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Epithelial cell fate in the nephron tubule is mediated by the ETS transcription factors *etv5a* and *etv4* during zebrafish kidney development

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ABSTRACT

Kidney development requires the differentiation and organization of discrete nephron epithelial lineages, yet the genetic and molecular pathways involved in these events remain poorly understood. The embryonic zebrafish kidney, or pronephros, provides a simple and useful model to study nephrogenesis. The pronephros is primarily comprised of two types of epithelial cells: transportive and multiciliated cells (MCCs). Transportive cells occupy distinct tubule segments and are characterized by the expression of various solute transporters, while MCCs function in fluid propulsion and are dispersed in a "salt-and-pepper" fashion within the tubule. Epithelial cell identity is reliant on interplay between the Notch signaling pathway and retinoic acid (RA) signaling, where RA promotes MCC fate by inhibiting Notch activity in renal progenitors, while Notch acts downstream to trigger transportive cell formation and block adoption of an MCC identity. Previous research has shown that the transcription factor *ets variant 5a* (*etv5a*), and its closely related ETS family members, are required for ciliogenesis in other zebrafish tissues. Here, we mapped *etv5a* expression to renal progenitors that occupy domains where MCCs later emerge. Thus, we hypothesized that *etv5a* is required for normal development of MCCs in the nephron. *etv5a* loss of function caused a decline of MCC number as indicated by the reduced frequency of cells that expressed the MCC-specific markers *outer dense fiber of sperm tails 3b (odf3b)* and *centrin 4 (cetn4)*, where rescue experiments partially restored MCC incidence. Interestingly, deficiency of *ets variant 4 (etv4)*, a related gene that is broadly expressed in the posterior mesoderm during somitogenesis stages, also led to reduced MCC numbers, which were further reduced by dual *etv5a/4* deficiency, suggesting that both of these ETS factors are essential for MCC formation and that they also might have redundant activities. In epistatic studies, exogenous RA treatment expanded the *etv5a* domain within the renal progenitor field and RA inhibition blocked *etv5a* in this populace, indicating that *etv5a* acts downstream of RA. Additionally, treatment with exogenous RA partially rescued the reduced MCC phenotype after loss of *etv5a*. Further, abrogation of Notch with the small molecule inhibitor DAPT increased the renal progenitor *etv5a* expression domain as well as MCC density in *etv5a* deficient embryos, suggesting Notch acts upstream to inhibit *etv5a*. In contrast, *etv4* levels in renal progenitors were unaffected by changes in RA or Notch signaling levels, suggesting a possible non-cell autonomous role during pronephros formation. Taken together, these findings have revealed new insights about the genetic mechanisms of epithelial cell development during nephrogenesis.

Abbreviations: C, cloaca; CAKUT, congenital and acquired diseases of the urinary tract; *cetn4*, centrin 4; cRNA, capped RNA; CS, corpuscle of Stannius; DAPT, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; DE, distal early; DEAB, 4-diethylaminobenzaldehyde; DL, distal late; ETS, E26 transformation-specific; *etv4*, *ets variant 4*; *etv5a*, *ets variant 5a*; *etv5b*, *ets variant 5b*; FISH, fluorescent in situ hybridization; hpf, hours post fertilization; IF, immunofluorescence; IM, intermediate mesoderm; MCC, multiciliated cell; MO, morpholino; N, neck; *odf3b*, outer dense fiber of sperm tails 3b; ORF, open reading frame; P, podocyte; Pea3, polyomavirus enhancer activator 3; PCT, proximal convoluted tubule; PD, pronephric duct; RA, retinoic acid; PST, proximal straight tubule; ss, somite stage; WISH, whole-mount *in situ* hybridization; WT, wild-type

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1. Introduction

The vertebrate kidney maintains fluid homeostasis within the body, a function essential for survival. In contrast to the development of most vertebrate organs, kidney ontogeny involves the progressive formation of several structures, such that the earliest forms are transient and become degraded when more complex kidney structures are generated (Saxen, 1987). The pronephros is the first kidney structure to form, and it is derived from bilateral stripes of intermediate mesoderm (IM). Although the pronephros has become a vestigial organ in mammals, lower vertebrates such as frogs and fish require this structure during embryonic development as a functioning excretory organ (Dressler, 2006). The second kidney structure, known as the mesonephros, serves as the functional fetal kidney in mammals and is drained by a nephric duct (Dressler, 2006). The third kidney emerges from an outgrowth of the nephric duct, termed the ureteric bud, which undergoes branching morphogenesis and complex interactions with the surrounding IM that induce mesenchymal-to-epithelial transitions to generate the final adult kidney, or metanephros (Little and McMahon, 2012). All of the three stages are primarily composed of a conserved functional unit known as the nephron, which is structured into three parts: a blood filter, a segmented epithelial tubule that reabsorbs nutrients and secretes solutes, and a collecting duct that drains the nephron and also participates in electrolyte and fluid balance (Vize et al., 1997; Cheng et al., 2015; Desgrange and Cereghini, 2015).

Failure of nephron function leads to kidney disease, the 8th leading cause of death in the United States alone. Over 20 million Americans have kidney disease, of which 200,000 are adolescents (Center for Disease Control and Prevention, 2014; Saran et al., 2015). Further, congenital and acquired diseases of the urinary tract (CAKUT) account for 20–30% of genetic malformations diagnosed during gestation, and are a major cause of morbidity and chronic kidney disease in children worldwide (dos Santos Junior et al., 2014). Despite the increasing global prevalence of renal disease, many aspects of the genetic and molecular pathways that control nephron development are poorly understood (Cheng and Wingert, 2014; Marra and Wingert, 2014). Furthermore, the mammalian kidney contains millions of intricately arranged nephrons, making nephrogenesis challenging to study (Costantini and Kopan, 2010).

Recently, the embryonic zebrafish (*Danio rerio*) kidney, or pronephros, has emerged as a useful nephrogenesis model that is highly amenable to experimental analysis (Ebarasi et al., 2011). The zebrafish pronephros is comprised of two nephrons that share a single blood filter and common collecting duct (Drummond, 2005), and exhibits a conserved segmentation pattern when compared to the mammalian nephron (Wingert et al., 2007; Wingert and Davidson, 2008). Recent studies have established the timing of renal progenitor development and continued to identify essential patterning factors (O'Brien et al., 2011; Naylor et al., 2013; Gerlach and Wingert, 2014; McKee et al., 2014; Cheng and Wingert, 2015), demonstrating that the zebrafish pronephros provides an excellent opportunity to delineate the fundamental genetic and molecular pathways that are relevant to nephrogenesis.

At just 24 hours post fertilization (hpf), the zebrafish pronephros is fully segmented and contains a mixture of two functionally distinct populations of epithelial cell types: transportive cells and MCCs (Fig. 1A) (Kramer-Zucker et al., 2005; Wingert et al., 2007). Transportive cells have a single primary cilium and recover ions based on their expression of ion transporters. Like populations of these cells make up the different tubule segments of the nephron, which include the podocytes (P), neck (N), proximal convoluted tubule (PCT), proximal straight tubule (PST), distal early (DE), corpuscle of Stannius (CS), distal late (DL), pronephric duct (PD) and cloaca (C) (Fig. 1A) (Wingert et al., 2007). By comparison, the epithelial population of MCCs functions in fluid propulsion and they are dispersed in a "salt-and-pepper" fashion within the tubule, located in the caudal portion of the PCT and throughout the PST and the DE segments (Liu et al., 2007; Ma and Jiang, 2007; Li et al., 2014). Transporter versus MCC fate choice is mediated by Notch signaling (Liu, et al., 2007; Ma and Jiang, 2007), and recent work has also revealed that the transcription factor *mecom* acts through the Notch signaling pathway to restrict MCC formation (Li et al., 2014). Further, RA signaling acts upstream to regulate the expression domain of *mecom* in the renal progenitors and thereby promote MCC fate (Li et al., 2014). However, much still remains unknown about the other factors that regulate MCC identity.

To date, a complex renal transcription factor code has been established for the zebrafish pronephros at 24 hpf (Wingert and Davidson, 2011), but the location of gene expression does not necessarily discern functionality. In fact, the role(s) of most genes expressed in the renal progenitors that form the pronephros are undetermined at present (Gerlach and Wingert, 2013). Of these factors, *etv5a* stood out to us as an intriguing candidate for MCC development due to its expression in the central region of the developing nephrons (Wingert and Davidson, 2011).

etv5a is a member of the conserved family of E26 transformation-specific (ETS) transcription factors, categorized within the polyomavirus enhancer activator 3 (Pea3) subfamily (Oh et al., 2012), which have been shown to have diverse roles in tissue patterning and ciliogenesis (Wasylyk et al., 1998; Kobberup et al., 2007; Eo et al., 2008; Mao et al., 2009; Znosko et al., 2010; Chen et al., 2013; Janesick et al., 2013; Akagi et al., 2015). Furthermore, the mammalian homolog of *etv5a*, *Etv5,* is known to be required for development of the murine kidney. Elegant genetic studies in the developing mouse metanephros have identified important roles for *Etv5,* as well as *Etv4*, another Pea3 subfamily member, in development of the ureteric bud and nephric duct (the latter also known as the Wolffian duct) (Lu et al., 2009; Kuure et al., 2010; Costantini, 2010; Costantini and Kopan, 2010; Little and McMahon, 2012). In contrast, the possible roles of these factors in nephron patterning have not been investigated to date. Previous studies have documented the homology between zebrafish *etv5a* and mammalian *Etv5* (Chen et al., 2013), which suggests they are orthologous genes. Also, *etv5a* is one of the few transcription factors that map principally to a lone nephron segment at the 24 hpf stage (Wingert and Davidson, 2011). However, the role of *etv5a* in zebrafish nephrogenesis has not been examined until now.

Here, we confirm that *etv5a* is expressed predominantly in the PST segment of the zebrafish pronephros, and that *etv5a* expression correlates to the MCC domain within the tubule. Through loss of function studies, we demonstrate that *etv5a* is required to promote MCC identity. Interestingly, the deficiency of its related gene *etv4* also caused reduced MCC numbers that were further reduced when in double *etv5a/etv4* deficient embryos, suggesting redundancy between these factors. The overexpression of *etv5a* alone was not sufficient to produce an MCC phenotype, adding further support to the notion that *etv5a* is just one target in the developmental pathway of MCCs. Through a combination of traditional molecular and chemical genetic approaches, we have placed *etv5a* downstream of RA in promoting MCC fate, where RA is a positive regulator of *etv5a* expression in the developing pronephros. Further, we ascertained that Notch signaling inhibits *etv5a* to restrict MCC identity. The concept that *etv5a* is induced by RA and inhibited by Notch was supported by the rescue of MCC density in *etv5a* morphants after treatment with exogenous RA, and by inhibition of Notch via DAPT. Interestingly, the expression domain of *etv4* suggests that this factor may serve non-cell