FGF8 is required for cell survival at distinct stages of nephrogenesis and for regulation of gene expression in nascent nephrons

Uta Grieshammer[1,*], Cristina Cebrián[2,*], Roger Ilagan[3,*], Erik Meyers[3], Doris Herzlinger[2,†] and Gail R. Martin[1]

[1]Department of Anatomy and Program in Developmental Biology, School of Medicine, University of California at San Francisco, San Francisco, CA 94143-2711, USA
[2]Department of Physiology, Biophysics and Systems Biology, Weill Medical College of Cornell University, New York, NY 10021, USA
[3]Departments of Pediatrics and Cell Biology, Duke University Medical Center, Durham, NC 27710, USA
*These authors contributed equally to this work
†Author for correspondence (e-mail: daherzli@med.cornell.edu)

Accepted 17 June 2005

Development 132, 3847-3857
Published by The Company of Biologists 2005
doi:10.1242/dev.01944

Summary

During kidney morphogenesis, the formation of nephrons begins when mesenchymal nephron progenitor cells aggregate and transform into epithelial vesicles that elongate and assume an S-shape. Cells in different regions of the S-shaped body subsequently differentiate into the morphologically and functionally distinct segments of the mature nephron. Here, we have used an allelic series of mutations to determine the role of the secreted signaling molecule FGF8 in nephrogenesis. In the absence of FGF8 signaling, nephron formation is initiated, but the nascent nephrons do not express *Wnt4* or *Lim1*, and nephrogenesis does not progress to the S-shaped body stage. Furthermore, the nephron progenitor cells that reside in the peripheral zone, the outermost region of the developing kidney, are progressively lost. When FGF8 signaling is severely reduced rather than eliminated, mesenchymal cells differentiate into S-shaped bodies. However, the cells within these structures that normally differentiate into the tubular segments of the mature nephron undergo apoptosis, resulting in the formation of kidneys with severely truncated nephrons consisting of renal corpuscles connected to collecting ducts by an abnormally short tubular segment. Thus, unlike other FGF family members, which regulate growth and branching morphogenesis of the collecting duct system, *Fgf8* encodes a factor essential for gene regulation and cell survival at distinct steps in nephrogenesis.

Key words: Cell death, FGF signaling, *Fgf8*, Kidney, *Lim1*, Nephrogenesis, *Wnt4*

Introduction

The mature nephron, the functional unit of the kidney (metanephros), is a complex structure comprising a renal corpuscle, where blood is filtered, and a tubular segment, where solutes and fluids required for homeostasis are reabsorbed (see Fig. 1A). Nephron formation is initiated by signals released from the tips of the nascent branches of the ureteric bud-derived collecting duct system (Saxen, 1987). These signals induce cells in the metanephric mesenchyme to condense into pre-tubular aggregates that develop into epithelial renal vesicles, which subsequently elongate and develop into S-shaped bodies (see Fig. 1A). Cells in the proximal domain of the S-shaped body differentiate into specialized epithelial cell types of the mature renal corpuscle, i.e. podocytes and Bowman's capsule cells. The more distal domain of the S-shaped body differentiates into the tubular portion of the nephron, which is segmented into proximal tubular, loop of Henle, and distal tubular domains (see Fig. 1A). The final stages of nephron maturation are characterized by extensive elongation of the tubular nephron segments and the expression of specialized nephron segment-specific transport proteins (Nakai et al., 2003).

The process of nephron induction and maturation is continually occurring in the cortical region of the developing kidney, which is found progressively further away from the original site of kidney induction as the organ grows. Nephrogenesis is dependent on the maintenance of a nephron progenitor cell population that is able to respond to the signals for nephron formation from the collecting duct branch tips. These nephron progenitors are thought to reside in the outermost region ('peripheral zone' in Fig. 1A) of the expanding kidney, which also contains cells that give rise to the stroma (Hatini et al., 1996).

At present, little is known about the molecules that control the early steps in nephrogenesis. Genetic analysis in mice has identified WNT9b, released from the developing collecting ducts, as a signaling molecule that induces nephrogenesis (T. Carroll and A. McMahon, personal communication). Another WNT family member, WNT4, produced by nephron progenitors within the pre-tubular aggregate, is thought to be required for the mesenchymal to epithelial transition that leads to the formation of renal vesicles (Stark et al., 1994; Kispert et al., 1998), whereas the transcription factor LIM1 (LHX1 –

Mouse Genome Informatics) is necessary for the progression of renal vesicles to the S-shaped body stage (Kobayashi et al., 2005). Presenilins, which are necessary for the proteolytic activation of Notch, appear to function at a slightly later stage in nephrogenesis, as S-shaped bodies develop in the absence of *Psen1* and *Psen2*, but the nephrons that form are severely truncated, and lack renal corpuscles and proximal tubules (Cheng et al., 2003; Wang et al., 2003). Similarly, embryos that lack *Brn1* (*Pou3f3* – Mouse Genome Informatics), a POU-domain transcription factor, initiate nephrogenesis but form truncated nephrons that lack much of the loop of Henle (Nakai et al., 2003).

Members of the Fibroblast Growth Factor (FGF) family of secreted signaling molecules are also known to play a role in kidney morphogenesis, but thus far genetic analyses have revealed only an indirect role for FGF signaling in nephron formation. Thus, in the absence of either FGF7 or FGF10 (Qiao et al., 1999; Ohuchi et al., 2000), or the receptor they activate (FGFR2-IIIb) (Revest et al., 2001; Zhao et al., 2004), the number of collecting duct branches is reduced. This in turn results in a decrease in the number of nephrons that form. Another FGF family member, *Fgf8*, is expressed in the developing kidney (Crossley and Martin, 1995; Mahmood et al., 1995), but its function in kidney development has not been assessed. In this study, we have used an *Fgf8* conditional-null allele (Meyers et al., 1998) to demonstrate that FGF8 is essential for the earliest steps in nephrogenesis, and an *Fgf8* hypomorphic allele (Meyers et al., 1998) to show that the survival of cells that develop into the tubular segment of the nephron is dependent on FGF8.

Materials and methods

Mouse mutants

All mouse lines were maintained on mixed genetic backgrounds. Two different null alleles, $Fgf8^{\Delta 2,3}$ (Meyers et al., 1998) and $Fgf8^{lacZ}$ (D. Brown and G.R.M., unpublished), were used interchangeably in this study; both are referred to as $Fgf8^{null}$. Embryos were genotyped for *Fgf8* alleles and for the presence of Cre as previously described (Sun et al., 1999; Sun et al., 2002), and for $Fgf8^{lacZ}$ by staining the tissue remaining after metanephros removal for β-galactosidase activity. Wild-type metanephroi for analysis of *Fgf8*, *Wnt4* and FGF receptor gene expression were isolated from outbred mice (CD1, Charles River). To stage embryos, noon of the day when a vaginal plug was detected was considered to be embryonic day (E) 0.5.

Quantification of *Fgf8* RNA

Both metanephroi from each embryo analyzed were homogenized in DEPC-treated phosphate-buffered saline (PBS) with 0.1% Triton X-100 and total RNA was extracted using TRIzol Reagent (Invitrogen 15596-026). Following isopropanol precipitation and resuspension in 15 μl water, reverse transcription was performed using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT; Invitrogen 28025-013) to synthesize cDNA. Quantitative PCR was performed using an ABI Prism 7900 HT Sequence Detection System. The primers used to amplify *Fgf8* sequences spanned exons 2 and 3 (forward primer, 5′-TCTCCAGCACGATCTCTGTGAA-3′; reverse primer, 5′-GGAAG-CTAATTGCCAAGAGCAA-3′). The TaqMan® probe sequence was 5′-ACGCAGTCCTTGCCT-3′. *Gapdh* sequences were amplified and the amount of product was used for normalizing samples.

In situ hybridization

RNA in situ hybridization on whole mounts or on 10 μm cryosections were performed according to standard protocols. For analysis of vibratome sections, kidneys were fixed in 4% paraformaldehyde (PFA), embedded in 4% low-melting point agarose in PBS and sectioned at 100 μm. For each gene analyzed, all sections collected from a single kidney were processed together using a whole-mount in situ hybridization protocol. The images presented here show a representative section from each kidney. To generate digoxigenin-labeled probes, we used plasmids containing mouse *Fgf8*, *Wt1*, *Gdnf*, *Foxd1*, *Lim1* and *Umod* sequences for in vitro transcription, whereas probes for *Slc34a1*, *Slc12a3*, *Wnt4* and *Podxl* were transcribed using PCR products as templates (see Table S1 in the supplementary material).

X-Gal staining to detect *lacZ* activity

Prior to X-Gal staining, tissues were embedded in a 1:1 mix of 20% sucrose and OCT (Tissue-Tek 4583), and cryosectioned at 10 μm. Sections were then fixed in 0.5% PFA for 10 minutes on ice, rinsed three times for 10 minutes each in PBS, stained overnight at 37°C using standard X-gal staining protocols, then counterstained with Eosin.

Immunofluorescence assays

Cryosections or vibratome sections of kidneys were prepared as described above, and then stained with antibodies against PAX2 (1:50, BAbCO PRB-276P), smooth muscle actin (1:300, Cy3-conjugated, Sigma C6198), WT1 (1:80, Santa Cruz Biotechnology sc-192), PECAM (1:50, BD Biosciences 553370), Calbindin D_{28K} (1:1000, Oncogene PD253L), Phospho-Histone H3 (1:100, Cell Signaling Technology 9706), and E-cadherin (10 μg/ml, R&D Systems AF748).

Detection of cell death

LysoTracker (Molecular Probes L-7528) labels acidic compartments within apoptotic cells themselves, as well as in healthy cells that are engulfing apoptotic debris (Zucker et al., 1999; Schaefer et al., 2004). For LysoTracker analysis, embryonic kidneys were isolated in Hank's balanced salt solution (HBSS), incubated with 5 μl/ml LysoTracker solution in HBSS at 37°C for 45 minutes, rinsed in HBSS, fixed in 4% PFA in PBS and stored at –20°C in 100% methanol. Following rehydration into PBS, LysoTracker-labeled kidneys were vibratome sectioned and processed for anti-PAX2 immunofluorescence. Images were photographed using either a stereomicroscope or a confocal microscope. For TUNEL analysis, embryonic kidneys were fixed in 4% PFA. After overnight incubation in 20% sucrose, kidneys were mounted in OCT and cryosectioned. Apoptotic cell death was detected with the In Situ Cell Death Detection Kit, TMR red, following manufacturer's instructions (2156792 Roche). When required, immunofluorescence assays were performed after TUNEL analysis.

Embryonic kidney explant cultures

Kidney rudiments were isolated from ~E11.5 mouse embryos. For experiments involving isolated metanephric mesenchyme (MM), the ureteric bud (at no later than the early T-stage) was removed from the kidney rudiment using fine tungsten needles, following treatment with collagenase and DNAse A, as previously described (Qiao et al., 1995). Intact kidney rudiments and isolated MMs were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum on polycarbonate membrane filters (0.4 μm pore size, Transwell®, Corning). For experiments on the effects of anti-FGF8 antibody, either normal goat serum (control) or blocking antibody against FGF8 (10 μg/ml, R&D Systems, AF423NA) was added to the culture medium. For experiments with MMs, dorsal spinal cord was added to the cultures. After 2 or 3 days, the tissues were fixed in 4% PFA and processed for immunofluorescence analysis in whole mount.

Isolation of nephrons

Kidneys were isolated from E18.5 mouse embryos, fixed in 4% PFA and vibratome sectioned at 200 μm. Sections were incubated in 0.2% collagenase (from *Clostridium Histolyticum*) with 50 units/ml DNase

I for 30 minutes at 37°C, rinsed in PBS and further digested in concentrated HCl for 15 minutes at 37°C. After extensive washing, nephrons were dissected in cold PBS under a stereomicroscope using fine tungsten needles.

Results

Fgf8 is expressed in the developing nephron

The earliest stage at which we detected *Fgf8* RNA in the kidney primordium was ~E12 [the 48-somite stage (som)], when it was observed in a continuous domain immediately surrounding the emerging ureteric bud (Fig. 1B,C, and data not shown). By ~E12.5 (52 som), *Fgf8* expression appeared to have resolved into numerous discrete spots near the periphery of the kidney primordium (Fig. 1D). This punctate staining pattern was observed until at least E18.5 (Fig. 1E, and data not shown). Analysis of sections at E16.5, which contain nephrons at all stages in their development, revealed that the punctate staining in whole mounts reflected abundant *Fgf8* RNA in pre-tubular aggregates and early renal vesicles (Fig. 1E′, and data not shown). Because these structures are sometimes difficult to distinguish in histological section, we will refer to them collectively as nascent nephrons. We found that *Fgf8* RNA became restricted to a subset of cells within the epithelium as the nascent nephrons matured (Fig. 1E″), and to the tubule progenitors in S-shaped bodies; *Fgf8* RNA appeared to be

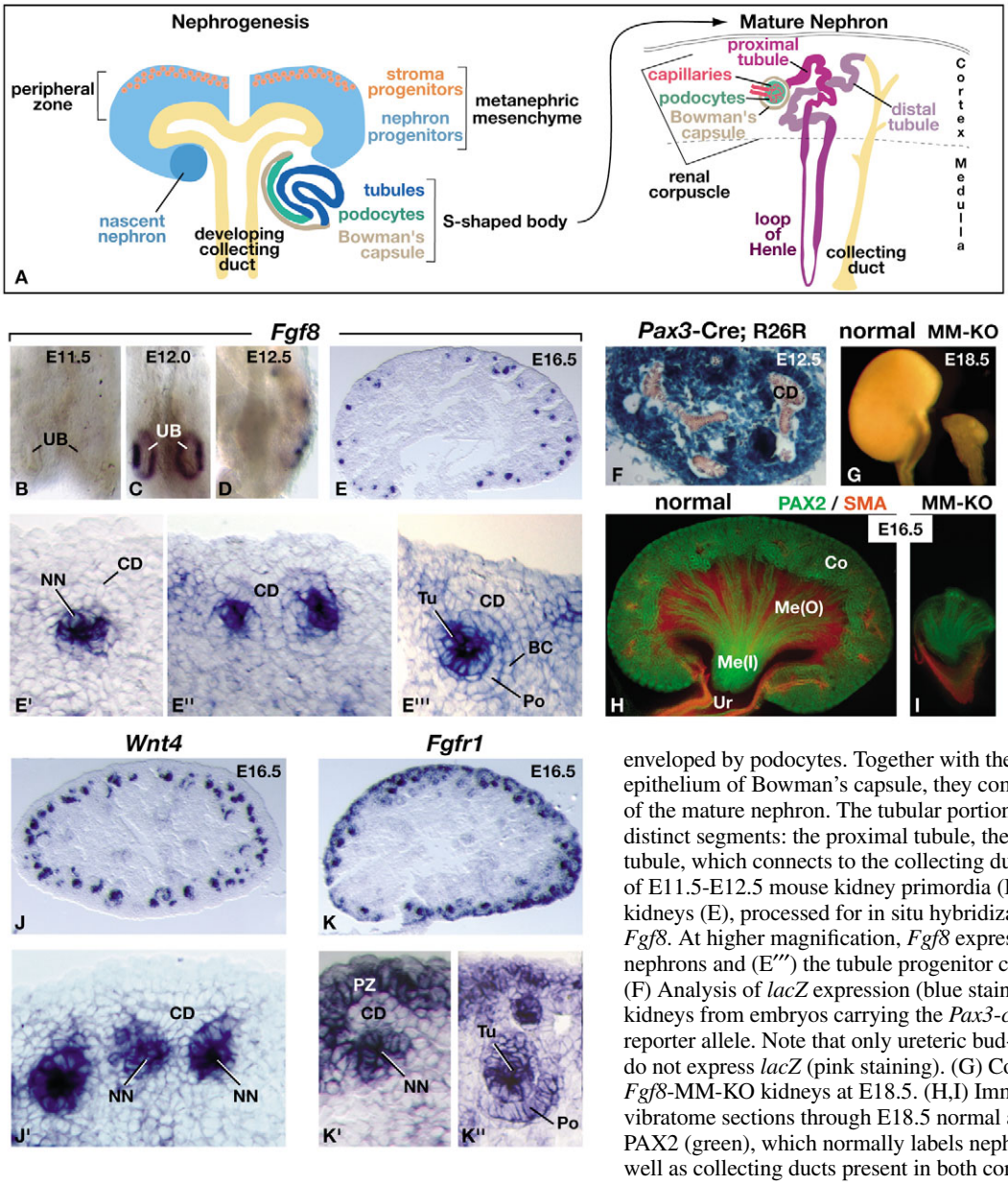

Fig. 1. Expression of *Fgf8* in the developing kidney and phenotype of *Fgf8*-MM-KO kidneys at E18.5. (A) Schematic diagrams illustrating the early stages of nephron formation (nephrogenesis; left) and the mature nephron (right). During development, nephron and stroma progenitors are localized in the peripheral zone near the outermost edge of the kidney primordium, whereas the branching collecting duct system is localized to the central region of the rudiment. Nascent nephrons form adjacent to collecting duct tips and develop into S-shaped bodies that differentiate into the various cell types of the nephron. During nephron maturation, invasion of the podocyte layer by endothelial cells (not illustrated) leads ultimately to the formation of a capillary tuft that becomes enveloped by podocytes. Together with the surrounding squamous epithelium of Bowman's capsule, they comprise the renal corpuscle portion of the mature nephron. The tubular portion of the nephron consists of three distinct segments: the proximal tubule, the loop of Henle, and the distal tubule, which connects to the collecting duct system. (B-E) Whole mounts of E11.5-E12.5 mouse kidney primordia (B-D), and cryo-sections of E16.5 kidneys (E), processed for in situ hybridization to localize expression of *Fgf8*. At higher magnification, *Fgf8* expression is detected in (E′,E″) nascent nephrons and (E‴) the tubule progenitor cells within S-shaped bodies. (F) Analysis of *lacZ* expression (blue staining) in sections through E12.5 kidneys from embryos carrying the *Pax3-cre* transgene and the R26R reporter allele. Note that only ureteric bud-derived collecting duct epithelia do not express *lacZ* (pink staining). (G) Comparison of intact normal and *Fgf8*-MM-KO kidneys at E18.5. (H,I) Immunofluorescence assays in vibratome sections through E18.5 normal and *Fgf8*-MM-KO kidneys for PAX2 (green), which normally labels nephron progenitors in the cortex as well as collecting ducts present in both cortex and medulla, and smooth muscle actin (SMA, red), which normally labels the stroma in the medulla, the vasculature in the cortex, and the ureter. (J-K″) Low (J,K) and high (J′,K′,K″) power views of cryo-sections, showing expression of *Wnt4* and *Fgfr1* in E16.5 kidneys. *Wnt4* and *Fgfr1* transcripts are detected in nascent nephrons. In addition, *Fgfr1* transcripts are detected in the peripheral zone and throughout the S-shaped bodies. BC, Bowman's capsule progenitors; CD, collecting duct progenitors; Co, cortex; Me(I), inner medullary region; Me(O), outer medullary region; NN, nascent nephron; Po, podocyte progenitors; PZ, peripheral zone; Tu, tubule progenitors; UB, ureteric bud; Ur, ureter.

absent from future podocytes and Bowman's capsule cells (Fig. 1E‴). As nephrogenesis proceeded, *Fgf8* expression appeared to be downregulated, as evidenced by the absence of a hybridization signal in cortical regions containing nephrons that had matured past the S-shaped body stage (Fig. 1E).

Inactivation of *Fgf8* in the metanephric mesenchyme

Because *Fgf8* null embryos die before kidney formation initiates (Sun et al., 1999), we employed a Cre-mediated tissue-specific knock-out strategy to study the consequences of loss of *Fgf8* function in the metanephric mesenchyme. To identify an appropriate source of Cre activity, we tested a *Pax3-cre* transgene (Li et al., 2000) that has been reported to function in the newborn kidney (Chang et al., 2004), as well as in the neural crest (Li et al., 2000), for Cre activity at early stages of kidney development. In embryos carrying both *Pax3-cre* and R26R, a reporter allele in which *lacZ* is expressed only after Cre-mediated recombination (Soriano, 1999), reporter gene expression was detected at E11.5 and E12.5, in what appeared to be all metanephric mesenchyme cells (Fig. 1F, and data not shown). A similar analysis using the Z/eG reporter, which expresses *lacZ* before but not after Cre-mediated recombination (Novak et al., 2000), revealed that very few *lacZ*-expressing cells were detected in the metanephric mesenchyme of *Pax3-cre*;Z/eG double transgenic embryos (not shown). Together, these data show that *Pax3-cre* activity leads to the virtually complete recombination of floxed reporter alleles in the metanephric mesenchyme, and thus should be useful for eliminating *Fgf8* function in the developing kidney by the stage when expression of *Fgf8* is initiated (see Fig. 1B,C).

To assess the efficiency at which *Pax3-cre* activity deletes *Fgf8* coding sequences during kidney development, *Pax3-cre*;$Fgf8^{null/+}$ males were crossed to females homozygous for $Fgf8^{flox}$, an *Fgf8* conditional allele with wild-type activity that is converted by Cre-mediated recombination to an *Fgf8* null allele lacking exons 2 and 3 ($Fgf8^{\Delta 2,3}$) (Meyers et al., 1998). cDNA prepared from extracts of kidney primordia isolated from their offspring at E11.5 and E12.5, was used to perform quantitative PCR for the exon 2/3 sequences that are flanked by *loxP* sites in the $Fgf8^{flox}$ allele. An *Fgf8* exon 2/3 amplification product was detected in all samples prepared from control (normal) embryos that were either $Fgf8^{flox/null}$ or carried one copy of $Fgf8^{+}$ (n=6 at E11.5, n=4 at E12.5), but no exon 2/3 amplification product was detected in samples prepared from the *Pax3-cre*; $Fgf8^{flox/null}$ embryonic kidneys (n=4 at E11.5, n=7 at E12.5; data not shown). These results demonstrate that *Pax3-cre* activity results in a complete loss of *Fgf8* function in *Pax3-cre*; $Fgf8^{flox/null}$ embryonic kidneys, although we cannot rule out the possibility that there is a small amount of transient *Fgf8* expression. Hereafter, we will refer to such embryos as *Fgf8*-MM-KO (Metanephric Mesenchyme-Knock Out) mutants. Although born at the expected Mendelian frequency, *Fgf8*-MM-KO mutants died shortly after birth.

At E18.5, all *Fgf8*-MM-KO kidneys were severely malformed (Fig. 1G). At that stage, normal kidneys comprise an inner medullary region, an outer medullary region and a cortical region (Fig. 1H), each containing part of the PAX2-expressing collecting duct system. The inner medullary region consists of mostly collecting ducts, whereas the outer medullary region also contains abundant smooth muscle actin (SMA)-expressing stroma, as well as the loop of Henle segment of the nephron. The cortical region contains all other domains of the nephron (see Fig. 1A). By contrast, *Fgf8*-MM-KO kidneys were composed solely of what appeared to be an inner medullary region containing very short PAX2-positive collecting ducts, but lacked SMA-positive outer medullary stroma and all portions of the nephron (Fig. 1I, and data not shown). The ureters of *Fgf8*-MM-KO and normal kidneys were indistinguishable, as determined by the presence of a well-developed smooth muscle coat and terminally differentiated urothelium (not shown). Collectively these data indicate that FGF8 signaling is essential for normal kidney development, although the earliest events in kidney morphogenesis, including ureteric bud formation and at least some collecting duct branching, occur in its absence.

Nephrogenesis is initiated in *Fgf8*-MM-KO kidneys but does not progress to the S-shaped body stage

To determine the cause of these abnormalities, we analyzed *Fgf8*-MM-KO kidneys from the earliest stages of development. At E12.5, the mutant kidneys appeared to be grossly normal (data not shown), but at E13.0 and E14.5, they were markedly smaller than kidneys from their normal littermates (Fig. 2). Despite this size reduction, the patterns of expression of genes thought to mark nephron progenitors in the peripheral zone, including *Wt1*, a transcriptional regulator required at early stages of kidney development (Kreidberg et al., 1993; Donovan et al., 1999) (Fig. 2A,B), *Gdnf*, a signaling molecule essential for ureteric bud formation and branching (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996) (Fig. 2C,D) and PAX2, a transcription factor required for *Gdnf* expression (Brophy et al., 2001) (Fig. 2Q,R), appeared to be normal in *Fgf8*-MM-KO kidneys at E13.5 to E14.5. Likewise, the expression of *Foxd1* (formerly known as *Bf2*), which marks stroma precursor cells (Hatini et al., 1996) appeared normal at E14.5 (Fig. 2E,F). Collecting ducts, marked by staining for Calbindin (CB) (Liu et al., 1993), were also present (Fig. 2Q,R), but the number of collecting duct tips was reduced in the *Fgf8*-MM-KO kidneys to ~45% of the normal number at E13.5 (see Table S2 in the supplementary material), suggesting that by that stage collecting duct tips had on average undergone one less branching event.

Nephrogenesis was initiated in *Fgf8*-MM-KO kidneys, as evidenced by the presence of *Wt1*- (Fig. 2A,B) and PAX2- (Fig. 2Q,R) positive nascent nephrons, at least some of which appeared to have reached the renal vesicle stage (Fig. 2B,R), and also by the expression of *Fgf8*, a marker for cells at early stages of nephrogenesis (see Fig. 1E). The latter was detected using a full-length *Fgf8* probe that includes sequences present in $Fgf8^{null}$ transcripts (Meyers et al., 1998) (Fig. 2G,H). These nascent nephrons normally co-express *Fgf8* (Fig. 1E′) and *Wnt4* (Stark et al., 1994) (Fig. 1J′). However, *Wnt4* RNA was not detected in *Fgf8*-MM-KO kidneys at E13.5 (n=9) or E14.5 (n=3) (Fig. 2I,J and data not shown). Likewise, *Lim1* expression was not detected in *Fgf8*-MM-KO nascent nephrons at E13.5 (n=2) or E14.5 (n=3; Fig. 2K,L). Consistent with the lack of *Wnt4* and *Lim1* expression, both of which are required for progression through the early stages of nephrogenesis (Stark et al., 1994; Kispert et al., 1998; Kobayashi et al., 2005), few if any S-shaped bodies were observed in the *Fgf8* mutant kidneys at any of the stages examined. It is likely that FGFR1

transduces the FGF8 signal required for *Wnt4* and *Lim1* expression, as *Fgfr1*, but not other FGF receptor family members, is abundantly expressed in nascent nephrons (Stark et al., 1991; Peters et al., 1992; Peters et al., 1993; Chi et al., 2004) (Fig. 1K,K′, and Fig. S1 in the supplementary material). Together, our data show that, from early stages, *Fgf8*-MM-KO kidneys are smaller than normal, but all progenitor cell types thought to be essential for further development are present, including nephron and stroma progenitors. Furthermore, nephrogenesis initiates in the mutant kidneys, but rarely, if ever, progresses to the S-shaped body stage.

As *Wnt4* function appears to be required for *Lim1* expression (Kobayashi et al., 2005), we performed an experiment to determine whether nephrogenesis in *Fgf8*-MM-KO kidneys could be rescued by providing a source of WNT signaling. We therefore co-cultured metanephric mesenchyme isolated from *Fgf8*-MM-KO kidney primordia with dorsal spinal cord, a tissue that has previously been shown to rescue nephrogenesis in metanephric mesenchyme isolated from *Wnt4* null kidneys (Kispert et al., 1998). Staining for E-Cadherin (E-CAD), which in these cultures marks nephron epithelia (Cho et al., 1998), showed that after two days of co-culture with dorsal spinal cord, metanephric mesenchyme isolated from control littermates contained numerous intensely E-CAD-positive vesicular structures (n=10; Fig. 3A). By contrast, under the same culture conditions, metanephric mesenchyme isolated from *Fgf8*-MM-KO embryos contained only weakly E-CAD-positive cell aggregates (n=7; Fig. 3B), presumably representing the cells we detected in *Fgf8*-MM-KO kidneys that have initiated nephrogenesis but then fail to develop into S-shaped bodies. Significantly, no E-CAD-positive cells were detected when metanephric mesenchyme was isolated from wild-type embryos and cultured without dorsal spinal cord (see Fig. S2 in the supplementary material). These data provide evidence that a source of signals that can rescue nephrogenesis in *Wnt4* null mesenchyme does not rescue this process in *Fgf8* null mesenchyme, suggesting that the lack of nephrogenesis observed in the absence of FGF8 signaling is not due solely to a lack of WNT4 signaling.

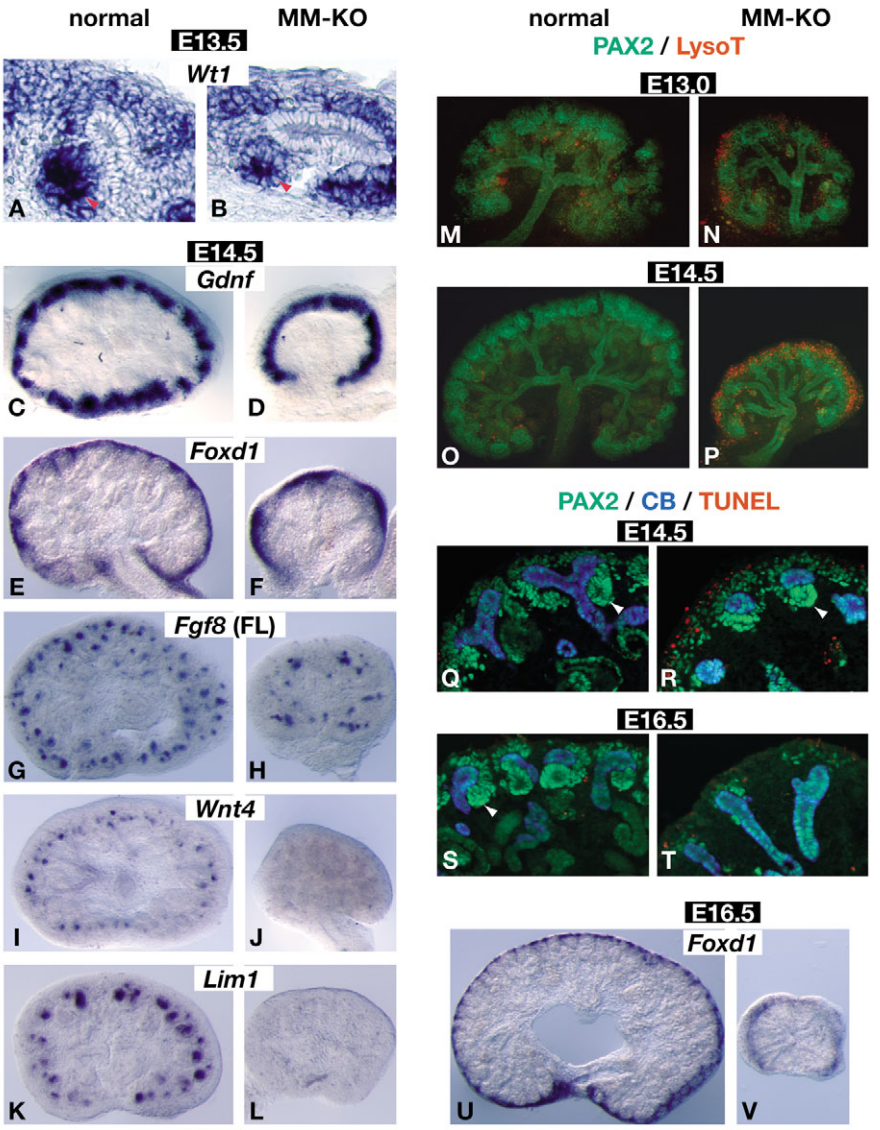

Fig. 2. Nephrogenesis arrests and cells in the peripheral zone die in the absence of FGF8. Marker analysis in (A,B,Q-T) cryosections or (C-P,U,V) vibratome sections of kidneys at the stages indicated. (A-L,U,V) In situ hybridization for the genes indicated. The *Fgf8* (FL) probe we used contained the full-length coding sequence, and therefore detected *Fgf8* RNA produced by the $Fgf8^{null}$ allele. (M-P) Immunofluorescence assays for PAX2 (green) to identify the developing nephrons and collecting ducts, co-stained with LysoTracker (LysoT, red) to identify regions containing dying cells. (Q-T) Immunofluorescence assays for PAX2 (green) and Calbindin (CB, blue), which identifies collecting ducts, and for TUNEL staining (red), which detects dying cells. Arrowheads point to nascent nephrons, which are present in normal kidneys and also in *Fgf8*-MM-KO kidneys at E13.5 (A,B) and E14.5 (Q,R), but not at E16.5 (S,T). Note that the nascent nephrons in *Fgf8*-MM-KO kidneys (B,R) have formed an epithelial structure surrounding a lumen, i.e. they have reached the renal vesicle stage.

Cells in the peripheral zone die at an early stage in *Fgf8*-MM-KO kidneys

In view of the small size of the *Fgf8*-MM-KO kidneys at early stages (Fig. 2), we explored the possibility that cell death contributes to this phenotype. In contrast to normal kidneys at E14.5, which displayed sporadic cell death mostly in the region where nephrons are forming (Koseki et al., 1992; Coles et al., 1993) (Fig. 2O,Q), *Fgf8*-MM-KO kidneys at this stage contained a large number of cells immediately underlying the capsule with the characteristics of apoptotic cells, as determined by staining for LysoTracker (Fig. 2P) and by TUNEL (Fig. 2R) assays. Abnormal cell death in the peripheral

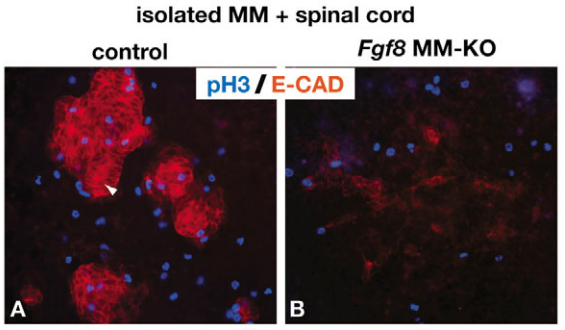

Fig. 3. Signals from the dorsal spinal cord are not sufficient to induce nephrogenesis in *Fgf8*-deficient metanephric mesenchyme. (A,B) Metanephric mesenchyme (MM) was isolated from E11.5 (A) $Fgf8^{flox/null}$ (control) or (B) *Pax3-cre*;$Fgf8^{flox/null}$ (*Fgf8*-MM-KO) littermates and cultured in the presence of dorsal spinal cord. After 48 hours of culture, the samples were processed for immunohistochemistry to detect phospho-Histone H3, which marks cells in mitosis (blue) and E-Cadherin, which marks epithelia (red). The arrowhead in A indicates a region where the tubular nature of the E-CAD-positive structures is particularly evident.

zone, although less widespread, was already observed in the mutant kidneys at ~E13.0 (Fig. 2M,N). Consistent with the extensive cell death observed at E14.5, we found that by E16.5, PAX2-positive and *Gdnf*-expressing peripheral cells were no longer present in *Fgf8*-MM-KO kidneys (Fig. 2S,T and data not shown). Significantly, numerous *Foxd1*-expressing cells were still detected in the peripheral zone of the mutant kidneys at E16.5 (Fig. 2U,V), indicating that the stroma progenitor cell population persists for longer than the nephron progenitor cell population in the absence of FGF8. These results strongly suggest that *Fgf8* is required for the survival of nephron progenitor cells in the peripheral zone, in which *Fgfr1*, but not other FGFR genes, is abundantly expressed (Stark et al., 1991; Peters et al., 1992; Peters et al., 1993; Chi et al., 2004) (Fig. 1K-K″, see also Fig. S1 in the supplementary material).

Tubular nephron segments are truncated in *Fgf8* hypomorphic kidneys

Because the complete loss of *Fgf8* function during kidney development results in a failure of nephron formation prior to the S-shaped body stage, we were unable to determine whether FGF8 signaling is essential for subsequent steps in nephrogenesis using *Fgf8*-MM-KO mutants. Therefore, we analyzed the renal phenotype of mouse embryos in which functional *Fgf8* RNA is expressed at lower than normal levels in all tissues throughout embryonic development. Embryos that are homozygous for $Fgf8^{neo}$, a hypomorphic allele ($Fgf8^{neo/neo}$ embryos; referred to as mild *Fgf8* hypomorphs), or compound heterozygotes for $Fgf8^{neo}$ and an *Fgf8* null allele ($Fgf8^{neo/null}$ embryos; referred to as severe *Fgf8* hypomorphs), have been roughly estimated to produce ~40% and ~20% of wild-type levels of *Fgf8* RNA, respectively (Meyers et al., 1998). At E18.5, the kidneys of these mutants were markedly smaller than control kidneys, but were larger than *Fgf8*-MM-KO kidneys (compare Fig. 4A and Fig. 1G).

Interestingly, we detected *Wnt4*-expression in nascent nephrons in E15.5 kidneys from severe *Fgf8* hypomorphs (Fig. 4B,C), demonstrating that even the relatively low level of functional *Fgf8* expression in these mutants is sufficient to induce and/or maintain some *Wnt4* expression. Nephrogenesis was able to progress in these mutants, as shown by the presence of renal corpuscles containing PECAM-positive capillary tufts (Baldwin et al., 1994) and WT1-positive podocytes (Armstrong et al., 1993) at E15.5 (Fig. 4D,D′). Furthermore, podocytes were identified in E18.5 mild and severe *Fgf8* hypomorph kidneys by in situ hybridization assays for *Podxl* (Doyonnas et al., 2001) (Fig. 5A-C). However, morphometric analyses performed at E15.5 and E18.5 showed that the number of renal corpuscles was reduced in mild (from ~70% to ~35% of normal), and even more reduced in severe (from ~50% to ~20% of normal), *Fgf8* hypomorph kidneys (see Table S3 in the supplementary material). This reduction can presumably be explained, at least partially, by a reduction in collecting duct system branching (see Table S2 in the supplementary material).

The presence of renal corpuscles in kidneys from *Fgf8* hypomorphs suggested that the tubular segment of the nephron might also be present. To address this possibility, we performed an in situ hybridization analysis at E18.5, using markers that identify various regions of maturing nephron tubules (see Fig. 1A): *Slc34a1*, which encodes a Na/P_i co-transporter expressed in the proximal tubular segment (Murer et al., 2004); Uromodulin, which encodes the Tamm-Horsfall protein produced in the loop of Henle (Bachmann et al., 1990); and *Slc12a3*, which encodes a Na/Cl co-transporter expressed in the distal tubular segment (Hebert et al., 2004). This analysis showed that proximal convoluted tubules and distal tubules

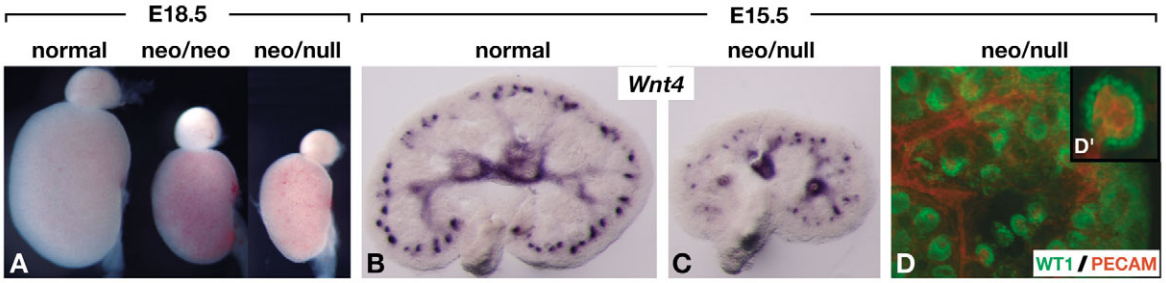

Fig. 4. Kidneys of *Fgf8* hypomorphs are smaller than normal, but contain nephrons. (A) Comparison of normal, $Fgf8^{neo/neo}$ (mild hypomorph) and $Fgf8^{neo/null}$ (severe hypomorph) kidneys at E18.5. (B,C) In situ hybridization assay for *Wnt4* RNA in vibratome sections of E15.5 kidneys. (D) Immunofluorescence assays at E15.5 for PECAM-positive (red) vasculature and WT1-positive (green) podocyte progenitors in vibratome sections. (D′) A higher magnification view of a renal corpuscle in D.

were substantially reduced in mild hypomorphs and barely detectable in severe hypomorphs, whereas the loop of Henle was not present in either mutant (Fig. 5D-L).

To determine whether this reduction or lack of gene expression was caused by a failure of nephron segments to express these differentiated tubule segment markers or whether it was a reflection of the absence of the tubule segments, we performed an immunofluorescence assay to detect nephron tubules. Because there is no single antibody that labels only nephron tubules, we double stained with two antibodies: anti-E-CAD, which labels both nephron tubules and collecting ducts (red) (Cho et al., 1998); and anti-CB, which labels collecting ducts only (green). Thus, the nephron tubules that stain for E-CAD only (red) can be distinguished from the collecting ducts that stain for both E-CAD and CB (yellow) (Fig. 6A). This analysis showed that at E18.5, the nephron tubules were indeed virtually absent in kidneys from severe *Fgf8* hypomorphs (Fig. 6C). Furthermore, an inspection of nephrons isolated from severe hypomorphs showed that the renal corpuscles were connected to the collecting system by a severely truncated tubular segment (Fig. 6B,D). These data demonstrate that low levels of FGF8 signaling support the formation of a limited number of nephrons with severely truncated tubular segments.

We also found that wild-type kidney rudiments cultured in the presence of function-blocking antibodies against FGF8 had much shorter tubule segments than were observed in control cultures (Fig. 6E-F′), phenocopying the *Fgf8* hypomorph nephron tubule phenotype. These data demonstrate that *Fgf8* is required within the developing kidney to support full tubule development, and argue against the possibility that the *Fgf8* hypomorph phenotype is secondary to an early defect in the development of the intermediate mesoderm, caused by reduced *Fgf8* expression during gastrulation in the hypomorphs.

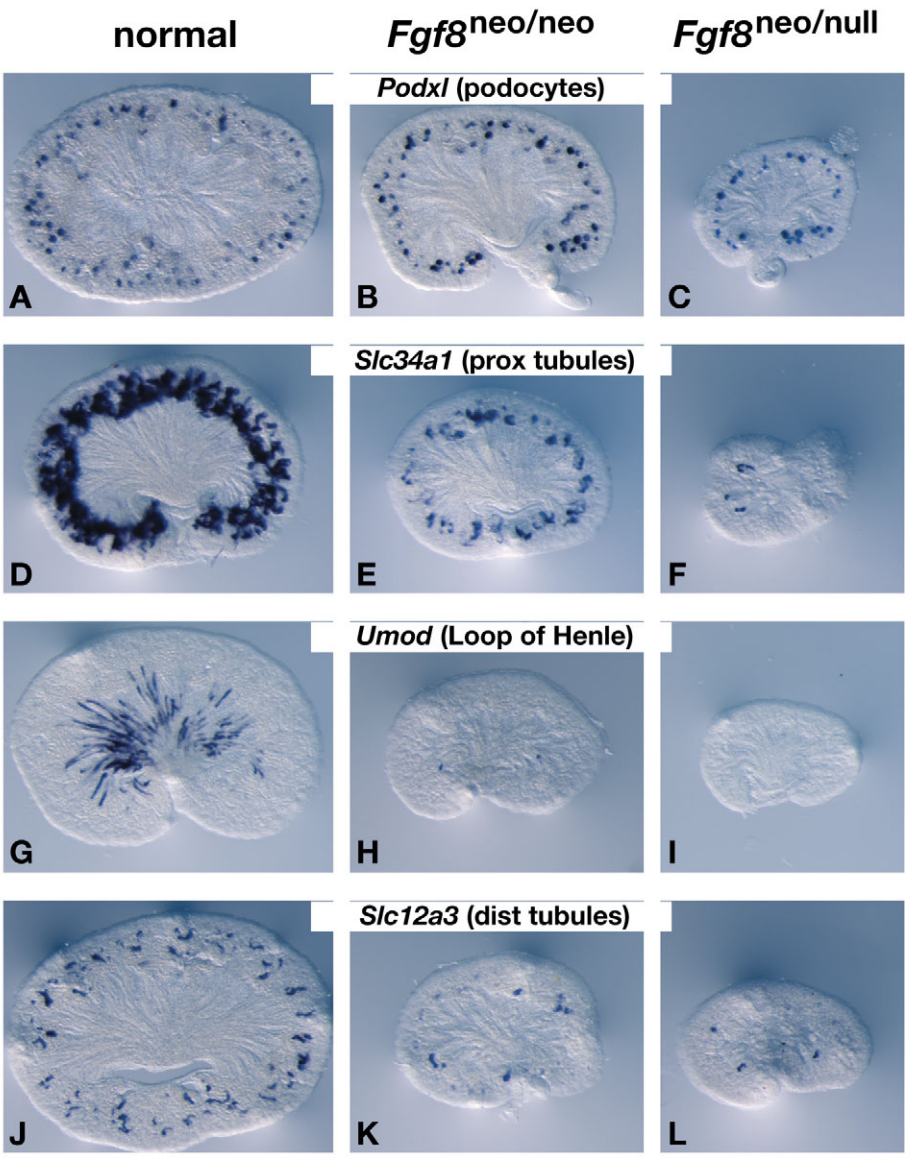

Fig. 5. Nephrons in *Fgf8* hypomorphs lack the loop of Henle. (A-L) In situ hybridization assays in vibratome sections of E18.5 normal and *Fgf8* hypomorph kidneys, for expression of the genes indicated, identifies podocytes (A-C), proximal tubules (D-F) loop of Henle (G-I) and distal tubules (J-L).

Fgf8 is required for cell survival within developing nephrons

To identify the cause of nephron tubule truncation, we focused our attention on the more severely affected $Fgf8^{neo/null}$ kidneys. Assays for cell death at E14.5 (Fig. 7A-B′) revealed that, as in normal kidneys, some dying cells were observed in the developing renal corpuscle in severe *Fgf8* hypomorphs (arrows in Fig. 7A′,B′). However, in addition, there were numerous dying cells in the tubule progenitors in the S-shaped bodies of the mutant kidneys (arrowhead in Fig. 7B′), in which *Fgfr1*, but not other FGFR genes, is abundantly expressed (Stark et al., 1991; Peters et al., 1992; Peters et al., 1993; Chi et al., 2004) (Fig. 1K,K″, and Fig. S1 in the supplementary material). We did not detect the extensive peripheral cell death that was observed in *Fgf8* MM-KO kidneys at this stage (compare Fig. 7B with Fig. 2P). These data suggest that the reduced tubular length observed in E18.5 hypomorphic kidneys is due to the death of tubule progenitors at the S-shaped body stage. At E15.5, the $Fgf8^{neo/null}$ kidneys continued to display abnormal cell death in S-shaped bodies, but, in addition, cell death was also observed in the peripheral zone (Fig. 7C-D′). These data indicate that *Fgf8* plays a role in sustaining the survival of the tubule progenitors present in S-shaped bodies at early stages of nephrogenesis, as well as of progenitor cells in the peripheral zone of the developing kidney.

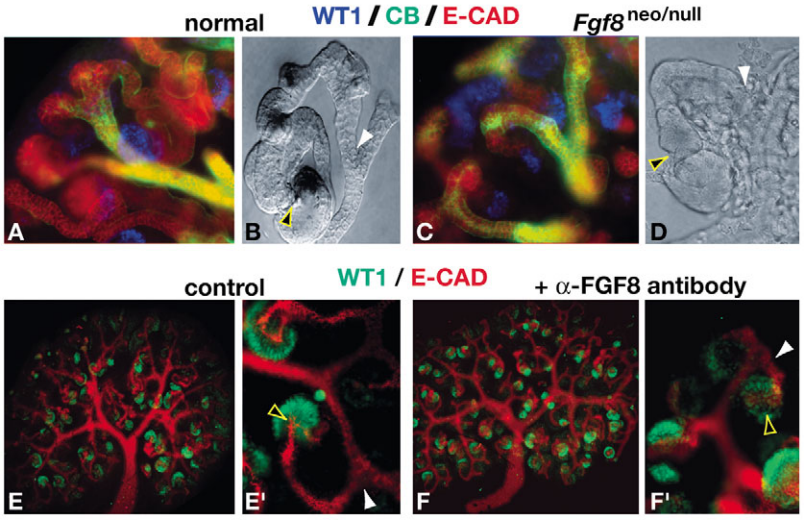

Fig. 6. Nephrons are truncated in severe *Fgf8* hypomorphs and in cultures of wild-type kidney explants treated with anti-FGF8 antibody. (A,C) Immunofluorescence assays in vibratome sections of E18.5 kidneys from normal and severe *Fgf8* hypomorphs. Podocytes are marked by staining for WT1 (blue). Collecting ducts are marked by staining for both E-CAD (red) and Calbindin (green), and thus appear yellow. The tubular portion of the nephron is marked by staining for E-CAD only. Note that the cortex of the hypomorphic kidney lacks the abundant, red tubular structures present in the normal kidney. (B,D) Representative nephrons isolated from E18.5 kidneys better reveal the lengths of the tubules. (E,F) Immunofluorescence assays for E-CAD (red) to identify epithelia, and WT1 (green) to identify podocyte progenitors of (E) control and (F) anti-FGF8 antibody-treated cultures of wild-type kidney explants. (E′,F′) Higher magnification views. The black arrowheads outlined in yellow indicate the proximal end of a tubule, where it is connected to the renal corpuscle. The white arrowheads indicate the nearest branch point of the collecting duct to which the tubule is connected. Note the decrease in tubule length in the nephrons from the *Fgf8* severe hypomorph kidney and the anti-FGF8 antibody-treated cultures.

Discussion

Using an allelic series of *Fgf8* mutants, we have investigated the role of FGF8 in kidney development. We found that complete inactivation of *Fgf8* in the metanephric mesenchyme results in the formation of kidneys that are severely malformed at birth, lacking both the outer medullary and cortical regions, and thus are devoid of nephrons and most of the collecting duct system. Our analysis of *Fgf8*-MM-KO kidneys at early stages of development indicated that this final phenotype is the consequence of two defects: a failure of nascent nephrons to progress to the S-shaped body stage and death of nephron progenitors in the peripheral zone. These defects are attributable, at least in part, to a lack of *Wnt4* and *Lim1* expression in the absence of FGF8. Using a different *cre* transgene to eliminate *Fgf8* function, Perantoni et al. (Perantoni et al., 2005) came to similar conclusions about FGF8 function in kidney development. In hypomorphic mutants, *Fgf8* expression is severely reduced but sufficient to allow nephrogenesis to proceed. However, cells in the tubular portion of the developing nephrons die, resulting in severe truncation of the nephrons. Thus, analysis of *Fgf8* null and hypomorphic embryonic kidneys in which FGF8 signaling is either absent or severely reduced has enabled us to demonstrate roles for FGF8 in the regulation of gene expression and in cell survival essential for nephron formation.

Fgf8 is required at early stages of nephrogenesis

Nephron formation is initiated in the absence of FGF8, as evidenced by the finding that renal vesicles, marked by *Wt1*, PAX2 and non-functional *Fgf8* expression, are present at E13.5 and E14.5. However, we detected neither *Wnt4* nor *Lim1* RNA, suggesting that FGF8 is required to induce and/or maintain expression of these genes. Alternatively, FGF8 may affect *Wnt4* and *Lim1* expression less directly; for example, by

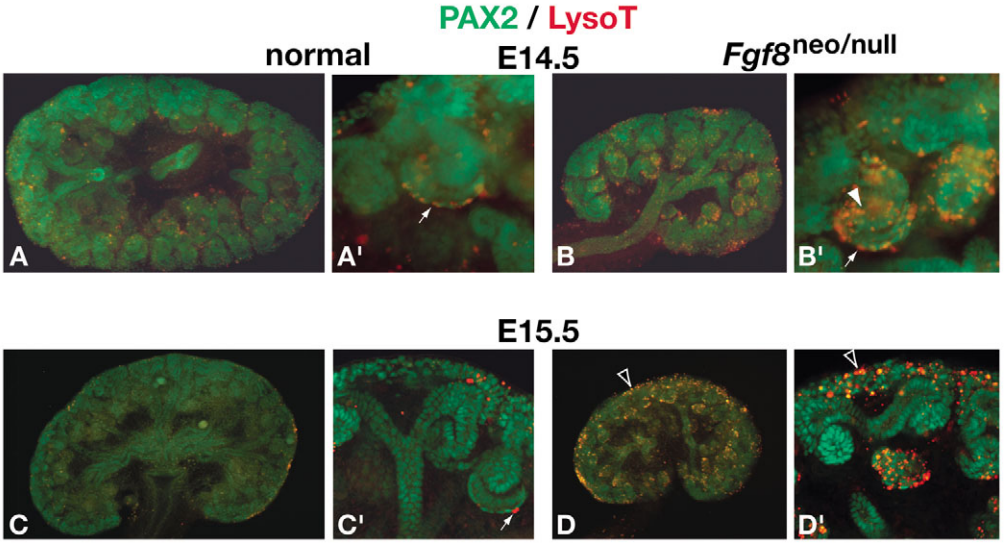

Fig. 7. Cells within S-shaped bodies die in *Fgf8* hypomorphs. (A-D) Immunofluorescence assay for PAX2 (green), to identify the developing nephrons and collecting ducts, and LysoTracker (LysoT, red) staining to identify regions containing dying cells in vibratome sections of normal and *Fgf8* severe hypomorph kidneys at the stages indicated. (A′-D′) Higher magnification views. Arrows indicate regions where Bowman's capsule progenitors are dying in normal and mutant kidneys; white arrowhead indicates regions where tubule progenitors are dying in the mutant kidney; open arrowheads point to regions in the peripheral zone of the mutant kidney where cells are dying.

ensuring that cells in the nascent nephrons are competent to express these genes. In either case, the data suggest a model whereby FGF8 produced in the nascent nephron acts in an autocrine fashion in a genetic pathway(s) upstream of *Wnt4* and *Lim1*. Consistent with this model, nascent nephrons in kidneys that lack *Wnt4* (Perantoni et al., 2005) or *Lim1* (Kobayashi et al., 2005) function still express *Fgf8*. Because *Lim1* expression depends on *Wnt4* function (Kobayashi et al., 2005), the lack of *Lim1* expression and the consequent arrest of nephrogenesis in *Fgf8*-MM-KO kidneys might be caused entirely by the lack of *Wnt4* expression. However, this seems unlikely because we found that a source of WNT signals (dorsal spinal cord) did not rescue nephrogenesis in cultures of *Fgf8*-MM-KO metanephric mesenchyme. Therefore, the effect of FGF8 on *Lim1* expression is not solely due to its effect on *Wnt4* expression. Instead, FGF8 may act in conjunction with WNT4 to regulate *Lim1* expression.

The lack of *Lim1* expression readily explains the failure of the nascent nephrons in *Fgf8*-MM-KO mutant kidneys to progress to the S-shaped body stage, as the same phenotype is observed when *Lim1* is inactivated in the metanephric mesenchyme (Kobayashi et al., 2005). What is more puzzling is why renal vesicles are present in the apparent absence of *Wnt4* expression in *Fgf8*-MM-KO mutant kidneys, as *Wnt4* is thought to be required for nephron progenitors to undergo a mesenchymal to epithelial transition, i.e. to form renal vesicles (Stark et al., 1994; Kispert et al., 1998). One possible explanation is that *Fgf8* function is not required to induce *Wnt4* expression, but only to upregulate/maintain it. If so, then there may be transient *Wnt4* expression in the *Fgf8*-MM-KO kidneys that we did not detect, but which is sufficient to allow renal vesicles to form. Alternatively, it may be that differences in genetic background between the *Wnt4* null and the *Fgf8*-MM-KO mutant mice may influence the penetrance of the block to renal vesicle formation caused by the absence of *Wnt4*. In this context, it is interesting to note that a small number of S-shaped bodies are present in *Wnt4* null kidneys at E14.5 (Kobayashi et al., 2005). This indicates that at least some nephron progenitors do undergo a mesenchymal to epithelial transition in the *Wnt4* mutants, perhaps in response to other WNT signals. Limited nephrogenesis can subsequently occur in *Wnt4* null kidneys (Kobayashi et al., 2005; Perantoni et al., 2005), presumably because FGF8 is still produced and available to induce *Lim1*. By contrast, neither S-shaped bodies nor nephrons can form in *Fgf8*-MM-KO kidneys because FGF8 is not available to induce *Lim1* expression.

Fgf8 is required for cell survival in the developing kidney

By examining animals carrying the $Fgf8^{neo}$ hypomorphic allele, in which sufficient FGF8 is produced to support the formation of S-shaped bodies, we were able to uncover a function for FGF8 at later stages of nephrogenesis. We found that a substantial number of tubule precursor cells in the S-shaped bodies of $Fgf8^{neo/null}$ and $Fgf8^{neo/neo}$ kidneys die (Fig. 7, and data not shown). We therefore conclude that FGF8 signaling is required within S-shaped bodies for cell survival, thus providing an explanation for our finding that nephron tubule length is shorter than normal in $Fgf8^{neo/neo}$ kidneys, which produce less FGF8 than normal, and is dramatically reduced in $Fgf8^{neo/null}$ kidneys, which produce even less FGF8.

FGF8 signaling is also required for the survival of cells in the peripheral zone, as we detected abnormal cell death in *Fgf8*-MM-KO kidneys as early as E12.5 (data not shown), presumably accounting for the reduced size of the null mutant kidneys at early stages. At E14.5, a large number of cells in this region are dying, although both PAX2- and *Foxd1*-expressing peripheral cell populations are still present. However, by E16.5, the PAX2-positive nephron progenitor cell population is absent, presumably because it is eliminated by cell death at earlier stages. By contrast, *Foxd1*-expressing stroma progenitors are still detected at E16.5. However, even if they continue to remain viable, these cells never form SMA-positive stroma because the entire outer medullary and cortical regions of the mutant kidneys, where SMA-positive cells normally reside, fail to develop in *Fgf8*-MM-KO kidneys. The progressive loss of nephron progenitors, which produce the GDNF required for collecting duct branching, may also account for the reduction in the number of collecting duct tips that we observed in *Fgf8*-MM-KO kidneys at all stages analyzed (see Table S2 in the supplementary material). Thus all of the defects that we observed in the neonatal *Fgf8*-MM-KO kidneys can be explained by the requirement for FGF8, both for gene expression essential for nephrogenesis and for the survival of nephron progenitors in the peripheral zone.

Because kidney development depends on reciprocal interactions between its tissue components, it is difficult to determine whether a specific defect observed in mutant kidneys reflects the primary function of the mutated gene. This is especially true when the gene in question encodes a secreted protein such as FGF8, which could act locally or at a distance to exert its effects. Thus, it is unclear whether the death of nephron progenitors observed in *Fgf8*-MM-KO kidneys is directly or indirectly due to the lack of FGF8. If it is a direct effect, then the FGF8 signal produced in nascent nephrons is probably transduced by FGFR1, the only FGF receptor gene abundantly expressed in that zone (Stark et al., 1991; Peters et al., 1992; Peters et al., 1993; Chi et al., 2004) (Fig. 1K-K″; see also Fig. S1 in the supplementary material). Moreover, the effect presumably occurs over a relatively long distance, because at the stage when there is extensive cell death in the *Fgf8*-MM-KO peripheral zone *Fgf8* expression in normal embryos appears to be restricted to the nascent nephrons.

In support of a direct effect, previous studies have shown that FGF signaling can prevent cell death in the metanephric mesenchyme. Thus, addition of FGF2 to metanephric mesenchyme isolated at E11.5 and cultured in the absence of the ureteric bud prevents the cell death that is normally observed in such cultures, although it does not induce nephrogenesis (Perantoni et al., 1995; Barasch et al., 1997; Dudley et al., 1999). We have found that FGF8 can likewise sustain cell survival and proliferation, but is not sufficient to induce the formation of nascent nephrons in isolated wild-type metanephric mesenchyme (see Fig. S2 in the supplementary material). Because no kidney defect has been reported in *Fgf2* null mice (Dono, 1998; Ortega et al., 1998), whereas we show that FGF8 is required in vivo for survival of nephron progenitor cells, it appears that the effects of FGF2 in vitro may in fact be mimicking the survival function of FGF8 in normal kidney development.

Another secreted factor, BMP7, is capable of supporting the survival of isolated metanephric mesenchyme without inducing nephrogenesis, and BMP7 has been shown to act synergistically with FGF2 in that assay (Dudley et al., 1999). During kidney development, *Bmp7* is normally expressed in the peripheral zone, in the developing nephron at early stages, and also in the branching collecting ducts (Godin et al., 1998). Although the phenotype of *Bmp7* null kidneys appears to be less severe than that of *Fgf8*-MM-KO kidneys (Dudley et al., 1995; Luo et al., 1995), *Bmp7* null and *Fgf8*-MM-KO kidneys both display abnormal cell death in the peripheral zone starting at E12.5-E13.5, and by E16.5 the nephron progenitors in the peripheral zone are eliminated (Luo et al., 1995; Dudley and Robertson, 1997). These observations raise the possibility that, in vivo, BMP7 and FGF8 cooperate to maintain progenitor cell populations in the peripheral zone.

Alternatively, cell death in the peripheral zone caused by lack of *Fgf8* function may be due to the observed absence of *Wnt4* expression in nascent nephrons. Support for this suggestion comes from a recent observation that there is extensive death in the peripheral zone in *Wnt4* mutant kidneys at E13.5 and E14.5 (S. Vainio and P. Itäranta, personal communication), similar to that observed in *Fgf8*-MM-KO kidneys. These findings are consistent with the possibility that the role of FGF8 in cell survival in the peripheral zone is to induce and/or maintain the expression of *Wnt4* in nascent nephrons, and that WNT4 alone, or acting in conjunction with FGF8 or some other factor, promotes the survival of nephron progenitors.

Concluding remarks

The concept that FGF8 is required for cell survival during organogenesis has been a recurrent theme in studies on *Fgf8* function in development. For instance, loss of *Fgf8* function in developing limb buds (Sun et al., 2002; Boulet et al., 2004), first branchial arch (Trumpp et al., 1999), forebrain (Storm et al., 2003), midbrain/anterior hindbrain (Chi et al., 2003), and, as shown here, developing kidneys, results in extensive cell death. In some of these cases, the regions in which dying cells are found in the absence of FGF8 signaling are distant from the site of FGF8 synthesis. A challenge for the future will be to determine precisely how FGF signaling functions to promote cell survival in these different developmental settings.

We thank Drs. J. Epstein and P. Soriano for kindly providing the *Pax3-cre* and R26R mouse lines. We are grateful to C. Ahn and P. Ghatpande for excellent technical assistance. We also thank T. Carroll, M. Lewandoski, A. McMahon, and S. Vainio for sharing unpublished data and helpful discussion, and to our laboratory colleagues for critical reading of the manuscript. C.C. was supported by a postdoctoral fellowship from Ministerio Español de Educación Cultura y Deporte. This work was supported by NIH PO1 HD39948 (E.N.M.) and RO1 grants HD42803 (E.N.M.), DK45218 and DK56365 (D.H.) and HD34380 (G.R.M.).

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/17/3847/DC1

References

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.

Bachmann, S., Metzger, R. and Bunnemann, B. (1990). Tamm-Horsfall protein-mRNA synthesis is localized to the thick ascending limb of Henle's loop in rat kidney. *Histochemistry* **94**, 517-523.

Baldwin, H. S., Shen, H. M., Yan, H. C., DeLisser, H. M., Chung, A., Mickanin, C., Trask, T., Kirschbaum, N. E., Newman, P. J., Albelda, S. M. et al. (1994). Platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31): alternatively spliced, functionally distinct isoforms expressed during mammalian cardiovascular development. *Development* **120**, 2539-2553.

Barasch, J., Qiao, J., McWilliams, G., Chen, D., Oliver, J. A. and Herzlinger, D. (1997). Ureteric bud cells secrete multiple factors, including bFGF, which rescue renal progenitors from apoptosis. *Am. J. Physiol.* **273**, F757-767.

Boulet, A. M., Moon, A. M., Arenkiel, B. R. and Capecchi, M. R. (2004). The roles of Fgf4 and Fgf8 in limb bud initiation and outgrowth. *Dev. Biol.* **273**, 361-372.

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.

Chang, C. P., McDill, B. W., Neilson, J. R., Joist, H. E., Epstein, J. A., Crabtree, G. R. and Chen, F. (2004). Calcineurin is required in urinary tract mesenchyme for the development of the pyeloureteral peristaltic machinery. *J. Clin. Invest.* **113**, 1051-1058.

Cheng, H. T., Miner, J. H., Lin, M., Tansey, M. G., Roth, K. and Kopan, R. (2003). Gamma-secretase activity is dispensable for mesenchyme-to-epithelium transition but required for podocyte and proximal tubule formation in developing mouse kidney. *Development* **130**, 5031-5042.

Chi, C. L., Martinez, S., Wurst, W. and Martin, G. R. (2003). The isthmic organizer signal FGF8 is required for cell survival in the prospective midbrain and cerebellum. *Development* **130**, 2633-2644.

Chi, L., Zhang, S., Lin, Y., Prunskaite-Hyyrylainen, R., Vuolteenaho, R., Itaranta, P. and Vainio, S. (2004). Sprouty proteins regulate ureteric branching by coordinating reciprocal epithelial Wnt11, mesenchymal Gdnf and stromal Fgf7 signalling during kidney development. *Development* **131**, 3345-3356.

Cho, E. A., Patterson, L. T., Brookhiser, W. T., Mah, S., Kintner, C. and Dressler, G. R. (1998). Differential expression and function of cadherin-6 during renal epithelium development. *Development* **125**, 803-812.

Coles, H. S., Burne, J. F. and Raff, M. C. (1993). Large-scale normal cell death in the developing rat kidney and its reduction by epidermal growth factor. *Development* **118**, 777-784.

Crossley, P. H. and Martin, G. R. (1995). The mouse Fgf8 gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. *Development* **121**, 439-451.

Dono, R., Texido, G., Dussel, R., Ehmke, H. and Zeller, R. (1998). Impaired cerebral cortex development and blood pressure regulation in FGF-2-deficient mice. *EMBO J.* **17**, 4213-4225.

Donovan, M. J., Natoli, T. A., Sainio, K., Amstutz, A., Jaenisch, R., Sariola, H. and Kreidberg, J. A. (1999). Initial differentiation of the metanephric mesenchyme is independent of WT1 and the ureteric bud. *Dev. Genet.* **24**, 252-262.

Doyonnas, R., Kershaw, D. B., Duhme, C., Merkens, H., Chelliah, S., Graf, T. and McNagny, K. M. (2001). Anuria, omphalocele, and perinatal lethality in mice lacking the CD34-related protein podocalyxin. *J. Exp. Med.* **194**, 13-27.

Dudley, A. T. and Robertson, E. J. (1997). Overlapping expression domains of bone morphogenetic protein family members potentially account for limited tissue defects in BMP7 deficient embryos. *Dev. Dyn.* **208**, 349-362.

Dudley, A. T., Lyons, K. M. and Robertson, E. J. (1995). A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye. *Genes Dev.* **9**, 2795-2807.

Dudley, A. T., Godin, R. E. and Robertson, E. J. (1999). Interaction between FGF and BMP signaling pathways regulates development of metanephric mesenchyme. *Genes Dev.* **13**, 1601-1613.

Godin, R. E., Takaesu, N. T., Robertson, E. J. and Dudley, A. T. (1998). Regulation of BMP7 expression during kidney development. *Development* **125**, 3473-3482.

Hatini, V., Huh, S. O., Herzlinger, D., Soares, V. C. and Lai, E. (1996). Essential role of stromal mesenchyme in kidney morphogenesis revealed by targeted disruption of Winged Helix transcription factor BF-2. *Genes Dev.* **10**, 1467-1478.

Hebert, S. C., Mount, D. B. and Gamba, G. (2004). Molecular physiology of cation-coupled Cl- cotransport: the SLC12 family. *Pflüger's Arch.* **447**, 580-593.

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.

Koseki, C., Herzlinger, D. and al-Awqati, Q. (1992). Apoptosis in metanephric development. *J. Cell Biol.* **119**, 1327-1333.

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, J., Chen, F. and Epstein, J. A. (2000). Neural crest expression of Cre recombinase directed by the proximal Pax3 promoter in transgenic mice. *Genesis* **26**, 162-164.

Liu, L., Dunn, S. T., Christakos, S., Hanson-Painton, O. and Bourdeau, J. E. (1993). Calbindin-D28k gene expression in the developing mouse kidney. *Kidney Int.* **44**, 322-330.

Luo, G., Hofmann, C., Bronckers, A. L., Sohocki, M., Bradley, A. and Karsenty, G. (1995). BMP-7 is an inducer of nephrogenesis, and is also required for eye development and skeletal patterning. *Genes Dev.* **9**, 2808-2820.

Mahmood, R., Bresnick, J., Hornbruch, A., Mahony, C., Morton, N., Colquhoun, K., Martin, P., Lumsden, A., Dickson, C. and Mason, I. (1995). A role for FGF-8 in the initiation and maintenance of vertebrate limb bud outgrowth. *Curr. Biol.* **5**, 797-806.

Meyers, E. N., Lewandoski, M. and Martin, G. R. (1998). An Fgf8 mutant allelic series generated by Cre- and Flp-mediated recombination. *Nat. Genet.* **18**, 136-141.

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.

Murer, H., Forster, I. and Biber, J. (2004). The sodium phosphate cotransporter family SLC34. *Pflüger's Arch.* **447**, 763-767.

Nakai, S., Sugitani, Y., Sato, H., Ito, S., Miura, Y., Ogawa, M., Nishi, M., Jishage, K., Minowa, O. and Noda, T. (2003). Crucial roles of Brn1 in distal tubule formation and function in mouse kidney. *Development* **130**, 4751-4759.

Novak, A., Guo, C., Yang, W., Nagy, A. and Lobe, C. G. (2000). Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. *Genesis* **28**, 147-155.

Ohuchi, H., Hori, Y., Yamasaki, M., Harada, H., Sekine, K., Kato, S. and Itoh, N. (2000). FGF10 acts as a major ligand for FGF receptor 2 IIIb in mouse multi-organ development. *Biochem. Biophys. Res. Commun.* **277**, 643-649.

Ortega, S., Ittmann, M., Tsang, S. H., Ehrlich, M. and Basilico, C. (1998). Neuronal defects and delayed wound healing in mice lacking fibroblast growth factor 2. *Proc. Natl. Acad. Sci. USA* **95**, 5672-5677.

Perantoni, A. O., Dove, L. F. and Karavanova, I. (1995). Basic fibroblast growth factor can mediate the early inductive events in renal development. *Proc. Natl. Acad. Sci. USA* **92**, 4696-4700.

Perantoni, A. O., Timofeeva, O., Naillat, F., Richman, C., Pajni-Underwood, S., Wilson, C., Vainio, S., Dove, L. F. and Lewandoski, M. (2005). Inactivation of Fgf8 in early mesoderm reveals an essential role in nephron development. *Development* **132**, 3859-3871.

Peters, K. G., Werner, S., Chen, G. and Williams, L. T. (1992). Two FGF receptor genes are differentially expressed in epithelial and mesenchymal tissues during limb formation and organogenesis in the mouse. *Development* **114**, 233-243.

Peters, K., Ornitz, D., Werner, S. and Williams, L. (1993). Unique expression pattern of the FGF receptor 3 gene during mouse organogenesis. *Dev. Biol.* **155**, 423-430.

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.

Qiao, J., Cohen, D. and Herzlinger, D. (1995). The metanephric blastema differentiates into collecting system and nephron epithelia in vitro. *Development* **121**, 3207-3214.

Qiao, J., Uzzo, R., Obara-Ishihara, T., Degenstein, L., Fuchs, E. and Herzlinger, D. (1999). FGF-7 modulates ureteric bud growth and nephron number in the developing kidney. *Development* **126**, 547-554.

Revest, J. M., Spencer-Dene, B., Kerr, K., De Moerlooze, L., Rosewell, I. and Dickson, C. (2001). Fibroblast growth factor receptor 2-IIIb acts upstream of Shh and Fgf4 and is required for limb bud maintenance but not for the induction of Fgf8, Fgf10, Msx1, or Bmp4. *Dev. Biol.* **231**, 47-62.

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.

Saxen, L. (1987). *Organogenesis of the Kidney*. Cambridge: Cambridge University Press.

Schaefer, K. S., Doughman, Y. Q., Fisher, S. A. and Watanabe, M. (2004). Dynamic patterns of apoptosis in the developing chicken heart. *Dev. Dyn.* **229**, 489-499.

Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* **21**, 70-71.

Stark, K. L., McMahon, J. A. and McMahon, A. P. (1991). FGFR-4, a new member of the fibroblast growth factor receptor family, expressed in the definitive endoderm and skeletal muscle lineages of the mouse. *Development* **113**, 641-651.

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.

Storm, E. E., Rubenstein, J. L. and Martin, G. R. (2003). Dosage of Fgf8 determines whether cell survival is positively or negatively regulated in the developing forebrain. *Proc. Natl. Acad. Sci. USA* **100**, 1757-1762.

Sun, X., Meyers, E. N., Lewandoski, M. and Martin, G. R. (1999). Targeted disruption of Fgf8 causes failure of cell migration in the gastrulating mouse embryo. *Genes Dev.* **13**, 1834-1846.

Sun, X., Mariani, F. V. and Martin, G. R. (2002). Functions of FGF signalling from the apical ectodermal ridge in limb development. *Nature* **418**, 501-508.

Trumpp, A., Depew, M. J., Rubenstein, J. L., Bishop, J. M. and Martin, G. R. (1999). Cre-mediated gene inactivation demonstrates that FGF8 is required for cell survival and patterning of the first branchial arch. *Genes Dev.* **13**, 3136-3148.

Wang, P., Pereira, F. A., Beasley, D. and Zheng, H. (2003). Presenilins are required for the formation of comma- and S-shaped bodies during nephrogenesis. *Development* **130**, 5019-5029.

Zhao, H., Kegg, H., Grady, S., Truong, H. T., Robinson, M. L., Baum, M. and Bates, C. M. (2004). Role of fibroblast growth factor receptors 1 and 2 in the ureteric bud. *Dev. Biol.* **276**, 403-415.

Zucker, R. M., Hunter, E. S., 3rd and Rogers, J. M. (1999). Apoptosis and morphology in mouse embryos by confocal laser scanning microscopy. *Methods* **18**, 473-480.