Generation of the podocyte and tubular components of an amniote kidney: timing of specification and a role for Wnt signaling

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ABSTRACT
Kidneys remove unwanted substances from the body and regulate the internal body environment. These functions are carried out by specialized cells (podocytes) that act as a filtration barrier between the internal milieu and the outside world, and by a series of tubules and ducts that process the filtrate and convey it to the outside. In the kidneys of amniote vertebrates, the filtration (podocyte) and tubular functions are tightly integrated into functional units called nephrons. The specification of the podocyte and tubular components of amniote nephrons is currently not well understood. The present study investigates podocyte and tubule differentiation in the avian mesonephric kidney, and presents several findings that refine our understanding of the initial events of nephron formation. First, well before the first morphological or molecular signs of nephron formation, mesonephric mesenchyme can be separated on the basis of morphology and the expression of the transcription factor Pod1 into dorsal and ventral components, which can independently differentiate in culture along tubule and podocyte pathways, respectively. Second, canonical Wnt signals, which are found in the nephric duct adjacent to the dorsal mesonephric mesenchyme and later in portions of the differentiating nephron, strongly inhibit podocyte but not tubule differentiation, suggesting that Wnt signaling plays an important role in the segmentation of the mesonephric mesenchyme into tubular and glomerular segments. The results are discussed in terms of their broader implications for models of nephron segmentation.

KEY WORDS: Wnt signaling, Chick embryo, Kidney, Mesonephros, Podocyte, Renal vesicle

INTRODUCTION
Vertebrate kidneys are composed of functional units called nephrons (Saxen, 1987; Dressler, 2006). Each nephron has several distinct functional and morphological compartments. At the proximal end of each nephron is a glomerulus, which filters the blood. The glomerulus contains vascular capillaries and specialized epithelial cells, the podocytes. The combination of fenestrated slits between the capillary endothelial cells, the basement membrane between endothelial cells and podocytes, and specialized foot processes of the podocytes together form the glomerular filtration barrier. Small molecules can pass through the barrier into the interior of the nephron, whereas larger molecules and cells remain in the vascular system. After passing the glomerular filtration barrier, the filtrate enters the tubule, where it is processed by absorption and secretion carried out by tubular epithelial cells. Finally, the processed filtrate, now called urine, enters the collecting system, a system of ducts that drains the urine to the outside.

Because of the crucial role that kidneys play in regulating the internal body environment, it is not surprising that the structure of kidney tissues varies greatly between different species, as well as at different stages of the life cycle of the same organism (Fraser, 1950). Classically, three types of vertebrate kidneys are discussed: the pronephros, which serves as the embryonic kidney in amniotes and is a transient, largely non-functional structure in amniotes; the mesonephros, which is the adult kidney in anamniotes and the functional embryonic/fetal kidney in amniotes; and the metanephros, which is the functional amniote adult kidney and which is not formed in anamniotes. Each of these kidneys comprises collections of nephrons, which can vary in number from a single nephron in the zebrafish pronephros to ~1 million nephrons in the human metanephros (Vize et al., 2003b).

Understanding how nephrons form is crucial for developing approaches to repair or regenerate damaged kidney tissue. The most common model system for studying vertebrate nephron formation has been the rodent metanephros. Metanephric nephrons form as the consequence of mutual inductive interactions between the metanephric mesenchyme and an epithelial tube called the ureteric bud (UB) (Yu et al., 2004; Dressler, 2009; Costantini and Kopan, 2010). According to current models, as a result of initiating signals from the UB, the mesenchyme undergoes a sequence of morphological changes that leads to nephron formation. First, the mesenchyme undergoes localized condensation to form pretubular aggregates (PTAs), which then undergo a mesenchymal-to-epithelial transition (MET) to form renal vesicles (RVs). The RVs subsequently undergo elongation and differentiation, with the proximal end interacting with local endothelial cells to form the glomerulus, the middle part forming the tubule, and the distal end connecting to the branching UB, which will form the collecting system.

Knowledge of the signaling pathways that regulate specification of nephrogenic mesenchyme into glomerular and tubular fates is still incomplete. Wnt signaling is required for nephron formation and is thought to provide an initial inductive signal for PTA and RV formation (Herzlinger et al., 1994; Stark et al., 1994; Carroll et al., 2005; Park et al., 2007), and one report has linked Wnt4 expression to induction of glomerular fates (Naylor and Jones, 2009). Notch signaling has been found to be required for generation of glomerular and proximal tubular nephron components in several species (McLaughlin et al., 2000; McCright et al., 2001; Cheng et al., 2003; Taelman et al., 2006; Cheng et al., 2007; Wingert et al., 2007; Naylor and Jones, 2009), but the stage(s) at which Notch signaling...
acts during this process is not yet clear. Retinoic acid has also been found to promote glomerulus formation at the expense of tubules in zebrafish embryos (Wingert et al., 2007).

In most current models of metanephric nephron formation, podocytes as well as tubular epithelium are thought to be derived from a uniform pool of undifferentiated cells that constitutes the PTA and that is subsequently patterned to form the different nephron segments (Costantini and Kopan, 2010). However, genetic fate-mapping studies in the mouse metanephros (Boyle et al., 2008; Kobayashi et al., 2008) have not yet yielded a clear picture of when the podocyte and tubular lineages diverge. Interestingly, there are numerous examples in both vertebrates and invertebrates of kidney tissues in which the glomerular and the tubular functions are not tightly integrated (Ruppert, 1994). In the Xenopus pronephros (Vize et al., 1997), the kidneys of oligochaete worms (Ruppert, 1994), and insect nephrocytes (Weavers et al., 2009), filtration of blood or body fluids takes place in one compartment, and the processing of the filtrate and its excretion to the outside takes place in a separate compartment. The two compartments are typically separated by the internal body cavity or coelom (Ruppert, 1994). This raises the question of whether distinct developmental pathways of the glomerular and tubular components of the nephron are a general property of nephrons, a feature that may have become obscured in the highly compact and integrated metanephric nephron.

The development of the avian mesonephros may be able to shed light on the specification of filtration and tubular components of integrated nephrons. Avian mesonephric nephrons are similar in morphology to their metanephric counterparts and pass through similar stages in their development, including PTA and RV stages (Hamilton, 1952) (see also Results, below). However, the avian mesonephros is much simpler in structure than the metanephros, being a linear organ with only a few nephrons per body segment, and thus easier to manipulate and analyze. The current study investigated the initial stages of podocyte and tubule specification in the avian mesonephros. It was found that, well before the appearance of PTAs or RVs, tubule and podocytes precursors can be separated from each other and can differentiate independently. Evidence is also presented that canonical Wnt signaling plays a role in distinguishing between these two alternative mesonephric cell fates by repressing differentiation of podocytes.

RESULTS

Initial stages of nephron formation are similar in the avian mesonephros and mammalian metanephros

Qualitative analysis of early avian mesonephros development was performed in order to evaluate how closely nephron formation in the mesonephros resembles that in the more widely described mammalian mesonephros. The chick mesonephros develops in the intermediate mesoderm (IM) at axial levels between somites 15 and 30 (Hamilton, 1952). At the onset of mesonephric nephron formation, the future mesonephros consists of the nephric duct (which constitutes the future drainage system of the mesonephros) and the nephrogenic cord, a strip of mesoderm that lies ventral to the nephric duct and dorsal to the aorta (Fig. 1F). As mesonephros differentiation proceeds, the region undergoes a dorsal-to-lateral rotation, so that eventually the nephric duct lies lateral to the differentiating mesonephric nephrons (Fig. 1I).

Because chick development proceeds from anterior to posterior, examination of a single embryo during the period of mesonephros formation allows one to observe nephrons at various stages of maturation, with the less mature nephrons located in more posterior segments and more mature nephrons being found more anteriorly.

Figure 1A-C shows plastic sections through the region of the mesonephros at axial levels from posterior to anterior in a single Hamburger-Hamilton (HH) (Hamburger and Hamilton, 1951) stage 18 embryo, and illustrates the morphological changes that occur during nephron formation. Initially, mesenchyme adjacent to the nephric duct begins to condense to form PTAs (Fig. 1A, ag) and subsequently undergoes epithelialization to form RVs (Fig. 1B, rv). In more anterior sections, ‘S-shaped bodies’ can be found, in which an emerging tubule can be seen with a distal end connecting to the nephric duct and a proximal end beginning to show signs of glomerular differentiation (Fig. 1C, sb). These stages are morphologically similar to the stages that have been well described during nephron formation in the mouse and rat metanephros (Iino et al., 2001).

We also examined a set of molecular markers that have been shown to play key roles during mouse metanephric nephron formation. As in the mouse metanephros (Dressler et al., 1990), Pax2 is expressed in both the nephric duct and in the undifferentiated mesonephric mesenchyme (Fig. 1D) and Wnt4 (Stark et al., 1994) is expressed in newly condensing mesonephric pretubular aggregates (Fig. 1E).
Shortly thereafter, Lhx1 (Lim1) (Fujii et al., 1994; Shawlot and Behringer, 1995) begins to be expressed as the aggregates undergo MET to form renal vesicles (data not shown). At later stages, markers of specific nephron segments can be found, including Lhx1 in the nephric duct and tubules (Fig. 1G) and Nphs2 (podocin) (Boute et al., 2000), a component of the slit diaphragm that is expressed exclusively in podocytes (Fig. 1H).

Thus, with respect to expression of markers, as well as morphological appearance, nephron formation in the avian mesonephros appears to be very similar to that of the mouse metanephros and can therefore potentially serve as a useful model for studying formation of the type of integrated nephron seen in the mammalian metanephros.

**Distinct dorsal and ventral zones within the mesonephric IM**

Close inspection of plastic sections through the mesonephric IM before the onset of PTA formation revealed that the IM at this stage is not homogeneous, but appears to consist of two morphologically distinct zones: a dorsal zone adjacent to the nephric duct, which typically appears to be more compact, and a looser ventral zone adjacent to the dorsal aorta (Fig. 2A,A'). The dorsal and ventral IM regions appear to be continuations of the somatopleure and splanchnopleure layers of the lateral plate, respectively, with the difference that the dorsal and ventral layers of the IM are not separated by a coelomic space as they are in the lateral plate.

In a survey of genes known to be expressed in the developing mammalian kidney, the transcription factor gene Pod1 (also called Tcf21 and capsulin) (Hidai et al., 1998; Lu et al., 1998; Quaggin et al., 1999) was found to be expressed in the mesonephric region (Fig. 2C), and in sections was found to be expressed in the ventral IM and the adjacent splanchnic (ventral) portion of the lateral plate, but not in the dorsal IM or the splanchnopleure lateral plate (Fig. 2B). Upon appearance of differentiated nephrons, Pod1 was expressed specifically in glomerular podocytes (Fig. 2D).

**Dorsal and ventral IM zones generate distinct segments of the nephron in culture**

The existence of two distinct zones in the early mesonephric IM, and the observation that the ventral IM zone specifically expresses a marker (Pod1) that is later expressed in podocytes but not tubular parts of the nephron, raised the question of whether the ventral and dorsal zones of the IM might give rise to separate parts of the nephron. In order to begin to address this question, we developed an in vitro system for studying mesonephric nephron differentiation. Slices ~150 microns in thickness (~1.5 somites in thickness) were cut through the undifferentiated mesonephric region at the axial level of the most newly formed somite and cultured on a filter at the air-medium interface. A micrograph of a representative slice at the beginning of the culture period is shown in Fig. 3A. Importantly, the mesonephric mesenchyme at the start of the culture period does not express Wnt4, the earliest known molecular marker of PTA initiation (Kispert et al., 1998; Carroll et al., 2005). The slices were taken from the axial level of the most newly formed somite, where the nephrogenic cord is Wnt4 negative (Fig. 2E). Wnt4 expression is initiated in vivo approximately 6 hours later, after four more somites have formed (Fig. 2F). The nephrogenic cord at the axial level at which the slices were taken is also negative for the tubular marker Lhx1, which is expressed at this stage only in the nephric duct (Fig. 2G), and is also negative for the podocyte marker Nphs2, which is not expressed in the embryo at all at this stage (data not shown).

When whole slices were cultured for two days, robust nephron differentiation occurred. By bright-field microscopy, multiple tubular structures were detected (Fig. 3B). By immunofluorescence, abundant differentiation was seen of Lhx1-expressing nephric duct and tubule cells and Nphs2-expressing podocytes (Fig. 3C; 100% of 40 cultures expressed both Lhx1 and Nphs2). The podocytes were typically arranged in clusters resembling glomeruli and located at the periphery of the explants, immediately adjacent to the Lhx1-expressing tubules, suggesting that integrated nephrons had formed in culture.

In order to determine the developmental potential of the dorsal and ventral zones of the IM, the embryo slices were dissected into ventral and dorsal halves (Fig. 3A) and cultured separately. Dissection was aided by the fact that the more compact dorsal and looser ventral regions of the nephrogenic cord could be visually distinguished under the dissecting microscope. After two days in culture, dorsal cultures exhibited essentially normal Lhx1-expressing tubule formation, but...
Podocyte differentiation in the absence of duct or tubules: experimental blockade of nephric duct migration

The avian nephric duct originates in the pronephric region, adjacent to somites 8-10. Subsequently, the duct extends posteriorly into the mesonephric and metanephric regions, where it induces nephron formation (Obara-Ishihara et al., 1999; Schultheiss et al., 2003; Attia et al., 2012). Classical experiments have demonstrated that if posterior extension of the nephric duct is prevented, mesonephric tubules will not form (Gruenwald, 1937; Waddington, 1938; Soueid-Baumgarten et al., 2013). We utilized this knowledge in order to investigate whether podocytes can differentiate in the absence of both the nephric duct and tubules. A foil barrier was placed in the migration path of the nephric duct so that the duct was prevented from reaching the mesonephric zone (Fig. 4A). The opposite side of the embryo did not receive a barrier and served as a control. Eggs were incubated for a further 48 hours and then examined for expression of tubule and podocyte markers. Examination of Lhx1 expression found that tubule formation was completely blocked on the side with the barrier (data not shown). However, even in the complete absence of a nephric duct or tubules, Nphs2-expressing cells could be identified (Fig. 4B) (five out of five embryos). These Nphs2-expressing cells were often found within large cystic structures, which could possibly represent a greatly enlarged Bowman’s space, although this is uncertain. The number of Nphs2-expressing cells generated on the operated side was typically smaller than on the control side. These results indicate that presence of a nephric duct or kidney tubule is not required for formation of at least some podocyte-like cells in the mesonephric region.

Podocyte differentiation in the absence of tubules in vivo: external glomeruli

In addition to the typical nephrons of the mesonephros and metanephros, avian embryos have long been known to form structures in the pronephric region called external glomeruli (Hamilton, 1952; Hiruma and Nakamura, 2003; Vize et al., 2003a). In these structures, the glomerulus is not integrated into a nephron but instead bulges into the coelomic space. Often, a tubular opening is located nearby. Thus, these external glomeruli resemble the pronephric tubules of some anamniotes, in that there is a separation of glomerular and tubular functions.
The formation of external glomeruli in avians has not been examined at the molecular level. We reasoned that the study of the formation of a natural structure that exhibits a separation of glomerulus and tubule components of the nephron could yield insights into the relationship between these components during nephron differentiation. Chick embryos were examined at stage 18 in the pronephric region for expression of Nphs2, the podocyte marker, and Lhx1, which marks both tubules and the nephric duct. Serial sections of multiple embryos revealed many cases in which well-developed glomeruli bulging into the coelom were found in the absence of nearby tubular structures (Fig. 4C,D; the Lhx1-expressing structure in Fig. 4D is the nephric duct). Other examples were found of Nphs2-expressing cells embedded within mesenchyme (not bulging into the coelom), also in the absence of adjacent tubules (Fig. 4E). Thus, it appears that formation of these podocytes does not depend on a process that simultaneously generates tubules. It should be emphasized that the Nphs2-expressing cells in the duct block experiment shown in Fig. 4A,B are not external glomeruli, because external glomeruli develop in the pronephric region, whereas the duct-blocked embryos were analyzed in the mesonephric region.

**Canonical Wnt signaling selectively inhibits podocyte differentiation**

It has been well established that Wnt signaling components, particularly Wnt9b and Wnt4, are required for nephron formation in both the metanephros and mesonephros (Stark et al., 1994; Kispert et al., 1998; Carroll et al., 2005). As Wnt9b is expressed in the nephric duct (Carroll et al., 2005), which is located on the dorsal side of the mesonephric mesenchyme (Fig. 2), we explored whether Wnt signals might be involved in patterning the mesonephros into tubular and glomerular domains. Wnt9b is thought to work via the canonical Wnt signaling pathway (Carroll et al., 2005; Park et al., 2007). Mesonephric slices were taken from embryos before the initiation of PTA formation, as in Fig. 3, and cultured in the presence or absence of 6-bromoindirubin-3'-oxime (BIO), which inhibits the enzyme Gsk3 and therefore mimics activation of the canonical Wnt signaling pathway (Meijer et al., 2003; Sato et al., 2004). Addition of BIO to the culture medium resulted in loss of the podocyte marker Nphs2 in a dose-dependent manner (Fig. 5A-D), without significantly affecting expression of the duct/tubule marker Lhx1 (Fig. 5E-H). At higher doses of BIO, expression of Nphs2 was completely repressed (Fig. 5C,D). The podocyte-repressing effects of BIO were similar to those obtained by inhibition of Notch signaling (Fig. 5M,N), which has been found to repress glomerulus formation (Cheng et al., 2003; Cheng and Kopan, 2005). In complementary experiments, explants were treated with IWR-1-endo, an inhibitor of Axin degradation and thus an inhibitor of the canonical Wnt signaling pathway (Chen et al., 2009). In preliminary experiments (data not shown), an IWR-1-endo concentration of 20-50 μM was found to be capable of activating cardiac gene expression in cultures of posterior gastrula-stage chick mesoderm, a known effect of Wnt inhibition (Marvin et al., 2001). Consistent with the results of BIO treatment, IWR-1-endo-treated mesonephric explants exhibited stronger expression of Nphs2, whereas expression of the duct/tubule marker Lhx1 was modestly reduced (Fig. 5I-L).

**DISCUSSION**

In developing nephrons of the mammalian metanephros, distinct glomerular and tubular regions can first be detected at the S-shaped body stage, using both morphological and molecular criteria (Dressler, 2006; Quaggin and Kreidberg, 2008; Georgas et al., 2009). The history of the glomerular and tubular nephron components prior to this stage is not clear. Current models of nephron segmentation generally hold that the pretubular aggregate and the renal vesicle are composed of equivalent uncommitted precursor cells that subsequently, under the influence of poorly defined regionalizing signals, differentiate into glomerular and tubular cell types (reviewed by Costantini and Kopan, 2010). The current work modifies and supplements this model in several ways.

First, the studies reported here provide several strands of evidence indicating that the developmental pathways of the podocyte and tubular components of the nephron are distinct earlier than previously thought. Well before the formation of the first PTA or RV, the IM can be separated into dorsal and ventral regions, with the dorsal region generating tubules but no podocytes, and the ventral region exhibiting podocyte but not tubular gene expression (Fig. 3). In addition, blocking the formation migration of the nephric duct and the subsequent formation of tubules does not completely prevent the appearance of Nphs2-expressing podocyte-like cells in vivo (Fig. 4). The current experiments do not establish when the podocyte and tubular lineages become specified. It is possible that the lineages are already specified at the time when dorsal and ventral parts of the IM were separated in the experiments shown in Fig. 3A-G. The expression of Podl in the ventral but not the dorsal IM (Fig. 2B), as well as the morphological differences between the two IM regions (Fig. 2A,A'), supports the idea that the podocyte and tubular lineages may already be somewhat distinct at this stage. However, it is also possible that one or both of the IM regions...
continues to receive essential signals during the culture period from other tissues included in the dorsal and ventral explants. It does appear to be the case, however, that neither tubule nor podocyte lineages require the presence of the other lineage in order to undergo initial differentiation.

Second, the finding that canonical Wnt signaling is a strong repressor of podocyte formation suggests that Wnt signaling is likely to play a role in nephron segmentation. Wnt9b is expressed in the nephric duct and is required and sufficient for induction of nephrons (Carroll et al., 2005; Park et al., 2007). The current results suggest that Wnt9b (or other sources of canonical Wnt signaling) not only initiates PTA formation, but also is involved in patterning the nephron, with regions that receive high levels of Wnt signaling becoming tubules and regions that receive lower levels of signaling becoming podocytes. In support of this model, Wnt4, which is also required for nephron formation (Stark et al., 1994), is expressed in the PTA, RV and S-shaped bodies of the mouse metanephros, with a bias towards the future dorsal side of the nephron and away from the proximal end where the podocytes are located (Stark et al., 1994; Kispert et al., 1998) [see also GUDMAP Embryo #8209 (McMahon et al., 2008)]. Opposing results have been obtained in the Xenopus pronephros, in which overexpression of Wnt4 leads to an expansion of glomerular gene expression (Naylor and Jones, 2009). The difference between the chick and the Xenopus results could be due to timing (in Xenopus, Wnt4 RNA was introduced into the eight-cell embryo), or to differences between pronephric and mesonephric patterning. A working model for initial events in nephron segmentation in the mesonephros is presented in Fig. 6A. In the model, Wnt signaling from the nephric duct induces formation of tubules and represses formation of podocytes. Podocyte formation may be a ‘default’ differentiation pathway that occurs in the absence of Wnt signaling, or may require active inductive signaling, perhaps from the aorta or other tissue adjacent to the ventral IM. Notch and retinoic acid signaling have also been found to be involved in regulation of podocyte formation (McLaughlin et al., 2000; McCright et al., 2001; Cheng et al., 2003; Taelman et al., 2006; Cheng et al., 2007; Wingert et al., 2007; Naylor and Jones, 2009). An important aim in future work will be to understand the relationship between Wnt, Notch and retinoic acid signaling in regulating segmentation of the nephron.

A third issue raised by these studies is the question of the relationship of the PTA and the RV to the fully developed nephron (Fig. 6B,C). Because the current studies find that podocyte and tubule specification begin prior to PTA and RV formation, they disfavor a model in which both podocytes and tubules derive from a common unsegmented precursor structure, such as the PTA or RV, which then subsequently differentiates into podocyte and tubular lineages under local signals. It is possible, however, that the PTA and/or RV are hybrid structures that are built from two separate sources of already-patterned podocyte and tubule precursors.

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Fig. 5. Regulation of podocyte formation by manipulation of Wnt signaling. (A-N) Whole-slice explants were taken from the axial level of the most newly formed somite of stage 15-16 chick embryos and cultured in control medium (A,E,I,K,M), or in medium containing the canonical Wnt pathway activator BIO (B,D,F-H), the Wnt pathway inhibitor IWR-1-endo (J,L) or the Notch inhibitor Compound E (N) at the indicated concentrations. After 48 hours, cultures were analyzed by whole-mount in situ hybridization for Nphs2 (A-D,I,J,M,N) or Lhx1 (E-H,K,L). BIO produced a dose-dependent inhibition of Nphs2 expression but not of Lhx1 expression, while IWR-endo-1 produced an increase in Nphs2 expression and a modest decrease in Lhx1 expression (Lhx1 is also expressed in a subset of neurons in the neural tube). Compound E also inhibited Nphs2 expression. IWR produced an increase in the podocyte marker Nphs2 expression and a mild decrease in the duct/tubule marker Lhx1. n, neural tube; p, podocytes; t, tubules.
reported that individual Six2-expressing cells can give rise to both tubules; however, it must be said that there is also no definitive prepattern of proximal and distal components of metanephric be different in the two cases. There is currently no evidence for a of the establishment of podocyte and tubule precursors are likely to significantly more expansion than the mesonephros, and thus aspects the mammalian metanephros. The metanephros undergoes formation of the mammalian mesonephros, which has received and it is important to consider how these results apply to the understanding the factors that promote differentiation and maturation of the podocyte precursors as well as the integration of other morphological stages of nephron formation and expression of molecular markers. The finding in the current work that podocyte differentiation. The ability of this same ventral IM to form well-differentiated podocytes when cultured together with the tubule-forming dorsal IM (Fig. 3B,C) suggests that podocyte precursors require interaction with a component of the dorsal IM in order to fully differentiate. An important future goal will be to understand the factors that promote differentiation and maturation of the podocyte precursors as well as the integration of other glomerular components, including mesangial cells and endothelium.

The focus of the current experiments was the chick mesonephros, and it is important to consider how these results apply to the formation of the mammalian mesonephros, which has received much more attention. We have shown here (Fig. 1) that nephrogenesis in the chick mesonephros resembles closely the formation of nephrons in the mouse metanephros, both in terms of morphological stages of nephron formation and expression of molecular markers. The finding in the current work that podocyte and tubule develop along distinct developmental pathways from very early stages in kidney formation must now be investigated in the mammalian metanephros. The metanephros undergoes significantly more expansion than the mesonephros, and thus aspects of the establishment of podocyte and tubule precursors are likely to be different in the two cases. There is currently no evidence for a prepattern of proximal and distal components of metanephric tubules; however, it must be said that there is also no definitive evidence against the possibility. A genetic lineage-tracing study reported that individual Six2-expressing cells can give rise to both podocyte and tubular nephron components (Kobayashi et al., 2008), but because Six2 is expressed very early in the whole mesonephric mesenchyme (Oliver et al., 1995), this does not rule out the subsequent segregation of progeny of the original clone into separate podocyte and tubule precursor pools. One potentially interesting point to investigate will be the expression of Pod1, which is expressed specifically in the chick ventral mesonephric IM (Fig. 2). In the mouse metanephros, Pod1 (also known as Tcf21) is expressed in a subset of metanephric mesenchyme precursor cells and is later expressed in podocytes and stroma, but not tubular epithelia (Quaggin et al., 1998; Quaggin et al., 1999). Genetic lineage studies using mouse Pod1-cre lines have been somewhat conflicting regarding the fate map of Pod1-expressing cells, probably owing to differences in the timing and efficiency of cre induction and reporter activation (Acharya et al., 2011; Maezawa et al., 2012). A thorough re-examination of Pod1-cre mice will help to determine whether Pod1 marks a pre-podocyte and/or pre-stroma lineage in the mouse metanephros, and whether there is any connection between the podocyte and stromal lineages.

Although the mesonephric and metanephric nephrons of amniotes have glomeruli directly connected to tubules, this is not generally the case in kidneys throughout the animal kingdom (Goodrich, 1895; Goodrich, 1930; Ruppert, 1994). In many cases, the podocyte/blood filtration function is spatially separated from the tubule/processing and excretion functions. Typically, these two components are separated by an expanse of the body cavity, or coelom, with podocytes filtering body waste into the coelom, and tubules and nephric duct processing and draining the filtrate from the coelom to the outside. In Xenopus, evidence exists for the separability of glomerular and tubular developmental programs (Urban et al., 2006). In organisms requiring a high kidney throughput, the podocyte and tubules have been brought closer together, eventually reducing the coelomic space to the Bowman’s capsule that directly links the glomerulus to the entrance to the tubule, as seen in amniote vertebrates. The current results indicate that, despite this closer and closer apposition of the glomerular and tubular nephron components, a distinction between the initial developmental pathways of the podocyte and the tubule appears to be conserved in amniote integrated nephrons. Thus, the existence of separate developmental pathways of the filtration and tubular components of the nephric system might be a fundamental feature of animal excretory systems.
MATERIALS AND METHODS

Chick embryos

Fertile White Leghorn chick eggs were incubated at 38.5°C in a humidified incubator and staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951).

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as previously described (Wilkinson and Nieto, 1993; Schultheiss et al., 1995; Attia et al., 2012). Anti-sense probe to chick Pod1 was generated from a plasmid generously provided by S. Dietrich (von Scheven et al., 2006). Following signal development, embryos were embedded in sucrose/gelatin, cryosectioned at 20-μm intervals, and examined using a Zeiss Axiomager microscope with differential interference contrast (DIC) optics.

Section in situ hybridization

Embryos were fixed overnight at 4°C in 4% paraformaldehyde, dehydrated through a series of graded ethanol, and embedded in paraffin. Sections of 10-μm thickness were collected, and section in situ hybridization was performed for Pax-2 alkaline phosphatase detection as previously described (Murtaugh et al., 1999; Nissim et al., 2007). Digoxigenin-labeled probes were generated for Pax-2 (Burrill et al., 1997), Lhx1 (Tsuchida et al., 1994), Wnt4 (GenBank Locus BU418913, MRC Geneservice Clone ChEST934q18), Pod1 (von Scheven et al., 2006) and podocin (Nphs2) (GenBank Locus BU377446, ARK Genomics Clone ChEST790p13).

Nphs2 antibody

A rabbit polyclonal antibody to the N-terminus of chick Nphs2 was generated to the peptide RKRESPQARTQRKQEKRERSKEASK (Sigma-Aldrich). Antiserum was affinity purified against the target peptide using an Aminolink column (Pierce) according to the manufacturer’s instructions.

Immunofluorescence

For immunofluorescence on sections, embryos were fixed in 4% paraformaldehyde for 1 hour at room temperature. Fixed embryos were embedded in 7.5% gelatin and 15% sucrose in PBS, and cryosectioned at 15 μm intervals. After permeabilization in 0.25% Triton X-100 in PBS for 15 minutes and blocking (1% bovine serum albumin, 1% goat serum, 1% horse serum and 0.02% Tween 20 in PBS) for 15 minutes, sections were incubated with the primary antibodies rabbit anti-Nphs2 (1:4000) and mouse anti-Lhx1 (1:10) (Developmental Studies Hybridoma Bank, Clone 4F2). Alexa Fluor 488-, Cy3- and DyLight 649-conjugated secondary antibodies (Jackson ImmunoResearch) were used at 1:250 in blocking solution. DAPI (Jackson ImmunoResearch) were used at 1:250 in blocking solution. DAPI (1 μg/ml) was used to visualize nuclei. Images were collected on a Zeiss Axioimager microscope with a Qimaging ExiBlue digital camera and Image Pro plus imaging software.

For immunofluorescence of explant cultures, explants were fixed in 4% paraformaldehyde for 24 hours, washed three times in PBS and permeabilized in 0.5% Triton X-100 and 0.1% saponin in PBS (TSP) for at least 24 hours. Explants were blocked in TSP with 1% sheep serum for several hours and then incubated overnight at 4°C with the primary antibodies rabbit anti-Nphs2 (1:4000) and mouse anti-Lhx1 (1:10) (Developmental Studies Hybridoma Bank, Clone 4F2). The next day, the explants were washed in TSP (three × 15 minutes), and incubated in secondary antibodies Alexa Fluor 488-, Cy3- and DyLight 649-conjugated secondary antibodies (Jackson ImmunoResearch) at 1:250 in blocking solution.

Plastic sections

After overnight fixation in 3% glutaraldehyde, embryos were embedded in epon resin and 1-μm-thick sections were cut using a diamond knife. Slides were stained with Methylene Blue and coverslipped using methacrylate mounting medium.

Explant culture and microdissection

Explants were pinned to a silicone dish dorsal side up using insect pins and slices (~1.5 somites in thickness (150 μm) were cut with a microscalpel (Feather) at the axial level of the most newly formed somites. In some cases, slices were further microdissected using a microscalpel (see Results). Explants were incubated in drops of chilled growth medium (DMEM with 4.5 g/ml glucose, 10% fetal bovine serum, 2% chick extract, 2 mM glutamine and 50 μg/ml PenStrep) until all explants were collected, and were then grown for 48 hours at the air-medium interface on Millicell cell culture inserts (Millipore). In some cases, the canonical Wnt pathway activator 6-bromoindirubin-3’-oxime (BIO) (Calbiochem), the canonical Wnt pathway inhibitor IWR-1-endo (Calbiochem), or the γ-secretase inhibitor Compound E (Calbiochem) were added to the medium at the indicated concentrations.

DIO fate mapping

SP-DiO (Molecular Probes) was diluted in 0.3 M sucrose to a final concentration of 0.1 μg/μl. To mark the intermediate mesoderm, DIO was injected into the ventral IM in slice explant cultures using a pulled glass capillary (1.0 mm outer diameter; Drummond) with a 20-μm diameter tip attached to a Picospritzer III (Parker-Hannifin), with settings of pressure=10 psi; duration=20 mseconds. For coelom injections, DIO was injected into the embryonic coelom in ovo, and subsequently slices were prepared and cultured as described above.

Nephric duct barriers

A thin aluminium foil sheet was placed between two pieces of parafilm and small rectangles were cut (about 1.5 mm × 3 mm) using a scalpel knife. A small crosswise cut was made on one side of stage 9-11 embryos in ovo, at the axial level of about two somites posterior to the last somite using a thin tungsten needle (0.005 inch diameter). The aluminium barrier, isolated from the parafilm, was gently pushed to position using a thicker tungsten needle (0.01 inch diameter). Embryos were cultured for 48 hours in ovo at a 38.5°C humidified incubator.

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Competing interests

The authors declare no competing financial interests.

Author contributions

M.G., R.Y. and T.M.S. conducted the experiments. All the authors were involved in designing and analyzing the experiments and writing the paper.

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