Developmental Biology 386 (2014) 111–122

Contents lists available at ScienceDirect

Developmental Biology

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

Zebrafish nephrogenesis is regulated by interactions between retinoic acid, *mecom*, and Notch signaling ☆

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ARTICLE INFO

Article history:
Received 16 August 2013
Received in revised form
19 November 2013
Accepted 20 November 2013
Available online 3 December 2013

Keywords:
Pronephros
Nephrogenesis
Segmentation
Retinoic acid
mecom
Notch

ABSTRACT

The zebrafish pronephros provides a conserved model to study kidney development, in particular to delineate the poorly understood processes of how nephron segment pattern and cell type choice are established. Zebrafish nephrons are divided into distinct epithelial regions that include a series of proximal and distal tubule segments, which are comprised of intercalated transporting epithelial cells and multiciliated cells (MCC). Previous studies have shown that retinoic acid (RA) regionalizes the renal progenitor field into proximal and distal domains and that Notch signaling later represses MCC differentiation, but further understanding of these pathways has remained unknown. The transcription factor *mecom* (*mds1/evi1 complex*) is broadly expressed in renal progenitors, and then subsequently marks the distal tubule. Here, we show that *mecom* is necessary to form the distal tubule and to restrict both proximal tubule formation and MCC fate choice. We found that *mecom* and RA have opposing roles in patterning discrete proximal and distal segments. Further, we discovered that RA is required for MCC formation, and that one mechanism by which RA promotes MCC fate choice is to inhibit *mecom*. Next, we determined the epistatic relationship between *mecom* and Notch signaling, which limits MCC fate choice by lateral inhibition. Abrogation of Notch signaling with the γ-secretase inhibitor DAPT revealed that Notch and *mecom* did not have additive effects in blocking MCC formation, suggesting that they function in the same pathway. Ectopic expression of the Notch signaling effector, Notch intracellular domain (NICD), rescued the expansion of MCCs in *mecom* morphants, indicating that *mecom* acts upstream to induce Notch signaling. These findings suggest a model in which *mecom* and RA arbitrate proximodistal segment domains, while MCC fate is modulated by a complex interplay in which RA inhibition of *mecom*, and *mecom* promotion of Notch, titrates MCC number. Taken together, our studies have revealed several essential and novel mechanisms that control pronephros development in the zebrafish.

Introduction

Vertebrate kidney organogenesis proceeds through the formation and regression of several successive structures, each comprised of excretory units known as nephrons (Dressler, 2006). The first structure is the pronephros, composed of rudimentary nephrons formed next to the nephric cord, a bilateral epithelial tubule derived from the intermediate mesoderm (IM). Whereas the pronephros is a vestigial organ in mammals, it serves as the embryonic excretory organ in lower vertebrates such as fish and frogs (Dressler, 2006). During mammalian development, a mesonephros forms posteriorly to the pronephros and functions transiently in fetal life, then subsequently a third structure, the metanephros, is formed that functions as the definitive adult kidney (Dressler, 2006). The metanephros arises when the ureteric bud grows out of the caudal end of the nephric duct, invades the surrounding metanephric mesenchyme, and induces a mesenchyme-to-epithelial transition (MET) in cell aggregates adjacent to the ureteric bud tips (Little and McMahon, 2012). Mesenchymal cells undergoing MET form a polarized renal vesicle, which develops sequentially into a comma-shaped body, S-shaped body, and eventually into a segmented nephron tubule (Little and McMahon, 2012). Highly elaborate branching of the ureteric bud along the radial axis of the metanephric mesenchyme generates a complicated network within the collecting duct system, with thousands of nephrons situated in an intricate, arborized three-dimensional arrangement.

Abbreviations: CS, corpuscle of Stannius; DE, distal early; DEAB, 4-diethylaminobenzaldehyde; DL, distal late; dpf, days post-fertilization; G, glomerulus; hpf, hours post-fertilization; *mecom*, *mds1/evi1 complex*; IM, intermediate mesoderm; MCC, multiciliated cell; MET, mesenchymal to epithelial transition; N, neck; NICD, Notch intracellular domain; PCT, proximal convoluted tubule; PD, pronephric duct; PM, paraxial mesoderm; PST, proximal straight tubule; RA, retinoic acid; RARE, retinoic acid response element

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http://dx.doi.org/10.1016/j.ydbio.2013.11.021

There is currently a limited understanding of how nephron tubules are patterned into segments, due in part to the complexity of mammalian nephrogenesis and kidney anatomy (Costantini and Kopan, 2010). However, in recent years the zebrafish has emerged as a useful vertebrate model to study nephron segmentation (Gerlach and Wingert, 2013). Zebrafish embryos form a pronephros with two nephrons that originate from bilateral stripes of IM, from which renal progenitors are generated (Drummond, et al., 1998). The rostral renal progenitors give rise to a single glomerulus that the nephrons share, while the remaining renal progenitors undergo a MET to generate tubules that fuse at the cloaca (Drummond, et al., 1998; Serluca and Fishman, 2001). Gene expression profiling, largely based on genes encoding solute transporter proteins that account for the exquisite functions of each segment, revealed that the zebrafish pronephros segment composition is analogous to other vertebrates (Wingert, et al., 2007; Wingert and Davidson, 2008; Wingert and Davidson, 2011). By 24 hours post-fertilization (hpf), the zebrafish pronephros exhibits eight segments: the glomerulus (G), neck (N), proximal convoluted tubule (PCT), proximal straight tubule (PST), distal early (DE), corpuscle of Stannius (CS), distal late (DL), and pronephric duct (PD) (Fig. 1A) (Wingert, et al., 2007).

Studies of zebrafish nephrogenesis have identified several transcription factors and signaling pathways that are crucial for renal progenitor patterning (Gerlach and Wingert, 2013). Among them, the diffusible morphogen retinoic acid (RA) is essential for proximal–distal regionalization of the renal progenitor field (Wingert, et al., 2007; Wingert and Davidson, 2011). In target tissues, RA regulates gene expression by entering the nucleus and binding to its nuclear receptors, which upon RA interaction directly bind to retinoic acid response elements (RARE) to modulate transcription (Duester, 2008). Zebrafish genetic mutants lacking key RA synthesizing enzymes or wild types treated with diethylaminobenzaldehyde (DEAB), a chemical that blocks RA biosynthesis, develop a pronephros with reduced proximal segments and expanded distal segments (Wingert et al., 2007; Wingert and Davidson, 2011). These studies established that RA induces proximal segment identities during the early somite stages, and may directly repress distal segments. Downstream of RA, the terminal boundaries of each segment are defined by the expression of domain-specific genes and appear to be controlled by the activity of multiple downstream transcription factors, presently known to include *irx3b* and *hnf1β* (Wingert and Davidson, 2011; Naylor et al., 2013). Further, Notch signaling restricts MCC number by modulating the fate choice between transporting epithelium and MCC during mid-to-late somitogenesis (Ma and Jiang, 2007; Liu et al., 2006). Despite these findings, many questions remain concerning how each nephron segment is precisely established during nephrogenesis – including the identity of other key factors involved in segmentation.

In searching for additional factors that may control nephron segmentation, we identified the zinc finger transcription factor *Mecom* as an intriguing candidate gene. *Mecom* is a splice variant of the *ecotropic virus integration site 1* (*Evi1*) and *myelodysplastic syndrome 1* (*Mds1*) genes, which results in an N-terminal extension of the Evi1 protein (Wieser, 2007). Targeted disruptions that result in the loss of both transcripts cause embryonic lethality in mice associated with defects in neural, heart, and blood development – which suggests that this locus has multiple essential roles during ontogeny (Goyama, et al., 2008; Wieser, 2007; Hoyt, et al., 1997). More recent work has demonstrated that *Mecom* is required for long-term hematopoietic stem cell maintenance (Zhang, et al., 2011).

With respect to vertebrate kidney development, transcripts encoding *Mecom* have been detected in the pronephros distal tubule and duct in *Xenopus* (Mead, et al., 2005) and zebrafish *mecom* has also been reported in the pronephros (Wingert, et al., 2007; Wingert and Davidson, 2011). In the zebrafish, *mecom* is initially expressed in the renal progenitor field, but its domain changes dynamically during nephrogenesis (Wingert et al., 2007; Wingert and Davidson 2011). *mecom* marks a broad caudal domain in early stages, then is later restricted to the DL and PD at 24 hpf. A genome-scale *in situ* analysis of mammalian transcriptional regulatory factors reported expression of murine *Mecom* in nascent nephron S-shaped bodies in the developing metanephric kidney (Yu et al., 2012), thus further suggesting it could be involved in nephron patterning across vertebrates. However, the mechanism of how *mecom* modulates vertebrate nephron segmentation and the signaling pathways that may interact with *mecom* in renal progenitors remain unclear.

Through the present study, we found that interactions between RA, *mecom*, and Notch signaling are essential for zebrafish pronephros development. We show that *mecom* expression is extremely dynamic in zebrafish renal progenitors and is negatively regulated by RA. Using both loss and gain-of-function approaches, we found that *mecom* is necessary for proper DL segment formation, and that the absence of *mecom* activity expands the PST segment and MCC numbers. Moreover, *mecom* and RA have opposing roles in PST and DL formation, as *mecom* morphants treated with exogenous RA had a more expanded PST and an abrogated DL, while DEAB treatment rescued segmentation in *mecom* morphants. Consequently, we discovered a previously unappreciated role for RA in MCC development, since DEAB treatment prevented MCC formation while *mecom* knockdown in DEAB-treated embryos rescued MCCs. These data indicate that RA regulates MCC fate choice by inhibiting *mecom*. Furthermore, we established the epistatic relationship between Notch signaling and *mecom* during MCC differentiation, where *mecom* acts upstream to promote Notch activity. Taken together, our data suggest a model in which *mecom* and RA function during early nephrogenesis stages to arbitrate proximodistal segment pattern, and reveal that MCC fate choice is modulated by a complex interplay between RA, *mecom*, and Notch signaling to precisely define the MCC domain and density in the nephron.

Materials and methods

Zebrafish husbandry and ethics statement

Zebrafish were maintained in the Center for Zebrafish Research at the University of Notre Dame Freimann Life Science Center. Wild type embryos were raised and staged as described (Kimmel et al., 1995). The Institutional Animal Care and Use Committee at the University of Notre Dame supervised experimental procedures under protocols 13-021 and 16-025.

Morpholino knockdown, cRNA synthesis, and heat shock experiments

All morpholinos were purchased from Gene Tools, LLC (Philomath, OR), and were solubulized as recommended and stored at a 4 mM concentration. The *mecom* morpholino *e1SD* (5′-CTGAGTGACTTACATATGAAGGGCT-3′) was designed to target the splicing donor site of zebrafish *mecom* (XM_001920912) exon 3, and *e1SA* (5′-TTGTGGCAGACCTCACGACGGTGTT-3′) targets the splicing acceptor of exon 4. The *mecom* mismatch morpholinos (5′-CTGATTGACGTACAAATGATGGGCA-3′ and 5′-TTGTAGCAGGCCTCGCGACTGTGTA-3′) were used as controls. A combination of *mecom e1SD* and *e1SA* morpholinos produced fully penetrant effects and was used for all *mecom* knockdown experiments. One-cell stage wild type embryos were injected with 1–5 nl 0.2 mM *mecom* morpholinos and raised to the desired stages at 28 °C. For gene expression analysis, embryos were fixed in 4% paraformaldehyde/1 × PBST and stored in methanol at −20 °C. Synthetic *mecom* cRNA was synthesized from a *mecom.*