Canonical Wnt9b signaling balances progenitor cell expansion and differentiation during kidney development

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SUMMARY
The mammalian kidney is composed of thousands of individual epithelial tubules known as nephrons. Deficits in nephron number are associated with myriad diseases ranging from complete organ failure to congenital hypertension. A balance between differentiation and maintenance of a mesenchymal progenitor cell population determines the final number of nephrons. How this balance is struck is poorly understood. Previous studies have suggested that Wnt9b/β-catenin signaling induced differentiation (mesenchymal-to-epithelial transition) in a subset of the progenitors but needed to be repressed in the remaining progenitors to keep them in the undifferentiated state. Here, we report that Wnt9b/β-catenin signaling is active in the progenitors and is required for their renewal/proliferation. Using a combination of approaches, we have revealed a mechanism through which cells receiving the same Wnt9b/β-catenin signal can respond in distinct ways (proliferate versus differentiate) depending on the cellular environment in which the signal is received. Interpretation of the signal is dependent, at least in part, on the activity of the transcription factor Six2. Six2-positive cells that receive the Wnt9b signal are maintained as progenitors whereas cells with reduced levels of Six2 are induced to differentiate by Wnt9b. Using this simple mechanism, the kidney is able to balance progenitor cell expansion and differentiation insuring proper nephron endowment. These findings provide novel insights into the molecular mechanisms that regulate progenitor cell differentiation during normal and pathological conditions.

KEY WORDS: Wnt, Progenitor cells, Kidney, Six2, Mouse

INTRODUCTION
During organogenesis, a balance must be struck between progenitor cell proliferation/renewal and differentiation to ensure proper organ size. The precarious nature of this equilibrium is particularly evident in the developing metanephric kidney. During development, signals from the ureteric bud epithelium stimulate the survival, proliferation and differentiation of an adjacent population of progenitor cells known as the metanephric mesenchyme. In addition, a bud-derived signal(s) causes a subset of the progenitors to differentiate into an epithelial structure known as a renal vesicle. The renal vesicle will undergo significant morphogenesis to form a nephron, a vascularized tubule that maintains blood chemistry. As a result of these inductive interactions, each mouse kidney will form up to 20,000 nephrons (~1,000,000 in an adult human (Hoy et al., 2003; Keller et al., 2003; Nyengaard and Bendtsen, 1992)) all derived from an initial progenitor population of approximately 12,000 cells (Kobayashi et al., 2008).

An essential step in kidney development is establishment of a balance between the expansion and differentiation of the nephron progenitor cell population. Tipping this balance in favor of one or the other results in a reduction in nephron endowment and can have dire consequences, including the formation of progenitor cell cancers (Wilms’ tumors), renal hypoplasia, chronic hypertension and kidney failure (Keller et al., 2003; Koesters et al., 2003; Koesters et al., 1999; Li et al., 2004). Although several factors regulating progenitor cell expansion and differentiation have been identified (Barasch et al., 1999; Blank et al., 2009; Carroll et al., 2005; Dudley et al., 1995; Griesshammer et al., 2005; Perantoni et al., 2005; Schmidt-Ott et al., 2007; Stark et al., 1994), it remains unclear how the balance between these two events is controlled.

Nephron induction requires the sequential activity of two Wnts: Wnt9b and Wnt4 (Carroll et al., 2005; Stark et al., 1994). Wnt9b is secreted from the ureteric bud and induces a subset of the renal progenitor cells to aggregate and express Wnt4. Wnt4, by signaling through the canonical Wnt/β-catenin pathway, further induces these pre-tubular aggregates (PTAs) to transition into epithelial structures known as renal vesicles (RVs) (Park et al., 2007). Although the molecular nature of the Wnt4 signal has been determined, the pathway used by Wnt9b is still uncertain, although it has been suggested that it also signals through β-catenin (Park et al., 2007).

Wnt4, and other characterized Wnt9b targets such as Pax8 and Fgf8, are expressed in only a small proportion of the progenitor cells (those undergoing differentiation), while the remaining ‘uninduced’ progenitors do not express these genes. This observation, and others, has led to a model suggesting that the Wnt9b signal is not active in the renewing progenitor cells, which keeps them from differentiating. How Wnt9b signaling is repressed in these cells is unclear, although one hypothesis, that has some experimental support, suggests that the transcription factor Six2 blocks the ability of cells to receive the Wnt9b signal. Six2 is expressed in the renewing progenitors and its loss results in the precocious differentiation of the entire progenitor population in a Wnt9b-dependent manner (Kobayashi et al., 2008; Self et al., 2006). How Six2 affects Wnt9b activity is unclear.
In this study, we have identified several novel targets of Wnt9b. Our data suggest that, contrary to current models, Wnt9b/β-catenin signaling is active in both the differentiating and renewing progenitor cells. We provide evidence that, rather than inhibiting Wnt9b, Six2 appears to cooperate with it to promote progenitor cell proliferation. Based on our results, we propose a model whereby Wnt9b is the key factor that regulates the balance between progenitor cell proliferation and differentiation. In the renewing progenitor cells, Wnt9b/β-catenin cooperates with Six2 to elicit progenitor cell expansion. In cells where Six2 activity is low or absent, Wnt9b/β-catenin promotes differentiation. These results modify our current understanding of the molecular mechanisms that regulate progenitor cell expansion and differentiation during normal and abnormal kidney development.

MATERIALS AND METHODS

Mice

All mouse alleles (Wnt9b+, Wnt9bneo, Wnt9bflx, Six2+, Rarb2Cre; KspCre, RosaYFP, catnbcatnbneo and catnbcatnb) are as previously described (Brault et al., 2001; Carroll et al., 2005; Harada et al., 1999; Kerner et al., 2009; Kobayashi et al., 2005; Self et al., 2006; Shao et al., 2002; Stark et al., 1994).

Ex vivo organ culture

Organ culture, mesenchymal isolation and inhibitor of Wnt (I/W) or lithium treatment were as previously described (Carroll et al., 2005; Karner et al., 2009). Briefly, E11.5 kidneys or isolated mesenchyme were grown on transwell filters at the air/media interface for 24, 48 or 72 hours. The media was supplemented with LiCl (15 mM), IWR1 (100 μM), and IWP2 (5 μM) or DMSO as a negative control. The media was replaced with fresh media containing the above listed compounds every 12 hours. All treatments were repeated at least three times with a minimum of six individual kidneys from six distinct embryos assayed per replicate.

Heterochronic recombination

Metanephric mesenchyme from either E11.5 wild-type or E13.5 Wnt9b+/– animals was isolated as previously described (Carroll et al., 2005). Once isolated, both wild-type and Wnt9b+–/– metanephric mesenchyme were individually recombined with the E11.5 wild-type ureteric bud and cultured for 48 hours. Control mesenchymes were recombined with E13.5 Wnt9b+–/– ureteric bud.

Microarray analysis

Total mRNA was extracted from 30-35 wild-type or Wnt9b+–/– E11.5 mesenchymes to provide 1 μg total RNA. This combined mRNA constitutes a single replicate. Total RNA was hybridized to the Affymetrix Mouse Genome 430 2.0 Array by the UT Southwestern Genomics & Microarray Core facility. This analysis was repeated for five total replicates. The results of these replicates were analyzed using the Gene Set Analysis Toolkit (http://bioinfo.vanderbilt.edu/webgestalt) (Zhang et al., 2005).

In situ hybridization

Both whole-mount and section in situ hybridization was performed as previously described (Carroll et al., 2005; Self et al., 2006). Tissue was hybridized with antisense probes for the following genes: C1qc2 (Fam132a – Mouse Genome Informatics), Cited1, Pla2g7, Taf5a (Fam19a5 – Mouse Genome Informatics), Uncx4.1 (Uncx – Mouse Genome Informatics), Rsop1, Pax9, Parc2, Six2, Gdf6 and Eya1. Restriction enzymes and polymerase have been previously described, except for C1qc2 (Accession BC026393), Cited1 (Accession BC052030), Pla2g7 (Accession BC010726), Taf5a (Accession BC015306), Uncx4.1 (Accession AJ001116), Rsop1 (Accession BQ933737) and Eyal (Accession BC060260), that were cut with EcoRI and transcribed with T3 Polymerase (Cited1 and Eya1), EcoRI and T7 (C1qc2, Taf5a and Rsop1), EcoRV and T7 (Pla2g7) or XhoI and T7 (Uncx4.1).

Immunohistochemistry

Tissue fixation, staining and image capture were as previously described (Kerner et al., 2009). Tissue was incubated with the following antibodies: anti-phospho-histone H3 (Ser10) (1:500, Sigma, St Louis, MO, USA), anti-Six2 (rabbit; 1:500, ProteinTech, Chicago, IL, USA), anti-Pax2 (rabbit; 1:500, Covance, Princeton, NJ, USA) and anti-DBA (Biotinylated; 1:500, VectorLabs, Burlingame, CA, USA), anti-Cited 1 (rabbit; 1:500, NeoMarkers, Fremont, CA, USA) and anti-ampiphysin (rabbit; 1:500, ProteinTech). Nuclei were counterstained using either Topro-3 (1:200, Invitrogen, Carlsbad, CA, USA) or Sytox Green (1:5000, Invitrogen). Cell death was quantitated using Lysotracker (1:200, Invitrogen) as previously described (Zucker et al., 1999). Results shown are representative examples from one of at least three different stainings of three different kidneys.

Chromatin immunoprecipitation

Approximately 20-25 E15.5 mouse kidneys were isolated, homogenized and crosslinked in 1% formaldehyde. Crosslinked tissues were homogenized into a single-cell suspension. Nuclei from crosslinked cells were resuspended in Tris-EDTA buffer and sonicated. The soluble chromatin was transferred into radioimmunoprecipitation assay (RIPA) buffer and precleared. Immunoprecipitation was performed with 5 μg of rabbit anti-β-catenin (Santa Cruz, sc-7199) or isotype-specific IgG as a negative control (Schmidt-Ott et al., 2007), and the immune complexes were absorbed with protein A/G beads (Pierce) and blocked with bovine serum albumin. Purified genomic DNA was amplified using promoter-specific primers and visualized on a 2% agarose gel. One percent of the input DNA was amplified to normalize results.

To quantify the fold enrichment, the chromatin from two separate pull-downs was pooled and the relative amplification values were identified using an iCycler (Bio-Rad) real-time PCR detection system. The amplification was determined by normalizing the expression levels of the β-catenin pull-down samples to the input. The relative fold change of expression was further calculated with respect to the amplification in the IgG pull-down samples, which were arbitrarily assigned a value of 1 for each primer set. A 150 bp fragment with no consensus β-catenin-binding sites was amplified as the non-specific internal control site. The data provided are the averages of the fold change values relative to the IgG control from two independent runs (three replicates per run) and the error bars represent the standard deviation. As the IgG controls are always assigned a value of 1, no standard deviation is assigned.

Primer sequences for ChIP

Primer sequences were as follows: C1qc2 site 1 (forward) 5'-TGTCTCCTCCATTTCAGGTTG-3' and (reverse) 5'-GAGGACGTTTGTGAGAAGCTAC-3'; C1qc2 site 2 (forward) 5'-CGTCTCATGTCGAGATATCTC-3' and (reverse) 5'-ATTCCTCCCACAAAGACCC-3'; Tafa5 site 2 (forward) 5'-TCTTCAAAGTTCGACCC-3' and (reverse) 5'-TGGTCAAATCTCCGACCC-3'; Tafa5 site 3 (forward) 5'-GTGAGGTGGTGTAAC-3' and (reverse) 5'-TGCAGC-3'; Pla2g7 site 1 (forward) 5'-TAGGAGAGGAATGTGAAGCT-3' and (reverse) 5'-CAGCAGGACACACACAC-3'; Pla2g7 site 2 (forward) 5'-GAGGAGGAGGATGTGAGTT-3' and (reverse) 5'-CCCAAGGACCCATTTGAGT-3'.

Statistics

All statistical analysis was performed using Student’s t-test.

RESULTS

Identification of novel Wnt9b target genes

We previously showed that Wnt9b is necessary and sufficient for the induction of renal vesicles in the metanephric mesenchyme (Carroll et al., 2005). To identify novel molecular targets of Wnt9b, microarray analysis was performed comparing E11.5 wild-type and
Wnt9b−/− mesenchymes. Analysis of this data resulted in the identification of 30 putative Wnt9b targets that were downregulated at least twofold in the absence of Wnt9b function (Table 1).

The expression of the Wnt9b target genes was validated in wild-type E11.5 kidneys using in situ hybridization. We generated riboprobes for 28 of the 30 target genes identified. Twenty-seven

Table 1. Wnt9b target genes

<table>
<thead>
<tr>
<th>Gene description</th>
<th>Gene name</th>
<th>Fold change</th>
<th>Class</th>
<th>Expression in Wnt9b−/−</th>
<th>Expression in Wnt4−/−</th>
<th>LiCl induction</th>
<th>Expression in IWR1 treated explants</th>
<th>Expression in IWP2 treated explants</th>
<th>Lef/Tcf sites</th>
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<tr>
<td>C1q domain containing 2</td>
<td>C1qdc2</td>
<td>0.481</td>
<td>PTA/I</td>
<td>---</td>
<td>+/–</td>
<td>Yes</td>
<td>---</td>
<td>---</td>
<td>Yes†</td>
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<tr>
<td>Cadherin 4</td>
<td>Cdbh4</td>
<td>0.362, 0.328</td>
<td>PTA/I</td>
<td>---</td>
<td>+/–</td>
<td>ND</td>
<td>---</td>
<td>---</td>
<td>Yes†</td>
</tr>
<tr>
<td>Fibroblast growth factor 9</td>
<td>Fgf9</td>
<td>0.399</td>
<td>PTA/I</td>
<td>---</td>
<td>---</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Yes†</td>
</tr>
<tr>
<td>Glutathione peroxidase 6</td>
<td>Gpx6</td>
<td>0.203</td>
<td>PTA/I</td>
<td>---</td>
<td>---</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Yes†</td>
</tr>
<tr>
<td>Lymphoid enhancer binding factor 1</td>
<td>Lef1</td>
<td>0.44</td>
<td>PTA/I</td>
<td>---</td>
<td>---</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Yes†</td>
</tr>
<tr>
<td>Musashi homolog 2</td>
<td>Msi2</td>
<td>0.486</td>
<td>PTA/I</td>
<td>---</td>
<td>+/–</td>
<td>Yes</td>
<td>---</td>
<td>---</td>
<td>Yes†</td>
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<tr>
<td>Paired box gene 8</td>
<td>Pax8</td>
<td>0.49</td>
<td>PTA/I</td>
<td>---</td>
<td>+/–</td>
<td>Yes</td>
<td>---</td>
<td>---</td>
<td>Yes†</td>
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<tr>
<td>Phospholipase C-like 3</td>
<td>Plc3I</td>
<td>0.496</td>
<td>PTA/I</td>
<td>---</td>
<td>---</td>
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<td>ND</td>
<td>ND</td>
<td>Yes†</td>
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<td>Riken cDNA 0610010D24</td>
<td>Daple</td>
<td>0.494</td>
<td>PTA/I</td>
<td>---</td>
<td>---</td>
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<td>ND</td>
<td>ND</td>
<td>Yes†</td>
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<tr>
<td>Solute carrier family 45, member 3</td>
<td>Scl45a3</td>
<td>0.399</td>
<td>PTA/I</td>
<td>---</td>
<td>---</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Yes**</td>
</tr>
<tr>
<td>Sorbin and SH3 domain containing 2</td>
<td>Sorbs2</td>
<td>0.434</td>
<td>PTA/I</td>
<td>---</td>
<td>+/–</td>
<td>Yes</td>
<td>---</td>
<td>---</td>
<td>Yes†</td>
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<tr>
<td>Winless-related MMTV integration site 4</td>
<td>Wnt4</td>
<td>0.38, 0.347</td>
<td>PTA/I</td>
<td>---</td>
<td>---</td>
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</table>
out of 28 showed expression in the metanephric mesenchyme at E11.5 (Table 1). Based on spatial differences in the observed expression patterns, the genes were grouped into two distinct classes. Class I targets represented genes expressed in the differentiating pre-tubular aggregates (PTA, see Table 1). This class included the previously identified Wnt9b targets Pax8 and Wnt4 (Carroll et al., 2005), as well as several newly identified targets including cadherin 4 (Cdh4), Lef1 and C1qdc2 (Fig. 1A; Table 1). Class II targets were characterized by broader expression in the metanephric mesenchyme. This class of genes included Cited1, phospholipase A2 group 7 (Pla2g7), amphiphysin (Amph) expressed sequence AW049604 (Tafa5) and Uncx4.1 (Fig. 1D,G,J,M; Table 1).

In situ hybridization and immunofluorescence on adjacent sections of E11.5 and P1 wild-type kidneys revealed that the class II target genes had significant overlap in expression with the bona fide progenitor cell marker Six2. In fact, one Class II target, Cited1, has previously been shown to be a marker of the self-renewing mesenchymal progenitor cells (Boyle et al., 2008). Although several of the Class II targets appeared to be specifically expressed in the progenitor cells, others had patterns that were more expansive and included the PTA domain and later structures. By contrast, Class I targets showed little if any expression in the progenitor cells (see Fig. S1A in the supplementary material and data not shown).

To verify that the identified genes were indeed targets of Wnt9b, we examined their expression in Wnt9b mutants using in situ hybridization. Irrespective of their expression domain, the expression of most genes examined was noticeably reduced or undetectable in Wnt9b mutant mesenchyme at E11.5 (Fig. 1; Table 1 and data not shown).

The expression of Wnt9b target genes in the progenitor population was unexpected. Previous studies have suggested that Wnt9b was only active in the subpopulation of mesenchymal progenitor cells that was induced to differentiate and blocked in the remaining progenitors to allow expansion (Carroll et al., 2005; Kobayashi et al., 2008; Self et al., 2006). We therefore investigated the molecular basis of the progenitor signaling.

β-Catenin signaling induces the expression of Wnt9b target genes

Wnt signals can be transduced down β-catenin-dependent or -independent pathways. Although previous studies have suggested that Wnt9b signaled through β-catenin to induce tubule differentiation, this has not been directly demonstrated (Park et al., 2007). The identification of cell type-specific Wnt9b target genes allows us to address more precisely Wnt9b pathway usage in both the PTAs and the progenitor cells.

To determine which pathway was responsible for the activation of Class I and II target genes, we used ex vivo organ culture. E11.5 metanephric mesenchyme was isolated and cultured in the presence or absence of the Gsk-3beta antagonist LiCl (Klein and Melton, 1996), which results in stabilization of β-catenin. Mesenchymes were cultured for 48 hours in 15 mM LiCl, a treatment that effectively induces tubulogenesis in cultured kidneys (Davies and Garrod, 1995), and assayed for expression of Wnt9b targets. As expected, LiCl treatment was sufficient to induce the expression of the Class I genes C1qdc2 and Pax8, whereas DMEM alone was not (Fig. 2A,B; Table 1). Next, we evaluated the expression of Class II genes. Mesenchymes cultured with LiCl expressed every Wnt9b progenitor target tested, including Cited1, Pla2g7 and Tafa5, whereas mesenchyme cultured in DMEM alone exhibited no
expression (Fig. 2C-F; Table 1). These data show that activation of β-catenin is sufficient to stimulate expression of both Class I and II Wnt9b target genes.

**β-Catenin activity is necessary for expression of Wnt9b targets**

The studies listed above suggest that β-catenin activation is sufficient to activate expression of Class I and II targets. We next sought to determine whether it was necessary. Recently identified small molecule inhibitors of the Wnt pathway have been used to study the role of Wnt signaling in kidney organ culture (Chen et al., 2009; Huang et al., 2009). To test pathway requirements in the activation of Wnt9b targets, we used two individual inhibitors, IWP2 and IWR1, that function at distinct points in the Wnt pathway. IWP2 inhibits porcupine, a molecule necessary for Wnt ligand secretion (Kadowaki et al., 1996). Application of this molecule blocks both canonical and non-canonical Wnt signaling. IWR1 specifically inhibits the canonical/β-catenin pathway through inhibition of tankyrase 1 and 2 (Chen et al., 2009; Huang et al., 2009). Both of these factors act specifically and effectively in the context of kidney organ culture (Karner et al., 2010).

E11.5 wild-type kidneys were cultured for 24, 48 or 96 hours in the presence of either DMSO or IWP2 and assayed for the expression of Wnt9b targets. As expected, the class I targets C1qdc2 and Pax8, and the class II targets Cited1, Pla2g7, Tafa5 and Uncx4.1 were all expressed in the induced mesenchyme of kidneys cultured in DMSO (Fig. 2G,J,M,P; Table 1). However, inhibition of Wnt ligand secretion using IWP2 resulted in a significant reduction of all Wnt9b target genes examined (Fig. 2, compare G with H, J with K, M with N and P with Q; data not shown.). This was not a delay in kidney development as markers were still undetectable after 96 hours of culture (Karner et al., 2010). These data validate the efficacy of this approach and set a baseline for further comparison.

The data above verify that Wnt production is necessary for the expression of Wnt9b target genes. To test whether the loss of the target genes can be specifically attributed to inhibition of the canonical Wnt pathway, we cultured kidneys in the presence of IWR1. IWR1 administration leads to the complete inhibition of the Class I Wnt9b target gene Pax8 within 16 hours (Karner et al., 2010) (data not shown). Similarly, IWR1 co-culture inhibited the expression of other class I genes, including C1qdc2 and Cadh4, and all Class II target genes tested, including Cited1, Pla2g7, Tafa5 and Uncx4.1 (Fig. 2, compare G with I, J with L, M with O and P with R; Table 1). The kinetics of gene loss after treatment with IWR1 were indistinguishable for Class I and II targets. Together, these data suggest that β-catenin-dependent Wnt signaling is necessary for expression of both Class I and II Wnt9b target genes.

**Wnt9b/β-catenin cell autonomously activates progenitor target genes**

Although the data presented above indicate that β-catenin is required for the expression of Wnt9b target genes in the progenitors, treatment of isolated mesenchymes with LiCl or the IW inhibitors does not provide the cell type specificity to determine the precise cellular targets of pathway activation. These compounds could be affecting Wnt4 activity in the PTAs, which might secondarily affect expression of target genes in the progenitors.

To test the possibility that a signal(s) from the PTAs regulates expression of genes in the progenitor cells, we evaluated the expression of Class I and II Wnt9b targets in E11.5 Wnt4−/− kidneys (Stark et al., 1994). Not surprisingly, 6/12 class I targets (PTA markers) were completely absent from Wnt4−/− metanephric mesenchyme (MM) (Table 1), suggesting that they were indirect targets of Wnt9b identified due to the loss of Wnt4 activity in Wnt4−/− mutants. However, the majority of Wnt9b target genes, including the Class I targets C1qdc2 and Cadh4, and the Class II targets Cited1, Pla2g7, Tafa5 and Uncx4.1, were still expressed in Wnt4−/− MM at E11.5 (Fig. 1, compare A with C, D with F, G with I, J with L, M with O; Table 1). In fact, one class II target, Cited1, appeared to be expanded into what would normally be the PTA domain in Wnt4 mutants. This expansion most probably reflects a failure to induce differentiation in the mutant progenitor cells. Cited1, and all other class II targets examined, are still expressed through at least E13.5 (Table 1), even though all Class I targets examined were lost from Wnt4 mutants by E12.5.

To provide further evidence that β-catenin signaling was required in the progenitors, we determined whether activation of β-catenin in these cells was sufficient to rescue the expression of progenitor targets in a Wnt9b mutant. To accomplish this, we expressed an activated allele of β-catenin [catnb integrates (Harada et al., 1999)] in the progenitor cells of Wnt9b mutants using the Rarβ2Cre transgene (Kobayashi et al., 2005). Rarβ2Cre is active throughout the
nephrogenic mesenchyme (including the mesonephric and metanephric mesenchyme) from E10.5 through birth as well as in a subset of the cortical stroma at E12.5 (Kobayashi et al., 2005). Activation of β-catenin with Rarb2Cre in otherwise wild-type animals resulted in an anterior expansion of all evaluated genes, including C1qdc2, Tafa5 and Pla2g7, relative to wild-type littermates (Fig. 3A,B,E,F,I,J). This expansion is probably reflective of the expression of Rarb2Cre in the mesonephric mesenchyme. Consistent with this hypothesis, we also observed ectopic expression of C1qdc2, Tafa5 and Pla2g7 in the mesonephric tubules in Rarb2Cre;catnb1exon3flox animals (data not shown).

To test whether activation of β-catenin was sufficient to drive expression of Wnt9b target genes in the absence of Wnt9b, we generated Rarb2Cre;catnb1exon3flox;Wnt9b–/– mice. Activation of β-catenin in the Rarb2Cre expression domain of Wnt9b mutants was sufficient to rescue the expression of both the class I target gene C1qdc2 (compare Fig. 3C with 3D) and the class II targets Tafa5 and Pla2g7 (compare Fig. 3G with 3H and 3K with 3L).

We next asked whether β-catenin activity was required in the progenitors for the expression of Wnt9b targets using a floxed null allele of β-catenin (Brault et al., 2001) and the Rarb2Cre strain (Kobayashi et al., 2005). We found that most Rarb2Cre;β-catenin–/– pups (8/10) had severely hypoplastic kidneys and died within 2 days of birth (not shown). At E12.5, Rarb2Cre;catnb1exon3flox kidneys appeared normal or were slightly smaller than wild type (Fig. 4A-D). Both Pla2g7 and Tafa5 were significantly down regulated in the MM of Rarb2Cre;catnb1exon3flox animals at E12.5, with some areas showing a complete loss of expression (arrows in Fig. 4C,D). Immunofluorescence with a β-catenin antibody revealed mosaic expression of β-catenin protein in mutants at E12.5, indicating that recombination with this line was inefficient (data not shown). By E13.5, we were able to find large domains of progenitors (as marked by Six2 protein) that lacked detectable levels of β-catenin protein (Fig. 4E-H). Although mutant cells continued to express markers of the progenitor domains (such as Six2), they showed greatly reduced protein levels for two Class II
β-Catenin is required cell-autonomously for the expression of progenitor markers. (A-J) Expression analysis of Wnt9b target genes in wild-type (A,B,E-G) or Rab2Cre;Catnb−/flox mutant (C,D,H-J) E12.5 kidneys. A and C are images of whole-mount kidneys hybridized with antisense probes to Tafa5 (A,C) or Pla2g7 (B,D). E-J are images of sections stained with antibodies to β-catenin (red), Six2 (green in E,H), Cited1 (green in F,I) and amphiphysin (green in G,J). All sections were counterstained with the nuclear marker To-Pro 3 (blue). Arrows indicate progenitor cells. Removal of β-catenin from the progenitors results in cell-autonomous loss of Wnt9b target genes.

Wnt9b targets, amphiphysin and Cited1 (Fig. 4I,J). In some cases, we were able to find small clusters of wild-type cells expressing the Class II targets that were surrounded by mutant cells, suggesting that loss of expression was cell-autonomous (data not shown). The loss and gain of expression studies suggest that β-catenin activity in the progenitors is necessary and sufficient for the expression of Class II Wnt9b target genes in the progenitors.

β-Catenin is associated with progenitor gene promoters

Collectively, our data supports the hypothesis that Wnt9b signals to both the PTAs and progenitor cells via β-catenin. However, the data still do not indicate whether the Wnt9b target genes identified here are direct targets of β-catenin. To identify prospective β-catenin regulatory elements, we scanned the genomic loci of Wnt9b targets using the genome alignment tool in the ECR browser (http://ecrbrowser.dcode.org) looking for consensus Lef/Tcf-binding sites [5′-(A/T)(A/T)CAAAG-3′]. Of the genes evaluated, most (23/25) contained multiple consensus Lef/Tcf-binding sites within 50 kb of the transcriptional start (Table 1).

Previous studies showed that β-catenin was associated with the promoter of at least one of our Class I targets, Pax8 (Schmidt-Ott et al., 2007). Examination of another Class I target, C1qdc2, revealed that it contained three conserved Lef/Tcf-binding sites. Two of the sites were located in the first intron, while a third site was located downstream of the 3′ UTR (Fig. 3M). To determine whether β-catenin was physically associated with these sites, we performed chromatin immunoprecipitation (ChIP) with an antibody to β-catenin and real time-PCR with primers flanking each of these sites. ChIP against β-catenin significantly enriched for the DNA surrounding Lef/Tcf-binding sites 1 and 2 in C1qdc2 over a control (IgG) IP (6.3±0.8 and 2.8±0.9 fold, respectively) (Fig. 3M). No enrichment was observed for site 3 (not shown).

We next evaluated the genomic loci of the class II targets Tafa5 and Pla2g7. Tafa5 contained three conserved Lef/Tcf sites. The first site was located ~20 kb upstream of the first exon, while sites 2 and 3 were located in the second intron. Again, using site-specific primers, we found that the second site was not enriched in the β-catenin precipitated chromatin (relative to IgG control), whereas the first and third sites were (3.1±0.7 and 6.1±1.6 fold, respectively) (Fig. 3N). Pla2g7 contained two conserved Lef/Tcf sites. The first site was located upstream of the 5′ UTR, whereas the second site was located in the first intron. Both site 1 and 2 were enriched by β-catenin precipitation (5.5±0.4 and 5.1±0.2 fold relative to control, respectively), while a randomly chosen site upstream of the 5′ UTR that did not contain a consensus site was not enriched (Fig. 3O). These data indicate that β-catenin is physically associated with DNA surrounding the loci of both classes of Wnt9b target genes. Based on these studies, as well as the genetic and ex vivo culture data, we propose that Wnt9b signals through β-catenin to both the pre-tubular aggregates and the progenitor cells.

Wnt9b and Six2 act cooperatively to regulate expression of target genes

Previous studies suggested that Six2 inhibited Wnt9b and β-catenin signaling within the progenitor cells (Kobayashi et al., 2008). Loss of Six2 resulted in the expansion of several Wnt9b target genes including Pax8, Sfrp2 and Wnt4. However, our data suggest that Wnt9b actively signals to the Six2-expressing cells. To gain insight into these seemingly contradictory results, we determined what effect loss of Six2 had on our newly identified Wnt9b target genes. As expected, in situ hybridization revealed that, similar to previous observations (Kobayashi et al., 2008; Self et al., 2006), several Class I targets of Wnt9b, including Pax8, Wnt4 and C1qdc2, were expanded into the progenitor domain in Six2−/− mesenchyme at E11.5 [see Fig. 5A,B, Kobayashi et al. (Kobayashi et al., 2008) and Self et al. (Self et al., 2006)].

We next evaluated the expression of Class II targets in Six2−/− mutants. Somewhat surprisingly, we found that the Wnt9b targets Cited1, Pla2g7 and Tafa5 were absent from Six2−/− mesenchyme at E11.5 (Fig. 5C-H). Importantly, the loss of Wnt9b targets did not reflect a loss of the progenitor domain as several Wnt9b-independent progenitor markers, including Eya1 and Pax2, were still present in both Wnt9b and Six2−/− mutants at this stage (Fig. 5I-K) (Carroll et al., 2005; Kobayashi et al., 2008; Self et al., 2006). Furthermore, Tafa5 mRNA is absent from the mesenchyme of both Wnt9b−/− and Six2−/− embryos at E10.5 (see Fig. S2 in the
supplementary material), a time point prior to the manifestation of morphological defects in either mutant (Carroll et al., 2005; Self et al., 2006). These results suggest that Wnt9b and Six2 are both required for the expression of Wnt9b target genes in the progenitor population.

In both Wnt9b and Six2 mutants, the initial branching of the ureteric bud to form a T shape occurs normally (Carroll et al., 2005; Self et al., 2006). By contrast, E11.5 Wnt9b−/−;Six2−/− ureteric buds failed to branch within the metanephric mesenchyme (Kobayashi et al., 2008) (Fig. 5P). Branching of the ureteric bud is mediated by several factors expressed in the progenitor cells, including glial-derived neurotrophic factor (Gdnf). Although Gdnf mRNA is expressed in the mesenchyme of Wnt9b−/− and Six2−/− embryos at E11.5, the domain of expression is slightly reduced in both mutants relative to wild type (Fig. 5M-O). To determine whether the reduced branching phenotype was caused by a further decrease in Gdnf levels, we examined its expression in Wnt9b−/−;Six2−/− kidneys. Gdnf expression was completely absent from the mesenchyme of Wnt9b−/−;Six2−/− animals at E11.5 (Fig. 5P). To ensure that the loss of Gdnf was not due to a loss of progenitor cells, we evaluated the expression of an additional progenitor marker, Eya1, the expression of which is independent of Wnt9b and Six2 activity. Eya1 was expressed in the mesenchyme of Wnt9b−/−;Six2−/− animals at E11.5 (Fig. 5L). These data indicate that Six2 and Wnt9b act cooperatively within the mesenchymal progenitor cells to activate expression of target genes.

Wnt9b regulates progenitor cell proliferation

The data presented above suggest that canonical Wnt9b signaling is active in the progenitor cells. Previous studies have demonstrated roles for Wnt signaling in progenitor cell specification, survival/maintenance and renewal/proliferation (Chenn and Walsh, 2002; Reya et al., 2003; Zechner et al., 2003). As mentioned Six2, Eya1, Gdnf and Pax2 are all expressed in Wnt9b mutant mesenchyme at E11.5. Pax2 and Six2 are still expressed in Wnt9b mutants at E13.5, although relative to wild type, the domain of expression is greatly reduced (see Fig. S3 in the supplementary material). Furthermore, heterochronic recombination and culture of the E13.5 mutant mesenchyme with wild-type E11.5 ureteric bud results in activation of class II targets Cited1 and Class I targets Pax8 and C1qdc2 (see Fig. S3 in the supplementary material and data not shown). Together, these results indicate that the Wnt9b mutant progenitors are properly specified and retain competence to respond to the inductive signal.

The failure of the progenitor population to expand in Wnt9b mutants suggested that Wnt9b might regulate apoptosis or cell proliferation of the mesenchyme. Using Lyso-tracker, we were unable to detect differences in the rate of apoptosis between wild-type and Wnt9b-mutant mesenchymal cells at E11.5 (see Fig. S4 in the supplementary material). By contrast, wild-type E11.5 mesenchymal cells had a rate of proliferation approximately five times greater than those of Wnt9b mutants (2.48% versus 0.48% of cells positive for phospho-histone H3 staining; P=0.007, t-test, n=3 animals and 2376 or 2122 total cells for wild type and Wnt9b null, respectively (see Fig. S4 in the supplementary material)).

To ensure the effect on proliferation was a direct result of loss of Wnt9b and not a secondary consequence of failure to induce renal vesicles, we also evaluated proliferation in Wnt4−/− mesenchyme. Although loss of Wnt4 also resulted in a significant decrease in proliferation (3.12% and 1.81% in wild type and null, respectively; P=0.006, t-test, n=3 animals and 2922 or 2076 total cells for wild type and Wnt4 null), the rate of proliferation in Wnt4 mutants was much higher than observed in Wnt9b mutants. These data suggest that Wnt9b regulates proliferation/renewal of the mesenchymal progenitor cells.

Ablation of Wnt9b results in premature exhaustion of the progenitor cells

Wnt9b is expressed in mouse ureteric bud/collection ducts through the embryonic period and into adult stages (Karner et al., 2009). However, despite continued presence of active Wnt9b ligand, renal tubule formation ceases by postnatal (P) day 5 (Hartman et al., 2007). For most of the developmental period, progenitor cell renewal outpaces differentiation. However, around P3, the rate of progenitor cell expansion decreases, and by P5, the progenitor cell renewal rate is decreased further.
population is exhausted after a wave of differentiation (Hartman et al., 2007). A reasonable hypothesis based on the data presented in this study is that a loss in ability of the progenitor cells to respond to Wnt9b may play a causal role in the failure of progenitor cells to self-renew postnatally.

To test this hypothesis, we compared the expression of Wnt9b target genes to the progenitor expression of Six2 in wild-type mice from P3 to P4. As expected, Six2 mRNA and protein were present in P3 kidneys, although the progenitor expression is reduced and, in some cases, Six2 appeared to be only expressed in the PTAs (see Fig. S5A in the supplementary material). This probably represents the period where the last progenitors are being converted to tubules. Six2 mRNA and protein were completely undetectable by P4 (see Fig. S5B in the supplementary material). The Wnt9b target genes Pla2g7 and Cited1 were both present in the progenitor cells at P3 but completely absent (for Cited1) or present only in epithelial structures (for Pla2g7) at P4, indicating that, although Wnt9b was still expressed, it no longer actively signaled to the progenitor population (see Fig. S5C-F in the supplementary material). Importantly, the class I targets Cldn5 and Pax8 were still expressed at P4, a full day after Cited1 was lost, indicating that Wnt9b is still active and inducing tubule formation (see Fig. S5H in the supplementary material and not shown). These data suggest that a loss of Wnt9b signaling to the progenitor cells normally coincides with or immediately follows loss of Six2 expression in the progenitors.

We have previously shown that removal of Wnt9b from the kidney between E15.5 and E17.5 resulted in significantly smaller kidneys than in wild type (Karner et al., 2009). We originally attributed this hypoplastic phenotype to a deficit in tubule induction; however, our new findings suggest this phenotype may be equally attributable to a deficit in progenitor cell expansion. To test this hypothesis, we assessed progenitor cell maintenance/ expansion in KspCre;Wnt9b-/- mice. Prior to E15.5, KspCre is active only in the distal collecting ducts and has no apparent effect on the expression of Wnt9b target genes (Karner et al., 2009) (data not shown). At some time between E15.5 and E17.5, KspCre activity expands to include the ureteric bud tips (adjacent to the progenitors). We therefore examined the expression of Wnt9b-dependent and -independent progenitor markers in KspCre;Wnt9b-/- kidneys between E15.5 and P1. Pax2, Six2 and the Wnt9b target genes Pla2g7, Tafa5 and Uncx4.1 were all present and expressed at comparable levels with wild-type kidneys at E15.5 (data not shown). At E17.5, Pax2 and Six2 levels were reduced in the progenitors cells of KspCre;Wnt9b-/- animals (Fig. 6A,B; data not shown), whereas Pla2g7 and Uncx4.1 were completely lost (Fig. 6C,D,G,H). Levels of Tafa5 were highly reduced at E17.5 and completely absent at P1 (Fig. 6E,F,M,N). The loss of expression for these genes was specific to progenitor cells as Pla2g7 expression in the medullary stroma was maintained in mutants (not shown). By P1, the progenitor population (as indicated by Six2 expression) appeared to be completely lost (Fig. 6I-P).

We next tested the effects of late Wnt9b ablation on progenitor cell proliferation. At E17.5, there was a significant reduction in cell proliferation rates in Wnt9b mutants (2.44% and 1.75% for wild type and KspCre;Wnt9b-/-, respectively; P=0.027). However, as the progenitor cells in Wnt9b mutants are not being converted into new tubules (as determined by Cldn5 expression) decreases in proliferation alone cannot explain the complete absence of the progenitor population by P1. We also evaluated the rate of apoptosis in KspCre;Wnt9b-/- kidneys at E17.5. Somewhat surprisingly, this analysis revealed a significant increase in cell death upon loss of Wnt9b (1.62% and 26.83% apoptosis in wild-type and KspCre;Wnt9b-/- animals, respectively, P=0.0039, t-test), even though apoptosis was not affected by Wnt9b loss at earlier stages. These data suggest that Wnt9b plays an additional (most probably secondary) role in survival of the progenitor population.

DISCUSSION

In this study, we present data suggesting that Wnt9b is actively signaling to the kidney progenitor cells where it cooperates with Six2 to mediate expansion and/or self-renewal. Multiple pieces of data suggest that the role for Wnt9b in the progenitors is direct and through the canonical/β-catenin-dependent pathway. First, we show

**Fig. 6. Removal of Wnt9b results in a premature loss of progenitor cells.** (A-P) In situ hybridization evaluating the expression of Six2 (A,B,L,J), Pla2g7 (C,D,K,L), Tafa5 (E,F,M,N) and Uncx4.1 (G,H,O,P) in wild-type (A,C,E,G,I,K,M,O) or KspCre;Wnt9b(-) (B,D,F,H,J,L,N,P) kidneys at E17.5 (A-H) or P1 (I-P). Wnt9b is necessary for the maintenance of the progenitor population at later stages.
that canonical activity is required for normal expression of progenitor targets. Second, we show that activation of the canonical pathway is sufficient to induce expression of these genes, even in the absence of Wnt9b. That this activation is not mimicking signaling downstream of Wnt4 (and tubule differentiation) is supported by the observation that progenitor targets are expressed normally in Wnt4 mutants and are lost prior to the cessation of renal vesicle formation (and Wnt4 loss) in wild-type post-natal kidneys. Finally, several Wnt9b progenitor target genes (11/13) contain highly conserved consensus Lef/Tcf-binding sites within their loci and several of these elements are associated with β-catenin in vivo.

Our data suggest that Wnt9b signals through β-catenin to both cell populations with distinct cellular and molecular results. In the progenitors, it promotes renewal/proliferation, whereas in the PTAs it promotes differentiation. Reiterative use of β-catenin in phases of stem cell activation and differentiation has been shown in other systems, most notably the hair follicle stem cells (Lowry et al., 2005). How does Wnt9b induce disparate responses (self-renewal versus differentiation) in different cell types using the same signal transduction cascade? Our data suggest that, in the kidney, Six2 is an important player in this decision.

A simple model that explains all of the genetic and molecular analysis in this and previous studies is that a combination of β-catenin and Six2 results in progenitor renewal, while β-catenin alone (or in combination with another factor) results in differentiation. Cells that express Six2 and receive a Wnt9b signal are induced to proliferate and to maintain the progenitor pool, while cells that receive the Wnt9b/β-catenin signal but do not have active Six2 (and/or do express another, unknown factor) are induced to differentiate.

The molecular nature of the interaction between β-catenin and Six2 is unclear. It is plausible that Six2 regulates the expression of a separate set of progenitor specific factors that interacts with and/or alters the response to Wnt9b/β-catenin, perhaps amplifying or dampening the signal, as has been suggested in the hair follicles. It is also possible that Six2 directly (or indirectly) interacts with β-catenin to drive expression of individual genes. Determining which, if any, of the above scenarios exist within the kidney mesenchyme will certainly enhance our understanding of progenitor cell differentiation during normal development, as well as in pathological states.

It is important to note that, although levels are highest in the progenitors, Six2 also shows low level expression in the PTAs. It is possible that Six2 cooperates with β-catenin in both cell types to activate expression of target genes. This is consistent with our finding that many of the Wnt9b targets are expressed in both the progenitors and the PTAs, and are completely lost in Six2 mutants. However, some, such as Cited1, are expressed only in the progenitors whereas others, such as C1qdc2, are expressed only in the PTAs (and are expanded in Six2 mutants). These observations suggest that there are additional cell type-specific transcriptional regulators that cooperate with Six2 and β-catenin to drive expression in the various cell types.

In summary, we have identified a novel mechanism by which renal progenitor cells respond to the same Wnt9b signal with distinct results: self-renewal versus differentiation. By using this relatively simple mechanism, the kidney is able to balance tubule induction and progenitor cell maintenance, insuring that the proper numbers of nephrons form. These findings are highly relevant to human diseases that affect kidney progenitors, such as aplasia, hypoplasia, decreased nephron endowment and renal progenitor tumors. Furthermore, they provide novel insights into the molecular mechanisms that regulate kidney progenitor cell differentiation and will enhance future attempts to repair or replace missing or damaged organs.

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The authors declare no competing financial interests.

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