INTRODUCTION

Kidney development in the mouse begins at 11.5 d.p.c. (days postcoitum) and depends on reciprocal inductive interactions between the ureteric bud epithelium and metanephric mesenchymal cells. Branching morphogenesis of the renal collecting system is dependent on inductive signals from the surrounding metanephric mesenchyme (Grobstein, 1955). Simultaneously, the tips of the ureteric bud induce nephrogenic mesenchymal cells to condense and undergo a mesenchymal-to-epithelial transformation forming glomerular and tubular epithelial cells (podocytes). To determine the function of Pod1 in vivo, we have generated a lacZ-expressing null Pod1 allele. Null mutant mice are born but die in the perinatal period with severely hypoplastic lungs and kidneys that lack alveoli and mature glomeruli. Although Pod1 is exclusively expressed in the mesenchyme and podocytes, major defects are observed in the adjacent epithelia and include abnormalities in epithelial differentiation and branching morphogenesis.

Pod1 therefore appears to be essential for regulating properties of the mesenchyme that are critically important for lung and kidney morphogenesis. Defects specific to later specialized cell types where Pod1 is expressed, such as the podocytes, were also observed, suggesting that this transcription factor may play multiple roles in kidney morphogenesis.

Key words: Podocyte, Type II pneumocyte, Alveoli, Branching morphogenesis, Kidney, Mouse, Lung

SUMMARY

Epithelial-mesenchymal interactions are required for the development of all solid organs but few molecular mechanisms that underlie these interactions have been identified. Pod1 is a basic-helix-loop-helix (bHLH) transcription factor that is highly expressed in the mesenchyme of developing organs that include the lung, kidney, gut and heart and in glomerular visceral epithelial cells (podocytes). To determine the function of Pod1 in vivo, we have generated a lacZ-expressing null Pod1 allele. Null mutant mice are born but die in the perinatal period with severely hypoplastic lungs and kidneys that lack alveoli and mature glomeruli. Although Pod1 is exclusively expressed in the mesenchyme and podocytes, major defects are observed in the adjacent epithelia and include abnormalities in epithelial differentiation and branching morphogenesis.

Pod1 therefore appears to be essential for regulating properties of the mesenchyme that are critically important for lung and kidney morphogenesis. Defects specific to later specialized cell types where Pod1 is expressed, such as the podocytes, were also observed, suggesting that this transcription factor may play multiple roles in kidney morphogenesis.

Key words: Podocyte, Type II pneumocyte, Alveoli, Branching morphogenesis, Kidney, Mouse, Lung

The basic-helix-loop-helix protein Pod1 is critically important for kidney and lung organogenesis

Susan E. Quaggin1,2,*, Lois Schwartz1, Shiyong Cui1, Peter Igarashi3, Julie Deimling4, Martin Post4 and Janet Rossant1

1Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Ave., Toronto, Ontario, Canada M5G 1X5
2Division of Nephrology, St. Michael’s Hospital, 30 Bond St., Toronto, Ontario, Canada
3Yale University School of Medicine, 333 Cedar St., New Haven, CT, USA
4Department of Developmental Lung Biology, Hospital for Sick Children, 555 University Ave., Toronto, Ontario, Canada

*Author for correspondence (e-mail: quaggin@mshri.on.ca)

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INTRODUCTION

Kidney development in the mouse begins at 11.5 d.p.c. (days postcoitum) and depends on reciprocal inductive interactions between the ureteric bud epithelium and metanephric mesenchymal cells. Branching morphogenesis of the renal collecting system is dependent on inductive signals from the surrounding metanephric mesenchyme (Grobstein, 1955). Simultaneously, the tips of the ureteric bud induce nephrogenic mesenchymal cells to condense and undergo a mesenchymal-to-epithelial transformation forming glomerular and tubular epithelial cells of the nephron (Saxen, 1987). At the molecular level, a number of soluble factors have been identified that are important for these interactions including hepatocyte growth factor, transforming growth factor β (Stuart et al., 1995), bone morphogenetic proteins (Dudley et al., 1995; Luo et al., 1995), members of the Wnt family of signalling molecules (Stark et al., 1994) and extracellular matrix components such as α8 integrin (Stuart et al., 1995; Thesleff et al., 1995; Muller et al., 1997; Lechner and Dressler, 1997). During nephrogenesis, specific transcription factors must be involved in both upstream regulation of intercellular signalling and in downstream responses. A number of transcription factors have been identified that are important for nephrogenesis and include: WT1, Pax2 and BF2. WT1 is a zinc-finger-containing transcription factor that was identified as the Wilms tumour suppressor gene. It is expressed in metanephrogenic mesenchyme and in induced mesenchyme as well as in podocyte precursors in S-shaped bodies and capillary loop-stage glomeruli (Mundlos et al., 1993). In the absence of WT1 activity, the metanephric mesenchyme cannot respond to inductive signals from the ureteric bud and no kidneys are formed (Kreidberg et al., 1993). Pax2 has a paired-domain and is expressed in metanephric mesenchyme, mesenchymal condensates and comma-shaped bodies. Its expression decreases in S-shaped bodies specifically in podocyte precursors and is absent in mature nephrons. Mutant Pax2 mice do not form kidneys, mesonephric tubules or genitourinary tracts most likely due to defects in Wolffian duct formation (Torres et al., 1995). BF2 is a member of the winged helix family of transcription factors and is expressed in mesenchymal cells that will become stromal cells. Mice that lack BF2 activity die within 24 hours of birth with small and fused kidneys. Although mesenchymal condensates form in BF2 null mice, they fail to epithelialize and thus no nephrons are formed (Hatini et al., 1996).
Epithelial-mesenchymal interactions are also essential for lung development. In the mouse, lung development begins at 9.5 d.p.c. when the lung buds grow out from foregut endoderm and invade the surrounding mesenchyme. Branching morphogenesis occurs during the pseudoglandular stage (9.5–16 d.p.c.), followed by dilation of terminal lung buds during the canalicular stage (16–17 d.p.c.) and thinning of the mesenchyme and formation of blood vessels in apposition to the air spaces during the saccular stage (17 d.p.c. to birth). Alveogenesis occurs postnatally from the 5th to 30th day. Differentiation of airway epithelium, including the formation of type II pneumocytes, occurs from 14.2 d.p.c. onwards (Ten Have-Opbroek, 1991). Autocrine and paracrine signalling between the mesenchyme and epithelium are required for branching morphogenesis, terminal airway remodelling and differentiation of respiratory epithelial cells along a proximodistal axis (Minoo and King, 1994). Signalling molecules that have been identified to be important for lung development include TGF$\beta_1$, FGFs (Peters et al., 1994), bone morphogenetic proteins (Bellusci et al., 1996) and sonic hedgehog (Litingtung et al., 1998).

A number of transcription factors have been identified in the lung that are responsible for regulating some of these proteins and include: HNF3$\beta$, Ttf1 (thyroid transcription factor, Nkx2.1), Gli2 and Gli3. HNF3$\beta$ is expressed in foregut endoderm; mice that lack HNF3$\beta$ activity fail to develop lung buds. Ttf1 (Nkx2.1) contains a homeobox domain and a 17-amino acid domain that classifies it as a member of the Nkx family. In the developing human lung, Ttf1 is restricted to epithelial cells of the airways. After birth, it is expressed in type II pneumocytes in alveoli and in subsets of bronchiolar epithelial cells. Ttf1 is able to transactivate lung-cell-specific surfactant protein expression in vitro (Bohinski et al., 1994) and, in Ttf1 null mice, the lungs are replaced by sac-like structures and a rudimentary bronchial tree (Kimura et al., 1996). Gli2 and Gli3 are zinc-finger-containing transcription factors that are expressed in lung mesenchyme and are required for transduction of the Shh signal from the adjacent endoderm (Motoyama et al., 1998). Gli mutant mice demonstrate foregut malformations that include defects in lobar branch (secondary branch) formation.

Basic-helix-loop-helix (bHLH) proteins are transcriptional regulatory proteins that govern cell fate determination and differentiation in a variety of tissues (Edmondson and Olson, 1993; Murre et al., 1994). Members of the bHLH family include the myogenic protein, MyoD, and the related proteins, Myf-5, MRF4 and myogenin. These proteins have the capacity to activate muscle-specific genes when ectopically expressed in non-muscle cell types (Davis et al., 1987; Edmondson and Olson, 1989). Other bHLH proteins have been shown to be essential for neuronal differentiation (Ma et al., 1996), cardiac development (Riley et al., 1998; Srivastava et al., 1997), hematopoietic differentiation (Porcher et al., 1996) and placental development (Guillemot et al., 1994), but none have been shown to be essential during organogenesis of the kidney and lung.

Pod1 (Tcf21, capsulin, epicardin) is a bHLH protein that is most highly expressed in the mesenchyme of developing organs including the lung, kidney and gut. Additionally, Pod1 is expressed in the proepicardium, in the pericardium and in the podocyte, a highly specialized cell type in the renal glomerulus (Quaggin et al., 1998; Lu et al., 1998; Hidai et al., 1998). Antisense inhibition of Pod1 disrupted branching morphogenesis of the epithelium in murine embryonic kidney (Quaggin et al., 1998) and lung explants (M. P., unpublished data) suggesting a role for Pod1 in epithelial-mesenchymal interactions in these organs.

To understand the role of Pod1 during organogenesis, we created a $\text{lacZ}$-expressing null allele in murine embryonic stem cells.
Pod1 is required for kidney and lung morphogenesis

In this paper, we demonstrate that Pod1 activity is not required for specification of the mesenchyme or podocyte lineages although podocyte differentiation is arrested and conversion of condensing mesenchymal cells (cm) to epithelium of the nephron is delayed. Instead, major defects were observed in the adjacent epithelia and demonstrate an essential role for Pod1 in epithelial-mesenchymal interactions in kidney and lung morphogenesis that include epithelial differentiation and branching morphogenesis. Mutant lungs are hypoplastic, lack alveoli and type II pneumocytes and demonstrate severe defects in proximodistal differentiation of the airway epithelium. Mutant kidneys are markedly hypoplastic/dysplastic and lack mature podocytes and glomeruli.

**MATERIALS AND METHODS**

**Construction of Pod1 targeting vector**

Genomic clones for the murine Pod1 gene were isolated from a 129 SV genomic DNA library (Stratagene) by hybridization to a Pod1 cDNA EST clone (GenBank Accession no. W08124). Genomic fragments were subcloned and sequenced and a genomic map of Pod1 was constructed. Two exons were identified with splice donor/acceptor sites at the boundary of the single internal intron. The entire bHLH motif was contained within the first exon. The targeting vector was constructed by subcloning a 4.3 kb genomic fragment from the 5' flanking region of exon 1 into the HindIII site of the vector pSDKlacZpA (Puri et al., 1995) after HindIII sites had been added at both ends by PCR amplification. For the short homology arm, a 1.8 kb KpnI-SacI and a 1.3 SacI-KpnI fragment were subcloned into the KpnI site of the PNTloxP vector following addition of a KpnI site at the 5' end of the 1.8 kb fragment by PCR (Puri et al., 1995). The

**Fig. 2. Pod1 lacZ expression in the genitourinary tract.**

(A) lacZ expression is detected in the condensing metanephric mesenchyme (m) at 10.5 d.p.c. (B) At 13.5 d.p.c., lacZ expression is limited to condensing mesenchymal cells (cm) adjacent to ureteric bud epithelium (ub). Developing epithelium in the renal vesicle (v) and metanephrogenic mesenchyme (nm) does not express Pod1. (C) In 14.5 d.p.c. metanephroi, lacZ expression persists in condensing mesenchymal cells (cm) and is seen in a population of ‘spindle-shaped’ mesenchymal/interstitial cells (sp) at the developing cortical-medullary junction. In the developing nephron, lacZ is detected in podocyte precursors (p) but is absent from all other cell types. (D) At 8 weeks postnatal, lacZ expression defines a subset of interstitial cells (ic) around tubules (t) in the kidney. Magnification: ×80 (A-C), ×200 (D).

**Fig. 3. Genotype and gross phenotype of null mutant mice.**

(A) Southern blot analysis of Pod1 F2 generation. Genomic DNA was isolated from tails, digested with HindIII and hybridized with the 5' probe shown in Fig. 1. The wild-type allele measures 7.5 kb and the mutant allele measures 4.3 kb. (B) Northern blot analysis of mutant and wild-type littermates. Total RNA was isolated from lungs and kidneys of homozygous null mutant (−/−) and wild-type littermates (+/+). No Pod1 transcript was seen in homozygous animals. (C) Western blot analysis of F2 mice. Protein was isolated from lungs and kidneys of wild-type (+/+), heterozygous (+/−) and mutant (−/−) littermates. 2.5 μg of total protein was loaded on a 15% SDS Page gel and the presence of Pod1 protein was detected using a polyclonal Pod1 antibody. Full-length Pod1 protein was translated in vitro and 2.5 μg loaded as a control (tr). The Pod1 protein measures 22 kDa. No protein is present in nulls. (D) Genotype analysis of F2 litters. 228 mice were genotyped: (21%) were homozygous, (53%) heterozygous and (26%) wild type. (E) Lungs dissected at P0. Null mutant mice (−/−) have severely hypoplastic lungs compared with heterozygous (+/−) littermates. (F) Kidneys dissected at P0. Homozygous (−/−) kidneys are severely hypoplastic compared with heterozygous (+/−) littermate kidneys. Magnification: (E,F) ×10.
ES cell culture and generation of chimeras

The Pod1 vector was linearized at the Not I site and electroporated into R1 ES cells as described previously (Puri et al., 1995; Joyner, 1993). After selection with G418 and gancyclovir, resistant clones were picked and their DNAs analyzed by Southern analysis using a 3 ′ Pod1 genomic 1.5 kb Kpn I fragment probe outside the region of homology that recognized a 13 kb and a 17 kb Xho I fragment for the mutant and wild-type alleles, respectively. Homologous recombinant clones were verified by Southern blot analysis at the 5 ′ end using an internal genomic 1.5 kb Eco RI fragment as the probe. Three ES cell clones were correctly targeted at both 5 ′ and 3 ′ ends out of 1500 cell lines screened (targeting frequency = 0.13%).

Embryo manipulations and aggregation of the ES cell clones with ICR 8-cell embryos was carried out as described elsewhere (Joyner, 1993). One ES cell line generated chimera that gave germline transmission. Tail DNA was purified from F1 and F2 offspring and Southern analysis was performed to genotype the mice as described elsewhere (Puri et al., 1995). The internal 5 ′ Eco RI fragment was used as a probe to identify a 4.3 kb or a 7.5 kb Hind III fragment for the mutant and wild-type alleles, respectively.

X-gal staining, northern and western blot analysis

Embryos or embryonic tissues were dissected and fixed, washed in lacZ wash buffer and stained from 2 hours to overnight in X-gal stain as described elsewhere (Partanen et al., 1996). Tissues were paraaffin-embedded and 5 μm sections were cut.

Total RNA and protein were extracted from kidneys and lungs of mutant or wild-type littermates with Trizol according to manufacturer’s directions (Gibco, BRL). RNA gel and northern blot of mutant or wild-type littermates with Trizol according to embedded and 5 ′ as described elsewhere (Partanen et al., 1996). Tissues were paraffin-embedded and 5 ′ lacZ X-gal staining, northern and western blot analysis of mutant or wild-type littermates with Trizol according to modified and allowed to attach in the presence of 1 ml of DMEM-15 with 10% FBS (fetal bovine serum). Kidneys were then fixed in 4% paraformaldehyde for 1 hour and processed for in situ hybridization as described. Probes used for in situ were: BF2 (kind gift of Eseng Lai), c-Rel (kind gift of Frank Constatini), Pax2 cDNA (Dressler et al., 1990), Wnt 4 (EST # A1323302), CC-10 (Motoyama et al., 1998), SP-C probe (Motoyama et al., 1998), Shh probe (Pepicelli et al., 1997), FGF10 probe (Bellusci et al., 1997) and a BMP4 probe (Bellusci et al., 1996). Digoxigenin-labelled probes were prepared according to the Boehringer Mannheim protocol.

Whole kidneys were photographed using Nomarski optics; kidneys and lungs were also embedded in paraaffin and 10 μm sections cut.

Quantification of proximal airway cell types in the lung

Paraffin blocks containing the entire left lung lobe from a heterozygous or a homozygous pup at P0 were selected for serial sectioning. For light microscopy, tissues were photographed at a magnification of ×200 and enlarged to a final magnification of ×400 on the screen of a computer. 39 photos from mutant lung and 109 pictures from the heterozygous lung were taken at random for quantitative analysis. A coherent lattice square test system was used for point counting and intersection counting (Weibel, 1979). The distance between the heavy lines was 25 mm corresponding to approximately 62.5 μm. The number of proximal cell types (columnar, CC-10-positive cells) was counted on the screen of the computer in every secondary square. Profiles were taken in every fourth square of every sixth section from each slide. The volume density (Vv) was expressed as a percentage (100 mm³/mm³) by point counting and the surface density (Sv) expressed in mm²/mm³ by intersection counting. The volume density (Vv) of proximal cell types was calculated by the formula \( V_v = P_v / P_t \), in which \( P_v \) is the number of points and \( P_t \) is the number of points of reference area (Weibel, 1979). The surface density of proximal cell types was calculated by the formula \( S_v = 2 h / L_t \), in which \( h \) is the number of intersections and \( L_t \) is the length of test line (Weibel, 1979). Finally, the volume of the whole left lobe of each lung was also measured.

Histologic, immunohistochemical and in situ analysis

Embryonic tissues for histologic analysis were dissected and fixed in 10% formalin/phosphate-buffered saline, paraaffin-embedded and 4 μm-thick sections were cut. Sections were stained with hematoxylin and eosin, examined and photographed on a Leica microscope. The number of primordial tubules in the left lobe of the lung of 14.5 d.p.c. mutant and heterozygous littersates was determined by counting on histologic sections. 28 wild-type and 15 mutant lungs were included for histologic analysis. 28 wild-type and 15 mutant lungs were included for histologic, immunohistochemical and in situ analysis. These studies also provided more details on the identity of cells that express the centre of each kidney (that contained the ureteric bud) were stained with DBA as described above and the number of ureteric tips at the periphery of each kidney were counted. Samples were obtained from 6 mutant and 6 heterozygous littersates and statistical significance was determined using a paired Student’s t-test. Staining for the lectin DBA was also performed on whole embryonic kidneys as described (Quaggin et al., 1998).

Whole-mount in situ hybridization of embryonic kidneys was performed as described (Conlon and Rossant, 1992) with the following modifications. Metanephri from 14.5 d.p.c. embryos were dissected and plated on P.E.T. tissue culture inserts (Falcon #3104) and allowed to attach in the presence of 1 ml of DMEM-15 with 10% FBS (fetal bovine serum). Kidneys were then fixed in 4% paraformaldehyde for 1 hour and processed for in situ hybridization as described. Probes used for in situ were: BF2 (kind gift of Eseng Lai), c-Rel (kind gift of Frank Constatini), Pax2 cDNA (Dressler et al., 1990), Wnt 4 (EST # A1323302), CC-10 (Motoyama et al., 1998), SP-C probe (Motoyama et al., 1998), Shh probe (Pepicelli et al., 1997), FGF10 probe (Bellusci et al., 1997) and a BMP4 probe (Bellusci et al., 1996). Digoxigenin-labelled probes were prepared according to the Boehringer Mannheim protocol.

Whole kidneys were photographed using Nomarski optics; kidneys and lungs were also embedded in paraaffin and 10 μm sections cut.

RESULTS

Generation of a lacZ-expressing null allele for Pod1

The targeting construct for Pod1 was designed to replace the predicted initiation codon and the entire basic-helix-loop-helix domain (Fig. 1A) with an SDK-lacZ marker and neomycin cassette. A TK cassette was included for positive selection. Murine ES cell clones were screened by Southern blot analysis using an Xho I digest and 3 ′ and 5 ′ probes outside and inside the region of homology, respectively (Fig. 1B). Three independent ES cell clones were used to generate chimeric mice and one of the three lines provided germline transmission. A TK cassette was included for positive selection. Murine ES cell clones were screened by Southern blot analysis using an Xho I digest and 3 ′ and 5 ′ probes outside and inside the region of homology, respectively (Fig. 1B). Three independent ES cell clones were used to generate chimeric mice and one of the three lines provided germline transmission. A TK cassette was included for positive selection. Murine ES cell clones were screened by Southern blot analysis using an Xho I digest and 3 ′ and 5 ′ probes outside and inside the region of homology, respectively (Fig. 1B). Three independent ES cell clones were used to generate chimeric mice and one of the three lines provided germline transmission. A TK cassette was included for positive selection. Murine ES cell clones were screened by Southern blot analysis using an Xho I digest and 3 ′ and 5 ′ probes outside and inside the region of homology, respectively (Fig. 1B). Three independent ES cell clones were used to generate chimeric mice and one of the three lines provided germline transmission. A TK cassette was included for positive selection. Murine ES cell clones were screened by Southern blot analysis using an Xho I digest and 3 ′ and 5 ′ probes outside and inside the region of homology, respectively (Fig. 1B). Three independent ES cell clones were used to generate chimeric mice and one of the three lines provided germline transmission. A TK cassette was included for positive selection. Murine ES cell clones were screened by Southern blot analysis using an Xho I digest and 3 ′ and 5 ′ probes outside and inside the region of homology, respectively (Fig. 1B). Three independent ES cell clones were used to generate chimeric mice and one of the three lines provided germline transmission. A TK cassette was included for positive selection. Murine ES cell clones were screened by Southern blot analysis using an Xho I digest and 3 ′ and 5 ′ probes outside and inside the region of homology, respectively (Fig. 1B). Three independent ES cell clones were used to generate chimeric mice and one of the three lines provided germline transmission.
The earliest detectable expression of Pod1 is at 8.5 d.p.c. in mesodermal cells of the first and second branchial arches (not shown). In the developing genitourinary tract, Pod1 is expressed in the condensing metanephric mesenchyme that surrounds the invading ureteric bud on day 10.5 (Fig. 2A). By day 12.5 d.p.c., Pod1 is highly expressed in condensing metanephric mesenchyme (MM) but is absent from renal vesicles and metanephrogenic mesenchyme that has yet to undergo aggregation (Fig. 2B). In the 13.5 d.p.c. metanephros, Pod1 expression persists in condensing MM cells and is highly expressed in ‘spindle-shaped’ interstitial/mesenchymal cells at the developing cortical-medullary junction (Fig. 2C). In the developing nephron, Pod1 expression is first detected in podocyte precursors at the S-shaped body stage and in differentiating and mature podocytes (Fig. 2C) but is excluded from all other epithelial cell types in the nephron. In the adult kidney, Pod1 expression is seen in peritubular interstitial cells (Fig. 2D). Also, Pod1 is expressed in smooth muscle cell precursors and smooth muscle cells of the ureter (data not shown). In the lung, Pod1 expression is detected in the mesenchyme of the lung bud at E10.5 (data not shown) and in the mesenchyme of the lung at E12.5 and E14.5. In the adult lung, Pod1 is expressed in interstitial cells.

Pod1 is also expressed in the mesenchyme throughout the developing gastrointestinal (GI) tract from the esophagus to the rectum and is expressed in smooth muscle cells in the adult GI tract (not shown). In the heart, lacZ expression was seen in the proepicardium (future site of coronary vessels) and, in the pericardium, in cells surrounding the coronary arteries and in a subset of interstitial cells of the mature myocardium (data not shown).

Pod1 null mutant mice die on postnatal Day 0

Heterozygous Pod1/lacZ mice were mated and the F2 generation was analyzed. One-quarter of each litter died within minutes following birth after taking only a few agonal breaths. Genotype analysis of the newborn litters was performed using the 5’ internal probe and a shorter HindIII RFLP (Fig. 3A) and confirmed that the pups dying at the time of birth were homozygous null for Pod1. Absence of Pod1 mRNA was confirmed in null mutant animals by northern blot analysis (Fig. 3B). Western blot analysis was performed with a polyclonal antibody to Pod1 and confirmed the absence of Pod1 protein in null mutants (Fig. 3C). 228 mice were genotyped at P0. 120 (53%) were homozygous wild type and 48 (21%) were heterozygous, 60 (26%) were homozygous null for Pod1 (Fig. 3D). A number of mice that died in the perinatal period were eaten by the mother and were not able to be genotyped. However, the observed genotypes are not significantly different from expected Mendelian ratios by $\chi^2$ analysis ($P=0.61$).

Dissection of newborn null Pod1 pups demonstrated severely hypoplastic lungs (Fig. 3E) and hypoplastic kidneys (Fig. 3F) despite similar birthweights. Other features noted on gross dissection included abnormal surface vasculature of the kidney and lungs and hemopericardium. Dissections at earlier staged time points did not reveal any embryonic resorptions.

Pod1 is not required for mesenchyme formation but tubulogenesis is delayed in mutant kidneys

lacZ-expressing mesenchyme forms in mutant lungs and kidneys and demonstrates that the early Pod1-positive mesenchymal cell lineages are correctly specified (Fig. 4B,D). At 12.5 d.p.c., no differences are seen between null and wild-type kidneys (not shown). In the null 14.5 d.p.c. metanephros, lacZ-positive condensates are visible around the tips of the ureteric bud epithelium (Fig. 4F); thus, the mutant Pod1-positive mesenchyme is able to respond to induction and undergoes condensation. In fact, an increase in the quantity of Pod1-positive condensing mesenchyme (CM) with an increase in the number of cell layers of CM surrounding ureteric bud tips (Fig. 4H) is observed in null animals. In mutant 15.5 d.p.c. kidneys, up to 15 cell layers of CM can be identified around ureteric bud tips compared with two or three layers in wild-type littermates (Fig. 4G,H). Conversion of this CM into tubular structures of the nephron is delayed as no S-shaped bodies or capillary-loop glomeruli can be identified in 14.5 d.p.c. null metanephroi compared with wild type (Fig. 4C,D). Although the conversion of CM to epithelia of the nephron is delayed, this stage of nephrogenesis is not completely blocked as a reduced number of capillary loop glomeruli and nephrons can be found in mutant kidneys at P0.

Molecular markers of condensing metanephric mesenchyme, such as Pax2 and Wnt4, are expressed appropriately in the CM of mutant kidneys (Fig. 5C,D and data not shown). The winged-helix transcription factor, BF2, is a marker of the stromal cell lineage within the mesenchyme and is present in both wild-type and mutant kidneys (Fig. 5E-H). The nephrogenic mesenchyme, which is located at the periphery of the metanephros, contains stem cells that undergo induction and condensation to form nephrons. This mesenchymal cell population does not normally express Pod1 and appears unchanged in null metanephroi. In addition, we were unable to detect any difference in glial-derived neurotrophic (GDNF) expression. Furthermore, nephrogenesis is ongoing at the time of birth in both null and wild-type kidneys as evidenced by the presence of mesenchymal condensates (Fig. 5A,B). Thus, Pod1 appears to act downstream of these factors, mutations in which block earlier kidney development.

In contrast to the mesenchymal markers, expression of c-ret in the ureteric bud (UB)-derived epithelium is abnormal in mutants. Early in nephrogenesis, c-ret is expressed throughout the UB epithelium but at 13.5 d.p.c., its expression is restricted to UB tips at the periphery of the kidney (Fig. 5I). In nulls, c-ret expression is also seen in UB tips in the medulla of the kidney and extends along some UB branches (Fig. 5H).

Pod1 activity is required for terminal epithelial differentiation and branching morphogenesis

Although the conversion of CM to tubular epithelium is delayed in mutants, a reduced number of nephrons are formed. Thus, we also looked at later stages of tubular epithelial development in our mutants. During nephrogenesis, segmental tubular epithelial differentiation occurs along a proximal-to-distal axis; morphologic change characteristic of proximal tubular differentiation includes development of a brush border and is evident by 15 d.p.c. (Laitinen et al., 1987). In addition, they begin to express specific carbohydrates that are recognized by the lectin TP. The brush border of proximal tubular cells is necessary to allow reabsorption of ions (Na, K, Ca), glucose and other essential substances from the luminal (urinary) space back to the bloodstream. In mutant kidneys,
developing tubular epithelium is clearly visible and expresses Pax2 (Figs 5D, 6D); however, brush borders fail to develop and they do not express the lectin TP (Fig. 6F,I) demonstrating an arrest in segmental tubular differentiation.

In addition to the tubular defects, lack of Pod1 caused a clear defect in branching morphogenesis of the ureteric branches. Heterozygous and mutant kidneys were stained with the lectin DBA that specifically stains branches of the ureteric bud epithelium. Fig. 7 demonstrates a reduction in the number of branches at 14.5 d.p.c. that becomes more severe at 17.5 d.p.c.
Pod1 is required for kidney and lung morphogenesis

In addition to an overall reduction in the number of branches, a defect in the branch pattern is apparent (Fig. 7D,F) as ureteric branches are crowded together in mutants and UB tips can be identified in the medulla (Fig. 5J). The number of ureteric branch tips at the periphery of wild-type (n=6) and mutant kidneys (n=6) was reduced by 61% in nulls at P0 (P<0.001).

Pod1 activity is required for lung morphogenesis

In the lung, the airway epithelium develops as an outgrowth of foregut endoderm. The second order of branching gives rise to four pulmonary lobes on the right and one lobe on the left and is unaffected in Pod1 mutants. During the early pseudoglandular stage, (9.5-14.2 d.p.c.), tertiary branching occurs that gives rise to primordial tubules (segmental bronchi) lined by columnar epithelium. We performed serial dissections of heterozygous and mutant embryos from 10.5 to 14.5 d.p.c. By 14.5 d.p.c., a reduction in the total number of tubules was evident; the average number of primordial tubules in the left lobe of heterozygous lungs was 33 (n=28) compared to 14 in mutant lungs (n=15) and represents a 57% reduction (see Figs 4B, 7H). During the late pseudoglandular stage (14.2-16.6...
and found that the absolute number of proximal airway cells is versus distal cell types in mutant versus heterozygous lungs occur and the pups die. We quantified the number of proximal without air sacs or type II pneumocytes, air exchange cannot specifically in the development of type II pneumocytes. Without air sacs or type II pneumocytes, air exchange cannot occur and the pups die. We quantified the number of proximal versus distal cell types in mutant versus heterozygous lungs and found that the absolute number of proximal airway cells is greatly increased in the left lobe of mutant lungs (5.4x10^5) compared to heterozygous lungs (2.1x10^5) despite a severe reduction in total size of the lobe (1.96 mm^3 versus 5.40 mm^3) (see Table 1).

We also looked at molecular markers of lung development known to be involved in epithelial differentiation and branching morphogenesis and found a marked reduction in the level of BMP4 expression in the distal tips of branching airway epithelia of mutant lungs (Fig. 9). In contrast, we were unable to detect any differences in the level of expression of other markers including Shh, FGF7 and FGFI0 (Fig. 9 and data not shown).

Together, these defects are indicative of a failure of normal branching morphogenesis of the lung at both the early and late pseudoglandular stages, a process that requires a series of interactions between the underlying mesenchyme and the epithelium. In addition, the absolute increase in bronchiolar epithelium demonstrates a defect in cell fate specification/differentiation of the epithelium with disruption of normal ‘patterning’ of the airway epithelium.

Pod1 activity is also required for podocyte and glomerular differentiation

Glomeruli are the ‘filtering units’ of the kidney and are composed of podocyte (visceral epithelial cells), mesangial cells and endothelial cells. Podocytes are mesodermally derived cells that are largely responsible for maintaining the glomerular filtration barrier between the blood and urinary space (Mundel and Kriz, 1995). Pod1 is expressed in developing and mature podocytes but is absent from all other epithelial cell types in the nephron. Similar to the defects seen in the mesenchyme, lacZ-expressing podocyte precursors form and begin to differentiate in the mutant glomeruli. However, mutant podocytes fail to undergo terminal differentiation. In heterozygous littermates, podocytes flatten and develop characteristic structural features such as foot processes that line the glomerular basement membranes (GBM) of glomerular capillaries. In mutant glomeruli, podocytes remain columnar shaped and develop few rudimentary foot processes that do not line the GBM of capillaries. Intercellular junctions between podocytes appear to form normally (Fig. 10) (Reeves et al., 1978).

In Pod1 mutant kidneys, the total number of glomeruli is markedly reduced and fully differentiated glomeruli are absent. The most mature glomerular stage seen in the null kidneys represents the capillary loop stage (Abrahamson, 1991); mesangial cells are present as are endothelial cells, although the complexity of capillary looping is markedly reduced (only a single capillary loop can be identified within mutant glomeruli). These findings demonstrate a block in glomerular differentiation.

DISCUSSION

The molecular mechanisms that control differentiation of specific cell types and branching morphogenesis in the lung and kidney are incompletely understood. Tissue recombination experiments have shown a requirement of mesenchymal ‘factors’ to direct some of these processes in vitro. Pod1 is the first bHLH transcription factor to be identified in the developing lung and kidney. Although Pod1 is exclusively expressed in the mesenchyme and podocytes, major defects are observed in the adjacent epithelia of mutant mice. In the lung, Pod1 is required to correctly ‘pattern’ the proximodistal axis of airway epithelium and for normal branching to occur. In the kidney, Pod1 is required for conversion of CM to epithelium of the nephron, branching morphogenesis and terminal differentiation of tubular epithelium. We also observed defects in later specialized renal cell types such as the podocyte, where Pod1 is also expressed, that suggest Pod1 plays multiple roles in kidney morphogenesis. Pod1 therefore is essential for regulating properties of the mesenchyme that are critically important for several aspects of lung and kidney morphogenesis.

Pod1 mutants demonstrate defects in tubular epithelial differentiation and branching morphogenesis

In the kidney, mesenchymal cells are induced to condense by the ureteric bud epithelium and then undergo a mesenchymal-to-epithelial transformation. Pax2 is expressed in the earliest metanephric mesenchymal condensates and is required for nephron formation (Rothenpieler and Dressler, 1993). In addition, induction of the mesenchyme results in the expression of Wnt4 in the condensed mesenchymal cells that is necessary for nephron tubule formation (Stark et al., 1994). A stromally induced signal under the regulation of the winged-helix transcription factor BF2 is required for later events that include tubulogenesis (Hatini et al., 1996). In the absence of BF2, mesenchymal cells condense and express Wnt4 but the conversion to tubular epithelium of the nephron is delayed with few nephrons formed. Similar to BF2 mutants, Pod1 mutant metanephroi demonstrate an increase in the amount of CM surrounding ureteric bud tips and a delay in formation of nephrons (no S-shaped bodies can be identified at 14.5 d.p.c.
Pod1 is required for kidney and lung morphogenesis

**Pod1 is required for branching morphogenesis and proximodistal differentiation of the airway epithelium**

Epithelial-mesenchymal interactions are also essential for branching morphogenesis in the lung and Pod1 mutant mice demonstrate defects in both secondary and tertiary branching. The airway epithelium develops as an outgrowth of the foregut endoderm and is dependent upon HNF3β activity. Secondary branching gives rise to four pulmonary lobes on the right and one on the left. Signalling through the sonic hedgehog (Shh) pathway is essential for secondary branch formation as Shh and compound Gli mutants form only single lung lobes (Motoyama et al., 1998; Litingtung et al., 1998; Pepicelli et al., 1998). Interestingly, terminal airway formation and epithelial differentiation of the airways is undisturbed in Gli mutants and suggests that the genetic pathways required at different branching timepoints are distinct. FGF signalling also plays a role in secondary branching as shown by the complete block in branching morphogenesis in mice that overexpress a dominant-negative FGFRIB receptor in lung epithelia (Peters et al., 1994) and the presence of pulmonary agenesis in FGF 10 nulls. Although these initial stages of branching are unaffected in Pod1 mutant mice, defects become evident by 14.5 d.p.c. The total number of tertiary branches (segmental bronchi) is reduced as demonstrated by the reduced number of primordial tubules at 14.5 d.p.c. Terminal branching is also abnormal as demonstrated by the lack of acinar tubules and their derivatives (terminal air sacs, alveoli) in mutant lungs.

In contrast, bronchiolar tubules form in mutant lungs and express the marker CC-10 (Shannon, 1994; Minoo and King, 1994; Borok et al., 1998). In fact, the number of airways lined by bronchiolar epithilium is increased. Thus, it appears that some terminal branches are formed but that the epithelium lining these branches has adopted a new fate (i.e. bronchiolar rather than alveolar). Recently, FGF and BMP signalling have been identified as important components that are required to pattern the airway epithelium along a proximodistal axis. Proximal airways are lined by CC-10-expressing bronchiolar epithelium and terminal airways are lined by cuboidal and squamous epithelium that include type I and type II pneumocytes. When combined with distal lung mesenchyme, tracheal epithelial cells can be reprogrammed to become type II pneumocytes in vitro (Shannon, 1994). More recently, the same investigators have demonstrated that transdifferentiation of tracheal epithelium to a type II pneumocyte fate can occur in a mesenchyme-free environment in the presence of FGF7 and an unidentified factor(s) in charcoal-stripped bovine serum (Shannon et al., 1999). Weaver et al. (1999) have also demonstrated an essential role for BMP4 signalling in proximodistal airway differentiation. Transgenic mice that overexpress the BMP antagonist Noggin or a dominant negative BMP4 receptor (dnAlk6) in distal airway epithelium, demonstrate a marked reduction in the number of distal airway types and a coincident increase in the amount of proximal airway epithelium. BMP4 is normally expressed in the tips of terminal branches from 11.5 d.p.c. onwards and at lower levels in the adjacent mesenchyme. Weaver et al. (1999) hypothesize that high levels of BMP4 in distal airway epithelium are required to maintain cells in a relatively undifferentiated state and, once cells leave this area of high BMP4 expression, they differentiate into proximal CC-10-expressing cells.

In Pod1 mutant lungs, there is a disruption of airway differentiation along the proximodistal axis with an absolute increase in the number of proximal airway cell types despite a severe reduction in lung size. At the same time, there is a lack of type II pneumocytes. Although we have not been able to demonstrate a difference in FGF7 expression between wild-type and mutant lungs, there is a marked reduction in BMP4 expression at 13.5 d.p.c. (Fig. 9). Although Pod1 is not
expressed in the epithelium, these results suggest that Pod1-expressing mesenchyme is required for appropriate BMP4 expression in the adjacent epithelium. The expression pattern of Shh and FGF10 in mutant lungs demonstrates that distal cell types are present in nulls and that the decrease in BMP4 expression plays a direct role in generation of the lung phenotype.

Taken together, these results support the following model: in the absence of Pod1, perturbations in the mesenchyme lead to defects in the adjacent epithelia. These defects include an arrest in terminal epithelial differentiation (renal proximal

Fig. 8. Epithelial differentiation and branching morphogenesis of the airways are defective in mutant lungs. Light microscopy and in situ analysis of heterozygous (A,C,E,G,I) and homozygous (B,D,F,H,J) lungs at P0. (A) Heterozygous lungs have fully developed airways while (B) mutant lungs are much smaller and have dilated immature airways. (C) Mature distal epithelial cell types (ep) line the airways in heterozygous mice and the interstitial cells (ic) are tightly packed between the airways. In addition, a complex capillary (c) network is seen in close apposition to the airways. (D) Bronchial epithelium (br) lines the airways in mutant mice and the interstitial cells (ic) are loosely packed. The capillaries (c) are dilated and are not in direct apposition to airways. (E) A digoxigenin-labelled CC-10 probe stains bronchial epithelial cells (arrowhead) in heterozygous lungs. (F) The number of CC-10-positive tubules appears to be increased in mutant lungs. (G) A digoxigenin-labelled SP-C probe recognizes mature type II pneumocytes lining the airways of heterozygous lungs (arrowhead). (H) SP-C expression is seen only in the periphery of mutant lungs (arrowhead) demonstrating an absence of type II pneumocytes within the interior of mutant lungs. (I) SP-C-positive type II pneumocytes are seen scattered throughout mature airways of heterozygous lungs. (J) No SP-C-positive cells are seen in the interior of homozygous lungs. Magnification: ×20 (A,B), ×50 (C,D), ×25 (E-H), ×65 (IJ).

Fig. 9. Molecular markers of lung differentiation at 14.5 d.p.c. (A,C) A digoxigenin-labelled BMP4 probe stains the distal tips of branching airway epithelium (arrowheads) in wild-type lungs. (B,D) There is a marked reduction in BMP4 expression in mutant lungs. (E,F) Shh is expressed in both (E) wild-type and (F) mutant lungs. (G,H) FGF10 is expressed in the lung mesenchyme of both (G) wild-type and (H) null mice. Magnification: ×20 (A,B), ×40 (C,D) ×80 (E,F) ×200 (G,H).
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tubular epithelium), transdifferentiation of airway epithelium
(conversion of distal to proximal cell types) and a disruption
in branching morphogenesis in both lung and kidney. Presently, it is not known whether the epithelial defects are
due to the absence of mesenchymal molecules that are
transcriptionally regulated by Pod1 or due to an absence of a
subset of differentiated mesenchymal cells that are required to
produce these factors. In addition to the epithelial defects, we
observed a delay in conversion of Pod1-expressing CM to
nephric structures similar to that seen in BF2 mutants.
Although Pod1 is expressed early in lung buds and
metanephric mesenchyme, its role is not essential at these
timepoints as initial stages of lung and renal organogenesis
appear unaffected and various molecular markers are expressed
in mutant mice (Pax2, Wnt4, BF2). Thus, the molecular and
phenotypic analysis allow us to place Pod1 genetically
downstream of other known transcription factors important for
renal and lung organogenesis but upstream of BMP4 in
patterning the airway epithelium (Fig. 11).

Our data also demonstrate that Pod1 is required for terminal
differentiation of podocytes in the kidney. The observation that
glomerular differentiation is arrested at the capillary loop stage
reveals an essential role of the podocyte and/or factors that are
regulated by Pod1 to set up the glomerular filtration barrier and
functional architecture of the mature glomerulus. Disruptions
in the glomerular filtration barrier are clinically important as
they lead to proteinuria and morbidity (Smoyer and Mundel,
1998). Other factors that have been shown to be important for
glomerular development include Pax2 and the ligand PDGF-B
and its receptor, PDGF-βR. When Pax2 is ectopically
expressed in podocytes, mice demonstrate fusion of podocyte
foot processes and proteinuria (Dressler et al., 1993). However,
glomerular maturation is unaffected. In contrast, mutations in
the PDGF-βR receptor or its ligand result in glomeruli that lack
mesangial cells and have a poorly developed glomerular
capillary system although the podocytes appear to develop
normally (Lindahl et al., 1998; Soriano, 1994; Leveen et al.,
1994).

The present phenotype demonstrates definitively that
differentiated mesenchyme and/or mesenchymal factors
regulated by Pod1 play a critical role in epithelial-
mesenchymal interactions during kidney and lung

Table 1. Quantitative analysis of proximal cell types from
left lobe of the lung

<table>
<thead>
<tr>
<th></th>
<th>Heterozygous</th>
<th>Homozygous</th>
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<tbody>
<tr>
<td>Volume of left lobe of lung</td>
<td>5.4 mm³</td>
<td>1.96 mm³</td>
</tr>
<tr>
<td>Number of proximal cells</td>
<td>2.1×10⁵</td>
<td>5.4×10⁵</td>
</tr>
<tr>
<td>Volume density (Vv, %)</td>
<td>2.7 μm³/μm³</td>
<td>14.6 μm³/μm³</td>
</tr>
<tr>
<td>Surface density</td>
<td>4.8 μm²/μm³</td>
<td>17.3 μm²/μm³</td>
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Fig. 10. Podocyte and glomerular differentiation is arrested in mutant kidneys. (A) Fully differentiated glomeruli (g) and podocytes (p) are seen in heterozygous kidneys but (B) glomerular differentiation is blocked in mutant kidneys. The most mature glomerular stage that can be identified is the capillary-loop stage (g) as shown. Podocytes are present but remain columnar shaped. (C) Transmission electron micrograph of a heterozygous kidney demonstrates mature morphology of podocytes (p) that include well-developed foot processes (fp) in direct apposition to the glomerular basement membrane (arrowheads). Red blood cells (rbc) are visible within glomerular capillaries. (D) Mutant podocytes (p) remain columnar-shaped; no foot processes can be identified in this section. An intercellular junction (j) is seen. Magnification: ×200 (A,B), ×1300 (C,D).

Fig. 11. Pod1 acts upstream of BMP4 to regulate airway epithelial differentiation along the proximodistal axis. Model that demonstrates a requirement for Pod1 in lung mesenchyme (blue cells) for expression of BMP4 in the adjacent distal airway epithelium (pink cells). In the absence of Pod1, mesenchymal cells still form (blue cells) but BMP4 expression is severely reduced. As a result, distal airway epithelium adopts a ‘proximal’ fate to become CC-10-expressing, bronchiolar cells (green cells). P, proximal; D, distal.
morphogenesis from mid-gestation onwards and that the podocyte is required for glomerular maturation. The role of the mesenchyme in regulating terminal epithelial differentiation in these organs in vivo has not been previously reported. In addition, Pod1 is the first transcription factor identified that is required for correct patterning of the adjacent airway epithelium. Identification of the target genes regulated by Pod1 will provide insight into a variety of essential events that are required during organogenesis and allow us to dissect the molecular events that are essential for correct proximodistal patterning of the airway epithelium.

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