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Chapter 9

RENAL SYSTEM DEVELOPMENT IN THE ZEBRAFISH: A BASIC NEPHROGENESIS MODEL

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ABSTRACT

The zebrafish has proven to be an important model organism for investigating numerous developmental topics, especially organogenesis. To date, the zebrafish has played an expanding role in aiding our understanding of the genetic intricacies that underlie renal development, a process that has been poorly understood in the past. The kidney is a vital organ that is responsible for maintaining fluid homeostasis and removing waste metabolites from the body. Current research has established that the segmentation patterning of the nephrons, which are the basic functional units of the kidney, is conserved between this teleost species and higher vertebrates, including mammals. This concept of conservation within the kidney was further exemplified by the identification of orthologous genes that are expressed during the dynamic cellular and morphological processes that occur throughout nephrogenesis. Continuing advances in molecular techniques, from morpholinos to TALENs and now CRISPRs, have fueled the increasing appeal of employing zebrafish in research. The future application of these technologies offers a valuable venue to study kidney development and holds promise to elucidate clinical interventions for a variety of renal diseases.

Keywords: Zebrafish, nephrogenesis, retinoic acid, *cdx* genes, HNF1β, *irx3b*, morpholino, TALENs, CRISPR-Cas

ABBREVIATIONS

5’ UTR	5’ untranslated region
A-P axis	anterior-posterior axis

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AKI	acute kidney injury
AS	antisense
CAKUT	congenital abnormalities of the kidney and urinary tract
CAR	coxsackie and adenovirus receptor
CaSR	calcium-sensing receptor
CD	collecting duct
cdx	*caudal*
CKD	chronic kidney disease
CNF	congenital nephrotic syndrome of the Finnish type
CRISPRs	clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
CS	Corpuscle of Stannius
DCT	distal convoluted tubule
DE	distal early
DEAB	4-diethylaminobenzaldehyde
DL	distal late
dpi	days post injection
DSB	double-stranded break
ESRD	end-stage renal disease
HDR	homology directed repair
hpf	hours post fertilization
IM	intermediate mesoderm
indels	insertion or deletion mutations
irx	*Iroquois*
MCC	multiciliated cell
MD	macula densa
MET	mesenchymal-to-epithelial transition
MKS	Meckel syndrome
NHEJ	non-homologous end joining
PAM	protospacer adjacent motif
PCT	proximal convoluted tubule
PD	pronephric duct
PKD	polycystic kidney disease
PST	proximal straight tubule
RA	retinoic acid
RALDH	retinaldehyde dehydrogenases
RAR	retinoic-acid receptor
RAREs	retinoic-acid response elements
RVDs	repeat-variable di-residues
RXR	retinoid X receptor
S	sense
SCC	single ciliated cell
sgRNA	single guide RNA
ssDNA	single-stranded DNA
ssODNs	single-stranded oligodeoxynucleotides
TAL	thick ascending limb

TALE	transcription activator-like effector
TALENs	transcription activator-like effector nucleases
tracrRNA	trans-activating crRNA
WISH	whole mount *in situ* hybridization
WT	wild-type

INTRODUCTION

Danio rerio, more commonly known as the zebrafish, is a freshwater fish native to the shallows of the Indian floodplains in the tropics. Once domestic aquarium strains were procured for laboratory settings, research involving the zebrafish initially focused on toxicology and environmental studies (Spence et al., 2008; Laale, 1977). However, work by Streisinger and colleagues (1981) pertaining to the application of molecular genetics within the zebrafish to investigate embryology revealed the potential of zebrafish for vertebrate developmental biology research (Spence et al., 2008; Streisinger et al., 1981; Kimmel, 1993). This concept was further established by studies that examined the patterning and differentiation of the vertebrate brain using the zebrafish (Kimmel, 1989; 1993). These studies, in addition to the discovery of genetic conservation between the zebrafish and other vertebrates, ultimately founded the importance of the zebrafish in cellular and molecular biology. To date, numerous findings regarding various developmental phenomena including neurogenesis, hematopoiesis, and organogenesis have been made by employing the zebrafish model (Kimmel, 1993; Barrallo-Gimeno et al., 2003; Galloway et al., 2005; Wingert & Davidson, 2011).

Overall, the zebrafish is an excellent organism for developmental studies. Not only does the zebrafish show significant genetic and anatomical conservation with that of higher vertebrates (Gerlach & Wingert, 2013; Lieschke & Currie, 2007; Goldsmith & Jobin, 2012; Howe et al., 2013), it is also very amenable to experimental analyses due to the attribute of optical clarity during its embryonic stages (Kimmel, 1993). Adult zebrafish undergo broadcast spawning with each adult capable of producing hundreds of embryos each week. This fecundity of zebrafish heightens their appeal for use in high-throughput experiments. Moreover, the external development of the zebrafish embryo offers researchers the ability to study the developmental processes that occur from the onset of fertilization (Driever et al., 1996; Haffter & Nusslein-Volhard, 1996; Drummond et al., 1998; Amsterdam et al., 1999; Westerfield, 2000; Swanhart et al., 2011). Advances made in molecular techniques (Nasevicius & Ekker, 2000; Gaj et al., 2013) suitable for use with the zebrafish have further stimulated the popularity of this particular organism to study development and model a plethora of diseases as well. Consequently, the zebrafish has both transformed the scientific community and enabled the advancement of biomedical knowledge in recent years (Spence et al., 2008; Santoriello & Zon, 2012; Seth et al., 2013). In terms of organogenesis, the zebrafish has begun to enhance our understanding of kidney development, which will be the focus of this chapter.

Among vertebrates, the kidney is a vital organ since it primarily functions to filter the blood and remove waste metabolites from the body. Thus, organismal homeostasis is maintained through the kidney's stringent regulation of fluids, pH, and blood pressure.

Furthermore, the ability of the kidney to control these important processes resides in the integral performance of its functional units known as nephrons. These nephrons are highly specialized epithelial tubules that are divided into proximal and distal regions based on their respective activities of solute reabsorption and urinary salt regulation (Reilly et al., 2007; Gerlach & Wingert, 2013) Until recently, the mechanisms responsible for vertebrate nephrogenesis remained largely unknown. However, the prospect for new insights into this complex process emerged once it was discovered that the patterning of the nephron segments was conserved across species including frogs, zebrafish, and mammals (Wingert et al., 2007; Wingert & Davidson, 2008). As a result, the zebrafish has become a key model organism for investigating the mechanisms that underlie renal development, regeneration, and disease.

Kidney dysfunction can lead to a variety of diseases in human adults and is typically categorized as either acute kidney injury (AKI) or chronic kidney disease (CKD) (McCampbell & Wingert, 2012; CDC, 2010). In addition, prenatal kidney defects, which comprise congenital abnormalities of the kidney and urinary tract (CAKUT), can also arise from genetic and/or environmental aberrations (Welham et al., 2005; Song & Yosypiv, 2011; Renkema et al., 2011; El-Dahr et al., 2000; Schwaderer et al., 2007). Most notably, the overall incidence of renal anomalies throughout the world is astounding. In the United States alone, it is estimated that approximately 1 out of 10 people are afflicted with some form of chronic kidney disease (NIH, 2012). Presently, there are only two viable forms of treatment for nonfunctioning kidneys: dialysis and transplantation. Dialysis is an inherently grueling and time-consuming process for the patient since it involves a lifetime of frequent blood filtrations performed by an external medical device. The prospects for a kidney transplant can also be quite disheartening since this procedure normally entails long waitlist times of several years. Furthermore, even after a kidney transplant is obtained, it is still possible that the patient will suffer from transplant rejection and/or eventual graft failure. Kidney transplant recipients may experience many side effects that are associated with the administration of immunosuppressant drugs as well (Poureetezadi & Wingert, 2013; Jacobs, 2009; Song et al., 2013; CDC, 2010; U.S. HHS, 2013).

Interestingly, in some cases of human AKI, varying degrees of regeneration have been documented. However, the general capability of the mammalian kidney to repair nephrons is minimal when compared to the robust rates of regeneration observed within the renal systems of lower vertebrates including the zebrafish (Humphreys et al., 2008; Hartman et al., 2007; Diep et al., 2011; Zhou et al., 2010). Currently, it is unknown why there is such disparity between the regenerative abilities of the kidney between species. Even so, by revealing the fundamental mechanisms that govern zebrafish kidney regeneration, it is believed that this knowledge could be extended to treat human renal diseases because of the high degree of genetic conservation exhibited within the kidney (McCampbell & Wingert, 2012; Li & Wingert, 2013). Nevertheless, it is essential to first characterize the basic processes that occur during kidney organogenesis because such pathways may also play significant roles in the regeneration events that transpire after injury. This chapter will demonstrate how the zebrafish is being used to elucidate the complex genetic intricacies that underlie the process of kidney development, thereby providing the basic understanding needed for prospective translational applications to disease and potential therapeutic treatments. This chapter will then review the recent advances in molecular techniques that are poised to transform future research using the zebrafish, not only in the field of nephrology but to further elucidate topics ranging from development to regeneration.

NEPHRON STRUCTURE AND ORGANIZATIONAL CONSERVATION AMONG VERTEBRATES

Previously, it was believed that the mammalian nephron was significantly more complex than that of lower vertebrate species, where the former consisted of highly specialized epithelial tubules that contained numerous distinct regions responsible for performing certain tasks related to maintaining homeostasis, nutrient reabsorption, and waste excretion (Figure 1A). Despite this preconceived notion within the field of nephrology, recent studies have demonstrated that the segmentation patterning exhibited by the mammalian nephron is actually conserved among other species, specifically *Xenopus* and the zebrafish (Figure 1B) (Wingert et al., 2007; Wingert & Davidson, 2008; Dressler, 2006). Orthologs of many key genes that had been implicated in kidney organogenesis from murine studies are also similarly expressed in these model organisms, establishing their importance in renal developmental research (Wingert et al., 2007; Wingert & Davidson, 2008; Drummond, 2005). For instance, renal progenitor differentiation in vertebrates is facilitated in part by the transcription factors Pax2 and Wt1 that are also expressed by renal precursors in zebrafish (Wingert & Davidson, 2008; Dressler, 2006). Therefore, the combination of structural simplicity and conserved specialization of the zebrafish pronephros offers great advantages for genetic investigations of nephrogenesis (Gerlach & Wingert, 2013; Drummond, 2003).

During early vertebrate development, a succession of up to three different kidney forms emerges from the intermediate mesoderm (IM). The pronephros is the first embryonic kidney and is vestigial in some species. Degeneration of the pronephros is accompanied by the formation of a second kidney, the mesonephros. The mesonephros functions transiently until the development of the metanephros, after which it will rapidly degenerate leaving the metanephros to serve as the adult kidney in higher vertebrates including birds, reptiles, and mammals. Lower vertebrates like fish never form a metanephros and instead utilize the mesonephros during adult life. Despite this difference, each kidney form is comprised of nephrons that exhibit a similar composition (Gerlach & Wingert, 2013; Wingert & Davidson, 2008; Dressler, 2006).

Each nephron consists of a renal corpuscle that connects to an epithelial tubule (sometimes via a ciliated neck segment) followed by a collecting duct (CD) (Schonheyder & Maunsbach, 1975; Hallgrimsson et al., 2003; Reilly et al., 2007). Within the renal corpuscle, the glomerulus, which serves as the blood filter, contains podocytes whose foot processes interdigitate with those of its neighbors to produce the fenestrated architecture of the slit diaphragm that is characteristic of this particular structure (Reilly et al., 2007). The adjoining nephron tubule is responsible for the reabsorption of specific solutes, such as salts and sugar, as well as the excretion of metabolic waste. In order to achieve these tasks, this specialized nephron tubule is further subdivided into distinct segments: a series of proximal, intermediate, and distal segments (Figure 1A) (Reilly et al., 2007). Solute reabsorption primarily occurs in the proximal regions through the utilization of solute transporters, while the distal segments are involved in the precise regulation of salt concentrations. Urinary waste will then exit the nephron at the collecting duct and be channeled into a centralized drainage system culminating at the bladder for final excretion from the body (Reilly et al., 2007; Hallgrimsson et al., 2003).

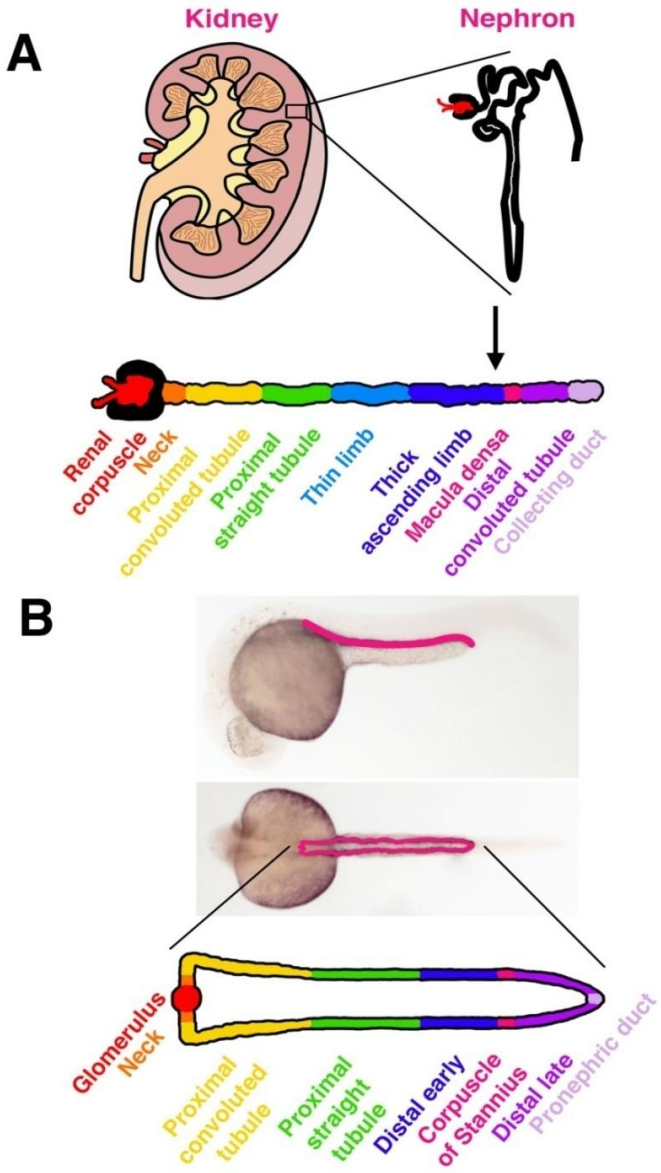

Figure 1. Nephron segmentation is conserved between mammals and zebrafish. (A) A generic mammalian kidney in sagittal section and its functional unit, the nephron, which has a characteristic coiled structure. Lower schematic indicates the distinct segments present within a "stretched out" nephron. (B) Top and middle images illustrate the zebrafish pronephric kidney (designated in pink) from lateral and dorsal views, respectively. Bottom schematic shows the zebrafish pronephros segmentation pattern viewed dorsally. Embryo anterior is located on the left. Analogous segments between the mammalian nephron (A) and the zebrafish pronephros (B) are color-coded. Note that there are variations in nephron structure among mammals, *e.g.* the presence of a neck segment (see text).

Historically, research about nephron segmentation has been impeded by the architectural complexity of the metanephros in mammals since this kidney form is composed of thousands to millions of nephrons (McCampbell & Wingert, 2012; Hallgrimsson et al., 2003). A new opportunity to study nephrogenesis emerged when scientific evidence was found supporting

the conclusion that the zebrafish nephron also consists of a similarly segmented tubule, contrary to past belief concerning its lack of specialization (Wingert et al., 2007; Wingert & Davidson, 2008). Further, the zebrafish pronephros is composed of two nephrons that share a single glomerulus and collecting duct at either end, which offers a simplified anatomical kidney model (Figure 1B) (Gerlach & Wingert, 2013; Wingert & Davidson, 2008). By evaluating gene expression patterns in the zebrafish kidney, Wingert et al. (2007) demonstrated that the segment composition of the nephron was conserved between mammals and the zebrafish with the notable exception of the intermediate segment known as the Loop of Henle (Gerlach & Wingert, 2013; Wingert et al., 2007; Wingert & Davidson, 2008). Since zebrafish have no need for water conservation as opposed to its mammalian counterpart, this difference is logically acceptable. Therefore, the mechanisms that govern kidney development can still be uncovered by investigating such processes within the zebrafish pronephros.

Several lines of evidence have demonstrated the similarity of the renal corpuscle between zebrafish and mammals (Ruotsalainen et al., 1999; Holthofer et al., 1999; Kramer-Zucker et al., 2005b; O'Brien et al., 2011). For example, earlier studies in humans (Ruotsalainen et al., 1999; Holthofer et al., 1999; Kestila et al., 1998) and murine models (Holzman et al., 1999; Putaala et al., 2001) showed that the transmembrane protein, nephrin, localized to the slit diaphragm of the glomerulus. Mutations in the gene encoding nephrin, NPHS1, lead to severe edema and proteinuria in mice (Putaala et al., 2001) reminiscent of patients suffering from Finnish type congenital nephrotic syndrome of the Finnish type (CNF) who also exhibit this genetic defect (Ruotsalainen et al., 1999; Holthofer et al., 1999; Kestila et al., 1998). Based on these findings, nephrin has been implicated in playing a crucial role in normal glomerular filtration (Ruotsalainen et al., 1999; Holthofer et al., 1999; Kestila et al., 1998; Holzman et al., 1999; Putaala et al., 2001). Additionally, Boute et al. (2000) discovered podocin, another podocyte specific membrane protein that is vital for the functionality of the glomerulus. In this respect, individuals displaying autosomal recessive steroid-resistant nephrotic syndrome, which is first characterized by proteinuria followed by end-stage renal disease (ESRD) and glomerulosclerosis, possess defects in the podocin gene NPHS2 (Boute et al., 2000). Correspondingly, zebrafish homologs of nephrin and podocin have since been identified, revealing the conserved function of these genes across species (Kramer-Zucker et al., 2005b) as well as their ability to signify mature glomeruli in studies evaluating the mechanisms of podocyte formation (O'Brien et al., 2011).

Not only does the zebrafish glomerulus exhibit genetic conservation with that of mammals, but the same situation has been found in terms of the specialized nephron tubule. Tubule similarities have been documented based on the differential expression of various solute transporters shared between analogous segments (Wingert et al., 2007; Wingert & Davidson, 2011). Most notably, nephrons within the zebrafish pronephros are comprised of a proximal convoluted tubule (PCT), proximal straight tubule (PST), distal early (DE), and distal late (DL) segment, which show correspondence to the mammalian PCT, PST, thick ascending limb (TAL), and distal convoluted tubule (DCT), respectively (Figure 1B) (Wingert, et al., 2007). In the murine nephron, *solute carrier family 5 (sodium/glucose cotransporter) member 2 (slc5a2*) (Reggiani et al., 2007) and *solute carrier family 20 (phosphate transporter) member 1 (slc20a1*) typically demarcate the PCT region of the nephron (Wingert et al., 2007; Wingert & Davidson, 2008). Likewise, the zebrafish PCT expresses *solute carrier family 20, member 1a (slc20a1a)* (Wingert et al., 2007; Gerlach & Wingert, 2013; Nichane et al., 2006). The PST in the mouse and zebrafish can be delineated

by expression of *solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 3 (slc13a3)* (Wingert et al., 2007). Both the mouse and zebrafish DE segments display *solute carrier family 12, member 1 (slc12a1)* expression while *solute carrier family 12 (sodium/chloride transporters), member 3 (slc12a3)* specifically indicates the DL region (Gerlach & Wingert, 2013; Wingert et al., 2007; Wingert & Davidson 2008). Finally, *GATA-binding protein 3 (gata3)* designates either the CD or pronephric duct (PD) of the mouse and zebrafish, respectively (Gerlach & Wingert, 2012; Wingert et al., 2007; Wingert & Davidson 2008). These observations ultimately led to the appreciation of organizational similarity between mammalian and zebrafish nephron segmentation, thereby exemplifying the utility of the zebrafish for the examination of renal development. In addition to the aforementioned tubule segments, zebrafish nephrons contain a neck segment, marked by the ciliogenesis regulator, *regulatory factor X, 2 (rfx2)*, which is also found in the nephrons of some mammals (Wingert et al., 2007; Dubruille et al., 2002; Lipton, 2005; Reilly et al., 2007).

Of interest, certain anatomical differences have been documented between the kidneys of mammals and zebrafish. Under normal conditions, the mammalian kidney contains primary cilia while both multiciliated cells (MCCs) and single ciliated cells (SCCs) are present within the zebrafish kidney (Ma & Jiang, 2007; Liu et al., 2007; Tammachote et al., 2009; Kramer-Zucker et al., 2005a). Furthermore, in zebrafish, the Jagged2a-Notch signaling pathway mediates the differentiation of these ciliated cell types, and these cell populations are responsible for facilitating fluid propulsion throughout the nephron (Ma & Jiang, 2007; Liu et al., 2007). In particular, *rfx2*$^{+}$ MCCs are dispersed throughout the zebrafish PST, DE, and anterior most region of the DL (Wingert et al., 2007; Ma & Jiang, 2007; Liu et al., 2007; Kramer-Zucker et al., 2005a). However, unlike the zebrafish kidney, the appearance of MCCs is typically associated with diseased states within the mammalian kidney. Thus, ciliary defects commonly result in human pathologies, such as polycystic kidney disease (PKD) and Meckel syndrome (MKS) (Tammachorte et al., 2009; Deane & Ricardo, 2007; 2012). Another intriguing distinction is the Corpuscles of Stannius (CS), a teleost-specific endocrine gland important for the regulation of calcium and phosphate concentrations (Greenwood et al., 2009; Krishnamurthy, 1976). Though, based on the close proximity of the zebrafish CS to the distal region of the pronephros resulting from supposed renal-derived progenitors, it has been hypothesized that the CS may be reminiscent of the mammalian macula densa (MD), a region of specialized cells found in the distal tubule (Wingert & Davidson, 2008). Nonetheless, studies have demonstrated the evolutionarily conserved roles of the calcium-sensing receptor (CaSR), expressed by the mammalian parathyroid glands and zebrafish CS, in organismal calcium regulation (Greenwood et al., 2009; Loretz, 2008).

While these observed differences are still being actively investigated, overall, the zebrafish pronephros still offers a simplified model to study how nephrons arise from renal progenitors during renal ontogeny, a process termed nephrogenesis. Nephrogenesis includes a constellation of complex events that include pattern formation, differentiation, tubulogenesis, and lumen formation, just to name a few. Anatomically the zebrafish pronephros provides several advantages for study because it is only comprised of two nephrons located in a linear orientation along the trunk (Wingert & Davidson, 2008). The location of nephron segments can be compared to the position of other anatomical features (Wingert et al., 2007). Namely, the somites, which are transient blocks of mesoderm that make up the embryonic trunk, can be used to map the location of renal progenitor subdomains and consequently the nephron segment boundaries (Figure 2) (Wingert et al., 2007). Corresponding nephrogenesis events in

the mouse embryo happen during stages of development that occur *in utero*, and kidney organ culture is severely limited in terms of how long cultures can be maintained. Thus, the zebrafish pronephros provides a number of unique opportunities to delineate the mechanisms of nephrogenesis, particularly to discover genes that are essential for the process and may be relevant in higher vertebrates like humans.

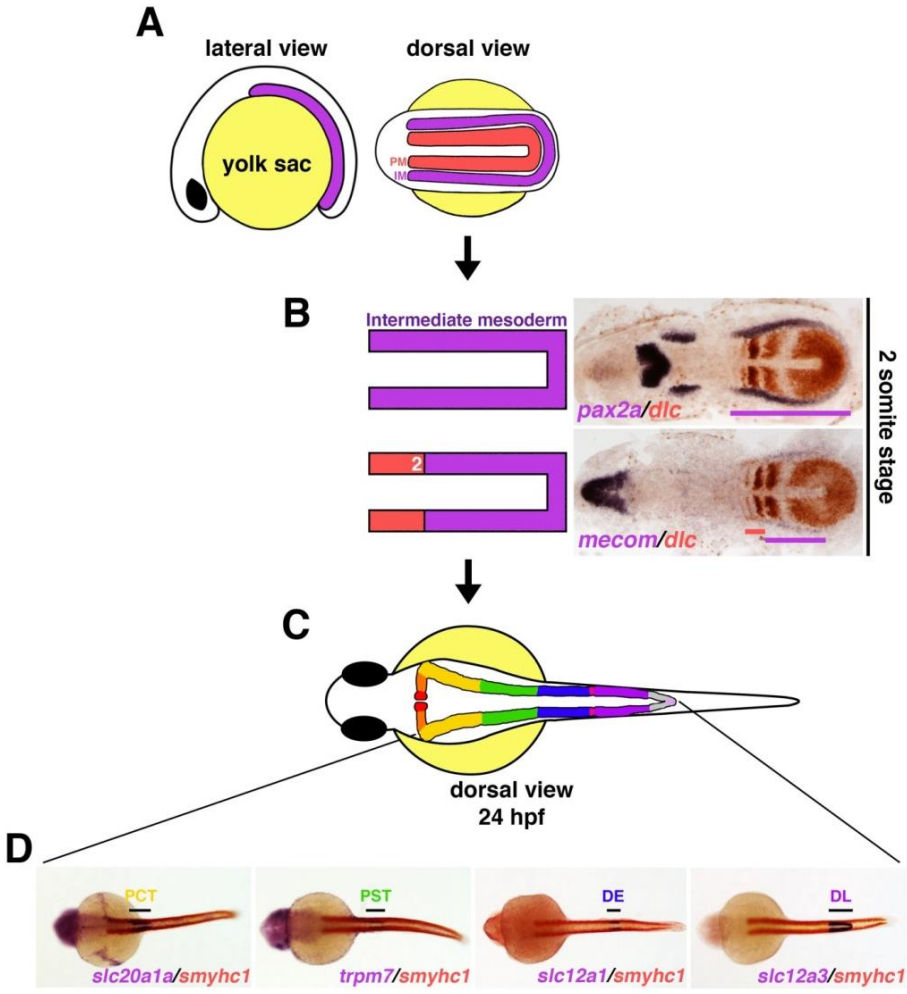

Figure 2. Nephron segmentation formation during zebrafish development. (A) Schematics depicting the intermediate mesoderm (IM) and paraxial mesoderm (PM) fields within a zebrafish embryo. (B) Renal progenitors arise from the intermediate mesoderm and can be demarcated by the expression of *pax2a* (top panels). At the 2 somite stage (indicated by *dlc* expression), exclusive rostral and caudal domains can be visualized by *dlc* and *mecom* transcripts, respectively (bottom panels). (C) Schematic of the segmented pronephros in a zebrafish embryo. (D) WISH of transcripts specific to each tubule segment is shown in embryos at 24 hpf – PCT (*slc20a1a*), PST (*trpm7*), DE (*slc12a1*), DL (*slc12a3*). Somites are marked by *smyhc1* expression. Embryo anterior is located on the left. Abbreviations: PM: paraxial mesoderm; IM: intermediate mesoderm; PCT: proximal convoluted tubule; PM: paraxial mesoderm; PST: proximal straight tubule; DE: distal early; DL: distal late.

INSIGHTS INTO NEPHROGENESIS USING THE ZEBRAFISH

Several prominent studies have momentously contributed to current knowledge of the genetic pathways that direct nephron segmentation during zebrafish nephrogenesis and will be discussed in depth in this chapter (Wingert et al., 2007; Wingert & Davidson 2011; Naylor et al., 2013). During zebrafish pronephric development, renal progenitors arise from bilateral stripes of IM located in embryonic trunk adjacent to the paraxial mesoderm (Figure 2A) (Drummond, 2003). Genes encoding transcription factors implicated in nephron development, such as *paired box genes 2a (pax2a)* and *8 (pax8)* and *LIM homeobox 1a (lhx1a)*, are expressed throughout these early stages as well (Wingert & Davidson, 2011; Ma & Jiang, 2007; Dawid et al., 1998; Pfeffer et al., 1998; Toyama et al., 1998). Subdivision of the renal progenitor field commences during early somitogenesis (Figure 2B), and has been associated with highly dynamic spatiotemporal changes in the gene expression domains of numerous transcription factors throughout the remainder of somitogenesis, though the functions of most still remain unknown (Wingert & Davidson, 2011; Li, et al., 2014). By 24 hours post fertilization (hpf), nephron segmentation of all proximal and distal domains can be visualized (Figure 2C, Figure 2D), though dynamic gene expression patterns and morphological changes within the pronephros will continue to occur during subsequent development (Wingert et al., 2007; Drummond, 2003). Renal progenitors ultimately progress through a mesenchymal-to-epithelial transition (MET). The two resulting epithelial tubules will eventually become connected, as anterior renal progenitors will differentiate into populations of podocytes that migrate and fuse at the midline into a single glomerulus, and the posterior ends of the nephrons join at the cloaca where waste will exit (Drummond, 2003). At approximately 48 hpf, signified by the midline fusion of the podocyte progenitors, blood filtration begins, and the pronephros is then considered to be functional. Furthermore, between 72-144 hpf, a progressive coiling of the PCT is observed, driven partially by the proliferation and collective cell migration of the distal segments (Wingert et al., 2007; Vasilyev et al., 2009). While these morphological events became well documented, the underlying mechanisms behind these phenomena were still poorly understood until recent studies, which have provided a framework of many genetic components of nephrogenesis (Figure 3).

The Role of Retinoic Acid in Proximodistal Renal Progenitor Patterning

To shed insight into the mechanisms that mediate segment patterning within the zebrafish pronephros, the retinoic acid (RA) pathway (Wingert et al., 2007; Wingert & Davidson, 2011) and *caudal (cdx)* (Wingert et al., 2007) gene family were first investigated since these factors are essential in the development of tissues located near or adjacent to the renal precursors. Among vertebrates, the establishment of the anterior-posterior (A-P) axis in ectoderm, mesoderm, and endoderm-derived tissues is largely dependent on the signaling of RA, a vitamin A metabolite, that regulates *Hox* genes during development. This transpires by the binding of RA to the retinoic-acid receptor (RAR) and retinoid X receptor (RXR) heterodimer, enabling subsequent interactions with the retinoic-acid response elements (RAREs) of the *Hox* genes. Spatiotemporal bioavailability of RA is influenced through sources and sinks of RA synthesis and degradation, respectively. RA synthesis relies on

retinol dehydrogenase (RDH) and retinaldehyde dehydrogenase (RALDH) enzymes, and RA removal is mediated by retinoic acid hydroxylases (Cyp26) that degrade RA (Holland, 2007).

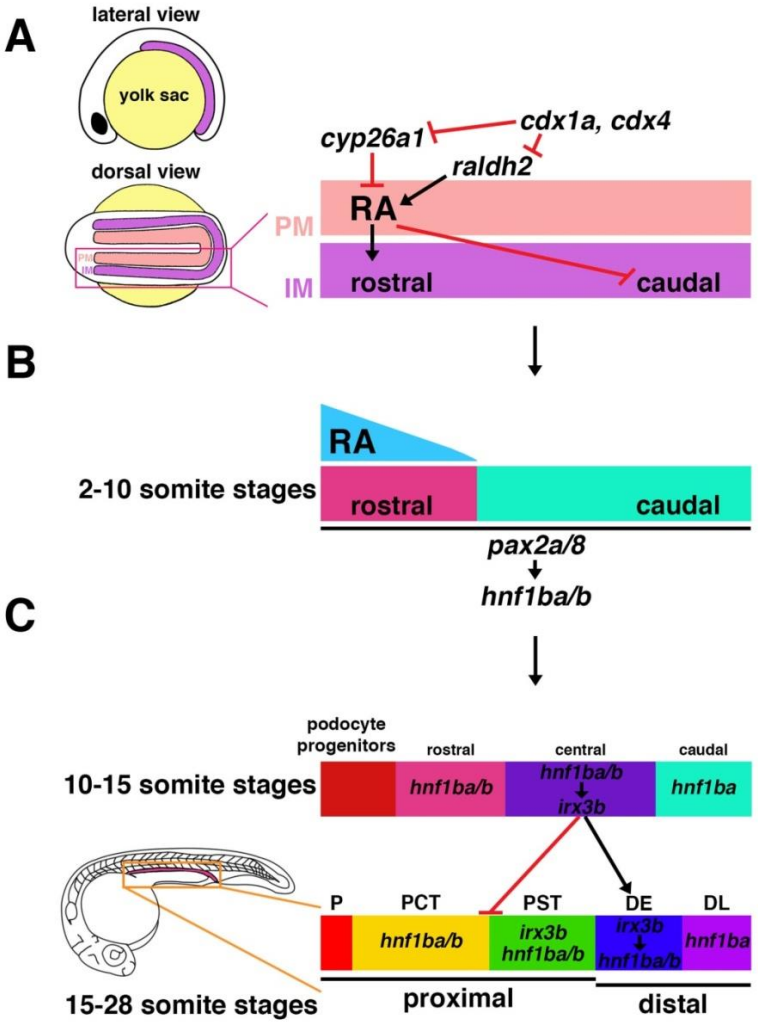

Figure 3. Functional roles of RA, *cdx* genes, and the HNF1β factors in the segmentation programming of the zebrafish pronephros. (A) Schematics of the intermediate mesoderm (IM) and paraxial mesoderm (PM) regions in a zebrafish embryo. The *cdx* genes regulate *cyp26a1* and *raldh2* to mediate the concentration of RA along the anterior-posterior (A-P) axis of the embryo. RA promotes the formation of the rostral domain while it inhibits the caudal field. (B) Exclusive rostral and caudal domains appear around the 2 somite stage under the influence of an RA gradient along the A-P axis. HNF1β factors act downstream of RA to initiate the segmentation patterning among renal progenitors and are also dependent on *pax2a/8*, which are early regulators of nephrogenesis. (C) HNF1β factors are essential for inducing the segmentation programming in the zebrafish pronephros. During early somite stages, *hnf1ba/b* is required for the expression of *irx3b*, which will facilitate the differentiation of the DE segment and mediate the PCT/PST boundary. At later stages, *hnf1ba/a* is dependent on *irx3b* for the specification and maintenance of the DE. Embryo anterior is located on the left. Abbreviations: A-P: anterior-posterior; PM: paraxial mesoderm; IM: intermediate mesoderm; RA: retinoic acid; P: podocytes; PCT: proximal convoluted tubule; PM: paraxial mesoderm; PST: proximal straight tubule; DE: distal early; DL: distal late.

Previous studies have shown that precisely regulated RA production and degradation are vital for the dynamic patterning events during somitogenesis and ontogeny of the hindbrain, pancreas, and even hematopoiesis (Dubrulle & Pourquie, 2004; Stafford et al., 2006; Stafford & Prince, 2002; Ross et al., 2000; Gavalas & Krumlauf, 2000; Chanda et al., 2013).

The role of RA signaling during zebrafish pronephros development was assessed by performing morpholino inactivation of *retinaldehyde dehydrogenase 2 (raldh2)* and by treating wild-type (WT) embryos with the reversible RALDH inhibitor 4-diethylaminobenzaldehyde (DEAB). It was observed that the distal regions expanded at the expense of the proximal segments (Wingert et al., 2007). The same result was obtained when WT embryos were treated with an antagonist targeting the RAR-α receptor (Wingert & Davidson, 2011). Conversely, opposite effects were seen when embryos were exposed to exogenous RA (Wingert et al., 2007) or when RAR-α was administered to WT embryos (Wingert & Davidson, 2011). These findings indicated that a functional RA pathway (RA and RAR-α receptors) is essential for normal nephron segmentation to occur by promoting proximal fates and restricting distal domains (Wingert et al., 2007; Wingert & Davidson, 2011). Furthermore, results obtained from a DEAB timecourse suggested that various segments had differential RA requirements that were temporally modulated. Interestingly, the impact of RA on proximo-distal patterning was found to be greatest between gastrulation and the 5-somite stage. Moreover, during this time period, varying RA levels could influence renal progenitor gene expressions of *dlc*, *jag2a*, and *mecom* in the IM as well. Thus, RA signaling is necessary for proper proximodistal nephron patterning during early development (Wingert et al., 2007; Wingert & Davidson, 2011).

During early embryogenesis, the entire renal progenitor field can be visualized by the expression of *pax2a* and *pax8* (Wingert & Davidson, 2011). It is now known that by the end of the 2-somite stage, the renal progenitors can be demarcated into two exclusive domains, termed rostral and caudal, by the transcripts *deltaC (dlc)* and *MDS1 and EVI1 complex locus (mecom*, formerly known as *evi1)* respectively (Li, et al., 2014). Previously, other genes such as *jagged 2a (jag2), POU class 3 homeobox 3a (pou3f3a),* and *empty spiracles homeobox 1 (emx1),* were known to show overlapping domains in the renal progenitor field at various early stages (Wingert & Davidson, 2011). These dynamic gene expression patterns suggested that the segmentation program might be activated among the renal progenitors prior to somitogenesis. Therefore, in order to evaluate the effect of RA on nephron segmentation during early development, the *lightbulb (lib)* mutant containing a defect in RA production was utilized. Indeed, while the renal progenitor domains were still visible, there was a noticeable reduction of the rostal domain and expansion of the distal domain, leading to an overall shift of these regions towards the anterior based on *dlc*, *jag2*, and *mecom* transcript localization in *lib* mutants at the 8-somite stage. Together, these findings suggest that RA mediates the early rostal and caudal domains (Figure 3B) (Wingert & Davidson, 2011).

Caudal Gene Activity Positions the Renal Progenitor Field by Influencing the Locale of Retinoic Acid Activity in the Zebrafish Embryo

Like RA, *cdx* transcription factors can directly regulate *Hox* genes to influence posterior fates in an embryo along the A-P axis (Lohnes, 2003). For this reason, the relationship between the *cdx* genes and RA were evaluated in the developing zebrafish pronephros

(Wingert et al., 2007). The *cdx* transcription factors were found to influence the location of the pronephros along the A-P axis (Wingert et al., 2007). In embryos deficient for either *cdx4* or *cdx1a/4*, *Wilms tumor protein 1a (wt1a)* expressing podocytes were located more posteriorly. However, the podocytes of *cdx1a/4* doubly-deficient embryos failed to fuse into a single glomerulus at 48 hpf, resulting in the eventual development of cysts and extreme pericardial edema unlike WT and *cdx4*$^{-/-}$ embryos (Wingert et al., 2007). The tubules of both of these morphant types were also more caudally positioned as indicated by the pan-tubule and duct marker *cadherin 17* (*cdh17*). Although *cdh17* expression was continuous in *cdx4* morphants, this was not the case in *cdx1a/4* deficient embryos, indicating that the pronephros was unable to form normally (Wingert et al., 2007). In agreement with this latter observation, *slc20a1a* expression in the PCT was intermittent while markers for the other segments were absent (Wingert et al., 2007). Alternately, all segments (PCT, PST, DE, CS, DL, PD) were present in *cdx4*$^{-/-}$ embryos, but the PCT domain increased by approximately two somites whereas the distal segments became slightly reduced (Wingert et al., 2007).

Intriguingly, data was found in support of the *cdx* genes regulating the expression of *raldh2* and *cyp26*, the synthesizing and degrading enzymes of RA respectively, to further direct nephron segmentation (Figure 3A) (Wingert et al., 2007). The gene expression domains of both *raldh2* in the paraxial mesoderm and *cyp26a1* in the upper trunk became more posteriorly shifted in *cdx4*$^{-/-}$ and *cdx1a/4* deficient embryos, with more dramatic effects observed in the latter (Wingert et al., 2007). When these embryos were treated with DEAB, distal fates were rescued (Wingert et al., 2007). Consequently, these analyses suggested that along the A-P axis, the *cdx* genes regulate *raldh2* and *cyp26a1* to specify RA levels enabling RA to spatially promote proximal fates while limiting distal domains (Wingert et al., 2007). Of importance, these results also indicated that this proximodistal patterning is largely enabled by the paraxial mesoderm, which is the predominant source of RA (Figure 3A) (Wingert et al., 2007; Wingert & Davidson, 2011).

Renal Progenitor Pattern Specification and Differentiation: The Influence of the Homeobox Transcription Factor Genes *hnf1ba/b* and *irx3b*

Additional regulation of nephron segmentation in the zebrafish pronephros is facilitated by the paralogues, *HNF1 homeobox Ba (hnf1ba)* and *HNF1 homeobox Bb (hnf1bb)* (Figure 3C) (Naylor et al., 2013). Typically, HNF1β factors have been associated in the early development of various organs (Chauveau et al., 2013; Sun & Hopkins, 2001; Ulinski et al., 2006), especially the differentiation of epithelia (Sun & Hopkins, 2001; Coffinier et al., 1999; D'Angelo et al., 2010). With respect to the kidney, genetic defects in HNF1β are commonly detected among individuals suffering from developmental renal abnormalities that arise prenatally or in later life (Massa et al., 2013; Decramer et al., 2007; Heidet et al., 2010; Ulinski et al., 2006). Correspondingly, murine models have recently shown the role of HNF1β in regulating nephron segmentation with links to the *Iroquois (Irx)* transcription factors (Heliot et al., 2013; Massa et al., 2013).

In WT zebrafish embryos, *hnf1ba* is expressed throughout the entire nephron while *hnf1bb* is only found in the proximal and DE segments (Wingert & Davidson, 2011). Embryos lacking these homeodomain transcription factors exhibited normal cloaca development but failed to express proximal and distal markers at 24 hpf, which suggested the

absence of segmentation events upon tubule formation (Naylor et al., 2013). Thus, *hnf1ba/b* appears to be essential for the activation of the segmentation pathway but not tubule epithelialization (Naylor et al., 2013). When embryos deficient for *hnf1ba/b* were treated with DEAB (Naylor et al., 2013), podocytes were lost and tubules adopted entirely distal fates as seen in DEAB treated WT embryos (Wingert et al., 2007). This implicated that the HNF1β factors act downstream of the RA pathway to initiate nephron patterning (Naylor et al., 2013). In contrast, *hnf1ba/b* expression was dependent on that of early nephrogenesis regulators, *pax2a* and *pax8* (Naylor et al., 2013). This was indicated though the observation of similar phenotypes in *hnf1ba/b* and *pax2a/8* morphants, where the expression of markers for mature nephron segments was significantly decreased in the latter (Naylor et al., 2013). Moreover, epistasis experiments demonstrated a temporal feedback loop where *hnf1ba/b* factors are first needed for *iroquois homeobox protein 3b (irx3b)* expression in the DE, but at later stages of development, *irx3b* is required for a *hnf1ba*-expressing DE (Naylor et al., 2013). Based on these findings, HNF1β factors operate downstream of RA to initiate the segmentation programming in the nephron while dynamic interactions between *irx3b* and *hnf1ba/b* are needed for the specification and maintenance of the DE (Figure 3C).

In concurrence with this model establishing the relationship between RA, the HNF1β factors, and *irx3b* (Naylor et al., 2013), corresponding evidence places *irx3b* downstream of RA signaling to induce DE differentiation (Figure 3C) (Wingert & Davidson, 2011). WT embryos injected with an *irx3b* morpholino failed to develop a DE segment at 24 hpf leading to a caudal expansion of the proximal regions (Wingert & Davidson, 2011). In support of this finding, the domain of *hepatocyte nuclear factor 4, alpha (hnf4a)*, a rostral and PST marker, elongated and the *pou3f3a*-expressing region was found to undergo a posterior shift (Wingert & Davidson, 2011). However, the transcripts of *mecom* (DL and PD) and *gata3* (CS and PD) retained their WT phenotypic expression in the *irx3b* morphants (Wingert & Davidson, 2011). Therefore, it was thought that *irx3b* imparts late acting functions of inhibiting proximal boundaries and initiating DE specification (Wingert & Davidson, 2011). In adherence to these proposed roles of *irx3b, lib* mutants injected with morpholinos targeting *irx3b* displayed a lack of DE with substantially expanded PCT and PST segments (Wingert & Davidson, 2011). This revealed that *irx3b* is still able influence nephron segmentation once RA has already initiated proximal programming in the progenitors of these segments, thereby showcasing the dynamism of this intricate process (Wingert & Davidson, 2011). Furthermore, in DEAB treated embryos, *irx3b* expression was comparable to that of *slc12a1* in the DE (Wingert & Davidson, 2011). In embryos doubly treated with DEAB and the *irx3b* morpholino, a region corresponding to the DE as delineated by *irx3b* was visualized by the DL marker, *slc12a3* (Wingert & Davidson, 2011). The authors concluded that *irx3b* plays a prominent part in the later stages of DE segment differentiation, such that *irx3b* activity is required for the expression of DE-specific solute transporters, but in its absence, DL markers will be expressed instead (Wingert & Davidson, 2011).

Overall, these studies exemplify the genetic complexities that drive the fundamental mechanisms of nephron segmentation. The dynamic interplay between RA signaling, HNF1β factors, and *irx3b* illustrate the elaborate processes that coordinate the initial boundaries of proximal and distal segments to the refinement of gene expression patterns for the final specification of various domains (Wingert et al., 2007; Wingert & Davidson, 2011; Naylor et al., 2013). While considerable research has already been conducted to elucidate the pathways that control nephrogenesis, the function of many genes in the kidney are still unknown as

implicated by the recently annotated zebrafish genome. Additional analyses within the zebrafish model will undoubtedly contribute significantly to the current working model of renal development.

FURTHER DELINEATION OF RENAL DEVELOPMENTAL PATHWAYS WITH ESTABLISHED AND EMERGING MOLECULAR TOOLS IN ZEBRAFISH

Newly developed molecular techniques pertinent to the zebrafish system have further enhanced its application as a powerful model organism for developmental studies. In particular, the generation of loss of function (e.g. morpholinos) and genome editing techniques (e.g. TALENs and CRISPRs) has fueled the advancement of gene function analyses in the zebrafish (Figure 4) (Nasevicius & Ekker, 2000; Ekker, 2000; Gaj et al., 2013). Specifically, within the context of this chapter, the current use of morpholinos to reveal the underlying mechanisms that govern kidney organogenesis (Wang et al., 2012; Ihalmo et al., 2003; Nishibori et al., 2011; Perner et al., 2007) and the potential of TALENs and CRISPRs to revolutionize renal research will be discussed in the following sections.

		Advantages	Disadvantages

Transient Gene Manipulation	Morpholino	• *in vivo*
		• Effective gene knockdown
		• Broad gene targeting range
		  • ATG or splice sites
		• High target specificity
		• Reproducible results
		• Nuclease resistant
		• Water soluble
		• Affordable
			• Transient
			  • Inability to evaluate post-embryonic phenotypes (e.g. metabolic disease)
			• Off-target effects
			• Need for additional controls to confirm target specificity
			  • 5 base pair mismatch morpholino control
			  • Rescue by co-injection with exogenous mRNA
			  • p53 morpholino co-injection
			• Lack of spatiotemporal control*
			
			* Applies to standard morpholinos only

	Photo-morpholino	• Includes all morpholino advantages
		• Spatiotemporal control
		  • Whole embryo or select groups of cells
		  • Can turn gene expression on or off	

Genome Editing	TALENs	• *in vivo*
		• Site-specific gene targeting
		• Effective, permanent mutations
		• Can induce insertions or deletions
		  • At gene loci or chromosomally
		• Heritable mutations
		• Generation of mutant lines
		• Recognition of a single nucleotide by each TALE repeat domain**
		• Can target multiple genes at once***
		• Fast, easy to use technique***
		• Short sgRNAs***
		
		** TALEN specific
		*** CRISPR-Cas specific
			• Requirement of a 5′ thymine base in target sequences
			• Time-consuming
			  • Construction of 2 nucleases per target site
			• Highly repetitive sequences in TALEN vectors
			  • Increased risk of complications in synthesis, vector delivery, and/or sequencing

	CRISPR-Cas		• Requirement of a PAM sequence in the target DNA and a “seed” region in the crRNA • Varying degrees of specificity • Toxicity**** • Off-target effects**** • Certain sites cannot be mutagenized**** **** Similarly seen in TALENs

Figure 4. Chart detailing the advantages and disadvantages of current molecular techniques amenable to the zebrafish model for investigating gene function.

Morpholino Mediated Knockdown of Target Genes to Investigate Kidney Development

One fundamental method used to study gene function by targeted knockdown in the zebrafish is the utilization of antisense morpholino oligonucleotides (Nasevicius & Ekker, 2000; Bill et al., 2009). A morpholino is synthesized as an oligomer containing approximately 25 morpholine bases that are "antisense" to the target "sense" RNA (Ekker, 2000; Summerton & Weller, 1997; Summerton, 1999). The morpholino is then able to bind the RNA strand of interest through complementary base pairing, thereby functioning to sterically inhibit the translation of that RNA into a functional protein *in* vivo (Bill et al., 2009; Summerton & Weller, 1997). In many cases, morpholinos are generated to bind mRNA at the 5' untranslated region (5' UTR) near the start site resulting in the complete inhibition of gene expression by preventing the assembly of the cell's translational machinery (Bill et al., 2009; Summerton 1999). Alternately, morpholinos can be designed to target splice junctions. In this respect, these splice blocking morpholinos are able to deter proper RNA splicing by interfering with the binding ability of the spliceosome during pre-mRNA processing (Summerton & Weller, 1997; Morcos, 2007). Accordingly, both of these morpholino targeting strategies have been found to be quite effective in the zebrafish (Nasevicius & Ekker, 2000; Ekker, 2000) and are currently being widely used to evaluate gene function and confirm morphant phenotypes (Bill et al., 2009; Yuan & Sun, 2009).

In zebrafish, the delivery of a morpholino occurs through microinjection directly into the 1- to 2- cell stage of embryonic development (Yuan & Sun, 2009). Based on previous studies that employed the use of morpholinos in zebrafish, the effects of morpholinos have been documented to be fully penetrant during the first 48 hours of development (Nasevicius & Ekker, 2000) and can even last as long as 5 days post injection (dpi) (Bill et al., 2008; 2009; Smart et al., 2004; Kimmel et al., 2003). An example of a typical readout of a morpholino experiment, aside from the observation of morphological morphant phenotypes, is a whole mount *in situ* hybridization (WISH) where gene expression is detected by the localization of mRNA transcripts in the zebrafish embryo. This is achieved by labeled antisense riboprobes that are complementary to the mRNA of interest (Jacobs et al., 2011). As a result, this technique enables the visualization of aberrant mRNA expression patterns within the injected embryo when compared to that of the endogenous mRNA in a WT embryo.

Morpholinos are excellent for the investigation of gene function during development and are also particularly valuable to scientists because they are affordable, water soluble, and unaffected by nuclease activity (Summerton & Weller, 1997). Additionally, for the most part, morpholinos (i) can be designed to target any gene and show sequence specificity, (ii) are efficient at eliminating proteins of interest by preventing RNA translation, (iii) exhibit potency in all cells in terms of gene knockdown, and (iv) results obtained by morpholinos are typically reproducible (Ekker, 2000). However, as with any molecular technique, while morpholinos provide developmental biologists with a critical means for studying gene function in the zebrafish, several drawbacks are still associated with this method. For example, an inherent disadvantage of morpholinos is the inability to evaluate phenotypes (e.g. metabolic diseases) that manifest during post-embryonic stages since morpholinos tend to lose potency after the first 48 hours (Seth et al., 2013). Furthermore, in some circumstances, the injection of a translational blocking morpholino can lead to the ectopic upregulation of the p53 apoptotic pathway resulting in neuronal cell death as an off-target pheonotype, but this

effect can be alleviated in part by a concomitant p53 morpholino co-injection (Bill et al., 2009; Bedell et al., 2011; Robu et al., 2007; Ekker & Larson, 2001). Another common issue with morpholinos is the observation of off-target effects (e.g. creation of "monster" embryos with truncated tails, grossly dysmorphic bodies, and shrunken heads) (Bill et al., 2009; Bedell et al., 2011).

Nevertheless, the risk of nonspecific activity can typically be reduced by the standardization of morpholino doses within an experiment (Bill et al., 2009; Bedell et al., 2011). Control RNA rescue experiments can also be performed for further validation of morpholino induced phenotypes by verifying the specificity of the morpholino. In a rescue, exogenous mRNA coding for the protein of interest is co-injected with the antisense morpholino (Bill et al., 2009; Eisen & Smith, 2008). Essentially, this injected mRNA contains the same coding region as the morpholino, but lacks or possesses a different 5' UTR (Eisen & Smith, 2008). If the morpholino is specific for its target locus, the presence of the exogenous mRNA should be able to offset the effects of the morpholino knockdown, thereby restoring the WT phenotype and demonstrating morpholino target specificity in the zebrafish. Another strategy for testing morpholino specificity is the creation of a five base pair mismatch morpholino. In this case, five nucleotides are changed in the original morpholino, which should impair its ability to bind to its target mRNA, resulting in a WT phenotype (Eisen & Smith, 2008). In combination, dosage adjustments and these specificity controls can help reduce the amount of nonspecific and off-target effects often seen in morpholino experiments (Bill et al., 2009; Bedell et al., 2011; Eisen & Smith, 2008).

Even so, the most significant disadvantage associated with conventional morpholinos is the lack of spatial and temporal control during gene knockdown, especially when such loss of function results in an embryonic lethal phenotype. These limitations have been obviated through the recent development of photo-morpholinos (Shestopalov et al., 2007; Tallafuss et al., 2012; Wolf & Ryu, 2013; Schweitzer et al., 2013) where gene expression can be activated or de-activated in the whole embryo or select groups of cells (Tallafuss et al., 2012; Wolf & Ryu, 2013; Schweitzer et al., 2013). These photo-morpholinos can be synthesized as either antisense (AS) or sense (S) oligomers and gene expression will either be inhibited or activated, respectively. In the AS-photo-morpholino strategy, a photo-cleavable unit is inserted into the middle of the AS-photo-morpholino. Upon injection into the zebrafish embryo, gene expression is silenced by the binding of the AS-photo-morpholino to the target mRNA. Once the embryo is exposed to 365 nm UV light, the photo-cleavable unit is severed releasing the AS-photo-morpholino from its target and allowing protein expression of the targeted gene to ensue (Tallafuss et al., 2012).

Alternatively, the S-photo-morpholino is annealed to the conventional AS-morpholino strand and injected into the embryo. In this scenario, gene expression is still active because the AS-morpholino is inhibited from blocking translation since it is bound to the S-photo-morpholino. When exposed to UV light, the S-photo-morpholino will be cleaved enabling the AS-morpholino to target its complementary endogenous RNA sequence, thus turning gene expression off (Shestopalov et al., 2007; Tallafuss et al., 2012; Wolf & Ryu, 2013; Schweitzer et al., 2013). This S-photo-morpholino technique has been successfully utilized in studies examining the role of various genes, such as *no tail* (Tallafuss et al., 2012), *FEZ family zinc finger 2* (Wolf & Ryu, 2013), and *single-minded homolog 1a* (Schweitzer et al., 2013), in zebrafish neurogenesis. Consequently, temporal control is achieved through the presence of a photo-sensitive unit in AS- and S-photo-morpholinos (Tallafuss et al., 2012;

Wolf & Ryu, 2013; Schweitzer et al., 2013). Additional studies have shown it is possible to attain spatial control as well by using a laser to limit UV exposure to a small subset of cells in the zebrafish embryo (Shestopalov et al., 2007; Tallafuss et al., 2012). These modified morpholino targeting strategies provide researchers with the flexibility to control gene expression both spatially and temporally, which permits the evaluation of when and where a gene's activity is essential during development (Tallafuss et al., 2012; Wolf & Ryu, 2013; Schweitzer et al., 2013). Thus, the use of photo-morpholinos has high potential to provide insights into the mechanisms of renal progenitor development, for example.

Morpholino knockdown has become a crucial tool for evaluating gene function in zebrafish (Bedell et al., 2011; Yuan & Sun, 2009; Tallafuss et al., 2012; Draper et al., 2001), and this method has provided considerable insight into kidney organogenesis as well (Wang et al., 2012; Mitra et al., 2012; Raschperger et al., 2008; Wang et al., 2013). In particular, morpholinos have helped elucidate the roles of genes with unknown functions in kidney development through gene silencing resulting in the abrogation of normal nephron phenotypes. Current literature contains numerous examples of morpholinos to establish gene function in the complex processes of glomerulus and nephron formation. For instance, zebrafish *kin of IRRE like 3 like (kirrel3l*, previously named *neph3)*, an ortholog of human *neph3*, was found to be important in the early developmental stages of the glomerulus, the blood filter of the renal system, but not for glomerular function after pronephros maturation (Wang et al., 2012). In WT embryos, *neph3* encodes a transmembrane protein that is expressed in the glomerular podocyte epithelial cells (Ihalmo et al., 2003). Upon *neph3* morpholino injection, embryos displayed body curvature and transient pericardial edema detected at 4 dpi, representative of renal dysfunction. Histological analysis of these *neph3* morphant embryos revealed an expanded Bowman's space within the glomerulus, but interestingly, the expression of *nephrin* and *podocin*, both of which are glomerular markers of maturity and differentiation, were not perturbed. These findings suggest that *neph3* may regulate early glomerular formation rather than its structural integrity and function following maturation (Wang et al., 2012). Alternately, another morpholino study showed that *glucocorticoid-induced transcript 1 (glcci1)* was imperative for podocyte formation and structural maintenance, as the knockdown of this gene caused extensive effacement of podocyte foot processes and collapse of glomeruli (Nishibori et al., 2011). Strikingly, the inactivation of *wt1a* by morpholinos in zebrafish embryos lead to the complete loss of podocytes and glomerular differentiation indicated by the lack of *nephrin* and *podocin* expression (Perner et al., 2007).

In the tubules of the zebrafish pronephros, the apical surfaces of the epithelial cells express *uroplakin 3l (upk3l)* (Mitra et al., 2012). When *upk3l* function is obliterated by morpholinos, drastic functional and morphological events occur in the pronephros. *upk3l* morpholino dose dependent phenotypes were observed with regards to pericardial edema beginning at 3 dpi and axial curvature where severity correlated with increased dosage. While *upk3l* morpholino inactivation did not alter the development or organization of the pronephros, epithelial cell polarization was significantly disrupted, as was the presence of their brush borders. Not surprisingly, *upk3l* morphants exhibited lower rates of renal clearance based on dextran injections, indicative of faulty kidney function (Mitra et al., 2012). Similarly, knockdown of the coxsackie and adenovirus receptor (CAR), which is expressed at the tight junctions of epithelial cells, resulted in the loss of close cell-to-cell contacts throughout the tubule and severely reduced or abrogated apical brush borders. The latter

finding indicates the potential role of CAR in microvilli organization within the proximal regions of the nephron (Raschperger et al., 2008). Additionally, in another study relating to proximal tubule characterization, *microRNA 34b (mir24b)* was determined to be important for multiciliogenesis and the convolution of this segment (Wang et al., 2013). Taken together, these examples demonstrate how morpholinos have enabled the characterization of many genes during nephrogenesis and will undoubtedly continue to do so in the future.

Genomic Engineering: Implications of TALENs in Nephrology

The recent development of transcription activator-like effector nucleases (TALENs) for genome editing is now beginning to emerge within the zebrafish community (Moore et al., 2012; Bedell et al., 2012b; Sander et al., 2011; Huang et al., 2011; Dahlem et al., 2012; Joung & Sander, 2013). In general, TALENs consist of a FokI nuclease domain fused to transcription activator-like effector (TALE) proteins, which are naturally present in *Xanthomonas*, a genus of phytopathogenic bacteria (Moore et al., 2012; Gaj et al., 2013; Boch & Bonas, 2010). The DNA binding ability of TALENs occurs through the TALE repeat domains which contain DNA-binding domains comprised of a series of 33-35 conserved amino acid repeat domains. Within each TALE DNA-binding domain, two hypervariable positions, termed repeat-variable di-residues (RVDs), are able to recognize a single DNA nucleotide, conferring TALEN sequence specificity (Mak et al., 2012; Deng et al., 2012; Boch & Bonas, 2010).

Upon TALEN dimerization on the DNA, the FokI nuclease domains are able to induce double-stranded breaks (DSBs), causing the cell to undergo non-homologous end joining (NHEJ) as a repair response (Moore et al., 2012; Bedell et al., 2012b). Since NHEJ is a highly error-prone pathway, the resulting creation of insertion or deletion mutations (indels) at the site of the DSB can cause shifts in the reading frame often leading to a premature stop during translation (Cade et al., 2012; Dahlem et al., 2012). Notably, studies examining the mechanism of modified TALENs in editing endogenous genes discovered that homology directed repair (HDR) (Gaj et al., 2013) can occur after a TALEN induced DSB in the presence of a donor DNA fragment (Miller et al., 2011) and be inherited by the offspring (Moore et al., 2012). This finding unlocks the possibility of inserting a sequence of interest into a homologous region near the location of the DSB, which would be used during HDR to repair the DNA and therefore introduce that sequence into the desired area of the genome (Jasin, 1996).

Extensive chromosomal insertions and deletions mediated by pairs of TALENs in livestock (Carlson et al., 2012), silkworm (Ma et al., 2012), and zebrafish (Xiao et al., 2013) have been successful as well. In the latter organism, two sites in zebrafish *semaphorin 3fb (sema3fb)* were targeted by TALENs, one pair per location (Xiao et al., 2013), resulting in heritable deletions and inversions of that chromosomal region (Xiao et al., 2013; Ma et al., 2012). Five other endogenous zebrafish loci were tested in the same manner and showed that this outcome was effective for segments up to 1Mb (Xiao et al., 2013). Together, these findings reveal the ability of TALENs to not only disrupt protein-coding regions by the creation of indels (Moore et al., 2012; Bedell et al., 2012b; Huang et al., 2011), but to also perturb sequences containing regulatory elements or non-coding genes by sizeable genomic inversions or deletions (Xiao et al., 2013; Carlson et al., 2012; Ma et al., 2012).

Due to its mechanism of action, TALENs represent a class of molecular techniques that is particularly appealing in terms of gene function analyses. The construct of TALENs enables the effective disruption of gene expression in a highly specific and predictable manner (Huang et al., 2011; Cade et al., 2012), which has been demonstrated in various model organisms including *Drosophila* (Katsuyama et al., 2013), *Xenopus* (Suzuki et al., 2013), mice (Qiu et al., 2013; Sung et al., 2013; Panda et al., 2013), and zebrafish (Sander et al., 2011; Huang et al., 2011; Bedell et al., 2012b). TALEN gene inactivation has phenocopied mutations caused by morpholinos, providing additional confirmation of TALEN specificity (Moore et al., 2012). In essence, the engineering of different combinations of TALE repeat domains containing the RVDs can significantly increase the ability of TALENs to recognize the correct target gene sequence within the genome (Moore et al., 2012; Bedell et al., 2012b; Dahlem et al., 2012). Since it is possible to design and manipulate the arrangements of RVDs for personalized applications, this method ultimately provides researchers with the flexibility to customize TALENs to fundamentally target any number of sequences within the genome of an organism (Gaj et al., 2013; Joung & Sander, 2013). A major caveat to the targeting range of TALENs is the requirement of a 5' thymine base located at position 0 of the target site (Mak et al., 2012; Katsuyama et al., 2013; Bochtler, 2012: Boch et al., 2009). Consensus in the literature indicates that this thymine base is critical for TALEN target recognition occurring through two degenerate folds at the N-terminus (Mak et al., 2012; Katsuyama et al., 2013). If this conserved thymine site is altered, TALEN activity at that location will substantially decrease (Boch et al., 2009; Romer et al., 2010). Regardless, the relative frequency of thymines in a genome should help alleviate this limitation on TALEN targeting to some degree.

Subsequent murine (Qiu et al., 2013; Sung et al., 2013) and zebrafish (Huang et al., 2011; Cade et al., 2012) studies have also shown that mutations induced by TALENs can be transmitted through the germline with high frequencies of up to 100% (Moore et al., 2012; Cade et al., 2012; Dahlem et al., 2012) depending on the targeted site to produce mutant progeny. This high level of germline transmission can typically be achieved with GoldyTALENs, a modified TALEN construct resulting in greater efficiencies (Moore et al., 2012). However, the repeated outcrossing of mutant lines is still necessary to remove any unrelated mutations that might have been inadvertently introduced. Confirming observed phenotypes from TALEN mutagenesis by at least two independent lines is an added precaution that is highly recommended for these types of experiments as well (Hwang et al., 2013a).

While TALENs are a considerably powerful technique for site-specific genome engineering, and have exciting implications for the advancement of both developmental and therapeutic studies, TALEN technology has not yet been used to extensively study kidney development. In fact, the majority of published studies pertaining to the utilization of TALENs in zebrafish typically addressed the ability of this new technology to recapitulate the mutagenic efficiencies seen in other model organisms in this teleost species (Huang et al., 2011; Zu et al., 2013; Huang et al., 2012). The findings of these analyses suggest a favorable outlook for TALEN based approaches for elucidating the genetic mechanisms that direct nephrogenesis. Provided that the sequence of interest begins with a thymine (Bochtler, 2012; Romer et al., 2010), virtually any genetic mutant could be created for kidney studies. As opposed to the more time-consuming approach of morpholino induced gene knockdown, which is only effective during early embryonic stages (Smart et al., 2004; Bill et al., 2008),

TALENs would enable targeted gene knockouts with lifelong effects in the zebrafish. Gene inactivation can be achieved in somatic cells (Bedell et al., 2012b; Sander et al., 2011) and transmitted to the F1 generation as well (Dahlem et al., 2012; Zu et al., 2013), the latter of which is unattainable by the transient-lasting morpholinos (Seth et al., 2013). It has also been reported that approximately two weeks in total are needed from TALEN construct design to obtaining mutants with germline transmission capabilities (Dahlem et al., 2012). Therefore, the establishment of mutant lines harboring defective genes that are important for kidney development would enable quicker analyses of gene function. Moreover, by exploiting HDR repair, TALEN facilitated insertions (Miller et al., 2011; Jasin, 1996) of essential nephrogenesis genes, such as *pax2a* and *wt1a/b*, could mimic gene duplication events resulting in either beneficial or deleterious effects on this process.

The use of TALENs to study kidney development has already been demonstrated (Bedell et al. 2012a; 2012b). Normal glomerulus formation in the zebrafish has been found to be dependent on *ponzr1* based on data obtained from morpholino knockdowns (Bedell et al., 2012a). As a result, TALENs were designed to target *ponzr1* in order to test the efficacies of pTAL (Cermak et al., 2011), a standard TALEN construct, and GoldyTALENs (Bedell et al., 2012b). Both types of TALENs produced similar types of indels at the targeted *ponzr1* site, but the efficiencies of both mutagenesis and transmission to the progeny were significantly higher when GoldyTALENs were used. Successful HDR mediated integrations of an EcoRV restriction site and *mloxP* into the *ponzr1* locus was achieved when GoldyTALENs targeting *ponzr1* was introduced with single-stranded DNA (ssDNA) containing either sequence, and these mutations were passed through the germline as well (Bedell et al., 2012b). These studies ultimately establish the abilities of TALENs to contribute additional temporal control, as seen by the introduction of an *mloxP* site, and to make stable kidney mutant lines with relative ease. In any event, it remains to be seen how TALENs will transform the world of zebrafish nephrology. However, these initial studies offer promise based on the overall effectiveness of this technique for site-specific gene targeting.

Prospects for CRISPR-Cas Facilitated Genome Editing in the Analyses of Nephrogenesis

An alternate, newly emerging technique for genome editing is the CRISPR-Cas system where a RNA guide strand is used to mediate a double-stranded break in DNA by a Cas9 endonuclease causing repair by the error-prone pathway of NHEJ. This strategy is derived from an adaptive defense mechanism against foreign nucleic acids that is found in Bacteria and Archaea species: clustered regularly interspaced short palindromic repeats (CRISPRs) and Cas proteins that possess nuclease activity (Hwang et al., 2013b; Horvath & Barrangou, 2010). In nature, the CRISPR loci contain several direct repeats that are interspersed with hypervariable regions known as spacers. These spacers are short segments of exogenous DNA sequences obtained from previously encountered viruses and plasmids that have become integrated into the CRISPR loci (Blackburn et al., 2013; Wiedenheft et al., 2012). A CRISPR RNA (crRNA), containing the target sequence, can then be transcribed and, in a type II system, will also base pair to a trans-activating crRNA (tracrRNA) and foreign DNA harboring complementary sequences adjacent to a protospacer adjacent motif (PAM) (Blackburn et al., 2013; Jao et al., 2013; Widenheft et al., 2012). Of note, the PAM sequence

in the target DNA and a "seed" region in the crRNA are required for Cas9 nuclease activity and target recognition (Jao et al., 2013; Jinek et al., 2012). Correspondingly, functioning in combination with the Cas9 endonuclease, this CRISPR-Cas complex is able to cleave exogenous DNA resulting in DSBs (Blackburn et al., 2013; Wiedenheft et al., 2012).

Following the discovery of this naturally occurring CRISPR-Cas system, efforts have been made to manipulate this mechanism for site-specific genomic engineering *in vitro* and *in vivo* (Hwang et al., 2013a; 2013b; Jao et al., 2013; Gaj et al., 2013). By fusing the crRNA and tracrRNA together *in vitro* to produce a synthetic single guide RNA (sgRNA), successful cleavage of target sequences by Cas9 can be attained (Hwang et al., 2013b; Jinek et al., 2012; Cho et al., 2013). This complex is designated as sgRNA:Cas9 and has been found to cause targeted indels in human cells (Cho et al., 2013; Jinek et al., 2013; Mali et al., 2013) varying from frequencies of 2-4% for induced pluripotent stem cells (Mali et al., 2013) and up to 33% in the K562 myelogenous leukemia line (Cho et al., 2013). Promising results from an *in vivo* study by Hwang and colleagues (2013b) indicated that the CRISPR-Cas system was able to produce directed modifications in the zebrafish genome at rates comparable to those caused by TALEN technology (Hwang et al., 2013b) as well thereby proposing the likely success of this method in mutagenizing the genome of any organism as long as RNA can be administered (Hwang et al., 2013b; Jao et al., 2013). Jao et al. (2013) reported efficient and effective knockdown of multiple genes in one zebrafish embryo further implicating the power of this method. Transmission of induced CRISPR-Cas mutations through the germline is also thought to be probable since the frequencies of site-specific alterations are similar between CRISPRs and TALENs as previously mentioned (Blackburn et al., 2013; Hwang et al., 2013b).

In general, studies that have employed the CRISPR-Cas technique cited that it was a fairly fast process and easy to use because only two plasmids are typically needed: one plasmid encoding the Cas9 sequence and another with the sgRNA containing the sequence of interest (Blackburn et al., 2013; Jao et al., 2013). This latter component of the CRISPR-Cas system provides simplified re-targeting strategies since only a single sequence would need to be replaced (Cho et al., 2013; Mali et al., 2013). Consequently, these attributes are perhaps the most attractive aspects of the CRISPR-Cas system since other methods, such as TALENs, require the construction of two nucleases per target site making it a more costly and time-consuming approach (Cho et al., 2013; Jao et al., 2013; Hwang et al., 2013b). Additionally, shorter sequences of approximately 100 base pairs are needed to encode an sgRNA (Hwang et al., 2013b), which is advantageous over the high degree of repeats present in TALEN vectors (Mak et al., 2012; Deng et al., 2012; Moore et al., 2012), a possible risk factor causing complications in synthesis, vector delivery, and/or DNA sequencing (Hwang et al., 2013b; Cho et al., 2013).

Of interest, Hwang et al. (2013b) indicated that the CRISPR-Cas methodology permitted successful targeting of endogenous genes in the zebrafish that had been unaffected by TALENs, though the reasons behind this discrepancy have yet to be elucidated. One potential explanation for this difference could be the location of the gene on the chromosome. For instance, if a substantial amount of steric hindrance (e.g. epigenetic state, insulators, compact heterochromatin, etc.) were present near the targeted site, it could be less accessible to the long TALEN dimers in comparison to the short, sgRNAs of the CRISPR-Cas system. The lower stringency between the base paring of the sgRNA spacer and target strand (Hwang et al., 2013a; Jao et al., 2013; Jinek et al., 2012; Cong et al., 2013) could provide more

flexibility in these situations as well. Strikingly, it has also been recognized that some sites in the zebrafish genome are inaccessible to CRISPR-Cas induced mutagenization (Hwang et al., 2013b; Jao et al., 2013). In any event, the specific causes of this phenomenon are presently undetermined at this time and warrants future investigation.

Despite the apparent benefits of the CRISPR-Cas system, some constraints are yet to be revealed. One proposed limitation of CRISPR-Cas applications is the requirement of the PAM region for Cas9 function. This stipulation could essentially place restrictions on which endogenous sequences could be targeted and may play a role in why certain sequences are left unmodified by the CRISPR-Cas system, though the reasons behind this event are currently unknown (Hwang et al., 2013b; Gaj et al., 2013). Variable results pertaining to sgRNA toxicity and off-target effects have been documented, which indicate that further evaluation of such incidents is necessary (Blackburn et al., 2013; Cho et al., 2013). However, it has been found that the frequencies of dead or deformed zebrafish embryos were similar between CRISPR-Cas and TALENs, suggesting equal toxicity associated with both techniques (Hwang et al., 2011). A possibility for decreased sgRNA specificity could result from the fact that only 8-14 bases (Jao et al., 2013; Jinek et al., 2012; Cong et al., 2013) of the 20 base pair spacer near the 5' end of PAM is critical for Cas9 directed cleavage, thus increasing the risk of off-target effects (Jao et al., 2013; Cho et al., 2013; Mali et al., 2013). Therefore, whole-genome sequencing could be used to identify any off-target mutagenesis caused by CRISPRs (Cho et al., 2013). Nevertheless, the attributes of the CRISPR-Cas strategy for modifying genomes in a site-specific manner holds great promise for analyzing gene function and determining the epistatic relationships that exist during development.

Upon the advent of the CRISPR-Cas system, assessment of whether or not this powerful technology is amenable for use in the zebrafish has been quite rapid, and as previously alluded, favorable results have been obtained so far (Xiao et al., 2013; Chang et al., 2013; Jao et al., 2013). Several endogenous zebrafish genes, including *fumarate hydratase (fh)*, *glycogen synthase kinase 3 beta (gsk3b)*, and *dopamine receptor D3 (drd3)*, were mutated at relatively high frequencies comparable to that of TALENs (Hwang et al., 2013b). Not only were genes successfully targeted by the CRISPR-Cas system (Hwang et al., 2013a; 2013b; Xiao et al., 2013; Chang et al., 2013), but the transmission of these site-specific mutations through the germline reached astoundingly high frequencies (Hwang et al., 2013a; Jao et al., 2013), one of which was 100% (Hwang et al., 2013a). In this case, two zebrafish were injected with *fh* sgRNA and Cas9 mRNA, and all of their progeny were mutant for *fh*, implicating that they possessed bialleleic gametes (Hwang et al., 2013a). Similarly, mosaic phenotypes were observed in zebrafish injected with CRISPR-Cas constructs targeting pigmentation genes, *tyrosinase (tyr)* and *golden (gol)*. When founders with *gol*-mutations were intercrossed, a majority of their progeny exhibited null-like pigmentation, suggestive of biallelelic alternations in the germline of the founders and confirmed by sequencing of the progeny. Furthermore, when five sgRNAs targeting different genomic loci were co-injected with Cas9 mRNA into a zebrafish, site-specific CRISPR-Cas disruptions were found in all five locations by sequencing, and the mutagenesis efficiencies were similar to those seen in single-knockdowns for the most part. These findings indicate the use of CRISPR-Cas to knockout multiple genes in a single organism to produce distinct phenotypes, a promising finding for applications in epistasis experiments (Jao et al., 2013). In conjunction, these studies revealed that RNA-directed Cas9 cleavage of specific target loci could be achieved at

effective rates in both somatic and germ cells within the zebrafish (Hwang et al., 2013a; 2013b; Jao et al., 2013; Chang et al., 2013).

Significantly, modifications have already been made to the CRISPR-Cas system within the zebrafish. Based on prior adjustments made to the TALENs protocol (Bedell et al., 2012b), co-injections with CRISPR-Cas components and single-stranded oligodeoxynucleotides (ssODNs) lead to precise alterations at the desired genomic loci including single-nucleotide substitutions, though differing targeting efficiencies imply that locus-dependent factors may be at play. However, it was noted that a portion of the ssODN sequence could become integrated at the indel and that the presence of ssODNs could impede the effectiveness of the sgRNAs based on the observation of lower mutation frequencies (Hwang et al., 2013a). Therefore, these aspects should be considered during future applications of ssODNs and the CRISPR-Cas system. Chang et al. (2013) also established that insertions could be made in the zebrafish genome through the CRISPR-Cas technique. This was achieved by introducing an ssDNA with an *mloxP* site in addition to the Cas9 mRNA and sgRNA. Of importance, both the ssDNA and sgRNA contained sequences targeting the *ets variant gene 2 (etv2*, previously named *etsrp),* but no sequence overlap was permitted between these two RNA strands thus preventing inadvertent cleavage of the ssDNA. As a result, after CRISPR-Cas induced cleavage of the *etsrp* site, the *mloxP* site was successfully inserted via HDR. Even so, there are several caveats to this mechanism: after *mloxP* integration (i) some embryos exhibited partial *mloxP* deletions and (ii) additional sequences appeared next to the inserted *mloxP*, a possible artifact of other repair processes. Regardless, the benefits of CRISPR-Cas/HDR mediated insertions cannot be entirely negated since these downsides can easily be remedied through careful screening (Chang et al., 2013). Moreover, the combined implications of CRISPR-Cas/HDR and Cre-lox recombination are immense in terms of targeting specificity and temporal control over gene expression, which are important factors in developmental studies. Alternately, another study demonstrated that the CRISPR-Cas system could potentially be extended to create large deletions in the zebrafish genome to inactivate non-coding genes, gene clusters, and other regulatory sequences. Here, the co-injection of two sgRNAs and the Cas9 mRNA was sufficient to generate a targeted chromosomal deletion corresponding to the location of a miRNA gene cluster (Xiao et al., 2013). While most groups focused on utilizing the CRISPR-Cas system to disrupt protein-coding genes by generating site-specific indels by NHEJ (Blackburn et al., 2013; Hwang et al., 2013a; 2013b), this finding is rather exciting since it shows even broader applications for this revolutionary method *in vivo*.

Although CRISPR-Cas technology has been evaluated to some degree in the zebrafish model, additional characterization of this technique is required before its significance in genetic and developmental studies can be truly appreciated. Nonetheless, preliminary data suggests that the CRISPR-Cas system may indeed transform genetic engineering in the near future and advance the field of nephrology. One obvious benefit of CRISPR-Cas technology is the relative ease of construct design and production of directed gene mutagenesis (Hwang et al., 2013b; Cho et al., 2013; Mali et al., 2013). In conjunction with the short generation time of 3-4 months among zebrafish, the creation of numerous genetic kidney mutants would be fairly rapid and therefore excellent for high-throughput experiments, functional analyses, and gene characterization. The heritability of CRISPR-Cas induced genomic modifications (Hwang et al., 2013a; Jao et al., 2013) is certainly appealing for similar reasons by way of establishing mutant lines displaying specific kidney defects. Multiple, targeted mutations in

the zebrafish by the CRISPR-Cas system (Jao et al., 2013) would also enable further analysis of the epistatic relationships between different genes and molecular pathways which are characteristic of the genetically intricate processes of kidney development. More insight into the roles of genes during nephron patterning could be obtained as well by exploiting the mechanism of HDR and the directed insertion of Cre-lox components into the zebrafish genome once DSBs are induced via the CRISPR-Cas complex (Chang et al., 2013) ultimately providing researchers with an additional means of temporal control. Moreover, under the assumption that little overlap exists between the sequences that cannot be mutated by either CRISPRs or TALENs (Hwang et al., 2013b; Jao et al., 2013), virtually all genes important for kidney development and function should be able to be targeted. In essence, the implementation of CRISPR-Cas for genetic engineering could potentially offer alternate avenues for efficiently studying gene function during zebrafish nephrogenesis.

CONCLUSION

The zebrafish model has come to the forefront of medical and developmental research in recent years (Lieschke & Currie, 2007; Spence et al., 2008). This teleost species is of particular interest throughout the research community because it exhibits a high degree of genetic conservation with higher vertebrates, including humans (Lieschke & Currie, 2007; Howe et al., 2013). Significantly, studies have determined that there are functional zebrafish homologs for an estimated 70% of all genes associated with human diseases (Langheinrich, 2003). As such, the zebrafish has been used to model a variety of disease pathologies including hematological disorders, tumorigenesis, and central nervous system abnormalities (Lieschke & Currie, 2007; Santoriello & Zon, 2012). Numerous developmental processes, such as organogenesis and neurogenesis, have also been evaluated within the zebrafish (Wingert et al., 2007; Barrallo-Cimeno et al., 2003). Furthermore, novel biomedical research using zebrafish is enabled by the relative ease through which tools such as transgenic lines can be generated, in combination with the ability to perform classical and chemical genetic screens. The zebrafish is indeed a powerful model organism for a number of reasons and will continue to fuel the acquisition of exciting, novel discoveries in the near future.

As demonstrated, analogous segmental organization exists between the mammalian and zebrafish nephrons, which are the basic functional units of the kidney (Wingert et al., 2007). Research using zebrafish has provided several new insights into the complex pathways that direct nephron segmentation during renal development. The initial induction of RA permits the proximodistal regulation of nephron patterning, and this process is further refined along the A-P axis by the *cdx* genes (Wingert et al., 2007; Wingert & Davidson, 2011). Dynamic gene expression among the renal progenitors from early embryogenesis to somitogenesis also contributes to the formation of distinct segment boundaries as well as the differentiation of specific cell types within those regions (Wingert & Davidson, 2011). While substantial advances have been made to elucidate the genetic intricacies that underlie kidney organogenesis, such as the identification of the essential roles performed by the *hnf1ba/b* and *irx3b* transcription factors, the function of many genes within the kidney remain to be determined. The development of novel molecular technologies (e.g. TALENs and CRISPRs)

amenable to the zebrafish system can lead to the generation of new nephrology tools, thus enabling the practicality of future substantive kidney research using zebrafish.

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