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The zebrafish pronephros: A model to study nephron segmentation

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Nephrons possess a segmental organization where each segment is specialized for the secretion and reabsorption of particular solutes. The developmental control of nephron segment patterning remains one of the enigmas within the field of renal biology. Achieving an understanding of the mechanisms that direct nephron segmentation has the potential to shed light on the causes of kidney birth defects and renal diseases in humans. Researchers studying embryonic kidney development in zebrafish and *Xenopus* have recently demonstrated that the pronephric nephrons in these vertebrates are segmented in a similar fashion as their mammalian counterparts. Further, it has been shown that retinoic acid signaling establishes proximodistal segment identities in the zebrafish pronephros by modulating the expression of renal transcription factors and components of signaling pathways that are known to direct segment fates during mammalian nephrogenesis. These findings present the zebrafish model as an excellent genetic system in which to interrogate the conserved developmental pathways that control nephron segmentation in both lower vertebrates and mammals.

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The kidney functions to remove nitrogenous waste from the body as well as to regulate fluid balance, osmolarity, and pH. To accomplish these varied tasks, the individual nephrons are comprised of specialized segments that perform a unique combination of solute transport functions.[1–3] Until recently, there has been only a poor understanding of the developmental mechanisms that govern how nephrons are patterned into discrete segments.[4,5] New studies in zebrafish, as well as frogs, have discovered that the nephrons of the embryonic (pronephric) kidneys have a segmental organization similar to that found in adult (metanephric) mammalian kidneys, thus uncovering conservation between nephrons from different kidney types.[6–18] These findings raise the exciting prospect of utilizing the benefits of lower vertebrate models to dissect the developmental pathways controlling nephron segmentation. This review will focus on the segment pattern of the zebrafish pronephros, how it compares to frogs and mammals, and how segmental identity is established during zebrafish nephron development.

OVERVIEW OF KIDNEY ONTOGENY AND THE USE OF THE PRONEPHROS TO STUDY NEPHROGENESIS

During vertebrate development, a series of three kidney structures arises sequentially from the intermediate mesoderm (IM): the pronephros, the mesonephros, and the metanephros.[4] Each kidney is made up of nephrons that share a fundamental composition of three parts: a glomerulus for blood filtration, a tubule that reabsorbs and secretes solutes, and a collecting duct (CD) that transports the modified filtrate to a waste disposal site. In mammals and other amniotes, the pronephros and mesonephros exist as transient structures, and they are induced following the formation and posterior migration of the nephric duct.[4] A series of nephrons are derived from mesenchymal cells located next to the duct, with the anterior-most nephrons referred to as pronephros and the more posterior nephrons making up the mesonephros.[4] Whereas the pronephros is a vestigial organ, the mesonephros functions during fetal life; however, both of these structures will later degenerate or, in the case of males, become part of the reproductive system.[4] Meanwhile, the metanephros forms at the caudal end of the nephric duct from an outgrowth, known as the ureteric bud, which interacts with the adjacent mesenchyme and

undergoes an elaborate process of branching morphogenesis.[4] Ureteric bud branching generates an arborized network of CDs. Within the mesenchyme, aggregations of some cells undergo an epithelial conversion to form circular renal vesicles that develop into nephrons, and the remaining mesenchymal cells constitute a stromal population that plays essential signaling roles in regulating ureteric bud branching. Subsequent proliferation and elongation of the renal vesicle creates an 'S'-shaped tubular body that will be patterned along its proximodistal axis to generate the podocytes that contribute to the glomerulus, followed by a tubular epithelium made up of multiple specialized segments. These segments include the neck, proximal convoluted and straight tubules (PCT and PST), descending and ascending thin limb segments of the loop of Henle (also known as the intermediate tubule), distal straight (or thick ascending limb), distal convoluted tubule, and the connecting tubule, which joins to a CD[1–3] (Figure 1a). The epithelial cell populations that make up each tubule segment possess a distinctive ultrastructure and gene expression signature and perform discrete roles in modifying the glomerular filtrate.[1–3] Metanephros development thus produces an intricate kidney structure that will contain on the order of a million nephrons and serves as the functioning adult kidney. In lower vertebrates, such as fish and amphibians, the pronephric kidney is functional and comprises a pair of bilateral nephrons that work throughout embryonic and larval (juvenile) stages.[19–22] A mesonephros develops later, during larval life, as a result of additional nephrons being induced from the surrounding mesenchyme, and functions as the adult kidney.

Despite the fact that vertebrates utilize kidney types of differing organization and complexity, the genes that pattern these organs are highly conserved.[4] One explanation for this commonality may lie in the fact that their renal structures are composed of a common building block: the nephron. For example, studies of pronephros and metanephros development in various vertebrate models have demonstrated that the transcription factors Wt1 and Pax2 are functionally important for the differentiation of renal progenitors in both kidney types.[4] As such, the study of pronephros development has been a fruitful avenue to identify and investigate factors that are essential for metanephros development.[4,19–22] Amphibian pronephric models, in particular, have provided a system to test gene function using micromanipulation and grafting experiments and assay inductive factors using animal cap explants.[20,22]

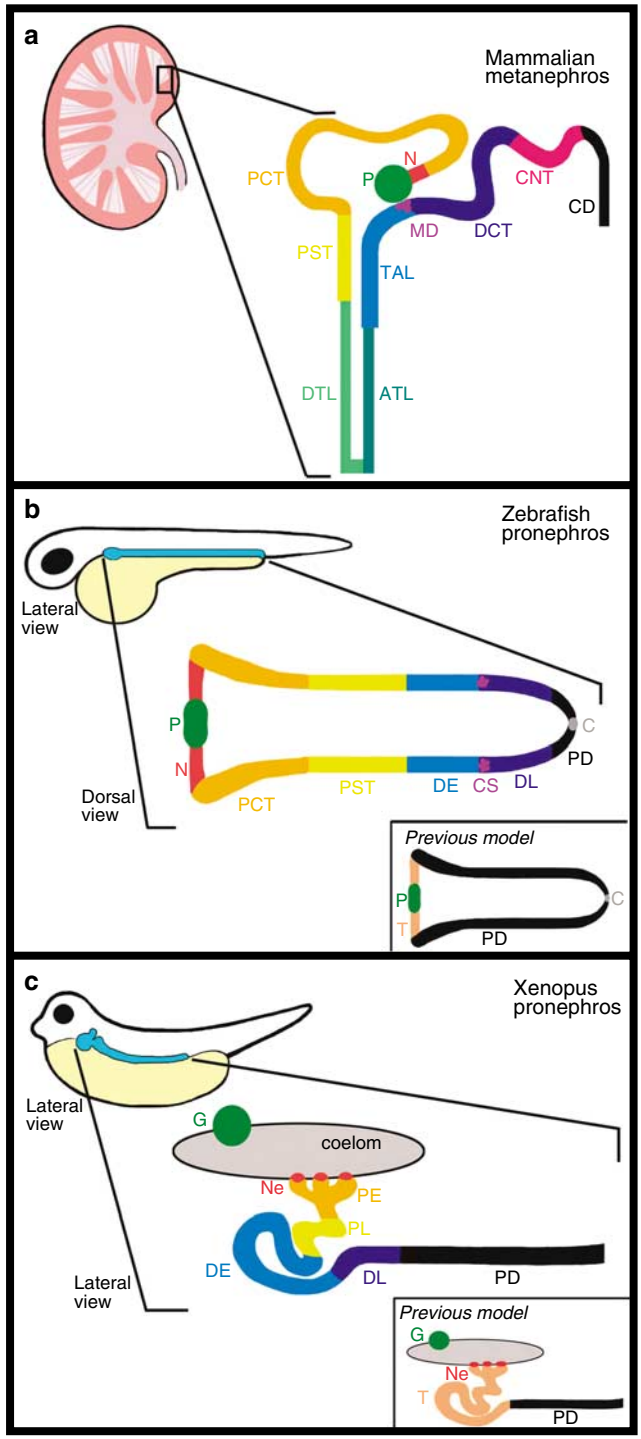

Figure 1 | Metanephric and pronephric nephrons share a similar segmentation pattern. (**a**) Mammalian metanephric kidney, with enlargement depicting the segmental organization of a single nephron. (**b**) Zebrafish embryo, shown in a lateral view, with the pronephros depicted in blue. Enlargement shows a dorsal view of the pronephros, with the segmental organization of each nephron as indicated. Inset shows the previous model of nephron organization. (**c**) *Xenopus* embryo, shown in a lateral view, with pronephros depicted in blue. Enlargement shows a lateral view of only one nephron. Inset shows the previous model of nephron organization. For all nephrons, color-coding of analogous segment identities is based on comparison of gene expressions[6–18] as depicted in Figure 2. Abbreviations: ATL, ascending thin limb; C, cloaca; CD, collecting duct; CNT, connecting tubule; CS, corpuscle of Stannius; DCT, distal convoluted tubule; DE, distal early; DL, distal late; DTL, descending thin limb; G, glomus; MD, macula densa; N, neck; Ne, nephrostome; P, podocytes of renal corpuscle; PCT, proximal convoluted tubule; PD, pronephric duct; PE, proximal early; PL, proximal late; PST, proximal straight tubule; T, tubule; TAL, thick ascending limb.

In the last decade, the zebrafish model has become a powerful developmental and genetic model. Pioneering work from several research groups has established the zebrafish pronephros as a useful system to study renal development.[19,23] Like other fish and amphibians, the zebrafish embryo forms a simple pronephros that is comprised of two nephrons that derive from bilateral stripes of IM.[19,24] The anterior-most cells give rise to podocytes that migrate to the midline and recruit endothelial cells to form a single vascularized glomerulus that the two nephrons share.[19,24–27] The remaining IM undergoes a mesenchymal-to-epithelial transition to form a tubular epithelium that fuses at the cloaca, a common exit portal for waste products from the gut and pronephros.[18,23,24] Analysis of several differentiated cell types in the zebrafish pronephros has found similarities with those in the mammalian metanephros. The endothelial cells of the vascular supply are fenestrated, and the podocytes possess extensive interdigitating foot processes.[19,24] At a molecular level, the podocytes express hallmark components of the slit diaphragm, including proteins such as nephrin and podocin.[28] These similarities to mammalian nephrons make the zebrafish pronephros a relevant and convenient tool for renal physiology studies.[6,29] In addition, defects in genes associated with polycystic kidney diseases in humans lead to pronephros cyst formation in zebrafish, highlighting the usefulness of fish to study this type of disorder.[23,30–34]

PROXIMODISTAL SEGMENTATION OF ZEBRAFISH PRONEPHRIC NEPHRONS

Until recently, it was thought that the one major drawback of the zebrafish pronephros as a model for studying nephron formation was the simple organization of the tubule itself. There was initially no evidence that the pronephric tubule was specialized into the prototypical series of proximal and distal segments that exist in metanephric nephrons. Indeed, only a short stretch of tubule was thought to exist in the pronephros, connecting the glomerulus to a long pronephric duct (see inset, Figure 1b). In the last few years, however, several reports suggested the existence of subdomains within the zebrafish pronephric duct.[6–9] For example, the expression domains of the solute transporter *slc4a4* and the endocytic receptor *megalin/lrp2* were found to be restricted within the anterior-most portion of the pronephric duct.[6–9] However, both of these genes mark specific proximal tubule segments in metanephric nephrons.[35,36] Thus, these results challenged the notion that the region of the zebrafish pronephros historically regarded as duct might actually be tubule-like in nature.

To investigate this possibility, our laboratory used a functional genomics-based strategy to isolate markers of differentiated renal cell types in zebrafish. From this approach, a number of genes were identified whose expression in the pronephros was restricted to one or more particular subdomains.[10] In total, the zebrafish pronephros was found to contain at least eight discrete regions[10] (Figure 1b). The expression profile of these regions likens them to many of the segments that exist in metanephric nephrons[10] (Figure 2). Based on this comparison, we redefined the organization of the pronephros. What was previously considered the tubule portion of the pronephros was reclassified as a neck segment, whereas the long stretch of tubular epithelium that has traditionally been considered duct was subdivided into two proximal tubule segments, two distal tubule segments, and a short duct.[10]

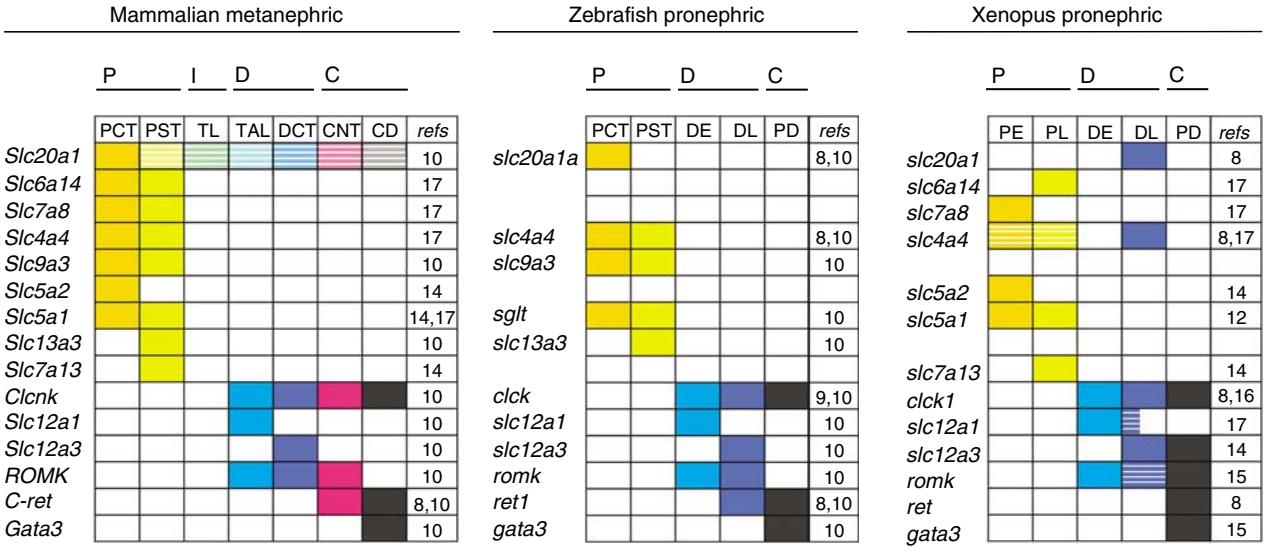

Figure 2 | Molecular segment anatomy in pronephric tubules of zebrafish and *Xenopus* compared to the metanephric tubule of mammals. The expression domain of numerous solute transporters as well as renal transcription factors are highly conserved between mammalian metanephric nephrons and the pronephric nephrons of fish and frogs. Solid color bars indicate strong expression and striped color bars indicate weak expression, as reported.[6–18] See text or Figure 1 for segment abbreviations in each species. Abbreviations: C, collecting system; D, distal; I, intermediate; P, proximal.

The zebrafish neck segment expresses *rfx2*, an established marker of ciliated cells, suggesting that it is ciliated similar to the neck segment found in a number of mammals, including humans.[1,10] The PCT and PST express genes found throughout the proximal tubule of mammals, like *slc9a3*.[10] The PCT segment undergoes morphogenesis beginning at 2 dpf to form a coiled loop on each side of the glomerulus, whereas the PST segment remains linear, morphologies similar to their mammalian counterparts. Transcripts for megalin are confined to the PCT domain in zebrafish (RAW and AJD, unpublished data), where megalin has been shown to perform metabolite recovery via endocytosis,[6] analogous to its role in the mammalian PCT. The zebrafish PST segment expresses the mammalian PST marker *slc13a3* in a discontinuous pattern that reflects the presence of muliticiliated and transporting cells.[10,37,38] Interestingly, immunostaining with the *Xenopus* 3G8 antibody, which detects the brush border, appears to label the region encompassing both proximal segments,[24] suggesting that they possess the apical microvilli that are a structural characteristic of the mammalian proximal tubule.[1] Following the proximal segments in the zebrafish is a distal region, marked by expression of *clck*, which includes the distal early (DE), distal late (DL), and pronephric duct (PD) segments. The DE exclusively expresses *slc12a1*, which specifically marks the thick ascending limb segment in mammals, whereas the DL expresses *slc12a3*, which is uniquely expressed in the mammalian distal convoluted tubule segment.[10] These observations suggest that the DE and DL are analogous to the mammalian thick ascending limb and distal convoluted tubule, respectively.[10] Finally, *gata3* expression demarcates the zebrafish PD segment and is similarly expressed in the mouse CD.[10]

Several differences between zebrafish and mammals are evident from this analysis. Notably absent from the zebrafish pronephros segmental series is a thin limb segment. This is to be expected because zebrafish are freshwater teleosts and therefore have no requirement to conserve water and concentrate their urine.[10] Further, the fish pronephric duct is a single, short segment that connects to the cloaca for drainage.[10] Mammalian kidneys possess an intricate collecting system that enables waste from thousands of nephrons to be funneled efficiently, an architecture that is not required for the two-nephron system in the fish pronephros. Lastly, the zebrafish pronephros gives rise to a unique cell type: a population that is located initially within the DL region, and later forms discrete clusters that become positioned just dorsal to the distal tubule, at the junction between the DE and DL segments.[10] These clusters form endocrine glands, known as the Corpuscles of Stannius, which are responsible for maintaining calcium and phosphate homeostasis.[39,40] The Corpuscles of Stannius have been observed in various fish,[39,40] and because of its origin in the field of tubule progenitors, we included it as part of the initial segmental plan of the pronephros.[10] It is presently unclear if the Corpuscle of Stannius cell population corresponds to any particular mammalian structure(s). In a similar region of the mammalian nephron is the macula densa, a cluster of regulatory cells that sense the composition of the filtrate flowing to the distal tubule and participate in triggering the release of the hormone renin to affect blood pressure changes in the renal corpuscle.[41] Studies in eels have linked the Corpuscle of Stannius to changes in blood pressure, raising the speculative possibility that there is an evolutionary tie between it and the macula densa.[42]

PRONEPHROS SEGMENTATION IS CONSERVED AMONG LOWER VERTEBRATES

The pronephros of amphibians exhibits a similar segmental organization as zebrafish, although the gross structure and development of their pronephros differs slightly (Figure 1c). The amphibian pronephros consists of a pair of nonintegrated nephrons, in which the blood filter (glomus) projects into a coelom rather than being directly attached to the tubule.[19,21] Filtrate delivered to the coelom enters the tubule via ciliated portals called nephrostromes and then passes through a coiled tubule domain into the duct.[19,21] During development, the renal progenitors in the amphibian IM give rise to a tubule anlage and a small region of duct precursors. The duct extends caudally by growing along the anteroposterior axis until it reaches the cloaca, a phenomenon similar to the caudal growth of the nephric duct in mammals.[4,43] In contrast, zebrafish renal progenitors are induced simultaneously from the IM, and little (if any) growth of the PD occurs during its formation.

As is the case in fish, the segmental organization of the amphibian pronephros has only been recently appreciated and led to a revised model of its structure[8,9,13–18] (see inset, Figure 1c). Distinct pronephros segment domains can be identified in amphibians based on their histological features.[13] Expression studies in the frog, *Xenopus laevis*, have shown that the tubule consists of at least two proximal segments and two distal segments, referred to as early and late, followed by a duct segment (Figure 1c). These five segments express solute transporters that liken them to the proximal and distal segments of both the zebrafish pronephros and the mammalian metanephros (Figure 2). For instance, *slc5a2* is expressed in the frog proximal early and *slc7a13* defines proximal late; the corresponding mammalian orthologs *Slc3a2* and *Slc7a13* are expressed in the PCT and PST, respectively.[14] As in fish, transcripts encoding the *clck1* transporter mark the entire distal tubule and duct domains, *slc12a1* expression defines the DE, *slc12a3* defines the DL, and *gata3* marks the long stretch of PD.[15–18]

The expression domain of several genes, however, is distinct between frogs and mammals. The amino-acid transporters *slc7a8* and *slc6a14* in frogs are confined to the proximal early and proximal late segments, respectively, whereas their mammalian counterparts are found throughout the proximal tubule.[17] Further, a recent report suggests that the frog DE and DL may contain subregions, based on the expression analysis and function of the transcription factors *irx1*, *irx2*, and *irx3*.[14] However, additional

investigation is needed to establish whether these domains express individual solute transporters that would confirm their unique segment status. Finally, the frog DL segment expresses *slc20a1* and *slc4a4*, which mark the proximal segments in fish and mammals, suggesting that this segment may possess nonconserved functions.[8,17] Despite these differences, the overall proximodistal specialization of pronephros appears remarkably similar across vertebrate species and demonstrates that these once-considered 'simple' nephrons also share many segment attributes with metanephric nephrons.

RETINOIC ACID ESTABLISHES THE SEGMENTATION PATTERN OF THE ZEBRAFISH PRONEPHROS

We have recently shown that retinoic acid (RA) is essential for directing the proximodistal patterning of the zebrafish pronephric nephron.[10] Zebrafish embryos deficient in RA synthesis form reduced numbers of podocytes and developed proximal segments that are either absent or shortened in length, whereas the distal segments are expanded in length[10] (Figure 3). These results suggest that RA acts to promote proximal segment identities and perhaps limits the size of the distal segments. In support of this, zebrafish embryos treated with exogenous RA develop a pronephros made entirely of proximal segments[10] (Figure 3). Changes in pronephros segmentation only occurred when RA levels were altered at the end of gastrulation, suggesting that this was the critical time for RA's patterning effect on renal progenitors.[10] During this time, RA is formed by the anterior domain of the paraxial mesoderm (PM), which is located laterally to the IM. Given its established role as a morphogen, we hypothesize that RA produced in the PM diffuses to the adjacent IM to affect its patterning in a graded fashion, such that high levels of RA generate proximal IM fates and low RA levels are permissive for distal IM fates[10] (Figure 3). The PM-produced RA source acts concomitantly to pattern the hindbrain segments,[44,45] endoderm precursors that form the pancreas,[46–50] and the mesoderm populations of the heart and limb bud.[51–53] Taken together, RA produced by the PM likely acts as an organizing center to pattern multiple tissues.

CONSERVATION OF RA IN THE MECHANISM OF NEPHRON SEGMENTATION IN OTHER VERTEBRATES?

Studies in *Xenopus* using chemical antagonists to RA receptors have shown that preventing RA signaling leads to a failure in the formation of the pronephros.[11] Whether RA has subsequent roles in proximodistal patterning of the *Xenopus* pronephros is not clear. Interestingly, other RA-blocking experiments in *Xenopus* indicate that the PM alone is not the sole source of RA needed to induce the pronephros.[11] This result indicates that other tissues, such as the IM and lateral plate mesoderm, may also be important sites of RA biosynthesis. In mammals, RA has been linked to kidney development for some time: it has been long appreciated that vitamin A deficiency leads to defects in metanephric development, including hypoplasia and

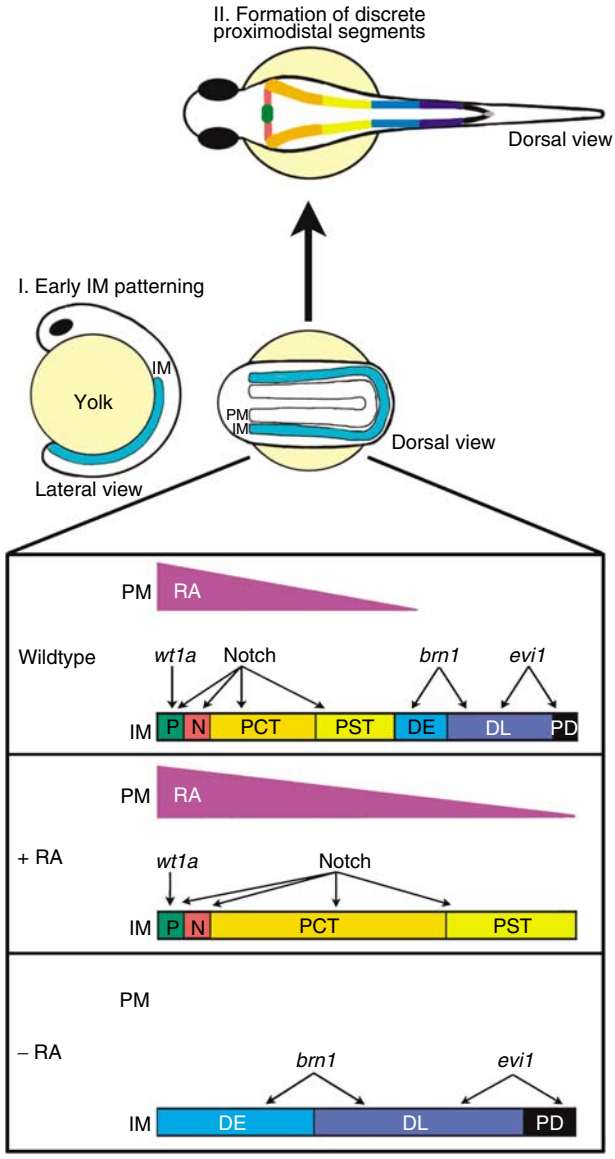

Figure 3 | Proposed model of how RA controls the proximodistal patterning of the intermediate mesoderm. (**a**) Early IM patterning. Drawings depict lateral and dorsal views of the zebrafish embryo at the end of gastrulation, showing how the PM is located adjacent to the IM. Renal progenitors in the IM will give rise to the pair of pronephric nephrons. Bottom enlargement: RA produced by the PM is proposed to pattern the IM by diffusing in a graded fashion, such that proximal progenitors are exposed to high RA levels, and distal progenitors developed are exposed to low RA levels. At the molecular level, high RA levels are proposed to lead to *wt1a* expression in the podocyte precursors and the expression of Notch pathway components in podocytes and proximal segments, whereas low RA levels are associated with the expression of *brn1* and *evi1* in distal IM progenitors. The mechanism by which RA directs the expression domains of these genes remains to be determined. Zebrafish embryos treated with exogenous RA (+RA) have expanded proximal segment identities and lack distal segments, changes coupled with expanded expression domains of *wt1a* and Notch pathway genes. In contrast, RA deficiency (−RA) in zebrafish embryos leads to the expansion of distal segment identities and the abrogation of proximal fates, changes coupled with expanded IM expression of the *brn1* orthologs and *evi1*. (**b**) Formation of discrete proximodistal segments. Drawing depicts dorsal view of the zebrafish embryo, after establishment of segment fates.

agenesis.[54] Much of the vitamin A-deficient phenotype is recapitulated by gene targeting of the RA receptors (*RARα,β*) in the mouse.[55] From these studies, it has been established that RA is essential for regulating ureteric bud branching by inducing expression of *c-Ret*, the receptor for the probranching factor GDNF (glial cell-derived neurotrophic factor).[56] To date, RA has not been attributed with directing proximodistal patterning of the mammalian metanephric nephron. However, in the S-shaped body, *Raldh2* is expressed in the glomerular anlagen, and *Raldh1* is expressed in the tubular anlagen and connecting tubule.[57] Further study is needed to determine whether either of these RA sources is involved in subsequent segmentation processes.

POTENTIAL DOWNSTREAM TARGETS OF RA IN THE PRONEPHROS PLAY ROLES IN METANEPHRIC SEGMENTATION

Gene-targeting studies in the mouse have identified some of the factors involved in the proximodistal segmentation program of the metanephric nephron.[4,5] Signaling through the Notch pathway has been shown to be essential for inducing proximal nephron fates.[4,5] Several Notch ligands and receptors show regionalized expression domains during nephrogenesis,[58,59] and the conditional knockout of *Notch2* was found to abrogate podocyte and proximal tubule fates without altering distal segment development.[60] Conversely, the function of the renal transcription factors *Lhx1* and *Brn1* has been linked to distal segment patterning.[5] The genetic inactivation of *Lhx1* or *Brn1* leads to metanephric nephrons that lack distal segments, and there is evidence to indicate that *Lhx1* acts upstream of *Brn1* during nephrogenesis.[5] In addition to these murine studies, recent work in *Xenopus* has shown that the renal transcription factor *evi1* is required for distal tubule and duct patterning, whereas *evi1* overexpression blocks development of proximal nephron fates.[9] In the mouse, the role of *evi1* in proximodistal patterning is not as clear. Transcripts are found in the nephric duct and metanephric tubules; however, a detailed analysis of expression along the proximodistal axis of the nephron has not been determined.[61,62] *Evi1*-null embryos die at E10.5, before metanephros formation, thus precluding an assessment of metanephric nephron patterning.[63] *Xenopus* studies have also demonstrated a role for the transcription factor *irx3* in the formation of the proximal late and DE segments, based on the loss/reduction of *slc7a13* and *slc12a1* expression.[14]

In the zebrafish, orthologs of these genes show restricted domains of expression in the IM, suggesting that they might play similar patterning roles. Initially, the IM field uniformly expresses *pax2a* when it is formed at the end of gastrulation. Within several hours, however, the IM field can be divided into two territories. First, there is a proximal territory that is delineated by the expression of the Notch ligands *deltaC*, *jagged1b (jag1b)*, and *jagged2a* (*jag2a*);[10] this region includes a subset of cells that express *wt1a*,[10] shown recently to be required for podocyte formation.[64] Second, there is a distal territory defined by transcripts for *evi1* and the *Brn1* orthologs, *zp12* and *zp23*.[10,65] The onset of expression of these territory genes occurs after the time-window when RA is needed for IM patterning, thus making them potential downstream targets of RA. Consistent with this notion, we found that changes in RA bioavailability are associated with changes in the expression domains of these IM genes. For example, RA-deficient embryos fail to express *wt1a*, *deltaC*, and *jag2a* in the proximal IM,[10] whereas the distal IM territories of *evi1*,[10] *zp12*, and *zp23* expression are expanded (RAW and AJD, unpublished data). Conversely, RA-treated embryos have expanded proximal IM domains of *wt1a*, *deltaC*, and *jag2a*,[10] and reduced distal domains of *evi1*,[10] *zp12*, and *zp23* (RAW and AJD, unpublished data). Thus, changes in the expression domains of these proximal and distal patterning genes correlate to subsequent expansions and reductions in nephron segment identities (Figure 3). Taken together, these observations suggest that RA acts to regulate regional gene expression in the IM precursors, although whether RA directly influences the expression of these particular genes is currently unknown.

FUTURE PROSPECTS

In conclusion, the emergence of common players linked to the establishment of proximal or distal segment fates in pronephric and metanephric nephrons suggests that many elements of the segmentation program may be conserved between kidney types. The ongoing challenge will be the identification of other genes involved in nephron segment patterning, as well as understanding the epistatic relationships and interactions between factors. Given the fundamental conservation in their molecular anatomy, pronephros models of nephron segmentation have great potential as a rapid means to discover the genes and pathways that act to compartmentalize nephrons in mammals. The zebrafish model provides a high-resolution system for analyzing gene expression patterns and the ability to test combinatorial gene function using morpholinos. In addition, zebrafish are a genetically tractable model, allowing unbiased forward genetic screens to be used to identify nephron segmentation mutants. With these tools in hand, we are at the threshold of an exciting era of developmental renal biology that has the potential to unlock the program of nephron segmentation.

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