rates that range between 30% and 80% world-wide.[12] AKI commonly results from ischemia/reperfusion insults of the kidney, the use of nephrotoxins such as aminoglycosides and cisplatin, circulatory shock, and sepsis.[13] In the United States, approximately 4% of AKI cases in critically ill patients require renal replacement therapies and this specific form of AKI has an in-patient mortality rate of 50%.[14] Renal replacement therapies (dialysis or organ transplantation) have significant limitations and require long-term medical care. The total number of deaths associated with AKI in which dialysis was required rose from approximately 18,000 in the year 2000 to nearly 39,000 by 2009, more than doubling in incidence in the United States alone.[15] Therefore, developing novel therapeutic treatments that are able to prevent kidney injury or trigger renal regeneration following injury has gained significant interest in the scientific community.

In a normal physiological setting, cells of the mammalian kidney have a very low basal turnover rate. Within nephrons, cell proliferation occurs through the division of cells that reside in the tubule, which has been documented through assays such as immunoreactivity for proliferating cell nuclear antigen and Ki-67.[16,17] A subpopulation of rare tubular epithelial cells are positive for markers of the G1 phase of the cell cycle (Fig 3, *A*). This data led to the hypothesis that nephrons contain resident cells that are poised to respond to damage through proliferation.[17] Indeed, proliferation rates change dramatically after epithelial injury; the vertebrate kidney possesses the remarkable ability to repair itself by epimorphic regeneration after an ischemic insult or exposure to nephrotoxins. The marked increase

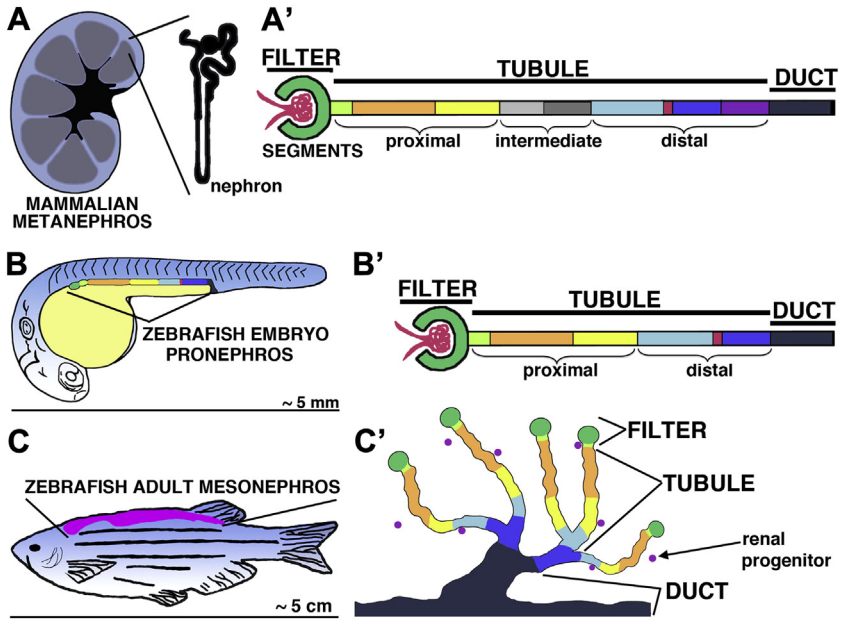

Fig 1. Kidney architecture varies between vertebrates, but the strategy for nephron segmental composition is broadly conserved. **A,** The prototypical mammalian metanephros is a kidney composed of nephrons ranging in number from thousands to millions, and each nephron is an epithelial tube with a macroscopic structure of stereotypical loops and convolutions. **A',** The nephron is drawn as a straightened epithelial tube. It is comprised of a filter, tubule, and duct, with a regular pattern of proximal, intermediate, and distal segments of epithelial cells that have discrete roles in modifying the filtrate during urine production. The mammalian nephron segments are as follows: blood filter (dark green surrounding red capillary network); neck (light green); proximal convoluted tubule (orange); proximal straight tubule (yellow); descending thin limb (light grey); ascending thin limb (dark grey); thick ascending limb (light blue); macula densa (red); distal convoluted tubule (dark blue); connecting tubule (purple); collecting duct (black). **B,** The zebrafish embryo initially develops a linear pronephros, with a pair of nephrons, and laterally a single nephron can be visualized. **B',** The zebrafish pronephric nephron has a blood filter, multisegmented tubule, and duct. Analogous segments to the mammalian nephron are indicated by color. The pronephros nephron segments are as follows: blood filter (dark green surrounding red capillary network); neck (light green); proximal convoluted tubule (orange); proximal straight tubule (yellow); distal early (light blue); Corpuscle of Stannius (red); distal late (dark blue); collecting duct (black). **C,** The zebrafish adult contains a single, flattened mesonephric kidney on the dorsal wall of the body cavity. **C',** Examination of the mesonephros nephron arrangement and constitution has found that nephrons are arranged in branched units and pinwheel-like arrays that connect to a central duct system. The mesonephric nephrons have similar segments as the embryonic nephrons, and intervening stroma contains renal progenitors that can form new nephrons after injury. The mesonephros nephron segments are as follows: blood filter (dark green circle); neck (light green); proximal convoluted tubule (orange); proximal straight tubule (yellow); distal early (light blue); distal late (dark blue); collecting duct (black).

in tubular cell proliferation is considered to be the driving force behind nephron repair as opposed to cellular hypertrophy.[18] Although the mammalian tubule epithelium has the capacity to self-renew, the generation of new nephrons has not been observed and many responses to injury involve the formation of fibrotic, nonfunctional tissue.[19]

The morphologic manifestations of AKI occur in multiple overlapping phases. Initially, cells at the injury site exhibit a dedifferentiated appearance associated with changes in proximal tubular cell polarity and a loss of the brush border (Fig 3, *B*). These cells also express genes that are associated with early nephron development, such as Paired box 2 and neural cell adhesion molecule, and mesenchymal markers like vimentin.[20-25] Consequently, cell detachment occurs and areas of the basement membrane are left denuded. Eventually, some cells undergo apoptosis or may become necrotic if an insult is severe and rapid. Detached cells can be seen in the lumen and can cause tubular obstruction downstream within the nephron. In rodent models of toxin and ischemia/reperfusion kidney injury, epithelial cell death occurs shortly after injury, and typically affects the S3 segment of the proximal tubule, although other proximal tubule regions can be damaged.[26]

The next major phase of AKI involves tubular regeneration (Fig 3, *C*).[18] This process involves the production of new epithelial cells from cells within the nephron.[18,24,27] Depending on the severity of the

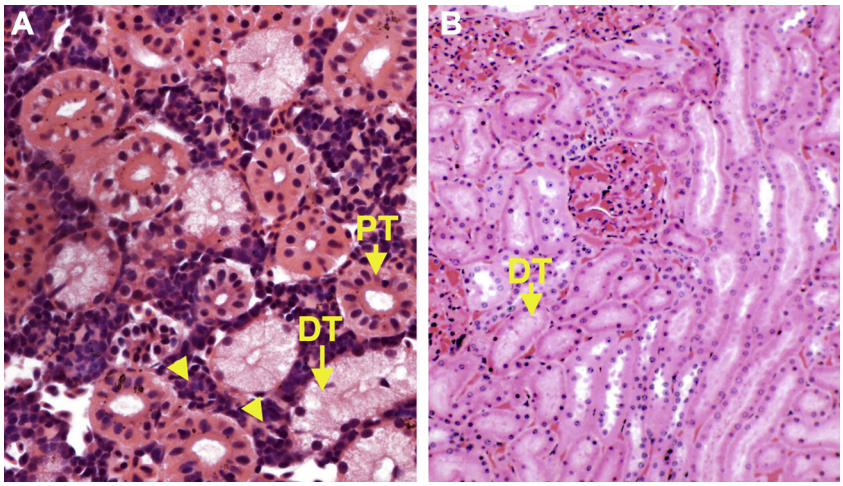

Fig 2. Comparison of renal histology between zebrafish and mouse. **A,** Zebrafish mesonephros and **B,** mouse metanephros sections stained with hematoxylin and eosin. **A,** This zebrafish section includes proximal tubule (PT) (dark pink) and distal tubule (DT) (light pink) cross-sections along with a dense interstitial stroma (arrowheads) with intensely-purple stained nuclei that includes hematopoietic cells and is also the proposed location of renal progenitors. This particular **B,** mouse section is dominated by distal tubules (DT) (light pink).

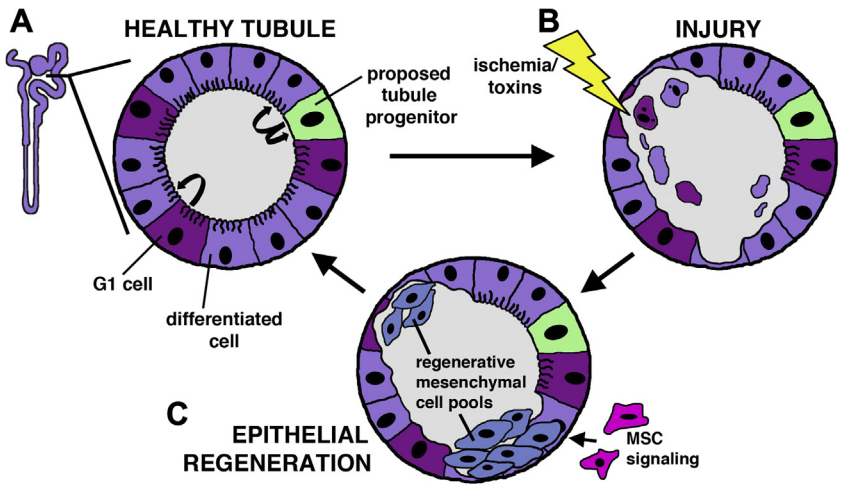

Fig 3. Acute kidney injury (AKI). **A,** (left) A schematic depiction of a nephron, with the level of cross section indicated. **A,** (right) Healthy tubule cross-section, with differentiated epithelial cells (purple) interspersed with currently debated regeneration sources: cells in G1 undergoing low rate of turnover (magenta), and the renal progenitor/stem cell (light green). **B,** Schematic after injury, with luminal debris and surviving epithelial cells. **C,** During renal epithelial regeneration, mesenchymal cells (blue) have been observed within the tubule. Whether these mesenchymal cells emerge from renal progenitors/stem cells that reside in the nephron, from differentiated cells (either in G1 or noncycling) that dedifferentiate, or some combination of these sources, is currently an active area of nephrology research. Cells located in the interstitial space between nephrons that can impact tubular regeneration include mesenchymal stem cells (MSC)s (pink). After the tubular regenerative cells proliferate, their offspring differentiate and re-establish tubular integrity.

injury, a normalization of kidney function occurs over a 15-day period.[27] The intratubular source is an active area of investigation, with several major cell mechanisms under scrutiny. One mechanism is hypothesized to involve a process of dedifferentiation that occurs in the initial phase after damage. In this model, surviving epithelial cells undergo a cell state change, or an epithelial to mesenchymal transition. Once dedifferentiated, the mesenchymal cells acquire migratory capacity and physically cover the denuded basement membrane in the areas where actual cell death occurred. Concomitantly, the mesenchymal cells undergo proliferation and these offspring will differentiate, undergoing a mesenchymal to epithelial transition that ultimately reconstitutes the

tubular epithelium.[28-30] A second mechanism is hypothesized to involve a different cell source than resident differentiated tubule cells: a dedicated renal stem or progenitor cell, with the distinction being the degree to which the cell might be able to self-renew and produce differentiated offspring.[11,19,31,32] It remains a subject of intense debate and ongoing research whether the true origin of new tubular epithelium comes from a resident stem cell, though there is exciting recent evidence for the existence of candidate tubule subpopulations that could serve this role.[33-38]

In addition to the intratubular events, another process that impacts tubular regeneration is signaling from stromal cells, such as mesenchymal stem cells that are located in or migrate into the interstitial space near damaged nephrons (Fig 3, *C*).[39] Researchers have documented that mesenchymal stem cells secrete factors that are capable of promoting the process of kidney repair, a process that has recently received much attention because it could become a vehicle for clinical treatment.[39]

CURRENT RESEARCH MODELS USED TO STUDY AKI

There are several widely researched systems for AKI research, which use different agents of injury and different animals as subjects of study. In the sections below we provide a broad overview of injury agents and mammalian models, so as to provide perspective on this field of research and set the stage for where zebrafish fit into the landscape of nephron regeneration studies.

Agents of damage: chemical toxicity by gentamicin. Gentamicin is an aminoglycoside antibiotic (AG) used in the treatment of a variety of bacterial infections mainly caused by Gram-negative organisms.[40] Because of its rapid bactericidal activity and low levels of resistance, gentamicin is an extremely useful drug when prompt control of a serious infection is necessary. However, gentamicin is both ototoxic and nephrotoxic.[41,42] In the kidneys, AGs like gentamicin specifically accumulate in the proximal tubule, resulting in undesirable side effects.[43] Despite these toxic consequences, gentamicin has remained in clinical use because it is the only effective therapy against organisms resistant to other antibiotics.[44] Thus, gentamicin has been widely used as a model drug for the AG family to study nephrotoxicity, both in animals and in humans.[45-47]

While the mechanisms underlying the cytotoxic effects of AGs are intertwined and multifactorial, gentamicin nephrotoxicity in humans is typically characterized by the death of tubular epithelial cells resulting in nephron damage and reduced functionality. As mentioned, tubular death is concentrated mainly in the proximal segment.[48] Exposure to gentamicin in rodents leads to apoptosis as well as necrosis of these epithelial cells.[49-52] However, the actual manifestation of death may depend on the concentration of the drug, similar to other cytotoxic compounds such as hydrogen peroxide.[53] A large complex formed by Lrp2 and Cubilin that is restricted to the proximal tubule leads to gentamicin uptake via endocytosis.[54] Gentamicin is trafficked through the endosomal compartments and accumulates mostly in the lysosomes, the Golgi body, and the endoplasmic reticulum.[55] As the concentration of the drug increases in these organelles, the membranes become disrupted and their contents spill out into the cytosol. Cytosolic gentamicin acts on mitochondria both directly and indirectly, activating the intrinsic pathway of apoptosis.[56] Other numerous disruptions take place, which further contributes to cell death.[48]

Other AKI agents: the example of ischemia. Renal ischemia/reperfusion injury (IRI) is a common cause of AKI. IRI results from the inability of oxygen and nutrients to be delivered to cells within the kidney tissue, and also because waste products cannot be carried away.[57-60] AKI resulting from ischemia is a common clinical occurrence that leads to high morbidity and mortality rates. Variables such as age, existing kidney disease, and proteinuria contribute to the increased risk of developing AKI after slight to moderate decreases in kidney perfusion.[61-63] The imbalance between oxygen supply and demand results in tubular epithelial cell injury, primarily in the proximal tubular segment of the nephron, leading to functional impairment of the organ.[60,64] The epithelial cells of the proximal tubules lose their polarity and brush border characteristics, leading to protein redistribution along the cell membrane. Intricate communications between epithelial cells, endothelial cells, and inflammatory mediators can result in persistent injury, which signal the initiation of apoptosis and necrosis, both mechanisms of cell death.[65]

Animal models. Mammalian models like the mouse and rat are considered extremely valuable models of disease that typically mimic human conditions. Their anatomy and cell biology are well conserved and techniques such as genetic fate mapping can facilitate the tracking of cell types during regeneration. Furthermore, these models are essential to evaluate efficacy and toxicity of pharmaceuticals for AKI treatment, and remain the gold standard in preclinical trials. Rodent AKI models include IRI as well as exposure to chemical agents such as gentamicin and, thus, can be used to model the outcomes of different insults.[66] However, scientists are still faced with several

limitations when studying AKI in these mammalian kidneys. Access to the rodent kidney requires surgery. For the most part, this eliminates real-time visual monitoring of the renal tissues in living animals, with the only current exception being a very small population of renal tubules and vessels near the surface of the organ.[67]

For a number of reasons, the zebrafish has emerged as a relevant vertebrate that can be used to address several voids in the AKI field. Research in zebrafish embryos and adults has shown that the pronephros and mesonephros kidney forms, respectively, are valid models for gentamicin-based AKI studies.[68-73] Zebrafish nephrons in embryos and adult animals show a conserved make-up with mammals (detailed further in following sections).[10,74] Zebrafish larvae are optically transparent, allowing microscopic observation along the entire length of the kidney. Additionally, zebrafish serve as a suitable experimental model in that they breed frequently, produce large numbers of progeny, and the embryos develop *ex utero*.[75] They also progress very rapidly through embryogenesis and organogenesis. For example, the embryonic kidney has formed 1 day after fertilization and the pronephric tubules begin filtration of the blood by the second day of life.[76]

One important aspect of AKI research resides in the possibility of identifying small molecules with therapeutic potential to aid in repair and regeneration. The zebrafish has become an appealing tool for such small molecule screens.[75,77,78] Because the embryo is small in size, relatively small quantities of compounds are needed for testing, and embryos can be kept alive for days without added nutrients because they utilize maternal food deposits. The adult zebrafish can be injected with small amounts of compounds to interrogate regeneration because of the small adult mass,[79] enabling findings from the embryo to be tested in an adult organ setting. Comparable screening of pharmaceutical molecules in rodents would require an extraordinary amount of time, chemical compounds, as well as residential space. In the following sections, we first elaborate on the work that has been done using zebrafish embryos to model kidney regeneration after AKI, and then move to a discussion of renal regeneration research in the adult zebrafish.

AKI MODELING IN THE ZEBRAFISH EMBRYO

Anatomy and physiology of the zebrafish embryo pronephros. The zebrafish embryonic kidney, or the pronephros, contains 2 nephrons that are formed from bilateral stripes of intermediate mesoderm that lie on either side of the embryo trunk.[10,76] The anterior-most renal progenitor cells give rise to podocytes, which will migrate to the midline and fuse to form a highly vascularized blood filter, or glomerulus that the nephrons share.[10,76] The remaining renal progenitors undergo a mesenchymal-to-epithelial transition and form tubules that fuse posteriorly at the cloaca, which is the exit portal for waste from both the pronephros and the gut.[74,76]

Recently, a functional genomics-based strategy to identify markers of differentiated renal cell types revealed that the zebrafish pronephros is composed of at least 8 discrete regions, including the glomerulus, a neck segment, 2 proximal segments, 2 distal segments, and a duct (Fig 1, *B* and *C*).[10] The expression profile of zebrafish nephron segments likens them to many of the distinct segments that exist in metanephric nephrons of higher vertebrates (refer to color-coded segments in Fig 1).[10] Based on this comparison, an updated model of zebrafish pronephros organization has been defined.[10] Functionally, the zebrafish kidney nephrons are essential for solute recovery, water homeostasis, and waste excretion, as in other vertebrates.[76] The zebrafish kidney begins to filter blood at approximately 48 hours postfertilization (hpf).[76] The glomerulus serves as a blood filter, collecting filtrate from the blood and passing it through the tubule where solutes are reabsorbed or secreted during the flow of fluid toward the cloaca.[76]

Consequences of gentamicin exposure in the zebrafish pronephros. Embryonic nephrons can be damaged by gentamicin or cisplatin, and show disrupted apical-basal tubule cell polarity and death.[68] After gentamicin is injected at early embryonic stages of development in the zebrafish, there is a substantial decline in renal function due to an inability to maintain water homeostasis.[68,72] Gentamicin-mediated injury results in flattening and loss of the pronephric tubule brush border, tubular and glomerular swelling, formation of debris in the tubular lumen, and peritubular accumulation of leukocytes.[68] Gentamicin injury also disrupts renal clearance, with injured animals unable to void 10 kDa rhodamine-labeled dextran.[68] In addition, the loss of cell polarity and disruption in damaged tubules was demonstrated through the visualization of the redistribution of the basolateral Na^+/K^+ATPase pump to the apical membrane.[72]

We have performed further analysis of the outcomes resulting from gentamicin exposure, and noted several additional phenotypes in zebrafish embryos that received an intramuscular injection at 48 hpf with gentamicin at a concentration of 2.5 mg/mL (Figs 4 and 5). At 24 hours postinjection (hpi), the yolk sac of the embryo showed a darkened hue, and some pericardial edema was evident, consistent with the phenotype of renal insufficiency observed by others[68,72] (Fig 4, *A*). By 48 hpi, the yolk sac had continued to darken and the edema increased

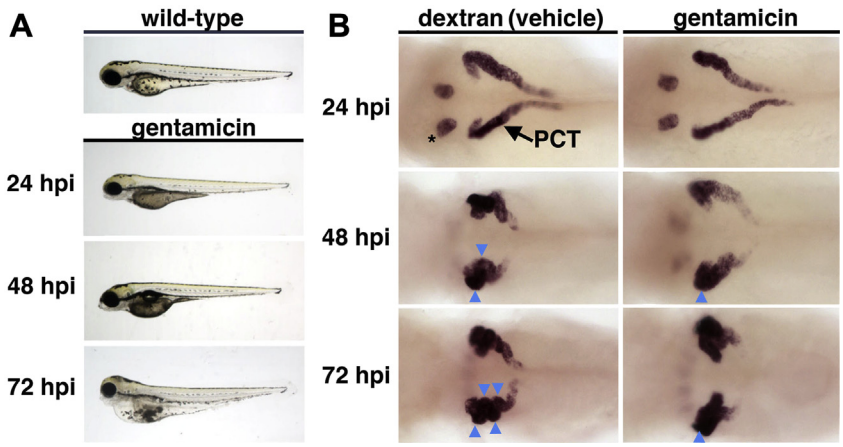

Fig 4. Acute kidney injury (AKI) modeling in the zebrafish embryo. Gentamicin exposure resulted in pericardial edema and hindered proximal tubule development. **A,** Live images of a wild-type and embryos injected with gentamicin at 48 hours postfertilization (hpf). The wild-type embryo and 24 hours postinjection (hpi) embryo shown were 72 hpf, with 48 and 72 hpi embryos shown to document the progression of the gentamicin phenotype. Gentamicin injected embryos displayed a darkened yolk sac and pericardial edema at 24–72 hpi (×4 magnification). **B,** A comparison of whole mount *in situ* hybridization of embryos following either dextran injection (vehicle control) or gentamicin injection at 48 hpf. Gentamicin injected embryos showed delayed proximal convoluted tubule (PCT) coiling (arrow), visualized with the *slc20a1a* transcript signal (purple); asterisk (*) indicates *slc20a1a* staining in trunk mesenchyme that is not associated with the pronephros. Blue arrowheads demarcate tubular folds on the left nephron in 48 and 72 hpi embryos, the number of which is reduced in gentamicin-injected embryos. Dorsal views are shown, with anterior to the left (×10 magnification).

to a moderate level. Severe pericardial edema and body curvature was observed in embryos at 72 hpi. Following documentation of live embryos, several zebrafish were selected for further analysis and processed through *in situ* hybridization with *slc20a1a*. The gene *slc20a1a* is a sodium dependent phosphate transporter that has previously been used to specifically distinguish the location of the proximal convoluted tubule (PCT) from the other segments in the zebrafish pronephros.[10] During normal development, the expression of *slc20a1a* can be detected by 24 hpf in parallel tracks of the PCT (Fig 4, *B*).[10] Between 24 and 20 hpf, *slc20a1a* transcripts continue to be highly expressed in the PCT, enabling its clear visualization. At approximately 48 hpf, the cells occupying the PCT begin morphogenesis from linear tubes into a compact coiled structure (Fig 4, *B*). Initially, the rostral-most PCT tubes display a lateral shift and form a characteristic 'Y' shape, and then between 96 and 120 hpf undergo progressive coiling to form a tightly packed unit located rostral to the yolk sac at 120 hpf. The driving force behind the coiling of the PCT segment is fueled by a combination of cellular division within the distal segments,[10] and collective migration of distal segments.[80,81] However, gentamicin exposure obviates this process of nephron morphogenesis. In our analysis, embryos fixed at three time points post-gentamicin injection (24, 48, and 72 hpi) and processed through whole mount *in situ* hybridization with *slc20a1a* revealed that gentamicin delayed the PCT coiling process (Fig 4, *B*). In addition, spotted staining of cells within the tubule was noted. This could indicate PCT cells that should have been stained with the marker had either undergone necrosis and sloughed off, or were too damaged for recognition by the *slc20a1a* RNA probe.

To further analyze the effects of gentamicin exposure on tubular integrity and epithelial cell architecture, immunohistochemistry was performed on tissue cryosections of injected zebrafish at 24 and 48 hpi (Fig 5). The use of a transgenic line that stably expresses green fluorescent protein in larval zebrafish (Tg:*enpep*:eGFP) enabled the visualization of the pronephric duct and tubules.[82] In healthy rat kidneys, phalloidin has been characterized as having an affinity for the actin in the apical brush border microvilli of proximal tubule epithelial cells.[83] Tissue cryosections of healthy and injured embryos were stained with phalloidin at 24 and 48 hpi (Fig 5). No disruption in tubule structure or epithelial polarity was noticeable in the healthy, uninjected control embryos at either time point; the lumen was clearly demarcated by a band of actin (Fig 5, *A*). In the gentamicin-injected embryos, tubules show disruption in the apical phalloidin band and cellular architecture at 24 hpi, as evidenced by discontinuities in the band of phalloidin-positive cells (Fig 5, *A*). The cells observed at the phalloidin gaps appeared to be dysmorphic, with fissures in anti-GFP staining suggestive of cytoplasmic disruptions. By 48 hpi, the luminal space within the tubules was collapsed (Fig 5, *B*) and some

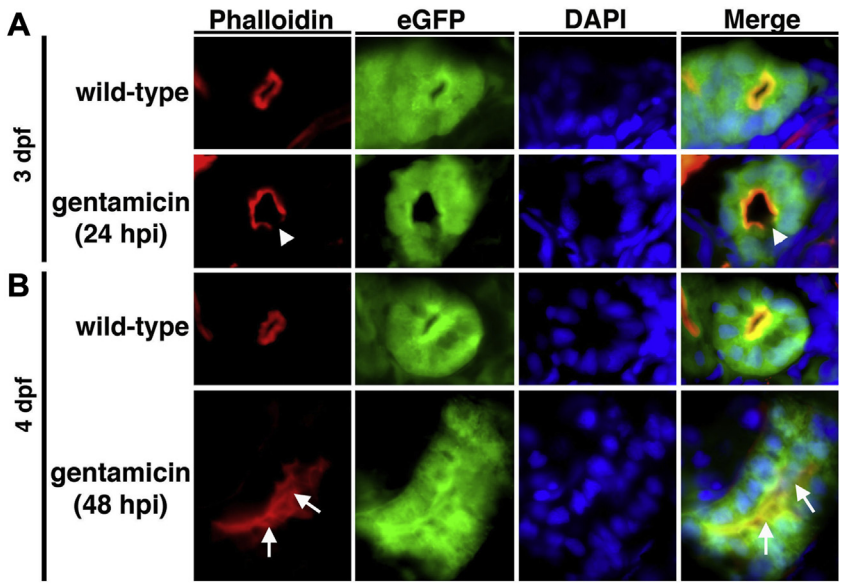

Fig 5. Analysis of tubule cell composition and architecture revealed that gentamicin disrupts the apical-basal polarity of renal tubules. Tubules of wild-type *enpep*:eGFP transgenic embryos and gentamicin injected embryos were analyzed with immunohistochemistry to detect the tubule cells, which were demarcated using anti-eGFP antibody (green). The apical surface of tubule cells was labeled with phalloidin (red) and nuclei were labeled with DAPI (blue). (A,B, top rows) Control embryos at 3 days postfertilization (dpf) and 4 dpf displayed an intact luminal border respectively, Embryos that received gentamicin showed structural disruptions in (A, lower row) the phalloidin staining of tubular epithelial cells at 24 hpi (white arrowhead), and (B, lower row) displayed collapsed lumens at 48 hpi (white arrows) (×60 magnification). All embryos were injected at 48 hours postfertilization (hpf).

areas appeared to be filled with cells and/or cellular debris (data not shown), suggestive of tubular disorganization and epithelial cell death. In addition, phalloidin staining was diffuse and disorganized although it was generally dispersed in regions closely adjacent to the debris-filled lumen. Thus, independent lines of evidence demonstrate that gentamicin triggers AKI, causing damage to the zebrafish pronephros that grossly mimics mammalian AKI damage, with disrupted apical-basal polarity of the tubular epithelium and massive tubule cell shedding.

Prospects for AKI regeneration studies in embryos with gentamicin or laser ablation damage. Although the injury following gentamicin is similar, several groups have now documented that gentamicin treatment is lethal to the zebrafish embryo.[68,72] We have also found through further testing of gentamicin doses that all embryos that developed edema were unable to survive. From these data, it appears that gentamicin exposure causes nephron tubular damage that is far too catastrophic for the embryo to recoup through any type of repair or regeneration without some form of intervention. The embryonic and larval zebrafish possess only two nephrons, and both are exposed during gentamicin systemic administration. Thus, the generalized damage to both nephrons may be one explanation for this outcome. Whether the embryo can repopulate its damaged pronephros epithelium in this context remains unknown.[68]

However, a very promising venue for future study has been demonstrated through an innovative approach to identify small molecules capable of rescuing gentamicin-induced edema. In a recent report, zebrafish larvae injected with gentamicin were treated with a specific histone deacetylase inhibitor (HDACi), methyl-4-(phenylthio)butanoate (m4PTB) beginning at 2 days postinjection (dpi), when AKI symptoms like edema and loss of cell polarity were first evident.[73] Results revealed that m4PTB treatment increased zebrafish embryo survival.[73] m4PTB treatment also led to elevated cell proliferation, and the dividing cells were found to express paired box 2—a long-appreciated hallmark of nephron tubule regeneration in the mouse.[73] While m4PTB enhances the functional recovery of the zebrafish kidney after gentamicin-induced AKI,[73] the same research group initially reported this HDACi was able to expand the embryonic renal progenitor cell field that initially produces the pair of pronephric nephrons.[84] They were spurred to test m4PTB in the setting of gentamicin-induced AKI with the rationale being that compounds that expand renal progenitors during development might be capable of enhancing recovery by driving cell cycle progression in fully-formed nephrons.[73]

The extremely exciting aspect of this zebrafish-centered research was the finding that m4PTB treatment was beneficial to mice with AKI from ischemia.[73] Mice with moderate IRI that were given m4PTB had accelerated recovery, and mice with severe IRI showed reduced interstitial fibrosis.[73] The researchers found that m4PTB treatment was associated with elevated cell cycling in tubular cells and a decrease of cells in G2/M arrest.[73] These results indicate that there are fundamental similarities in the response to AKI from chemical toxins between the zebrafish and mammalian kidney.[73,85] Thus, these data strongly suggest the practicality of using zebrafish as a simplified screening tool for drug discovery that can be relevant to mammals, but would at present be prohibitive for many labs working with mammalian models.

In addition, another promising injury model for future studies is laser ablation injury. While gentamicin-injury in the zebrafish embryo is lethal, focal tubule injury to a single nephron is typically not lethal.[69] Further, there is some evidence for tubular regeneration based on observations of gross cellular replacement that were documented following laser ablation injury of pronephros cells in the zebrafish embryo (Fig 6).[69] Laser ablation could potentially serve as a highly controlled *in vivo* model of AKI, as this protocol allows the induction of cell death in focal areas within the kidney tubule. Substantial work needs to be done to characterize this damage model. One intriguing potential with this approach is that different populations of cells throughout the nephron can be targeted, allowing analysis of injury and regeneration mechanisms in discrete nephron segment populations.

AKI MODELING IN THE ZEBRAFISH ADULT

The adult zebrafish kidney, or mesonephros. As previously mentioned, the embryonic zebrafish pronephros develops into the adult kidney known as the mesonephros.[4-6] The adult zebrafish mesonephric kidney is a single, flattened structure that is adherent to the dorsal body wall via connective tissues (Fig 1, *C*).[86] Anatomically, the kidney consists of 3 main parts: the head, the trunk or so-called saddle, and the tail. Nephrons in the mesonephros are similar to those found in the embryonic kidney; however, the adult kidney nephrons are highly bifurcated and are drained by 2 collecting ducts (Fig 1, *C'*).[10,70,71] As the zebrafish ages, new nephrons are continually added to the kidney, and arise from renal progenitors that are thought to be interspersed among the interstitial stroma located between nephrons.[70,71] This process of neonephrogenesis shares molecular hallmarks with the neonephrogenesis induced after renal injury (discussed in more detail below). Utilizing the adult zebrafish in experimental studies is beneficial because it enables the examination of hundreds of nephrons (approximately 300–500 depending on the age of the adult fish) compared with the 2 nephrons found in embryos.

Histology of the adult kidney renal structures compared to the mouse. Hematoxylin and eosin (H&E) staining is a basic method that distinguishes the proximal tubules from the distal tubules based essentially on the presence of a brush border: proximal tubules possess a brush border, whereas distal tubules do not.[87] The luminal surface of the epithelial cells of the proximal segment is lined with densely packed microvilli forming a border that greatly increases the surface area of the cells. When paraffin sections of adult zebrafish kidney between 9 and 12 months of age were stained with H&E, the brush border is prominent, along with the characteristic elongated cells and dilated lumen of the proximal tubule (Fig 2). In addition, the cells of the distal tubule formed a narrow lumen and appeared to stain a much lighter shade of pink, allowing further confirmation of segment identity. H&E staining in the mammalian kidney reveals a comparable staining result.[88]

Renal regeneration events in the adult zebrafish: epithelial replacement and neonephrogenesis. Research in adult zebrafish has documented several parallels in the processes of gentamicin-induced injury and regeneration compared with mammals. First, there is an initial phase of cell death and denuding of the basement membrane in the proximal tubule. Further, there is flattening and loss of the brush border followed by a repopulation of the basement membrane (Fig 7).[70] It is speculated that new cells emerge through proliferation of tubular epithelial cells, and the process of regeneration leading to functional restoration of the proximal tubule is complete in 2 weeks (Fig 7).[70] Gentamicin injections in the adult zebrafish resulted in damaged nephrons that failed to take up 40-kDa dextran (a test of functionality) and a downregulation of *slc20a1a*, the PCT segment solute transporter marker.[70] Over subsequent days, expression of *slc20a1a* was steadily regained in nephron tubules. By 15 dpi, the damaged nephrons had recovered to near-normal functional levels, as determined by *slc20a1a* staining and dextran uptake assessment, thereby suggesting regeneration had occurred.[70]

In addition to the injury phase and repair phase, adult fish have an additional phase that makes them a valuable model; they respond to injury with *de novo* nephron development.[89] Several days after gentamicin injury in zebrafish, clusters of cells (which have been also termed nephrogenic aggregates) appear and they grow and elongate in a process that recapitulates

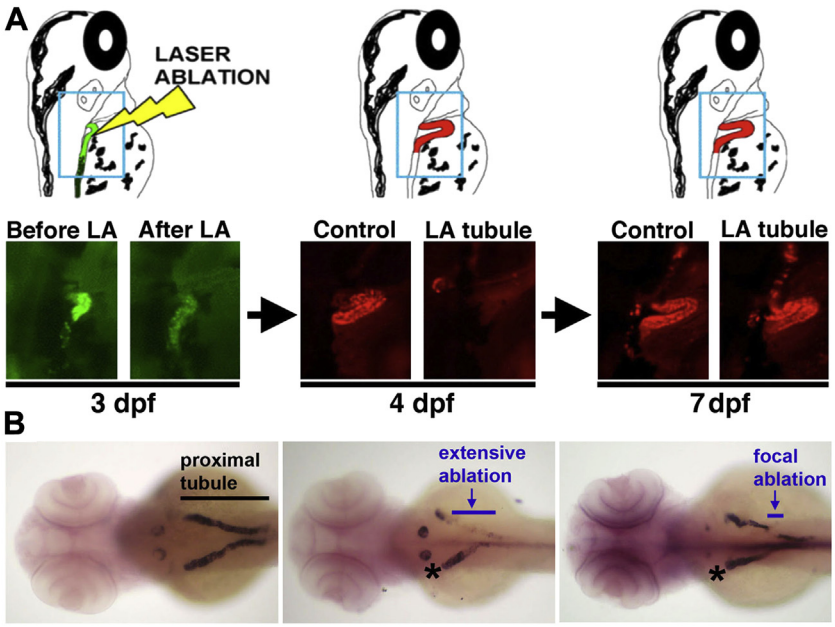

Fig 6. Laser ablation of the zebrafish pronephros. **A,** (top) zebrafish schematic showing region of proximal tubule laser ablation, with (bottom) tubule labeled with dextran-FITC (green) or dextran-rhodamine (red). Cells ablated at day 3 of development are replaced by day 7, suggestive of robust tubular proliferation that regenerates the ablated cell populace. **B,** Whole mount in situ hybridization for slc20a1a to demarcate the proximal tubule in a wild-type control (left), or embryos fixed immediately after ablation: an embryo with extensive cell ablation (center) and an embryo with a focal ablation (right). All views are dorsal. *Images reprinted with permission.[69]

mesonephric nephrogenesis.[70,71] Live imaging of nephron formation in zebrafish larvae reveals that nephrogenic aggregates form by merging cells, which then differentiate into nephrons.[70] Consistent with this, the source of new nephrons in the injured adult zebrafish has been traced to small cellular aggregates that are characterized as long-lived with a significant replicative potential.[70,71] The clusters can be identified through histological analysis as cells that appear a dark-purple hue because they are basophilic (Fig 7). Induced nephrotoxicity in the goldfish has similarly demonstrated that their kidneys are capable of developing new nephrons.[90] After nephrotoxin exposure, goldfish exhibit tubular necrosis with luminal debris. Subsequently, new nephrons were identified as arising from basophilic cell clusters that enlarge, form lumens, and eventually elongate into eosinophilic tubules reminiscent of a fully mature nephron.[90] Similarly, the renal tubular epithelium of the medaka kidney exhibited severe damage after exposure to the same nephrotoxin.[91] The initial response to the injury was repair of damaged nephrons, followed by a second regeneration phase in which numerous mesenchymal clusters and nephrogenic bodies were observed. The appearance of developing nephrons was established as a hallmark for the recapitulation of normal nephron development.[91]

In particular, the recent finding that zebrafish undergo neonephrogenesis means that this genetically tractable model can be used as a paradigm to dissect the molecular mechanisms of neonephrogenesis, which have been prohibitive in other species like goldfish. Another appealing avenue for future investigation is the application of chemical genetics to interrogate the role(s) for known signaling pathways in the tubular regeneration phase and neonephrogenesis process. Identification of markers that enable the isolation of scattered renal progenitors will also be crucial, so that the behavior and modulation of these cells can be studied. However, it should be kept in mind that the ability to continually add nephrons to the adult kidney attributable to the presence of renal progenitors is a feature of many teleost fish species. Because continual kidney growth of this nature is not an attribute of mammals, the mechanisms of neonephrogenesis may in fact be species-specific. Understanding the differences could also provide tremendous insights about whether mimicking neonephrogenesis in mammals will be possible.

CONCLUSION, FUTURE PERSPECTIVE

A fundamental understanding of zebrafish kidney regeneration may offer insights about how to stimulate

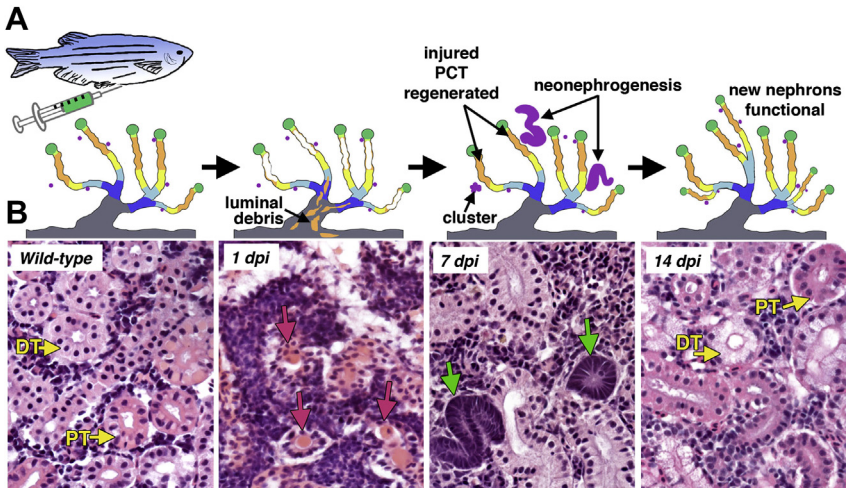

Fig 7. Acute kidney injury (AKI) modeling in the zebrafish adult; there are 2 regeneration responses. **A,** Intraperitoneal injection of gentamicin into the adult fish (schematic). **B,** (top) A timecourse of schematics and (bottom) histologic sections stained with hematoxylin and eosin showing the major cellular events. The uninjured kidney contains both proximal tubules (PT) and distal tubules (DT) (yellow arrows). At 1 day postinjection (dpi), luminal debris is seen as tubular casts (pink arrows) that fill surviving tubule lumens. At 7 dpi, proximal convoluted tubule (PCT) integrity is restored, and sections contain basophilic (dark purple) clusters and S-shaped tubular structures that correspond to new nephrons (green arrows). By 14 dpi, basophilic structures are infrequent, and the tissue is dominated by tubules with either proximal (dark pink) or distal (light pink) staining (yellow arrows).

regeneration in the setting of other kidney diseases. Although zebrafish, other fish models, and mammals display nephron regeneration, many questions have not been addressed in previous studies. The nature of reparative tubule epithelia, (eg, the contributions of surviving G1 tubular cells and prospective tubular stem cells) is still an issue to resolve and can be performed using genetic fate mapping and lineage analysis. It will likely prove informative to the nephrology field to perform such studies in both zebrafish and mouse models, as a comparative analysis of this regeneration process may reveal crucial similarities and differences. Transgenic injury models in zebrafish have also been developed, and these methods of nephron injury will also provide useful avenues for research. For example, transgenic injury models can target particular cell types and then evaluate regeneration. This has been reported recently for the podocyte cells that comprise the blood filter.[92,93] In addition, the zebrafish in particular provides a unique opportunity to visualize cell dynamics in real time; cells within the embryonic kidney can be recorded to document cell migration and proliferation. This will provide a useful *in vivo* way to study tubular regeneration in the context of the whole organism and, also, to interrogate the process in different injury models and when the environment is altered with small molecules.

A major question that remains is the identity and workings of the molecular events that regulate renal regeneration after acute injury. Identifying the pathways that regulate the behavior of reparative epithelia would address a major gap that exists in the field of nephrology. Through the success of using zebrafish chemical genetics approaches to gain insights into AKI and polycystic kidney disease,[73,94] it is clear that recent work has established the essential groundwork to study renal regeneration and disease using the zebrafish. The similarities in tubular regeneration events between zebrafish and mammals support the notion that many molecular signals and mechanisms may be conserved between these species.

Ultimately, the discovery of renal progenitors capable of neonephrogenesis in the zebrafish adult opens a new portal for clinical studies given the ability to induce cell type changes with defined factors. Knowledge of the critical regulators that define the renal progenitor identity could allow researchers to test if controlled expression of these genes can induce nephrogenesis in the mammalian kidney—which would constitute a major breakthrough for the treatment of kidney disease. Current and future studies in zebrafish are an exciting research area that may identify renal regeneration pathways and/or repair mechanisms, and therefore provide formative clues concerning the recipe of signals that are essential to mediate kidney regeneration in humans.

The authors thank the staffs of the Department of Biological Sciences and the Center for Zebrafish Research for their support, and the members of our research lab for stimulating conversations about this topic.

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