march/april 2015

genesis

The Journal of Genetics and Development

editor sally a. moody

ISSN 1526-968X

WILEY Blackwell

volume 53 nos. 3-4

genesis

The Journal of Genetics and Development

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Cover illustration: Formation of the adult zebrafish kidney involves clusters of *wt1b*$^{+}$ progenitor cells. These clusters, labeled in the Tg(*wt1b:GFP*) transgenic line shown here, proliferate and elongate into nascent nephrons, the functional units of the kidney.

ISSN 1526-968X (Online)

genesis 2015:257–269 (2015)

RESEARCH ARTICLE

Development of the Zebrafish Mesonephros

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Received 31 October 2014; Revised 4 February 2015; Accepted 8 February 2015

Summary: The vertebrate kidney plays an essential role in removing metabolic waste and balancing water and salt. This is carried out by nephrons, which comprise a blood filter attached to an epithelial tubule with proximal and distal segments. In zebrafish, two nephrons are first formed as part of the embryonic kidney (pronephros) and hundreds are formed later to make up the adult kidney (mesonephros). Previous studies have focused on the development of the pronephros while considerably less is known about how the mesonephros is formed. Here, we characterize mesonephros development in zebrafish and examine the nephrons that form during larval metamorphosis. These nephrons, arising from proliferating progenitor cells that express the renal transcription factor genes *wt1b*, *pax2a*, and *lhx1a*, form on top of the pronephric tubules and develop a segmentation pattern similar to pronephric nephrons. We find that the pronephros acts as a scaffold for the mesonephros, where new nephrons fuse with the distal segments of the pronephric tubules to form the final branching network that characterizes the adult zebrafish kidney. genesis 53:257–269, 2015. © 2015 Wiley Periodicals, Inc.

Key words: kidney; pronephros; nephron

INTRODUCTION

The vertebrate kidney contains functional units called nephrons that remove metabolic waste and maintain body fluid volume and composition. Nephrons contain a blood filter called the glomerulus, which integrates with the vasculature, and a segmented epithelial tubule that modifies the filtrate (Hoenig and Zeidel, 2014). In mammals, three progressively more complex kidney structures develop during embryogenesis (the pronephros, mesonephros, and metanephros). The mammalian pronephros is vestigial and consists of bilateral nephric ducts that extend down the trunk to the cloaca and induce the formation of the mesonephros during this transit. Mesonephric nephrons are also largely rudimentary and eventually degenerate. Near the cloaca, a ureteric bud (UB) protrudes from the nephric duct, and reciprocal interactions between the UB and the

Additional Supporting Information may be found in the online version of this article.

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Contract grant sponsor: Health Research Council (HRC), New Zealand; Contract grant sponsor: Harvard Stem Cell Institute; Contract grant sponsor: American Society of Nephrology; Contract grant sponsor: National Institutes of Health/National Institute of Diabetes and Digestive and Kidney Diseases, Contract grant number(s): DK069403-06; Contract grant sponsor: Massachusetts General Hospital (Fund for Medical Discovery); Contract grant sponsor: Indiana University of Pennsylvania (Startup Fund, USRC Small Grant, President's Advancing Grantsmanship Awards, the President's Strategic Initiative Fund, and the Professional Development Fund); Contract grant sponsor: Commonwealth of Pennsylvania University Biologists.

Published online 11 February 2015 in
Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/dvg.22846

neighboring metanephric mesenchyme initiates development of the metanephros (the definitive adult kidney). This involves reiterative elongation and branching of the UB, which becomes the collecting duct system of the kidney, and the induction of metanephric nephrons from the mesenchyme. Nephrogenesis follows a stereotypical pattern: a small cluster of mesenchymal cells (pretubular aggregates) epithelialize into a ball (renal vesicle) that fuses with the collecting duct tree and elongates via comma and S-shaped body stages into a nascent nephron (Dressler, 2006). During this process, the nephron progenitors become patterned along the proximodistal axis of the nephron into glomerular fates (podocytes and parietal epithelial cells), and the different segment fates of the tubule (proximal, intermediate, and distal tubule epithelial cells). Podocytes recruit angioblasts, wrap around the resulting blood vessels, and contribute to the sieve-like blood filter of the glomerulus (Costantini and Kopan, 2010).

Only the pronephric and mesonephric kidneys develop in zebrafish (Davidson, 2011). The pronephros is fully functional and essential for survival due to its role in osmoregulation in the free-swimming embryo. Structurally, the pronephros comprises bilateral nephrons that are fused rostrally at their glomeruli and again caudally at the cloaca (Drummond, 2005). Like mammalian nephrons, the pronephric tubules can be divided into functionally distinct segments. Proximally, there are two segments called the proximal convoluted tubule (PCT) and the proximal straight tubule (PST), while distally there are two segments called the distal early (DE) and the distal late (DL) (Wingert and Davidson, 2008).

Zebrafish undergo a postembryonic metamorphosis from larva to juvenile starting around 10 days postfertilization (dpf). This involves changes to many organs and tissues, including the formation of scales and fins, and remodeling of the gonads, gut, and nervous system (Parichy *et al*., 2009). Formation of the mesonephros also occurs during this transition, presumably to cope with higher osmoregulatory demands arising from increased body mass (Diep *et al*., 2011; Zhou *et al*., 2010). Mesonephric nephrons first form on top of, and fuse with, the pronephric tubules. Early events of mesonephrogenesis include the appearance of renal progenitor cells at the caudal end of the swim bladder that are fluorescently labeled in the Tg(*lbx1a:eGFP)* transgenic line (Diep *et al*., 2011). These cells aggregate into clusters and epithelialize into renal vesicle-like bodies that elongate into nascent nephrons and fuse with the underlying pronephric tubule.

The mesonephros remains as the permanent adult zebrafish kidney with an ongoing capacity for new nephron formation during normal growth as well as in response to injury (Diep *et al*., 2011; Zhou *et al*., 2010). However, not much is known about how mesonephric nephrons form or whether they have a similar segmentation pattern as pronephric nephrons. In this report, we examine in detail the development of the zebrafish mesonephros. We show that mesonephric nephrons arise from proliferating *wt1b*$^{+}$ cell clusters that first appear during metamorphosis. Mesonephric branching (the site of nephron formation and fusion) occurs at the two distal (DE and DL) segments of the pronephros. Analyses of the tubular segmentation pattern indicate that mesonephric nephrons have a similar proximodistal organization to pronephric nephrons. Overall, our results show that the distal pronephros serves as a scaffold for the developing mesonephros and that these two kidney structures integrate to form the definitive mesonephric kidney.

RESULTS

The First Functional Mesonephric Nephron Forms at the 6 mm Stage

To better characterize the development of the mesonephros we carried out histological analyses of larvae at the 5 mm stage (~11 dpf), around the time when the first nephron is reported to form (Diep *et al*., 2011; Zhou *et al*., 2010). As previously reported, we found a single cluster of cells on top of one of the pronephric tubules near the caudal end of the swim bladder where the first mesonephric nephron forms (Fig. 1A arrow). These cells stain prominently with methylene blue and closely resemble the basophilic clusters reported in adult goldfish kidneys (Reimschuessel *et al*., 1990). By the 5.5 mm stage (~13 dpf), a rudimentary basophilic tubule is found in the caudal swim bladder region, consistent with being derived from the cluster observed at the 5 mm stage (Fig. 1B arrow). This nascent nephron has not yet fused with the lumen of the underlying pronephric tubule, suggesting it is not yet fully mature. To determine when this first mesonephric nephron becomes functional, we injected a 40 kDa fluorescent dextran (dex-FITC) tracer into the circulation of the larvae. Nephrons integrated into the circulatory system will filter the dex-FITC and accumulate the tracer in the proximal tubule segments (Diep *et al*., 2011). Larvae at the 6 mm stage, but not at the 5.5 mm stage, showed uptake and accumulation of the tracer (Fig. 1C,D arrows). Taken together, these results indicate that the first functional mesonephric nephron forms between the 5.5–6 mm stages, most likely from a cluster of basophilic nephrogenic cells that arise on the pronephric tubules at the 5 mm stage.

Nephrogenic Clusters Contain Dividing Cells and Express *wt1b*

To investigate the growth of the basophilic clusters, which we anticipated is essential for nephron

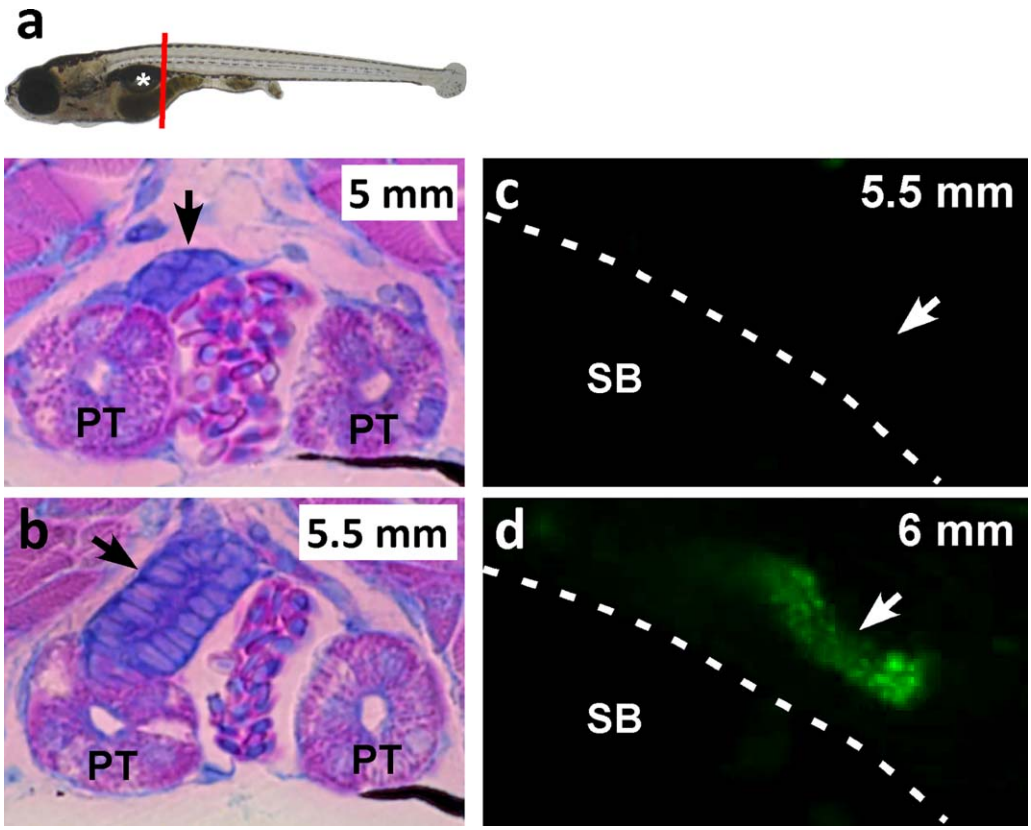

FIG. 1. Formation of the first mesonephric nephron. **A**: A cross section of a 5 mm larva stained with methylene blue and basic fuchsin shows a basophilic cluster sitting on top of the PT (arrow) at the caudal end of the swim bladder (white asterisk). The red line indicates the region of the cross sections for A-B. **B**: A cross section of a 5.5 mm larva stained similarly to A shows a nascent basophilic tubule making contact with the PT (arrow), but its lumen has not yet fused with the PT lumen. **C**: Injection of 40 kDa dextran-FITC into a 5.5 mm larva indicates that the nascent tubule is not yet functional and did not accumulate the fluorescent tracer (arrow). **D**: The tubule of a 6 mm larva did accumulate the fluorescent tracer (arrow), indicating that the first mesonephric nephron is functional at the 6 mm stage. PT: pronephric tubule; SB: swim bladder; dashed lines demarcate the swim bladder from the pronephric tubule.

formation, we injected the thymidine analog bromodeoxyuridine (BrdU) into the circulation of larvae. Serial sagittal sections were alternately stained with hematoxylin and eosin (H+E) and by immunohistochemistry with antibodies against BrdU, and revealed that the basophilic clusters contain many $BrdU^+$ cells (Fig. 2A,B arrows). In addition, we noted that the clusters appear to invade into the pronephric tubule, presumably as part of the morphological process underlying fusion of the nascent nephron with the pronephric tubule. A closer examination of this process by live imaging of Tg(*lhx1a:eGFP*)/Tg(*cdh17:mCherry*) double transgenic larvae revealed that the distalmost cells of the nascent nephron appear to invade and then retract over a four-day period, followed by a downregulation of the *lhx1a*:eGFP transgene (Fig. 3A). A similar downregulation of the endogenous *lhx1a* gene is also observed (Fig. 3B).

We next examined adult kidneys for the presence of basophilic clusters, given that mesonephric nephron formation continues throughout adulthood (Diep *et al.*, 2011; Zhou *et al.*, 2010). While clusters were rarely detected in tissue sections from undamaged fish kidneys (data not shown), we readily detected basophilic $BrdU^+$ clusters in regenerating kidneys following injection of the nephrotoxin gentamicin (Fig. 2C,D arrows). To demonstrate that the basophilic clusters correspond to the *wt1b*-expressing clusters observed in previous studies, we induced kidney damage in the *Tg(wt1b:GFP)* transgenic line (Perner *et al.*, 2007) and alternately stained serial sections with H+E and by immunohistochemistry with anti-GFP antibodies. This analysis confirmed that the basophilic clusters express *wt1b* and likely comprise nephron progenitors involved in both mesonephros development and adult mesonephros regeneration (Fig. 2E,F arrows).

Characterization of Mesonephros Development

Following the formation of the first mesonephric nephron, additional nephrons arise in more caudal locations along the pronephric tubules as well as rostrally in

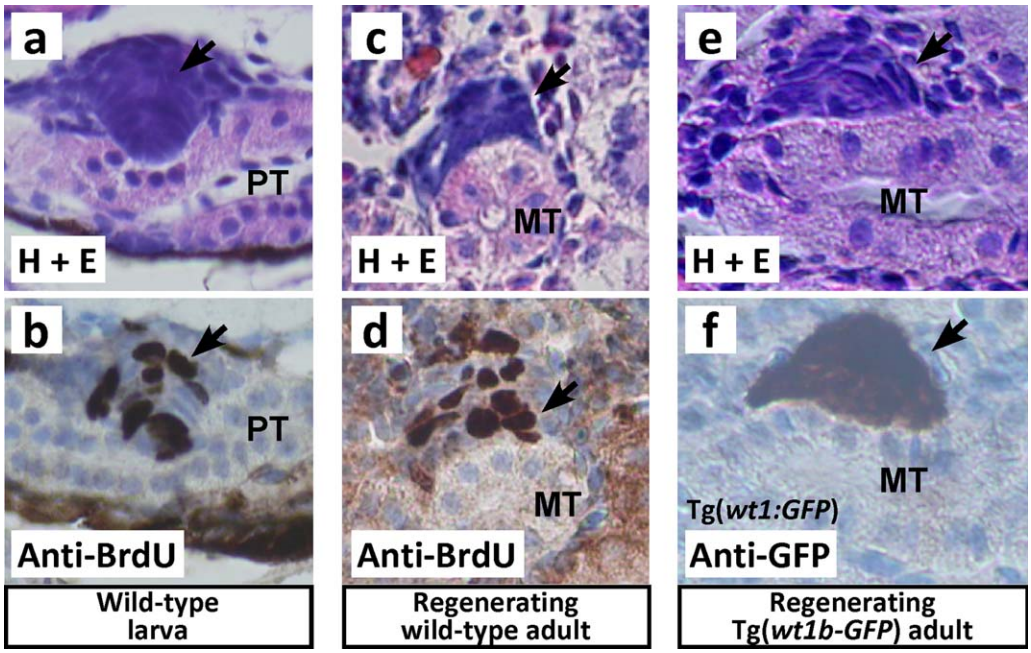

FIG. 2. The developing and regenerating mesonephros contain proliferating basophilic clusters that express *wt1b*. **A**–**D**: Larvae (A,B) and adult fish with kidney damage (C,D) were injected with BrdU and serial sections were alternately stained with H+E and anti-BrdU by immunohistochemistry. This shows that the developing mesonephros (A,B) and the regenerating adult mesonephros (C,D) both have basophilic clusters with $BrdU^{+}$ cells (arrows). **E**,**F**: Tg(*wt1b:GFP*) transgenic fish with kidney damage were also injected with BrdU and stained similarly to C,D. This shows that basophilic clusters in the regenerating kidney also express *wt1b*-GFP (arrows). PT: pronephric tubule; MT: mesonephric tubule; H+E: hematoxylin and eosin.

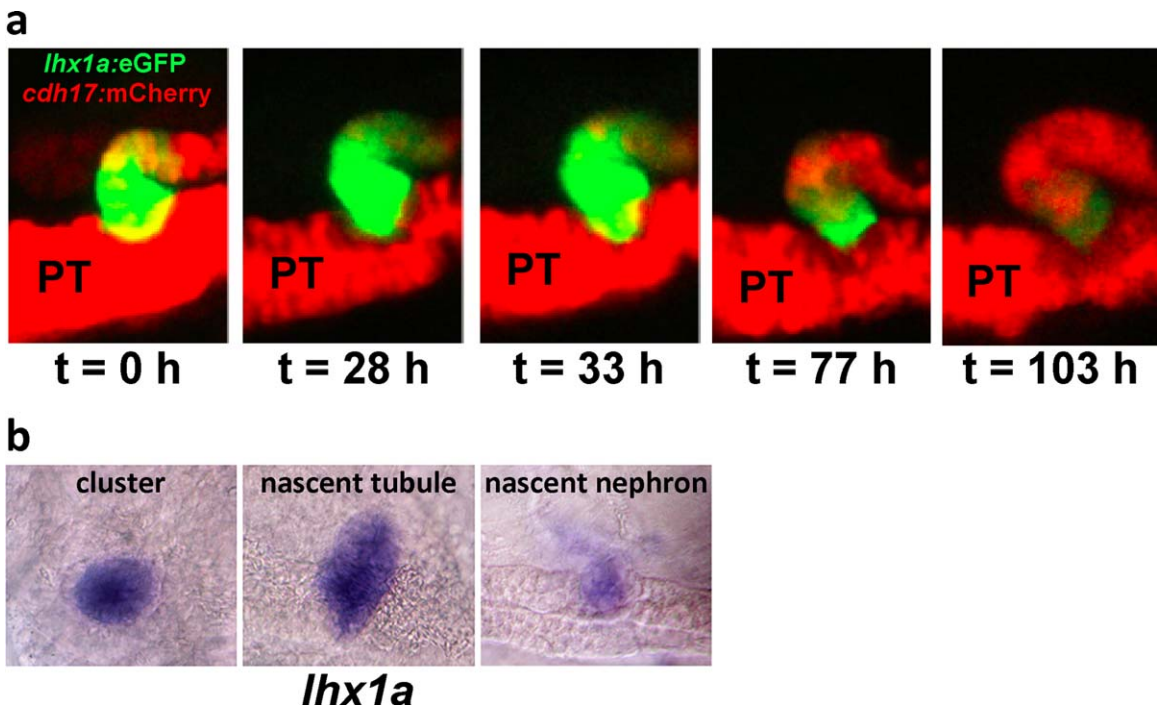

FIG. 3. Invasion of the mesonephric nephron into the pronephric tubule. **A**: A live time-course of Tg(*lhx1a:eGFP*)/Tg(*cdh17:mCherry*) double transgenic larvae shows that the nascent nephron infiltrates the underlying PT (middle panel), followed by downregulation of *lhx1a*:eGFP expression. B: Downregulation of endogenous *lhx1a* expression was also observed in nascent nephrons. PT: pronephric tubule; t: time; h: hours.

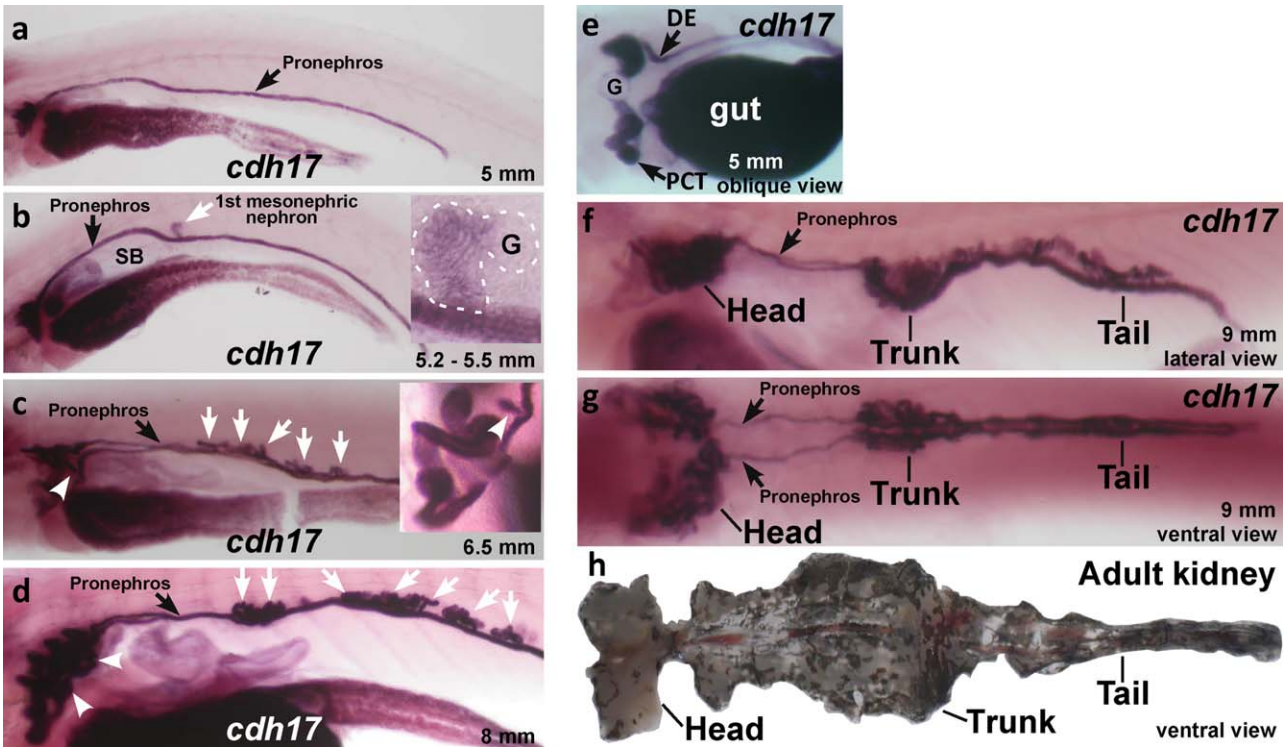

FIG. 4. Mesonephric expression of the pan-tubule *cdh17* marker. **A–G**: Whole mount in situ hybridizations of larvae show that the first mesonephric nephron expresses *cdh17* at the 5.2–5.5 mm stage (B white arrow and inset). Additional nephrons appear later in both caudal and rostral positions relative to the first nephron (C,D: white arrows, arrowheads, and inset). The inset in C is a magnified image of the arrowhead. At the 9 mm stage (~30 dpf), the developing mesonephros has three distinct regions (head, trunk, and tail; F,G), resembling the adult mesonephros in H. **H**: The mesonephros of a sexually mature adult fish (90 dpf) has the head, trunk, and tail regions. SB: swim bladder; G: glomerulus.

the region of the pronephric glomerulus (Diep *et al*., 2011; Zhou *et al*., 2010). To better characterize the formation of these nephrons we performed a time-course analysis of *cdh17* expression by whole mount in situ hybridization. At the 5 mm stage, *cdh17* transcripts mark the pronephric tubules and not the basophilic clusters (Fig. 4A,E). At the 5.2−5.5 mm stage, expression of *cdh17* becomes detectable in the first mesonephric nephron in presumptive tubular but not glomerular cells (Fig. 4B white arrow and inset). At the 6.5 mm stage, *cdh17* transcripts are found in several new nephrons that form in more caudal positions along the pronephros (Fig. 4C white arrows). At this stage, new nephrons are also detected near the pronephric glomerular region (Fig. 4C white arrowhead and inset). By the 8 mm stage, several more new nephrons are observed caudally (Fig. 4D white arrows) and rostrally (Fig. 4D arrowheads). As the larvae reach the 9 mm stage (~30 dpf), the young mesonephros (Fig. 4F,G) morphologically resembles the fully mature adult (~90 dpf) mesonephros (Fig. 4H), consisting of the "head," "trunk," and "tail" regions.

Consistent with each new nephron arising from a *wt1b*$^{+}$ basophilic cluster, we found that *wt1b* transcripts in larvae at the 5.2−5.5 mm stages labeled 1-2 clusters of cells on the top of the pronephric tubules (Fig. 5A arrows, D). At the 8 mm stage, the number of *wt1b*$^{+}$ clusters increased to 3–4 in the future head kidney region and 2–4 in the trunk and tails regions ($n = 5$, Fig. 5B arrowheads). Similar expression patterns were also found for other early acting renal transcription factors, *pax2a* and *lhx1a* (Fig. 5E,F, and data not shown). No regional differences in the expression of *wt1b*, *pax2a*, or *lhx1a* were found in the clusters that formed in the head, trunk, or tail regions of the developing mesonephros, suggesting a common process of nephrogenesis occurs throughout the kidney. Transcripts for *wt1b* were additionally found in presumptive glomerular cells of the nascent nephrons, most likely podocytes, as previously described (Diep *et al*., 2011). The expression pattern of the podocyte marker *nephrin* (*nphs1*) showed a time-course that paralleled that of *cdh17* and *wt1b* (Fig. 5C white arrows and arrowheads) with the earliest formed nephrons showing enlarged mature glomerular structures (Fig. 5G arrow) while the most recently formed nephrons displaying compacted immature glomeruli (Fig. 5H arrow).

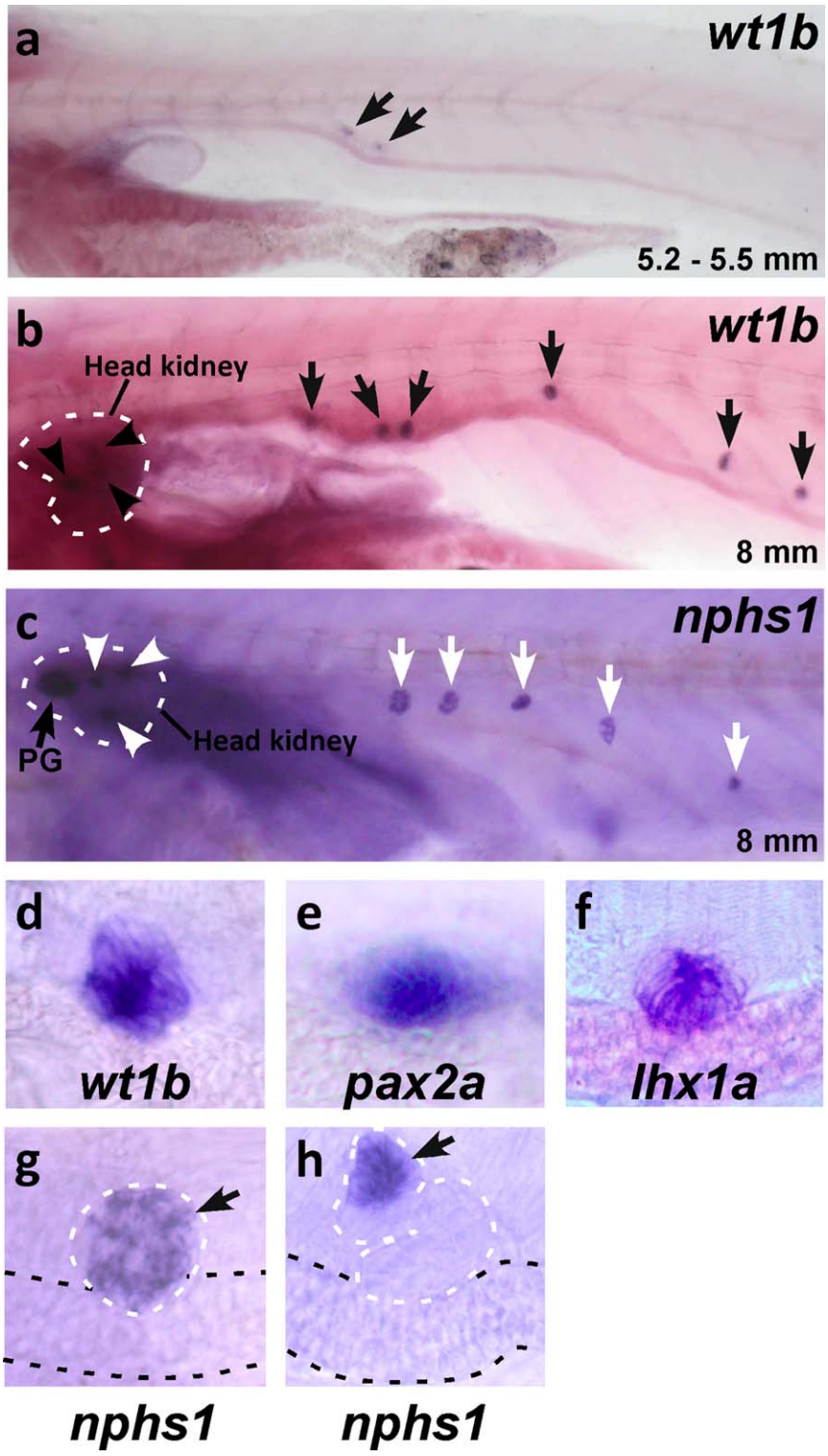

FIG. 5. Mesonephric expression of early and mature nephron markers. **A**,**B**: Whole mount in situ hybridizations of larvae show that the early acting transcription factor *wt1b* is expressed in clusters of nephron progenitor cells (arrows and arrowheads). **C**: The mature glomerular marker *nphs1* is expressed in mesonephric glomeruli (white arrows and arrowheads) in addition to being maintained in the PG (black arrow). **D**–**F**: Magnified images of nephron progenitor clusters expressing the early acting markers *wt1b*, *pax2a*, and *lhx1a*. **G**–**H**: Magnified images showing a mature glomerulus (G) and an immature presumptive glomerulus (H) expressing *nphs1* (arrows). PG: pronephric glomerulus.

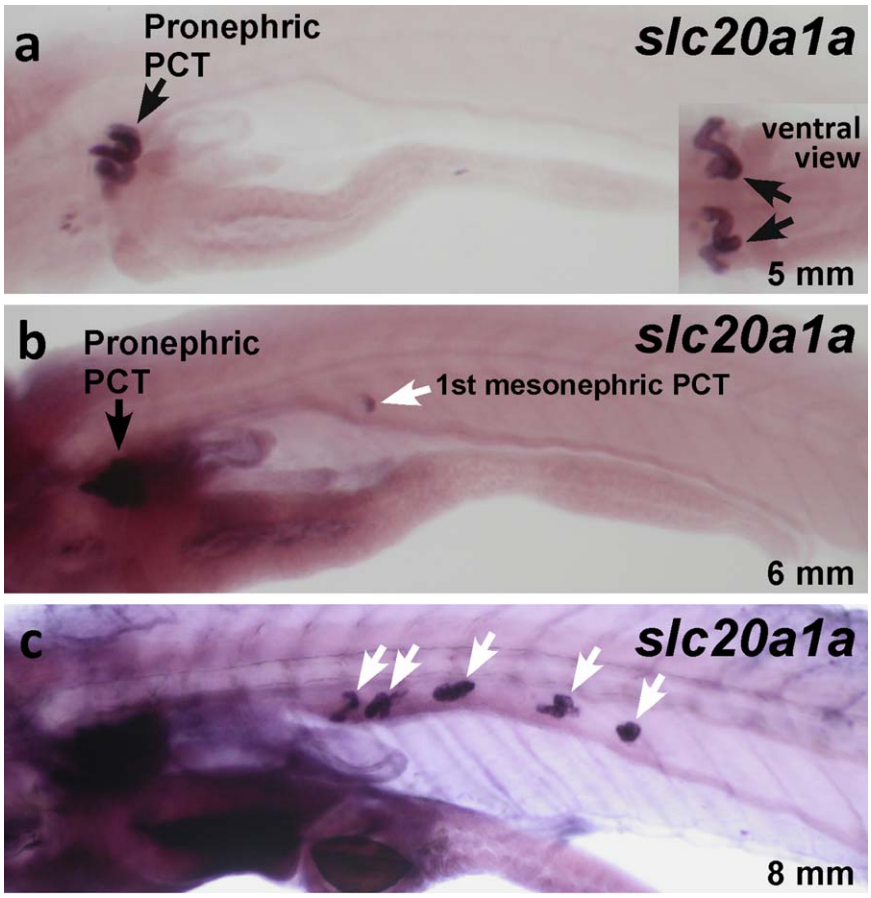

FIG. 6. Expression of *slc20a1a* in the PCT. **A–C**: Whole mount in situ hybridizations of larvae show that the first mesonephric PCT expresses *slc20a1a* at the 6 mm stage (B white arrow), with several more PCTs appearing at the 8 mm stage (C arrows). PCT: proximal convoluted tubule.

Mesonephric Nephrons Express Segment-Specific Markers and Fuse at the DE and DL Segments of the Pronephros

We previously showed that zebrafish pronephric nephrons are divided into two proximal and two distal segments (Wingert *et al*., 2007). To determine whether mesonephric nephrons show a similar segmentation pattern, we examined the expression of the segment-specific markers *slc20a1a* (PCT), *trpm7* (PST), *slc12a1* (DE segment), and *slc12a3* (DL segment) during mesonephrogenesis. In addition, this analysis allowed us to investigate, which of the pronephric segments were fusing with the mesonephric nephrons.

Transcripts encoding *slc20a1a*, *trpm7*, and *slc12a1* were detected in mesonephric nephrons at the 6 mm stage, initially in the first nephron and then sequentially in the more caudal and then rostral nephrons, consistent with the temporal pattern seen with *cdh17*, *wt1b*, and *nphs1* (Figs. 6–8; close-up views of the rostral nephrons at the 8 mm stage are shown in Supporting Information Fig. 1). Unexpectedly, expression of the DL marker *slc12a3* was not detected in the first nephron at the 6 mm stage (Fig. 9A,B, dashed box) but began appearing later, starting at the 8 mm stage (Fig. 9C–E, red and blue arrows). Closer examination of the first and rostral nephrons prior to the onset of *slc12a3* expression showed that their DE segments fused directly to the pronephric DE segment (Fig. 8C white arrows, red arrowhead and inset, and D). However, starting around the 8 mm stage, *slc12a1* transcripts downregulated in these nephrons near the junction with the pronephros, presumably coinciding with the initiation of *slc12a3* expression (Fig. 8E, arrow). The more caudal mesonephric nephrons were found to form on top of the pronephric DL segment and fused via short DL segments by the 8 mm stage (Fig. 9C blue arrow, and E). By the 10-11 mm stage, no difference was observed in the segmentation pattern of rostral and caudal nephrons, with both showing PCT, PST, DE and DL segments (data not shown). Taken together, these findings demonstrate that mesonephric nephrons fuse with the pronephric DE and DL segments and initially

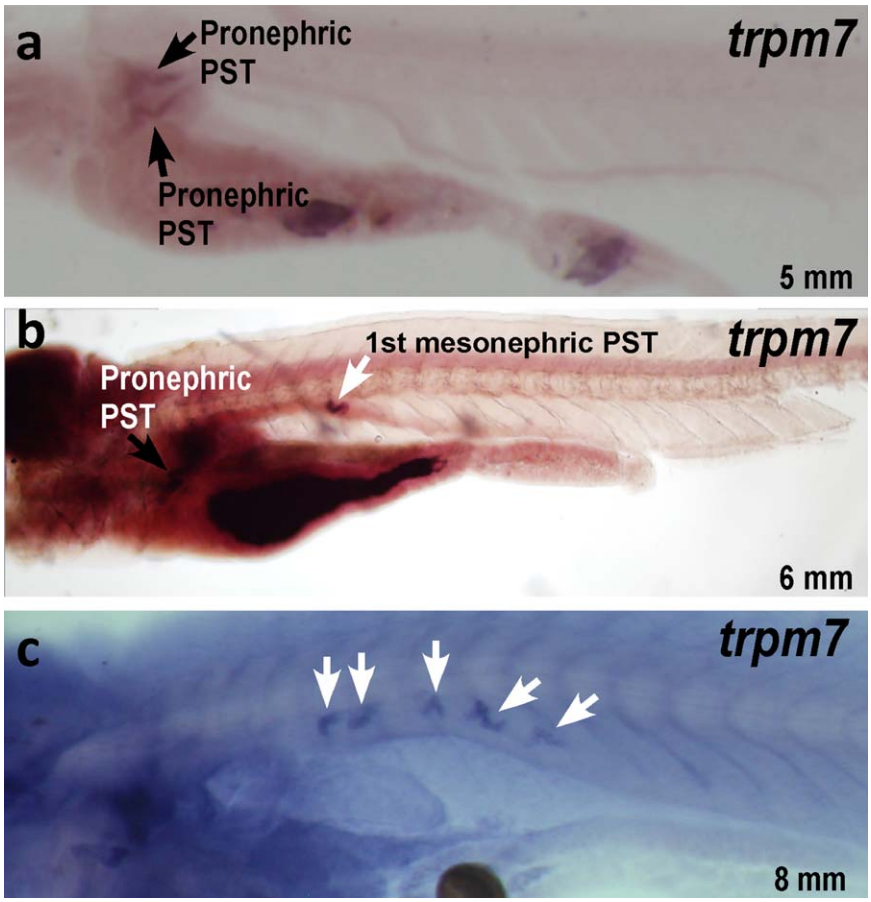

FIG. 7. Expression of *trpm7* in the PST. **A–C**: Whole mount in situ hybridizations of larvae show that the first mesonephric PST expresses *trmp7* at the 6 mm stage (B white arrow), with several more PSTs appearing at the 8 mm stage (C arrows). PST: proximal straight tubule.

develop the PCT, PST, and DE segments, with the DL segment arising during later stages of nephrogenesis. We also analyzed expression of *gata3*, which is expressed in the distal terminus of the DL segment of the pronephros during embryonic stages (Wingert *et al.*, 2007). The role of the *gata3*$^{+}$ segment is unclear but it may act as a common collecting duct or ureter. In support of the latter notion, expression of *gata3* is not observed in mesonephric nephrons (Fig. 10). A schematic of the segmentation patterns of pronephric and mesonephric nephrons is shown in Figure 11.

DISCUSSION

The zebrafish mesonephros undergoes neonephrogenesis throughout larval and adult life, making it a novel model to study nephrogenesis and to investigate unique mechanisms of kidney regeneration. However, our understanding of how fish mesonephric nephrons develop is very limited. It was previously reported that the first zebrafish mesonephric nephron forms in approximately two-week old larvae (5.2 mm) near the level of the caudal swim bladder (Diep *et al.*, 2011; Zhou *et al.*, 2010). Our data here show that this nephron forms on top of the pronephric DE segment and becomes functional by the 6 mm stage based on the filtration of a fluorescent tracer. Additional nephrons are progressively added during juvenile life, first caudal and then rostral to the first-forming nephron. The cellular source of mesonephric nephrons is likely clusters of basophilic *wt1b*-expressing progenitor cells. Our previous data indicated that these clusters arise from the aggregation of single cells marked by the *lhx1a:eGFP* transgene that then go on to activate *wt1b* expression (Diep *et al.*, 2011). Our finding that the *wt1b*$^{+}$ clusters are proliferating extensively is consistent with our prior observations that only 3-4 cells appear sufficient to initiate cluster formation (Diep *et al.*, 2011). As the cluster grows, it epithelializes into a renal vesicle-like structure (Diep *et al.*, 2011). In mammals, nephron induction involves a similar mesenchyme-to-epithelial transition (MET) that is dependent on *Wnt9b* expression from the ureteric epithelium (Carroll *et al.*, 2005). It is not yet known if a similar Wnt signal is operative during

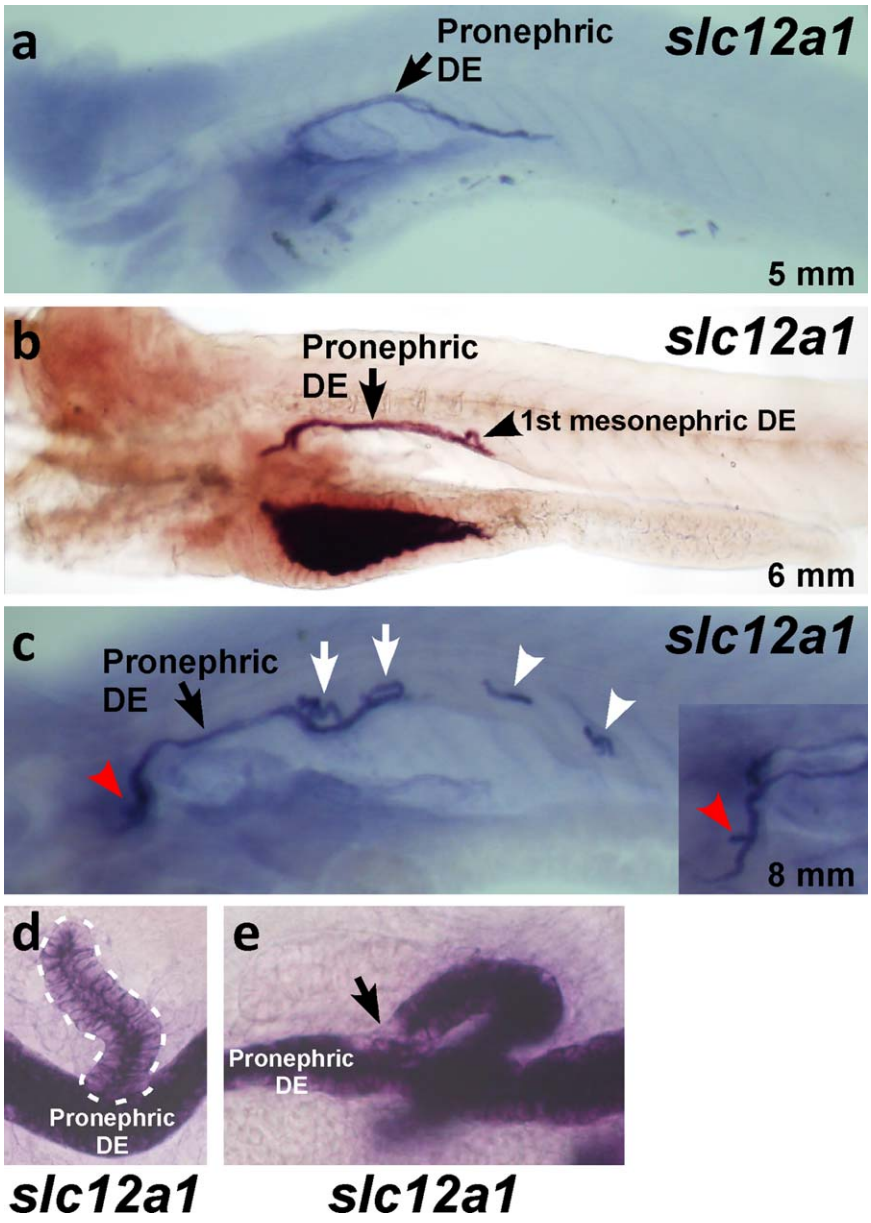

FIG. 8. Expression of *slc12a1* in the DE. **A**–**C**: Whole mount in situ hybridizations of larvae show that the first mesonephric DE forms on top of the pronephric DE and expresses *slc12a1* at the 6 mm stage (B arrowhead). At the 8 mm stage, several more DEs appear on top of the pronephric DE (C white arrows) and pronephric DL (C white arrowheads), and in the rostral region of the pronephric DE (C red arrowhead and inset). The inset is a magnified image of the red arrowhead at a different angle. **D**: A magnified image of a branch point between the mesonephric DE (dashed line) and the pronephric DE. **E**: At the 8 mm stage, *slc12a1* is downregulated at the junction point with the pronephric DE. DE: distal early segment; DL: distal late segment.

zebrafish nephrogenesis. However, the closely related *wnt9a* gene is expressed in the distal segments of the pronephros from 4 dpf and onwards, making this Wnt an excellent candidate for inducing the MET of $wt1b^{+}$ clusters (Curtin *et al*., 2011).

We found that the nascent mesonephric nephron invades into the underlying pronephric tubule, presumably as a part of the morphogenic process of nephron fusion and the establishment of a contiguous lumen. From live imaging of Tg(*lhx1a:eGFP*)/Tg(*cdh17:mCherry*) larvae we found that the *lhx1a*:eGFP transgene remains active in the invading cells during the fusion process and then downregulates. A similar invasive progression has been reported during mammalian nephrogenesis with distal renal vesicle cells penetrating the ureteric epithelium and entering the lumen of the adjacent collecting duct (Kao *et al*., 2012). Mammalian distal renal vesicle cells also express *Lhx1* and because

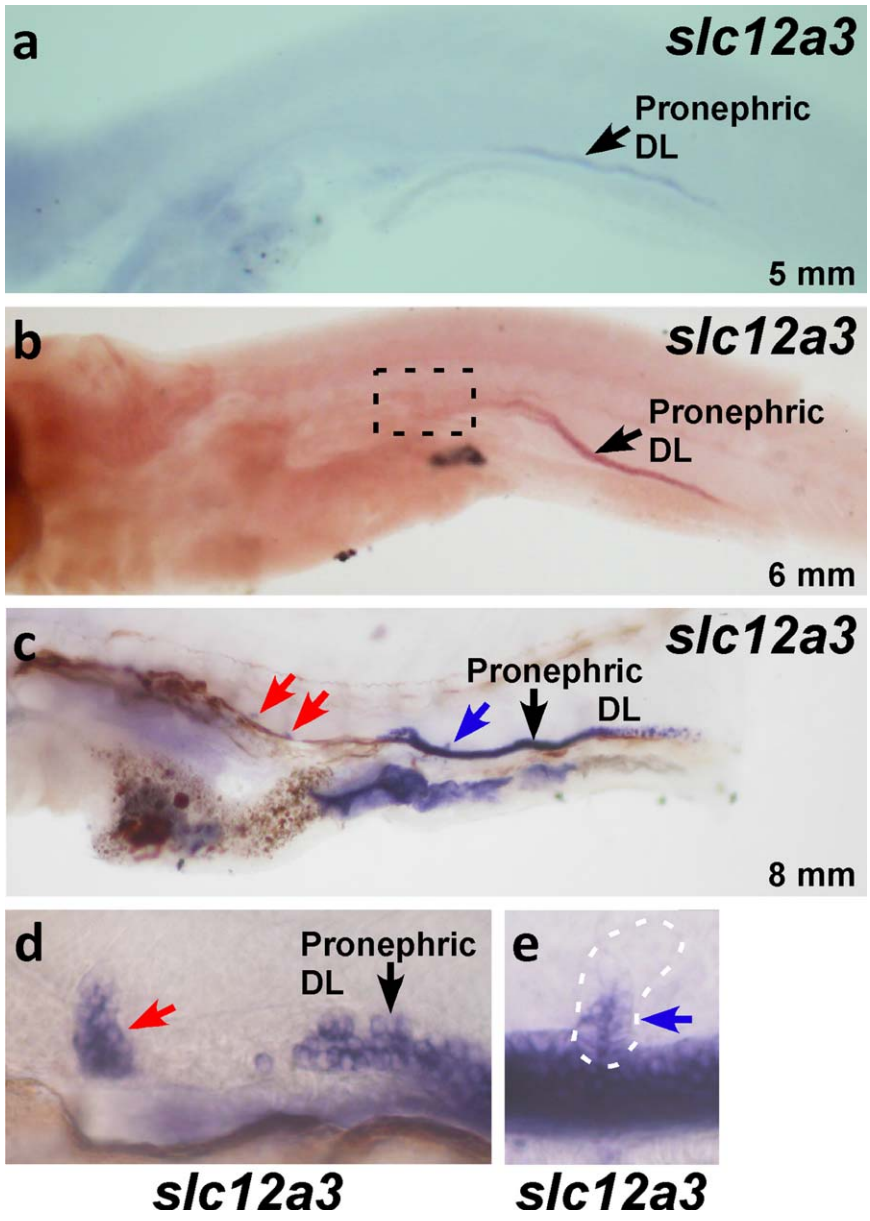

FIG. 9. Expression of *slc12a3* in the DL. **A,B**: Whole mount in situ hybridizations of larvae for the *slc12a3* marker show that the first mesonephric nephron does not form a DL the 6 mm stage (B dashed box). **C–E**: At the 8 mm stage, both rostral and caudal nephrons express *slc12a3* and form on top of the pronephric DE (red arrows) and pronephric DL (blue arrows). D and E are magnified representations of rostral and caudal nephrons at the 8 mm stage. DE: distal early segment; DL: distal late segment.

this transcription factor gene has been implicated as a regulator of migration in other contexts (Winchell and Jacobs, 2013), it raises the possibility that *Lhx1* controls a conserved program of invasive epithelial behavior (Georgas *et al*., 2009; Kao *et al*., 2012).

We showed that the mesonephric nephrons that form on the pronephric DE segment initially develop only the PCT, PST, and DE segments, using the latter to fuse with the pronephros. However, at later stages, *slc12a1* transcripts in these nephrons become downregulated at the junction with the pronephric DE segment, around the time that the DL marker, *slc12a3*, becomes expressed. This result suggests that the patterning of the distal mesonephric nephron is dynamic and raises the possibility that the DL segment arises from the DE segment, perhaps as a result of transdifferentiation. It will be interesting to ascertain whether the appearance of *slc12a3* expression corelates with when

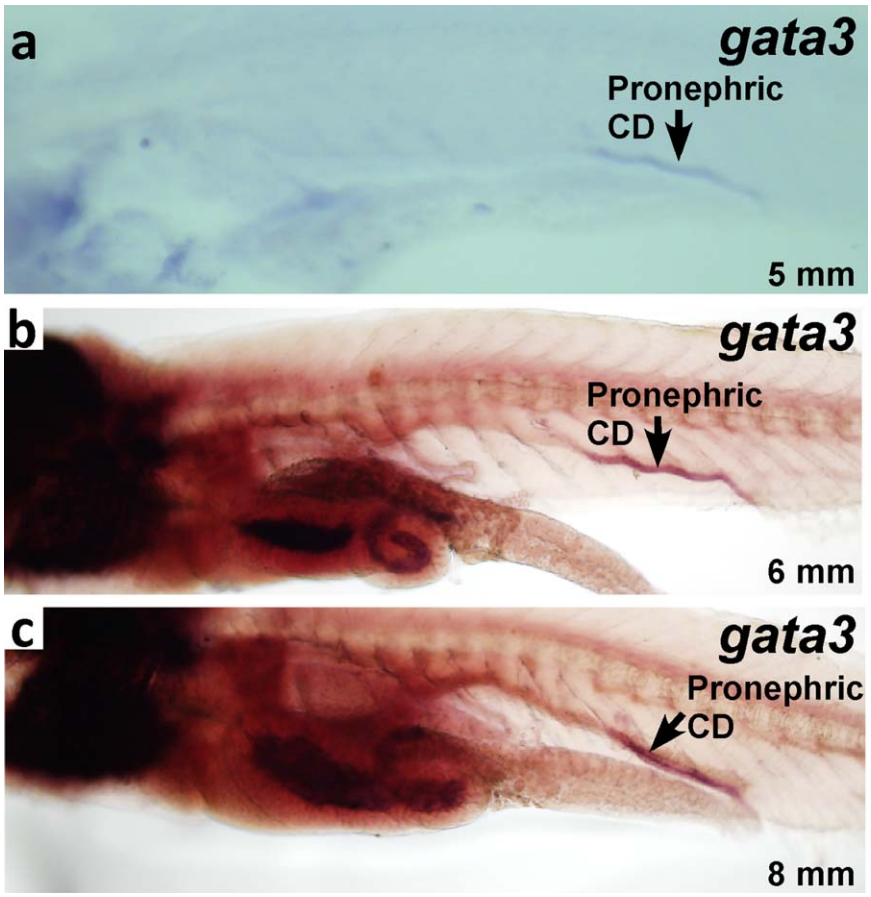

FIG. 10. Expression of *gata3* in the pronephric CD. **A–C**: Whole mount in situ hybridizations of larvae show that *gata3* is not expressed in mesonephric nephrons at the 6 mm or 8 mm stage, but is expressed in a terminal segment of the pronephros. CD: presumptive collecting duct or ureter segment.

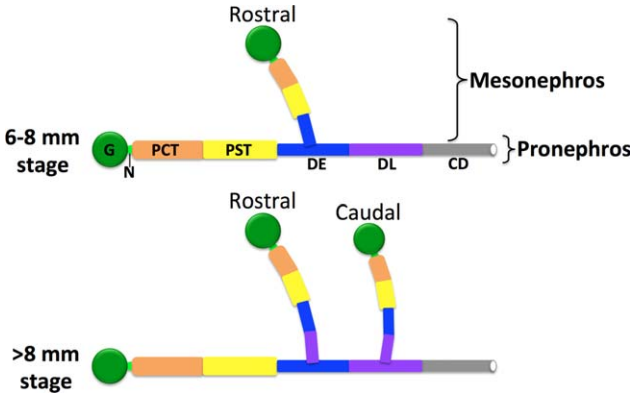

FIG. 11. Schematic representation of pronephric and mesonephric nephrons. Pronephric nephrons are subdivided into glomerulus (G), neck (N), proximal convoluted tubule (PCT), proximal straight tubule (PST), distal early segment (DE), and distal late segment (DL). Mesonephric nephrons initially fuse at the pronephric DE and DL segments and eventually acquire a similar segmentation pattern to pronephric nephrons. All nephrons drain via a common segment, possibly a collecting duct (CD) or ureter to the cloaca.

the nascent nephron becomes functional, as this would suggest that *slc12a3* is being induced in response to fluid flow and/or changes in salt composition.

Although the pronephros provides an essential function during larval life, our observations are consistent with it transitioning into a scaffold for mesonephros formation during metamorphosis. We hypothesize that the pronephric DE/DL segments are the origin of the two major collecting ducts that form along the midline of the adult kidney (Diep *et al.*, 2011; Zhou *et al.*, 2010). The adult mesonephros contains hundreds of nephrons that all need to plumb into these major collecting ducts (Diep *et al.*, 2011; Zhou *et al.*, 2010). We envision a process in which the first wave of mesonephric nephrons fuse with the pronephros directly, forming branches at the pronephric DE and DL segments but as new DL segments form, these would fuse with subsequent waves. Reiterations of this process would eventually lead to the highly arborized network of nephrons that is present in the adult mesonephros.

In summary, our study has provided further insights into the molecular and anatomical basis of zebrafish mesonephros formation. Because zebrafish, but not mammals, retain the ability to add new nephrons after birth, it is hoped that a greater understanding of fish nephrogenesis will help develop novel regenerative therapies in humans. Any such therapy would involve the formation of new nephrons that can functionally integrate with existing nephrons. In this context, the zebrafish mesonephros may serve as a valuable system for understanding how the invasive behavior of nascent nephrons is regulated during the functional integration of 'old' and new tubules.

METHODS

Zebrafish Husbandry

Maintenance of zebrafish was carried out as previously described (Westerfield, 2007). All experiments were approved by the Institutional Animal Care and Use Committee. The Tg(*wt1b:GFP*) and Tg(*lhx1a:eGFP*) transgenic lines were previously reported (Diep *et al*., 2011; Perner *et al*., 2007; Swanhart *et al*., 2010).

Adult and Larval Zebrafish Experiments

Epifluorescent and bright field images were taken from a Nikon Eclipse 80i microscope using the Hamamatsu ORCA-ER camera. Adults: Gentamicin (20 ul of 2 mg/ml) and BrdU (20 ul of 5 mg/ml) were administered by intraperitoneal injection (Diep and Davidson, 2011). The kidneys were dissected 4 h after BrdU injection and processed for histological staining and immunohistochemistry. Larvae: 40 kDa dextran-FITC (1-100 nl of 150 ug/ml) and BrdU (1-100 nl of 5 mg/ml) were injected near the tail region using glass capillary needles. Larvae were processed 4 h after BrdU injection for histological staining and immunohistochemistry.

Whole-Mount *In Situ* Hybridization

Whole-mount in situ hybridization was performed as previously described (Diep *et al*., 2011; Elizondo *et al*., 2005). The markers *cdh17*, *gata3*, *lhx1a*, *nphs1*, *pax2a*, *slc12a1*, *slc12a3*, *slc20a1a*, *trpm7*, and *wt1b* have been reported earlier (Bollig *et al*., 2006; Drummond *et al*., 1998; O'Brien *et al*., 2011; Toyama *et al*., 1995; Wingert *et al*., 2007). Anti-sense RNA probes (digoxigenin-labeled) were synthesized using T7 or SP6 RNA polymerase from Roche Diagnostics.

Histology and Immunohistochemistry

Hematoxylin and eosin: larvae and adult kidneys were fixed in 4% paraformaldehyde/1% DMSO, embedded in paraffin, sectioned, and stained with hematoxylin and eosin or antibodies against GFP and BrdU (Dana-Farber/Harvard Cancer Center Pathology Core Facility). Methylene blue and basic fuchsin: larvae were fixed in 4% paraformaldehyde/1% DMSO, embedded in JB4 resin, sectioned, and stained with methylene blue and basic fuchsin.

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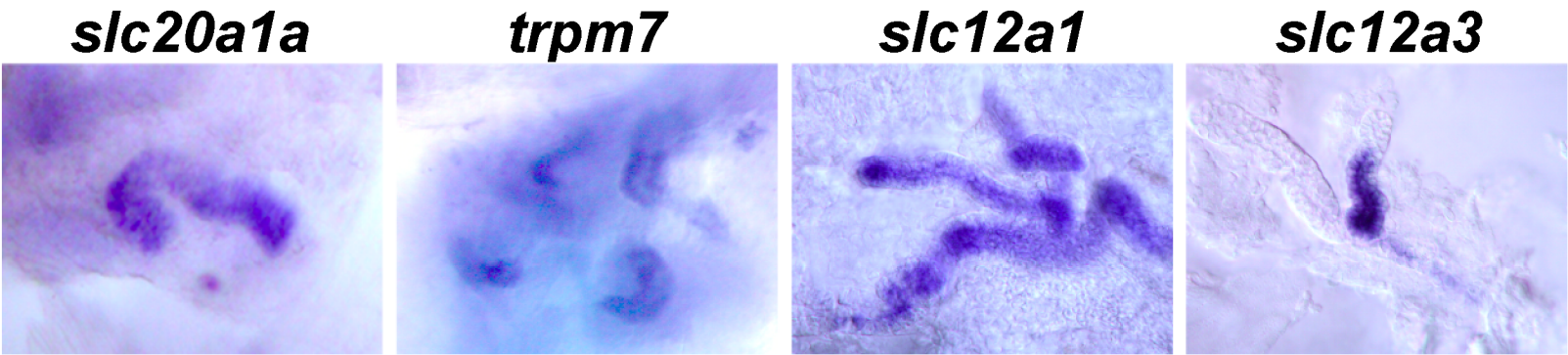
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