ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

DEVELOPMENTAL BIOLOGY

Developmental Biology 254 (2003) 262–276

www.elsevier.com/locate/ydbio

# *Pbx1* regulates nephrogenesis and ureteric branching in the developing kidney

Catherine A. Schnabel,[a] Robert E. Godin,[b] and Michael L. Cleary[a,*]

[a] Department of Pathology, Stanford University School of Medicine, Stanford, CA 94305-5342, USA

[b] Department of Molecular and Cellular Biology, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138, USA

Received for publication 20 February 2002, revised 28 October 2002, accepted 28 October 2002

**Abstract**

*Pbx1* encodes a TALE homeodomain transcription factor that regulates developmental gene expression in a variety of tissues. Loss-of-function studies have demonstrated a critical role for *Pbx1* in cellular proliferation and patterning and suggest its involvement in numerous regulatory pathways. In this study, examination of metanephric development in *Pbx1*$^{-/-}$ embryos was conducted to further elucidate *Pbx1*-dependent processes during organogenesis. Prior to death at E15.5, *Pbx1*$^{-/-}$ embryos displayed kidneys that were reduced in size, axially mispositioned, and in more severe cases, exhibited unilateral agenesis. Analysis with molecular markers revealed the effective induction of tubulogenic mesenchyme; however, *Pbx1*$^{-/-}$ kidneys contained fewer nephrons and were characterized by expanded regions of mesenchymal condensates in the nephrogenic zone. Despite the restricted expression of Pbx1 in metanephric mesenchyme, developing nephrons, and stroma, decreased branching and elongation of the ureter were also observed. Moreover, heterologous recombination studies with explant cultures verified that *Pbx1*$^{-/-}$ renal defects arose exclusively from mesenchymal dysfunction. Taken together, these data establish a role for *Pbx1* in mesenchymal–epithelial signaling and demonstrate that *Pbx1* is an essential regulator of mesenchymal function during renal morphogenesis.
© 2003 Elsevier Science (USA). All rights reserved.

*Keywords:* *Pbx1*; Homeodomain; Kidney; Epithelial–mesenchymal; Nephrogenesis; Stroma; Morphogenesis

## Introduction

Renal organogenesis is initiated when the ureteric bud sprouts from the posterior Wolffian duct and invades a caudal region of the intermediate mesoderm, the metanephric blastema. Ureteric induction establishes the stromal and nephrogenic populations within the blastema (Aufderheide et al., 1987) and promotes the survival and differentiation of the nephrogenic mesenchyme into nephrons (Grobstein, 1953, 1955; Saxen, 1987). In a reciprocal fashion, signals from the mesenchyme induce growth and branching of the ureter to ultimately form the renal collecting system (Grobstein, 1953, 1955; Saxen, 1987). Stromal mesenchyme which develops peripheral to the tubular population, and does not contribute to the formation of nephrons or collecting ducts, also provides important signals that regulate ureteric bud arborization and nephrogenesis (reviewed in Bard, 1996; Kuure et al., 2000). Therefore, the complex process of metanephric development relies on the integrated regulation of signals between the ureter, nephrogenic mesenchyme, and stroma.

Development of the metanephric kidney has served as a model system to establish the importance of inductive events during embryogenesis (Grobstein, 1953); however, the molecular mechanisms that coordinate renal morphogenesis remain poorly understood. Much of the current knowledge of metanephric regulators is based primarily on loss-of-function studies that have elucidated the roles of transcription factors, secreted factors and their receptors, signaling molecules, cell adhesion factors, and extracellular matrix components in kidney development (reviewed in Horster et al., 1999; Kuure et al.,

* Corresponding author. Fax: +1-650-498-6222.

*E-mail address:* michael.cleary@stanford.edu (M.L. Cleary).

0012-1606/03/$ – see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0012-1606(02)00038-6

2000). Continued identification of novel regulators, along with further characterization of molecular interactions and pathways, are fundamental to refining existing models of renal organogenesis.

*Pbx* genes (*Pbx1-4*) are the mammalian homologs of *Drosophila* extradenticle (Rauskolb et al., 1993) and encode a family of homeodomain transcription factors that participate in multiprotein complexes to regulate developmental gene expression (reviewed in Mann and Affolter, 1998). The prototypic *Pbx* family member, *Pbx1,* was originally identified as a chromosomal breakpoint product implicated in a subset of childhood leukemias (Kamps et al., 1990; Nourse et al., 1990). In comparison to its well-defined role as a transcription factor, the biological functions of *Pbx1* during embryogenesis have not been extensively examined. Pbx1 is expressed in a variety of tissues during embryonic development where it is predominantly associated with condensing mesenchyme and is detected at sites of mesenchymal–epithelial interaction (Schnabel et al., 2001). Recent studies demonstrate a critical role for *Pbx1* in developmental regulation whose loss-of-function results in embryonic lethality and multiple tissue abnormalities (Selleri et al., 2001).

In the present study, we define Pbx1 expression domains during renal development and demonstrate that loss of *Pbx1* leads to the absence or abnormal formation of metanephric kidneys. Although Pbx1 is primarily expressed in the nephrogenic mesenchyme and stroma, defects in ureteric branching and patterning were observed. In addition, we show that differentiation of the nephrogenic mesenchyme into nephrons is severely reduced in *Pbx1* mutants. Our findings establish a role for *Pbx1* in mesenchymal–epithelial inductive signaling and demonstrate that *Pbx1* is a critical regulator of mesenchymal function during renal morphogenesis.

## Materials and methods

### *Generation of Pbx1$^{-/-}$ mice*

Targeted disruption of *Pbx1* and generation of *Pbx1* mutant mice have been described (Selleri et al., 2001). Heterozygous (*Pbx1*$^{+/-}$) mice were intercrossed to obtain homozygous embryos on a mixed genetic background derived from strains C57BL6/6J and 129/SvTer. Phenotypes were analyzed from the fourth and fifth backcrossed generations. Renal phenotypes were similar in mice derived from two independent ES clones (38 and 176) used in these studies. No apparent haploinsufficiency was observed in *Pbx1*$^{+/-}$ animals; therefore, "wild type" refers to phenotypes examined in *Pbx1*$^{+/+}$ and *Pbx1*$^{+/-}$ kidneys. Gestational age was estimated from the time of mating, with noon of the day of plug detection as E0.5. Genotypic analysis was performed on DNA extracted from embryonic yolk sacs as described (Selleri et al., 2001).

### *Histology and immunohistochemistry*

Embryos were fixed overnight in 10% formalin, dehydrated to ethanol, and embedded in paraffin. Four-micron sections were either stained with hematoxylin and eosin or used for immunohistochemical analysis. Immunohistochemistry was performed as described (Schnabel et al., 2001). Monoclonal antibodies used in this study were anti-Pbx1b (Chang et al., 1997) and anti-BrdU (NeoMarkers); polyclonal antisera consisted of anti-Pax-2 (Zymed) and anti-WT1 (Santa Cruz Biotechnology). Secondary antibodies used were either biotin-conjugated goat anti-mouse IgG or goat anti-rabbit IgG and horseradish peroxidase-conjugated streptavidin (Jackson Immunoresearch), which were detected with DAB (Sigma). For in vivo BrdU labeling studies, pregnant mice were injected intravenously with 50 mg BrdU/gram of body weight 3 h before sacrifice. BrdU detection was performed as described previously (Nowakowski et al., 1989).

### *In situ hybridization*

In situ hybridization analysis was performed essentially as decribed (Wilkenson, 1992). Briefly, embryos were fixed in freshly prepared 4% paraformaldehyde in PBS (8 h to overnight), dehydrated to ethanol, and embedded in paraffin for sectioning. Hybridizations on tissue sections were performed by using digoxygenin-labeled antisense riboprobes prepared according to manufacturer's instructions (Boehringer-Mannheim), and as previously described: *BF-2* (Hatini et al., 1996), *Wnt-11* (Kispert et al., 1996), *c-ret* (Pachnis et al., 1993), and *Pod-1* (Quaggin et al., 1998). Detection was performed by using an alkaline phosphatase-conjugated monoclonal antibody against digoxygenin (Boehringer-Mannheim) and BCIP/NBT (Vector Laboratories).

### *Immunofluorescence*

Indirect immunofluorescence was performed on paraffin sections of E12.5 formalin fixed embryos. Sections were treated for antigen retrieval by using Antigen Unmasking Solution (Vector Laboratories) according to manufacturer's instructions. Slides were blocked with normal sheep sera for 1 h and incubated with anti-Pbx1b antibody (Chang et al., 1997) in PBS/1% sheep sera for 2 h at room temperature in a humidified chamber. Secondary detection was performed by using FITC-conjugated goat anti-mouse $IgG_1$ antisera (Cortex Biochem) for 1 h at room temperature. Slides were

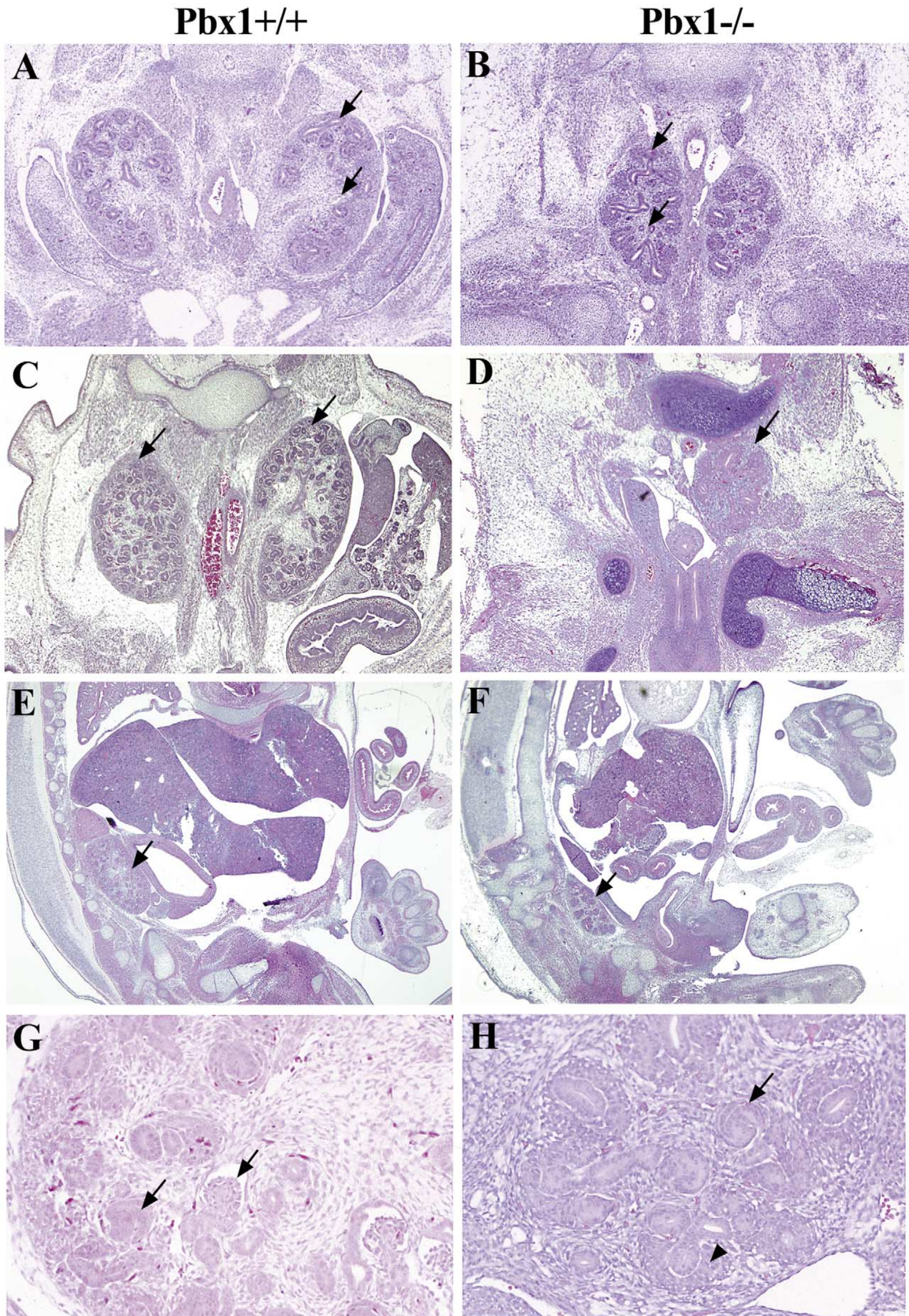



Fig. 1

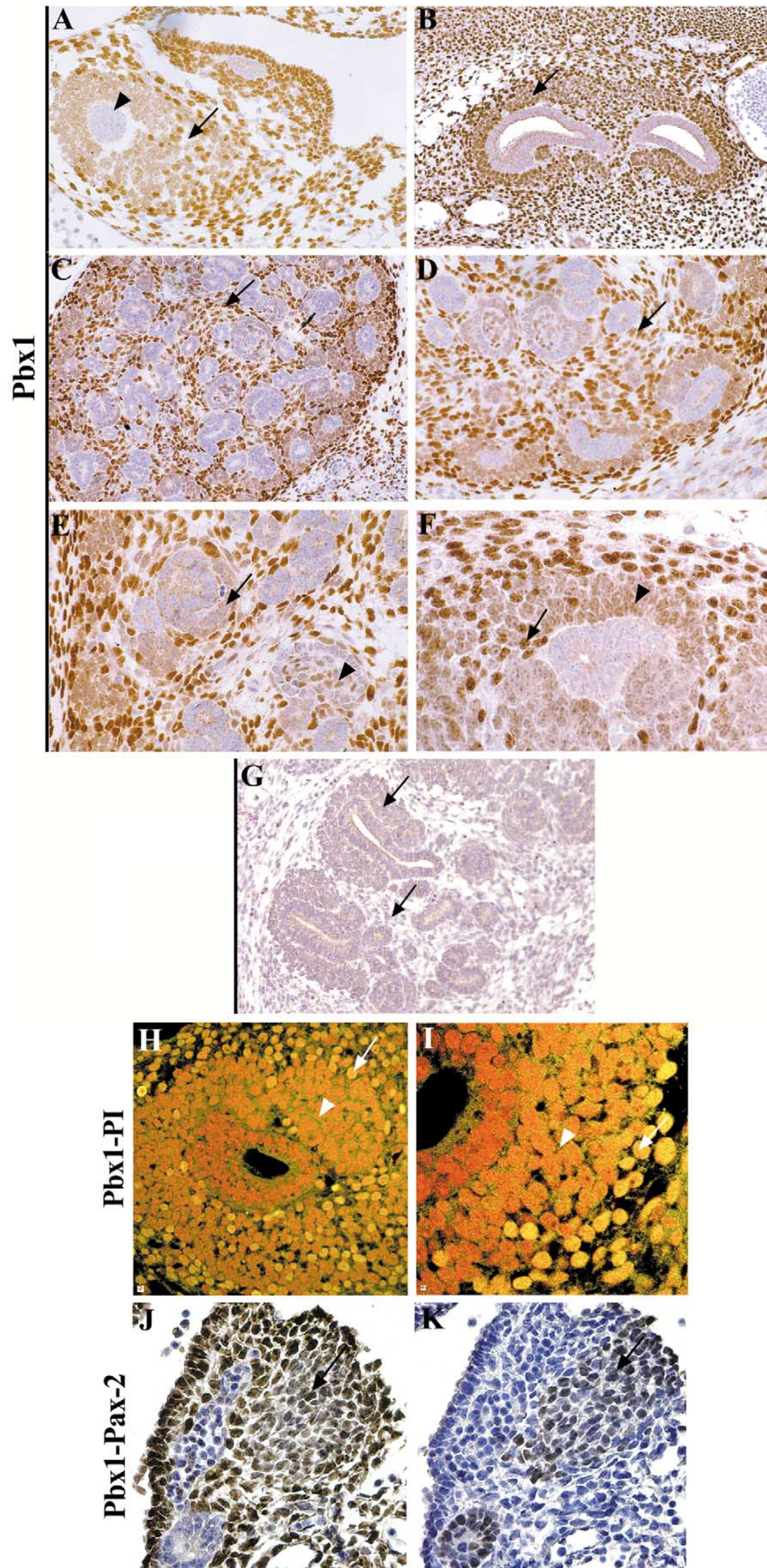



Fig. 2

mounted with Vectashield (Vector Laboratories) mounting media with propidium iodide (PI) and viewed on a Molecular Dynamics Multiprobe 2010 laser scanning confocal microscope.

### *Lectin staining and ureteric branch quantification*

Whole embryonic kidneys were dissected at E12.5, E13.5, and E14.5 and grown overnight at 37°C as raft cultures on Nuclepore polycarbonate filters (Whatman). Organ cultures were maintained in DMEM/F12 media (Gibco) without serum or growth factors. For whole-mount staining with fluorescein-coupled dolichous biflorus agglutinin (DBA) (Vector Laboratories), embryonic kidneys were fixed in 2% paraformaldehyde for 10 min, blocked for 1 h at 37°C in PBS/0.5% Triton X-100/3% BSA (PBS-TX-BSA), and incubated for 1 h at 37°C with DBA diluted 1:40 in PBS-TX-BSA. Ureteric bud ends visualized by lectin staining from wild type kidneys ($n = 4$) and *Pbx1*$^{-/-}$ kidneys ($n = 4$) for each developmental stage were counted.

### *Organ culture and explant treatments*

Metanephric explant cultures were established as described (Dudley et al., 1999; Saxen, 1987). Briefly, kidneys were dissected from E11.5 embryos in L-15 medium supplemented with 1% Cosmic Calf Sera (Hyclone). To isolate metanephric mesenchyme and ureter, kidneys were treated with 0.25% trypsin in dissection medium for 10 min on ice, and tissues were separated mechanically with 26G needles. Explants were grown in transfilter systems on Nuclepore membranes (Whatman) for 60 h in DMEM/F12 medium supplemented with 10% FBS (Hyclone) and penicillin/streptomycin/glutamine (Sigma).

## Results

### *Metanephric kidney defects in Pbx1*$^{-/-}$ *embryos*

Targeted disruption of *Pbx1* and generation of *Pbx1* homozygous mutant mice have been previously reported (Selleri et al., 2001). Loss of *Pbx1* function resulted in embryonic lethality at E15.5 and widespread developmental defects that were generally manifested as tissue hypoplasias (DiMartino et al., 2001; Kim et al., 2002; Selleri et al., 2001). Analysis of urogenital development in *Pbx1*$^{-/-}$ embryos demonstrated multiple organ abnormalities affecting mesodermal derivatives of the urogenital ridge (C.A.S., L. Selleri, and M.L.C., unpublished observations). Namely, *Pbx1*$^{-/-}$ embryos lacked adrenal glands and displayed impaired differentiation of the gonads, mesonephros, and kidneys. Mullerian ducts were absent in *Pbx1* mutants, whereas Wolffian ducts were formed and joined appropriately with the cloaca. These findings suggested that *Pbx1* serves multiple functions during urogenital ontogeny that are required both at early stage intermediate mesoderm development as well as later during tissue differentiation. In this study, further analyses of metanephric defects were conducted to elucidate *Pbx1* function during kidney organogenesis.

Histological analysis of *Pbx1*$^{-/-}$ embryos revealed defects in renal development with varied phenotypic severity (Fig. 1A–D). A majority of *Pbx1*$^{-/-}$ embryos exhibited bilateral formation of hypoplastic kidneys with unilateral agenesis detected in approximately 30% of *Pbx1* mutants analyzed (right, 36%; left, 64%) (Fig. 1A–D). In cases of unilateral agenesis, extension of the Wolffian duct into the cloacal region was undisrupted on the affected side (Fig. 1D). Further examination of E13.0 embryos showed that kidneys in *Pbx1*$^{-/-}$ embryos were reduced in size, rotated ventrally, and mispositioned caudally in the body cavity when compared with wild type (wt) embryos (Fig. 1A, B, E,

Fig. 1. Histological analysis of metanephric development in *Pbx1*$^{-/-}$ embryos. Matching sections of wt (A, C, E, and G) and *Pbx1*$^{-/-}$ littermates (B, D, F, and H) at E13.0 (A, B) and E14.5 (C–H). (A, B) Developing kidneys at E13.0 contain distinct cortical and medullary regions (arrows in A), which are poorly defined in *Pbx1*$^{-/-}$ kidneys (arrows in B). *Pbx1*$^{-/-}$ kidneys are smaller and rotated ventrally toward their inferior poles. (C, D) Arrows denote the presence of unilateral kidney formation observed in approximately 30% of *Pbx1*$^{-/-}$ embryos (D) vs bilateral formation in control embryos (C). (E, F) By E14.5, wt kidneys are ascended in the body cavity (arrow in E), whereas kidneys in *Pbx1*$^{-/-}$ embryos are positioned inappropriately more caudally (arrow in F). (G, H) E14.5 wt kidneys contained tubular structures at various stages of development (arrows in G). In comparison, *Pbx1*$^{-/-}$ kidneys were less than half the size of controls and contained few S-shaped bodies (arrow in H) and no glomerular structures. Expanded regions of mesenchymal condensates are present surrounding the ureteric buds (arrowhead in H).

Fig. 2. Expression of Pbx1 during metanephric development. (A–F) Expression of Pbx1 was evaluated by immunoperoxidase staining at E11.0 (A), E11.5 (B), E13.5 (C, D), and E14.0 (E, F). At metanephric induction, nuclear Pbx1 (brown stain) is present in the metanephric blastema (arrow in A) and surrounding mesenchyme but is absent in the ureter (arrowhead in A). As the ureter branches, Pbx1 is detected throughout the invaded mesenchyme (arrow in B). By E13.5, Pbx1 is seen thoughout the mesenchymal compartment in both the cortex and medulla (arrows in C and D). During nephrogenesis, low-level Pbx1 is present in pretubular aggregates and cells undergoing epithelial conversion (arrow in E) and is upregulated in glomerular cells (arrowhead in E). (F) Higher magnification field of ureteric induction showing differential levels of Pbx1 in mesenchymal populations, with higher levels observed in stromal cells (arrow in F) and lower levels detected in induced mesenchyme (arrowhead in F). (G) The absence of immunoperoxidase staining observed in the induced mesenchyme and stroma of E13.5 *Pbx1*$^{-/-}$ kidneys (arrows in G) confirms antibody specificity. (H, I) Confocal micrographs of immunofluorescent detection of Pbx1 in metanephric mesenchyme (green) costained with the nuclear marker PI (red) further demonstrate lower levels of nuclear Pbx1 in the induced mesenchyme (arrowheads in H, I) and higher levels associated with the surrounding stroma (arrows in H, I). (J, K) Adjacent sections from E11.0 embryos stained with Pbx1 (J) and Pax-2 (K) reveal their colocalization in the metanephric blastema (arrows in J and K).

and F). Distinct cortical and medullary regions, which were evident in wt kidneys at this stage, were poorly defined in *Pbx1*$^{-/-}$ kidneys (Fig. 1A and B). A striking feature of the *Pbx1*$^{-/-}$ kidneys was the decreased number of differentiating nephrons when compared with control littermates (Fig. 1G and H). At E14.5, the renal cortex of wt kidneys contained a nephrogenic zone with tubular structures at various stages of nephrogenesis, including comma-shaped bodies, S-shaped bodies, and glomeruli (Fig. 1G). In contrast, kidneys of E14.5 *Pbx1*$^{-/-}$ embryos showed few comma-and S-shaped structures and lacked mature glomeruli (Fig. 1H). Moreover, the nephrogenic zone of *Pbx1*$^{-/-}$ kidneys was severely attenuated and associated with the formation of large mesenchymal condensates surrounding a reduced number of ureteric bud ends in comparison to wt controls (Fig. 1G and H). Epithelial conversion of the mutant mesenchyme was delayed; however, nephrogenesis was not completely blocked as few glomerular structures were detected in E15.0 *Pbx1*$^{-/-}$ kidneys (data not shown). These findings demonstrate a critical role for *Pbx1* in renal organogenesis.

### *Nuclear Pbx1 levels delineate distinct mesenchymal populations in the developing kidney*

To characterize *Pbx1* function, its expression patterns were defined in the developing kidney by immunohistochemical analysis. At E11.0, when metanephric induction began, Pbx1 was detected as nuclear staining in the metanephric blastema and was colocalized with Pax-2, a marker of nephron progenitors (Fig. 2A, J, and K). Pbx1 was also apparent in the surrounding mesenchyme and coelomic epithelium, but was absent in the ureteric bud (Fig. 2A). High levels of Pbx1 were present at E11.5 in the induced mesenchyme and stroma as ureteric branching was initiated (Fig. 2B). By E13.5, pervasive expression of Pbx1 was seen throughout the mesenchyme (Fig. 2C and D). Pbx1 was detected in nephrogenic condensates adjacent to the ureteric bud epithelium and in the metanephrogenic mesenchyme that had yet to undergo aggregation (Fig. 2D). As tubulogenesis proceeded, Pbx1 was downregulated, with low level expression in pretubular condensates and podocyte precursors, but was increased in glomerular cells at the stage of capillary loop extension (Fig. 2E). The highest levels of Pbx1 were present in the stromal lineage (Fig. 2C–F). Unlike other stromal markers, which are associated with distinct metanephric regions (Hatini et al., 1996; Quaggin et al., 1998), Pbx1 was detected in the cortical progenitor population as well as the more mature stroma in the medullary regions of the developing kidney (Fig. 2C). In addition, specific detection of Pbx1 metanephric expression was confirmed by the absence of staining observed in sections from *Pbx1*$^{-/-}$ embryos (Fig. 2G). Therefore, the expression patterns of Pbx1 during kidney development suggest an important function for *Pbx1* in metanephric mesenchyme.

Further expression analysis demonstrated that levels of nuclear Pbx1 distinguished the induced mesenchyme from the stroma (Fig. 2F and Fig. 2H and I). Immunoperoxidase staining of E11.0 embryos revealed two levels of Pbx1 expression within the metanephric blastema upon ureteric induction. Lower levels of Pbx1 were detected in the induced mesenchyme compared with that observed in the surrounding stroma (Fig. 2A). Immunofluorescence confirmed that this distinctive pattern of Pbx1 mesenchymal expression persisted in the nephrogenic zone at later stages of development (Fig. 2H and I). Furthermore, double labeling to detect Pbx1 along with the nuclear stain, propidium iodide (PI), showed that mesenchymal expression patterns correlated with differential levels of nuclear Pbx1 (Fig. 2H and I). Therefore, highest levels of nuclear Pbx1 were associated with stromal cells, whereas lower levels of nuclear Pbx1 were detected in cells committed to nephrogenesis. These findings indicate that Pbx1 protein levels delineate distinct mesenchymal populations during metanephric development.

### *Early events in metanephric development are unaltered in Pbx1*$^{-/-}$ *embryos*

To establish the origin of *Pbx1*-associated renal defects, expression of early-stage markers of metanephric development was investigated. Metanephric induction ensues with ureteric bud outgrowth from the Wolffian duct, which requires *c-ret* and *Pax-2,* and mesenchymal competence, which requires *WT-1. Pax-2* plays an essential role in the development of the posterior Wolffian duct and, subsequently, ureteric bud formation (Torres et al., 1995). In sections of E11.0 embryos, appropriate expression of Pax-2 was observed in the Wolffian ducts and metanephric blastema of *Pbx1*$^{-/-}$ embryos and control littermates (Fig. 3A and B). Ureteric bud development was further inspected with the epithelial marker, *c-ret,* which is required for the outgrowth and subsequent branching of the ureter (Schuchardt et al., 1996). *c-ret* expression was detected at equivalent levels in the sprouting ureteric buds of wild type and *Pbx1*$^{-/-}$ embryos (Fig. 3C and D). Expression of WT-1, which functions to regulate mesenchymal signals essential for ureteric induction (Kriedberg et al., 1993), was also detected in the metanephric mesenchyme of both E11.0 wt and *Pbx1*$^{-/-}$ embryos (Fig. 3E and F).

Since previous studies implicated a role for *Pbx1* in cellular growth and expansion (DiMartino et al., 2001; Kim et al., 2002; Selleri et al., 2001), levels of proliferation were measured by in vivo BrdU labeling analysis. Comparable levels of proliferation were seen in the ureteric buds as well as the metanephric blastema of E11.0 *Pbx1*$^{-/-}$ and wt littermates (Fig. 3G and H), indicating that proliferative growth of the ureter and metanephric mesenchyme was not deficient in *Pbx1*$^{-/-}$ kidneys. Thus, *Pbx1*$^{-/-}$ embryos exhibit appropriate expression of genes that mark the early

stages of ureteric and mesenchymal differentiation. Furthermore, these results indicate that, despite earlier stage defects in urogenital development resulting from loss of *Pbx1* (unpublished observations), alterations during the initial events of metanephric induction were not observed in *Pbx1* mutants.

### *Pbx1*$^{-/-}$ *kidneys exhibit defects in ureteric branching*

In addition to tubular abnormalities, *Pbx1*$^{-/-}$ kidneys displayed defects in ureteric branching morphogenesis (Fig. 4A–E). Histological analysis of E14.5 kidneys demonstrated fewer ureteric tips in the renal cortex of *Pbx1*$^{-/-}$ kidneys when compared with controls (Fig. 1H). To better visualize the ureter, kidneys were dissected at various embryonic stages and stained with dolichous biflorus agglutinin (DBA), which labeled the ureteric epithelium (Fig. 4A–D). Differences in branching, which were estimated by counting ureteric bud ends, were not apparent in E12.5 *Pbx1*$^{-/-}$ and wt kidneys (Fig. 4E). However, branching defects became progressively accentuated such that, by E14.5, *Pbx1*$^{-/-}$ kidneys exhibited a threefold reduction in the number of cortical ureteric buds in comparison to wt littermates (*Pbx1*$^{+/+}$, 60 $\pm$ 3.5; *Pbx1*$^{-/-}$, 22 $\pm$ 1.4) (Fig. 4E). Aside from the quantitative reduction in overall branching, *Pbx1*$^{-/-}$ kidneys also showed ureteric patterning defects (Fig. 4D). Whereas the dichotomous branching pattern of wt ureters was clearly observed, the ureteric tree appeared disorganized in *Pbx1*$^{-/-}$ kidneys (compare Fig. 4C and D). *Pbx1*$^{-/-}$ epithelia maintained a dichotomous branching program; however, elongation of the ureter was impaired with shorter intervals between subsequent branches (Fig. 4D). As a result, bifurcations of the *Pbx1*$^{-/-}$ ureter were less defined and created a webbed appearance at branch points in comparison to the consistent pattern observed in wt kidneys (Fig. 4D). These results demonstrate a role for mesenchymal factor *Pbx1* in ureteric branching morphogenesis and indicate its function in the regulation of mesenchymal–epithelial interactions.

To further characterize defects in the collecting system, markers of ureteric development were examined in *Pbx1*$^{-/-}$ kidneys (Fig. 5A–D). Expression of *Wnt-11* was detected and appropriately confined to the tips of the branching ureter (Kispert et al., 1996) in both *Pbx1* null and wt kidneys (Fig. 5A and B). In contrast, alterations of *c-ret* expression were observed in *Pbx1* mutant kidneys. At E14.5, *c-ret* expression is restricted to the growing tips of wild type ureters (Fig. 5C). In *Pbx1*$^{-/-}$ kidneys, however, *c-ret* expression was ectopically detected in branches and the ureteric stalk in addition to its expected distribution at the ureteric tips at this stage (Fig. 5D). These observations suggest that misexpression of *c-ret* may be correlated with impaired ureteric development in *Pbx1*$^{-/-}$ kidneys.

### *Abnormal expansion of induced mesenchyme in Pbx1*$^{-/-}$ *kidneys*

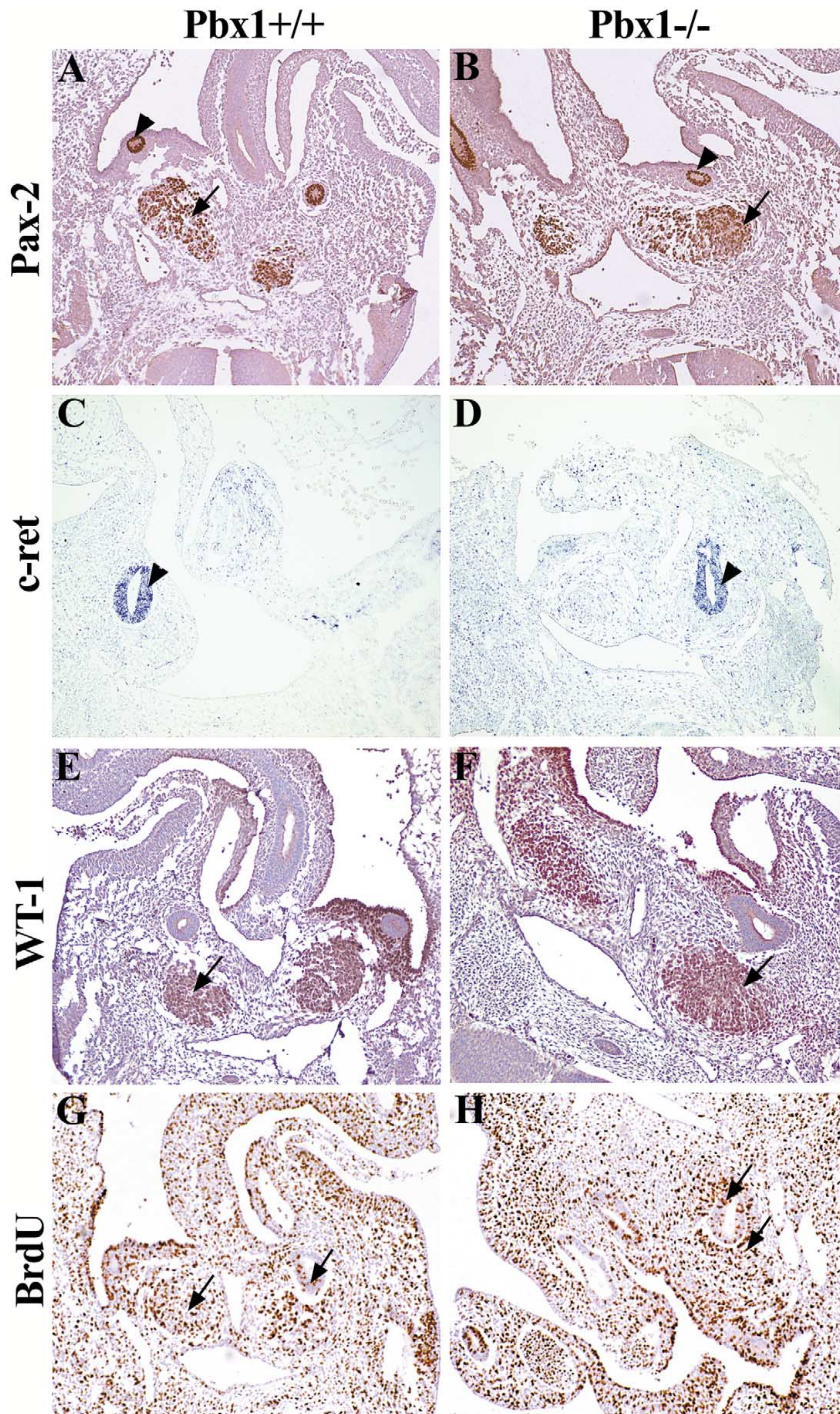
Morphological defects in *Pbx1*$^{-/-}$ metanephric mesenchyme included a delay in tubular differentiation as well as the accumulation of mesenchymal condensates surrounding the tips of the growing ureter. Further analysis of *Pbx1*$^{-/-}$ mesenchyme was conducted by using molecular markers for stromal precursors (*BF-2*), interstitial stroma (Pod-1), induced mesenchyme (Pax-2, WT-1), and cycling cells (BrdU).

Stromal factors, *BF-2* and *Pod-1,* have been shown to regulate epithelial differentiation and ureteric branching during kidney development (Hatini et al., 1996; Quaggin et al., 1999). Examination of *BF-2* expression at E14.0 demonstrated that the stromal lineage was established in *Pbx1*$^{-/-}$ kidneys, where predominant staining was detected in subcapsular regions (Fig. 6A and B). Analysis of *Pod-1* staining also revealed similar patterns of expression between *Pbx1*$^{-/-}$ and control embryos in medullary stroma and differentiating podocytes (Fig. 6C and D). Thus, specification and patterning of the stromal compartment was unaffected in *Pbx1* mutants.

Pax-2 and WT-1 are expressed in mesenchymal condensates and are required for nephrogenesis (Armstrong et al., 1993; Dressler et al., 1990; Moore et al., 1999; Rothenpieler and Dressler, 1993). Appropriate cell-specific expression of Pax-2 and WT-1 was detected; however, their distributions were altered such that abnormally expanded regions of mesenchymal induction were seen in *Pbx1*$^{-/-}$ kidneys (Fig. 6E–J). Newly formed mesenchymal condensations in wt kidneys contained 2 to 4 cell layers surrounding ureteric buds, which were increased up to 15 layers in *Pbx1*$^{-/-}$ kidneys (compare Fig. 6G and H). The presence of enlarged mesenchymal regions expressing Pax-2 and WT-1 in *Pbx1*$^{-/-}$ kidneys suggests that the associated tubular defects are not related to abnormalities in mesenchymal specification but, rather, in the regulation of mesenchymal differentiation.

Differences in the levels of proliferation and apoptosis were also investigated to account for the observed overall growth reduction in *Pbx1*$^{-/-}$ kidneys. By in situ TUNEL staining, comparable levels of cell death were detected in *Pbx1*$^{-/-}$ and control kidneys (data not shown). Further-

Fig. 3. Markers of early metanephric development. Comparison between the expression of molecular markers in wt (A, C, E, and G) and *Pbx1*$^{-/-}$ (B, D, F, and H) embryos at E11.0 (A–H). Competence for metanephric induction was examined by immunohistochemical detection of Pax-2 (A, B), WT-1 (E, F), and BrdU (G, H) and by in situ hybridization for *c-ret* (C, D). Pax-2 is expressed in the Wolffian ducts (arrowheads in A, B) and the metanephric blastema (arrows in A, B) in both wt (A) and *Pbx1*$^{-/-}$ embryos (B). Expression of *c-ret* is detected in the sprouting ureteric bud in wt (arrowhead in C) and *Pbx1*$^{-/-}$ (arrowhead in D) embryos. Bilateral expression of WT-1 is detected in the metanephric blastema of both control (arrow in E) and *Pbx1*$^{-/-}$ embryos (arrow in F). Comparable levels of proliferation detected by BrdU staining are observed in nephric tissues of wt (arrows in G) and *Pbx1*$^{-/-}$ embryos (arrows in H).


Pbx1+/+
Pbx1-/-
A
B
Pax-2
C
D
c-ret
E
F
WT-1
G
H
BrdU

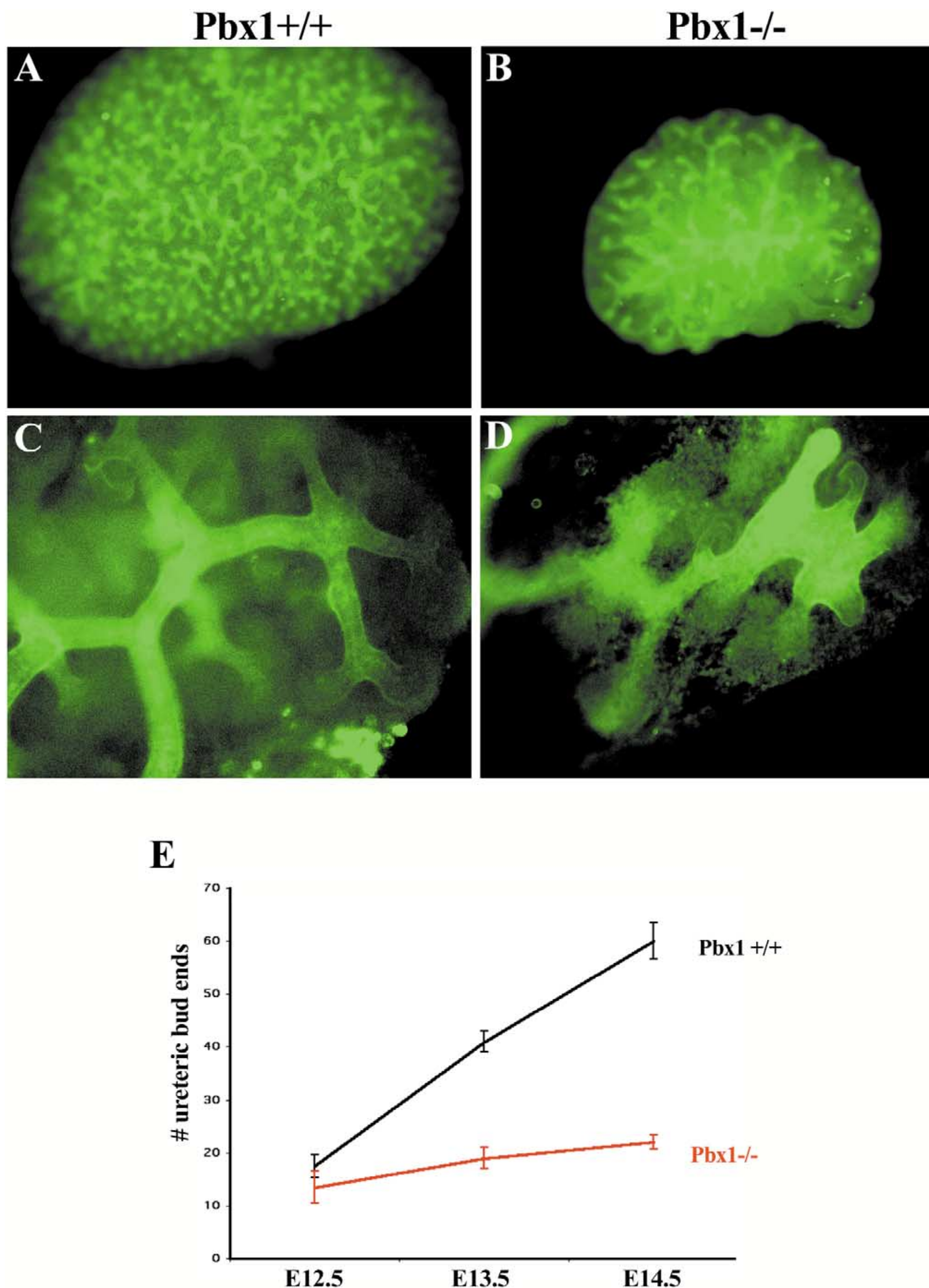



Fig. 4. Ureteric defects in $Pbx1^{-/-}$ kidneys. Comparison of DBA lectin staining, which labels ureteric epithelia, in wt (A, C) and $Pbx1^{-/-}$ embryonic kidneys (B, D) at E14.5 (A, B) and E13.5 (C, D). Wt kidneys at E14.5 contain a highly branched ureteric tree (A), whereas branching is reduced approximately threefold in $Pbx1^{-/-}$ kidneys (B). (C, D) Higher magnification of DBA-stained kidneys. The program of branching in $Pbx1^{-/-}$ kidneys remains dichotomous, but ureteric elongation is impaired and branches show a webbed pattern (D) in comparison to control kidneys (C). (E) Graphic representation of the progressive, quantitative reduction in ureteric branching in $Pbx1^{-/-}$ kidneys.

more, expression of *BMP-7,* which is necessary for survival of the nephrogenic mesenchyme (Dudley et al., 1995, 1999) was unperturbed in $Pbx1^{-/-}$ kidneys, and expression of *Pbx1* in $BMP\text{-}7^{-/-}$ kidneys was also unaltered (Fig. 5G and H; and data not shown). In vivo BrdU labeling to identify cells in mitotic S-phase revealed that the zones of induced $Pbx1^{-/-}$ mesenchyme correlated with high levels of proliferation (Fig. 5E and F). These results suggest that loss of *Pbx1* does not affect mesenchymal survival but results in the abnormal expansion of induced mesenchyme.

### *Mesenchymal deficiency in $Pbx1^{-/-}$ kidneys*

Disruption of *Pbx1* function resulted in developmental defects of both the ureter and metanephric mesenchyme. To determine which component was directly affected by loss of

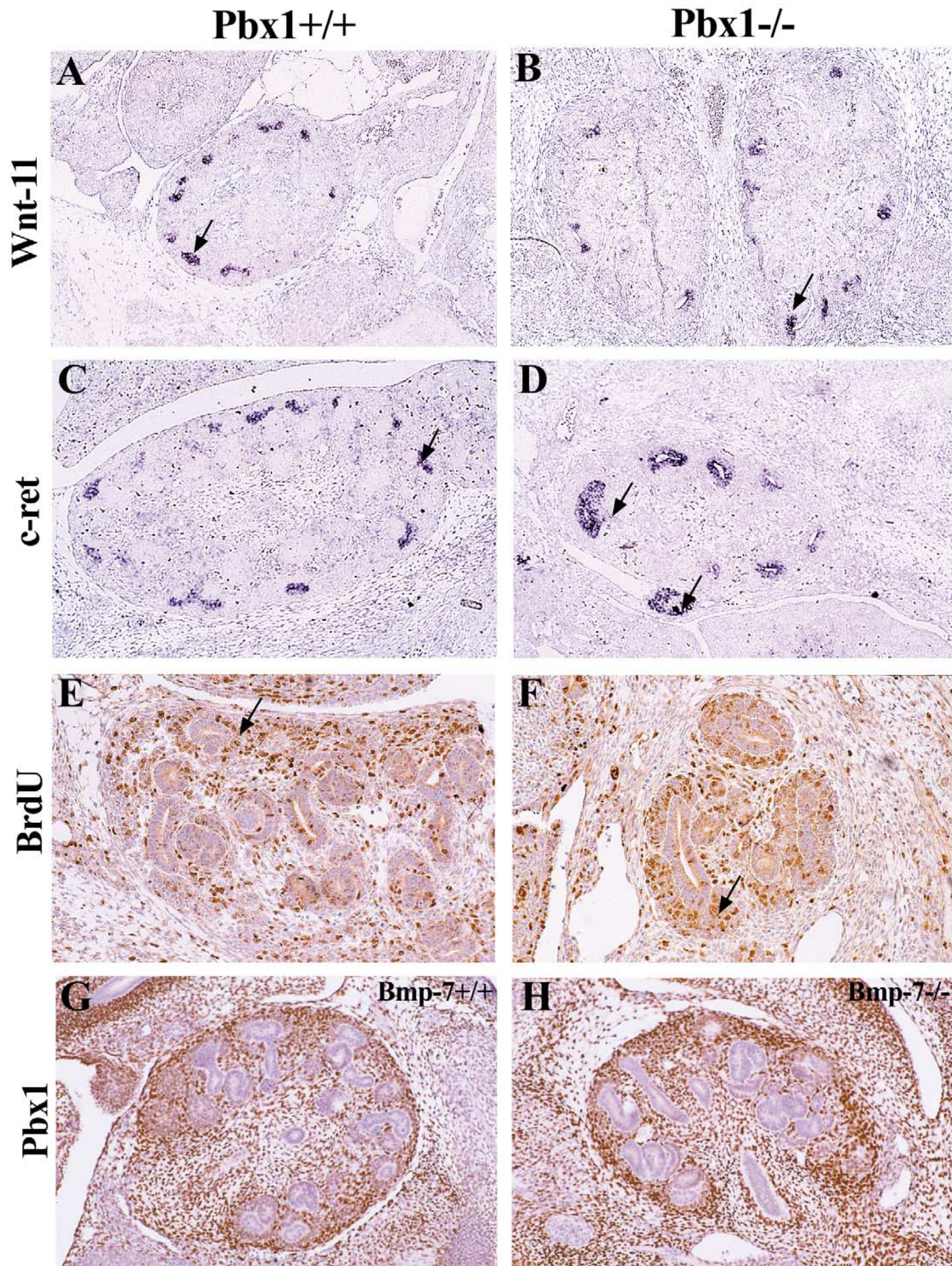



Fig. 5. Markers of ureteric development and cell growth. (A–D) Expression of ureteric markers *Wnt-11* (A, B) and *c-ret* (C, D) in E14.0 wt (A, C) and $Pbx1^{-/-}$ kidneys (B, D). *Wnt-11* expression was detected in the ureteric tips of both wt (arrow in A) and $Pbx1^{-/-}$ kidneys (arrow in B). *c-ret* expression in control kidneys was restricted to the ureteric tips (arrow in C), whereas ectopic expression of *c-ret* was present in ureteric branches and the ureteric stalk (arrows in D) of $Pbx1^{-/-}$ kidneys in addition to its expression at ureteric tips. (E, F) Comparison of BrdU-labeled cells between E14.5 wt (E) and $Pbx1^{-/-}$ kidneys (F) revealed the predominance of proliferating cells in the expanded regions of induced mesenchyme of $Pbx1^{-/-}$ kidneys (compare arrows in E and F). (G, H) Appropriate expression of Pbx1 in BMP-$7^{-/-}$ kidneys and control littermates at E13.5.

*Pbx1,* heterologous recombination studies were conducted with isolated ureter and metanephric mesenchyme. Both epithelialization of the mesenchyme and branching of the ureter occurred when E11.5 wt mesenchyme and ureter were cocultured (Fig. 7A and B). Similarly, ureteric buds isolated from $Pbx1^{-/-}$ embryos cocultured with wild type mesenchyme supported both ureteric branching and epithelial transformation comparable to that observed using wt

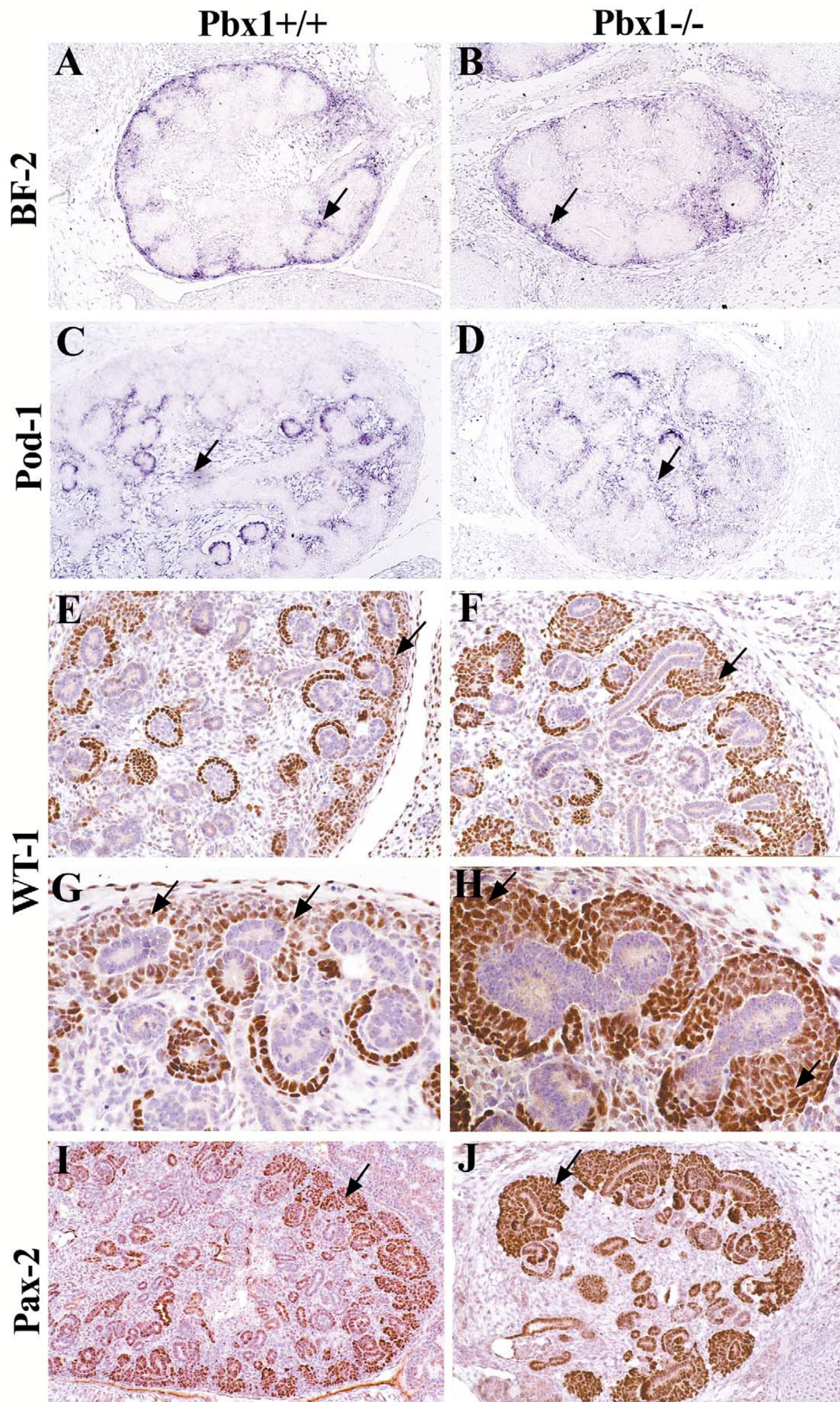



Fig. 6

ureter (Fig. 7C and D). In contrast, coculture of *Pbx1*$^{-/-}$ mesenchyme with wt ureter severely disrupted ureteric branching and mesenchymal differentiation (Fig. 7E and F). Therefore, *Pbx1*-associated defects in metanephric development arise from mesenchymal dysfunction.

## Discussion

The present study establishes a function for *Pbx1* in renal organogenesis, thereby extending its role as a developmental regulator. During kidney development, Pbx1 expression pervades in the metanephric mesenchyme, where its levels distinguish the stromal (high expression) and tubular compartments (low expression). In a majority of *Pbx1* mutants, bilateral kidneys are formed abnormally, which exhibit delayed nephrogenesis and defects in ureteric branching. Our observations support a role for *Pbx1* in the regulation of mesenchymal–epithelial inductive interactions that are critical for renal patterning and morphogenesis.

### *Pbx1 is critical for regulating mesenchymal function during renal morphogenesis*

The earliest metanephric phenotype detected with loss of *Pbx1* is unilateral kidney agenesis, but whether this results from defects in patterning of the urogenital axis is unknown. We find that *Pbx1*$^{-/-}$ nephric tissues are competent to initiate kidney development since expression of genes essential for metanephric induction, such as *c-ret* and Pax-2 in the ureter and WT-1 in the metanephric blastema, is unperturbed in *Pbx1*$^{-/-}$ embryos. In addition, ureteric function in *Pbx1*$^{-/-}$ embryos is uncompromised and can fully support renal development in vitro with wild type mesenchyme. Therefore, impaired differentiation of *Pbx1*$^{-/-}$ kidneys is not solely due to secondary effects of earlier stage urogenital abnormalities.

During kidney morphogenesis, loss of *Pbx1* leads to the progressive reduction of kidney growth and differentiation. We show that growth defects in *Pbx1*$^{-/-}$ kidneys are not attributed to an increase in apoptosis or a decrease in cellular proliferation overall but are indicative of perturbations in the reiterative developmental program. *Pbx1*$^{-/-}$ kidneys display a dramatic reduction in ureteric branching, resulting in the formation of a smaller collecting system. Although branching remained dichotomous in *Pbx1*$^{-/-}$ epithelia, the pattern was abnormal with diminished ureteric elongation. *Pbx1*$^{-/-}$ embryos also exhibited defects in tubular differentiation with few S-shaped bodies and glomeruli observed in E15.0 mutant kidneys. Mesenchymal abnormalities were characterized by an increase in condensates surrounding ureteric tips, which were composed predominantly of proliferating cells, along with those undergoing aggregation. Accumulation of cells in a transitional, proliferative stage of differentiation suggests that *Pbx1* function may be crucial in the nephrogenic mesenchyme prior to pretubular aggregation. *Pbx1*$^{-/-}$ kidneys also lacked the full complement of stroma in comparison to their wt counterparts, as evidenced by staining with BF-2 and Pod-1. Since *Pbx1*$^{-/-}$ kidneys did not display increased cell death or impaired proliferation of stromal cells, our findings suggest a role for Pbx1 in mediating signaling pathways that are important for appropriate subdivision of the mesenchyme throughout kidney morphogenesis.

In vitro, *Pbx1*$^{-/-}$ mesenchyme displayed limited developmental potential in tissue recombination studies, whereas *Pbx1*$^{-/-}$ ureter was unaffected. In addition, *Pbx1*$^{-/-}$ mesenchymal explants treated with LiCl, which elicits the early stages of mesenchymal aggregation (Davies and Garrod, 1995), exhibited a similar capacity for mesenchymal condensation when compared with wt mesenchyme (C.A.S. and M.L.C., unpublished observations). These findings demonstrate that *Pbx1* mutant mesenchyme is competent to initiate epithelialization and suggest that *Pbx1* activity is not required to promote early stage nephrogenesis. Taken together, our analysis clearly demonstrates that *Pbx1*$^{-/-}$ renal abnormalities arise from deficiencies in mesenchymal function that are required for differentiation of the nephrogenic mesenchyme and stroma, and to regulate signaling pathways necessary for development of the collecting system.

### *Pbx1 deficiency phenotypically overlaps with loss of stromal regulators*

Pbx1 expression during renal development is associated with mesenchymal populations, i.e., the stroma and nephrogenic mesenchyme, both of which mediate important functions in renal development. Although inductive interactions between the ureteric bud and metanephric mesenchyme were previously thought to be sufficient to induce nephrogenesis and ureteric branching, a growing body of evidence has revealed the importance of the stromal compartment as another signaling center (reviewed in Bard, 1996; Kuure et al., 2000). Regulation of nephrogenesis through stromal cell signaling to the nephrogenic mesenchyme has been demonstrated by studies of GD3, *BF-2,* and

Fig. 6. Markers of mesenchymal differentiation. (A–D) Stromal cell differentiation was examined in E13.5 (A, B) and E14.5 (C, D) comparing wt (A, C) and *Pbx1*$^{-/-}$ kidneys (B, D). Patterns of *BF-2* expression in stromal cell progenitors and *Pod-1* expression in interstitial stroma and differentiating podocytes were unaltered in *Pbx1*$^{-/-}$ kidneys (arrows in B, D) when compared with control littermates (arrows in A, C). (E–J) Expression of markers of induced mesenchyme, WT-1 (E–H) and Pax-2 (I, J), in wt (A, G, I) and *Pbx1*$^{-/-}$ kidneys (F, H, J). (E–H) WT-1 expression was detected in induced mesenchyme (arrows in E, F) and in differentiating podocytes of wt (E, G) and *Pbx1*$^{-/-}$ kidneys (F, H). (I, J) Pax-2 expression was detected in the ureter and induced mesenchyme of control (I) and *Pbx1*$^{-/-}$ kidneys (J). Arrows indicate regions of induced mesenchyme in wild type kidneys (E, G, I) that are expanded in *Pbx1*$^{-/-}$ kidneys (F, H, J).

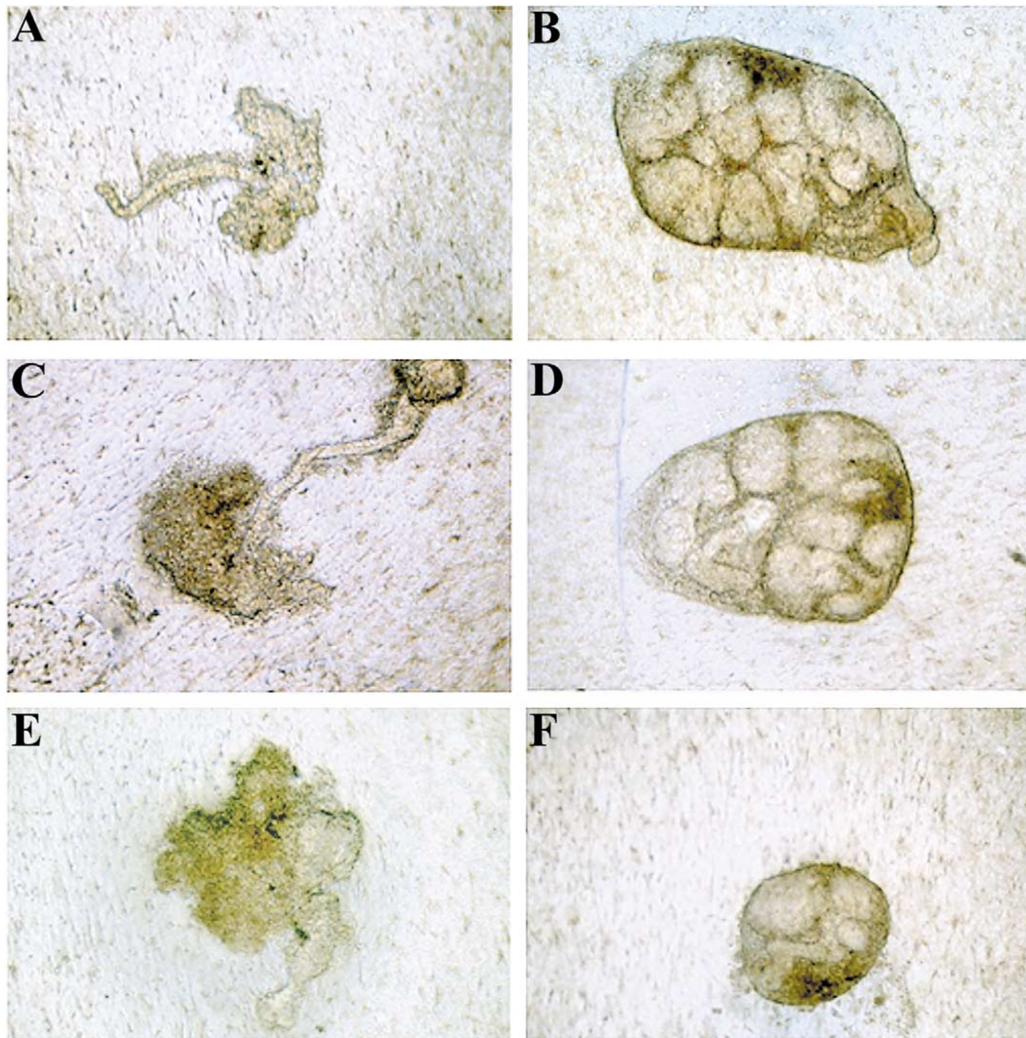



Fig. 7. Developmental potential of isolated *Pbx1*$^{-/-}$ metanephric tissues in explant cultures. Ureteric and mesenchymal tissues were isolated from E11.5 wt and *Pbx1*$^{-/-}$ kidneys and grown in vitro. Cultures are shown at initial plating (A, C, E) and after 60 h in culture (B, D, F). (A, B) Wt ureter was recombined with wt mesenchyme where nephrogenesis and ureteric branching were observed in five of six experiments. (C, D) Wt mesenchyme cocultured with *Pbx1*$^{-/-}$ ureter. In five of seven cultures, ureteric branching and epithelialization occurred. (E, F) *Pbx1*$^{-/-}$ mesenchyme was cocultured with wt ureter. The extent of ureteric branching and mesenchymal induction was impaired in all of six experiments. In two cultures, the ureter was present but did not branch, and in four experiments, the ureter had branched once or twice with limited mesenchymal induction seen in two cultures.

*Pod-1,* where loss or perturbation of stromal function resulted in tubulogenic defects (Hatini et al., 1996; Quaggin et al., 1999; Sariola et al., 1988). Signaling from the nephrogenic mesenchyme to the stroma has been defined by explant studies with FGF-2 and BMP-7, where effects on stromal cell expansion are mediated in part by BMP-7, a ligand produced by nephrogenic mesenchyme (Dudley et al., 1999). In addition, targeted disruption of the retinoic acid receptors, *RARa* and *RARb2,* revealed that reciprocal inductive interactions between the ureter and stroma are required for stromal cell patterning and ureteric bud growth (Batourina et al., 2001; Mendelsohn et al., 1999).

Our findings indicate that regulation of stromal activity is a critical component of *Pbx1* function during renal development. To a great extent, Pbx1 metanephric defects phenotypically overlap with loss of stromal regulators *BF-2* and *Pod-1* (Hatini et al., 1996; Quaggin et al., 1999). Similar to mice deficient for these transcriptional regulators, mesenchymal cells survive but nephron formation is reduced in *Pbx1* mutants as evidenced by few S-shaped bodies and glomeruli detected at E15.0. In addition, *Pbx1*$^{-/-}$ metanephroi display an increase in the condensing mesenchyme surrounding ureteric tips as is observed in *BF-2* and *Pod-1* mutants (Hatini et al., 1996; Quaggin et al., 1999). Another common feature is the disruption in ureteric branching morphogenesis and associated perturbations in *c-ret* expression (Hatini et al., 1996; Quaggin et al., 1999). During normal kidney development, *c-ret* expression becomes restricted to ureteric tips in the subcapsular region after ureteric branching is initiated (Pachnis et al., 1993). In *Pbx1*$^{-/-}$ kidneys, *c-ret* expression is present in ureteric tips but is also inappropriately expressed in the ureteric stalk and branches. A

similar feature is seen in *BF-2* and *Pod-1* mutants, which display ectopic expression of *c-ret* in ureteric branches (Hatini et al., 1996; Quaggin et al., 1999). Pertinent to these observations, misexpression of *c-ret* throughout the ureteric epithelia has been shown to inhibit ureteric growth and branching in transgenic mice (Srinivas et al., 1999). Notably, *BF-2* and *Pod-1* expression is unperturbed in $Pbx1^{-/-}$ kidneys. The similarity in phenotypes between $Pbx1^{-/-}$ and $BF\text{-}2^{-/-}$ kidneys also prompted us to examine Pbx1 expression in $BF\text{-}2^{-/-}$ embryos, which revealed that Pbx1 expression through various embryonic stages remained unchanged (R. Levinson and E. Lai, unpublished observations). Taken together, our results strongly suggest that disruption of *Pbx1* impairs stromal cell function on pathways that are parallel or downstream of *BF-2* and *Pod-1,* respectively.

A requirement for retinoic acid receptor function in regulating stromal signals has also been demonstrated (Batourina et al., 2001; Mendelsohn et al., 1999). Mutations in *RARa* and *RARb2* dramatically impaired ureteric branching; however, mesenchymal cell differentiation was not as severely affected as in $Pbx1^{-/-}$ embryos. Ureteric abnormalities in *RARab2*-deficient mice were associated with down-regulation of *c-ret* expression to undetectable levels in the ureteric buds and reduced expression of a *c-ret* signaling target, *Wnt-11* (Mendelsohn et al., 1999; Pepicelli et al., 1997). In contrast, *Wnt-11* expression was maintained at comparable levels as controls in the ureteric tips, and *c-ret* was ectopically expressed in the ureteric stalk and branches, of *Pbx1* mutants. Moreover, whereas an abnormal thickening of the stromal layer was seen in $RARab2^{-}$ kidneys, stromal cell patterning was unperturbed in $Pbx1^{-/-}$ kidneys as well as in *BF-2* and *Pod-1* mutants (Hatini et al., 1996; Mendelsohn et al., 1999; Quaggin et al., 1999). Thus, although *Pbx1*-dependent pathways appear to overlap with stromal regulators *BF-2* and *Pod-1,* our results suggest that *Pbx1* function is likely to be independent of retinoid signaling.

Metanephric defects directly attributable to loss of *Pbx1* in the tubular population are less clear. Abnormalities in ureteric branching and delayed nephron formation in $Pbx1^{-/-}$ kidneys can be accounted for by stromal cell dysfunction; however, we cannot exclude that some of these activities may be mediated through the nephrogenic mesenchyme. Therefore, nephrogenic defects could arise from loss of a cell-autonomous function for *Pbx1* in nephrogenic mesenchyme that consequently impairs ureteric development indirectly. However, loss of *Pbx1* in the nephrogenic mesenchyme does not lead to apparent exacerbation of nephrogenic defects than would be predicted from stromal insufficiency, suggesting that *Pbx1* may not be a limiting factor in these cells. Another possibility is that functional redundancy results from other Pbx family members, which are expressed in the metanephric mesenchyme (C.A.S. and M.L.C., unpublished observations). Thus, our findings cannot definitively exclude a requirement for *Pbx1* activity in the nephrogenic mesenchyme, and further studies are necessary to elucidate *Pbx1*-dependent functions in the tubular compartment.

### *Potential pathways regulated by Pbx1*

During normal kidney development, *Pbx1* expression in the nephrogenic mesenchyme is decreased upon induction, suggesting that down-regulation of *Pbx1* promotes mesenchymal differentiation. Loss of *Pbx1* in this case would be predicted to cause an increase in tubulogenesis. However, differentiation is inhibited in *Pbx1* mutants with expanded regions of mesenchymal condensates containing a preponderance of cycling cells. These findings indicate that $Pbx1^{-/-}$ kidneys initiate nephrogenesis but exhibit sustained proliferation of the mesenchyme without subsequent differentiation. Recent studies by Dudley et al. propose that stromal signals potentially mediate pathways opposing differentiation (Dudley et al., 1999). Based on this assertion, one possibility consistent with our results is that *Pbx1* functions in stromal cells to negatively regulate anti-tubulogenic signals. Along these lines, loss of *Pbx1* in the stroma would lead to an increase in signals that antagonize tubulogenesis, thereby preventing cells in the induced mesenchyme from continued differentiation. Misregulation of signaling pathways that mediate tubulogenesis and conserve stromal progenitors would also result in abnormal partitioning of the mesenchyme at each point of induction. The implication of these findings is that *Pbx1* functions in the stroma to define the boundary of induced mesenchyme and to coordinate pathways of proliferation and differentiation in the metanephric mesenchyme. Consistent with this notion, Pbx1 developmental expression is associated with proliferating cells as well as those undergoing terminal differentiation (Schnabel et al., 2001). In addition, *Pbx1* has been implicated in the programming of chondrocyte proliferation with terminal differentiation during skeletogenesis (Selleri et al., 2001). Along with the results presented here, recent advances in the understanding of *Pbx1* function during mammalian embryogenesis are indicative of its participation in numerous transcriptional pathways (DiMartino et al., 2001; Kim et al., 2002; Selleri et al., 2001). In future work, it will be interesting to examine Pbx1 expression in other loss-of-function models to potentially elucidate molecular interactions.

## Acknowledgments

We thank Andy McMahon, Frank Costantini, Susan Quaggin, and Eseng Lai for hybridization probes; Maria Ambrus and Carmencita Nicolas for technical assistance; Elizabeth Dominay and Roger Warnke for immunohistochemistry support; and Elizabeth Robertson and members of the Cleary lab for comments on the manuscript. These studies were supported by a fellowship from the Leukemia and Lymphoma Society (to C.A.S.) and grants from the

National Institutes of Health (CA42971, CA70404, and CA90735).

## References

Armstrong, J., Pritchard-Jones, K., Bickmore, W.A., Hastie, N.D., Bard, J.B., 1993. The expression of the Wilm's tumour gene, WT-1, in the developing mammalian embryo. Mech. Dev. 40, 85–97.

Aufderheide, E., Chiquet-Ehrismann, R., Ekblom, P., 1987. Epithelial–mesenchymal interactions in the developing kidney lead to expression of tenascin in the mesenchyme. J. Cell Biol. 105, 599–608.

Bard, J., 1996. A new role for stromal cells in kidney development. Bioessays 18, 705–707.

Batourina, E., Gim, S., Bello, N., Shy, M., Clagett-Dame, M., Srinivas, S., Constantini, F., Mendelsohn, C., 2001. Vitamin A controls epithelial/mesenchymal interactions through Ret expression. Nat. Genet. 27, 74–78.

Chang, C.P., Jacobs, Y., Nakamura, T., Jenkins, N.A., Copeland, N.G., Cleary, M.L., 1997. Meis proteins are the major in vivo DNA binding partners for wild type but not chimeric Pbx proteins. Mol. Cell. Biol. 17, 5679–5687.

Davies, J.A., Garrod, D.R., 1995. Induction of early stages of kidney tubule differentiation by lithium ions. Dev. Biol. 167, 50–60.

DiMartino, J.F., Selleri, L., Traver, D., Firpo, M.T., Rhee, J., Warnke, R., O'Gorman, S., Weismann, I.L., Cleary, M.L., 2001. The Hox cofactor and proto-oncogene *Pbx1* is required for maintenance of definitive hematopoiesis in the fetal liver. Blood 98, 618–626.

Dressler, G.R., Deutsch, U., Chowdhury, K., Nornes, H.O., Gruss, P., 1990. Pax2, a new murine paired box-containing gene and its expression in the developing excretory system. Development 109, 787–795.

Dudley, A., Lyons, K., 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., Robertson, E.J., 1999. Interaction between FGF and BMP signaling pathways regulates development of the metanephric mesenchyme. Genes Dev. 13, 1601–1613.

Grobstein, C., 1953. Inductive epithelio-mesenchymal interaction in cultured organ rudiments of the mouse. Science 113, 52–55.

Grobstein, C., 1955. Inductive interaction in the development of the mouse metanephros. J. Exp. Zool. 130, 319–340.

Hatini, V., Huh, S.O., Herzlinger, D., Soares, V.C., 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.

Horster, M.F., Braun, G.S., Huber, S.M., 1999. Embryonic renal epithelia: induction, nephrogenesis and cell differentiation. Phys. Rev. 79, 1157–1191.

Kamps, M.P., Murre, C., Sun, X.H., Baltimore, D.A., 1990. A new homeobox gene contributes the DNA binding domain of the t(1;19) translocation protein in pre-B ALL. Cell 60, 547–555.

Kim, S.K., Selleri, L., Lee, J.S., Zhang, A.Y., Gu, X., Jacobs, Y., Cleary, M.L., 2002. Pbx1 inactivation disrupts pancreas development and in Ipf1-deficient mice promotes diabetes mellitus. Nat. Genet. 30, 430–435.

Kispert, A., Vainio, S., Shen, L., Rowitch, D.H., McMahon, A.P., 1996. Proteoglycans are required for maintenance of *Wnt-11* expression in ureter tips. Development 122, 3627–3637.

Kreidberg, J.A., Sariola, H., Loring, J.M., Maeda, M., Pelletier, J., Housman, D., Jaenisch, R., 1993. WT-1 is required for early kidney development. Cell 74, 679–691.

Kuure, S., Vuolteenaho, R., Vainio, S., 2000. Kidney morphogenesis: cellular and molecular regulation. Mech. Dev. 92, 31–45.

Mann, R.S., Affloter, M., 1998. Hox proteins meet more partners. Curr. Opin. Genet. Dev. 8, 423–429.

Mendelsohn, C., Batourina, E., Fung, S., Gilbert, T., Dodd, J., 1999. Stromal cells mediate retinoid-dependent functions essential for renal development. Development 126, 1139–1148.

Moore, A.W., McInnes, L., Kreidberg, J., Hastie, N.D., Schedl, A., 1999. YAC complementation shows a requirement for Wt1 in the development of epicardium, adrenal gland and throughout nephrogenesis. Development 126, 1845–1857.

Nourse, J., Mellentin, J.D., Galili, N., Wilkinson, J., Stanbridge, E., Smith, S.D., Cleary, M.L., 1990. Chromosomal translocation t(1;19) results in synthesis of a homeobox fusion mRNA that codes for a potential chimeric transcription factor. Cell 60, 535–545.

Nowakowski, R.S., Lewin, B.S., Miller, M.W., 1989. Bromodeoxyuridine immunohistochemical determination of the length of the cell cycle and the DNA-synthetic phase for an anatomically defined population. J. Neurocytol. 18, 311–318.

Quaggin, S.E., Vanden Heuvel, G.B., Igarashi, P., 1998. *Pod-1,* a mesodermal-specific basic-helix- loop-helix protein expressed in the mesenchymal and glomerular epithelial cells in the developing kidney. Mech. Dev. 71, 37–48.

Quaggin, S.E., Schwartz, L., Cui, S., Igarashi, P., Deimling, J., Post, M., Rossant, J., 1999. The basic-helix–loop–helix protein Pod1 is critically important for kidney and lung organogenesis. Development 126, 5771–5783.

Pachnis, V., Mankoo, B., Costantini, F., 1993. Expression of the *c-ret* proto-oncogene during mouse embryogenesis. Development 119, 1005–1017.

Pepicelli, C.V., Kispert, A., Rowitch D.H., McMahon, A.P., 1997. GDNF induces branching and increased cell proliferation in the ureter of the mouse. Dev. Biol. 192, 193–198.

Rauskolb, C., Pfeifer, M., Wieschaus, E., 1993. Extradenticle, a regulator of homeotic gene activity, is a homolog of the homeobox-containing human proto-oncogene, pbx1. Cell 74, 1101–1112.

Sariola, H., Auferheide, E., Bernard, H., Henke-Fahl, S., Dippold, W., Ekblom, P., 1988. Antibodies to cell surface ganglioside GD3 perturb inductive epithelial-mesenchymal interactions. Cell 54, 235–245.

Saxen, L., 1997. Organogenesis of the Kidney. Cambridge University Press, Cambridge, UK.

Schnabel, C.A., Selleri, L., Jacobs, Y., Warnke, R., Cleary, M.L., 2001. Expression of Pbx1b during mammalian organogenesis. Mech. Dev. 100, 131–135.

Schuchardt, A., D'Agati, V., Pachnis, V., Costantini, F., 1996. Renal agenesis and hypodysplasia in ret-k- mutant mice result from defects in ureteric bud development. Development 122, 1919–1929.

Selleri, L., Depew, M.J., Jacobs, Y., Chanda, S.K., Tsang, K.Y., Cheah, K.S.E., Rubenstein, J.L.R., O'Gorman, S., Cleary, M.L., 2001. Requirement for *Pbx1* in skeletal patterning and programming chondrocyte proliferation and differentiation. Development 128, 3543–3557.

Srinivas, S., Wu, Z., Chen, C-M., D'Agati, V., Costantini, F. (1999). Dominant effects on RET receptor misexpression and ligand-independent RET signaling on ureteric bud development. Development 126, 1375–1386.

Torres, M., Gomez-Pardo, E., Dressler, G.R., Gruss, P., 1995. Pax-2 controls multiple steps of urogenital development. Development 121, 4057–4065.

Wilkenson, D., 1992. In: Wilkenson, D.G. (Ed.), In Situ Hybridization: A Practical Approach, IRL Press, Oxford, pp. 75–83.