Developmental Biology 222, 147–157 (2000)
doi:10.1006/dbio.2000.9642, available online at http://www.idealibrary.com on IDEAL®

The Zebrafish *floating head* Mutant Demonstrates Podocytes Play an Important Role in Directing Glomerular Differentiation

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In zebrafish, the pronephric glomerulus occupies a midline position underneath the notochord and is vascularized through angiogenic capillary ingrowth from the dorsal aorta. The midline mutants *floating head* (*flh*), *sonic you* (*syu*), and *you-too* (*yot*) provide the opportunity to study glomerular differentiation in the absence of the notochord and vascularization from the dorsal aorta. In *flh, syu,* and *yot* mutants, glomeruli differentiate at ectopic lateral positions within the embryo and contain morphologically identifiable podocyte and endothelial cell types. In the absence of the dorsal aorta, endothelia from an alternate source are recruited by podocytes during glomerular vascularization to make functional glomeruli. Our results suggest that midline signals are required for proper glomerular morphogenesis but not for the differentiation of podocytes. Podocytes appear to play an important role in directing cellular recruitment events leading to glomerular differentiation. Furthermore, we find defects in sclerotomal development that correlate with defects in glomerular morphogenesis suggesting a possible link between the formation of these embryonic structures. © 2000 Academic Press

Key Words: zebrafish; *floating head;* pronephros; glomerulus; podocyte; sclerotome.

INTRODUCTION

The nephron is the functional unit of the vertebrate kidney, performing the essential functions of blood filtration and fluid homeostasis. Nephron development and the formation of the glomerulus occurs as the result of a series of cellular interactions, giving rise to the complex arrangement of podocytes, capillary endothelial cells, and supporting mesangial cells that characterize the glomerular blood filter (Takahashi *et al.*, 1998). The sequential recruitment and assembly of glomerular cell types is thought to involve cell–cell signaling mediated by secreted factors. Tissue grafting experiments using mouse nephrogenic mesenchyme and avian chorioallantoic membrane (CAM) have implicated a requirement for differentiated renal epithelia for vascularization and the development of glomerular capillaries (Sariola *et al.*, 1983). Tissue grafting experiments have also demonstrated the importance of podocyte/endothelial cell interactions for glomerular basement membrane (GBM) synthesis during glomerular differentiation (Abrahamson, 1991; Sariola *et al.*, 1984). However, the absence of complete glomerular development in these experiments has precluded a closer investigation of cellular interactions during glomerulogenesis.

Subsequent studies have shown that glomerular podocytes express vascular endothelial growth factor (VEGF) while neighboring capillary forming endothelial cells express flk-1, a receptor for VEGF (Breier *et al.*, 1995; Millauer *et al.*, 1993; Simon *et al.*, 1995). The VEGF/flk signaling pathway has been shown to be required for angiogenesis and vasculogenesis (Neufeld *et al.*, 1999; Risau, 1998; Takahashi *et al.*, 1998). However, VEGF and flk-1 knock-out mice die prior to metanephric development due to severe circulatory system defects, thus preventing a genetic analysis of VEGF/flk signaling during kidney vascularization (Carmeliet *et al.*, 1996; Ferrara, 1999; Ferrara *et al.*, 1996; Shalaby *et al.*, 1995, 1997). Antibody blocking experiments using injections of anti-VEGF have to some extent circumvented this limitation and demonstrate a requirement for VEGF in formation of kidney microvasculature (Kitamoto *et al.*, 1997). These observations suggest an important role for VEGF/flk signaling between podocytes and endothelia in the glomerular vascularization process. The recruitment of glomerular mesangial cells, a smooth muscle-like cell

0012-1606/00 $35.00

type that stabilizes glomerular structure by interconnecting the glomerular capillary branches, has also been shown to depend on cell signaling. PDGFβ and PDGF-Rβ mutant mouse kidneys lack glomerular mesangial cells, indicating an essential role for PDGFβ signaling in the migration and differentiation of mesangial cells (Lindahl *et al.*, 1998).

In zebrafish, the embryonic pronephric glomerulus occupies a midline position ventral to the notochord and is vascularized through angiogenic capillary ingrowth from the dorsal aorta (Drummond *et al.*, 1998). Histological studies in fish have described the morphogenesis of the pronephros and the differentiation of the glomerulus, tubule, and duct components (Armstrong, 1932; Drummond *et al.*, 1998; Goodrich, 1930; Vize *et al.*, 1997). The pronephric nephron primordia are bilateral paired structures formed by a process of inpouching and septation of the coelomic lining. Between 30 and 40 h postfertilization (hpf), the initially lateral primordia coalesce such that their medial surfaces abut at the embryo midline. Vascularization of the glomerulus follows closely thereafter with angiogenic vessel ingrowth from the overlying dorsal aorta. Just prior to hatching (48 hpf) the pronephros is a functional, blood filtering kidney.

The proximity of the pronephric glomerulus to the notochord has focused attention on a possible role for midline signaling in pronephric glomerular differentiation. The notochord, in addition to its role in patterning the neural tube has been shown to direct development of ventral and lateral tissues such as the pancreas and derivatives of the somites (Dodd *et al.*, 1998; Hebrok *et al.*, 1998; Kim *et al.*, 1997; Placzek, 1995). Studies in medaka have also suggested a role for Sonic Hedgehog, a notochord-derived secreted protein, in the expression of a fish *spalt* homolog during pronephric differentiation (Koster *et al.*, 1997). In this report we have taken advantage of zebrafish midline mutants *floating head* (*flh*), *sonic you* (*syu*), and *you-too* (*yot*) to assess glomerular development in the absence of notochord signaling and the dorsal aorta. Owing to mutations in a notochord-specific helix–loop–helix transcription factor, *flh* mutant embryos lack a notochord and precursor cells are transfated to muscle (Halpern *et al.*, 1995; Melby *et al.*, 1996; Talbot *et al.*, 1995). As a consequence, *flh* mutants lack a dorsal aorta, the normal source of vasculature for the pronephros (Fouquet *et al.*, 1997; Sumoy *et al.*, 1997). *syu* mutants lack a functional sonic hedgehog gene and show a number of developmental patterning defects in the floor plate, somites, and fins, in addition to perturbation of motorneuron axon pathfinding (Schauerte *et al.*, 1998). In these mutants, we show that glomerular morphogenesis is disrupted and nephric primordia differentiate to give rise to morphologically identifiable podocytes in ectopic, lateral locations. In *flh* mutants, we find that endothelial cells are recruited by podocytes that proceed to form ectopic glomeruli capable of blood filtration. Our results suggest an important role for podocytes in directing glomerular differentiation. We also demonstrate an intimate association of the glomerulus with sclerotome derivatives and show that failed glomerular morphogenesis in midline mutants correlates with the absence of sclerotome tissue that normally surrounds the pronephric glomerulus.

MATERIALS AND METHODS

Zebrafish stocks. Zebrafish were grown and mated at 28.5°C. The *flh*^{n1} null allele was used for all experiments (Talbot *et al.*, 1995). *syu*$^{tbx392/t4}$ heteroallelic embryos exhibit a strong loss of *syu* function and were a kind gift from H. Schauerte and P. Haffter (Schauerte *et al.*, 1998). Homozygous *yot*^{ty119} embryos were also a gift from H. Schauerte and P. Haffter (Karlstrom *et al.*, 1999).

Histological sections. Zebrafish embryos were fixed in BT fix (Westerfield, 1993), dehydrated, and embedded in JB-4 (Polysciences) for plastic sectioning. Sections of 4-μm thickness were cut, stained using methylene blue/azure II (Humphrey and Pittman, 1974), and Permount (Sigma) mounted for light microscopy. Photographs were taken on Kodak Ektachrome 160T film on a Zeiss Axioplan microscope.

In situ hybridization. Whole-mount *in situ* hybridization was performed as described using digoxygenin-labeled antisense RNA probes and α-digoxygenin alkaline phosphatase-conjugated antibodies (Boehringer Mannheim) (Oxtoby and Jowett, 1993). Hybridized embryos were cleared in benzyl benzoate/benzyl alcohol 2:1 and photographed on Kodak 160 Tungsten film. Tissue sections were made by embedding embryos in JB-4 plastic, sectioning at 4 μm, and they were photographed using DIC optics. The following templates were linearized and transcribed with RNA polymerase (Boehringer Mannheim) to make antisense probes: Wilm's tumor suppressor gene *wt1 Not*I/T7, vascular endothelial growth factor (VEGF) *Eco*RI/T3, *flk-1 Eco*RI/T7, *tie-1 Eco*RI/T7, SM22 *Eco*RI/T7, *twist Xba*/T7, *Pax9 Not*/T7, and *you-too/gli2 Bam*HI/T7.

Dye filtration. A solution of 1% lysine fixable rhodamine dextran dye (Molecular Probes) was injected into the blood circulation at the sinus venosus of 2-day postfertilization (dpf) embryos. Dye was allowed to circulate for 7 min and embryos were then fixed in BT fix. Embryos were embedded and sectioned as described above. For wheat germ agglutinin (WGA, Vector Labs) staining, dye-injected, fixed embryos were stained with a 1:10 dilution of WGA for 2 h at room temperature and then embedded for sectioning. Sections were photographed using Kodak Elite 400 film on a Zeiss Axioplan microscope.

Electron microscopy. Embryos at 2 dpf were fixed in 1.5% glutaraldehyde/1% paraformaldehyde/70 mM $NaPO_4$ pH 7.2/3% sucrose at 4°C overnight. Embryos were washed in 0.1 M cacodylate buffer, pH 7.4, and postfixed in 1% OsO_4/1.5% postassium ferrocyanide for 3 h at room temperature without agitation. Embryos were washed in cacodylate buffer and taken through an ethanol dehydration series. Embryos were first infiltrated in propylene oxide and then infiltrated through a propylene oxide/Epon 812 series. Finally, embryos were embedded in Epon 812 and sectioned. For electron microscopy analysis, thin sections were cut on a Reichert Ultracut E ultramicrotome and collected onto Formvar-coated slot grids. Sections were poststained with uranyl acetate and lead citrate and viewed in a Phillips CM10 electron microscope at 80 keV.

RESULTS

Pronephric Glomerular Morphogenesis but Not Podocyte Differentiation Is Affected in floating head, sonic you, and you-too

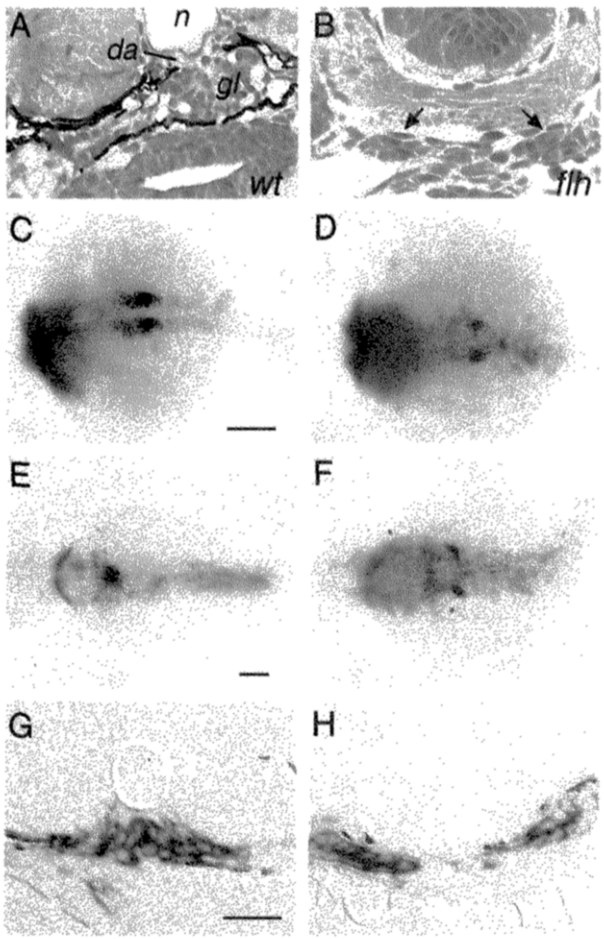
In wildtype zebrafish, the pronephric glomerulus occupies a midline position directly underneath dorsal aorta and notochord (Drummond *et al.*, 1998). Podocytes are intimately associated with capillary endothelial cells and a capsular parietal epithelium enclosing a Bowman's space is clearly visible (Fig. 1A). In histological sections from *flh* mutants, a midline glomerulus is not found (Fig. 1B). Instead, clusters of cells are found in ectopic lateral locations at the level of the third somite, the anteroposterior position where the kidney normally forms. Other components of the pronephros, the tubules and the ducts, are present in *flh* and appear normal (data not shown).

To follow glomerular development and determine whether podocyte differentiation was occurring in *flh*, we performed *in situ* hybridization on *flh* embryos with the Wilm's tumor suppressor gene *wt1* probe. *wt1* is first expressed by cells making up the pronephric primordia and *wt1* expression is later maintained only in differentiated podocytes (Drummond *et al.*, 1998). At 20 hpf, *wt1* labels the two nephric primordia (Fig. 1C). By 48 hpf, these primordia have coalesced at the midline where podocytes continue to express *wt1* (Figs. 1E and 1G). In *flh* mutants, nephric primordia are visualized by *wt1* at 20 hpf (Fig. 1D). However, these primordia never occupy a midline position and instead remain at ectopic lateral locations (Figs. 1F and 1H). The *wt1* expression is maintained at 48 hpf suggesting that podocytes are differentiating in *flh* but that they are doing so at lateral locations.

To explore potential mechanisms underlying the defective morphogenesis of the glomerulus, we examined pronephric development in the mutants *syu* and *yot*, which carry mutations in the zebrafish *sonic hedgehog* (*shh*) and *gli2* genes, respectively (Karlstrom *et al.*, 1999; Schauerte *et al.*, 1998). The *shh* gene encodes a well-characterized secreted factor expressed in the notochord and floor plate and has been shown to play an important role in patterning neighboring tissues (Dodd *et al.*, 1998; Placzek, 1995; Schauerte *et al.*, 1998). Owing to the absence of a notochord, *shh* expression is reduced in *flh* mutants (Talbot *et al.*, 1995). In contrast to *flh*, *syu* mutants have a morphologically normal notochord (Schauerte *et al.*, 1998). Histological analysis revealed that similar to *flh*, a midline glomerulus is not present in *syu* mutants and instead clusters of cells are found at lateral positions (Fig. 2A). *wt1*-expressing cells are also found in ectopic lateral locations in *syu* mutants indicating that *flh* and *syu* share a common pronephric phenotype (Fig. 2B). To further analyze whether sonic hedgehog acts directly on the pronephric primordium cells or whether it may have indirect effects on tissues that support pronephric structure, we examined development in *yot* embryos which lack a functional *gli2* gene (Karlstrom *et al.*, 1999). In several tissues, *gli2* is required cell autonomously in cells stimulated by sonic hedgehog to transduce transcriptional responses (van Eeden *et al.*, 1996). The mutant *yot* displays a bifurcated pronephric phenotype similar to *syu* and *flh* (Fig. 2C). Analysis of *gli2* expression by *in situ* hybridization revealed that although *gli2* is expressed in the fin bud and neural tube no expression is detected in the pronephric primordia (Fig. 2D). This result suggests that defects in the sonic hedgehog signaling pathway act indirectly to cause the observed disruption of pronephric morphogenesis. We therefore examined whether supporting tissues and structures in the environment of the forming glomerulus may be altered or missing in midline mutants. In histological examinations of older larvae we found that the pronephric glomerulus persists for at least 3 weeks and at this stage is supported by

FIG. 1. Glomerular development is altered in *flh*. Transverse histological sections (A, B). In wildtype zebrafish embryos at 2 days postfertilization (dpf) (A) a midline glomerulus (gl) is seen underneath the notochord (n) and dorsal aorta (da). A midline glomerulus is not seen in *flh* (B) at 2 dpf. Instead, clusters of cells are found in ectopic locations in *flh* (arrows in B). Whole-mount *in situ* hybridization with the *wt1* probe (C–F). *wt1* is expressed in nephric primordia in wildtype embryos at 20 h postfertilization (hpf) (C) and later at 48 hpf (E) in differentiated podocytes after pronephric primordia fuse in the midline. *wt1* is expressed in *flh* nephric primordia at 20 hpf (D), but *wt1*-expressing cells remain in ectopic lateral positions at 48 hpf (F). Transverse sections of *wt1* hybridized 36-hpf embryos (G, H). *wt1*-expressing cells are found at the midline in wildtype siblings (G) and in laterally positioned cells in *flh* (H). Some brown pigment granules are seen in these sections. Scale bars: (C, D) 200 μm; (E, F) 250 μm; (G, H) 50 μm.

FIG. 2. Glomerular defects in *sonic you* and *you-too* mutants correlate with failed sclerotome development. Transverse section of a 48-hpf $syu^{tbx392/t4}$ embryo (A) reveals the absence of a midline glomerulus and in its place are two lateral cell clusters (arrows). *In situ* hybridization of $syu^{tbx392/t4}$ embryos with a *wt1* probe (B) identifies the lateral cell clusters as podocytes. Similar histological section of a 36-hpf *you-too* embryo (C) shows a failed convergence of podocytes. *In situ* hybridization with *gli2* probe (D) shows expression in the neural tube and fin bud (dark arrows) but no expression in the pronephric primordia (light arrow). Transverse histological section through a wildtype 3-week-old zebrafish (E) shows the glomerulus (gl) is surrounded by bone and cartilaginous tissue (double arrows; da, dorsal aorta). *twist*-positive cells are found in the wildtype ventromedial somite at 18 hpf (F). *twist*-positive cells are absent in *flh* mutants (G). *Pax9*-expressing cells are found between neural tube and somite tissue in 24-hpf wildtype embryos (H). *Pax9*-expressing cells are reduced in *flh* (I). Scale bar equals 50 μm.



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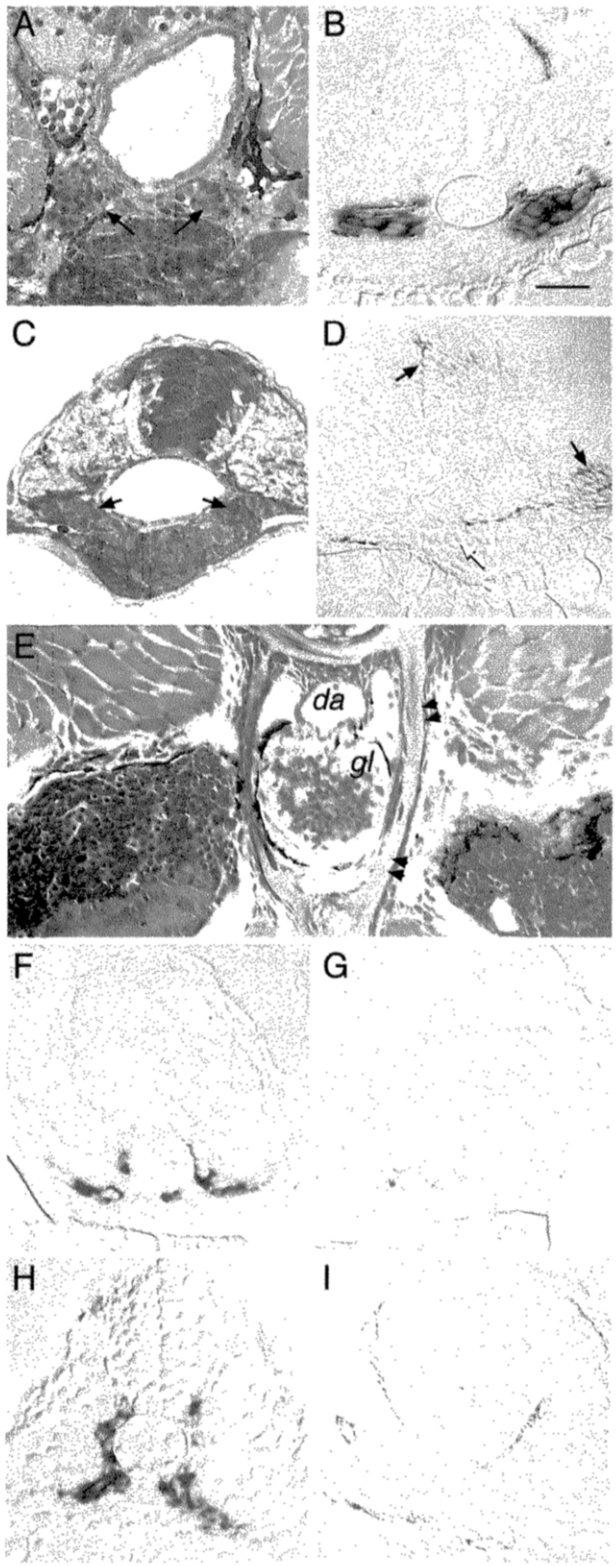

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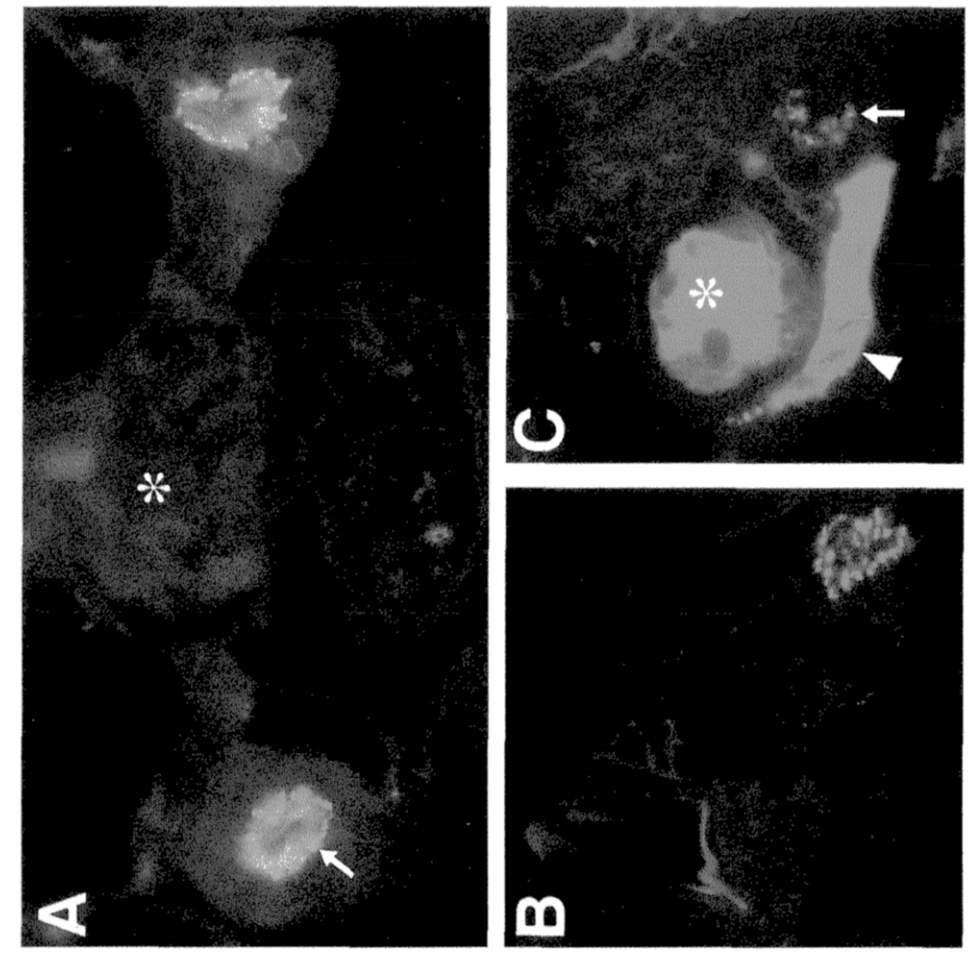

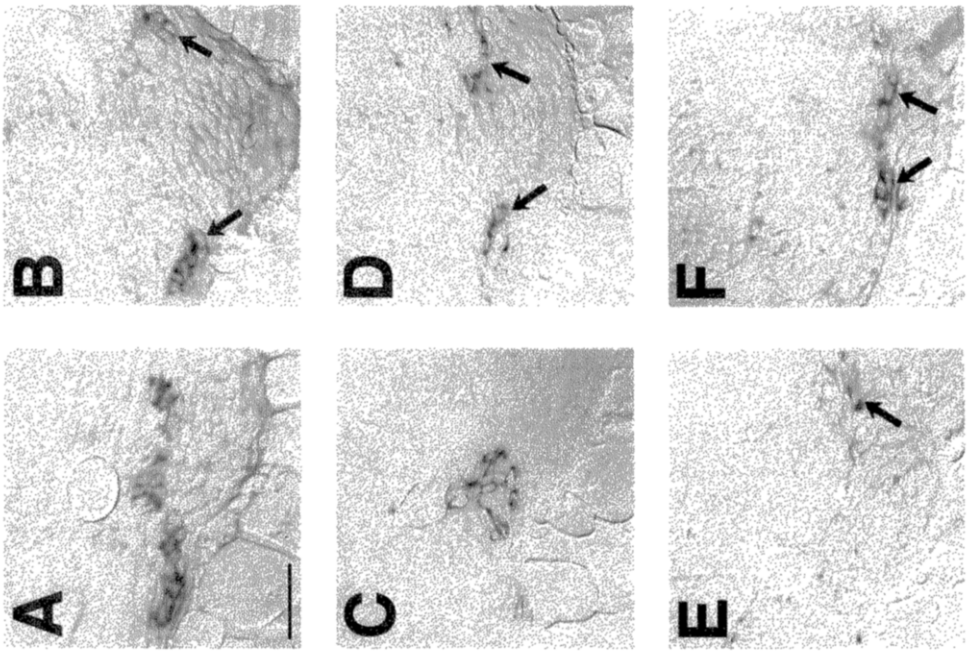

FIG. 3. Glomeruli differentiate at ectopic locations in *flh*. Wildtype (A, C, E) and *flh* (B, D, F) embryos hybridized with *in situ* probes: VEGF (A, B) at 40 hpf, *flk-1* (C, D) at 40 hpf, and *tie-1* (E, F) at 48 hpf. Differentiated podocytes express VEGF in wildtype embryos (A). VEGF-positive cells are found in ectopic lateral locations in *flh* (arrows in B). *flk-1* labels glomerular capillary-forming endothelial cells in wildtype (C). *flk-1*-positive cells surround laterally positioned nephric primordia in *flh* (arrows in D). *flk-1*-positive cells are found in between nephric primordia and cardinal veins. *tie-1* labels endothelia from cardinal veins (arrow in E) but not dorsal aorta in wildtype embryos at this time. In *flh*, *tie-1*-positive cells are associated with nephric primordia (arrows in F). Scale bar equals 50 μm.

FIG. 4. *flh* embryos make functional glomeruli as assayed by filtration of rhodamine dextran dye. Rhodamine dextran dye (red) has been injected into the sinus venosus, filtered through the glomerulus, and taken up into duct endosomes (arrow) in 2-dpf wildtype embryos (A, B). Note that no dye is retained in the glomerulus (asterisk in A). WGA staining (green) positively identifies the duct apical brush border. In 2 dpf *flh* embryos (C) injected with rhodamine dextran, dye is found in the cardinal vein (arrowhead), ectopic glomeruli (asterisk), and duct endosomes (arrow). Dye is frequently trapped in glomeruli. Sections in B and C have not been stained with WGA.

bone (Fig. 2E). Since the sclerotome, the progenitor of vertebral bone, is a somite derivative known to be influenced by hedgehog signaling in mouse and chick development, we examined whether the differentiation or migration of sclerotome cells may correlate with glomerular defects. Sclerotome development can be followed using the *Pax9* and *twist* markers (Morin-Kensicki and Eisen, 1997; Nornes *et al.*, 1996). In wildtype 18 hpf embryos, *twist* positive cells are found in the ventral region of each somite (Fig. 2F) migrating to an eventual midline position by 48 hpf (data not shown). In *flh* embryos, somite derived *twist* positive cells are not observed, suggesting that notochord signals are needed for sclerotome development (Fig. 2G). Similar results are seen with *Pax9* expression (Figs. 2H and 2I). The data suggest that sclerotome differentiation is defective in the *flh* mutant and that the absence of supporting tissue may result in the failure of pronephric primordia to fuse. Although midline signals are required for proper glomerular morphogenesis, the data also indicate that neither *shh* nor signals from a differentiated notochord are necessary for podocyte cell differentiation as assayed by *wt1* expression.

Glomerular Differentiation in flh

The expression of *wt1* in displaced podocytes suggested that glomerular differentiation may occur in *flh* despite the absence of supporting tissue and the dorsal aorta, the normal source of pronephric vasculature. To establish whether glomeruli could form in ectopic positions, we first established the normal course of events as ingrowth of vessels to the glomerulus occurred. Cell–cell signaling through the VEGF/flk receptor tyrosine kinase signaling pathway has been shown to be important for angiogenesis and vasculogenesis including the assembly of blood vessels during glomerular vascularization (Neufeld *et al.*, 1999; Risau, 1998; Takahashi *et al.*, 1998). A zebrafish VEGF has been shown to be expressed during vasculogenic events, while zebrafish *flk-1*, a putative VEGF receptor, has been shown to be expressed in endothelial cells at the early stages of vascular development (Fouquet *et al.*, 1997; Liang *et al.*, 1998; Liao *et al.*, 1997). Consistent with a role in wildtype pronephric glomerular development, we find that VEGF is expressed in differentiating podocytes in the pronephros (Fig. 3A). In a complementary fashion, *flk-1* is expressed in ingressing endothelial cells derived from the dorsal aorta (Fig. 3C). The results indicate that VEGF/flk-1 signaling coincides with glomerular angiogenesis in the pronephros and may play an important role in this process. The receptor tyrosine kinase *tie-1* is a later marker of endothelial differentiation and appears after *flk-1* (Dumont *et al.*, 1995; Lyons *et al.*, 1998). In wildtype embryos at 24 hpf *tie-1* is seen in both the dorsal aorta and the cardinal vein, but at 48 hpf *tie-1* expression is seen predominantly in endothelial cells of the cardinal veins but not in endothelial cells of the dorsal aorta (Fig. 3E).

In order to determine the extent of glomerular differentiation in *flh*, we used VEGF and *flk-1* as markers to assess whether endothelial capillaries are associated with podocytes in *flh* mutants. In *flh* embryos, VEGF-expressing cells are found in the region of the pronephros but at ectopic lateral locations, supporting the notion that podocyte differentiation occurs in *flh* at novel locations (Fig. 3B). Significantly, *flk-1*-positive cells are found surrounding VEGF-expressing cells in *flh*, suggesting that in the absence of their normal vascular input, podocytes may be capable of recruiting endothelial cells even at ectopic locations (Fig. 3D). Since the dorsal aorta is missing in *flh*, *flk-1*-positive cells observed in mutants must come from an alternate origin. *tie-1*-positive cells can also be observed around ectopic podocytes in *flh* providing further evidence for podocyte endothelial interactions (Fig. 3F).

flh Embryos Make Functional Glomeruli

Our results demonstrating that the expression of a variety of glomerular cell type differentiation markers could be observed even in ectopic lateral locations in *flh* suggested the possibility that complete development of a functional glomerulus may occur, despite the absence of the dorsal aorta. The observation that *flh* embryos only show evidence of edema at 4 days of development further implies that at hatching these mutant embryos must have an at least partially functional system for osmoregulation and the elimination of excess water. To test whether glomerular filtration was occurring in *flh* embryos, rhodamine dextran was injected into the circulatory system. Successful glomerular filtration was assayed by the presence of dye in apical endosomes of pronephric duct cells. Consistent with previous studies of glomerular function, wildtype embryos at 72 hpf showed prominent uptake of dye in pronephric duct cells, confirming that glomerular filtration is active at this stage (Figs. 4A and 4B; Drummond *et al.*, 1998). In *flh*, we find rhodamine dextran dye in the glomeruli and in duct endosomes demonstrating that mutant embryos make at least partially functional glomeruli (Fig. 4C). The cardinal veins, highlighted by injected dye, are found adjacent to ectopic glomeruli, suggesting a vascular continuity between the glomerular capillary tuft and the venous blood supply.

Ultrastructure of flh Glomeruli

To determine whether glomeruli in *flh* contained the appropriate cell types and to assay the extent of podocyte and endothelial morphological differentiation, we studied *flh* pronephroi by electron microscopy. By 48 hpf, the wildtype glomerulus is a stereotyped and organized structure with podocytes sitting on the outer aspect of the glomerular basement membrane (GBM) and endothelial cells lining the inner aspect of the GBM (Figs. 5A and 5B).

Podocytes contact the GBM through well-formed and regularly spaced foot processes.

In 48 hpf *flh* mutants, we find red-blood-cell-containing capillaries adjacent to the cardinal veins (Figs. 5C and 5D). Morphologically identifiable podocytes are seen surrounding the vasculature within the ectopic glomeruli. A Bowman's or urinary space is also seen separating podoctyes from the parietal epithelial layer. The presence of red blood cells within the capillary space demonstrates that *flh* glomeruli are receiving vascular input, confirming our rhodamine dye injection results.

Higher magnification views show podocytes and endothelial cells on opposite sides of a well-formed and continuous GBM (Fig. 5E). Podocytes appear fully differentiated and display well-formed and regularly spaced foot processes that contact the outer aspect of the GBM. The GBM appears morphologically normal, exhibiting a trilaminar structure with a robust lamina densa and neighboring lamina rarae.

Specialized endothelia with filtration and secretion functions have fenestrated membranes (Palade *et al.*, 1979; Risau, 1998). Glomerular capillary endothelial membranes in zebrafish normally have a fenestrated morphology that is visible at the ultrastructural level (Fig. 5A). Endothelial membranes touch the GBM at contact points that are separated by pores. In *flh*, capillary endothelial membranes are not fenestrated displaying a continuous membrane morphology (Fig. 5E). Membrane can be seen contacting the GBM, but there are many places where the membrane is detached and membrane processes can be seen extending into the vascular space. Although endothelia are recruited to ectopic podocytes to form capillaries, these results suggest that in *flh*, these endothelial cells are not competent to form a normal fenestrated glomerular capillary tuft.

DISCUSSION

The location of the zebrafish pronephric glomerulus in the midline raised the possibility that signals emanating from the notochord might be required for podocyte differentiation or proper glomerular morphogenesis. We tested the requirement for midline signaling in pronephric development with three molecularly and phenotypically well-characterized midline mutants, *flh*, *syu*, and *yot*, and have found that midline signals are required for proper glomerular morphogenesis but not podocyte differentiation. Our observation that podocytes are able to recruit appropriate cell types to yield functional glomeruli, even in novel locations in the embryo, leads us to the view that podocytes perform a primary role in driving glomerular differentiation.

Endothelial Cell Recruitment during Glomerular Differentiation

Classical studies employing mouse metanephric mesenchyme to chick chorioallantoic membrane chimeras suggested endothelial cells are recruited from an extramesenchymal source during glomerular capillary formation (Sariola *et al.*, 1983). However, more recent transplantation experiments suggest that formation of the glomerular capillary tuft occurs through both vasculogenic and angiogenic processes during metanephric kidney development (Abrahamson *et al.*, 1998; Hyink *et al.*, 1996; Robert *et al.*, 1996).

VEGF/flk signaling plays an important regulatory role in blood vessel growth during vasculogenic and angiogenic events associated with organogenesis and tumor formation. During vertebrate metanephric development, VEGF has been shown to be expressed by differentiated glomerular podocytes while the *flk-1* receptor is expressed on neighboring endothelial cells suggesting this signaling pathway may be used by podocytes to recruit endothelial cells to make capillaries (Breier *et al.*, 1995; Millauer *et al.*, 1993; Simon *et al.*, 1995; Takahashi *et al.*, 1998). In zebrafish, the glomerulus is normally vascularized by capillary ingrowth from the dorsal aorta through an angiogenic mechanism (Drummond *et al.*, 1998; Majumdar and Drummond, 1999). The conserved pattern of VEGF/*flk-1* expression in podocytes/endothelia during glomerular vascularization in both wildtype and mutant embryos suggests that this signaling pathway may also mediate glomerular capillary formation in zebrafish.

Glomerular capillary formation in *flh* could occur through either an angiogenic or a vasculogenic mechanism. The association of ectopic glomeruli with the cardinal vein suggests that the capillary-forming endothelial cells may arise as an angiogenic outgrowth of the cardinal vein. Alternatively, angioblasts distributed in surrounding mesenchyme could be recruited by VEGF-expressing podocytes to form capillaries by a vasculogenic process. Although at present we are not able to discriminate between these two possibilities, we do observe differences between the endothelium formed in ectopic glomeruli and the normal glomerular vasculature derived from the dorsal aorta.

Unlike wildtype glomerular capillaries, the capillary endothelium in *flh* lack fenestrations. Capillary fenestrations in the kidney and other organs are thought to increase vascular permeability and, in the case of the glomerulus, facilitate blood plasma filtration (Palade *et al.*, 1979; Risau, 1998). Wildtype glomeruli pass injected dye completely into pronephric ducts where it is taken up into endosomes while glomeruli in *flh* show a compromised ability to filter blood as evidenced by the retention of rhodamine dye in glomeruli. The lack of fenestration in *flh* glomerular capillaries may result in low transendothelial permeability and explain the glomerular trapping of injected rhodamine dye. The absence of fenestrations in *flh* glomerular capillaries could be due to the absence of appropriate podocyte-derived signals or to a lack of competence of endothelial cells to respond to podocyte-derived secreted factors. While it is possible that in the absence of a notochord podocyte development may not be completely normal, we do observe that

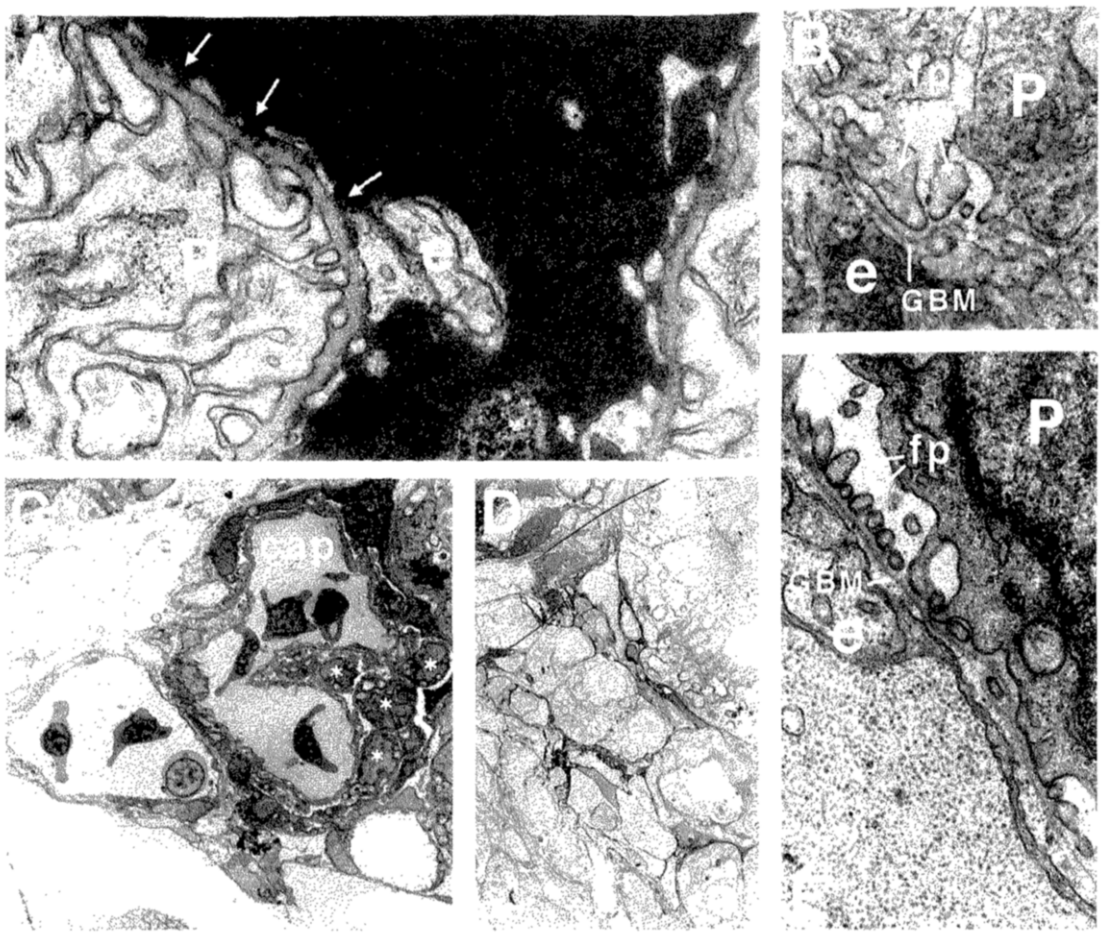

FIG. 5. *flh* glomeruli contain appropriate glomerular cell types. In wildtype (A, B), podocytes (P) and endothelial cells (e) are found on opposite surfaces of the trilaminar glomerular basement membrane (GBM). Podocytes make well-formed, evenly spaced foot processes. Capillary endothelia show a fenestrated membrane morphology and pores are present (arrows in A). In *flh* glomeruli (C, D, E), blood-cell-containing capillaries (cap) are found adjacent to the cardinal vein (cv). In D, a vascular continuity (shaded in red) between the capillary space and the cardinal vein is visible and is surrounded by podocytes (shaded in blue). Capillary endothelia are intimately associated with morphologically identifiable podocytes in *flh* (asterisks in C). Podocytes and endothelia are organized on opposite sides of a well-formed and continuous GBM with well-formed lamina densa and lamina rarae (E). Podocytes appear morphologically normal with well-formed foot processes (fp). Significantly, endothelial cell membranes are nonfenestrated and continuous. Pores are absent. Occasionally, the membranes are detached from the GBM. Original magnifications: (A, B, E) 40,000×; (C, D) 1600×.

ectopic podocytes express VEGF, which has been shown to be sufficient to induce the formation of endothelial fenestrations *in vivo* within 10 min of application (Roberts and Palade, 1995) and *in vitro* (Esser *et al.*, 1998). We therefore favor the alternative hypothesis that the lack of fenestrations may indicate that in *flh* glomeruli, the endothelial cells, whatever their origin, are not fully competent to respond to VEGF to form normal glomerular capillaries.

Notochord Signals, Sclerotome, and Glomerular Morphogenesis

The notochord has been demonstrated to function as a signal source in patterning neighboring tissues such as the neural tube, somites, and vasculature during embryonic development (Dodd *et al.*, 1998; Fouquet *et al.*, 1997; Placzek, 1995; Sumoy *et al.*, 1997). Sonic hedgehog (Shh), expressed by the notochord and floor plate, is important for somite patterning and is sufficient to induce sclerotomal markers in explant culture (Fan *et al.*, 1995; Fan and Tessier-Lavigne, 1994). Phenotypic analysis of the Shh knock-out mouse has also recently demonstrated a requirement for Shh in sclerotome survival (Borycki *et al.*, 1999).

Altered glomerular morphogenesis in *flh, syu,* and *yot* could be the result of a direct effect of the notochord on pronephric primordia or an indirect effect of the notochord on tissues that interact with or support glomerular struc-

ture. Based on several lines of evidence, a direct effect of the notochord and Shh acting on pronephric primordia drawing them to the midline is unlikely. *yot* is defective in *gli2*, a transcription factor required cell autonomously for reception of the *shh* signal (Karlstrom *et al.*, 1999; van Eeden *et al.*, 1996). The finding that *yot* embryos show a bifurcated pronephric phenotype while *yot* expression is not detectable in pronephric cells suggests that the glomerular morphogenesis defect is an indirect consequence of a disruption in the Shh signaling pathway. In addition, at the time when this morphogenetic event occurs, *shh* expression is not detectable in the notochord or floor plate (Krauss *et al.*, 1993, and A.M., unpublished observations). Finally, no expression of the *ptc1* or *ptc2* genes, which encode putative receptors for zebrafish Shh, has been reported in the pronephros (Concordet *et al.*, 1996; Lewis *et al.*, 1999). We favor the explanation that glomerular morphogenesis is indirectly affected in midline mutants through a primary defect in a neighboring tissue. The most obvious interacting tissue dependent on midline signaling, and therefore missing in *flh* is the dorsal aorta (Fouquet *et al.*, 1997; Sumoy *et al.*, 1997). While the absence of the dorsal aorta is likely to profoundly affect the function of pronephroi that do form in *flh*, its absence alone can not account for failed morphogenesis since in the mutant *cloche*, which lacks all vasculature, midline fusion of glomerular podocytes occurs normally (Majumdar and Drummond, 1999).

A number of observations suggest the sclerotome may play a role in normal pronephric glomerular morphogenesis. First, previous studies have shown that *twist*-positive sclerotomal cells undergo a lateral to medial migration similar to but temporally earlier than that of glomerular primordia (Morin-Kensicki and Eisen, 1997). Second, we find that *twist*- and *Pax9*-positive sclerotomal cells are intimately associated with the pronephric primordia at 24 hpf and later colocalize with the glomerulus in 48-hpf embryos (data not shown). Third, sclerotome defects correlate with glomerular morphogenesis defects we observe in *flh*. Finally, in 3-week-old fish, the glomerulus is supported by bone and cartilage, suggesting the morphogenesis of these two structures may be linked. However, the sclerotome and pronephric defects may simply be pleiotropic *flh* phenotypes. A mutant specifically affecting sclerotome development would be needed to test whether a causal link between the sclerotome and the pronephric glomerular morphogenesis exists.

Podocytes Play an Important Role in Glomerular Differentiation

Despite the failure of glomerular morphogenesis, the program of podocyte differentiation is not affected in *flh* or *syu*, demonstrating that the signals needed for podocyte development are independent of the presence of a notochord. Podocyte precursors express cell-type-specific markers *wt1* and VEGF and go on to differentiate as morphologically wildtype podocytes with foot processes and associated GBM. The involvement of other known zebrafish hedgehog genes in pronephric patterning is also unlikely since pronephric cell differentiation appears to occur normally in the mutants *no tail* and *cyclops* which lack expression of *echidna hedgehog* and *tiggy winkle hedgehog*, respectively (Chandrasekhar *et al.*, 1998, and unpublished observations; Currie and Ingham, 1996). Podocyte differentiation is also independent of the presence of a dorsal aorta, consistent with our earlier observations that podocytes differentiate apparently normally in the avascular mutant *cloche* (Majumdar and Drummond, 1999). The genetic requirements for podocyte differentiation are currently not known and our results would suggest that they most likely act during early somitogenesis stages coincident with the onset of *wt1* expression.

ACKNOWLEDGMENTS

We thank Heike Schauerte and Pascal Haffter for *syu* and *yot* embryos, Dr. Ruowen Ge for the VEGF template, Fabrizio Serluca for the *wt1* template, and Rolf Karlstrom for the *gli2* template. We are grateful to Colleen Boggs and Dr. Mark Fishman for use of the CVRC fish facility. Special thanks to Mary McKee for technical help with electron microscopy and printing negatives and to Jau-Nian Chen for critical reading of the manuscript. This work was supported by NRSA fellowship DK09763 to A.M. and NIH R01 DK53093 and P01 DK54711 to I.A.D.

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Received for publication October 8, 1999
Revised January 24, 2000
Accepted January 24, 2000