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REVIEW

(Zebra)fishing for nephrogenesis genes

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ABSTRACT

Kidney disease is a devastating condition affecting millions of people worldwide, where over 100,000 patients in the United States alone remain waiting for a lifesaving organ transplant. Concomitant with a surge in personalized medicine, single-gene mutations, and polygenic risk alleles have been brought to the forefront as core causes of a spectrum of renal disorders. With the increasing prevalence of kidney disease, it is imperative to make substantial strides in the field of kidney genetics. Nephrons, the core functional units of the kidney, are epithelial tubules that act as gatekeepers of body homeostasis by absorbing and secreting ions, water, and small molecules to filter the blood. Each nephron contains a series of proximal and distal segments with explicit metabolic functions. The embryonic zebrafish provides an ideal platform to systematically dissect the genetic cues governing kidney development. Here, we review the use of zebrafish to discover nephrogenesis genes.

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1. Introduction: a cellular tour of the human kidney

In humans, the metanephric kidneys are a pair of vital organs that are situated in the back of the abdomen and nestled beneath the ribcage on each side of the spine.[1] The kidney is responsible for performing numerous physiological tasks such as ion homeostasis, maintenance of acid–base balance, regulation of blood pressure, hormone production, and clearance of toxins, among others.[1,2] On average, each adult human kidney is comprised of approximately 1 million microscopic functional units called nephrons.[3] The nephron consists of two core compartments, the renal corpuscle (blood filter) and an epithelial tubule, which connects to a collecting duct.[4] Broadly, blood flows through the afferent arteriole into the glomerulus, which encases tiny capillaries that act as a molecular sieve. The resulting fluid, or filtrate, undergoes transit through the tubule and plumbs into a highly arborized collecting system for waste excretion. The multipartite anatomy of the nephron allows for stepwise modification of the filtrate, where essential nutrients are reabsorbed, and waste products are concentrated (or retained) to produce urine. Nephrons are surrounded by renal stroma, which provide a supportive framework by synthesizing extracellular matrix and growth factors. The renal stroma is a heterogenous interstitial population that consists of resident fibroblasts, vasculature, immune cells, and other cell types yet to be resolved,[4] whose developmental origins are under ongoing study.[5] In the following introductory sections, we discuss the unique functions of each nephron region in more detail.

1.1. Glomerulus: the head of the nephron

As mentioned previously, the nephron is a functional unit of the kidney and depends on a diverse inventory of differentiated cells to perform specialized physiological tasks.[4] Situated at the proximal end of the nephron, the glomerulus is a sphere-shaped filtering structure that serves as the first point of entry via an afferent arteriole.[6,7] The glomerulus encases a tuft of capillaries that drain into an efferent arteriole. Capillaries are comprised of endothelial cells that have fenestrations, approximately 70–100 nm in diameter, to allow the movement of larger molecules without

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membrane transporters. A collection of mesangial cells provides a supportive matrix around the glomerular microvasculature by secreting soluble factors. Mesangial cells send essential signals to podocytes, which are cells with interdigitating foot processes that are situated on the glomerular basement membrane, where they enwrap capillaries to form the slit diaphragm. Cell surface proteins like Nephrin (NPHS1) and Podocin (NPHS2) help to create the slit diaphragm, which serves to prevent passage of macromolecules, like plasma proteins. Insults to the slit diaphragm results in progressive glomerular disease and proteinuria, whereas damaged podocytes are believed to have a limited regenerative capacity.[8] Interestingly, parietal epithelial cells, which line the glomerular capsule, are believed to play a reparative role upon acute injury. Lineage tracing experiments performed in mice suggest parietal epithelial cells differentiate into podocytes and can ameliorate proteinuria and preserve renal function, though this remains a highly scrutinized and controversial topic.[9–13]

1.2. Proximal tubules: the major site of reabsorption

After passage through the glomerulus, the filtrate then transits to the nephron tubule, which in humans is compartmentalized into a series of proximal, intermediate (or loop of Henle), and distal segments which join to a collecting duct.[14] Each segment expresses a unique molecular signature defined by distinct solute transporters and tight junction proteins.[15] The proximal convoluted tubule (PCT or S1-S2 segments) and the proximal straight tubule (PST, which most closely corresponds to the S3 segment) perform the bulk of the reabsorption activities and undergo transepithelial transport of glucose, solutes, amino acids, and low molecular weight proteins.[16,17] Collectively, proximal tubule cells also play a major role in regulating acid–base balance by reabsorption of bicarbonate.[16,17] Another role unique to the proximal tubules involves glucose reabsorption via sodium-glucose cotransporters (SGLT).[16,17] Specifically, the PCT expresses SGLT2, and the PST expresses SGLT1, which are encoded by SLC5A2 and SLC5A1, respectively.[18–21] In terms of ultrastructure, the PCT cells have a wider brush borders, denser microvilli, and more endocytic vesicles as compared to PST cells.[16,17]

1.3. Loop of Henle: initiating a concentration gradient

Directly downstream of the PST is the loop of Henle, which functions to initiate an osmotic gradient via a countercurrent multiplication mechanism.[22,23] The loop of Henle is subdivided into three regions: the thin descending limb, thin ascending limb, and thick ascending limb (TAL), also referred to as the distal straight tubule.[22,23] The thin limbs are mutually known as the intermediate segment, are identified by aquaporin-1 expression, and perform transport of Na+, urea, and water.[24] In contrast, the TAL is impermeable to water and expresses apical channel proteins that transport Na+, Cl-, and K+ ions. Interestingly, the TAL is believed to contain two subtypes of cells: 1) rough surfaced cells (R cells) that exhibit high apical K+ conductance and weak basolateral Cl- conductance 2) smooth surfaced cells (S cells) that exhibit low apical K+ conductance and high basolateral Cl- conductance.[23] To this point, NKCC2 (Na-K-Cl cotransporter) is present on the apical surface of both R and S cells, however KCNJ1 (K+ channel) expression appears absent in S cells.[23] Our current understanding of the cellular heterogeneity of the TAL remains extremely limited and additional studies are needed to dissect unique molecular phenotypes and functions that further define these cell types.

1.4. Distal tubule: fine tuning the filtrate and drainage to the collecting duct

Adjoining the loop of Henle is the distal tubule that functions to fine tune the filtrate by balancing Na+, K+, Ca2+, and Mg2+.[25] The distal tubule is partitioned into two segments: the distal convoluted tubule (DCT) and connecting tubule (CNT).[25] The DCT reabsorbs Na+ mainly by employing the apical channel protein NCC, which is encoded by the SLC12A3 gene. Further, the DCT cells have abundant mitochondria, extensive basolateral infoldings, apically shifted nuclei, and high

Na^+K^+ATPase activity as compared to the CNT cells, which are less metabolically involved.[25] Both the DCT and CNT respond to secretion of the steroid hormone aldosterone by mineralocorticoid receptors.

The distal tubule attaches to the collecting duct (CD) and is responsible for water reabsorption and helps maintain acid–base balance by action of interspersed intercalated cells and principal cells. Intercalated cells are present in both the CNT and CD, reabsorb small amounts of residual bicarbonate, and excrete ammonium. Defining ultrastructural features of these cells include copious mitochondria, irregular apical microvilli, and absence of a central cilium.[26] Principal cells of the collecting duct epithelium constitutively express water channels, AQP2 and AQP3, which aid in urine concentration.[27,28] Taken together, the nephron exhibits tremendous cellular and functional diversity. With the advent of single-cell sequencing technologies, we will continue to elaborate a greater understanding of the molecular signatures and significance of these different nephron cell types.

2. Developmental origins: congenital anomalies of the kidney and urinary tract

Congenital anomalies of the kidney and urinary tract (CAKUT) occur in 1 in 500 births, lead to poor clinical outcomes, and are at the root of pediatric end-stage renal disease.[29–33] To this end, it is of utmost importance to fully understand the biological mechanisms underlying these conditions. CAKUT can manifest as an assortment of renal tract malformations of the kidney, ureter, bladder, and urethra and exhibit a broad spectrum of phenotypes often with variable penetrance. Perhaps the most severe class of these disorders is renal agenesis, which entails the complete absence of one or both kidneys upon birth. Another subtype encompasses renal hypoplasia where a reduced number of nephrons are formed during embryonic development commonly resulting in decreased kidney mass. Mild renal hypoplasia can still pose a threat later in adult life, as reduced nephron endowment predisposes patients to hypertension and chronic kidney disease.[34]

CAKUT may emerge as isolated incidents via de novo mutations or as part of a familial syndrome with extrarenal phenotypes such as neurodevelopmental and cardiovascular defects.[35] Despite the diverse spectrum of these renal anomalies, they all stem from one commonality: faulty or absent nephron function due to dysregulation of developmental gene networks, which involve transcription factors, signaling molecules, and growth factors. There are sequential steps fueled by genetic programs to facilitate nephrogenesis such as ureteric bud induction, mesenchymal-to-epithelial transition (MET), renal branching morphogenesis, and nephron segment patterning and elongation.[36,37] Previous studies in mice indicate perturbing any of these processes can mirror clinical features observed in CAKUT.[38] To date, more than 50 CAKUT-causing genes have been identified.[39,40] Although it is well-known single-gene mutations can initiate renal pathogenesis, overall poor genotype–phenotype correlation likely points to the involvement of multiple factors or modifier genes.

Only a handful of studies have screened large patient cohorts to identify specific mutations linked to CAKUT. A 2014 study conducted by Hwang et al.[35] utilized a targeted sequencing approach and identified 33 novel monogenic lesions associated with isolated pediatric CAKUT, where lesions in SALL1, HNF1B, and PAX2 loci were most prevalent. However, more recent investigations have focused on how genetic landscape and copy number variation (CNV) influence kidney anomalies. CNV contributes to genetic diversity in the human population but can produce negative outcomes by altering gene dosage triggering sporadic traits and complex diseases.[41] One research group surveyed approximately 3,000 CAKUT cases across the phenotype spectrum for CNVs that intersect genes by performing whole-genome genotyping of peripheral blood samples. Their deletion mapping and prioritization analyses revealed TBX6 as the chief genetic driver in CAKUT patients exhibiting heterozygous 16p11.2 microdeletion syndrome, where an allelic series of TBX6 mutant mice was generated to provide functional validation, though it should be noted that mice show phenotypes when mutations or deletions occur in both Tbx6 alleles.[39] Further, a 2020 investigation employed a chromosomal microarray

analysis strategy which identified six novel pathogenic CNVs upon cord blood testing of fetal CAKUT patients.[42] Single nucleotide polymorphism arrays revealed CNVs within HNF1B and CLDN16 gene regions. Interestingly, in this study, the highest incidence of CNVs occurred in fetuses with polycystic kidney dysplasia.[42] Collectively, these studies indicate that multiple genetic factors contribute to disease complexity and provide evidence that CNVs and genetic predisposition play a significant role in CAKUT pathogenesis. There is also an increasing appreciation of the impact from various environmental factors.[43] An ongoing challenge in the discovery of novel CAKUT-causing genes is the difficulties posed by genotype–phenotype heterogeneity and the high frequency of sporadic cases. To combat these issues, future large-scale genomics studies paired with functional assessment of vertebrate models holds promise in identifying genetic drivers of CAKUT.

3. The zebrafish pronephros: a window into genetic regulation of embryonic kidney development

Within the past decade, the zebrafish has ascended as a valuable model system to study nephron ontogeny, function, and model kidney diseases.[44–60] The structure of the zebrafish pronephros encompasses two parallel tubules that fuse with a glomerulus at the midline.[61] In addition to a simple two-nephron anatomical layout, embryonic zebrafish are optically transparent and develop rapidly *ex utero*, which enhances the ability to visualize cellular changes.[62,63] By comparison, mammals undergo internal embryonic development and contain thousands of nephrons arranged in a complex tissue architecture, which can present significant challenges to studying kidney organogenesis.[64,65] The zebrafish pronephros tubule is epithelialized and partitioned into distinct proximal and distal segments by 24-h post fertilization (hpf).[66,67] Subsequent morphogenesis leads to the formation of a single glomerulus at the midline connected to the bilateral nephron tubules.The glomerulus begins active filtration by approximately 48 hpf,[46,68–75] and further morphogenesis events transpire to drive progressive coiling of the proximal tubules, which occurs through a combination of distal tubule proliferation and rostrally directed collective cell migration.[76–78] In addition, the segmental organization of the pronephros displays considerable conservation with higher vertebrates (Figure 1).[79–81] Beginning at the rostral end, the pronephros is subdivided into the following territories: podocytes (P), neck segment (N), proximal convoluted tubule (PCT), proximal straight tubule (PST), distal early tubule (DE), distal late tubule (DL), and pronephric duct (PD).[79–81] The PD extends into a specialized opening called the cloaca (C) for waste excretion.[79–81] Pronephric segment domains are occupied by distinct cohorts of monociliated transporter cells that differentially express ion channel and tight junction genes[66,67], as well as interspersed multiciliated cells (MCCs) with distinct gene signatures[82–85]. The solute transporter signatures that define the different tubule segments within the zebrafish pronephros exhibit considerable overlap with mammalian nephron segment markers.[79–81]

3.1. Conserved segment properties of the zebrafish pronephric tubule

The zebrafish PCT and PST share many attributes with mammalian proximal tubules.[80,86–93] For example, the cells of the pronephric PCT apically project densely packed microvilli forming a well-defined brush border and actively endocytose traceable dextran conjugates.[86–89] Additionally, the expression of *megalin/lrp2*, *cubilin/cubn*, and *slc20a1a* are synonymous to mammalian proximal tubule markers.[80,86–89] The mammalian PCT, or S1-S2 segment, expresses *Lrp2*, *Cubn*, and *Slc20a1*, which encode transmembrane proteins that facilitate bulk reabsorption at this site.[4,92] From 3 to 5 days post fertilization (dpf), the zebrafish PCT undergoes coiling morphogenesis, which mimics the convolutions present in mammalian proximal tubules.[45] The embryonic zebrafish PCT and PST both express *slc5a1*, which produces a sodium/glucose cotransporter protein (SGLT).[91,94] This slightly deviates from mammalian nephrons, where two members of the SGLT family (*Slc5a1* and *Slc5a2*) are differentially expressed in the PST and PCT, respectively. The pronephric PST is also demarcated by *trpm7* and *slc13a3* genes.[80] In mammals, the PST expresses *Slc13a3*, however TRPM7

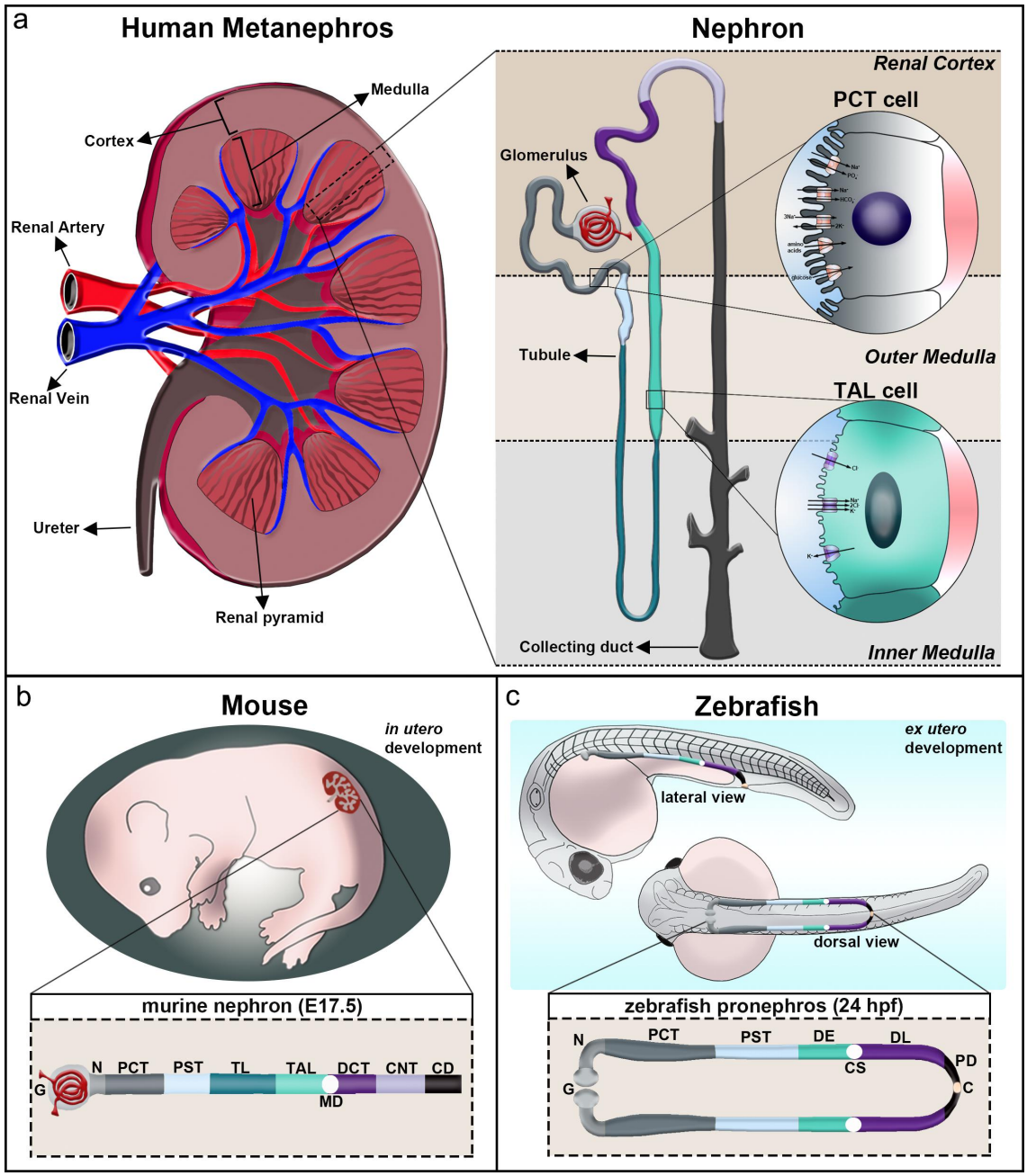

Figure 1. Nephron segmentation is conserved across vertebrate species. (a) Schematic of adult human kidney (left) and segmented nephron (right). Circle insets depicts a proximal tubule cell (above, gray) and thick ascending limb cell (below, teal) with specialized solute transport proteins (orange, purple) on the apical surfaces. (b) Developing mouse at embryonic day 17.5 and color-coded nephron segment compartments. (c) 24 hpf zebrafish embryo and color-coded pronephros segment compartments. Matching segment colors indicate the current working model of analogous structures between mammals and zebrafish. Abbreviations: PCT = proximal convoluted tubule, PST = proximal straight tubule, TAL = thick ascending limb, DE = distal early segment, MD = macula densa, CS = Corpuscle of Stannius, DCT = distal convoluted tubule, DL = distal late segment, CD = collecting duct, PD = pronephric duct, C = cloaca. Schematics adapted in part from.[79.]

along with TRPM6 are enriched in the DCT and modulate Mg2+ homeostasis.[95]

The pronephric DE segment is analogous to the mammalian TAL, which is known as the 'diluting segment' that decreases the osmolarity of the filtrate by playing a major role in Na+ reabsorption.[1,23] The DE is characterized by a conserved suite of solute transport genes: *slc12a1*, *kcnj1a.1*, and *clcnk*, which correspond to mammalian *Slc12a1*, *Kcnj1*, and *Clcnk*.[80] These DE/

TAL genes code for apically docked ion channels that transport Na+, K+, and Cl-, respectively. The DL segment expresses *clcnk* and *slc12a3* and parallels the mammalian DCT, which participates in fine tuning Na+ and Cl- absorption.[25,80] However, the DL also expresses *c-ret* and *gata3*, which are markers restricted to the collecting duct epithelium in mammals.[80] This difference is due to species divergence, as zebrafish are freshwater creatures that have no requirement for concentrating urine.

The DL curves around the yolk sac extension and merges with a short strip of tubule that is defined as the PD which opens to the external environment via the C.[80] Although there have only been a small number of studies examining PD and C development,[96–99] there is evidence that they share similar properties to mammalian CD epithelium.[80] The renal CD is typified by a distinct assemblage of tight junction proteins, which enforce epithelial barrier function and constitute paracellular anion channels.[100,101] One of such tight junction genes enriched in the CD is *Cldn8*, which is a member of the Claudin family and is believed to perform both pore and barrier-forming functions.[100,101] In the developing zebrafish pronephros, *cldn8* localization gradually restricts the DL, PD, and C by 24 hpf and shows diffuse expression in the DE and PST.[67] The PD and C also strongly express *occludin* tetraspan membrane genes *olcna* and *oclnb*.[67] Consistent with this expression pattern, human *OCLN* mapped to developing ureteric epithelium indicating a likely barrier function role in CD tissue (GUDMAP RID:165ZNM). Additionally, the zebrafish C expresses *aqp3*, and the mammalian homolog AQP3 marks principal cells within the CD.[80,102] AQP3 localizes to the basolateral membranes and transports water, glycerol, and H_2O_2.

3.2. Divergent features of the zebrafish pronephros

Although there are many parallels between the zebrafish pronephros and mammalian nephrons, key differences do exist. For example, the zebrafish pronephros tubules are associated with teleost-specific endocrine glands situated between the DE and DL segments, which are known as the Corpuscles of Stannius (CS).[80,103–106] This specialized cluster of cells buds off the pronephros by a gland extrusion mechanism, expresses *stanniocalcin 1* (*stc1*), and functions to maintain calcium and phosphate homeostasis.[80,103–106] To date, it is unclear if there is a mammalian equivalent of the teleost CS. Further, the zebrafish pronephros contains multiciliated cells (MCCs), which are interspersed in a "salt and pepper" fashion throughout the tubule.[82–85] MCCs mainly occupy the PST region, express markers such as *odf3b* and *cetn2*, and employ numerous motile cilia to propel fluid through the tubule.[82–85] They form in response to a steadily expanding list of factors including Gmnc, Mcidas, Etv5a, Etv4, E2f5, Ppargc1a, prostaglandin signaling, and others.[82–85,107–116] Interestingly, MCCs have been reported anecdotally in human fetal kidney tissue, but future studies are needed to confirm their presence and significance during renal development. The zebrafish pronephros also lacks the thin limbs of the Loop of Henle, a connecting tubule segment, and various collecting duct markers.[80,81] As mentioned earlier, these variations are consistent with the physiological needs of this freshwater species; therefore, the pronephros does not possess these segments and certain transporters because water conservation is not requisite. Despite these differences, the similarities in the overall nephron structure, segment attributes, and genetic profile of the pronephros are conserved with higher vertebrates, making the embryonic zebrafish a powerful system to study nephron development.

4. Tools for experimental study in the zebrafish model

Sophisticated molecular tools and protocols have been formulated to study gene function in the zebrafish.[54,117,118] Further, there continue to be exciting advances.[119,120] In the following sections, we provide an overview of several major experimental paradigms, which have been crucial for delineating mechanisms of organogenesis.

4.1. Chemical and genetic screening strategies in embryonic zebrafish

Forward chemical genetic screening is an approach where small-molecule libraries are applied to pinpoint molecular pathways that perturb a biological

process of interest.[121,122] Zebrafish have long been employed as an *in vivo* vertebrate system to perform high-throughput chemical screens and have the capacity to test greater than 10,000 different compounds. The first screen conducted to identify chemicals that affect developmental processes in zebrafish was carried out in 96-well plates.[123] Since then, hundreds of compounds have been identified to have conserved biological functions in fish and humans.[124] Zebrafish exhibit a constellation of features that make them amenable to high volume chemical and genetic manipulations. For example, one mating pair can produce greater than 100 embryos. In addition to high fecundity, zebrafish embryos are relatively small, approximately 5 mm in length, and most organs are formed within 24 hours. Once embryos are arrayed in a 96-well plate, small molecules can be added directly to the water and can readily penetrate the chorion and embryonic skin. Chemical treatments in zebrafish allow temporal control, as the drug can be added to the system at a chosen timepoint during embryonic development. Compared to cell culture-based screens, zebrafish offer a more comprehensive assessment by investigating the effects compounds have on a whole organism. Regarding drug activity, zebrafish mimic mammalian pharmacokinetic properties: absorption, distribution, metabolism, excretion, and toxicity (ADMET). In particular, screening libraries of known bioactive compounds is an approach widely used in zebrafish to conduct rapid genetic pathway analysis, as each of the small molecules tested has known mechanisms of action. Another advantage of the 'known bioactives screening strategy' is many of these drugs are FDA approved and could potentially be repurposed and fast-tracked through the clinical trial phases.

In addition to chemical screens, another route to reveal novel genes and pathways required for developmental processes is forward genetic screens. Significantly, the zebrafish genome contains homologs to approximately 70% of protein-coding human genes, therefore these organisms can facilitate the discovery of conserved biological mechanisms.[125] Large-scale genetic screens in zebrafish have been implemented successfully and identified thousands of genes implicated in embryonic development. In 1996, Boston and Tübingen groups reported exciting genome-wide mutagenesis screens conducted in zebrafish where the teams of researchers found embryonic phenotypes spanning differentiation, patterning, and organ defects.[126,127] Forward genetic screens involve isolating carriers of modified alleles that exhibit phenotypes of interest and subsequent mapping and/or sequencing approaches to identify the causative mutation. The standard chemical to induce heritable mutagenic lesions is ethylnitrosourea (ENU). When male zebrafish are exposed to this mutagen, hundreds of point mutations are generated in the spermatogonia.[128] The mutagenized male is crossed to a wild-type female, and the F1 generation is raised to sexual maturity, which is a process that takes approximately 3 months. Here, each F1 individual typically possesses one genetic lesion that will cause an embryonic phenotype.[118]

Many investigators have employed a haploid screen approach, which is an accelerated pipeline that does not require raising F2 and F3 generations to isolate recessive mutant alleles. To generate haploid embryos, eggs are harvested from F1 females and fertilized *in vitro* by treatment with ultraviolet inactivated sperm.[129] Because the paternal DNA is crosslinked, the sperm is unable to contribute viable DNA, but initiates events necessary for zygotic development. In the case of a recessive allele, the haploid progeny will consist of roughly 50% mutant animals; thus, this elevated penetrance enhances the detection of phenotypes.[129] Haploid embryos can survive for several days and have a similar morphology to diploid embryos. While haploid embryos have been documented to often display a shorter body axis, their body plans nevertheless enable assessment of numerous organs, such as the pronephros.[129] Upon detection of a haploid phenotype, the F1 female founder can be outcrossed to a wild-type male to generate a stable mutant line for further functional analysis.

Zebrafish are an accessible system to perform forward chemical and genetic screens because processes such as gastrulation and organogenesis can be directly observed with a basic stereomicroscope. In addition to examining live phenotypes, screen readouts can also consist of examining fixed embryos using whole mount *in situ* hybridization (WISH) or immunofluorescence to detect defects in a specific cell population or tissue.[130–133]

Over the years, a battery of zebrafish screens have revealed novel regulators of kidney development. In 2004, a forward genetic screen was conducted using an insertional mutagenesis strategy, where a pseudo-typed retrovirus served as the mutagen. The goal of this screen was to identify genes that initiate pronephric cyst formation, which is a phenotype that parallels human polycystic kidney disease (PKD). Because zebrafish embryos are transparent, kidney cysts can readily be visualized in the glomerular region between 2 and 5 dpf. Upon analysis of mutant zebrafish, 10 cilia-associated genes were linked to renal cystogenesis.[134] In 2005, a zebrafish ENU mutagenesis screen was performed to isolate alleles contributing to developmental defects. From this screen, the *kto* mutant was isolated that harbors a lesion in the *trap230* locus and displays abnormal development of the brain, neural crest, and kidney.[135]

Later, the advent of whole-exome sequencing technologies expedited the ability to perform large-scale genotyping of ENU-induced mutations in zebrafish. Ryan et al. (2013) utilized a publicly available Next-Generation Sequencing software processor, SNPtrack, to identify coding and splice site mutations that cause kidney cyst phenotypes.[136] Using this pipeline, four genes were rapidly identified that contribute to embryonic cystogenesis consisting of *ift172*, *lrrc6*, *kif3a*, and *dync2h1*.

A following study conducted an ENU F3 forward genetic screen and isolated zebrafish mutants that exhibited congenital edema.[79] Among the lines identified was the lightbulb mutant, which harbors a lesion in the retinoic acid (RA) biosynthesis gene *aldehyde dehydrogenase 1a2* (*aldh1a2*) and exhibits proximal-distal segment patterning defects consistent with reduced RA levels.[79–81] Subsequent cloning of the *zeppelin* mutant from this screen[79] by whole-genome sequencing analysis identified a mutation in *breast cancer 2, early onset* (*brca2*).[73] Upon examination for kidney defects, *zeppelin* displayed a loss of podocytes and elevated interrenal cells, which are akin to the mammalian adrenal gland.[73] This study indicated for the first time that the well-known tumor suppressor gene, *brca2*, is an essential component for glomerular development.[73]

Regarding chemical screen execution in zebrafish, manual approaches as well as automated imaging technology have been used to discover compounds that affect nephron development.[137–144] For example, in 2013 a pilot drug screen was performed utilizing the *Tg(wt1b:EGFP)* line that fluorescently labels podocytes and proximal tubules. Transgenic animals were positioned dorsally in microtiter plates for consistent live imaging of the pronephros. From this screen, acetaminophen, NSAIDS, and ACE inhibitors provoked gross morphological abnormalities of the pronephros.[138] Interestingly, a portion of the chemical screen 'hits' had been previously documented to exert adverse effects during human gestation, causing fetopathies and renal pathologies.[138]

Using a similar smart screening microscopy strategy, Pandey et al. (2019) profiled the effect of 1280 approved drugs from the Prestwick library on pronephric cyst development in *ift172* deficient zebrafish.[140] In this study, high-throughput image acquisition allowed the analysis of more than 20,000 zebrafish embryos for pronephric alterations. It is possible that automated zebrafish screening approaches may fast-track the discovery of compounds that alleviate PKD. In line with this idea, Metzner et al. (2020) conducted a chemical screen on *pkd2* mutant zebrafish.[143] This group selected the Spectrum library, which contains 2000 known bioactive compounds and FDA-approved drugs. After drug treatments, the degree of dorsal tail curvature was recorded via an automated 96-well plate microscope, which is a hallmark of ciliopathies in zebrafish. This screen identified 13 compounds that significantly altered the *pkd2* mutant curvature phenotype. One hit of interest consisted of a COX-2 inhibitor, diclofenac, which repressed tail curvature. Further, this screen brought to light that AR-independent androgen signaling and ALK5 are likely drivers of PKD progression.

In addition to automated live imaging of zebrafish, analysis of pronephric tissue in fixed samples by WISH can offer a more specific phenotype readout. For example, a 2016 study designed a chemical genetic screen to identify novel regulators of nephron patterning.[137,144] A total of 480 compounds from the ICCB Known Bioactives library were tested for effects on pronephric development.

After treatment, embryos were processed for multiplex WISH analysis, which involved the application of molecular probes that label alternating segments of the pronephros consisting of *wt1b*, *slc20a1a*, and *slc12a1*. After surveying the expression of these markers, the researchers found 16.25% of chemicals elicited nephron defects. A workup of several novel pathways from this screen has revealed that prostaglandin signaling, the transcriptional co-activator encoded by *ppargc1a*, and estrogen signaling all have discrete roles in regulating the nephron segment fate trajectory.[144–146]

In a separate study, the same ICCB Known Bioactive library was tested to define effectors of MCC development in the zebrafish pronephros.[111] The screen design involved processing treated animals for WISH and utilizing *odf3b* riboprobe to score differentiating MCCs.[111] It was determined that prostaglandin signaling promotes MCC ontogeny during pronephric development by influencing renal tubular cell fate choice, as well as promotes ciliogenesis consistent with findings of Jin and colleagues who previously demonstrated the effects of prostaglandin signaling on ciliary outgrowth.[111,147] Upon further dissection of this pathway, the investigators established novel roles for prostaglandin synthesis components *cox1* and *cox2* in MCC specification and ciliary outgrowth.[111] These chemical and genetic screen examples demonstrate the utility of the zebrafish pronephros in uncovering novel regulators of kidney development. In the future, combining chemical screening and emerging genetic engineering strategies, like CRISPR, in zebrafish could be employed to interrogate pharmacogenetic mechanisms and pursue personalized treatment options for kidney patients.

4.2. Classical reverse genetics in zebrafish: crispants, morphants, and misexpression

In recent years, CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats) technology has been adapted from a bacterial defense mechanism and applied to various model systems to make precise genome edits. The Cas9 protein can be guided to a specific site by a single guide RNA (sgRNA) and facilitates binding to the DNA at a protospacer adjacent motif (PAM). The Cas9 endonuclease is then able to uniquely cleave the DNA at a single site, causing a double-strand break and triggering endogenous repair mechanisms. Here, either homology-directed repair or nonhomologous end joining (NHEJ) results in sequence alterations that can disrupt the function of the gene of interest.[148] CRISPR-based approaches to create knockout and knock-in zebrafish models have revolutionized the field. A popular tactic to study loss of function in zebrafish is the generation of F0 CRISPR mutants otherwise known as 'crispants.' This method was first described by Jao et al. (2013) and typically entails the microinjection of a concoction containing Cas9 protein and multiple *in vitro* synthesized sgRNAs into the one-cell stage.[149] The delivery of various sgRNA sequences that target different locations in the same gene induces robust biallelic disruptions, and null phenotypes can be readily observed in the founder generation. The resulting F0 crispants are mosaic in nature, as this strategy relies on the NHEJ repair mechanism and generates random indels. Furthermore, the CRISPR-Cas9 system is not 100% efficient; therefore crispants can contain a mixture of wild-type and edited cells. Despite heterogeneity, F0 crispants can faithfully recapitulate true loss of function phenotypes.[150] With the optimization of crispant technology and ability to multiplex sgRNAs, reverse genetic screening in zebrafish has been conducted by various groups in a high throughput manner to identify regulators of developmental processes.[151,152] Additionally, F0 animals contain both somatic and germ-line mutations, thus they can be grown to sexual maturity and crossed to produce isogenic F1 heterozygotes. A stable F2 knockout line can then be generated for the in-depth analysis of gene function.[153] This process spans approximately 9 months from the initial injection of the F0 generation to the generation of a stable F2 CRISPR mutant zebrafish line.[154]

A molecular tool that has traditionally been the main option to knockdown gene function in zebrafish are antisense morpholino oligonucleotides (MO).[155,156] MOs are typically 25 nucleotides in length and are a synthetic derivative of DNA with a few structural alterations. For example, instead of containing the standard deoxyribose ring and phosphodiester bonds, MOs possess a six-membered morpholine ring and a nonionic

phosphorodiamidate linkage instead. Upon microinjection into the one-cell stage, the overall neutral charge and molecular small size facilitates diffusion throughout the developing embryo. MOs are designed to bind to a specific mRNA sequence, and either block translation or splicing resulting in the disruption of gene function. Because the effect of MOs is transient and does not alter the DNA sequence, this technique is comparable to cell culture knockdown methods such as small interfering RNAs (siRNAs) and short hairpin RNA (shRNAs). MO-injected animals (morphants) often phenocopy corresponding mutants, therefore MOs are widely used for genetic studies. However, MO use in zebrafish has been documented to display more severe phenotypes than mutants for several reasons: 1) MOs can have off-target effects, 2) translation-blocking MOs can target maternally deposited transcripts, 3) the mutant being examined is hypomorphic in nature, 4) genetic compensation can occur in mutants but not morphants. Because of these caveats, the zebrafish community has built general guidelines for employing MOs as knockdown reagents in research studies.[157]

Another rapid method to interrogate gene function is the injection of synthetic capped mRNA (cRNA) at the one-cell stage[158]. This transient pulse of cRNA induces global overexpression of a gene product and allows the investigator to probe for gain of function phenotypes. Because cRNA can easily degrade and may not diffuse evenly, this can lead to an uneven distribution throughout the developing embryo. However, cRNA injection is widely used in rescue experiments to validate loss of function phenotypes.[158] Perhaps a superior method to overexpress a gene product is applying the *heat shock 70* promoter (*hsp70*) in transgenic zebrafish. The *hsp70* promoter is approximately 1.5 kilobases and is responsive to the heat shock transcription factor (HSF), which is naturally produced upon physiological stressors. This strategy enables temporal control, as raising embryonic body temperature to 38.0°C activates the *hsp70* promoter and drives global transcription of multiple transgene copies.[159,160] The ectopic expression of downstream gene product can be visualized as soon as 30 minutes post heat exposure.[161] Although this wave of expression is transient, embryos can be subjected to serial heat shock treatments to amplify transgene expression for experimental purposes.[162] Taken together, the ease of genetic manipulation of zebrafish embryos paired with other previously discussed attributes makes them prime models for the discovery of novel nephrogenesis regulators.

5. Vertebrate kidney development: a nephron-centric perspective

During vertebrate embryogenesis, the kidney is derived from the intermediate mesoderm (IM), which is a narrow bilateral band of cells situated between the paraxial and lateral plate mesoderm[4,5,36,37]. In mammals, the IM begins to express Pax8 and Pax2, indicating renal lineage commitment. Further, Pax8 and Pax2 have redundant functions, as doubly deficient mouse embryos are unable to form later nephric structures.[163] In *Xenopus* and zebrafish, *pax8* expression precedes *pax2/pax2a* in the IM. *Xenopus* loss of function studies indicate *pax8* functions earlier to establish the pronephric anlage, and *pax2* is required for tubule differentiation.[164] In line with these observations, zebrafish *pax2a* mutants exhibit defects in tubule differentiation and cloacal morphogenesis.[165] Interestingly, *pax2a* mutants correctly initiate *pax8* transcription, but are unable to maintain this expression over the course of pronephric development, suggesting a genetic intersection of these two factors.[166] Regulation of renal progenitor fate choice is also mediated by the transcription factor *odd skipped related 1* (*osr1*) as well as antagonistic interplay between Osr1 and the bHLH transcription factor Hand2.[75,167–172] Further, *osr1* expression is necessary for the survival of progenitor cells and the proper establishment of the podocyte lineage.[75,173]

After IM specification, vertebrates undergo similar successive waves of kidney genesis. Mammalian kidney development entails the reiterative generation and degradation of three main kidney forms: pronephros, mesonephros, and metanephros.[36,37,174] Here, the pronephros and mesonephros are transient structures without renal function or limited function. In contrast, the mammalian metanephros is functional and the most architecturally complex

and final kidney form. In comparison, vertebrates such as amphibians and fish undergo two phases of renal development, where the mesonephros serves as the definitive version.[36,37,174] During embryonic and larval life, the pronephros is functional in frogs and fish unlike the mammalian pronephros, which is a vestigial structure.[36,37,174] Although the anatomical organization and level of complexity vary across kidney forms, each retains nephrons as core structural units. Because nephrons are a unifying element across vertebrate species, much can be learned about kidney development by employing model organisms like mice, *Xenopus*, and zebrafish.[14,81]

6. Nephron morphogenesis: parallels between mammals and zebrafish

Humans and mice cease to form new nephrons at birth or shortly after, and in general, mammalian kidneys exhibit limited regenerative capacity.[174] In comparison, following pronephros development the zebrafish develop a second mesonephros kidney form in the first 1–2 months, in which new nephrons form and connect to the existing pronephros.[175–177] While this process is robust during these months, it never completely ceases and so-called 'neonephrogenesis' occurs at a slow rate.[175–177] This kidney feature is thought to support the ongoing adult growth of zebrafish. Furthermore, in response to acute kidney injury, the rate of neonephrogenesis is enhanced, presumably to offset the loss of damaged tissue due to the presence of self-renewing renal progenitors.[175,176,178] These adult kidney properties are also shared with many other fish species,[179–182] and even some mammals such as the spiny mouse.[183]

Interestingly, the zebrafish renal progenitor pool exhibits a number of parallels with the Six2+ cap mesenchyme (CM) cells that give rise to nascent nephrons during mammalian metanephros kidney development (Figure 2). During the organogenesis of this third kidney form in mammals, two distinct progenitor compartments, the metanephric mesenchyme (MM) and ureteric bud (UB) epithelium, undergo reciprocal inductive signaling. The MM secretes Gdnf and Fgf10 molecules that initiate UB outgrowth and invasion.[184] As a result, the UB produces BMP7 and FGF2 to promote MM survival and condensation, forming a cap of Six2+ Cited1+ cells.[184] Throughout renal development, signals from the CM support dichotomous branching and differentiation of the UB into a highly arborized collecting duct system.[185]

The current working models of nephrogenesis involve the induction of nephron formation is driven by Wnt9b signals from the UB that stabilize β-catenin, which instructs the CM to form pretubular aggregates (PTA) below the ureteric branch tips.[186] Wnt4 activation then triggers PTAs to undergo a mesenchymal-to-epithelial transition to establish renal vesicles (RV).[186] The RV appears as a spherical epithelial structure that undergoes rapid polarization and de novo lumen formation, and similar events transpire during mesonephros development.[175–177] The zebrafish genes, *wnt9a* and *wnt9b*, appear to play conserved roles in neonephrogenesis within the mesonephros, and are upregulated upon kidney injury.[187] Disruption of Wnt/frizzled signaling in the adult zebrafish kidney leads to the inability to form polarized rosette structures resembling RVs.[187] After epithelialization and polarization, the RV elongates along the proximodistal axis and progresses through a series of morphogenetic events consisting of comma-shaped body (CSV), S-shaped body (SSB), and mature nephron tubule formation. Taken together, neonephrogenesis in fish closely echoes the morphogenetic steps of mammalian metanephric development (Figure 2).[52]

Throughout tubule morphogenesis, dynamic cell–cell interactions facilitated by the regional expression of cadherins, laminins, kinesins, and integrin molecules regulate nephron elongation and maturation. For example, in the developing mammalian SSB, K-cadherin expression restricts to the proximal domains, E-cadherin expression restricts to the distal domain, and the intermediate region (presumptive LOH) co-expresses these factors.[188] Because adhesion proteins are differentially expressed across the axes of developing nephrons, it is speculated that these factors help establish regional boundaries and physically separate different nephron segment populations.[189] In zebrafish, the pronephric tubule is defined by *cdh17* expression, which is orthologous to human

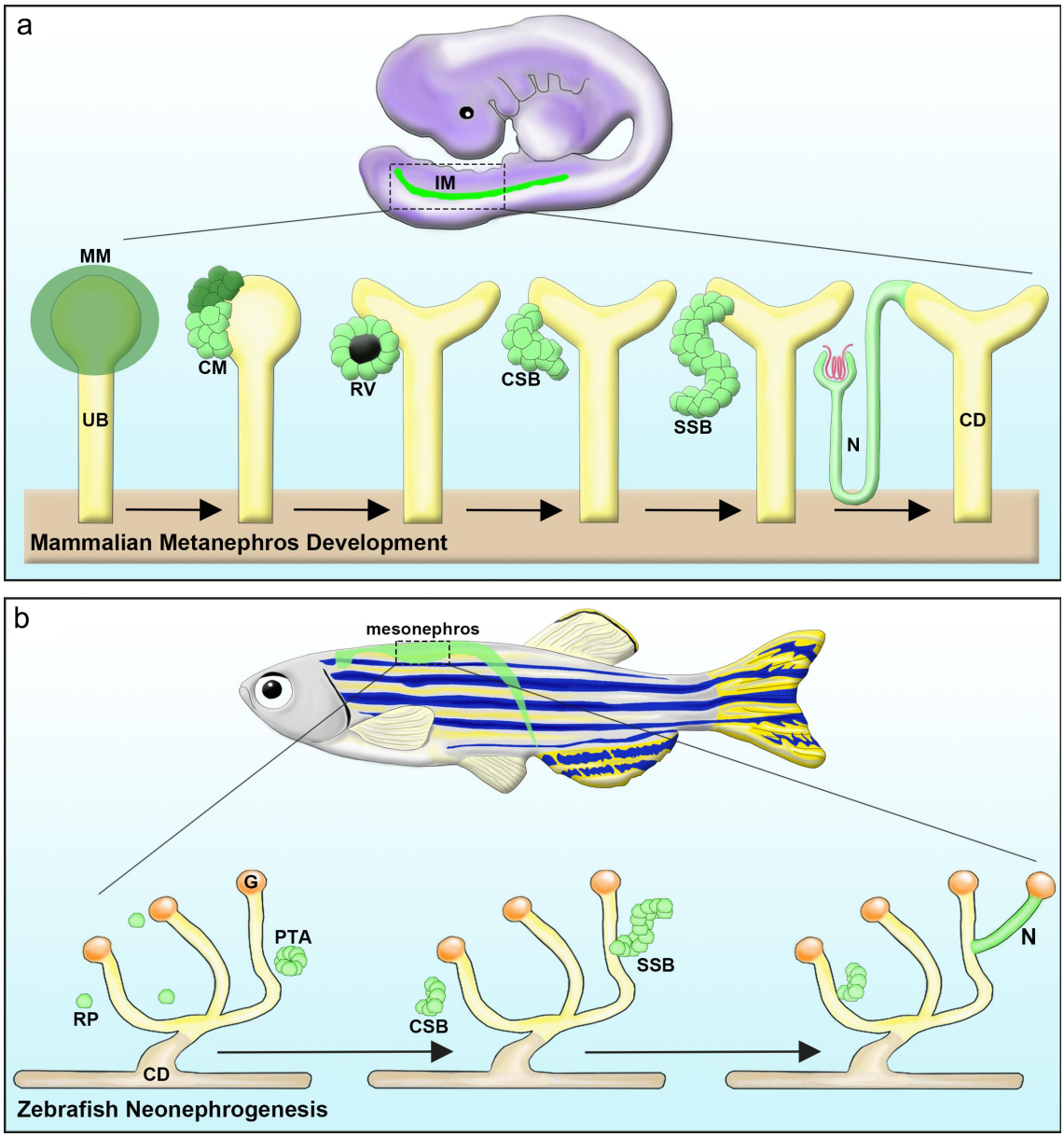

Figure 2. Mammalian nephrogenesis (top) and adult zebrafish neonephrogenesis (bottom) involve similar morphogenetic events. (a) During mammalian kidney development, the MM is derived from the IM. Upon UB (yellow) invasion, the MM (dark green) condenses to form the CM, a renal progenitor population. These progenitors receive signals to self-renew (dark green) or differentiate (light green). Cells receiving differentiation signals are organized into an epithelialized RV. Upon further maturation, these cells form the CSB, then an SSB, and finally the N. Concurrently, the UB undergoes progressive branching to form the CD system (yellow). (b) Adult zebrafish possess the unique ability to generate new nephrons during adulthood. Neonephrogenesis in the zebrafish kidney mimics the cellular dynamics of mammalian nephrogenesis. RPs (green) cluster to create a PTA, which polarizes and undergoes epithelialization. This structure changes morphology and forms a CSB, SSB, and eventually a mature N structure. IM: Intermediate mesoderm; MM: Metanephric mesenchyme; UB: Ureteric bud; CM: Cap mesenchyme; RV: Renal vesicle; CSB: Comma-shaped body; SSB: S-shaped body; N: Nephron; CD: Collecting duct; Renal Progenitor: RP; PTA: Pre-tubular aggregate; G: Glomerulus. Schematics adapted from.[52]

CDH17. Unlike mammalian CDH17, zebrafish *cdh17* maintains tubule integrity during pronephric duct development.[190] Comparable to mammalian nephrons, the zebrafish pronephros also expresses epithelial cell adhesion molecule, EpCAM, during development. Upon profiling zebrafish DL tubules, this transmembrane glycoprotein was found to control differentiation programs such as transmembrane transporter activity and cilium morphogenesis.[191]

During the RV to SSB transition, nephron morphogenesis is accompanied by the spatiotemporal expression of transcription factors that pattern the

nephron into the specialized segments discussed in detail earlier. These transcription factor cues are necessary for the specification and terminal differentiation of distinct nephron cell types. Recent studies have employed immunolabeling and single-cell RNA sequencing strategies to catalog conserved and divergent transcription factor programs of mouse and human nephron development.[192–196] Overall, these studies suggest similar molecular processes orchestrate nephron patterning and downstream differentiation of unique segment identities.[197] Notably, many of these transcription factors exhibit conserved localization patterns and function among vertebrate species.[14]

7. Lessons from lower vertebrates in nephron segment development

The formation of nephron segments during embryonic development is required for proper renal function. Genetic programs that control the expression of unique solute transporters, tight junction proteins, adhesion molecules, and other cellular features are ultimately responsible for the segment-specific modification of the filtrate. While there have been significant strides in identifying factors that are required for nephron segment development, there remain significant gaps in knowledge including 1) how these pathways relate to one another; 2) how distinct processes such as segment patterning, maturation, and terminal differentiation are regulated; 3) if key regulators of pronephros segment development in lower vertebrates exhibit conserved functions during mammalian nephrogenesis. There are many instances where homologous genes exhibit parallel expression patterns in both the pronephros and developing metanephric nephrons, hinting that their roles are preserved or partially conserved across species. In recent years, genetic studies using the zebrafish and *Xenopus* pronephros have provided substantial insight into nephron segment ontogeny. The following sections primarily discuss these findings from work in the zebrafish pronephros.

7.1. Nephron segment pattern formation via RA

One of the most potent morphogens that patterns the renal progenitor field in zebrafish is RA.[79–81] It was previously found that RA and *cdx (caudal)* transcription factors are responsible for the proximo-distal patterning and axial positioning of the pronephros.[80,81] RA promotes proximal segment fates at the expense of distal segment fates and *cdx* genes define the positioning of the pronephros on the embryonic axis.[80,81] Mechanistically, *cdx* factors control the expression boundaries of *aldh1a2* and *cyp26a1*, which are enzymes that control the synthesis and degradation of RA, respectively.[80,81] In accordance with these observations, inhibition of RA severely impairs zebrafish pronephros formation. Additionally, *Raldh2* null mouse embryos undergo abnormal specification of the pronephric lineage.[198] A separate study determined that RA is activated downstream of BMP to pattern non-axial mesoderm and induce anterior kidney fates.[199] RA has also been linked to proximo-distal nephron patterning in mammals,[37] and it has been supplemented along with BMP4 to kidney organoid cultures to stimulate IM differentiation.[200]

7.2. Transcription factors hnf1ba/b

The transcription factors *hnf1ba* and *hnf1bb* function downstream of RA signaling and are required for global nephron segmentation. Zebrafish deficient in *hnf1ba/b* fail to express both proximal and distal nephron solute transport markers, however pronephric tubules retain normal epithelial characteristics indicated by the presence of *laminin*, *cdh1*, and *epcam* transcripts.[201] Additionally, the cytoplasmic sequestration of Hnf1b is necessary for CS gland development.[105] In zebrafish, *hnf1ba* and *hnf1bb* exhibit broad expression patterns throughout developing renal progenitors. These patterns parallel observations in nascent mouse nephron tubules, which express *Hnf1b* in a proximo-distal gradient at the SSB stage. *Hnf1b*-deficient mouse kidneys exhibit severe tubular defects and undergo failure to develop all nephron segments.[202,203] Furthermore, *HNF1B* mutations in humans are associated with CAKUT and renal cystic disease.[204]

7.3. Segment fate regulators: roles and emerging interactions

7.3.1. Sim1a/Ppargc1a and the PST identity

A novel role for the *sim1a* transcription factor, traditionally known as the master regulator of midline cell development in the central nervous system, was unveiled by performing genetic manipulations in zebrafish. Cheng et al. (2015) discovered *sim1a* functions downstream of RA signaling and patterns proximal segments during pronephros development.[205,206] Notably, zebrafish deficient in *sim1a* suffer losses of the PST and CS populations and an expanded PCT.[205,206] Although *Sim1* is a key factor for early Wolffian duct formation, it has yet to be determined if it also functions to pattern mammalian proximal nephron fates.[207]

A separate study that arose from this initial chemical genetic study entailed workup of the 'screen hit,' *ppargc1a*, a master regulator of mitochondrial biogenesis.[145] Loss of *ppargc1a* results in pronephric phenotypes such as an expanded PST and severely reduced DL.[145] Through genetic rescue studies, Chambers et al. (2018) determined *ppargc1a* controls nephron patterning by antagonizing *sim1a* to delineate the PST segment boundary.[145] In mice, Ppargc1a exerts renoprotective effects in tubular cells upon acute kidney injury by controlling metabolic processes such as NAD biosynthesis.[208,209] Future studies in developing mammalian nephrons are needed to determine if Ppargc1a controls segmental identity, and if metabolic changes influences cell fate decisions in this context.

7.3.2. Mecom—Tbx2a/b—Emx1 and navigating DE, CS, and the DL identities

Many new discoveries have been brought to light by conducting genetic manipulations of the zebrafish and/or frog pronephros but have yet to be confirmed in a mammalian system. For example, zebrafish *tbx2a* and *tbx2b* were found to regulate DL and CS fate downstream of Notch signaling,[210] and the corresponding ortholog Tbx2 defines the pronephros territory in *Xenopus*.[211] Morales et al. (2018) determined *empty spiracles homeobox gene 1* (*emx1*) promotes DL formation and inhibits DE as part of the genetic cascade downstream of *mecom* and *tbx2a/b* under the influence of RA signaling.[212] To determine the role of *emx1* during pronephros development Morales et al. utilized a combination of loss of function, rescue experiments, and chemical studies. *emx1* is initially expressed in a broad domain of renal progenitors and is then confined to the distal late domain by the 28-somite stage.[212] Gene expression studies revealed that *emx1* transcripts were colocalized in the distal domain with *mecom*, *tbx2a*, and *tbx2b*, therefore implying that *emx1* functions in conjunction with these transcription factors.[212] Interestingly, by treating zebrafish embryos with exogenous RA or the RA biosynthesis inhibitor DEAB tha RA negatively regulates *emx1* expression, as its domain in renal progenitors showed a reduction and expansion, respectively.[212] Knockdown of *emx1* utilizing morpholino oligonucleotides led to an expansion of the *slc12a1*+ DE segment and reduction of the *slc12a3*+ DL segment.[212] Further, there was an increase in the CS lineage based on expanded *stc1* expression.[212] Although there is a difference in the specific markers of the distal pronephros, there it was change in the pan-distal expression thus implying there may be transfating within the distal pronephros. This is supported by the lack of an increase in cell death or proliferation in the distal domain.[212] Morales et al. showed that while the knockdown of *emx1* does not affect *mecom* expression, the knockdown of *mecom* affects *emx1* expression and could be rescued with the addition of *emx1* cRNA in *mecom* morphants, therefore placing *emx1* downstream of *mecom* in the cascade.[212] A similar assay was done with *tbx2a* and *tbx2b* knockdown, which placed *tbx2* factors within the cascade. This work suggests that distal patterning is modulated by the RA signaling pathway that functions upstream of *mecom*, *tbx2a/b*, and *emx1* during pronephros development.[212] Interestingly, *ppargc1a* also modulates *tbx2b* to promote DL formation,[145] but future work is needed to delineate the relationship between Ppargc1a and these other distal promoting factors.

Perturbations in zebrafish *efhc2* were also determined to affect distal nephron compartments including the DE, CS, and DL, and to act independently of RA signaling.[109] In *Xenopus*, the gene *prdx1*, which codes for an antioxidant enzyme, influences RA and Wnt signaling to regulate pronephros development. Interestingly, knockdown of

this factor increases cellular levels of reactive oxygen species, impairs primary cilia formation, and inhibits proximal tubule formation.[213]

7.3.3. Prostaglandin signaling and segment fate

A zebrafish chemical genetic screen conducted by Poureetezadi et al. (2016) facilitated the discovery of additional pathways that alter nephron patterning.[144] For the first time, this study identified prostaglandin signaling balances nephron segment fates, where treatment with PGE_2 results in an expanded PST at the expense of the DL.[144] Manipulation of *cox1*, *cox2*, *ptger2a*, and *ptger4a* activity similarly affected DL segment development.[144] Prostaglandin signaling controls segment identity by functioning upstream of pronephros patterning factors *irx3b* and *sim1a*.[144] Physiologically, PGE_2 synthesis occurs across nephron segments and affects transepithelial transport in the adult mammalian nephron; however, additional studies are needed to determine if prostaglandins function in an embryonic setting to influence cell fate during mammalian nephrogenesis.[214] Interestingly, prostaglandin signaling was recently discovered to promote renal progenitor proliferation during neonephrogenesis in the adult zebrafish mesonephros following acute kidney injury.[215,216] In this context, PGE_2 produced from renal interstitial cells, which reside in proximity to the renal progenitors, is essential to promote the rapid induction of cell division.[215,216] Whether the duration or dosage of PGE_2 impacts nephron segment fate decisions in the renal progenitors will be an intriguing question to pursue in future studies.

7.3.4. Gldc and nephron segmentation

Glycine decarboxylase (GLDC) is a rate-limiting enzyme in the glycine cleavage system (GCS), a vital complex for regulating glycine levels. GLDC specifically functions to break down glycine and produce one-carbon units for folate metabolism. When GLDC function is disturbed, glycine accumulation occurs, and there are detrimental defects in humans and animals including seizures, abnormal brain patterning, and premature death.[217–224] In mice, *Gldc* is essential for neural tube closure and normal brain development, however, the exact mechanism it has in organogenesis is still unknown.[222,224,225] GLDC has been implicated in a variety of cellular processes including glutathione production in the liver, stem cell fate through regulation of senescence and epigenetic modifications, and cell proliferation in some cancer types.[226–228]

In a recent publication, Weaver et al. identified *gldc* as a novel regulator of nephrogenesis in which it controls glycine levels leading to normal segmentation.[229] Upon surveying of live phenotypes in *gldc* deficient animals, they noted hydrocephalus and pericardial edema suggesting impaired kidney function.[229] To analyze this further, Weaver et al. performed a functional assay and determined the *gldc* morphants were unable to excrete a fluorescent molecule as efficiently as their wild-type counterparts.[229] Due to these phenotypes, they hypothesized that perturbation of *gldc* was leading to an increase of glycine globally which caused alterations in nephron segmentation and eventually, altered fluid homeostasis. Upon measurement of the nephron segments in *gldc* morphants, there were changes in several populations: proximally, the PCT was reduced; distally, the DE tubule expanded in length, while the DL was decreased.[229] After identifying changes in differentiated populations, they studied the basis of the distal segment changes, and found that *gldc* was working upstream of the transcription factors responsible for patterning these distal segments (*irx1a*, *irx3b*, *tbx2a*, and *tbx2b*).[229] Further studies are needed to ascertain the basis of the proximal changes.

Interestingly, the researchers tested glycine accumulation was disturbing segmentation, independent of *gldc* function. To determine whether excess glycine alone impacts kidney development, Weaver et al. treated animals exogenously with varying concentrations, and observed similar segment changes to *gldc* deficient animals in a dose-dependent fashion.[229] This suggests glycine may be the mechanistic action causing altered nephron patterning in *gldc* deficient embryos.[229] While glycine is a well-known neurotransmitter, it has previously been implicated in the development of the heart, liver, brain, and vascular system in zebrafish.[230–233] In the kidney, glycine has been found to impact ion uptake, renal plasma flow, and glomerular filtration rate in various contexts,

including kidney injury.[234–240] Both the GCS and glycine have been connected to differentiation and cell fate in various cell populations.[227,241] Thus, these studies support the conclusions that *gldc* deficiency causes glycine accumulation, and that both *gldc* and glycine are acting upstream to influence nephron segment differentiation.

7.3.5. Estrogen signaling and distal segment fate

A recent study by Wesselman et al. found that 17-beta (β) estradiol (or E2) influences pronephros development through following up on a related hit from a chemical screen.[146] Exposure to exogenous E2 beginning at the shield stage (about 6 hpf) led to significant alterations in distal segment development, where the DL was reduced and DE was expanded.[146] These changes were also repeated using additional DE and DL markers, whilst effects on the proximal segments were not observed.[146] Further, these distal segment change were not associated with changes in cell death or cell proliferation, which implied that exogenous E2 treatment caused changes in nephron patterning. Previous studies have shown that xenoestrogens such as ethinylestradiol, bisphenol A (BPA) and genistein also activate E2 signaling pathways similar to E2.2[44,242],, [243] Wesselman et al. found specifically that exposure to ethinylestradiol or genistein caused an increased DE domain and a decreased DL domain, similar to E2 treatment.[146] Additionally, they observed similar phenotypes with other DE and DL markers as well, while seeing no differences in PCT and PST formation.[146] Taken together, these results suggest that estrogen signaling is sufficient to impact distal nephron segmentation.

To query which components of E2 signaling were involved in proper nephrogenesis, the researchers designed and conducted a selective estrogen receptor modulator (SERM) screen, including an Esr1 agonist (PPT), an Esr1 antagonist (MPP), an Esr2 agonist (DPN) and an Esr2 antagonist (PHTPP).[146] Treatment with PHTPP caused embryos to exhibit an increase in DL domain and a decrease in DE domain.[146] However, DPN treatment did not result in increased DE domain.[146] Additionally, MPP and PPT did not result in changes in distal domains. Additionally, using additional markers such as *kcnj1a.1* and *tbx2b* for DE and DL, respectively, we observed similar trends in PHTPP-treated embryos, and no changes in proximal segments.[146] To further investigate the role of each receptor in the zebrafish pronephros, gene knockdown studies were performed to target *esr1*, *esr2a*, and *esr2b*, respectively. *esr2b* knockdown resulted in decreased DE and increased DL, similar to the phenotypes observed with PHTPP treatment.[146] On the other hand, *esr1* and *esr2a* knockdown did not affect proximal-distal segmentation.[146] To answer the question whether *esr2a* may serve a redundant role together with *esr2b* in distal cell development, they used morpholino to both dual knockdown of *esr2a* and *esr2b* was performed. This led to a similar decrease in the DE domain and an increase in DL domain, but not more significant than the *esr2b*-deficient embryos alone.[146] These findings strongly suggest that Esr2a and Esr2b do not function redundantly in nephron development. While analysis of a stable *esr2b*^{uab127} mutant line revealed that there were no changes in DE or DL domain in the homozygous embryos, this is likely due to the presence of maternal transcripts coding Esr2b.[146] Nevertheless, similarities between the results of *esr2b* MO and PHTPP treatment establish that the Esr2b receptor is most likely required for estrogen signaling during distal segmentation.[146]

Overall, these results suggest a crucial role of estrogen signaling in nephron segmentation of zebrafish embryonic kidneys, and suggest a mechanism of E2 signaling in nephron segmentation.[146] At its active stage, E2 diffuses from yolk into the adjacent renal progenitor cells and binds to Esr2b. Esr2b then leads to changes in genes that dictate DE/DL differentiation, such as Irx3b and Irx1a (discussed in section 7.4.2). When E2 is inactive whether via morpholino or PHTPP treatment, this causes inhibition of Esr2b, which causes reduction in activity of Irx3b and Irx1a, thus causing favored DL fate over DE fate. Overall, this study provides a crucial insight into the role of hormonal signaling in zebrafish kidney, with greater implication for understanding kidney development and treating kidney dysfunction due to hormonal imbalance.[146]

7.3.6. Pou3f3 transcription factors and the intermediate segments

The POU domain transcription factor *Brn1 (Pou3f3)* is another known effector of LOH fate.[244] *Brn1*

mutant mice form truncated nephrons lacking bona fide TAL segment structures as indicated by the disappearance of *Umod*, *Slc12a1*, *Bsnd*, *Kcnj1*, and *Ptger3* expression.[244] Mutant TAL cells exhibited undifferentiated ultrastructural features such as simple cell membranes and scarce mitochondria. During metanephric development, *Brn1* is localized to the intermediate and distal regions of the SSB.[244] Similarly, in zebrafish, *pou3f3a* and *pou3f3b* expression is concentrated in the central-distal pronephric regions.[79] However, the function of these factors in the zebrafish kidney has not yet been tested.

7.4. Control of segment differentiation

7.4.1. Dynamin binding protein

Recent studies in the frog pronephros, which is another excellent model of nephrogenesis,[245,246] have revealed new insights into the mechanisms of segment differentiation. Researchers discovered that dynamin-binding protein, *dnmbp*, does not function in pronephros specification or patterning, but rather controls tubular differentiation.[247] Animals deficient in *dnmpb* displayed defects in both proximal and distal pronephros solute transporters, exhibiting reduced expression of *slc5a1* and *clcnkb*, respectively. Additionally, knockdown of *dnmbp* caused disorganized pronephric cilia, a hallmark of defective tubular differentiation.[246] Further exploration of these factors in mammalian and organoid models will yield significant insights into embryonic nephron development, specifically concerning the mechanisms guiding specification, patterning, and terminal differentiation of distinct segment cell types.

7.4.2. Irx genes

The Iroquois homeobox genes also appear to exert conserved functions during nephron segmentation. During zebrafish development, *irx3b* and *irx2a* are expressed in the central renal progenitor field.[79,112,248] Specifically, *irx3b*-deficiency elicits an expansion of the PCT, PST, and CS, however, abrogates DE differentiation.[79] Intriguingly, *irx3b* is required to maintain *hnf1ba* expression in the DE domain, unveiling a segment-specific circuit.[201] Family member *irx2a* works upstream of *etv5a* and downstream of RA signaling to regulate PST and DL pronephric fates.[112] During pronephric development, *irx1a* is present in presumptive DE precursors, and by 24 hpf colocalizes with the *slc12a1*+ DE domain.[249] Overexpression studies indicate *irx1a* likely plays a prominent role in DE segment differentiation and is sufficient to activate *slc12a1* and *kcnj1a.1* expression.[249] Additionally, regulation of *irx1b* by the homeobox transcription factor Mnx2b has been associated with proper nephron tubule morphogenesis.[250] In agreement with these observations, studies in *Xenopus* and mice show similar nephron localization of *Irx1*, *Irx2*, and *Irx3* during kidney development.[251] Loss of function studies in frogs have also suggested conserved roles of *Irx1* and *Irx3* in segment differentiation.[251,252] In mice, Hnf1b promotes *Irx1* and *Irx2* expression in the intermediate SSB domain, which gives rise to the loop of Henle (LOH).[202] However, *Irx* knockdown studies in mammals are needed to confirm these roles are biologically conserved among vertebrates.

7.4.3. Tfap1a/B transcription factors and autoregulatory repression via Kctd15a/b

A novel determinant of DE terminal differentiation was discovered by a forward haploid genetic screen conducted in zebrafish. Mutant analysis revealed *transcription factor AP-2 alpha (tfap2a)*, a classical regulator of neural crest fate, controls the differentiation of the DE, CS, and DL. This transcription factor acts as the conductor of a multitiered genetic regulatory network including family member *tfap2b*.[249] In this network, Tfap2a operates a circuit consisting of *tfap2b* and *irx1a* to activate the expression of distal nephron solute transporter genes *clcnk*, *slc12a1*, *kcnj1a.1*, and *slc12a3*. In a subsequent study, the KCTD15 paralogs, *kctd15a* and *kctd15b*, were found to be key components of the Tfap2a distal nephron network.[253] By employing CRISPR-Cas9 and knockdown strategies, *kctd15a/b* loss was shown to prime the nephron cells to adopt a DE segment signature.[253] Mechanistically, *kctd15a/b* restricts DE differentiation by repressing Tfap2a activity in developing nephrons.[253] Further interrogation of this signaling axis revealed Tfap2a can reciprocally promote *kctd15* transcription.[253] These data revealed a transcription factor-repressor feedback module where nephron segment fate is controlled by precise regulation of Tfap2a-Kctd15 kinetics.

8. 8. Summary/Conclusions

Employing the zebrafish to perform genetic studies cultivates the identification of novel nephron regulators. By employing ENU mutagenesis, morpholino, transgenic, and CRISPR-Cas9 technologies in the genetically tractable zebrafish model, researchers have divulged valuable insights into the molecular pathways controlling nephrogenesis. Looking forward, zebrafish models exhibit many connections to renal conditions observed in humans, specifically congenital anomalies of the kidney and urinary tract (CAKUT). For example, the loss of *tfap2a/b* results in abrogated expression of distal nephron solute transporters *slc12a1*, *kcnj1a.1*, *clcnk*, and *slc12a3*, which is comparable to renal channelopathies in humans.[249] Neonatal Bartter's syndrome type 1, neonatal Barrter's syndrome type 2, classic Barrter's syndrome type 3, and Gitelman's syndrome are all classified as inherited disorders affecting TAL/DCT and are caused by defects in SLC12A1, KCNJ1, CLCNKB, and SLC12A3, respectively. Furthermore, there are extrarenal defects associated with loss of the *tfap2a/b* transcription factors in the zebrafish, including neural crest and ear abnormalities.[249] These phenotypes indicate human mutations in these genes are likely associated with a larger syndrome affecting multiple tissue types. To this point, patients with ear malformations have a higher incidence of kidney anomalies; thus, in these cases, renal sonography is often pursued by pediatricians as a screening measure.[254]

To date, the functions of many zebrafish pronephros regulators have not yet been examined in higher vertebrates, such as the mouse. In some cases, interesting distinctions have been observed. For example, several early studies have noted that both *Tfap2a* and *Tfap2b* mutant mice exhibit renal anomalies, suggesting that these factors likely play significant roles in mammalian kidney development.[255–257] Additionally, human mutations in TFAP2A cause branchio-oculo-facial syndrome, and a portion of these patients experience severe kidney defects.[258] More recent inactivation studies in the mouse indicate that TFAP2A is important for medullary collecting duct development, but not nephron segment differentiation, and that TFAP2B controls the terminal differentiation of the TAL and DCT.[259,260] Therefore, there are likely other distinctions in gene functions which have evolved across vertebrates. Nevertheless, identification of genes with critical functions in renal development of lower vertebrates provides a useful starting point to delineating such pathways in higher vertebrates like mammals.

Future studies of these and other genetic factors are of utmost importance to fully understand their roles across vertebrate species. In most cases, global knockout of these factors in mice results in perinatal lethality and impedes meaningful renal analysis. One approach to overcome this obstacle is to generate tissue-specific knockout mice, however this method can be time-consuming and expensive. Luckily, a system has recently emerged that enables researchers to study gene function and model human diseases in an organ-specific manner *in vitro*. Renal organoid technologies involve culturing miniaturized kidneys in a 3D matrix, recapitulates embryonic nephron development, and provides an excellent platform to explore the function of candidate genes in human cells.[261]

Taken together, the studies discussed in this review have illustrated the utility of the zebrafish pronephros in catalyzing the discovery of novel genetic mechanisms that orchestrate embryonic kidney development. Key developmental insights from these and future zebrafish studies will support the assembly of the genetic blueprint required to fashion a nephron, and in turn support efforts to advance kidney organoid technology, further develop precision medicine, and deepen our understanding of congenital renal syndromes.

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Author Contributions

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