

## RESEARCH ARTICLE

# Wnt signaling orients the proximal-distal axis of chick kidney nephrons

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## ABSTRACT

The nephron is the fundamental structural and functional unit of the kidney. Each mature nephron is patterned along a proximal-distal axis, with blood filtered at the proximal end and urine emerging from the distal end. In order to filter the blood and produce urine, specialized structures are formed at specific proximal-distal locations along the nephron, including the glomerulus at the proximal end, the tubule in the middle and the collecting duct at the distal end. The developmental processes that specify these different nephron segments are not fully understood. Wnt ligands, which are expressed in the nephric duct and later in the nascent nephron itself, are well-characterized inducers of nephrons, and are both required and sufficient for initiation of nephron formation from nephrogenic mesenchyme. Here, we present evidence that Wnt signaling also patterns the proximal-distal nephron axis. Using the chick mesonephros as a model system, a Wnt ligand was ectopically expressed in the coelomic lining, thereby introducing a source of Wnt signaling that is at right angles to the endogenous Wnt signal of the nephric duct. Under these conditions, the nephron axis was re-oriented, such that the glomerulus was always located at a position farthest from the Wnt sources. This re-orientation occurred within hours of exposure to ectopic Wnt signaling, and was accompanied initially by a repression of the early glomerular podocyte markers *Wt1* and *Pod1*, followed by their re-emergence at a position distant from the Wnt signals. Activation of the Wnt signaling pathway in mesonephric explant cultures resulted in strong and specific repression of early and late glomerular markers. Finally, cytoplasmic  $\beta$ -catenin, indicative of active canonical Wnt signaling, was found to be enriched in the distal as compared with the proximal region of the forming nephron. Together, these data indicate that Wnt signaling patterns the proximal-distal axis of the nephron, with glomeruli differentiating in regions of lowest Wnt signaling.

**KEY WORDS:** Kidney development, Nephron patterning, Glomerulus, Mesonephros, Chick embryo, Wnt signaling

## INTRODUCTION

The kidney is a vital, bilateral organ that is the central component of the body's excretory system. Three kidneys are formed sequentially during mammalian and avian development: the largely non-functional pronephros, the embryonic/fetal mesonephros and the functional adult kidney, the metanephros (Saxen, 1987). These three kidneys differ in complexity but all comprise the same basic functional unit, the nephron. The number of nephrons varies from a single nephron in the zebrafish pronephros to ~1 million nephrons in the human

metanephros (Vize et al., 2003). Each nephron is built from three main components: the glomerulus, the tubule and the collecting duct. The glomerulus, located at the proximal end of the nephron, is a bundle of capillaries and specialized epithelial cells, called podocytes, that filters the blood and acts as a semi-permeable barrier between the internal and external environment of the body (Quaggin and Kreidberg, 2008). The filtration barrier allows small molecules to pass through to the interior of the nephron and leaves larger molecules in the circulatory system. The primary filtrate subsequently enters the tubule, which is segmented into several distinct regions, including the proximal tubule, the loop of Henle, the distal tubule and the connecting tubule, which is linked to the nephric duct (ND). While the filtrate travels through the tubule, tubular epithelial cells absorb and secrete substances from and to the filtrate to create the final urine. This urine leaves the tubule and enters the collecting duct system, from which it will be passed on to the outside. In anatomical and physiological terms, the collecting duct is the most distal part of the nephron, the glomerulus is the most proximal part and the tubule is located between them. Establishing and maintaining the structural organization and polarity of the nephron is crucial for urine production and proper kidney function (Costantini and Kopan, 2010).

Nephrons of the mesonephros and metanephros are formed through inductive interactions between the nephric duct and nephrogenic mesenchyme of the mesonephric and metanephric regions (Dressler, 2006). In the mammalian metanephros, where kidney induction has been most extensively studied, the ureteric bud (UB), a branch of the nephric duct, undertakes a series of mutual interactions with the nephrogenic mesenchyme (NM) in order to generate nephrons. First, the UB undergoes a series of sequential branches to form a tree-like structure. Each UB branch will become the collecting duct of a single nephron (Costantini, 2006). The branching UB induces localized aggregation of NM to form pretubular aggregates (PTAs), which in turn undergo epithelial-to-mesenchymal transition (EMT) and form a structure called the renal vesicle (RV) (Iino et al., 2001). The RVs continue to expand, forming the morphologically identifiable 'comma-shaped bodies' (CBs) and later 'S-shaped bodies' (SBs). Around the SB stage, the distal part of the forming tubule fuses with the adjacent UB (Georgas et al., 2009), and signals from the proximal part of the nascent nephron attract endothelial cells from the surrounding mesenchyme that interact with differentiating podocyte cells at the proximal end of the SB to initiate glomerulus formation (Quaggin and Kreidberg, 2008). A similar series of events occurs in the avian mesonephros, with the difference that no UB is formed: the nephric duct itself induces nephron formation, and the distal ends of the mesonephric tubules insert into the duct itself (Soueid-Baumgarten et al., 2014).

Wnt signaling plays an essential role during nephron development. Wnt9b ligand is produced by the ND and UB and is required for NM aggregation and for the subsequent formation of the PTAs and RVs (Carroll et al., 2005). In *Wnt9b* null mice the NM fails to aggregate, resulting in failure of nephron formation in both

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Received 5 March 2015; Accepted 18 June 2015

the mesonephros and metanephros. *Wnt4* is expressed in PTAs and RVs in response to *Wnt9b* signals from the ND. *Wnt4* null mice also show severely impaired tubulogenesis and kidney hypoplasia (Stark et al., 1994).

Although much has been learned regarding the initiation of nephron formation, relatively little is known about how nephrons are patterned along their proximal-distal axis. Notch signaling has been found to be required for formation of proximal nephron elements, including the glomerulus and proximal tubule (Cheng et al., 2007, 2003; Cheng and Kopan, 2005). The transcription factors *Irx5* and *Irx3b* have been reported to be required for formation of the proximal tubule (Alarcon et al., 2008; Reggiani et al., 2007), and *Caudal* has been implicated in promoting distal tubulogenesis (Wingert et al., 2007). Recently, we have reported that Wnt signaling inhibits podocyte formation in mesonephric explants (Grinstein et al., 2013), suggesting that Wnt signaling may play a role in patterning the nephron, in addition to its known role in nephron induction.

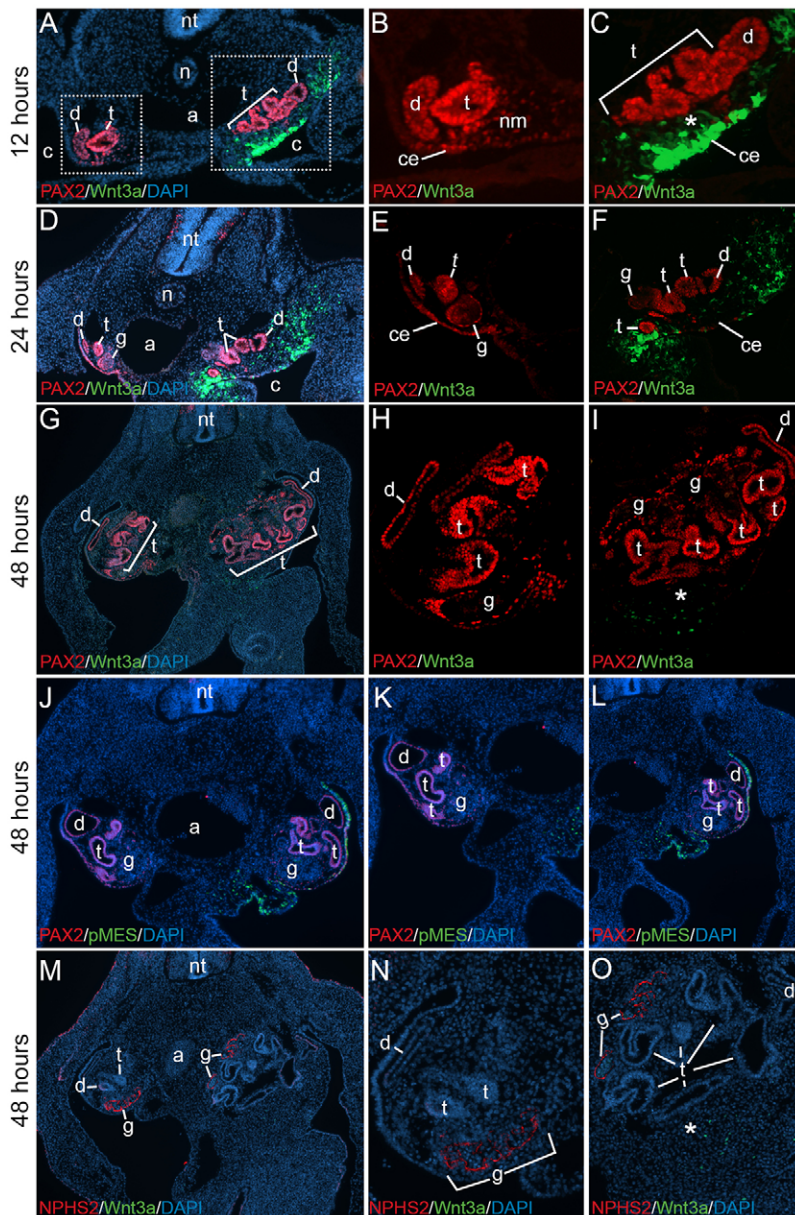
In the current study, we investigated the role of Wnt signaling in regulation of the overall pattern of the nephron. Using the chick

mesonephros model system, we find that exogenous expression of a canonical Wnt ligand in the coelomic epithelium reorients the nephron, such that the glomerulus is consistently located at the position that is furthest away from the Wnt source. In addition, the portions of the tubule that are closest to the exogenous Wnt source exhibit distal characteristics, in particular insertion into the coelomic epithelium as opposed to their normal insertion site in the nephric duct. We conclude that Wnt signaling, in addition to its known function in nephron induction, also patterns the nephron along the proximal-distal axis.

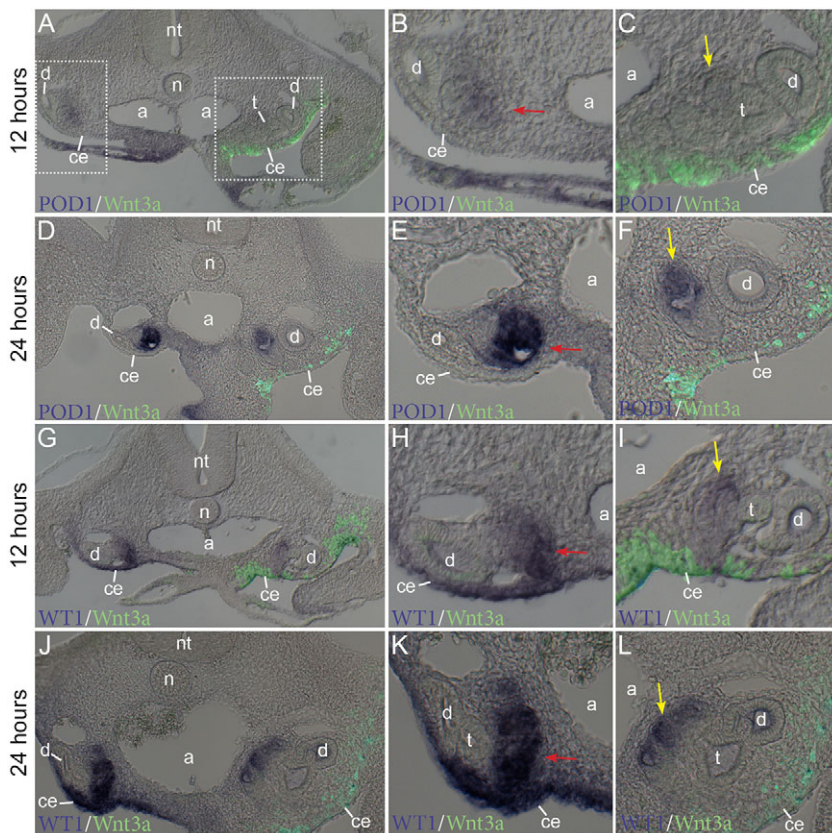
## RESULTS

### An ectopic source of canonical Wnt signaling reorients mesonephric nephron polarity

At Hamburger–Hamilton (HH) Stage 17–18, a section through the normal mesonephros reveals a nephric duct and one or two nascent tubules (Fig. 1A, left side; 1B). After a further 12 h of development (~HH Stage 19), additional tubular loops have developed, and glomeruli have begun to appear (Fig. 1D, left side; 1E). By Stage 22,



**Fig. 1. An ectopic source of canonical Wnt signaling reorients mesonephric nephron polarity.** (A–O) The right side of the coelomic epithelium was electroporated with pMES-Wnt3a (A–I, M–O) or with pMES (J–L), incubated for the indicated lengths of time, and analyzed for expression of Pax2 (A–L, red), Nphs2 (M–O, red), GFP (green) and DAPI (blue). The left, non-electroporated side served as a control. The middle and right panels in each row are higher magnifications of the left and right sides, respectively, of the section shown on the left side of that row. Asterisks denote expanded coelomic epithelium-derived mesenchyme in response to Wnt electroporation. Representative examples are shown from nine pMES-Wnt3a electroporated and nine pMES electroporated embryos examined for each timepoint. a, aorta; ce, coelomic epithelium; d, nephric duct; g, glomerulus; n, notochord; nm, nephrogenic mesenchyme; nt, neural tube; t, tubule.



**Fig. 2. Reorientation of nephron polarity is an early response to Wnt signaling and is accompanied by temporary repression of early glomerular/podocyte markers.** (A–L) The right side of the coelomic epithelium was electroporated with pMES-Wnt3a, incubated for the indicated lengths of time, and analyzed by *in situ* hybridization for expression of Pod1 (A–F) or Wt1 (G–L), and by immunofluorescence for GFP (green). The left, non-electroporated side served as a control. The middle and right panels in each row are higher magnifications of the left and right sides, respectively, of the section shown on the left side of that row. Red arrows in B, E, H and K indicate normal position of the nascent glomeruli on the medial side of the nephron, whereas the yellow arrows in C, F, I and L indicate the dorsal shift of these glomerular markers in response to ectopic Wnt expression in the coelomic epithelium. Representative results are shown from seven embryos examined for each timepoint. a, aorta; ce, coelomic epithelium; d, nephric duct; n, notochord; nt, neural tube; t, tubule.

the mesonephros has grown in size, with multiple tubules and well-developed glomeruli (Fig. 1G,J,M, left side; 1H,K,N). At all stages, collections of undifferentiated Pax2-expressing nephrogenic mesenchyme progenitor cells can also be seen (Fig. 1B,E,H).

Our previous studies found that activation of the canonical Wnt signaling pathway inhibits podocyte formation in a chick mesonephros explant system (Soueid-Baumgarten et al., 2014). In order to investigate the effects of ectopic canonical Wnt signaling in an *in vivo* context, a plasmid expressing *Wnt3a* was electroporated into the coelomic lining on one side of the embryo in the mesonephros region (Galceran et al., 1999; Willert et al., 2003). The *Wnt3a* expression plasmid also expressed eGFP so that electroporated cells could be identified. Electroporation using this approach initially labels only the epithelial cells lining the coelomic cavity (A.A.A. and T.M.S., unpublished). Twelve hours after electroporation, many of the epithelial cells of the coelomic lining strongly expressed GFP. In addition, GFP-expressing cells could be seen in the mesenchyme adjacent to the coelomic lining (Fig. 1A, right side; 1C). By 12 h after electroporation, there were already significantly more Pax2-expressing tubules on the Wnt3a-GFP-electroporated side compared with the non-electroporated control side (Fig. 1A–C). Concomitantly, a decrease could be seen in the number of undifferentiated Pax2-expressing nephrogenic mesenchyme cells on the electroporated side, suggesting that some of the nephrogenic progenitor cells were utilized to generate the additional tubules. GFP was never seen in mesonephric nephron elements, indicating that the coelomic epithelial cells and their derivatives do not take part in nephron formation.

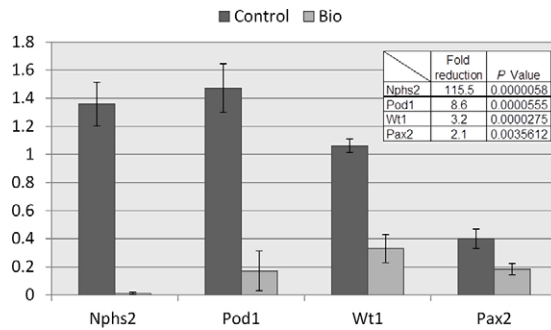
By 24 h after electroporation, there was a large increase in the number of GFP-expressing mesenchyme cells, with the result that the epithelial elements of the mesonephros became separated from the coelomic lining (Fig. 1F). By 48 h, these mesenchymal cells persisted,

although levels of GFP were significantly decreased owing to the multiple rounds of replication that occurred in the electroporated cells (Fig. 1I,O). At 48 h, a large expansion in the number of tubules could be seen on the electroporated side (Fig. 1G,M, right side; 1I,O). In addition, there was a change in the location of the glomeruli in relation to the tubules. On the control side, glomeruli were located medially to the tubules, and adjacent to the coelomic lining epithelium (Fig. 1G, M, left side; 1H,N). By contrast, on the Wnt3a-GFP-electroporated side, the glomeruli were typically located dorsal to the tubules and far from the coelomic epithelium (Fig. 1G,M, right side; 1I,O). By analyzing nine embryos, it could be seen that the nephrons were oriented such that the glomeruli were always located furthest from the Wnt-expressing cells, with the tubules located between the Wnt-expressing cells and the glomeruli (Fig. 1G,I,M–O). None of these changes was seen in embryos electroporated with a plasmid expressing GFP alone (Fig. 1J–L; nine embryos).

### Reorientation of nephron polarity is an early response to Wnt signaling and is accompanied by temporary repression of early glomerular/podocyte markers

After observing the shift in glomerulus orientation in accordance with the location of the Wnt-expressing cells 48 h post-electroporation, we wanted to trace this process back to earlier stages of glomerulus formation. As Nphs2 is not detected before defined glomerular structures are established (Soueid-Baumgarten et al., 2014), we used the early podocyte markers Pod1 (Quaggin et al., 1999, 1998) and Wt1 (Armstrong et al., 1993; Kreidberg et al., 1993) to investigate the effects of Wnt signaling on nephron orientation 12 and 24 h post-electroporation.

In the normally developing mesonephros 12 h after electroporation, Wt1 is expressed preferentially on the medial side of the developing mesonephros as well as in the coelomic epithelium (Fig. 2G, left side;



**Fig. 3. Repression of podocyte genes in mesonephric explant culture.**

Embryo slices through the mesonephric region were cultured in the presence or absence of BIO and analyzed by RT-PCR for expression of the indicated genes. Eight BIO-treated and eight control explants were analyzed. Expression levels were normalized with respect to *Gapdh* expression, and statistical analysis was by two-tailed Student's *t*-test. Error bars represent s.d.

2H). By 24 h after electroporation (HH Stage 18–19), *Wt1* expression in the mesonephros has intensified, and the position and morphology of the *Wt1*-expressing cells is consistent with a pre-podocyte identity (Fig. 2J, left side; 2K). At 12 h, *Pod1* is also expressed in the medial mesonephros (Fig. 2A, left side; 2B), although more weakly than *Wt1*. By 24 h, *Pod1* is expressed strongly in the medial-most part of the developing nephron (Fig. 2D, left side; 2E), also consistent with a podocyte identity.

Twelve hours after *Wnt3a* electroporation into the coelomic epithelium, there was a marked reduction in both *Pod1* and *Wt1* in the mesonephros, accompanied, in the case of *Wt1*, by a dorsal shift in the position of these cells (Fig. 2A,G, right side; 2C,I). After an additional 12 h of development (24 h post-electroporation) the levels of *Pod1* and *Wt1* were still lower than on the non-electroporated side, although they were markedly higher than was seen 12 h post-electroporation (Fig. 2D,J, right sides; 2F,L). Most significantly, 24 h post-electroporation, the *Wt1* and *Pod1* expression domains are seen to have shifted markedly to a location that is furthest away from the *Wnt3a*-expressing coelomic epithelium. These data suggest that *Wnt* signaling represses podocyte gene expression, but that eventually podocytes do develop at a location that is predicted to be relatively low in *Wnt* signaling.

### Wnt signaling selectively represses podocyte formation

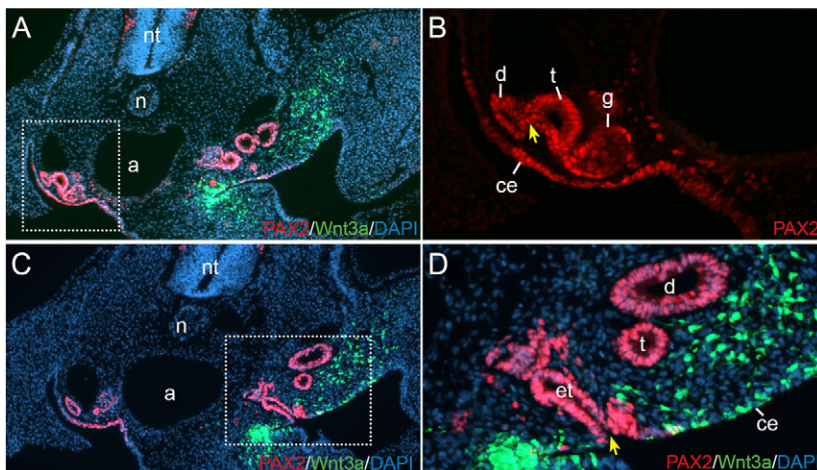
The data shown in Figs 1 and 2 indicate that nephron polarity can be reoriented by an ectopic source of *Wnt* signaling, with tubular

differentiation occurring in high-*Wnt* zones, and glomeruli developing in zones with lower levels of *Wnt* signaling. Theoretically, this result could be achieved by cell fate changes of nephron precursors, or by a spatial rearrangement of already-determined tubule and podocyte precursors. In order to analyze the mechanism of nephron re-orientation in a more controlled environment, an *in vitro* explant system was used (Soueid-Baumgarten et al., 2014). Mesonephric explants were grown for 48 h in control medium or in medium supplemented with 6-bromoindirubin-3'-oxime (BIO), which represses glycogen synthase kinase 3 (GSK3) activity and therefore promotes *Wnt* signaling (Meijer et al., 2003; Sato et al., 2004). Glomerular and tubular gene expression levels were assessed by semi-quantitative PCR. All tested glomerular marker expression levels were significantly reduced after continuous exposure to *Wnt* signals (Fig. 3). Notably, the effect on *Nphs2*, a specific marker of differentiated podocytes, was most strongly repressed, exhibiting a 115.5-fold reduction in gene expression levels (Fig. 3;  $P < 1 \times 10^{-5}$ ). The transcription factor *Pod1* showed an 8.6-fold reduction in gene expression (Fig. 3;  $P < 1 \times 10^{-4}$ ) and *Wt1* showed a significant, although more modest, 3.2-fold reduction in expression levels (Fig. 3;  $P < 1 \times 10^{-4}$ ). *Pax2*, a transcription factor that marks nephrogenic mesenchyme as well as kidney epithelial elements (Dressler et al., 1990; James et al., 2006), showed a 2.1-fold reduction in gene expression (Fig. 3;  $P < 1 \times 10^{-2}$ ) that could be attributable to many factors, including the reduction in glomerular tissue, utilization of undifferentiated nephrogenic mesenchyme cells for premature tubule formation, or general cell survival.

These results show that in an *in vitro* environment in which the *Wnt* signal is spread homogeneously throughout the growth media, allowing for no 'low *Wnt*' areas, there is a strong, selective inhibitory effect of *Wnt* on glomerulus differentiation. This result does not favor a model in which nephron orientation in response to *Wnt* signaling is caused only by spatial rearrangement of glomerulus and tubule precursors, and is more consistent with a model in which *Wnt* signaling actively patterns nephron progenitors along a proximal-distal axis (see Discussion).

### Ectopic distal nephron fusion events in response to ectopic *Wnt* signaling

During the course of normal mesonephric nephron formation, the distal part of the developing nephron inserts into the nephric duct at the S-shaped body stage, in order to form a drainage channel for urine generated by the tubule (Fig. 4A,B) (Georgas et al., 2009).



**Fig. 4. Ectopic distal nephron fusion events in response to ectopic *Wnt* signaling.**

(A–D) The right side of the coelomic epithelium was electroporated with pMES-*Wnt3a*, incubated for 24 h, and analyzed for expression of *Pax2* (red), GFP (green) and DAPI (blue). The left, non-electroporated side served as a control. B shows the distal end of a nephron fusing (yellow arrow) with the nephric duct on the control (left) side of the embryo shown in A. D (a higher magnification view of the right side of C) shows a tubule that has fused ectopically to the *Wnt3a*-expressing coelomic epithelium (yellow arrow). a, aorta; ce, coelomic epithelium; d, nephric duct; et, ectopic tubule; g, glomerulus; n, notochord; nt, neural tube; t, tubule.

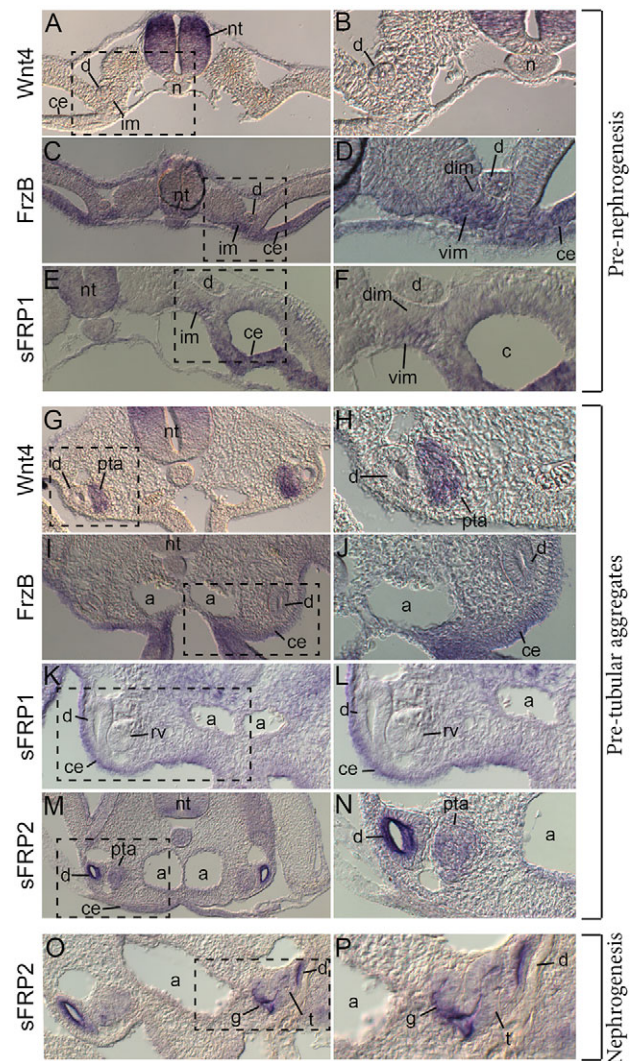
When Wnt3a was electroporated into the coelomic lining, on several occasions nephrons were observed to insert into the coelomic lining and not into their normal target, the nephric duct (Fig. 4C,D). This observation suggests that tubules are competent to insert into epithelia other than that of the nephric duct, and raises the possibility that Wnt signaling also plays a role in promoting the fusion process. In addition, because fusion is a property of the most distal segment of the tubule, this result is further evidence of a re-patterning of the nephron along the proximal-distal axis in response to Wnt signals.

### Expression of Wnt signals and inhibitors in the mesonephros

Given the ability of ectopic Wnt signaling to re-orient the proximal-distal axis of mesonephric nephrons, it was of interest to document the state of Wnt signaling during normal nephrogenesis. The expression of Wnt4 (Kispert et al., 1998; Stark et al., 1994) as well as that of the Wnt inhibitors FrzB (Leyns et al., 1997; Wang et al., 1997), Sfrp1 and Sfrp2 (Rattner et al., 1997) was examined at three developmental stages: Stage I before the onset of nephrogenesis, Stage II during the condensation of pretubular aggregates and Stage III during the appearance of glomerular and tubular nephron elements. At Stage I, Wnt9b is expressed in the nephric duct (Soueid-Baumgarten et al., 2014) but Wnt4, the earliest marker of pretubular aggregates is not yet expressed (Fig. 5A,B). At this stage, the Wnt inhibitors FrzB and Sfrp1 are expressed preferentially in the intermediate mesoderm farthest from the duct (Fig. 5C–F), which has been shown to have podocyte differentiation capabilities (Grinstein et al., 2013). Thus, already at this stage, there is a potential gradient across the intermediate mesoderm from dorsal, which expresses Wnt9b, to ventral, which expresses several secreted Wnt inhibitors. Between Stages I and II, the intermediate mesoderm undergoes a 90° rotation, such that the duct moves from a dorsal to a lateral position, whereas the ventral IM moves from a ventral to a medial position (Grinstein et al., 2013). At Stage II, when pretubular aggregates appear, they express both Wnt4 (Fig. 5G,H), which is essential for future nephron differentiation (Stark et al., 1994), and the secreted Wnt inhibitor Sfrp2 (Fig. 5M,N). In addition, the inhibitors Sfrp1 and FrzB are expressed in the coelomic epithelium adjacent to the mesonephros (Fig. 5I–L). The expression of these genes, together with the expression of Wnt9b in the nephric duct (Carroll et al., 2005; Soueid-Baumgarten et al., 2014), is consistent with the production of a gradient of Wnt signaling, from high nearest the nephric duct to low in the medial and ventral regions of the IM where the early glomerular markers Pod1 and Wt1 are expressed (Fig. 2B,E; see also Fig. 6, below). Finally, by Stage III, Wnt4 continues to be expressed in portions of the tubules (data not shown), whereas Sfrp2 is expressed preferentially in the most medial part of the nephron (Fig. 5O,P), where differentiated glomeruli are forming (Fig. 2H,K). Thus, at several stages during mesonephric nephron differentiation, Wnt signals are found in more dorsal/lateral regions of the nephron, where the distal nephron components are forming, whereas Wnt inhibitors are expressed in more ventral/medial regions, where proximal/glomerular tubular elements are developing.

### Evaluation of $\beta$ -catenin expression during normal nephron formation and reprogramming supports a role for canonical Wnt signaling during nephron segmentation

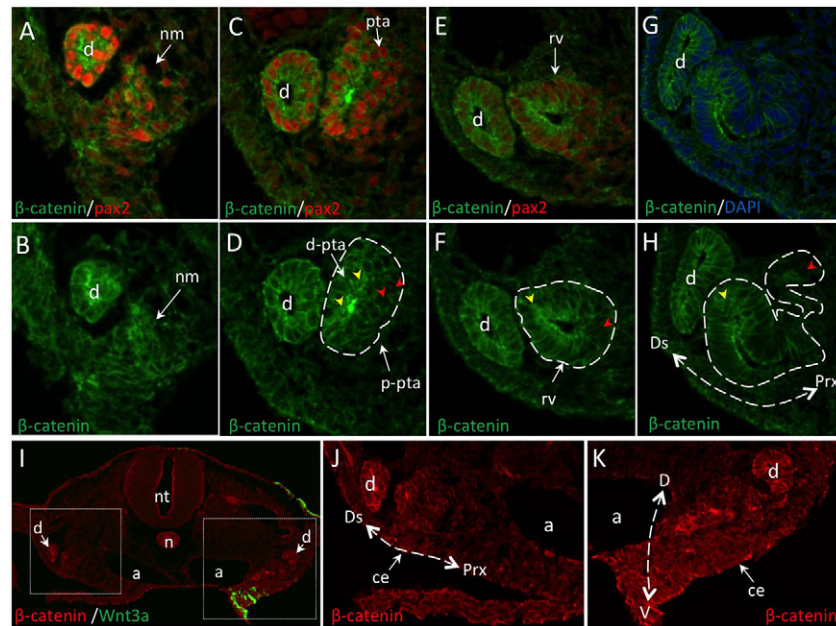
During canonical Wnt signaling,  $\beta$ -catenin is translocated from the cell membrane into the cytoplasm and eventually into the nucleus



**Fig. 5. Expression of Wnt signals and inhibitors in the mesonephros.**

(A–P) Expression of Wnt4, FrzB, sFRP1 and sFRP2 at three stages during nephron formation, as indicated. The right-hand column is a higher magnification view of one of the sides of the section shown in the left column. See text for description of expression patterns. a, aorta; ce, coelomic epithelium; d, nephric duct; dim, dorsal intermediate mesoderm; g, glomerulus; im, intermediate mesoderm; n, notochord; nt, neural tube; pta, pre-tubular aggregate; rv, renal vesicle; t, tubule; vim, ventral intermediate mesoderm.

(Krieghoff et al., 2006; MacDonald et al., 2009). In order to obtain a more direct assessment of the state of canonical Wnt signaling during mesonephric nephrogenesis, immunostaining for  $\beta$ -catenin was performed. As shown in Fig. 6A,B, before the initiation of PTA formation a region of increased  $\beta$ -catenin immunoreactivity that includes cytoplasmic as well as membrane localization can already be seen adjacent to the nephric duct, suggesting active canonical Wnt signaling in the mesonephric mesenchyme. By the PTA stage (Fig. 6C,D), cytoplasmic levels of  $\beta$ -catenin are distinctly higher in the region of the PTA closest to the nephric duct. This asymmetrical distribution of cytoplasmic  $\beta$ -catenin continues through the renal vesicle stage (Fig. 6E,F). At the S-shaped body stage (Fig. 6G,H),  $\beta$ -catenin expression at the proximal end of the nephron is much lower than at the distal end. These data suggest that throughout the period of nephron patterning and segmentation, there is differential activation of



**Fig. 6.  $\beta$ -Catenin immunoreactivity during normal nephron formation and upon canonical Wnt ligand misexpression.** (A-H) Confocal images of the mesonephric region of Stage 14 (A,B), Stage 16 (C-F) and Stage 19 (G,H) embryos showing nephrogenic mesenchyme (A,B), pretubular aggregate (C,D), renal vesicle (E,F) and S-shaped body (G,H) stages of nephrogenesis, stained for  $\beta$ -catenin (green), Pax2 (red) and DAPI (blue). B, D, F and H show the  $\beta$ -catenin channel alone of A, C, E and G, respectively. Note the higher levels of diffuse  $\beta$ -catenin immunoreactivity near the nephric duct (yellow arrowheads) as opposed to regions farther from the duct (red arrowheads). Dashed regions in D, F and H indicate the boundaries of the forming nephron. (I-K) The right side of the coelomic epithelium was electroporated with pMES-Wnt3a-GFP, incubated for 24 h and analyzed by confocal microscopy for expression of  $\beta$ -catenin (red) and GFP (green). The left, non-electroporated side served as a control. J and K are higher magnifications of the boxed areas of the left (control) and right (experimental) sides of I, respectively. Note the greatly increased levels of diffuse  $\beta$ -catenin immunoreactivity in the ventral side of region adjacent to the electroporated area. Representative results are shown from six embryos examined for each timepoint. a, aorta; ce, coelomic epithelium; d, nephric duct; Ds, dorsal; d-pta, distal region of the pta; Prx, proximal; pta, pretubular aggregate; rv, renal vesicle; V, ventral.

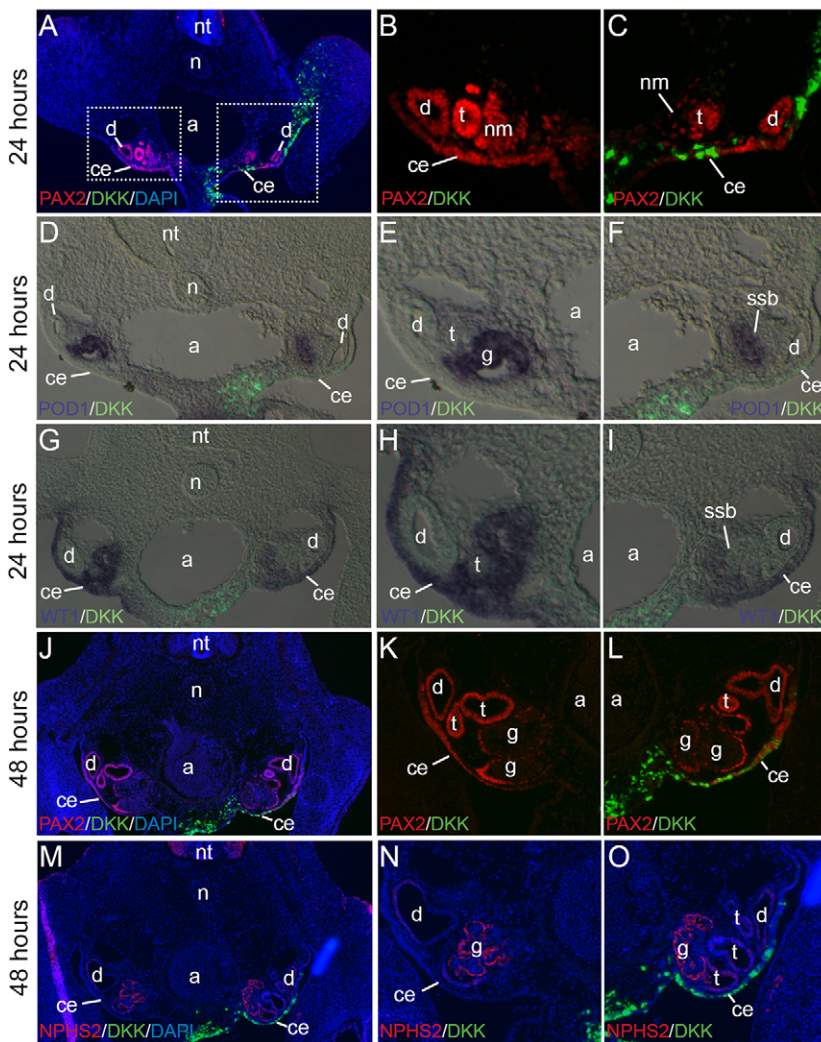
the canonical Wnt pathway across the developing nephron, consistent with a model in which proximal nephron elements differentiate in an environment of low Wnt signaling.

We also investigated the state of  $\beta$ -catenin immunoreactivity during the nephron reprogramming that occurs in response to ectopic expression of a canonical Wnt ligand. Wnt3a was electroporated into the coelomic lining in the mesonephric region (Fig. 6I-K) in the same manner as was performed for the experiments shown in Figs 1 and 2. After 24 h, on the control side (Fig. 6I, left; 6J), increased cytoplasmic  $\beta$ -catenin was seen in the mesenchyme adjacent to the nephric duct where nephrogenesis is initiating, whereas mesenchyme further from the duct exhibited very low  $\beta$ -catenin immunoreactivity. By contrast, electroporation of Wnt3a into the coelomic lining produced a strong increase in cytoplasmic  $\beta$ -catenin reactivity throughout the mesenchyme adjacent to the Wnt3a-expressing cells (Fig. 6I, right; 6K). This indicates that during the nephron re-patterning that occurs in response to ectopic Wnt signaling (as seen in Figs 1 and 2), the canonical Wnt pathway is active in those regions of the mesonephric mesenchyme that suppress expression of glomerular markers (compare Fig. 2D,F,J,L with Fig. 6I,K). Furthermore, examination of Fig. 1D-F and Fig. 2D-F,J-L (which are all taken at the same developmental time point as Fig. 6I-K, 24 h after electroporation of Wnt3a) shows that on the electroporated side glomerular markers are seen only at the level of the nephric duct or more dorsally (Fig. 2F,L, yellow arrows). Examination of Fig. 6K shows that the area of strong ectopic cytoplasmic  $\beta$ -catenin in response to ectopic Wnt extends to the level of the nephric duct but not more dorsally. Thus, ectopic Wnt administration induces strong cytoplasmic

$\beta$ -catenin localization, but glomerular genes are expressed only in regions of low Wnt activity.

### Effects of lowering canonical Wnt signaling in the mesonephros

Given the effects on nephron patterning observed upon localized ectopic activation of the canonical Wnt pathway, it was of interest to investigate the effects of lowering canonical Wnt signaling on patterning of the nephron. The secreted canonical Wnt signaling inhibitor Dickkopf (Dkk) was electroporated into the coelomic lining, and embryos were examined after 24 or 48 h of development (Fig. 7). Twenty-four hours after Dkk electroporation, the mesonephros appeared smaller in size (Fig. 7A-C), and the tubular component of the nephron appeared to be diminished. In addition, Pod1 and Wt1 were expressed more weakly, pointing to a reduction in glomerulus-associated gene expression (Fig. 7D-I). Thus, lowering of canonical Wnt signaling appears to repress nephron formation of all parts of the nephron, rather than affecting parts of the nephron differentially, as was the case with Wnt activation (Figs 1,2). As discussed in the Discussion, these results are likely to be connected to the earlier requirement for Wnt signaling for initiation of nephron formation. In addition, Wnt inhibitors are normally expressed in the coelomic lining (Fig. 5), so addition of Dkk might not generate a significantly lower localized concentration of Wnt signaling that could in turn influence patterning the nephron. Forty-eight hours after Dkk electroporation, mesonephric nephrons appeared normal with respect to size and pattern (Fig. 7J-O), indicating recovery from the disturbances that were apparent after 24 h.



**Fig. 7. Effects of canonical Wnt inhibition on mesonephros development *in vivo*.**

The right side of the coelomic epithelium was electroporated with pMES-Dkk, incubated for the indicated lengths of time, and analyzed by immunofluorescence for expression of Pax2 (A–C, J–L, red), Nphs2 (M–O, red), GFP (green) and DAPI (blue); or by *in situ* hybridization for expression of Pod1 (D–F) or Wt1 (G–I), and by immunofluorescence for GFP (green). The left, non-electroporated side served as a control. The middle and right panels in each row are higher magnifications of the left and right sides, respectively, of the section shown on the left side of that row. Representative results are shown from nine embryos examined for each timepoint. a, aorta; ce, coelomic epithelium; d, nephric duct; g, glomerulus; n, notochord; nm, nephrogenic mesenchyme; nt, neural tube; ssb, S-shaped body; t, tubule.

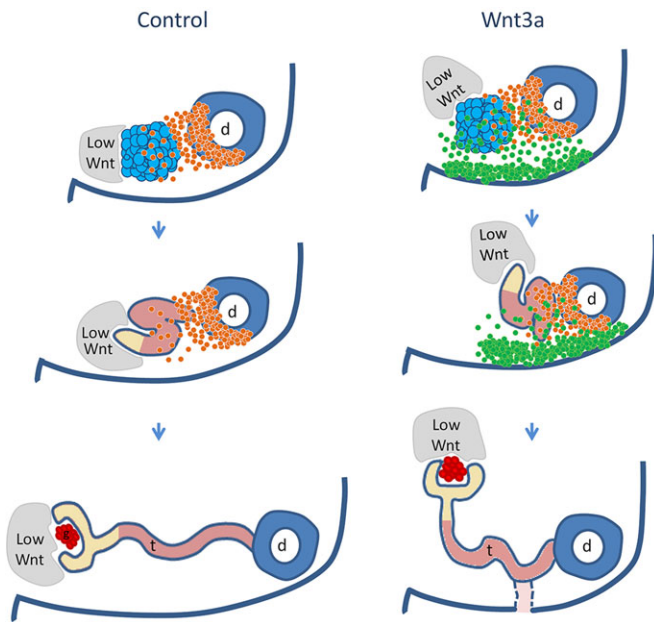
## DISCUSSION

Glomeruli normally develop at the proximal end of each nephron, at the opposite end of the nephron from the insertion of the nephron to the nephric duct. In the avian mesonephros, the duct is located at the lateral edge of the mesonephros and glomeruli develop on the medial side of the organ. The current study found that an ectopic source of canonical Wnt signal reoriented the nephron so that the glomeruli became located as far as possible from the Wnt source. As the nephric duct is a known source of Wnt signaling, this suggests that Wnt signals orient the nephron, with glomeruli developing in areas with the lowest Wnt signal.

One question that arises is whether the reorientation of nephrons in response to Wnt signaling is a result of re-specification of the pool of nephron progenitors or of migration of already specified glomerular precursors. The *in vitro* culture data, in which Wnt pathway activation leads to severe reduction of podocyte markers with relatively little effect on tubules (Fig. 3; Grinstein et al., 2013), indicates that Wnt signaling represses podocyte formation, and is most consistent with a model in which Wnt signaling actually affects nephron cell-type specification. Published reports indicating that some Wnt response genes are expressed preferentially in the distal part of the renal vesicle and S-shaped body (Georgas et al., 2009) are consistent with the proposed role of Wnt signaling in patterning the nephron and inhibiting podocyte formation. A working model of the establishment of nephron polarity is presented in Fig. 8.

The current study found that Wnt signaling has an inhibitory effect on podocyte formation. Several published studies have reported an antagonistic relationship in mature podocytes between Wnt signaling and the transcription factor Wt1, which is essential for podocyte function.  $\beta$ -catenin is activated during podocyte injury (Dai et al., 2009; Li and Siragy, 2014; Wang et al., 2011) and leads to ubiquitin-mediated degradation of Wt1 (Zhou et al., 2015), and Wnt inhibition restores podocyte integrity in an induced nephropathy model (He et al., 2011). Thus, inhibition of Wt1 by Wnt signaling, which was observed in the current study in the nephrogenic mesenchyme as well as in the coelomic epithelium within hours of Wnt delivery (Fig. 2), might be a general feature of both podocyte formation and maintenance.

During normal nephrogenesis, the distal-most end of the nascent nephron inserts into the nephric duct or, in the case of the mammalian metanephros, into a branch of the ureteric bud (UB), which is a derivative of the nephric duct. The process by which the distal tubule inserts into the duct or UB is not well understood (Georgas et al., 2009). The current study finds that when a source of canonical Wnt signaling is expressed in the coelomic epithelium, tubules can be seen inserting into the coelomic epithelium instead of into the nephric duct. This suggests that kidney tubules are competent to insert into other epithelium besides the nephric duct, and that Wnt signaling may be important for the insertion process. The expression of multiple secreted Wnt inhibitors in the coelomic



**Fig. 8. Model of the role of Wnt signaling in patterning the proximal-distal axis of the nephron.** Three stages in nephron formation are illustrated: pretubular aggregate (top), S-shaped body (middle) and mature nephron (bottom). The left column indicates the normal situation, where the source of Wnt is the nephric duct and the forming nephron is exposed to a gradient of Wnt activity. Regions exposed to high Wnt levels form tubular components, whereas areas exposed to low Wnt levels form glomerular elements. The right column illustrates the effects of ectopic expression of Wnt3a in the coelomic epithelium. In this case, the forming nephron is exposed to Wnt signals from both the duct and the coelomic epithelium. Glomeruli develop in the area furthest from the combined sources of Wnt signal, dorsal to their normal location. Occasionally, tubules can be seen fusing to the coelomic epithelium instead of the nephric duct (light pink in right bottom panel). d, nephric duct; g, glomerulus; t, tubule. Blue circles, pretubular aggregate cells; orange circles, Wnt9b; green circles, ectopic Wnt3a.

epithelium (Fig. 5I–L) may be important for preventing inappropriate insertion of tubules into coelomic epithelium during normal development.

Several aspects of the chick mesonephros experimental system facilitated these investigations, in particular the linear arrangement of mesonephric nephrons, which made it possible to recognize nephron re-orientation, and the ability to easily introduce genes into the coelomic epithelium, adjacent to the developing mesonephros. The question arises regarding how these results can be applied to other kidney types, particularly the mammalian metanephros. Although the metanephros is a more complex tissue, metanephric nephrons at the S-shaped body stage have a similar orientation to those of the avian mesonephros, with glomerular precursors located at the proximal end furthest away from the Wnt-expressing UB (Dressler, 2006). In addition, known Wnt response genes are expressed preferentially in more distal parts of nephron progenitor structures as early as the renal vesicle stage (Brunskill et al., 2008; Georgas et al., 2009). Thus, the model presented in Fig. 8 is consistent with what is known to occur in the developing metanephric nephron. Direct investigation of the role of Wnt signaling in patterning the mammalian metanephros is an important subject for future research. In agreement with the current results, a recently published study in mouse reports a connection between low Wnt signaling and the generation of proximal nephron elements, including the glomerulus (Lindstrom et al., 2014).

The current report documents a role for Wnt signaling in patterning the proximal-distal axis of the nephron, with the

formation of proximal nephron segments requiring an environment of low Wnt signaling. Previous reports have documented a requirement for Notch signaling for the formation of proximal components of mouse metanephric nephrons (Cheng et al., 2007, 2003; Cheng and Kopan, 2005). The relationship between Wnt and Notch signaling in patterning the nephron, particularly the proximal part of the nephron, is not yet clear. The data in the current paper with respect to  $\beta$ -catenin immunoreactivity (Fig. 6) indicate the existence of differential canonical Wnt pathway activity along the proximal-distal axis of the nephron throughout the period of nephron formation. This is not suggestive of a simple linear model in which Wnt from the nephric duct provides an initiating signal for nephron orientation, with subsequent patterning carried out by Notch signaling, although it does not rule it out. One possible scenario is that high Wnt signaling represses the activity of a Notch pathway that is required for proximal nephron fates. In future studies, it will be important to investigate the interactions between the Wnt and Notch pathways during nephron segmentation, particularly with respect to the glomerulus and other proximal nephron regions.

As Wnt signaling from the nephric duct is known to be required and sufficient for nephron induction (Carroll et al., 2005; Herzlinger et al., 1994), the current results present an interesting situation, in which the same signal both induces a structure (the nephron) and also selectively represses the subsequent differentiation of part of that structure (the podocyte/glomerulus). This earlier role for Wnt signaling in nephron induction could explain the observed effects of Dkk electroporation (Fig. 7), which produced a general, transient reduction in the generation of all nephron components, as opposed to a specific effect on patterning within the nephron. Presumably, there must be intrinsic mechanisms that ensure proper coordination between nephron induction and patterning. The induction of Wnt4 as well as the Wnt antagonist *Srpf2* in pretubular aggregates may be part of such a mechanism to ensure that each nephron contains a region in which Wnt signaling is sufficiently low that glomeruli are permitted to develop.

The current observations have potential relevance for kidney tissue engineering. Attempts to develop kidney tissue from embryonic or other stem cells are still at very early stages, but many published methods include Wnt signaling as part of the induction protocol (Taguchi et al., 2014; Takasato et al., 2014). This is reasonable in light of the known Wnt signaling requirement for the initiation of nephron formation. However, the current results indicate that general activation of the Wnt signaling pathway, although it might induce tubules efficiently, might also prevent formation of an essential component of the nephron, the glomerulus. Efficient formation of complete engineered nephrons will require careful spatial and temporal control of Wnt signaling.

## MATERIALS AND METHODS

### Plasmids

pMES (Swartz et al., 2001) contains CMV/ $\beta$ -actin enhancer/promoter elements driving universal expression and an IRES element driving eGFP expression. pMES-Wnt3a and pMES-dkk were generated by cloning the mouse *Wnt3a* or *Xenopus dkk* genes (Fonar et al., 2011; Glinka et al., 1998; Wolda et al., 1993) upstream of the IRES element.

### Electroporation

Fertile White Leghorn chick eggs were incubated at 38.5°C in a humidified incubator until HH Stage 13 (Hamburger and Hamilton, 1951). Three milliliters of albumin was drawn from the pointed side of the egg using a 10-ml syringe and a 21-gauge needle. The syringe hole was taped shut and an oval window was opened on the top of the egg to allow access to the

embryo. India Ink was injected beneath the embryo to allow for better visualization. Plasmid (1.2 µg/µl) was injected using a glass mouth micropipette into the coelomic cavity at the axial level of the vitelline veins (future somite 22), the area adjacent to the prospective mesonephros. Immediately after injection, the embryos were electroporated using tungsten electrodes (Momose et al., 1999). After electroporation, the top hole was taped with Scotch Magic tape (catalog number 810), and thoroughly sealed with melted beeswax to prevent the embryos from dehydrating. Embryos were incubated for 12, 24 or 48 h after electroporation, cut from the egg, washed with 1× PBS and fixed overnight in 4% paraformaldehyde.

### Immunofluorescence

For section immunofluorescence, embryos were fixed in 4% paraformaldehyde for 24 h at 4°C. Fixed embryos were embedded in 7.5% gelatin and 15% sucrose in PBS, and cryosectioned at 10 µm intervals. After permeabilization in 0.25% Triton X-100 in PBS for 15 min and blocking (1% bovine serum albumin, 1% goat serum, 1% horse serum and 0.02% Tween 20 in PBS) for 15 min, sections were incubated overnight at 4°C with primary antibody. The following primary antibodies were used: rabbit anti-Nphs2 (1:4000; Grinstein et al., 2013), rabbit anti-Pax2 (1:250; Covance, PRB-276P); mouse anti-GFP (1:500, Invitrogen) and mouse anti-β-catenin (1:1000; Sigma, clone c7082). Following 3×5 min washes in PBS, sections were incubated for 1 h at room temperature with secondary antibodies (donkey anti-rabbit Cy3 and donkey anti-mouse Alexa Fluor 488, Fab fragments, 1:250; Jackson ImmunoResearch). All antibodies were diluted in blocking solution. Following washing, DAPI (1 µg/ml) was used to visualize nuclei, and slides were mounted using Fluoromount (Dako). Images were taken on a Zeiss Axioimager M1 fluorescence microscope with a Qimaging ExiBlue digital camera and ImageJ and Micro Manager Software, except for images in Fig. 6, which were taken on a Zeiss LSM 700 confocal microscope.

### In situ hybridization

Whole-mount *in situ* hybridization was performed as previously described (Attia et al., 2012; Wilkinson and Nieto, 1993). The following anti-sense probes were used: Pod1 (von Scheven et al., 2006), Wt1 (Kent et al., 1995), Sfrp1 (Esteve et al., 2000) and Wnt4 (Soueid-Baumgarten et al., 2014). In addition, the following probes were generated by RT-PCR from chick mesonephros cDNA: Sfrp2 (primers ACAATGACCTTGCATCCCG and GGGCACAGTCTTTATGGTGG) and Frzb (primers ATCTGCACCATC-GACTTCCA and ATGGGCTGCACTAGATCTCC). Following signal development, embryos were embedded in sucrose/gelatin, cryosectioned at 20-µm intervals, and examined using a Zeiss Axioimager microscope with differential interference contrast (DIC) optics.

### Combined in situ hybridization/immunofluorescence

After whole-mount *in situ* signal development and sectioning as described above, slides were then stained with rabbit polyclonal anti-GFP antibody (1:1000, Invitrogen) as described above. The slides were mounted with Fluoromount (Dako) and photographed using a Zeiss Axioimager microscope with DIC optics.

### Explant culture and microdissection

Embryos were pinned to a silicone dish dorsal side up using insect pins and tissue slices, ~1.5 somites in thickness (150 µm), were cut with a micro-scalpel (Feather) at the axial level of somites 23–24. Slices were further micro-dissected to remove the neural tube, and explants were transferred with a P20 pipette to a culture dish cover containing 20 µl medium drops (DMEM 4.5 g/ml glucose; 10% fetal bovine serum; 2% chick extract; 2 mM glutamine, 50 µg/ml PenStrep). The explant-containing drops were incubated on ice until all required samples were collected. A Millicell cell culture insert (Millipore) was placed in a 35-ml dish containing 1.2 ml of medium so that the filter was soaked but there was no excess liquid on the top side of the filter to avoid unnecessary movement of the explants. BIO (Calbiochem), was diluted in DMSO and added to the medium at a concentration of 50 µM. DMSO alone was added to the medium of the control group. All explants were transferred to the filter and incubated at the medium-air interface for 48 h.

### RT-PCR

Cultured mesonephros explants were harvested in 200 µl of Trizol solution (Invitrogen). RNA was prepared by extraction with Chloroform (Bio Lab) and precipitation with ethanol, with 20 µg glycogen added as a carrier. Reverse transcription was carried out for 2 h at 42°C in 6 µl of 5× transcription buffer (Promega), with 0.33 mM of each dNTP, 200 ng random hexamers, 3.3 mM dithiothreitol, 4 units ribonuclease inhibitor and 200 units Moloney Murine Leukemia Virus Reverse Transcriptase (Promega). PCR was carried out in a volume of 25 µl, using REDTaq ReadyMix PCR Reaction Mix (Sigma-Aldrich), 500 ng of each primer and 1 µl of template cDNA (from a 30 µl RT reaction). Thermal cycling was performed in an Eppendorf Mastercycler as follows: (1) initial denaturation at 94°C for 3 min, (2) cycling for the indicated number of cycles for each primer (see supplementary material Table S1 for primer sequences) between 94°C for 30 s, 60°C for 30 s and 72°C for 90 s. The number of cycles was chosen so that the amplification remained within the linear range as assessed by quantification of ethidium bromide-stained gels using an ImageQuant LAS 4000 gel scanner.

The PCR products were electrophoresed on a 2% agarose gel, soaked in 0.5 µg/ml ethidium bromide solution for 20 min and washed in distilled water for another 20 min. Quantification was performed using the ImageQuant LAS 4000 gel scanner and software, and statistical analysis was performed using Excel software (two-tailed *t*-test).

### Acknowledgements

We thank S. Dietrich, P. Bovolenta and D. Frank for generous gifts of plasmids.

### Competing interests

The authors declare no competing or financial interests.

### Author contributions

J.S., A.A.A., M.G. and T.M.S. conceived and designed the experiments. J.S., A.A.A. and R.Y. conducted the experiments. J.S., R.Y., M.G. and T.M.S. contributed to analysis of the data. J.S. and T.M.S. prepared and edited the manuscript.

### Funding

This research was supported by grants from the March of Dimes [FY12-343 to T.M.S.]; the Israel Science Foundation [1573/12 to T.M.S.]; the Niedersachsen-Israel Research Program [VWZN2917 to T.M.S.]; and the Rappaport Family Foundation (to T.M.S.).

### Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.123968/-/DC1>

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