Development 133, 2995-3004 (2006) doi:10.1242/dev.02442

Odd-skipped related 1 is required for development of the metanephric kidney and regulates formation and differentiation of kidney precursor cells

Richard G. James[1,2,*], Caramai N. Kamei[1,2,*], Qingru Wang[3], Rulang Jiang[3] and Thomas M. Schultheiss[1,2,†]

Formation of kidney tissue requires the generation of kidney precursor cells and their subsequent differentiation into nephrons, the functional filtration unit of the kidney. Here we report that the gene *odd-skipped related 1* (*Odd1*) plays an important role in both these processes. *Odd1* is the earliest known marker of the intermediate mesoderm, the precursor to all kidney tissue. It is localized to mesenchymal precursors within the mesonephric and metanephric kidney and is subsequently downregulated upon tubule differentiation. Mice lacking *Odd1* do not form metanephric mesenchyme, and do not express several other factors required for metanephric kidney formation, including *Eya1, Six2, Pax2, Sall1* and *Gdnf*. In transient ectopic expression experiments in the chick embryo, *Odd1* can promote expression of the mesonephric precursor markers *Pax2* and *Lim1*. Finally, persistent expression of *Odd1* in chick mesonephric precursor cells inhibits differentiation of these precursors into kidney tubules. These data indicate that *Odd1* plays an important role in establishing kidney precursor cells, and in regulating their differentiation into kidney tubular tissue.

KEY WORDS: Kidney, Metanephros, Mesonephros, Chick embryo, Mouse, Osr1, Odd1

INTRODUCTION

Vertebrate kidney tissue is derived from the intermediate mesoderm (IM), a strip of tissue located adjacent to the somites in the developing embryo (Saxen, 1987). In amniotes, the IM gives rise to three types of kidney tissue, called (from anterior to posterior) the pronephros (a transient embryonic structure), the mesonephros (the functional embryonic kidney, which also contributes to the male reproductive system) and the metanephros (the definitive adult kidney). While all kidney types are comprised of the same fundamental functional unit (the nephron), the size and organization of these kidney types is very different: the mouse metanephros can contain up to 11,000 nephrons (Yuan et al., 2002), while the mesonephros has merely twelve (Sainio, 2003).

Kidney formation can be conceptualized as consisting of two stages: establishment of nephrogenic mesenchyme within the IM, and differentiation of that mesenchyme into functional nephrons. Through numerous studies over many years, much has been learned concerning the second stage: differentiation of nephrogenic mesenchyme. In the mouse metanephros, which is the best studied example, kidney tissue differentiates as the result of interaction between the metanephric mesenchyme, which is derived from the most posterior region of the IM, and the nephric duct, another IM derivative that migrates into the metanephric zone from a more anterior region of the embryo (Sariola and Sainio, 1997; Schultheiss et al., 2003; Vainio and Muller, 1997). Reciprocal signaling between the metanephric mesenchyme and a derivative of the nephric duct known as the ureteric bud results in branching of the ureteric bud and condensation of metanephric mesenchyme at its tips. The condensed mesenchyme is thought to form a precursor cell population, which both maintains itself at the tips of the ureteric bud (via proliferation and/or addition from the surrounding non-condensed mesenchyme) and gives off cells that differentiate into pretubular aggregates and renal vesicles, the precursors of the kidney tubules (Cho and Dressler, 2003).

The mechanisms that regulate the earlier phase of kidney development – formation of nephrogenic mesenchyme – are less understood. Multiple genes have been identified that are expressed specifically in the undifferentiated metanephric mesenchyme; many of these are required for proper differentiation of the metanephric kidney, including *Pax2* (Torres et al., 1995), *Wt1* (Kreidberg et al., 1993), *Eya1* (Xu et al., 1999), *Six1* (Xu et al., 2003), the *Hox11* paralogous group (Wellik et al., 2002) and *N-myc* (*Mycn* – Mouse Genome Informatics) (Bates et al., 2000). However, with the exception of *Eya1*, all of the above genes are dispensable for the initial formation of the metanephric mesenchyme, and are instead required for its subsequent differentiation and/or survival.

The current study characterizes the role during kidney formation of *odd-skipped related 1* (*Odd1*) (*Osr1* – Mouse Genome Informatics), a zinc finger-containing transcription factor related to the *Drosophila* pair rule gene *odd skipped* (*Odd*). *Odd1* is expressed before all previously described kidney regulatory genes, and its expression is confined to undifferentiated kidney precursor tissue. Loss-of-function studies in the mouse revealed a requirement for *Odd1* in the establishment of the metanephric mesenchyme and in the activation of a set of the earliest known genes expressed during metanephric kidney formation. Gain-of-function studies in the chick embryo revealed that *Odd1* can activate early markers of kidney precursor cells but inhibits the production of differentiated kidney tubules. On the basis of these studies, we propose that *Odd1* plays an important role in the establishment and maintenance of the nephrogenic mesenchyme, the precursor population from which the kidney is derived.

MATERIALS AND METHODS

In situ hybridization

RNA probes were generated for chicken *Odd1* (James and Schultheiss, 2005), *Pax2* (Burrill et al., 1997; Herbrand et al., 1998), *Lim1* (Tsuchida et al., 1994) and mouse *Pax2* (Dressler et al., 1990), *Lim1* (Fujii et al., 1994),

[1]Molecular and Vascular Medicine Unit, Beth Israel Deaconess Medical Center, 330 Brookline Ave, RW-663, Boston, MA 02215, USA. [2]Program in Biological and Biomedical Sciences, Harvard Medical School, Boston, MA, USA. [3]Center for Oral Biology and Department of Biomedical Genetics, University of Rochester School of Medicine and Dentistry, Rochester, NY, USA.

*These authors contributed equally to this work
†Author for correspondence (e-mail: tschulth@bidmc.harvard.edu)

Accepted 15 May 2006

Eya1 (Xu et al., 1999), *Six2* (Xu et al., 1999), *Gdnf* (Pichel et al., 1996), *Sall1* (Nishinakamura et al., 2001) and *Wt1* (Armstrong et al., 1993) using standard methods described previously (Schultheiss et al., 1995). Whole-mount in situ hybridization was performed as described previously (Schultheiss et al., 1995). For in situ hybridization on sections, paraformaldehyde-fixed cryostat sections were post-fixed, treated with 10 μg/ml proteinase K (Roche), and hybridized overnight with labeled RNA probe (1 μg/ml) at 70°C. The sections were then washed in 2× SSC (70°C), incubated with RNAse (1 μg/ml, 2× SSC, 37°C) and washed in 0.2× SSC (65°C). After blocking in PBT (PBS with 2 mg/ml BSA, 0.1% Triton X-100), the sections were incubated in alkaline phosphatase antibody solution (Roche, 1:2000, in PBT + 10% sheep serum) overnight. Lastly, sections were washed and the color was visualized using NBT and BCIP.

Immunohistochemistry and *lacZ* histochemistry

Immunofluorescence microscopy on paraformaldehyde-fixed cryostat sections was performed as previously described (James and Schultheiss, 2003). The following primary antibodies were used: polyclonal guinea pig anti-Odd1 [1:750 (James and Schultheiss, 2005)], rabbit anti-Pax2 (BabCo, 1:250), mouse monoclonal and rabbit polyclonal anti-Gfp (1:100, Molecular Probes) and mouse monoclonal anti-Wt1 (1:100, Dako). Apoptotic cells were detected by TUNEL assay (Sigma) as per the manufacturer's instructions. Staining for *lacZ* expression was adapted from Lobe et al. (Lobe et al., 1999).

Odd1 knockout mice

Generation of mice carrying a targeted in-frame fusion of a *lacZ* gene into the first coding exon of the *Odd1* gene has been described (Wang et al., 2005). Embryos were harvested at the indicated times and processed for in situ hybridization, immunofluorescence or *lacZ* histochemistry as described above.

Plasmid and retroviral expression vectors

For electroporation studies, full-length chicken *Odd1* (James and Schultheiss, 2005) was cloned into the *pMES* expression vector (Swartz et al., 2001), which drives expression from a CMV/chicken β-actin promoter/enhancer, and which also expresses *Gfp* from an IRES element. For retroviral expression studies, full-length *Odd1* was cloned into the *Cla*I site of the *RCAS* expression vector (Fekete and Cepko, 1993). The resulting *RCAS-Odd1* vector was transfected into chick embryo fibroblasts, and retroviral particles were harvested from culture supernatant, concentrated and titered using previously described methods (Fekete and Cepko, 1993). *RCAS-Gfp* retroviral particles for control experiments were produced in a similar manner (*RCAS-Gfp* plasmid was a kind gift from Cliff Tabin).

Electroporation and infection of chicken embryos

Electroporation and culture of chicken embryos was performed as previously described (James and Schultheiss, 2003; Wilm et al., 2004). Briefly, embryos were collected at Stage 5 or younger (Hamburger and Hamilton, 1951) and attached to a doughnut-shaped paper ring (P5, Fisher). The embryo was suspended in Tyrode's saline, ventral side down, above a 1 mm gauge platinum wire (positive electrode). Through a small hole in the vitelline membrane, 1 μl of DNA solution (0.6 μg/μl) was injected into the space between the embryo and the membrane. A 20 μm gauge tungsten wire (negative electrode) was lowered above the embryo until it was submerged in Tyrode's and the embryos were pulsed three times for 25 ms at 12 V using an electro-square porator BTX-830 (BTX). Subsequently, embryos were incubated endoderm-side up on albumin-agar culture dishes [50% Albumin, 1.5% Glucose, 0.9% NaCl (Sundin and Eichele, 1992)] at 38°C for 24-48 hours.

Methods for infecting unincubated chicken eggs with retroviral vectors were adapted from previous work (Andacht et al., 2004; Sang, 2004). Unincubated fertilized white-leghorn chicken eggs, obtained from Hy-Line International (Elizabethtown, PA), were placed on their sides at room temperature for 2 hours before microinjection. A small hole (approximately 5 mm in diameter) was ground into the shell using a blunt needle (cat. # 08-965A Fisher), while leaving the underlying membrane intact. A drop of Tyrode's saline was placed on top of the shell membrane, which was subsequently removed using a microscalpel. In order to prevent air from entering the egg, Tyrode's was continuously added to the hole; any eggs that developed air bubbles were discarded. After locating the embryo, a pulled glass capillary (100-200 μm in diameter), Picospritzer II injector and Leitz micromanipulator were used to inject 1-2 μl of retrovirus (10^7-10^8 infectious particles/ml) through the center of the embryo into the subgerminal space. The hole was then sealed with hot glue from a Superbonder glue gun (FPC). The glue was allowed to harden for 5 minutes, and the eggs were incubated glue-side down at 38°C until they were harvested for analysis.

Quantification of mesonephros, tubule and duct size

For each embryo, two images were collected using a Zeiss Axiophot microscope with a SPOT camera: (1) 10× DIC image of the entire section; and (2) a 20× fluorescent image of the urogenital ridge stained with anti-Pax2 antibody. For standardization, the ImageJ software package was used to calculate the two-dimensional area of the notochord from the first image. The freehand drawing tool was used to trace a line that encircled the notochord borders, and the measure command was executed to calculate its area in pixels. To calculate the total area of all tubular tissue from the second image, we calculated the area of the individual Pax2-positive epithelial condensates (groups of cells containing polarized nuclei) and lumenized tubules and summed them. Duct area was determined in the same manner. Finally, to control for variability of embryo size, the size of the notochord was used to standardize the tubular tissue and duct area measurements. The graph in Fig. 7 shows the mean of all embryos analyzed and the error bar represents one standard error. Differences between the mean values were tested for statistical significance at the 0.05 level by the two-sample Student's *t*-test.

RESULTS

Odd1 is expressed specifically in kidney precursor cells

Odd1 expression in the developing nephric system was examined in chicken and mouse embryos. Examination of developing chicken embryos revealed that *Odd1* is expressed in nephrogenic tissue before previously characterized kidney genes. Transcription of *Odd1* is initiated in the intermediate mesoderm immediately after gastrulation at Hamburger Hamilton (HH) Stage 5 (Hamburger and Hamilton, 1951) just lateral to Henson's node (Fig. 1A). Shortly thereafter, its expression pattern broadens to include the intermediate mesoderm and the medial lateral plate (Fig. 1B,E). By contrast, other markers of kidney differentiation, such as *Pax2*, are initially expressed later (HH9, Fig. 1C), and in a more spatially restricted pattern (Fig. 1D) (see James and Schultheiss, 2003; Mauch et al., 2000; Obara-Ishihara et al., 1999).

Cellular morphogenesis begins shortly after kidney-specific gene expression is initiated. Analysis of sections during the early stages of kidney morphogenesis revealed that *Odd1* is expressed only in the mesenchymal components. This is true at several different stages during the development of the urogenital region: initially at the onset of nephric duct differentiation (Fig. 1E), and later during mesonephric tubule differentiation (Fig. 1F,G). *Pax2* and *Odd1* have complementary expression patterns in the mesonephros: while *Pax2* is detectable in the differentiated epithelium of the nephric duct and tubules (Mauch et al., 2000), *Odd1* is limited to small regions of mesenchyme adjacent to the tubules (Fig. 1G).

That *Odd1* is present in kidney mesenchyme, but absent in differentiated epithelium at several stages of development, suggested that *Odd1* is expressed in kidney precursors and subsequently downregulated as they undergo epithelial morphogenesis. To investigate this idea further, Odd1 expression during kidney tissue differentiation was examined at single cell resolution. Immediately before nephric duct formation, Pax2 expression is initiated in the medial part of the urogenital region (Fig. 1J). Subsequently, the nephric duct rudiment is formed, as evidenced by a subset of the Pax2-expressing cells that migrate toward the ectodermal layer (Fig. 1M). Odd1 protein is detectable only in Pax2-expressing cells that

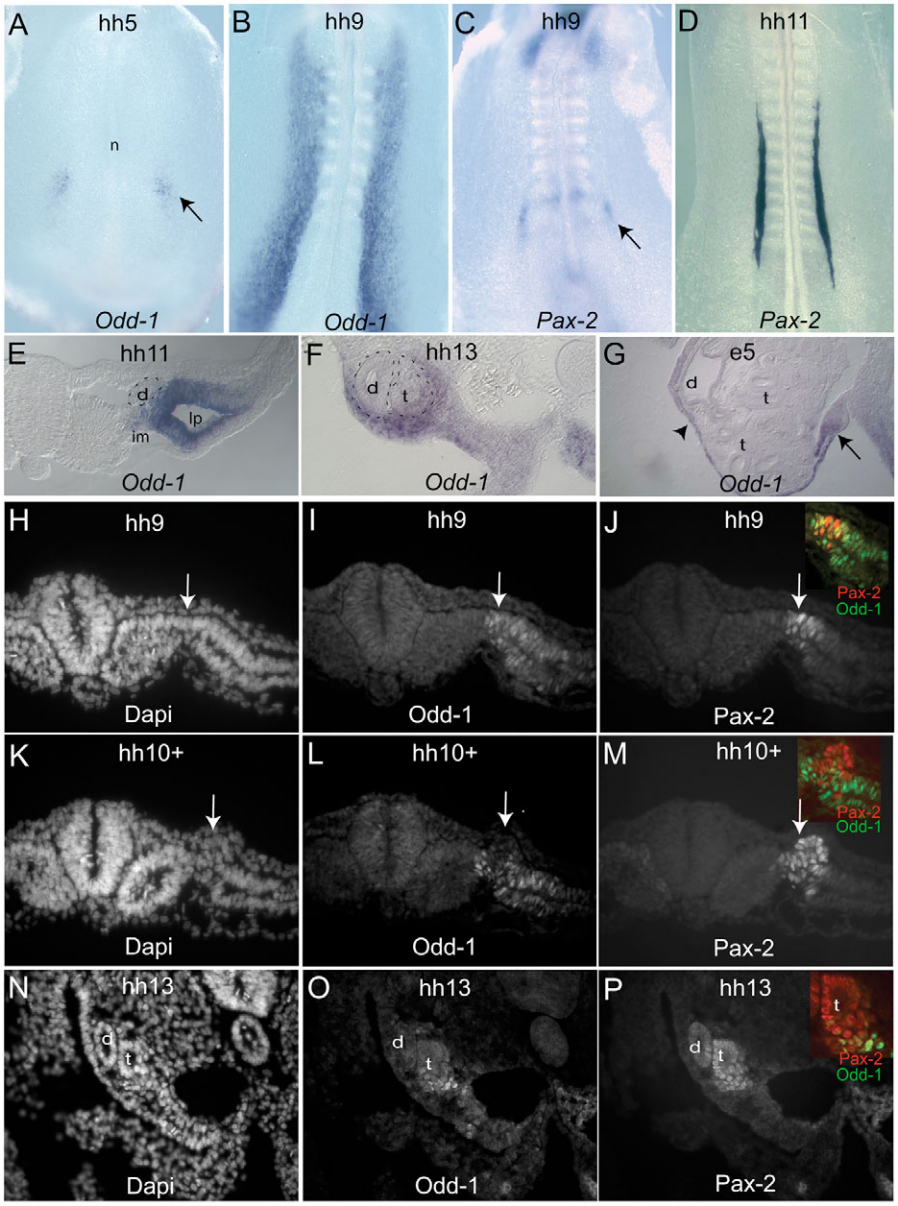

Fig. 1. Expression of *Odd1* in the chick embryo. (**A**,**B**) Whole-mount in situ hybridization for *Odd1* in HH Stage 5 (A) and Stage 9 (B) chick embryos. (**C**,**D**) In situ hybridization for *Pax2* at Stage 9 (C) and Stage 11 (D). Note that *Odd1* is expressed earlier than *Pax2*, and that it extends more broadly than *Pax2* in lateral, anterior and posterior directions. Arrows in A and C indicate nascent *Odd1* and *Pax2* expression, respectively. (**E-G**) Sections of whole-mount in situ hybridization for *Odd1*. At Stage 11 (E) *Odd1* is expressed in the intermediate mesoderm and extends into the lateral plate but is not found in the nephric duct. At Stage 13 (F), *Odd1* is expressed in mesenchymal cells adjacent to mesonephric tubules and also in splanchnic mesoderm derivatives, including the dorsal mesentery, but not in epithelialized nephric duct or tubules. At embryonic day 5 (G), *Odd1* is expressed in the coelomic lining (arrowhead) and in the rudimentary somatic gonad (arrow). (**H-M**) Expression of Odd1 and Pax2 protein during formation of the nephric duct rudiment. At Stage 9 (H-J), before the duct rudiment is morphologically detectable, most Pax2-expressing cells (arrow) also express Odd1. Inset in J shows merged detail from I and J. By Stage 10 (K-M), the nephric duct rudiment has begun to bulge dorsally (arrow) and continues to express Pax2 but no longer expresses Odd1. Inset in M shows merged detail from L and M. (**N-P**) In the developing mesonephros, Odd1 protein is expressed in mesenchymal cells adjacent to the tubules, but not in the Pax2-expressing tubules themselves. Inset in P shows merged detail from O and P. d, nephric duct; im, intermediate mesoderm; lp, lateral plate; n, Hensen's node; t, tubules.

have not migrated dorsally (compare Fig. 1I,J with 1L,M), indicating that Odd1 is downregulated in cells that have begun to differentiate. Similarly, in the mesonephros Odd1 expression is detectable in Pax2-expressing nephrogenic mesenchyme, but is absent in cells that have organized into tubules (Fig. 1O,P).

Expression of *Odd1* during mouse kidney development was examined in embryos heterozygous for a *lacZ* knock-in at the *Odd1* locus (Wang et al., 2005). At embryonic day (E) 9.5, *lacZ* is localized to the intermediate and lateral plate mesoderm (Fig. 2A), similar to the localization of the *Odd1* transcript described in chicken embryos (Fig. 1). *LacZ* expression is much higher in undifferentiated mesonephric mesenchyme than in the tubules of the mesonephric kidney (Fig. 2B). Starting at E10.5, the mouse metanephric kidney develops via reciprocal interaction between the ureter and metanephric mesenchyme (reviewed by Bard, 2003; Saxen, 1987). Initially, the ureteric bud forms as a thickening of the nephric duct, which bulges into the metanephric mesenchyme primordia (Fig. 2C, left). At this stage, *Odd1* is expressed in the metanephric mesenchyme and is absent in the ureteric bud (Fig. 2D). Shortly thereafter, signals from the mesenchyme induce branching within the ureter (Fig. 2C middle, and *lacZ* localization Fig. 2E,F). Lastly, tubular condensates begin to epithelialize adjacent to the branching ureteric bud trunks (yellow circles, Fig. 2C, right). At all stages of ureteric bud branching, *lacZ* is detectable only in the condensing mesenchyme that surrounds the branching ureteric bud and not in the tubular tissue that differentiates from this mesenchyme (Fig. 2E-I). As in the mesonephros, *Odd1* expression is downregulated in cells that have formed pre-tubular aggregates (arrow, Fig. 2H) and comma/S-shaped bodies (arrow, Fig. 2I). In summary, in both mouse and chick embryos, and during nephric duct, mesonephros and metanephros formation, *Odd1* is expressed in kidney precursor cells and downregulated upon the initiation of epithelial differentiation.

Odd1 is required for metanephric mesenchyme formation

In order to determine whether *Odd1* is required for development of the kidney, mice were examined that carry a targeted disruption of the *Odd1* locus (Wang et al., 2005). The majority of mice that are

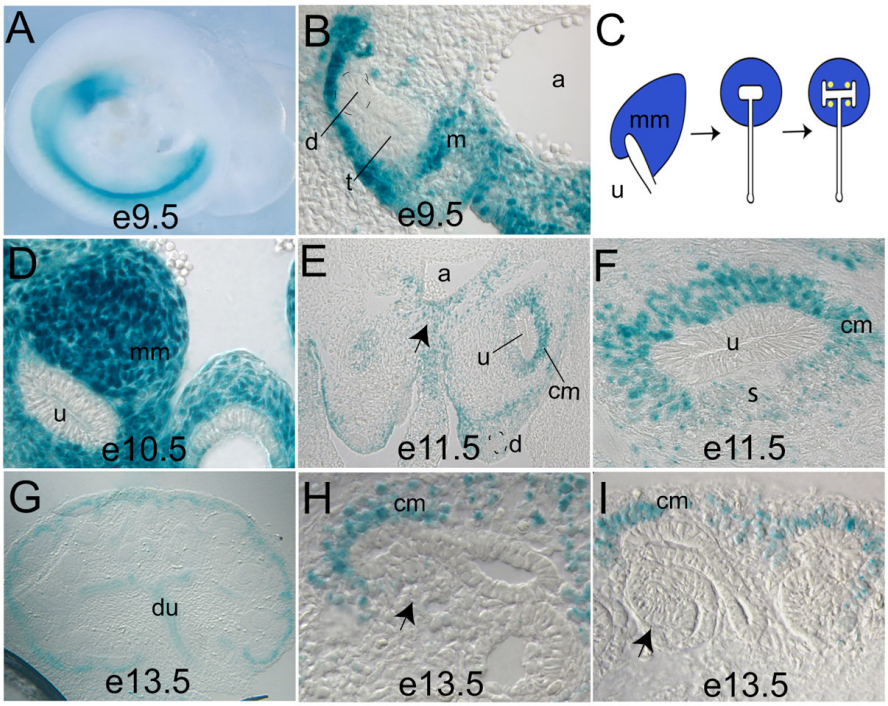

Fig. 2. Expression of *Odd1* in the mouse embryo. *LacZ* staining of mouse embryos heterozygous for the β-galactosidase gene inserted into the *Odd1* locus. At E9.5, *Odd1* is expressed in the intermediate and part of the lateral plate mesoderm (**A**). In a section through the mesonephros (**B**), *lacZ* staining is present in the coelomic lining and mesenchyme, but is absent from the nephric duct and largely absent from the mesonephric tubules. (**C**) Schematic of events in metanephros formation: (left) invasion of the ureteric bud (white) into the metanephric mesenchyme (blue); (center) initial branching of the ureteric bud; (right), continued branching of the ureteric bud and formation of epithelial vesicles (yellow). See text for details. (**D**) At E10.5, *Odd1* is expressed in the metanephric mesenchyme but not in the ureteric bud. (**E**,**F**) At E11.5, *Odd1* is expressed in the undifferentiated condensed mesenchyme of the metanephros and at lower levels in mesenchyme surrounding the ureteric stalk, but not in ureteric bud derivatives. *Odd1* is also expressed in mesenchyme adjacent to the ventral aorta (arrow in E). At E13.5 (**G-I**), *Odd1* continues to be expressed in the condensed mesenchyme, but not in pretubular aggregates (H, arrow) or comma-shaped bodies (I, arrow). The *lacZ* staining in the ureter epithelium in G is non-specific. a, aorta; cm, condensed mesenchyme; d, nephric duct; du, ureter epithelium; m, mesonephric mesenchyme; mm, metanephric mesenchyme; s, ureteric stalk; t, mesonephric tubules; u, ureteric bud.

homozygous for loss of *Odd1* function die at E11.5 due to cardiac abnormalities (Wang et al., 2005). At E11.5 the ureteric bud has already invaded the condensed metanephric mesenchyme in wild-type and *Odd1* heterozygous embryos, but the ureteric bud and metanephric mesenchyme condensation are completely absent in the homozygous mutant embryos (Fig. 3A,B).

In wild-type embryos, several factors required for kidney development are expressed in the metanephric mesenchyme before ureteric bud outgrowth, including: Six2 (Fig. 3C) (Xu et al., 1999); Eya1 (Fig. 3E) (Kalatzis et al., 1998; Xu et al., 1999); Gdnf (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996); Pax2 (Fig. 3H) (Dressler et al., 1990; Torres et al., 1995); and Sall1 (Nishinakamura et al., 2001). Embryos mutant for Odd1 completely lack expression of Six2, Eya1, Gdnf, Pax2 and Sall1 in the metanephric region (Fig. 3 and data not shown). Previous reports have shown that Eya1 mutants have similar defects in metanephric mesenchyme formation (Sajithlal et al., 2005). The data reported here implicate Odd1 as epistatic to Eya1 and as the earliest expressed factor known to be required specifically for metanephric mesenchyme formation.

Nephric duct and mesonephros defects in Odd1 mutant embryos

The ureteric bud forms as an outcropping of the nephric duct (Saxen, 1987). As ureteric bud defects were observed in *Odd1* mutant embryos (Fig. 3B), the earlier development of the nephric duct was analyzed. At E9.5, *Lim1*, which is required for formation of the nephric duct (Kobayashi et al., 2005), is a specific marker for the nephric duct primordia (Fig. 4A,B). In *Odd1* mutant embryos (Fig. 4A,B), *Lim1* is expressed, but at significantly lower levels than in heterozygous control embryos (Fig. 4C,D). Analysis of serial sections of *Odd1* mutants demonstrated that fewer cells express *Lim1* at E9.5 relative to the number seen in control embryos (Fig. 4B,D), and whole-mount in situ hybridization for *Lim1* revealed significant gaps in the *Odd1* mutant nephric ducts (Fig. 4C,D). Decreased diameter of the nephric duct as assessed by *Lim1* and *Pax2* expression was also seen at E8.5 (data not shown), indicating that defects in the nephric duct are present from close to the initiation of nephric duct formation. Visualization of embryos stained for *Pax2* at E10.5 also showed that the nephric duct does not migrate toward the cloaca and metanephric mesenchyme in *Odd1* mutants (Fig. 4F) as it normally does in wild-type embryos (Fig. 4E, arrow points to cloaca).

A small number of mesonephric tubules differentiate in *Odd1* mutants (Fig. 4F, arrowhead). Normally, two types of mesonephric tubule form in mouse embryos: anterior tubules, which are fused to the nephric duct, and posterior tubules, which remain separate from the duct (Sainio et al., 1997). Only anterior mesonephric tubules form in *Odd1* mutant mice (Fig. 4F), supporting the suggestion that anterior and posterior mesonephric tubules form via different mechanisms (Kobayashi et al., 2005; Sainio et al., 1997). In order to determine whether the mesonephric tubules in *Odd1* null embryos exhibit normal patterning, immunofluorescence was used to localize the expression of Wt1 and Pax2. Normally, the duct and the distal tubule express only Pax2 (Fig. 4G), while the proximal tubule coexpresses Pax2 and Wt1 (arrow Fig. 4G). *Odd1* mutant embryos exhibited normal regionalization of Pax2 and Wt1 expression within the duct and tubule (Fig. 4H), although the number and size of tubules was significantly smaller than in control embryos.

Gene expression defects precede apoptosis in *Odd1* mutants

In order to determine whether apoptosis was contributing to cell loss in the developing meso- and metanephros, TUNEL assays were performed on sectioned mouse embryos. At E8.5, the nephric duct

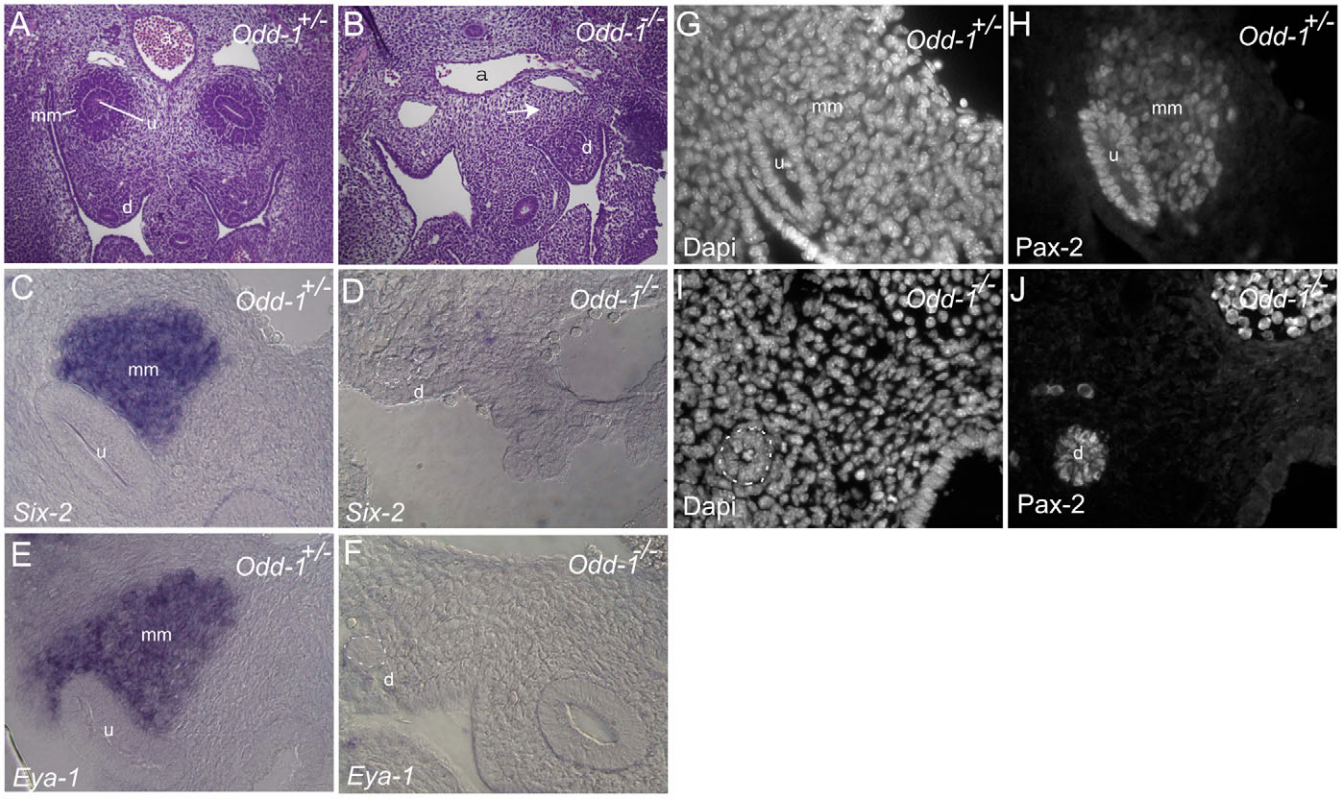

Fig. 3. The metanephric mesenchyme fails to form in *Odd1*$^{-/-}$ embryos. (**A**,**B**) Sections through the metanephric region of E11.5 heterozygous and homozygous null embryos stained with hematoxylin and eosin. In heterozygotes (A), a nephric duct, ureteric bud and condensed metanephric mesenchyme are seen. Homozygous null embryos (B) completely lack a ureteric bud and condensed mesenchyme (arrow). A duct is often present (on right side but not left in B), but smaller than normal. The region between the aorta and nephric duct is also significantly smaller in the *Odd1* null embryos. (**C-J**) Sections through the metanephric region of mouse embryos stained by in situ whole-mount hybridization (C-F) or immunofluorescence (G-J) for the indicated gene products (Dapi nuclear stains in G and I correspond to sections in H and J, respectively). At E10.5, the metanephric mesenchyme of *Odd1* heterozygous embryos expresses *Six2* (C), *Eya1* (E) and Pax2 (H). All three gene products are absent from the metanephric mesenchyme region of *Odd1* null mutants (D,F,J). The mutant nephric duct does express Pax2 (J). The duct in mutant embryos is outlined with a dotted line (D,F,I). The fluorescence in the upper right corner in J is autofluoresc;ence from blood cells in the aorta. a, aorta; d, nephric duct; mm, metanephric mesenchyme; u, ureteric bud.

of *Odd1* mutant embryos, as assessed by Lim1 and Pax2 expression, is already thinner than in control embryos, while the levels of apoptosis in the mesonephric region of mutant embryos are similar to those observed in controls (data not shown). At E9.5, there is some normal apoptosis at the border of the proximal tubule and the mesonephric mesenchyme (Fig. 5A, arrowhead). *Odd1* mutants exhibit significant inappropriate apoptosis in the mesenchyme surrounding the mesonephric tubules (compare Fig. 5A,B), indicating that increased programmed cell death is a probable contributor to the observed mesonephric size decreases in *Odd1* mutant embryos.

Expression of the metanephric mesenchyme genes *Pax2* (Bouchard et al., 2002; Dressler et al., 1990), *Pax8* (Bouchard et al., 2002), *Eya1* (Sajithlal et al., 2005) and *Wt1* (Armstrong et al., 1993) is initiated by E9.5 (see also Fig. 5C,E). The levels of apoptosis in metanephric mesenchyme precursor cells at E9.5 are similarly low in *Odd1* mutant and control embryos (compare Fig. 5C,D). Therefore, apoptosis begins in *Odd1* mutant mice after metanephric mesenchyme gene expression is normally initiated (see absence of mesenchymal Pax2 in Fig. 5D and *Eya1* in Fig. 5F). This implies that cell death cannot explain the initial metanephric mesenchyme gene expression defects observed in *Odd1* mutant embryos, and that *Odd1* is required autonomously for normal gene expression in the metanephric mesenchyme. Apoptosis does increase in *Odd1* mutant mesenchyme by E10.5 (Fig. 5G,H), and this may contribute to the metanephric size decreases observed in *Odd1* mutants. Even after the levels of apoptosis increase in the metanephric mesenchyme at E10.5, there are still many mesenchymal cells in the meso/metanephric region of *Odd1* mutant embryos that express nephrogenic genes, as evidenced by expression of Wt1 (Fig. 5I,J) and the detection of many *lacZ*-positive cells exhibiting intact *Odd1* promoter activity (Fig. 5K,L).

Odd1 promotes kidney precursor gene expression

As described above, *Odd1* is expressed in non-epithelial kidney precursor cells and is required for expression of many metanephric mesenchyme-specific factors. To test if *Odd1* could function to promote expression of kidney-specific genes, an *Odd1-IRES-Gfp* plasmid was electroporated into HH4 (gastrula) chick embryos, specifically targeting kidney and somite precursors. Morphologically, ectopic *Odd1* often disrupted somite formation (8/16 embryos; see Fig. 6B,C,E). Ectopic *Odd1* promoted expression of the kidney transcription factors Pax2 (11/14 embryos; Fig. 6A,B) and *Lim1* (4/5 embryos; Fig. 6D,E) in somitic cells within 24 hours of electroporation. Upregulation of Pax2 and Lim1 did not occur in all cells that were electroporated. In

particular, Gfp-labeled cells present in the lateral plate did not express Pax2 (arrows in Fig. 6B,C). This is consistent with the absence of kidney gene expression in the medial part of the lateral plate where *Odd1* is normally expressed (Fig. 1E,I,L), and indicates that the ability of *Odd1* to promote kidney gene expression is context-dependent.

Odd1 represses kidney tubule differentiation

We have found that *Odd1* is normally downregulated as intermediate mesoderm derivatives differentiate into epithelial structures (Figs 1, 2). In order to determine whether downregulation of *Odd1* is required for normal kidney tubule differentiation, chicken mesonephroi were infected with RCAS retrovirus expressing *Odd1*, which allows for the stable ectopic expression of *Odd1*. Unincubated (pre-gastrula) chicken embryos were infected with the *RCAS-Odd1* retrovirus and, after incubation for 4-5 days, adjacent sections were stained for *Odd1* by in situ hybridization or for Pax2 by immunofluorescence. For analysis, 'epithelialized tubules' were defined as Pax2-positive cells arranged around a distinct lumen, while the broader term 'tubular tissue' was defined as Pax2-positive cells either in epithelialized tubules or in cellular aggregates with polarized nuclei. *RCAS-Gfp*-infected embryos served as controls.

Several observations emerged from these experiments. First, cells that express ectopic *Odd1* did not differentiate into nephric duct or epithelialized tubules (Fig. 7D), while duct and tubules typically formed from adjacent non-*Odd1*-expressing cells (Fig. 7D, outline). As *RCAS-Gfp* efficiently infected the mesonephric duct and epithelial tubules (Fig. 7A), the lack of *Odd1*-infected epithelial cells cannot be due to infection artifact and probably reflects a reduced ability of *Odd1*-expressing cells to form duct and tubules. Second, visualization of urogenital ridges by DIC or Pax2 immunostaining showed that there was less tubular tissue overall in *Odd1*-infected embryos than in controls (compare Fig. 7E,F with 7B,C). The average total 'tubular tissue' area per section in *RCAS-Odd1*-infected embryos was only 49% of that of control embryos (Fig. 7G, $P<0.005$). Interestingly, the size of the nephric duct epithelium was similar in control and *RCAS-Odd1* infected embryos (Fig. 7H). Increased levels of TUNEL staining were not seen in the mesonephric regions of *RCAS-Odd1* infected embryos, indicating that the decrease in the area of tubular epithelium in *RCAS-Odd1*-infected embryos was not caused by apoptosis (data not shown). Together, these data indicate that ectopic expression of *Odd1* can promote kidney precursor gene expression, but inhibits organization of precursors into tubular tissue and epithelialized tubules.

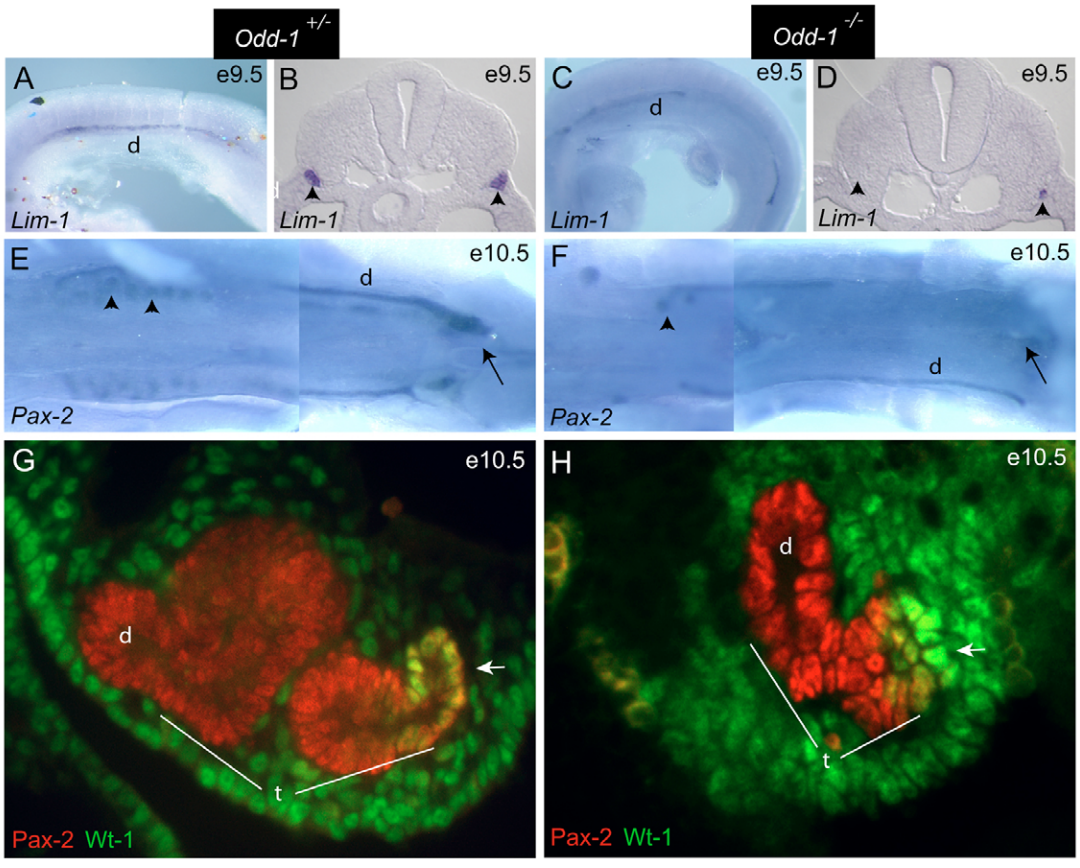

Fig. 4. Effects of loss of *Odd1* on nephric duct and mesonephric tubule development. *Odd1* heterozygous (A,B,E) and homozygous null (C,D,F) mouse embryos stained by whole-mount in situ hybridization for *Lim1* (**A-D**) or *Pax2* (**E**,**F**). B and D show sections through the mesonephric region. In homozygous null embryos, the nephric duct is thinner and often interrupted (C, left arrowhead in D). A few mesonephric tubules form in the null mutants (F, arrowhead), but fewer than in the heterozygotes (E, arrowheads). In *Odd1* mutants, the nephric duct also does not turn normally toward the midline at the posterior end (arrows in E,F). (**G**,**H**) Sections through the mesonephric region of E10.5 embryos stained with immunofluorescence against the indicated antigens. Tubules are smaller in mutant (H) compared with control (G) embryos. In both mutant and control embryos, Pax2 is expressed in the nephric duct and the tubules, while Wt1 is expressed in the proximalmost part of the tubule (arrow), as well as in the mesenchyme and coelomic lining. d, nephric duct; t, tubules.

DISCUSSION

Odd1 is required for metanephric mesenchyme formation

The metanephric kidney develops from interactions between the ureteric bud and the metanephric mesenchyme. In the absence of *Odd1*, many of the genes that mark the early metanephric mesenchyme rudiment are never expressed, including *Eya1, Six2 and Pax2* (Fig. 3). Absence of *Odd1* results in a more profound defect in the development of the metanephric mesenchyme than has been previously described in other loss-of-function studies, such as in mutations of *Six1* (Li et al., 2003; Xu et al., 2003), *Pax2* (Torres et al., 1995), *Gdnf* (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996), *Sall1* (Nishinakamura et al., 2001), *Wt1* (Kreidberg et al., 1993) and *Wnt4* (Kispert et al., 1998; Stark et al., 1994), all of which show some evidence of metanephric mesenchyme formation and expression of at least a subset of metanephric genes. Additionally, we have shown here that *Odd1* is epistatic to *Eya1*, which has also been shown to be crucial for the formation of the metanephric mesenchyme (Sajithlal et al., 2005; Xu et al., 1999). These data implicate *Odd1* as one of the earliest regulators of formation of the metanephric mesenchyme. These findings are consistent with the report that rare *Odd1* null embryos surviving beyond E11.5 have no morphologically detectable metanephric kidneys (Wang et al., 2005).

There are several possible alternative explanations for the absence of metanephric mesenchyme gene expression in the *Odd1* null mice. One possibility is that the defects in metanephric mesenchyme gene expression are secondary to defects in the formation of the nephric duct and its derivative, the ureteric bud, which would lead to problems in the inductive interactions between ureteric bud and metanephric mesenchyme (Saxen, 1987). However, the nephric duct is not required for initial expression of genes in the metanephric mesenchyme, as seen for example in the *Gata3* knockout mouse, where *Pax2*-expressing metanephric mesenchyme cells are found in the metanephric zone despite failure of the nephric duct to migrate into this region of the embryo (Grote et al., 2006; Sainio, 2003). Thus the effects of *Odd1* on nephric duct development cannot explain the effects of *Odd1* loss on early metanephric gene expression.

Another possibility is that, in the absence of *Odd1*, the tissue that would give rise to the metanephric mesenchyme undergoes apoptosis before the onset of metanephric mesenchyme gene expression, and that such apoptosis eliminates all metanephric mesenchyme precursor cells. Several lines of evidence point away from this explanation. While there is apoptosis in the metanephric region in *Odd1* null mice, the apoptosis begins after E9.5, which is after the time that the early metanephric mesenchyme markers *Pax2* and *Eya1* begin to be expressed (Fig. 5). Also, abundant cells with

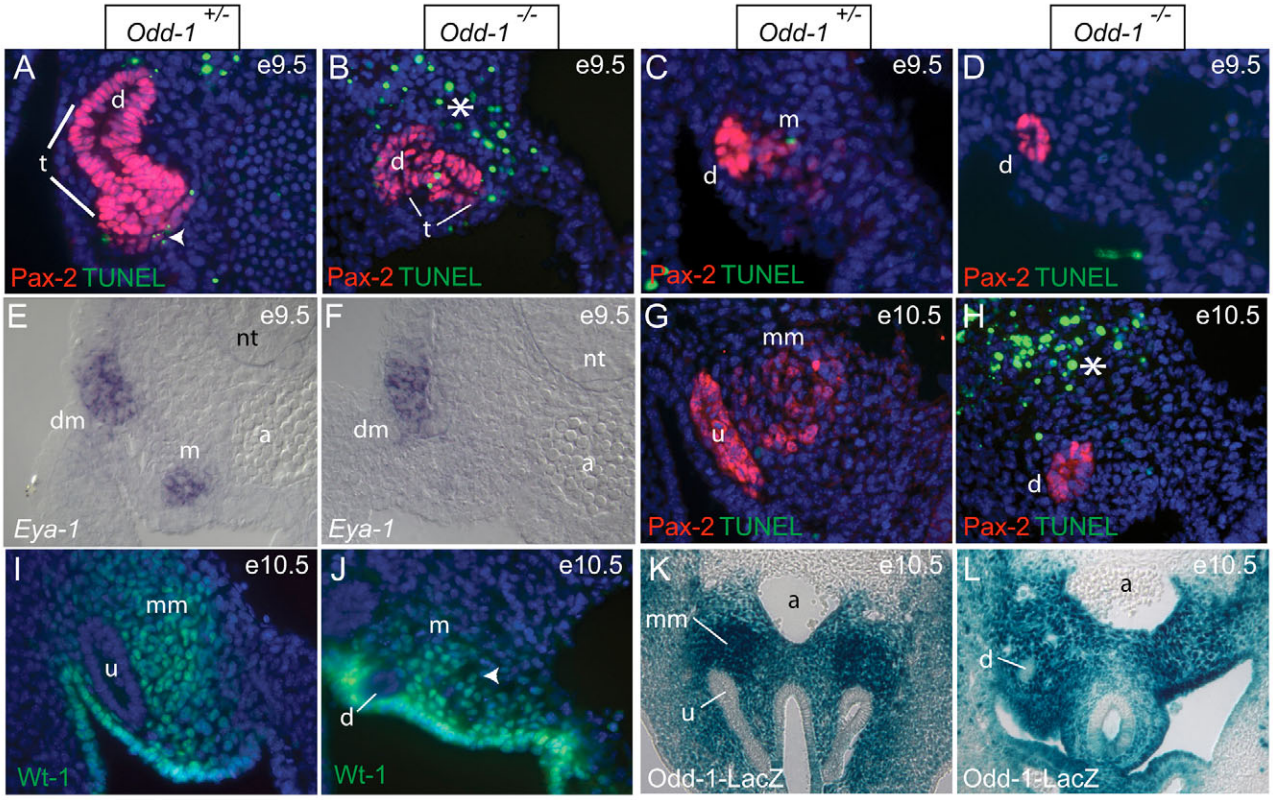

Fig. 5. Timing of apoptosis and loss of nephric gene expression in *Odd1* mutant embryos. *Odd1* heterozygous (**A**,**C**,**G**) and homozygous null (**B**,**D**,**H**) embryos were analyzed by TUNEL to detect apoptotic cells and stained with antibodies against Pax2. In the mesenchyme surrounding anterior mesonephric tubules, levels of apoptosis are significantly elevated in *Odd1* mutant (B, asterisk) compared with control embryos (A). By contrast, levels of apoptosis in E9.5 metanephric mesenchyme precursor cells are low in both *Odd1* mutants (D) and controls (C). At this stage and axial level, Pax2 (D) and *Eya1* (F) expression in prospective metanephric mesenchyme are absent in mutant embryos, while dermamyotomal *Eya1* expression is normal (**E**,**F**). By E10.5, *Odd1* null mutants (H) have increased levels of apoptosis in the metanephric mesenchyme (asterisk) compared with heterozygotes (G). At E10.5, *Wt1* is expressed in the mesenchyme of both mutant (**J**, arrowhead) and wild-type (**I**) embryos. Similarly, *Odd1* mutant embryos (**L**) exhibit substantial levels of *Odd1* promoter activity, as detected by *lacZ* expression driven by the *Odd1* promoter (heterozygote shown in **K**). a, aorta; d, duct; m, nephric mesenchyme; mm, metanephric mesenchyme; t, tubule; u, ureter.

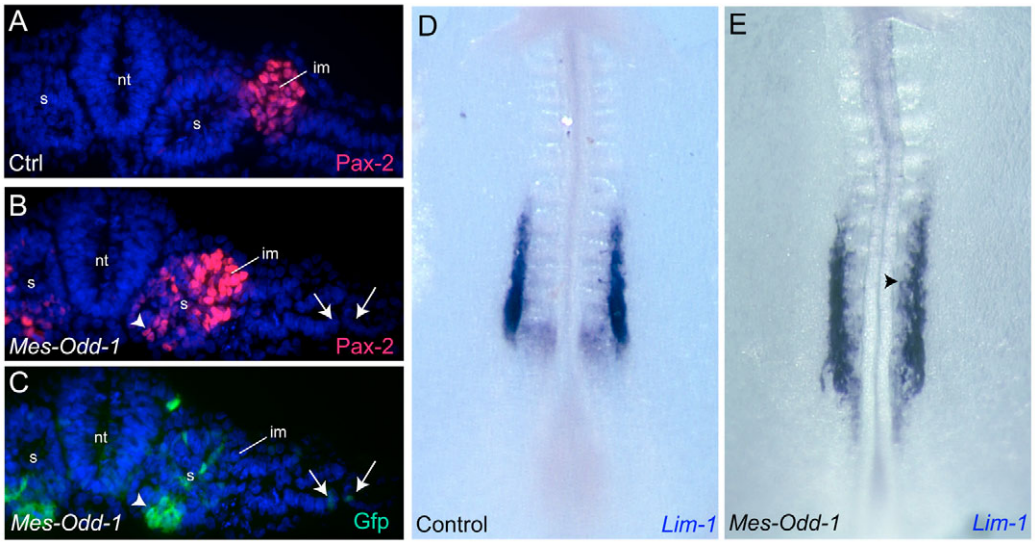

Fig. 6. *Odd1* can induce early nephric markers when ectopically expressed in the somites. Control chicken embryos (**A**,**D**) and embryos electroporated with *Mes-Odd1* expression vector (**B**,**C**,**E**) were stained with immunofluorescence against Pax2 (A,B) or GFP (C), or by whole-mount in situ hybridization for *Lim1* (D,E). Ectopic cells expressing Pax2 or *Lim1* are found in the somite region (arrowheads in B,E). In B and C, note that GFP cells in the lateral plate (arrows) do not express ectopic Pax2. im, intermediate mesoderm; nt, neural tube; s, somite.

Odd1 promoter activity are found in *Odd1* null mice at E10.5, after the initiation of apoptosis (Fig. 5L), indicating that many cells that normally express *Odd1* survive in the absence of *Odd1*. Many Wt1-expressing cells are present in the metanephric region of *Odd1* null mice (Fig. 5J), further indicating that cells with nephrogenic potential exist in the absence of *Odd1*, even if such cells do not proceed to express other kidney markers. Metanephric mesenchyme apoptosis also occurs in mouse lines mutant for *Pax2*, *Wt1*, *Six1* and *Eya1* (Kreidberg et al., 1993; Torres et al., 1995; Xu et al., 1999; Xu et al., 2003). The apoptosis seen in the *Odd1* null metanephros after E9.5 could be due to a combination of the absence of *Odd1* as well as of these other metanephric mesenchyme genes. Taken together, these data support an essential role for *Odd1* in the initial formation of metanephric mesenchyme.

Defects in nephric duct and pro/mesonephric tubule formation in *Odd1* null nice

By contrast to the complete absence of the metanephros, the nephric duct and some pro/mesonephric tubules do form in *Odd1* null mice (Fig. 4). The nephric duct phenotype in *Odd1* mutants is more severe than that seen in mutants for a number of other kidney genes, including *Eya1*, *Six1*, *Wt1* and *Sall-1*, all of which have morphologically normal ducts (Kreidberg et al., 1993; Nishinakamura et al., 2001; Xu et al., 1999; Xu et al., 2003). However, *Odd1* mutant nephric duct defects are less severe than those of double mutants for *Pax2* and *Pax8*, which have no duct at all (Bouchard et al., 2002). *Pax2* and *Pax8* are expressed in the nephric duct itself, suggesting a cell-autonomous requirement for *Pax2/8* activity in the nephric duct. By contrast, *Odd1* is expressed in nephric duct precursors and in nephrogenic mesenchyme, but not in the nephric duct itself (Fig. 1). Thus, the nephric duct defects seen in *Odd1* mutant mice can be attributed either to defects in duct precursor cells, or to defects in interactions between the duct and *Odd1*-expressing mesenchyme, which may be necessary for nephric duct maintenance (Obara-Ishihara et al., 1999). Wang et al. (Wang et al., 2005) have reported that the nephric duct defects are consistently more severe on the left side than on the right, suggesting an interaction of *Odd1* activity with laterality regulation pathways.

While the number of mesonephric tubules that form in *Odd1* mutants is greatly reduced compared with wild type, it is nevertheless significant that a few anterior tubules form in *Odd1* mutant mice. It has been reported that two types of mesonephric tubules develop in mice: anterior tubules that fuse with the nephric duct and posterior tubules that remain unfused with the duct (Sainio et al., 1997). In *Odd1* mutants, as well as in *Wt1* mutants (Sainio et al., 1997), only the anterior, fused tubules differentiate. It has been suggested that the anterior, fused tubules differentiate simultaneously with the nephric duct from common precursor cells or by direct extension from the developing duct (Hiruma and Nakamura, 2003; Sainio et al., 1997). Our data indicate that *Odd1* function is not required for the formation of this anterior group of pro/mesonephric tubules.

By contrast, posterior mesonephric tubules develop from a band of *Pax2*-expressing nephrogenic mesenchyme adjacent to the nephric duct. In *Odd1* mutants, there is no evidence for mesenchymal Pax2 expression or formation of this nephrogenic mesenchyme (Fig. 4), which would explain the severe defects in posterior mesonephric tubule formation. Mutants for *Eya1*, *Six1* and *Sall1*, which are required for metanephros development, have normal mesonephric tubule formation (Nishinakamura et al., 2001; Sajithlal et al., 2005; Xu et al., 1999; Xu et al., 2003). This suggests that *Odd1* is required for common processes that underlie both mesonephric and metanephric tubule formation, while genes such as *Eya1*, *Six1* and *Sall1* are required specifically for formation of the metanephros.

The *Odd1* mutant mesonephric tubule phenotype is less severe than that seen in *Pax2* mutants, which have no mesonephric tubules (Brophy et al., 2001; Torres et al., 1995). However, *Pax2*, unlike *Odd1*, is expressed in tubules themselves and in the nephric duct (Dressler et al., 1990). Thus, the lack of all pro/mesonephric tubules in *Pax2* mutants may reflect an autonomous requirement for *Pax2* at a later stage in tubule differentiation or in duct formation.

Odd1 may promote a nephric precursor state

Odd1 can activate the transcription of *Pax2* and *Lim1* when transiently expressed ectopically by electroporation in the paraxial mesoderm (Fig. 6). However, when *Odd1* is stably expressed ectopically via a retroviral vector, and embryos are examined after 4 to 5 days, relatively little ectopic expression of *Pax2* or *Lim1* is seen. Rather, the predominant effect of long-term ectopic expression is the inhibition of tubule formation in the mesonephric region (Fig. 7). *Odd1*-expressing mesonephric cells express *Pax2* but do not adopt an epithelial morphology. Indeed, *Odd1*-

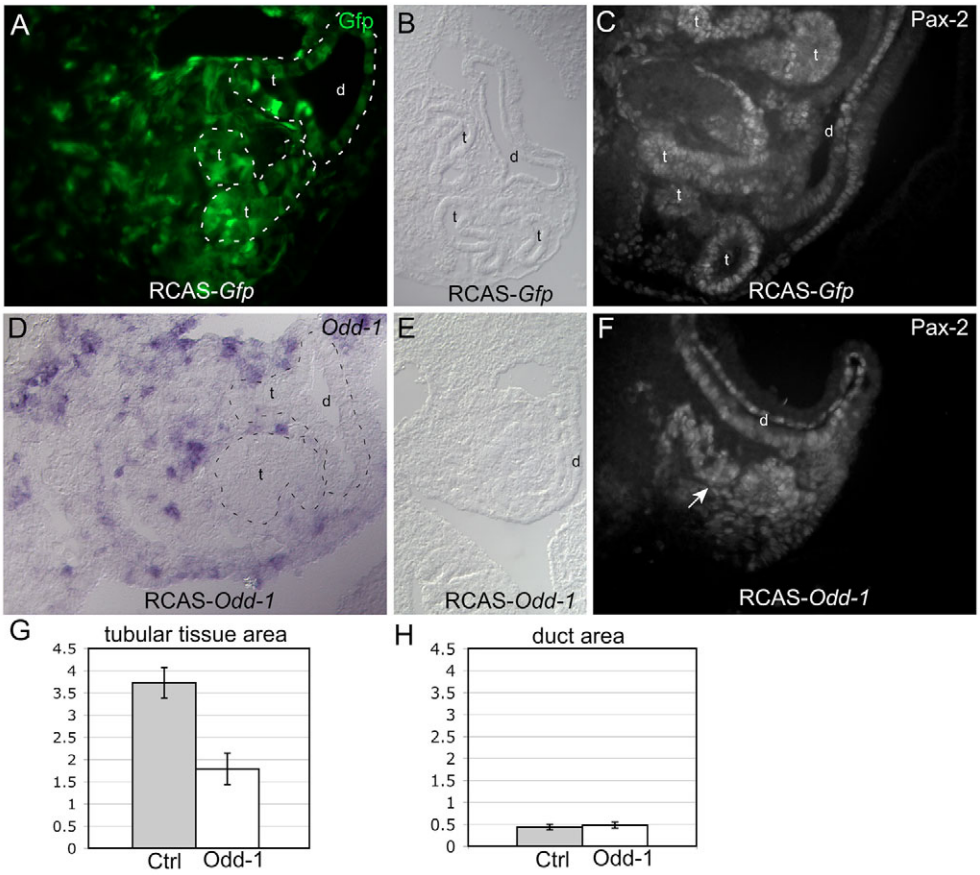

Fig. 7. Expression of *Odd1* inhibits kidney tubule formation. (**A-C**) Representative control chicken embryos infected with *RCAS-Gfp* at day 0 and harvested at day 5, showing extensive infection (A) and robust mesonephric tubule formation (A-C). (**D-F**) *RCAS-Odd1* infected embryos analyzed by section in situ hybridization for *Odd1* to document extent of infection (D), by Nomarski microscopy (E) and with immunofluorescence for Pax2 (F). The mesonephroi of *RCAS-Odd1* infected embryos contain fewer epithelialized tubules (compare B,C with E,F), but do contain significant numbers of Pax2-expressing non-epithelialized cells (arrow in F). When epithelialized tubules are present in *RCAS-Odd1* infected embryos, the tubules tend to be uninfected (D), whereas tubules are readily infected by control *RCAS-Gfp* virus (A) (in situ detection conditions were calibrated to detect only ectopic and not endogenous *Odd1* message). The area of total tubular tissue (defined as Pax2-expressing cells arranged either as lumenized epithelia or as aggregates with polarized nuclei) in *RCAS-Odd1* infected embryos is significantly smaller than in control embryos (**G**), but the nephric ducts are approximately the same size (**H**). Bar graphs show mean and standard error of 8 *RCAS-Gfp* and 10 *RCAS-Odd1* samples. The *y*-axis is in arbitrary units. d, nephric duct; t, tubule.

expressing cells are selectively excluded from epithelial tubules (Fig. 7). One way to reconcile the results of short-term versus longer-term ectopic expression of *Odd1* is to postulate that *Odd1* promotes the establishment of a *Pax2*-positive nephric precursor fate. *Odd1* may be able to both produce such a state and maintain it, thereby blocking subsequent tubule differentiation. The low degree of ectopic *Pax2* expression in the retrovirus-infected embryos compared with the electroporated embryos could be due to levels of expression (the RCAS retroviral vector integrates only one copy of the gene per cell, while electroporation can introduce many gene copies per cell) or to the absence of factors needed to maintain kidney gene expression in regions outside the intermediate mesoderm.

These data suggest that while *Odd1* is necessary for the activation of early kidney gene expression, downregulation of *Odd1* may be necessary for differentiation of nephric tissue into epithelial structures. Such a role for *Odd1* is consistent with the *Odd1* expression pattern, as *Odd1* is expressed in all nephric precursors but is downregulated upon tubule formation (Figs 1, 2). In future work, it will be important to understand the molecular mechanisms by which *Odd1* is downregulated during epithelium formation, as well as the mechanisms through which *Odd1* regulates kidney gene expression and inhibits epithelial differentiation.

We gratefully acknowledge A. Bober, P. Danielian, M. Goulding, T. Jessell, J. Kreidberg, R. Maas, R. Nishinakamura and C. Tabin for sharing probes and expression plasmids, and D. Herzlinger for providing an improved *lacZ* staining protocol. We also thank Mozhgan Afrakhte, Iain Drummond, Doris Herzlinger and Sasha Petrova for critical reading of the manuscript and for many helpful discussions and suggestions. R.G.J. was supported by a Predoctoral Fellowship from the Howard Hughes Medical Institute, and C.N.K. is supported by a Predoctoral Fellowship award from the National Science Foundation. This work was supported by grants R01 DK59980 and R01 DK71041 from the NIH (NIDDK) to T.M.S., and R01 DE013681 (NIH/NIDCR) to R.J.

References

Andacht, T., Hu, W. and Ivarie, R. (2004). Rapid and improved method for windowing eggs accessing the stage X chicken embryo. *Mol. Reprod. Dev.* **69**, 31-34.

Armstrong, J. F., Pritchard-Jones, K., Bickmore, W. A., Hastie, N. D. and Bard, J. B. (1993). The expression of the Wilms' tumour gene, WT1, in the developing mammalian embryo. *Mech. Dev.* **40**, 85-97.

Bard, J. B. L. (2003). The Metanephros. In *The Kidney: From Normal Development*

to Congenital Disease (ed. P. Vize, A. S. Woolf and J. B. L. Bard), pp. 139-148. San Diego: Academic Press.
Bates, C. M., Kharzai, S., Erwin, T., Rossant, J. and Parada, L. F. (2000). Role of N-myc in the developing mouse kidney. *Dev. Biol.* **222**, 317-325.
Bouchard, M., Souabni, A., Mandler, M., Neubuser, A. and Busslinger, M. (2002). Nephric lineage specification by Pax2 and Pax8. *Genes Dev.* **16**, 2958-2970.
Brophy, P. D., Ostrom, L., Lang, K. M. and Dressler, G. R. (2001). Regulation of ureteric bud outgrowth by Pax2-dependent activation of the glial derived neurotrophic factor gene. *Development* **128**, 4747-4756.
Burrill, J. D., Moran, L., Goulding, M. D. and Saueressig, H. (1997). PAX2 is expressed in multiple spinal cord interneurons, including a population of EN1+ interneurons that require PAX6 for their development. *Development* **124**, 4493-4503.
Cho, E. A. and Dressler, G. R. (2003). Formation and development of nephrons. In *The Kidney: From Normal Development to Congenital Disease* (ed. P. Vize, A. S. Woolf and J. B. L. Bard), pp. 195-210. San Diego: Academic Press.
Dressler, G. R., Deutsch, U., Chowdhury, K., Nornes, H. O. and Gruss, P. (1990). Pax2, a new murine paired-box-containing gene and its expression in the developing excretory system. *Development* **109**, 787-795.
Fekete, D. M. and Cepko, C. L. (1993). Replication-competent retroviral vectors encoding alkaline phosphatase reveal spatial restriction of viral gene expression/transduction in the chick embryo. *Mol. Cell. Biol.* **13**, 2604-2613.
Fujii, T., Pichel, J. G., Taira, M., Toyama, R., Dawid, I. B. and Westphal, H. (1994). Expression patterns of the murine LIM class homeobox gene lim1 in the developing brain and excretory system. *Dev. Dyn.* **199**, 73-83.
Grote, D., Souabni, A., Busslinger, M. and Bouchard, M. (2006). Pax2/8-regulated Gata3 expression is necessary for morphogenesis and guidance of the nephric duct in the developing kidney. *Development* **133**, 53-61.
Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49-92.
Herbrand, H., Guthrie, S., Hadrys, T., Hoffmann, S., Arnold, H. H., Rinkwitz-Brandt, S. and Bober, E. (1998). Two regulatory genes, cNkx5-1 and cPax2, show different responses to local signals during otic placode and vesicle formation in the chick embryo. *Development* **125**, 645-654.
Hiruma, T. and Nakamura, H. (2003). Origin and development of the pronephros in the chick embryo. *J. Anat.* **203**, 539-552.
James, R. G. and Schultheiss, T. M. (2003). Patterning of the avian intermediate mesoderm by lateral plate and axial tissues. *Dev. Biol.* **253**, 109-124.
James, R. G. and Schultheiss, T. M. (2005). Bmp signaling promotes intermediate mesoderm gene expression in a dose-dependent, cell-autonomous and translation-dependent manner. *Dev. Biol.* **288**, 113-125.
Kalatzis, V., Sahly, I., El-Amraoui, A. and Petit, C. (1998). Eya1 expression in the developing ear and kidney: towards the understanding of the pathogenesis of Branchio-Oto-Renal (BOR) syndrome. *Dev. Dyn.* **213**, 486-499.
Kispert, A., Vainio, S. and McMahon, A. P. (1998). Wnt-4 is a mesenchymal signal for epithelial transformation of metanephric mesenchyme in the developing kidney. *Development* **125**, 4225-4234.
Kobayashi, A., Kwan, K. M., Carroll, T. J., McMahon, A. P., Mendelsohn, C. L. and Behringer, R. R. (2005). Distinct and sequential tissue-specific activities of the LIM-class homeobox gene Lim1 for tubular morphogenesis during kidney development. *Development* **132**, 2809-2823.
Kreidberg, J. A., Sariola, H., Loring, J. M., Maeda, M., Pelletier, J., Housman, D. and Jaenisch, R. (1993). WT-1 is required for early kidney development. *Cell* **74**, 679-691.
Li, X., Oghi, K. A., Zhang, J., Krones, A., Bush, K. T., Glass, C. K., Nigam, S. K., Aggarwal, A. K., Maas, R., Rose, D. W. et al. (2003). Eya protein phosphatase activity regulates Six1-Dach-Eya transcriptional effects in mammalian organogenesis. *Nature* **426**, 247-254.
Lobe, C. G., Koop, K. E., Kreppner, W., Lomeli, H., Gertsenstein, M. and Nagy, A. (1999). Z/AP, a double reporter for cre-mediated recombination. *Dev. Biol.* **208**, 281-292.
Mauch, T. J., Yang, G., Wright, M., Smith, D. and Schoenwolf, G. C. (2000). Signals from trunk paraxial mesoderm induce pronephros formation in chick intermediate mesoderm. *Dev. Biol.* **220**, 62-75.
Moore, M. W., Klein, R. D., Farinas, I., Sauer, H., Armanini, M., Phillips, H., Reichardt, L. F., Ryan, A. M., Carver-Moore, K. and Rosenthal, A. (1996). Renal and neuronal abnormalities in mice lacking GDNF. *Nature* **382**, 76-79.
Nishinakamura, R., Matsumoto, Y., Nakao, K., Nakamura, K., Sato, A., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Scully, S., Lacey, D. L. et al. (2001). Murine homolog of SALL1 is essential for ureteric bud invasion in kidney development. *Development* **128**, 3105-3115.
Obara-Ishihara, T., Kuhlman, J., Niswander, L. and Herzlinger, D. (1999). The surface ectoderm is essential for nephric duct formation in intermediate mesoderm. *Development* **126**, 1103-1108.
Pichel, J. G., Shen, L., Sheng, H. Z., Granholm, A. C., Drago, J., Grinberg, A., Lee, E. J., Huang, S. P., Saarma, M., Hoffer, B. J. et al. (1996). Defects in enteric innervation and kidney development in mice lacking GDNF. *Nature* **382**, 73-76.
Sainio, K. (2003). Development of the Mesonephric Kidney. In *The Kidney: From Normal Development to Congenital Disease* (ed. P. Vize, A. S. Woolf and J. B. L. Bard), pp. 75-86. San Diego: Academic Press.
Sainio, K., Hellstedt, P., Kreidberg, J. A., Saxen, L. and Sariola, H. (1997). Differential regulation of two sets of mesonephric tubules by WT-1. *Development* **124**, 1293-1299.
Sajithlal, G., Zou, D., Silvius, D. and Xu, P. X. (2005). Eya1 acts as a critical regulator for specifying the metanephric mesenchyme. *Dev. Biol.* **284**, 323-336.
Sanchez, M. P., Silos-Santiago, I., Frisen, J., He, B., Lira, S. A. and Barbacid, M. (1996). Renal agenesis and the absence of enteric neurons in mice lacking GDNF. *Nature* **382**, 70-73.
Sang, H. (2004). Prospects for transgenesis in the chick. *Mech. Dev.* **121**, 1179-1186.
Sariola, H. and Sainio, K. (1997). The tip-top branching ureter. *Curr. Opin. Cell Biol.* **9**, 877-884.
Saxen, L. (1987). *Organogenesis of the Kidney*. London: Cambridge University Press.
Schultheiss, T. M., Xydas, S. and Lassar, A. B. (1995). Induction of avian cardiac myogenesis by anterior endoderm. *Development* **121**, 4203-4214.
Schultheiss, T. M., James, R. G., Listopadova, A. and Herzlinger, D. (2003). Formation of the nephric duct. In *The Kidney: From Normal Development to Congenital Disease* (ed. P. Vize, A. S. Woolf and J. B. L. Bard), pp. 51-60. San Diego: Academic Press.
Stark, K., Vainio, S., Vassileva, G. and McMahon, A. P. (1994). Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by Wnt-4. *Nature* **372**, 679-683.
Sundin, O. and Eichele, G. (1992). An early marker of axial pattern in the chick embryo and its respecification by retinoic acid. *Development* **114**, 841-852.
Swartz, M. E., Eberhart, J., Pasquale, E. B. and Krull, C. E. (2001). EphA4/ephrin-A5 interactions in muscle precursor cell migration in the avian forelimb. *Development* **128**, 4669-4680.
Torres, M., Gomez-Pardo, E., Dressler, G. R. and Gruss, P. (1995). Pax-2 controls multiple steps of urogenital development. *Development* **121**, 4057-4065.
Tsuchida, T., Ensini, M., Morton, S. B., Baldassare, M., Edlund, T., Jessell, T. M. and Pfaff, S. L. (1994). Topographic organization of embryonic motor neurons defined by expression of LIM homeobox genes. *Cell* **79**, 957-970.
Vainio, S. and Muller, U. (1997). Inductive tissue interactions, cell signaling, and the control of kidney organogenesis. *Cell* **90**, 975-978.
Wang, Q., Lan, Y., Cho, E. S., Maltby, K. M. and Jiang, R. (2005). Odd-skipped related 1 (Odd 1) is an essential regulator of heart and urogenital development. *Dev. Biol.* **288**, 582-594.
Wellik, D. M., Hawkes, P. J. and Capecchi, M. R. (2002). Hox11 paralogous genes are essential for metanephric kidney induction. *Genes Dev.* **16**, 1423-1432.
Wilm, B., James, R. G., Schultheiss, T. M. and Hogan, B. L. M. (2004). The forkhead genes, Foxc1 and Foxc2, regulate paraxial versus intermediate mesoderm cell fate. *Dev. Biol.* **271**, 176-189.
Xu, P. X., Adams, J., Peters, H., Brown, M. C., Heaney, S. and Maas, R. (1999). Eya1-deficient mice lack ears and kidneys and show abnormal apoptosis of organ primordia. *Nat. Genet.* **23**, 113-117.
Xu, P. X., Zheng, W., Huang, L., Maire, P., Laclef, C. and Silvius, D. (2003). Six1 is required for the early organogenesis of mammalian kidney. *Development* **130**, 3085-3094.
Yuan, H. T., Tipping, P. G., Li, X. Z., Long, D. A. and Woolf, A. S. (2002). Angiopoietin correlates with glomerular capillary loss in anti-glomerular basement membrane glomerulonephritis. *Kidney Int.* **61**, 2078-2089.