Mechanisms of Nephrogenesis Revealed by Zebrafish Chemical Screen: Prostaglandin Signaling Modulates Nephron Progenitor Fate

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Abstract

Nephron development involves the creation of discrete segment populations that are specialized to fulfill unique physiological roles. As such, renal function is reliant on the proper execution of segment patterning programs. Despite the central importance of nephron segmentation, the genetic mechanisms that regulate this process are far from understood, in large part due to the experimental complexities and cost of interrogating these events in the mammalian metanephros. For this reason, forward genetics utilizing phenotypic screening in the zebrafish pronephros provides an avenue to gain novel insights about the mechanisms of nephron segmentation in the vertebrate kidney. Discoveries from zebrafish can highlight possible conserved pathways and provide a useful starting point for reverse genetic analyses with other animal models or in vitro approaches. In this

review, we discuss the results of a novel chemical screen using the zebrafish to identify segmentation regulators. Through this screen, we identified for the first time that prostaglandin signaling can modulate nephron segmentation, and that it is normally requisite during development to mitigate segment fate choice in the embryonic kidney. We briefly discuss how these discoveries relate to current knowledge about nephron segmentation. Finally, we explore the possible implications of these findings for understanding renal ontogeny and disease, and how this knowledge may be useful for ongoing research initiatives that are aimed at deciphering how to build or rebuild the human kidney.

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Introduction

Nephrons possess an intricate cellular composition, where unique differentiated populations occupy regionally distinct domains, known as segments. The precise order and arrangement of segments within the nephron

is essential to kidney function [1]. Nephron segment composition is broadly conserved across vertebrate species, including fish, amphibians, reptiles, birds, and mammals [1]. In general, nephron anatomy consists of a blood filter, followed by a tubule that is comprised of a series of proximal, intermediate, and distal segments, where the last region is connected with the collecting duct system [1, 2]. Differences in nephron segment composition reflect the variations between organismal physiology and habitat [1, 2]. For example, avian and mammalian nephrons have a modified intermediate region, where the loop of Henle enables urine to be concentrated, and the length of this region varies dramatically among nephrons within each species, as well as between species [1, 2].

There have been continued advances in our knowledge about nephrogenesis in recent years due to complementary experimental studies in the zebrafish, frog, chick, and murine animal models, among others [2–4]. However, there is still much to learn about several aspects including segmentation, a term which refers to how nephrons are patterned along the proximo-distal axis, and how each specialized segment cell type is formed [2–4]. A deeper appreciation of the genetic regulation of nephron development is highly relevant to the origins and mechanisms underlying congenital abnormalities of the kidney [5]. Additionally, a greater command of nephrogenesis principles can be utilized to advance renal organoid research, where a current major challenge is the *in vivo* cultivation of functionally differentiated segment populations [6]. In this review, we discuss how our laboratory has leveraged the attributes of the zebrafish embryonic nephrons to perform a novel chemical screen that enabled the identification of regulators that affect nephron segment development and highlighted for the first time that prostaglandin signaling has essential roles during nephrogenesis in this vertebrate species.

Mechanisms of Nephron Segmentation: Progress and Ongoing Challenges

The process by which vertebrate nephron progenitors are fashioned into different segment populations has been the subject of growing research attention and has been reviewed in detail elsewhere [2]. Most recently, the molecular network for nephron segment patterning in the mammalian metanephros was shown to rely on a gradient of β -catenin activity that interacts with the BMP

pathway and Notch signaling [7], and levels of β -catenin also affect whether nephron progenitors differentiate or self-renew [8]. The interplay of these signaling pathways is thought to specify segment fates by controlling the expression of transcription factors, including *Hnf1b*, *Irx1/2*, and *Pou3f3*, for example [9, 10]. Conservation of these mechanisms during nephron segment development has been suggested by comparative studies in *Xenopus* [9] and the zebrafish [11]. However, an understanding of the complete program of segmentation is far from complete. Knowledge gaps persist regarding the identity of the essential genes that pattern segment cell types or control their differentiation. Further, the mechanisms that govern boundary establishment between adjacent segment domains are not understood.

In part, these knowledge gaps are being addressed through efforts to fully characterize the transcriptome of every nephron cell. Recent technological advances in RNA sequencing have enabled detailed characterization of nephron cell-type expression signatures from adult and embryonic kidneys of both the mouse and human [12, 13]. These exciting datasets proffer to reveal the inventory of genes that are requisite for developmental processes such as segmentation during nephrogenesis [12, 13]. Nevertheless, using these lists to further assemble the blueprint of instructions that build the nephron remains a long-range goal [12, 13]. A key impediment to reaching this goal remains how to prioritize the selection of genes for loss and gain of function analyses. Once selected, researchers must complete the functional assessments of the individual genes, which involve considerable time and cost when conducted in mammalian models. In most cases, this is also likely to require strategies to conditionally abrogate gene function within the nephron and/or subsets of the nephron populace. The reason for such measures is that many renal factors are expressed at multiple stages of kidney formation and/or in other embryonic tissues, which precludes analysis of their contributions to nephrogenesis through standard knockout strategies. Further study of the genetic pathways will also involve design of approaches to facilitate the intricate work of delineating the relationships and connections between genes to assemble the regulatory networks that accomplish segment specification and differentiation. The continued implementation of cross-species analyses with other vertebrate models offers many valuable opportunities to conduct rapid and low-cost assessments of genes in these mammalian nephron transcriptome repositories. Such strategies can help to uncover functionally important molecular components, and thereby triage candidate

genes of highest interest for further genetic studies in mammals.

Additionally, unbiased screening for nephrogenesis regulators through classical forward genetics and chemical genetics approaches affords the advantage of identifying important transcription factors and signaling pathways based solely on phenotypic outcomes. These forward approaches can identify novel components not evident from transcriptome data, and when convergent with such data can highlight the most likely candidates for the essential genetic components from among the dispensable. In particular, the zebrafish model is a powerful tool for unbiased phenotype screens to delineate the genetic pathways that control nephron development [14]. Zebrafish are vertebrates and possess high genetic similarity with humans [15]. Approximately 71% of human genes have a zebrafish orthologue [15]. Due to the teleost specific genome duplication, there are about 2 zebrafish genes for each human gene [15]. Therefore, study of human genes in the zebrafish must often consider multiple zebrafish orthologues, to determine whether redundant functions exist or subfunctionalization has occurred [15]. Despite these factors, several features of zebrafish embryogenesis facilitate their tractability for ontogeny research, including external fertilization, transparency during early development, and rapid organogenesis as most organs are formed over the first 24–72 hours post fertilization (hpf) [16]. These traits, along with fecundity and small adult size, support genetic studies because large numbers of animals can be generated and maintained with low costs and minimal husbandry space [16].

As such, the application of zebrafish for renal development [17–19] and disease studies [20–22] has grown tremendously in recent years. With respect to the kidney organ, zebrafish embryos develop an anatomically simple pronephros comprised of just 2 nephrons by 24 hpf [23]. By this time point, the nephrons are fully segmented and contain a suite of cell types that are conserved with humans, including a series of proximal and distal tubule segments that display analogous expression profiles of the solute transporters that dictate the explicit physiological roles of each populace [24–26]. Zebrafish nephron segment composition is not identical to mammals, however, with notable differences in that they lack intermediate tubule segments that enable water conservation – an unnecessary task for a freshwater aquatic species [24, 25]. Nevertheless, the zebrafish model provides an opportunity to delineate the genetic mechanisms of glomerular ontogeny, as well as the processes that direct emergence of several tubule segments [24–27].

Chemical Genetic Screen Links Prostaglandin Levels to Nephron Segment Patterning

To expand our knowledge about the signaling pathways that affect nephron segmentation, we hypothesized that a chemical screen could help to identify nephrogenesis regulators [28, 29]. We chose to implement a strategy utilizing a library of known bioactive molecules, the Screen-Well ICCB Known Bioactives Library (Enzo Life Sciences), which consists of nearly 500 compounds. The small molecules in the library include agents capable of affecting numerous cellular signaling pathways, and span such classes of compounds as kinase inhibitors, nuclear receptor ligands, protease inhibitors, and second messenger modulators [28]. The benefit of working with a known bioactive collection is that many or all aspects of the mechanism(s) of action for each compound have already been identified [9, 28]. This enables the researcher to bypass many challenges in delineating the molecular target(s) of the compound and quickly determine how they can further study the “hits” isolated from the screen approach [9, 28]. Therefore, our goal was to accomplish a comprehensive screen for agents that had the ability to alter nephron segmentation *in vivo*, which had the possibility of revealing essential molecular components of the normal segment development processes.

For the screen, we arrayed fertilized embryos into 96-well plates and incubated them with either vehicle control or an experimental drug treatment from the library between the 4 and 24 hpf time point [24] (Fig. 1a). The reasoning for this exposure window was that it overlapped with the entire period of time from when mesenchymal renal progenitors initially arise through the duration of their patterning into a pronephros organ comprised of 8 epithelial segment populations [24, 30, 31]. Embryo samples were then fixed for cell-type analysis, where whole mount *in situ* hybridization (WISH) was used to assess cell identities based on their expression signature of segment-specific transcripts. To gather information about multiple segments from each treatment, we used a multiplex WISH assay in which a cocktail of 3 cell-specific riboprobes was applied to detect 3 corresponding segment populations. The segments we screened were the podocytes, which contribute to the blood filter, the proximal convoluted tubule (PCT, the first of 2 proximal segments in the pronephros), and the distal early (DE, the first of 2 distal segments in the pronephros). Further, we selected these cell types for the multiplex WISH assay because they

Fig. 1. Chemical screen in zebrafish embryos identified prostaglandin signaling as a modulator of distal segment fates in the developing pronephros tubule. **a** The screening strategy involved the physical array of small groups of WT zebrafish embryos into 96-well plates, after which individual drugs from the Screen Well ICCB Chemical Library were arrayed for experimental treatment during a time window relevant to pronephros genesis. **b** Schematic depicts how exposure to prostaglandin antagonists (e.g., inhibitors of ligand synthesis or receptor activity) led to changes in the expression domains of several essential transcription factors in the developing nephron progenitors compared to WT embryos, specifically *sim1a* (purple), *irx3b* (green), and *mecom* (red) at the 20 somite (ss) of development. **c** **i** Nephrons within the zebrafish pronephros

organ have 2 distinct distal segments, the DE and DL marked by expression of the solute transporters encoded by *slc12a1* and *slc12a3*, respectively. The confocal image shows a merged view of a fluorescent in situ hybridization experiment in which riboprobes were used to detect *slc12a1* (green), *slc12a3* (red), and DAPI staining (blue) to mark nuclei. Embryo illustration depicts the 28 somite stage of development, which is approximately 24 hpf. Scale bar = 20 μ m. **ii** Prostaglandin signaling mitigates the balance between DE vs. DL segment fate choice in the zebrafish pronephros. In the absence of PG signaling, more nephron progenitors adopt a DE fate and fewer adopt the DL fate. WISH, whole mount in situ hybridization; WT, wild-type; DE, distal early; DL, distal late; ss, somite stage.

comprise alternating nephron segments, which would facilitate clear evaluation of the 3 compartments and also allow for some indirect assessment of the intervening populations based on the physical appearance (size, distribution) of the populations that were being directly assessed.

From this approach, we scored approximately 16% of library compounds as “hits” associated with causing nephron phenotypes [24]. Each compound was recorded as to whether it had no effect at the dosage tested (e.g., wild-type) or was capable of promoting (+) or inhibiting (–) each discrete lineage (P+, PCT+, DE+) or (P–, PCT–, DE–). The high percentage of flagged hits was expected given the multiplex nature of the assay that we employed, which was effectually 3 screens bundled together.

Of the hits obtained, there were numerous chemicals that corresponded to agonists or antagonists of the retinoic acid (RA) signaling pathway [29], which is an important regulator of development [32]. This result was especially encouraging to us, as it was a positive control from the vantage point that RA has been well established as an essential component of proximo-distal nephron segment patterning in the zebrafish [17, 24–26, 33–38]. RA is a vitamin A derivative that is processed into a secreted signaling molecule and binds to the RA receptors and retinoid X receptors, which are nuclear hormone receptors that effect transcriptional changes in response to interacting with an RA ligand [32]. Previous studies had demonstrated that elevating exogenous RA levels by incubating zebrafish with all-trans RA-induced proximal segments and inhibited distal fates, while inhibition of RA biosynthesis with antagonists or genetic mutations that disrupted RA production caused opposite effects [24]. RA-dependent segment changes were subsequently linked to alterations in the spatial domains of transcription factors that direct segment fates [33–35, 37, 38]. These include *MDS1* and *EVII complex locus (meacom)*, which is essential for the distal late (DL) fate [33], and *SIM bHLH transcription factor 1a (sim1a)*, formerly known as *single minded 1a*, which is essential for proximal straight segment (PST) fate [34]. From our segment screen, an example RA hit was the compound 4-hydroxyphenylretinamide, which is a synthetic analog of all-trans RA that expanded the proximal segments [29].

Upon surveying the other screen hits, we were fascinated to discover that agonists of the prostaglandin signaling pathway had scored as nephron segment modulators [29]. Prostaglandin signaling has important functions in a staggeringly broad range of normal biological

contexts and disease states, in part due to the number and biochemical diversity of ligands and receptors [39, 40]. Prostaglandins are lipid mediators derived from arachidonic acid that can act in an autocrine or paracrine fashion through G protein-coupled receptors to influence cellular activities like gene transcription [39]. The net effects of prostaglandin signaling include the control of physiological processes such as inflammation, pain perception, vasoregulation, and complex renal functions such as blood pressure control and fluid retention [39, 40]. In addition to these myriad roles, several recent studies have also uncovered important tasks for prostaglandin signaling in development. These advances were made possible using the zebrafish as they develop ex utero and had been masked in prior studies with mammalian models due to maternal contributions of prostaglandins to the fetus [39, 40]. Developmental roles of prostaglandins are now known to include cell motility during gastrulation, angiogenesis, hematopoietic stem cell development, and binary fate choice between pancreas versus liver development [39–42]. Especially in light of its function in endoderm lineage decisions, we wondered if prostaglandin signaling similarly had a bona fide involvement in the cell fate choices of renal progenitors during nephrogenesis.

The prostaglandin moieties that had scored as hits in the screen corresponded to the ligands PGD₂, PGA₁, PGJ₂, and PGB₂ [29]. In each case, incubation with the prostaglandin during renal progenitor development led to a smaller PCT and a DE segment that was shifted caudally along the embryonic trunk, suggesting that a large intervening domain had formed—mostly likely corresponding to a larger PST. Because prostaglandins are notorious for possessing a short half-life, we sought to validate these screen hits by treating embryos with a long-acting derivative, 16,16-dimethyl-PGE₂ (dmPGE₂) followed by WISH to assess the effects on each specific nephron segment. Interestingly, we found that the PST was indeed significantly expanded with exogenous dmPGE₂, and that the DL segment, which is located caudal to the DE, was significantly reduced. These phenotypes occurred in the absence of other tissue defects, suggesting that renal development had been specifically affected. Further, we observed dosage-dependent effects of dmPGE₂, as well as PGB₂, on PST and DL segment size. These findings indicated to us that renal progenitors were exquisitely sensitive to alterations in prostaglandin levels in the embryo during nephrogenesis.

Prostaglandin Signaling Mediates Fate Choice between Distal Segment Lineages

Next, to explore whether prostaglandin signaling was actually a required component of normal nephron segmentation events, we performed loss of function studies [29]. Using parallel approaches of employing pharmacological inhibitors or gene knockdowns using morpholinos, we found that abrogation of the prostaglandin production machinery, in this case cyclooxygenase enzymes Cox-1 and Cox-2 (also known as prostaglandin-endoperoxide synthase 1 and 2a; Ptgs1, Ptgs2a, respectively), was sufficient to alter distal nephron segmentation. With disruption of Cox-1, Cox-2, or both enzymes, the DE segment was significantly expanded and the DL was significantly reduced. Further, we found that all of these disruptions could be rescued specifically by provision of dmPGE₂. This indicated that PGE₂ is likely the prostanoid that is required for pronephros development in the zebrafish.

Subsequently, we explored whether PGE₂-mediated prostaglandin signaling was required in renal progenitors by assessing the spatial distribution of the known PGE₂ G-protein-coupled receptors, prostaglandin E receptor 2a or 4a (Ptger2a, Ptger4a; also known as EP2 and EP4, respectively) [29]. We found that *ptger2a* and *ptger4a* transcripts were expressed in renal progenitors during somitogenesis stages, prior to the time when nephron segment pattern is established. These results were consistent with the hypothesis that Ptger2a and/or Ptger4a work in renal progenitors to affect their development.

To test this further, we abrogated each of these PGE₂ receptors through parallel strategies involving genetic knockdown or pharmacological inhibition in zebrafish embryos and then assessed nephron segmentation by WISH. Similar to the Cox-1/Cox-2 studies, loss of Ptger2a or Ptger4a led to an expanded DE segment and reduced DL segment. These results further strengthened our interpretation that prostaglandin signaling was affecting the balance of the DE and DL segment fates during nephron development. To identify the developmental window when prostaglandin signaling affected segment fates, we performed loss or gain of function experiments with dmPGE₂ and indomethacin, a nonselective Cox enzyme inhibitor, at different times of addition which enabled us to narrowly define the time of signaling [29]. Consequently, this led us to question how renal progenitors were altered during this developmental window that could lead to the DE/DL alterations.

We explored the expression patterns of several essential transcription factors and discovered that their domains in the renal progenitor field were dramatically altered in response to changes in prostaglandin levels during this critical developmental window (Fig. 1b). For example, in prostaglandin-deficient embryos, the expression domain of Iroquois transcription factor *irx3b* was significantly expanded. As *irx3b* is required for DE fate [26], this result suggested that prostaglandin signaling regulates the *irx3b* domain to control DE fate choice. Subsequently, because Cox enzyme inhibition was unable to promote DE fate in *irx3b*-deficient embryos, we were able to surmise that prostaglandin signaling works upstream of Irx3b to regulate the DE segment lineage.

Additionally, we found that the expression domain of *mecom* was reduced in prostaglandin-deficient embryos (Fig. 1b). Given that *mecom* is requisite for DL fate [33], this suggested to us that prostaglandin signaling promotes *mecom* expression within renal progenitors. However, we were unable to assess this relationship with additional studies because overexpression of *mecom* is toxic to embryogenesis [28] and would require engineering of a transgenic system to exact control over the spatiotemporal domain of *mecom* in renal progenitors.

Finally, in gain of function studies with dmPGE₂ application, we discovered that the domain of the essential PST factor *sim1a* [34] was significantly expanded. In contrast, *sim1a* expression was unchanged in prostaglandin-deficient embryos (Fig. 1b). This leads to effects on DE vs. DL fate (Fig. 1c). Taken together, this suggested the hypothesis that prostaglandin mediated promotion of *sim1a* underlies the expansion of the PST lineage when prostaglandin levels are elevated. In accordance with this, knockdown of *sim1a* was sufficient to abrogate PST expansion in dmPGE₂-treated embryos.

Mechanisms of Prostaglandin Signaling and Relation to Other Segmentation Pathways

Our studies of renal progenitor development revealed that alterations in prostaglandin levels had potent effects on the regional gene expression domains of critical factors that mediate segment identities in the zebrafish pronephros. This suggests that prostaglandin signaling leads to patterning changes that specify the fate of the distal segment populations. However, more analyses are needed to ascertain whether these alterations involve cell proliferation, apoptosis, or changes in migration of the nephron progenitors. Furthermore, while our experi-

ments have delineated that the transcription factors *Irx3b* and *Mecom* are key targets of normal prostaglandin signaling during zebrafish embryonic kidney development, and that elevating prostaglandin levels via exogenous PGE_2 is sufficient to affect *Sim1a* expression in renal progenitors, we hypothesize that other genes are affected as well.

Continued efforts to determine the process of pronephros segment development using the zebrafish model can help to discover conserved pathways and mechanisms of segmentation. Toward this end, continued work is needed to uncover whether PGE_2 -mediated signaling has direct or indirect effects on the expression of *irx3b*, *mecom*, and *sim1a* and other targets. For example, recent work has highlighted function of reciprocal antagonism between *Sim1a* and *Ppargc1a* that regulates PST segment size in the pronephros [43] and that *Irx2* is a requisite for proper PST and DL segment development [44]. With regard to the DL segment, the T-box transcription factors *tbx2a* and *tbx2b* are essential to regulate DL fate [37]. Further, *tbx2b* acts downstream of *mecom*, and in turn the homeogene *emx1* acts downstream of *tbx2b* to promote DL fate [45]. Interestingly, however, only *emx1*-deficient embryos (not *tbx2a/b* or *mecom*) develop an expanded DE and reduced DL segment phenotype, which fully mimics the loss of prostaglandin signaling observed in the context of Cox enzyme or *Ptger2a/4a* deficiency [45]. Additionally, our recent research has revealed a cascade of Iroquois factors that interact with the transcription factor *AP-2 alpha* to coordinate a gene network that controls DE differentiation [46]. Interestingly, prostaglandin signaling also affects the fate choice between a transporter cell or multiciliated cell identify within the nephron [47]. Genetic studies that delineate the relationships between all of these nephron lineage networks will continue to grow our understanding of how nephron segmentation is controlled in the zebrafish, which provides inroads for parallel investigations in other vertebrates.

The larger question is whether prostaglandin signaling is a conserved mechanism that dictates nephron segmentation during renal development in mammals, and in particular whether it is relevant to human ontogeny or kidney disease. Whether prostaglandins have roles in mammalian development has not been ascertained in murine knockout studies to date because of maternal contributions – which has made the roles of this pathway historically underappreciated [39, 40]. An intriguing place to test whether elimination of prostanoid production or receptor function has any effects on nephron segmentation

would be in vitro systems such as organoid culture. Related to this topic, another aspect of interest is exploring how other mechanisms of nephron segmentation in the zebrafish, frog, and chick relate to mammals. For example, the *Brn1* transcription factor has been linked for many years now to the establishment of distal segments in the developing murine metanephric kidney [48]. Conservation between mouse and zebrafish nephrogenesis has been suggested by the report that the zebrafish have 2 orthologues of *Brn1*, *pou3f3a* and *pou3f3b*, which are regionally expressed in the renal progenitors that give rise to the pronephros distal segments [26]. Future work to follow-up on such leads will cast further insight on the degree of conserved or divergent mechanisms by which vertebrate kidneys generate functional nephron segments.

Summary

There is an urgent need to identify innovative ways to repair, regenerate, and replace kidney functions for patients with congenital and acquired renal diseases. Knowledge about the mechanisms of nephron development can provide crucial insights for identifying new therapies for pathological conditions. Toward this end, information both about which molecules are sufficient to modulate nephrogenesis and how developmental programs normally control the process of nephron segmentation has many useful applications, such as enhancing the production of more biologically faithful renal organoids.

Our chemical screen for nephrogenesis regulators has led to the discovery that the prostaglandin signaling is normally required during nephrogenesis in the zebrafish embryonic kidney to dictate the balance between distal segment fates. Interestingly, alterations in COX-2 gene dosage in the mouse cause kidney development defects including hypoplastic glomeruli and cortical thinning, though nephron segment ontogeny has yet to be assessed [49]. Only continued research on this topic will reveal whether prostaglandin signaling has conserved roles in nephron segmentation, and/or whether prostaglandin targets are conserved across any other vertebrates. Nevertheless, it is our hope that the research community can put to good use the information from our zebrafish screen, available within the publicly accessible database of zebrafish kidney chemical screen hits [29], to hunt for other key nephrogenesis mechanisms, or even to identify relevant pharmaceuticals for clinical applications.

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Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

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Author Contributions

B.E.C. and R.A.W. wrote and revised the article. B.E.C. created the original illustrations and performed the fluorescent in situ hybridization and confocal photography of distal segments in the zebrafish pronephros.

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