Developmental Biology 399 (2015) 100–116

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Nephron proximal tubule patterning and corpuscles of Stannius formation are regulated by the *sim1a* transcription factor and retinoic acid in zebrafish

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ARTICLE INFO

Article history:
Received 9 July 2014
Received in revised form
24 November 2014
Accepted 17 December 2014
Available online 25 December 2014

Keywords:
Pronephros
Kidney
Zebrafish
Nephron
Segmentation
sim1a
Retinoic acid
Nephrogenesis
Proximal tubule
Corpuscles of Stannius

ABSTRACT

The mechanisms that establish nephron segments are poorly understood. The zebrafish embryonic kidney, or pronephros, is a simplified yet conserved genetic model to study this renal development process because its nephrons contain segments akin to other vertebrates, including the proximal convoluted and straight tubules (PCT, PST). The zebrafish pronephros is also associated with the corpuscles of Stannius (CS), endocrine glands that regulate calcium and phosphate homeostasis, but whose ontogeny from renal progenitors is largely mysterious. Initial patterning of zebrafish renal progenitors in the intermediate mesoderm (IM) involves the formation of rostral and caudal domains, the former being reliant on retinoic acid (RA) signaling, and the latter being repressed by elevated RA levels. Here, using expression profiling to gain new insights into nephrogenesis, we discovered that the gene *single minded family bHLH transcription factor 1a* (*sim1a*) is dynamically expressed in the renal progenitors—first marking the caudal domain, then becoming restricted to the proximal segments, and finally exhibiting specific CS expression. In loss of function studies, *sim1a* knockdown expanded the PCT and abrogated both the PST and CS populations. Conversely, overexpression of *sim1a* modestly expanded the PST and CS, while it reduced the PCT. These results show that *sim1a* activity is necessary and partially sufficient to induce PST and CS fates, and suggest that *sim1a* may inhibit PCT fate and/or negotiate the PCT/PST boundary. Interestingly, the *sim1a* expression domain in renal progenitors is responsive to altered levels of RA, suggesting that RA regulates *sim1a*, directly or indirectly, during nephrogenesis. *sim1a* deficient embryos treated with exogenous RA formed nephrons that were predominantly composed of PCT segments, but lacked the enlarged PST observed in RA treated wild-types, indicating that RA is not sufficient to rescue the PST in the absence of *sim1a* expression. Alternately, when *sim1a* knockdowns were exposed to the RA inhibitor diethylaminobenzaldehyde (DEAB), the CS was abrogated rather than expanded as seen in DEAB treated wild-types, revealing that CS formation in the absence of *sim1a* cannot be rescued by RA biosynthesis abrogation. Taken together, these data reveal previously unappreciated roles for *sim1a* in zebrafish pronephric proximal tubule and CS patterning, and are consistent with the model that *sim1a* acts downstream of RA to mitigate the formation of these lineages. These findings provide new insights into the genetic pathways that direct nephron development, and may have implications for understanding renal birth defects and kidney reprogramming.

Introduction

Organogenesis of the vertebrate kidney involves the formation of up to three distinct structures that develop in succession from the renal progenitors that emerge from the intermediate mesoderm (IM) (Saxen, 1987; McCampbell and Wingert, 2012; Romagnani et al., 2013). Across species, each kidney functions to various degrees in the regulation of waste excretion, fluid balance, and osmolarity. The pronephros is the first embryonic kidney to arise. While lower vertebrates (fish and amphibians) use the pronephros to perform vital excretory tasks, it is a transient, vestigial organ in higher vertebrates (reptiles, birds, and mammals). The mesonephros is the second

Abbreviations: AO, acridine orange; cRNA, capped RNA; CS, corpuscle of Stannius; DE, distal early; DEAB, 4-diethylaminobenzaldehyde; DL, distal late; dpf, days post fertilization; epi, epiboly; hpf, hours post fertilization; hpi, hours post injection; IM, intermediate mesoderm; MET, mesenchymal to epithelial transition; mmMO, mismatch morpholino; MO, morpholino; N, neck; ORF, open reading frame; P, podocytes; PCT, proximal convoluted tubule; PD, pronephric duct; PFA, paraformaldehyde; PM, paraxial mesoderm; PST, proximal straight tubule; PTU, 1-phenyl-2-thiourea; RA, retinoic acid; RARE, retinoic acid response element; RAR/RXR, retinoic acid receptors; *sim1a*, *single-minded family bHLH transcription factor 1a*; ss, somite stage; WISH, whole mount *in situ* hybridization; WT, wild-type.

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http://dx.doi.org/10.1016/j.ydbio.2014.12.020

vertebrate kidney structure that forms concomitant with the degeneration and/or remodeling of pronephric tissues, and performs excretory roles during embryonic life. In higher vertebrates, the mesonephros degenerates upon the formation of the third and final kidney, known as the metanephros, which functions throughout adulthood. Interestingly, fish and other lower vertebrates utilize their mesonephros during adulthood and never develop a metanephric kidney (Gerlach et al., 2011).

Despite these developmental differences, vertebrate kidney structures are all comprised of tubular functional units known as nephrons (Cheng and Wingert, 2014). Nephrons connect to the vascular system at their proximal end by surrounding a small bundle of capillaries, while the opposing distal end links to collecting ducts that drain waste into the urinary system. Nephrons are regionalized along their proximo-distal length with segments of discrete segment populations of epithelial cells that are specialized to perform precise modifications of fluid as it transits through the tubule (Reilly et al., 2007). Recent research has demonstrated that the segmental nature of the nephrons is fundamentally conserved across pro-, meso- and metanephric nephrons in humans and popular animal research models including the zebrafish, frog and mouse (Wingert and Davidson, 2008; Wessely and Tran, 2011; Kroeger and Wingert, 2014). At present, however, there is only a rudimentary understanding of the mechanisms that regulate renal progenitor patterning into the specific segment identities found within the nephron (Kopan et al., 2007; Schedl, 2007).

The zebrafish pronephros, in particular, is an excellent model for studying segmental patterning during nephrogenesis (Gerlach and Wingert, 2013). The pronephros is anatomically simple, as it consists of two nephrons (Drummond et al., 1998). These pronephric nephrons emerge from bilateral fields of renal progenitors that derive from the IM during somitogenesis, and then undergo a mesenchymal to epithelial transition (MET) to form tubular structures (Gerlach and Wingert, 2014). Each pronephric nephron contains the following segments: a blood filter comprised of podocytes (P), neck (N), proximal convoluted and straight tubules (PCT, PST), distal early and late tubules (DE, DL), and a pronephric duct (PD) (Fig. 1A) (Wingert et al., 2007). In addition, a small subset of cells situated in each renal progenitor field within the domain occupied by DL precursors will become the corpuscles of Stannius (CS), endocrine organs that regulate calcium and phosphorus in teleosts (Camp et al., 2003; Elizondo et al., 2005; Wingert et al., 2007). After the CS precursors emerge from the renal progenitor field, they undergo morphogenesis events that situate them into bilateral lobes that are located dorsal to the distal tubules (Camp et al., 2003; Elizondo et al., 2005; Wingert et al., 2007). The marked conservation of nephron segmentation between zebrafish and other vertebrates, in combination with the structural simplicity inherent to a two-nephron kidney, makes the zebrafish both a relevant and feasible experimental system for conducting genetic interrogations to discover nephrogenesis mechanisms.

To date, several major events that direct renal progenitor patterning during zebrafish pronephros formation have been identified. Recently, we found that renal progenitors undergo a regionalization that divides them into rostral and caudal domains, and that the materialization of this rostral domain requires a local source of retinoic acid (RA) secreted by the adjacent paraxial mesoderm (PM), which forms the embryonic somites (Wingert and Davidson 2011; Li et al., 2014). Interestingly, RA positively regulates the expression domains of rostral domain markers, such as the transcription factor *wt1a* (Wingert et al., 2007; Bollig et al., 2009), which is required for podocyte development (Perner et al., 2007; O'Brien et al., 2011). In addition, RA negatively regulates the transcription factor *mecom*, a caudal domain gene that is required for PST and DL development (Li et al., 2014). Further subdivisions, demarcated by the nested expression of other transcription factors and other genes, precede the emergence of discrete segments by 24 h post fertilization (hpf), which corresponds to approximately the 28 somite stage (ss) (Wingert and Davidson, 2011; McKee et al., 2014). However, while numerous transcription factors have been mapped to the emerging renal progenitor domains (Wingert and Davidson, 2011), the functional roles of most remain an enigma.

Among these, previous studies have documented the expression of the transcription factor *sim1a* in the zebrafish pronephric renal progenitors (Serluca and Fishman, 2001). *sim1a* encodes a basic helix-loop-helix and Period-Arnt-Sim (bHLH-PAS) transcription factor that is homologous to the *Drosophila single-minded* (*Sim*) gene, a master regulator of midline cell development in the central nervous system (Linne et al., 2012). In zebrafish, *sim1a* is likewise expressed in the developing central nervous system (Wen et al., 2002) where it is requisite for the formation of dopaminergic neurons from neural progenitors (Borodovsky et al., 2009; Mahler, et al., 2010; Wolf and Ryu, 2013), the creation of the neuroendocrine system (Eaton and Glasgow, 2006; Eaton et al., 2008; Löhr et al., 2009), as well as axon guidance (Schweitzer et al., 2013). Despite the assignation of these various *sim1a* functions in the nervous system across invertebrate and vertebrate species, the role of *sim1a* has not yet been explored during kidney establishment.

Here, we used a combination of expression and functional studies of *sim1a* to gain new insights into nephrogenesis, and elucidated essential roles of *sim1a* both in pronephros segmentation and CS development. Using whole mount *in situ* hybridization (WISH) to profile renal progenitor gene expression, we discovered that *sim1a* expression is highly dynamic during nephron construction. *sim1a* is one initial marker of the renal progenitor caudal domain, and that its expression later is maintained in both proximal tubule segments before becoming restricted to the CS. Since these findings suggested that *sim1a* might contribute to segment patterning and CS formation, we performed loss and gain of function studies to explore the role(s) of *sim1a* in renal ontogeny. *sim1a* deficiency caused an expansion of the PCT, which was minimally functional as indicated by a dextran-FITC uptake assay, and an abrogation of the PST and CS populations. However, the domains of both the DE and DL segments remained unchanged. These results suggest that *sim1a* activity is necessary to pattern the PST and CS, and that *sim1a* may negotiate the PCT/PST boundary. Consistent with these findings, *sim1a* overexpression was sufficient to some extent in promoting the formation of the PST and CS populations at the expense of the PCT. Further, we evaluated the relationship between *sim1a* and RA, and discovered that *sim1a* expression is reliant on RA levels. We found that elevations in RA were not sufficient to rescue PST formation in *sim1a* morphants, and that the abrogation of RA synthesis using 4-diethylaminobenzadehyde (DEAB) was not sufficient to rescue CS formation in *sim1a* morphants—indicating that RA cannot substitute for normal *sim1a* function in PST and CS development. Taken together, these data are consistent with the hypothesis that *sim1a* functions downstream of RA during renal progenitor patterning. In sum, these studies show for the first time that *sim1a* is essential for several aspects of nephron segmentation, and establish that *sim1a* is an essential component of CS formation.

Materials and methods

Zebrafish husbandry and ethics statement

Zebrafish were cared for and maintained in the Center for Zebrafish Research at the University of Notre Dame, with experimental procedures approved under protocols 13–021 and 16–025. Wild-type embryos of the Tübingen strain were raised and staged