Pax2 and Pax8 Regulate Branching Morphogenesis and Nephron Differentiation in the Developing Kidney

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Pax genes are important regulators of kidney development. In the mouse, homozygous Pax2 inactivation results in renal agenesis, a phenotype that has largely precluded the analysis of Pax gene function during metanephric kidney development. To address this later function, kidney development was analyzed in embryos that were compound heterozygous for Pax2 and for Pax8, a closely related member of the Pax gene family. Both genes are coexpressed in differentiating nephrons and collecting ducts. At the morphological level, Pax2−/−Pax8−/− metanephric kidneys are severely hypodysplastic and characterized by a reduction in ureter tips and nephron number in comparison with wild-type or Pax2+/− kidneys. In developing nephrons, the molecular analysis of Pax2+/−Pax8+/− kidneys reveals a strong reduction in the expression levels of Lim1, a key regulator of nephron differentiation, accompanied by an increase in apoptosis. At a more mature stage, the reduction of Pax2/8 gene dosage severely affects distal tubule formation, revealing a role for Pax genes in the differentiation of specific nephron segments. At the ureter tips, the expression of Wnt11, a target of glial cell–derived neurotrophic factor–Ret signaling, is significantly reduced, whereas the expression levels of Ret and GDNF remain normal. Together, these results demonstrate a crucial role for Pax2 and Pax8 in nephron differentiation and branching morphogenesis of the metanephros.


Kidney development proceeds through a complex series of mesenchymal–epithelial interactions whereby the epithelial ureteric bud initially evaginates from the nephric duct and invades the metanephric mesenchyme at the level of the hindlimb bud. The ureteric bud subsequently undergoes multiple branching cycles to form the collecting duct system, which is responsible for urine flow to the bladder. In turn, the ureter tips induce the surrounding mesenchyme to condense, epithelialize, and differentiate into mature nephrons through a succession of morphogenetic steps that are referred to as renal vesicle, comma-shaped body, and S-shaped body. Several signaling and transcriptional regulators are necessary for these early steps of kidney development. Among them, the glial cell–derived neurotrophic factor (GDNF)/Ret-GFRα1 pathway is crucial for ureter budding and branching (1–6), whereas Fgf8 and Wnt4 are necessary for nephron differentiation past the renal vesicle stage (7–9). At the transcriptional level, Lim1 acts downstream of Fgf8 in nephron differentiation and is part of an intricate network of transcriptional regulation in renal epithelia, together with Pax, Gata, and homeodomain family members (10–12). However, the cellular mechanisms by which this transcriptional network operates during kidney morphogenesis and differentiation are still largely unknown.

The Pax2 and Pax8 transcription factors are central regulators of kidney development. In the transient pro- and mesonephros of the mouse, they both are expressed in the mesenchymal primordium as well as in the epithelial components. In the metanephros, Pax2 is expressed in the collecting duct, nephron progenitors, and all epithelial components of the developing nephron. In contrast, Pax8 expression starts at the renal vesicle stage and is maintained, together with Pax2, until the end of nephron differentiation. In terms of function, Pax2/8 genes are necessary and sufficient to induce the nephric lineage. Embryos that are mutant for both genes indeed fail to generate the nephric (Wolffian) duct and therefore are defective in the formation of all three embryonic kidneys (pro-, meso-, and metanephros) (13). Both genes act in a cooperative and dosage-dependent manner as revealed by the correlation between the number of Pax2/8 alleles and the severity of renal defects. In contrast to double-knockout embryos, Pax2−/− embryos initially form the pro/mesonephros but fail to induce the metanephric kidney, largely because of the role of Pax2 in nephric duct maintenance (10,14) and GDNF regulation in the metanephric mesenchyme (15). A milder reduction in Pax gene dosage in Pax2−/− mice allows metanephros formation but results in hypoplastic kidneys (60 to 75% of the normal size), a phenotype also found in Pax2−/− patients with renal coloboma (16). Finally, Pax8 inactivation alone does not result in any kidney malformation (17). The relatively mild kidney phenotype of Pax2+/− mice and the complete absence of metanephros in Pax2−/− mice have largely obscured the role of Pax genes in branching morphogenesis and nephron differentiation. Organ culture experiments and Pax2 heterozygote kidney analysis could nonetheless reveal the role of Pax2 in cell survival and
mesenchymal-to-epithelial transition leading to nephron formation (18–21).

To understand the role of Pax genes in early metanephros development, we performed a detailed analysis of Pax2+/−/Pax8+/− mice, which consistently form kidneys that are severely reduced in size (25 to 50% of the normal size) (13) and therefore represent an intermediate between Pax2−/− and Pax2+/− phenotypes. We found that Pax2+/−/Pax8+/− kidneys show a significant reduction in nephron number, which correlates with a downregulation of the key developmental regulator Lim1 (11) and an increase in the apoptotic index of nephron progenitor cells. In mature nephrons, the reduction of Pax2/8 gene dosage results in a differentiation defect of the distal convoluted tubules. In addition, Pax2+/−/Pax8+/− kidneys display branching defects that are associated with a downregulation of Wnt11 gene expression, a target of Ret signaling. From these data, we conclude that Pax genes are important regulators of both nephron differentiation and branching morphogenesis in the developing kidney.

Materials and Methods

Mice

Pax2−/− and Pax8−/− mice were maintained in a C3H/He background and genotyped as described previously (13,18).

Immunohistochemistry and Terminal Deoxynucleotidyl Transferase–Mediated Digoxigenin–Deoxyuridine Nick-End Labeling Assays

Metanephric kidneys were dissected in cold PBS and fixed for 2 h in 4% paraformaldehyde at 4°C. Samples were processed for cytochemistry and subsequent immunohistochemistry as described previously (18). The following primary detection reagents were used: Rat anti-E-cadherin antibody (1:400; Zymed Laboratories, San Francisco, CA), rabbit anti-Laminin antibody (1:2000; Sigma, St. Louis, MO), rabbit anti-phospho-H3 antibody (1:200; Upstate Biotechnology, Lake Placid, NY), and biotinylated Lotus tetragonolobus lectin (1:500; Vector Laboratories, Burlingame, CA). Secondary detection was done with anti-rat or anti-rabbit secondary antibodies that were labeled with Alexa488, Alexa568 (1:200; Molecular Probes, Eugene, OR), or FITC-conjugated streptavidin (Zymed Laboratories). Sections were occasionally counterstained with hematoxylin or DAPI at 50 μg/ml in SlowFade Light mounting medium (Molecular Probes). Terminal deoxynucleotidyl transferase-mediated digoxigenin–deoxyuridine nick-end labeling (TUNEL) assay was performed using the In Situ Cell Death Detection Kit according to manufacturer’s instruction (Roche, Basel, Switzerland). Apoptotic indexes were obtained from 12-μm-thick frozen sections of embryonic day 16.5 (E16.5) wild-type, Pax2+/−, or Pax2+/−/Pax8−/− kidneys that were immunostained against E-cadherin and counterstained with DAPI. Peripheral mesenchyme was defined by dense DAPI staining, absence of epithelial morphology, and peripheral location relative to the ureter tips. Cellular morphology, low E-cadherin staining, and location in proximity to the ureter trunk identified early differentiating nephrons. High E-cadherin levels and cell morphology defined collecting duct cells. A minimum of 20 independent fields (>10 sections) from four kidneys of each genotype was used for statistical analysis (Student t test).

In Situ Hybridization and Histology

Metanephric kidneys were prepared as for immunohistochemical staining (see previous section). In situ hybridization with digoxigenin-dUTP RNA probes was performed as described previously (22), using the following probes: Pax2 and Pax8 (13), Wnt4 (23), Wt1 (24), Fgf8 (25), Ret (26), GDNF (27), and Wnt11 (28). Additional probes for Lim1 (NM_008498), Nkcc2 (NM_011389), Ncc (NM_019415), and Ncx1 (NM_011406) were generated from E18.5 kidney cDNA with the following primer pairs and cloned in pGEM-T-easy: Lim1 (Lxt1) 5’-TGGCTCTTCCTCCTTTGCCTGTG-3’ and 5’-CTTGGCCACACCTGGTGTACTC-3’; Nkcc2 (Skl2a5) 5’-TCATGCCCTCTCCTGGATTG-3’ and 5’-CCCTGACAAGACCTGGAAATAC-3’; Ncc (Skl2a5) 5’-TGGCTGACCTGATTCTCATC-3’ and 5’-GCTGGAGAATCTGGAATGC-3’; and Ncx1 (Skl8a1) 5’-ACGACCGTGAAAATCGTCAAGGC-3’ and 5’-CAAACAGCTGGTGTCACTGCG-3’.

Glomeruli and ureter tip counts were performed in situ hybridization with Wt1 and Ret probes, respectively. Wt1 and Ret stainings were obtained from adjacent 10-μm-thick cryosections while quantification was performed every 50 μm (minimizing double counts) on four entire kidneys of each genotype (15 to 25 sections per kidney). Samples were scored for high Wt1 expression (maturing nephrons) or Ret-positive ureter tips and subjected to statistical analysis (Student t test). Hematoxylin and eosin stainings were performed on 6-μm-thick paraffin sections using standard procedures.

Results

Growth Impairment in Pax2+/−/Pax8+/− Kidneys

Pax2 and Pax8 were previously shown to cooperate genetically as compound heterozygous mutations result in abnormal kidney development (13). To understand better the role of Pax2 and Pax8 in metanephros formation, we initially performed histologic analysis of Pax2+/−/Pax8+/− compared with Pax2+/− and wild-type kidneys at various developmental stages. By day 13.5 post coitum (E13.5), Pax2+/−/Pax8+/− kidneys were smaller than controls, with very few detectable nephron progenitors (Figure 1, A through C). At this stage, both wild-type and Pax2+/− kidneys already showed several comma- and S-shaped bodies (Figure 1, A and B). By E16.5, control kidneys had formed mature glomeruli, and proximal and distal tubules were readily visible (Figure 1, D and E). In contrast, Pax2+/−/Pax8+/− kidneys harbored only a few maturing nephrons and tubules at this stage, and certain areas were completely devoid of differentiating nephrons (Figure 1F). This marked dysplasia and delay in nephron differentiation was also observed at E18.5, when the number of differentiatied nephron structures was irregular and still significantly reduced (Figure 1, G through I, data not shown). Immunohistochemical analysis with the epithelial markers E-cadherin and laminin revealed that collecting ducts and nephrons epithelialized normally in Pax2+/−/Pax8+/− kidneys at E16.5 (Figure 1, J through L). The dysplastic phenotype and nephrogenesis delay that was specifically found in Pax2+/−/Pax8+/− but not in Pax2+/−/Pax8−/− kidneys prompted us to quantify both branching morphogenesis and nephron differentiation in these samples. For this, we counted the number of Ret(+) ureter tips and Wt1(+) glomeruli (including progenitors), throughout E16.5 wild-type, Pax2+/−, and Pax2+/−/Pax8−/− kidneys. Pax2+/−/Pax8−/− kidneys displayed a 51 and 33% reduction in ureter tip number when compared with wild-type (P = 0.007) and Pax2+/− (P = 0.092), respectively. In addition, the number of glomeruli was reduced by 63% (P = 0.002) compared with wild-type and 42% compared with Pax2+/− (P = 0.007; Figure 2). Importantly, the striking reduction in Pax2+/−/Pax8−/− glomeruli
Hematoxylin and eosin stainings (A through I) and immunohistochemical analysis (J through L) of wild-type (A, D, G, and J), Pax2+/− (B, E, H, and K), and Pax2+/−Pax8+/− (C, F, I, and L) kidneys at embryonic day 13.5 (E13.5; A through C), E16.5 (D through F and J through L), and E18.5 (G through I). (A through C) At E13.5, wild-type and Pax2+/− kidneys have initiated nephron differentiation, whereas Pax2+/−Pax8+/− kidneys are smaller and exhibit little signs of nephron differentiation. (D through E) By E16.5, both wild-type and Pax2+/− metanephros harbor several differentiated nephrons. (F) At the same stage, Pax2+/−Pax8+/− kidneys have initiated nephrogenesis, but few nephrons have matured past the S-shaped body stage. (G through I) At E18.5, Pax2+/−Pax8+/− kidneys develop differentiated nephrons, but these are reduced in number and disorganized when compared with control wild-type and Pax2+/− kidneys. (J through L) Immunohistochemical staining against E-cadherin (green) and laminin (red) reveal a normal epithelialization of the collecting duct and early differentiating nephrons at E16.5 in wild-type Pax2+/− and Pax2+/−Pax8+/− kidneys. cb, comma-shaped body; cd, collecting duct; g, glomerulus; sb, S-shaped body; pct, proximal convoluted tubule.

Figure 1. Metanephros development in Pax2Pax8 mutant embryos. Hematoxylin and cosin stainings (A through I) and immunohistochemical analysis (J through L) of wild-type (A, D, G, and J), Pax2+/− (B, E, H, and K), and Pax2+/−Pax8+/− (C, F, I, and L) kidneys at embryonic day 13.5 (E13.5; A through C), E16.5 (D through F and J through L), and E18.5 (G through I). (A through C) At E13.5, wild-type and Pax2+/− kidneys have initiated nephron differentiation, whereas Pax2+/−Pax8+/− kidneys are smaller and exhibit little signs of nephron differentiation. (D through E) By E16.5, both wild-type and Pax2+/− metanephros harbor several differentiated nephrons. (F) At the same stage, Pax2+/−Pax8+/− kidneys have initiated nephrogenesis, but few nephrons have matured past the S-shaped body stage. (G through I) At E18.5, Pax2+/−Pax8+/− kidneys develop differentiated nephrons, but these are reduced in number and disorganized when compared with control wild-type and Pax2+/− kidneys. (J through L) Immunohistochemical staining against E-cadherin (green) and laminin (red) reveal a normal epithelialization of the collecting duct and early differentiating nephrons at E16.5 in wild-type Pax2+/− and Pax2+/−Pax8+/− kidneys. cb, comma-shaped body; cd, collecting duct; g, glomerulus; sb, S-shaped body; pct, proximal convoluted tubule.

Figure 2. Ureter tips and glomerular counts. Counts of ureter tips and glomeruli were obtained throughout E16.5 kidneys (every 50 μm) using in situ hybridization with Ret and Wt1 cRNA probes, respectively. Glomerular progenitor counts, normally reaching a mean value of 839 in control kidneys (wild-type or Pax8+/−), are reduced by 63% to 306 in Pax2+/−Pax8+/− kidneys (P = 0.002). This reduction represents a 42% decrease in comparison with Pax2+/− kidneys (with 525 glomeruli progenitors; P = 0.007). The ureter tip count of Pax2+/−Pax8+/− kidneys (277 tips) is a 51% reduction in reference to wild-type (566 tips; P = 0.007) and by 31% compared with Pax2+/− (415 tips; P = 0.092). SD is indicated above each column; n = 4 entire kidneys for each genotype.

number was associated with dysplasia that was characterized by an unequal distribution of nephrons throughout the kidney (Figure 1F). This phenotype, which is not observed in Pax2+/− kidneys, is indicative of a nephrogenesis defect independent of the ureter branching delay that is also observed in Pax2+/−Pax8+/− kidneys. These data point to a role for Pax2 and Pax8 in both branching morphogenesis and nephron formation.

Pax2 and Pax8 Are Coexpressed and Regulated Independently within the Collecting Duct and Differentiating Nephrons

The involvement of Pax8 in branching morphogenesis was unexpected because, until recently, Pax8 expression had been reported only in the renal vesicle and subsequent steps of nephron differentiation (29,30). Although the expression in nephron progenitors could, in principle, influence collecting duct elongation and branching, the possibility remained that the defect is intrinsic to the collecting duct. To clarify the issue, we used in situ hybridization to reexamine Pax8 expression in the developing metanephros. As expected, we found high expression of Pax8 in renal vesicles, comma- and S-shaped bodies, and more differentiated structures (Figure 3, A and C). It is interesting, however, that Pax8 expression was also found at low levels in the collecting duct of E13.5 and E16.5 kidneys (Figure 3, A through D), in accordance with a recent report (31). This expression of Pax8 was thus similar to Pax2 expression in the metanephros, with the exception of the condensing mesenchyme (before renal vesicle stage), which is positive for Pax2 but negative for Pax8 (Figure 3, C and E). To verify whether the expression of Pax2 and Pax8 in the metanephros was subjected to auto- or cross-regulation, we then compared their expression levels in wild-type and Pax2+/−Pax8+/− and found no significant difference in staining intensity (Figure 3). These results are in agreement with the normal expression of Pax2 in Pax8+/− kidneys (data not shown) and Pax8 in Pax2+/− mesonephros (12,13). Together, these data suggest that Pax2 and Pax8 are coexpressed independently in the collecting duct and regulate branching morphogenesis within these cells rather than indirectly from differentiating nephrons.
Reduction of Pax2 and Pax8 Gene Dosage Is Associated with a Downregulation of Wnt11, a Target of Ret Signaling

Because the role of Pax2 and Pax8 in branching morphogenesis may be related to the GDNF-Ret signaling pathway, central to this process, we looked at Ret expression and signaling at E13.5 and E16.5. In the metanephros, Ret expression is restricted to the newly branched ureter tips (Figure 4, A and C). This restricted expression pattern was essentially unchanged in Pax2−/−Pax8−/− kidneys (B and D). In comparison, Pax2 is expressed in the collecting duct and differentiating nephrons as well as in the condensing mesenchyme of wild-type kidneys (E). This expression is unaltered in Pax2−/−Pax8−/− samples (F), arguing against an auto- or cross-regulation of Pax genes in this system.

cm, condensing mesenchyme; csb, comma-shaped body; lh, loop of Henle; rv, renal vesicle.

Figure 3. Expression of Pax8 and Pax2 in the metanephros. In situ hybridizations on wild-type and Pax2−/−Pax8−/− kidneys with Pax8 (A through D) and Pax2 (E and F) cRNA probes at E13.5 (A and B) and E16.5 (C through F). The expression of Pax8 is found in the differentiating nephrons but also in the collecting duct of wild-type embryos at E13.5 (A) and E16.5 (C). Despite a reduction in the number of differentiating nephrons, this expression is essentially unchanged in Pax2−/−Pax8−/− kidneys (B and D). In comparison, Pax2 is expressed in the collecting duct and differentiating nephrons as well as in the condensing mesenchyme of wild-type kidneys (E). This expression is unaltered in Pax2−/−Pax8−/− samples (F), arguing against an auto- or cross-regulation of Pax genes in this system.

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Figure 4. Regulation of branching morphogenesis in Pax2: Pax8 kidneys. In situ hybridization for Ret (A through D), Wnt11 (E through H), and GDNF (I and J) at the indicated stages. At E13.5, the expression of Ret is restricted to ureter tips of wild-type kidneys (A). This expression pattern is maintained in Pax2−/−Pax8−/− kidneys (B). At E16.5, the expression of Ret remains normal in Pax2−/−Pax8−/− kidneys (C and D). At E12.5, the Ret signaling target Wnt11 is downregulated in Pax2−/−Pax8−/− (F), when compared with wild-type expression pattern at the ureter tips (E) or with Pax2−/− (insert in E). At E16.5, Wnt11 downregulation is even more pronounced (H) in comparison with wild-type (G) or Pax2−/− (insert in G). GDNF expression levels are similar between wild-type (I) and Pax2−/−Pax8−/− (J) kidneys. The different thickness of the GDNF signal reflects an increase in metanephric mesenchyme resulting from reduced branching morphogenesis in Pax2−/−Pax8−/− kidneys. mm, metanephric mesenchyme; ut, ureter tip.
gous animals. For this, in situ hybridization with a GDNF cRNA probe was performed on E16.5 kidneys but revealed no significant alteration in expression levels in Pax2+/−Pax8+/− samples compared with controls (Figure 4, I and J). The GDNF expression domain was, however, more prominent in Pax2+/−Pax8+/− kidneys, reflecting a larger metanephric mesenchyme region. Similar expression results were obtained for the Ret co-receptor GFRα1 (data not shown). We therefore conclude that the GDNF-Ret target gene Wnt11 requires Pax gene for normal expression, which is in accordance with the branching morphogenetic defect observed in Pax2+/−Pax8+/− kidneys.

Pax Genes Regulate Nephron Differentiation

To investigate directly the reduction of nephron number that was observed in Pax2+/−Pax8+/− kidneys, we looked at the expression of the key nephron differentiation regulators Wnt4 (9), Lim1 (11), and Fgf8 (7,8). In the developing nephron, Wnt4 is predominantly expressed at the renal vesicle stage, in the medullary part of the collecting duct, and in the surrounding mesenchyme (Figure 5A). This expression was maintained in Pax2+/−Pax8+/− kidneys (Figure 5B). Similarly, Fgf8 expression was not affected in Pax2+/−Pax8+/− kidneys (Figure 5C and D), indicating that the initial formation of renal vesicles is not significantly perturbed by a reduction in Pax2/8 gene dosage. In contrast, the expression of Lim1, normally strong in nephron progenitors and weak in the collecting duct, was severely decreased in Pax2+/−Pax8+/− kidneys (Figure 5E).
downregulated in Pax2+/−Pax8+/− kidneys when compared with wild-type or Pax2+/− controls (Figure 5, E and F). These kidneys indeed showed large and irregular regions of low or absent Lim1 expression. Such distribution is consistent with the dysplastic distribution of differentiated nephrons that was observed in these kidneys (Figure 1, D through I). The genetic regulation of Lim1 by the Pax2/8 genes was confirmed in Pax2−/−Pax8−/− mesonephros, in which Lim1 expression was virtually lost despite the presence of differentiating mesonephric tissue (Y.G., unpublished data). Together, these results identify an important role for Pax genes in nephron formation through the regulation of Lim1, a key component of nephron differentiation and patterning (11).

Regulation of Cell Survival by Pax2/8

To elucidate further the nephron differentiation defects of compound heterozygote kidneys, we examined the apoptosis and proliferation indexes of Pax2+/−Pax8+/− kidneys using TUNEL and phospho-histone H3 assays, respectively. These experiments revealed a strong increase of apoptotic signals in Pax2+/−Pax8−/− kidneys compared with controls (Figure 6, A and B), whereas the proliferation index remained unchanged (Figure 6, C and D). To characterize better the effect of Pax genes on cell survival, we quantified the number of TUNEL-positive signals in different compartments of wild-type, Pax2+/−, and Pax2−/−Pax8−/− kidneys. As previously reported, Pax2+/− kidneys showed an increase in apoptotic signals in the peripheral mesenchyme, collecting duct, and differentiating nephrons (Figure 6E) (18–20). However, this effect was markedly higher in Pax2+/−Pax8−/− kidneys, with the strongest effect observed in differentiating nephrons. This finding is in agreement with the high expression levels of both Pax2 and Pax8 that are normally present in this structure (Figure 3). Interestingly, the increase in TUNEL-positive signals that was observed in the peripheral mesenchyme of Pax2+/−Pax8+/− kidneys revealed a non–cell-autonomous effect of Pax genes on cell survival as Pax8 is not expressed in these cells (Figure 3) (29,30). Together, these results demonstrate a high sensitivity of nephron progenitors to Pax gene–dependent cell survival and suggest a cellular explanation for the decrease in nephron number and overall kidney size that are observed in Pax2/8 compound heterozygote embryos.

Pax Genes Regulate Distal Convoluted Tubule Differentiation

The results presented so far involved primarily the early stages of nephron morphogenesis. To determine the developmental outcome of reduced Pax gene dosage on nephron differentiation, we looked at late differentiation markers for the main segments of the nephron (glomerulus, proximal convoluted tubule, loop of Henle, and distal convoluted tubule). As expected, Wt1 expression marking the podocytes of the glomerulus was not affected in the developing nephrons, in accordance with the absence of Pax gene expression in these cells (Figure 7, A and B). Similarly, the lectin Lotus tetragonolobus and the Nkcc2 gene, which mark the proximal convoluted tubule and the loop of Henle, respectively, were still expressed in

Figure 7. Late nephron marker analysis in Pax2/Pax8 kidneys. In situ hybridization (A, B, and E through J) and lectin staining (C and D) of the glomerular marker Wt1 (A and B), proximal tubule marker Lotus tetragonolobus (C and D), loop of Henle marker Nkcc2 (E and F), and distal tubule markers Ncc (G and H) and Ncx1 (I and J). A and B are E16.5 and E through H are E18.5 kidneys. (A and B) The expression of Wt1 is maintained at normal levels in Pax2−/−Pax8−/− kidneys, despite a general reduction in glomerular counts. (C and D) The number and patterning of proximal tubules are affected in Pax2−/−Pax8−/− kidneys, but the tubules are still detectable. Note the large dysplastic region in the left kidney side. (E and F) Nkcc2 shows a normal expression pattern in a reduced number of loops of Henle. (G and H) The expression of Ncc reveals a specific defect in the formation of distal convoluted tubules in Pax2−/−Pax8−/− kidneys compared with Pax2+/− and wild-type. (I and J) The distal convoluted tubule defect is also observed with Ncx1, an independent marker of this structure. alh, ascending loop of Henle; dct, distal convoluted tubule.
Pax2+/−Pax8+/− kidneys, despite the overall reduction in nephron number (Figure 7, C through F). Strikingly, however, the expression of the distal convoluted tubule marker Ncc was severely affected in Pax2+/−Pax8+/− kidneys, with very few and small regions of expression compared with controls (Figure 7, G and H). This result was confirmed by in situ staining with Ncx1, another marker primarily expressed in distal convoluted tubules (Figure 7, I and J). Hence, these results reveal a specific role for Pax2 and Pax8 in the differentiation of the distal part of the nephron.

Discussion

Pax genes have been implicated in several aspects of kidney development ranging from nephric lineage specification to mesenchymal-to-epithelial transition and control of cell survival (18–21). The crucial role that is played by Pax genes during mesonephros development and metanephros induction has hindered the study of their function during later kidney development. The hypoplastic phenotype of Pax2+/− kidneys has provided some important information, notably about the implication of Pax genes in cell survival. However, mainly because of the persistence of Pax8 expression, Pax2+/− mice only allow the study of some aspects of Pax gene function in the metanephros. To specifically address the role of Pax genes during metanephros development, we took advantage of the strong phenotype that is observed in Pax2+/−Pax8+/− metanephric kidneys (13). These kidneys were hypodysplastic and characterized by a strong reduction in branching morphogenesis associated with a failure to activate Wnt11. In addition, they showed a significant reduction in the potential of Pax2+/−Pax8+/− progenitor cells to differentiate into mature nephrons, a defect that is accompanied by a severe downregulation of Lim1 gene expression and by an increase in cell death of the progenitor population. It is interesting that the distal segment of the developing nephrons was especially affected by a reduction in Pax gene dosage. Together, these results identify a key role for Pax genes in branching morphogenesis and nephron differentiation.

Recent analyses using a conditional inactivation in the mesenchymal components of the metanephros showed that Lim1 is strictly required for nephron development past the renal vesicle stage (11). The observation that nephron differentiation occurred irregularly in Pax2+/−Pax8+/− kidneys correlates well with the absence or low expression of Lim1 that is observed in most Pax2+/−Pax8+/− nephron progenitor cells and thus indicates that Lim1 is sensitive to Pax gene dosage. Consistent with this is the loss of Lim1 expression from the mesonephros of Pax−/−Pax8−/− embryos in which the mesonephric tissue is present but Pax gene dosage is minimal (Y.G., unpublished results) (12). The genetic regulation of Lim1 by Pax genes in the mesonephros, however, is not observed in Pax2/Pax8 compound heterozygote embryos, which excludes an effect on the phenotype described here. Accordingly, Pax2+/−Pax8+/− embryos show a normal elongation and budding of the ureter (data not shown).

Our finding that Lim1 is genetically regulated by Pax genes adds to a recent model of early nephron differentiation implicating Fgf8, Wnt4, and Lim1 (7–9,11). According to this model, the autocrine activity of Fgf8 in renal vesicles is necessary for Lim1 and Wnt4 expression, both of which are required for nephrogenesis (9,11). Our results raise the question of whether the activity of Pax genes on Lim1 is mediated through this pathway. The persistent expression of Wnt4 in Pax2+/−Pax8+/− kidneys, however, does not support such a model. In addition, the expression of Fgf8 was found to be normal in compound heterozygote kidneys, suggesting that Pax genes do not act upstream of the Fgf8 pathway. A complete elimination of Pax genes from nephron progenitors would be necessary to definitively conclude on this issue. The other possibility suggested by the model is that Fgf8 would exert its regulatory effect on Lim1 through Pax gene activity. However, Pax2 is still expressed at normal levels, and Pax8 is only slightly downregulated in Fgf8-deficient renal vesicles (7) (U. Grieshammer and G. Martin, personal communication). From these data, we therefore conclude that Fgf8 and Pax genes act in parallel to activate Lim1 in renal vesicles, thereby allowing nephron differentiation to proceed.

The increase in apoptotic cell death that was observed in Pax2+/−Pax8+/− kidneys is in accordance with previous studies implicating Pax genes in cell survival (18–20). Because Pax8 expression is turned on at the renal vesicle stage, it was somewhat expected to find the highest increase in cell death in these cells. It is interesting, however, to correlate this increased apoptosis in nephron progenitor cells with the loss of distal convoluted tubules later during development. It is indeed tempting to speculate that some parts of the differentiating nephron are more sensitive to Pax-dependent cell survival. Alternatively, Pax proteins may regulate key factors such as Brn1, which is involved in the differentiation of specific nephron segments (32,33). Another interesting aspect of the cell survival analysis is the significant increase of apoptosis in the peripheral mesenchyme that does not express Pax8. This finding suggests that the survival signal that is regulated by Pax proteins in the ureter tip or renal vesicle has a non–cell-autonomous component. Such non–cell-autonomous survival signal was also proposed downstream of Pax2 and Pax8 in the intermediate mesoderm (13) and may be mediated by members of the FGF or TGF superfamily (7,34).

The specific effect of Pax genes on Ret signaling is still unclear. One interpretation of our results is that Pax genes are necessary for the expression of a critical component of the Ret signaling pathway. Alternatively, Pax genes could act in parallel to the Ret signal to mediate part of its transcriptional response, notably on Wnt11. The current data do not allow us to discriminate between these possibilities, which would require a detailed analysis of Ret signaling. Nonetheless, the downregulation of Wnt11 that was observed in Pax2+/−Pax8+/− kidneys is significant per se as this gene was shown to be necessary for normal branching morphogenesis in the kidney (28). Although some aspects of the role of Pax2/8 in metanephros development remain to be clarified, our results unambiguously identified a cooperative role for Pax2 and Pax8 in metanephric branching morphogenesis and nephron differentiation.
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Disclosures

None.

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