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ERK and p38 MAP kinase are required for rat renal development

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Background. We previously demonstrated that extracellular signal-regulated protein kinase (ERK) and p38 mitogen-activated protein (MAP) kinase (p38) are strongly expressed in the embryonic kidney. In the present study, we investigated the role of ERK and p38 during kidney development.

Methods. Rat metanephroi were cultured from 15-day-old embryos, and exposed to inhibitors of MEK, an activator of ERK, PD98059 (300 μmol/L), U0126 (10 μmol/L), or a p38 inhibitor SB203580 (30 μmol/L) 24 to 120 hours after the start of culture. Growth of metanephroi was measured by surface area and thymidine incorporation. Ureteric buds and glomeruli were identified by labeling with *Dolichos biflorus* lectin and peanut agglutinin, respectively. PCNA staining and TUNEL assay were performed on kidney sections. The level of apoptosis was evaluated by examining DNA ladder formation.

Results. Growth of metanephroi was significantly inhibited by SB203580 but not by PD98059 or U0126. Ureteric bud branching was not affected by SB203580 or MEK inhibitors. Glomerular number was markedly reduced by SB203580 and to a lesser extent by U0126 (14 ± 2 and 48 ± 10% of controls, respectively). On histological examination, the number of tubuloglomerular structures was reduced in MEK inhibitor-treated metanephroi compared to controls. Very few mesenchymal condensates were observed in kidneys incubated with SB203580. PCNA-positive cells were reduced in SB203580-treated metanephroi compared to control and PD98059-treated kidneys. Apoptosis was increased in SB203580-treated kidneys and to a lesser extent in PD98059-treated cultures.

Conclusions. Both ERK and p38 are required for renal development. ERK appears to play a role in nephrogenesis and p38 for kidney growth and nephrogenesis.

The mitogen-activated protein kinase (MAPK) family is serine/threonine kinases that comprise three major subgroups, namely extracellular signal-regulated protein kinase (ERK), p38 MAPK (p38), and c-Jun N-terminal kinase/stress-activated protein kinase (JNK) [1]. ERK is activated by various growth factors and promotes cell proliferation and differentiation. In contrast, p38 and JNK are activated by cytokines and cellular stresses and thought to inhibit cell growth and induce apoptosis [2]. Kidney development is determined by proliferation, differentiation, and apoptosis. We previously reported ERK and p38 were strongly expressed and activated in rat embryonic kidney, whereas JNK was abundantly expressed in the adult kidney [3]. Furthermore, the spatial expression of ERK correlated with kidney development. Thus, ERK was ubiquitously expressed in early metanephric kidneys. Thereafter, ERK expression was confined to the nephrogenic zone, and then shifted to deep cortical layer and medullary region, correlating with nephrogenesis and tubule maturation. While ERK expression was reduced but maintained in the adult kidney, expression of p38 was confined to the embryonic kidney. These results strongly suggest the role of ERK and p38 during kidney development.

In the embryonic kidney, nephrons develop by induced mesenchyme-epithelium transition, and collecting ducts develop by ureteric bud branching and elongation [4]. There is a reciprocal interaction between metanephric mesenchyme and epithelial ureteric buds. The undifferentiated metanephric mesenchymal cells are rescued from programmed cell death by the signal from ureteric buds, condense around the tip of ureteric buds, and differentiate into epithelial cells. In turn, ureteric buds are rescued from degeneration by the signal from metanephric mesenchyme, branch and elongate to form collecting ducts. Various growth factors have been implicated in the mesenchymal-to-epithelial conversion and branching morphogenesis of ureteric buds. These include fibroblast growth factor (FGF), transforming growth factor (TGF), bone morphogenetic protein-7 (BMP-7), hepatocyte growth factor (HGF), glial cell-line derived neurotrophic factor (GDNF), and others [5]. Some of these factors have been shown to activate the ERK or p38 cascade in various cell types.

The present study examined the role of ERK and p38

Key words: mitogen-activated protein kinase, extracellular signal-regulated protein kinase, p38 mitogen-activated protein kinase, kidney, nephrogenesis, ureteric bud.

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during renal development using rat metanephric organ culture system. Inhibition of these enzymes by specific inhibitors should reveal the function of each enzyme, which contributes to our understanding of kidney development.

METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and fungizone were purchased from Gibco BRL (Rockville, MD, USA). Transwell polycarbonate filters were from Corning Costar (Acton, MA, USA). PD98059 was from Calbiochem Co. SB203580 was kindly provided from Smith Kline Beecham. U0126 was from Promega Co. (Madison, WI, USA). Neuraminidase and gelatin were from Sigma (St. Louis, MO, USA). Saponin was from Research Organics Inc. (Cleveland, OH, USA). Fluorescein-*Dolichos biflorus* agglutinin (DBA) and rhodamine-coupled peanut agglutinin (PNA) were from Vector Laboratories (Burlingame, CA, USA). SlowFade® Antifade Kit was from Molecular Probes, Inc. (Eugene, OR, USA). A monoclonal antibody specific for proliferating cell nuclear antigen (PCNA), peroxide-conjugated rabbit anti-mouse and swine anti-rabbit immunoglobulins, monoclonal antibody specific for Wilms' tumor 1 (WT1), and DAKO Protein K Enzyme Digestion were from DAKO A/S (Glostrup, Denmark). Rabbit polyclonal antibody for single stranded DNA (ssDNA) was from DAKO JAPAN (Kyoto, Japan). ApopTag Peroxides In Situ Apoptosis Detection Kit was from Intergen Company (Purchase, NY, USA). Monoclonal anti-ERK activated (P-ERK) was from Sigma, and anti-phospho-p38 (P-p38) was from New England Biolab (Beverly, MA, USA).

Metanephric organ culture

Sprague-Dawley rats were purchased from Sankyo Labo-Service Co. (Tokyo, Japan). Pregnancy was determined by the detection of a vaginal plug. Before removal of the embryos, pregnant rats were sedated with an intraperitoneal injection of sodium pentobarbital. The method of organ cultures was based on that of Saxn and Lehtonen [6]. Briefly, metanephroi and associated ureteric buds were microdissected en block from 15-day-old rat embryos, and were cultured for 24 hours on polycarbonate filters (pore size, 3.0 μm) in DMEM, supplemented with 10% FBS, penicillin 100 U/mL, streptomycin 100 μg/mL, and fungizone 250 μg/mL under 5% CO_2 at 37°C. Kidneys were then treated with vehicle, MEK inhibitors PD98059 300 μmol/L, U0126 10 μmol/L, or a p38 inhibitor SB203580 30 μmol/L for 96 hours. PD98059 and U0126 inhibit MEK1 with IC_{50} values of 2 to 7 μmol/L and 0.1 μmol/L, respectively [7, 8]. SB203580 inhibits p38α and p38β with an IC_{50} of 0.3 to 0.6 μmol/L [9, 10]. Since tissue penetration of the inhibitors may be restricted, we determined the dose of inhibitors in a pilot study as the lowest concentration to affect the ureteric bud width for PD98059 and U0126, and growth of kidney size for SB203580. Medium was changed every 24 hours. The cultured kidneys were photographed, and the surface area was measured using NIH Image [11]. After 120 hours of culture, the metanephroi were processed for the following experiments. At least three independent experiments were performed for each study.

Evaluation of branching and nephron formation

Ureteric buds and nephrons were visualized after labeling the explants with DBA and PNA, respectively [12, 13]. For detection of ureteric buds, cultured metanephroi were fixed with cold methanol for 10 minutes, washed three times with PBT (0.1% Tween 20 in PBS), and incubated with DBA (dilution 1:25) for two hours at room temperature. Samples were washed three times with PBT, mounted in SlowFade® to obtain stable fluorescence. For detection of glomerular structures, metanephroi were fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) supplemented with 1 mmol/L $MgCl_2$ and 0.1 mmol/L $CaCl_2$ (PBS/CM) for two hours at 4°C, and were rinsed in PBS/CM. After overnight incubation in 50 mmol/L NH_4Cl at 4°C, specimens were permeabilized with 0.075% saponin detergent. After incubation with 0.2% gelatin for 30 minutes, the explants were washed twice in neuraminidase buffer (50 mmol/L Na acetate, 150 mmol/L NaCl, 9 μmol/L $CaCl_2$, pH 5.5) and incubated for three hours at 37°C with 1 IU/mL of *vibrio cholerae* neuraminidase. After rinsing with PBS/CM, explants were labeled for one hour with rhodamine-coupled PNA diluted in PBS/CM with saponin and gelatin. After several washes, explants were mounted in SlowFade®, and viewed under an OLYMPUS BX-50 (Olympus Optical Co., Ltd., Tokyo, Japan). The number of branching points and glomeruli were counted on photographs. Ureteric bud width in the third branch was measured using real-time computer-assisted monitoring device (Fujix Digital Camera HC-2500 and Fujix Photograb-2500 software, Fuji Photo Film Co., Ltd., Tokyo, Japan).

Immunohistochemistry

After fixation, cultured kidneys were embedded in paraffin. Immunohistochemical staining was performed on serial sections 3 μm thick, using the enzyme-labeled antibody method. Paraffin sections were deparaffined and rehydrated. Endogenous peroxide activity was quenched by incubating sections in 0.3% H_2O_2/methanol for 15 minutes in 10% citrate buffer (pH 6.0)/methanol. Sections were incubated with antibodies against PCNA (1:100), WT1 (1:50), or ssDNA (1:100). The incubation time was overnight at 4°C. After incubating with secondary antibody (1:100), immunoreaction products were developed

using 3,3′-diaminobenzidine (DAB) as the chromogen, with standardized development times. Sections were then counterstained with methyl green.

TUNEL assay

Cells undergoing apoptosis were identified using an in situ DNA labeling method. Paraffin embedded sections were deparaffined, and terminal deoxynucleotide transferase–mediated nick-end labeling (TUNEL) staining was performed using the Apoptag kit. Sections were counterstained with methyl green. TdT was omitted from the staining procedure in negative controls.

DNA fragmentation assay

DNA was isolated from cultured metanephroi and fragmentation was detected using TACS Apoptotic DNA Laddering Kit from TREVIGEN (Gaithersburg, MD, USA). Samples were subjected to electrophoresis on a 1.5% agarose gel, and transferred to nitrocellulose membrane (Trans-Blot; Bio-Rad Lab, Hercules, CA, USA). Nonspecific binding sites were blocked in PBS containing 5% skim milk at room temperature for 30 minutes. Blots were washed and processed for fragmentation detection with chemiluminescent system.

[^{3}H]-thymidine incorporation studies

Metanephroi were cultured for 24 hours, and then exposed to 1 μCi [^{3}H]-thymidine with or without MEK or p38 inhibitors for 96 hours. Explants were washed with cold PBS twice and treated with 5% trichloroacetic acid for 30 minutes at 90°C. The hydrolysates were centrifuged, and the supernatants were counted by a liquid scintillation counter.

Immunoblot analysis

Metanephroi were homogenized, and insoluble material was removed by centrifugation (10,500 × g, 10 min). The protein content of kidney lysates was measured using DC protein assay (Bio-Rad Laboratories, Tokyo, Japan). Lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidine difluoride (PVDF) membranes (Immobilon; Millipore Corp, Bedford, MA, USA). Non-specific binding sites were blocked in TBS buffer (10 mmol/L Tris-Cl, pH 7.4, 0.15 mol/L NaCl) containing 5% skim milk overnight at 4°C or for one hour at 25°C. Antibodies were added to TBS in saturating titers and incubated with mixing for two hours at 25°C. Blots were washed, and incubated with peroxidase-labeled secondary antibodies for one hour. Membranes were developed using the enhanced chemiluminescent (ECL) Western blotting system (Amersham, Buckinghamshire, UK). At least three independent experiments were performed with similar results.

Statistical analysis

The results are expressed as mean ± SEM. Statistical analysis was performed with analysis of variance (ANOVA) followed by multiple comparisons as appropriate. Statistical significance was determined as $P < 0.05$.

RESULTS

Effects of ERK or p38 inhibition on cultured kidney growth

During 120 hours of culture, control explants increased in size, as measured by surface area, by 2.9-fold ($P < 0.05$, Fig. 1 A, B). Control explants at the end of culture were about half the size of kidneys harvested from 20-day-old rat embryos. Metanephric kidneys exposed to ERK inhibitors PD98059 or U0126 displayed slightly decreased growth compared to controls, but the change was not statistically significant (90 ± 6 and 92 ± 8%, respectively). In contrast, the p38 inhibitor SB203580 significantly inhibited metanephros growth by 30 ± 7% ($P < 0.05$). Similarly, thymidine incorporation was not affected by MEK inhibitors, but was slightly but significantly decreased by SB203580 (Fig. 1C). The phosphorylated forms of ERK or p38 were significantly decreased in cultures treated with MEK or p38 inhibitors, respectively, compared with controls (Fig. 1D). The results confirmed the specific actions of PD98059, U0126, or SB203580.

Effects of ERK or p38 inhibition on ureteric bud growth

To investigate the effect of ERK or p38 inhibition on branching morphogenesis, we examined binding of DBA, a lectin specific for the ureteric bud. There was no significant difference in the number of branching points among controls, metanephroi treated with PD98059, U0126, or SB203580 after 120 hours of culture (Fig. 2 A, B). On the other hand, ureteric bud width was significantly smaller in kidneys treated with PD98059 or U0126, and larger in kidneys treated with SB203580 than that in controls (Fig. 2C).

Effects of ERK or p38 inhibition on glomerular number

To investigate the effect of ERK or p38 inhibition on nephron formation, kidneys were labeled with PNA, which stains podocytes of glomeruli (Fig. 3A). After 120 hours of culture, the number of glomeruli was markedly reduced in SB203580-treated metanephroi and to a lesser degree in U0126-treated cultures (14 ± 2 and 48 ± 10% of controls, respectively, Fig. 3 A and B, lower column). Since the size of metanephroi treated with SB203580 was significantly less than that of controls, the glomerular number was corrected by kidney surface area. The differ-

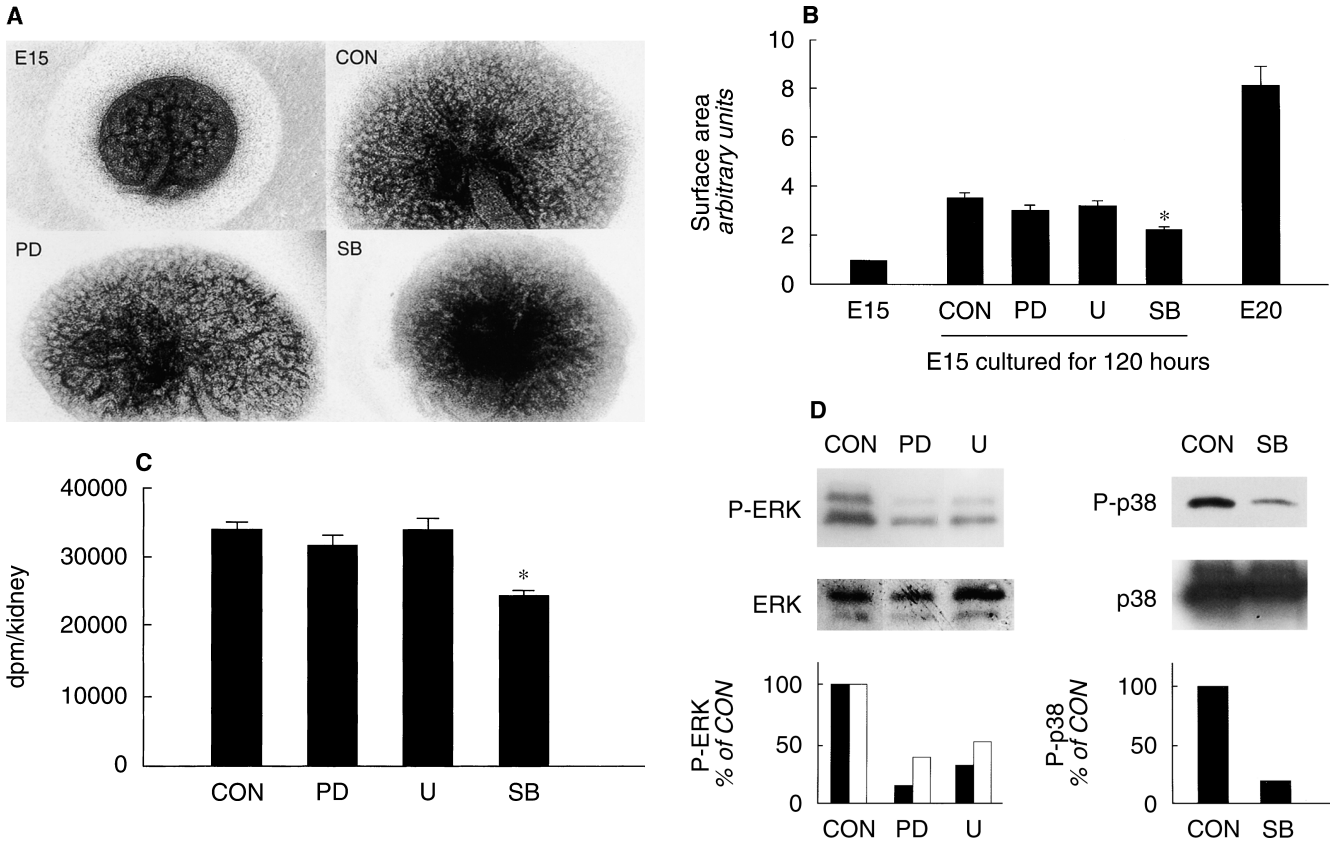

Fig. 1. Effects of ERK or p38 inhibition on cultured embryonic kidney growth. Metanephroi were freshly dissected from 15-day-old embryos (E15), cultured for 24 hours, and exposed to vehicle (CON), 300 μmol/L PD98059 (PD), 10 μmol/L U0126 (U), or 30 μmol/L SB203580 (SB) for 96 hours. (*A*) Photomicrographs of metanephroi. (*B*) Quantitative analysis of metanephros surface area. Kidneys freshly harvested from 20-day-old embryos (E20) were analyzed for comparison. Values are mean ± SEM. $^*P < 0.05$ vs CON, $N = 9$. (*C*) Thymidine incorporation. Values are mean ± SEM. $^*P < 0.05$ vs CON, $N = 4$. (*D*) Western blot analysis using anti-phospho-ERK (P-ERK) or anti-phospho-p38 (P-p38) antibody. Quantitative analysis is shown below. Symbols are: (■) p44 ERK; (□) p42 ERK.

ence was also statistically significant after correction (20 ± 3% of controls). To exclude the possibility of degeneration of already induced glomeruli, embryonic day 15 (E15) kidneys were exposed to the inhibitors from 24 to 48 hours of culture (Fig. 3B, upper column). Similar effects of U0126 and SB203580 were observed for shorter period of incubation.

Hematoxylin-eosin staining

Nephron formation was evaluated histologically after 120 hours of culture. In control kidneys (Fig. 4a), condensing mesenchyme, vesicles, comma- and S-shaped bodies were observed, which was similar to the metanephroi harvested from 20-day rat embryos (Fig. 4e). In PD98059-treated metanephroi, developing tubuloglomerular structures were observed, but the number was reduced in accord with the results of PNA binding (Fig. 4b). In SB203580-treated cultures, a few signs of mesenchymal condensations were observed, but kidneys were mostly filled with densely packed cells (Fig. 4c). Metanephroi exposed to SB203580 for 24 hours and then cultured in control medium for 72 hours showed mesenchymal condensates and distinct tubular structures (Fig. 4d).

PCNA staining

In control and PD98059-treated kidneys, PCNA-positive cells were distributed throughout the kidney (Fig. 5 a, b). Uninduced mesenchymal cells, condensing mesenchyme, and ureteric bud epithelial cells showed strong staining. Comma- and S-shaped bodies were weakly stained. In SB203580-treated kidneys, PCNA staining was reduced and restricted to mesenchymal cells in subcapsular cortical area, and ureteric bud epithelia (Fig. 5c). These results were in agreement with the results of thymidine incorporation.

Apoptosis

In control kidneys, TUNEL staining was observed weakly in the cortex and occasionally in ureteric bud epithelial cells (Fig. 6a). The proportion of apoptotic

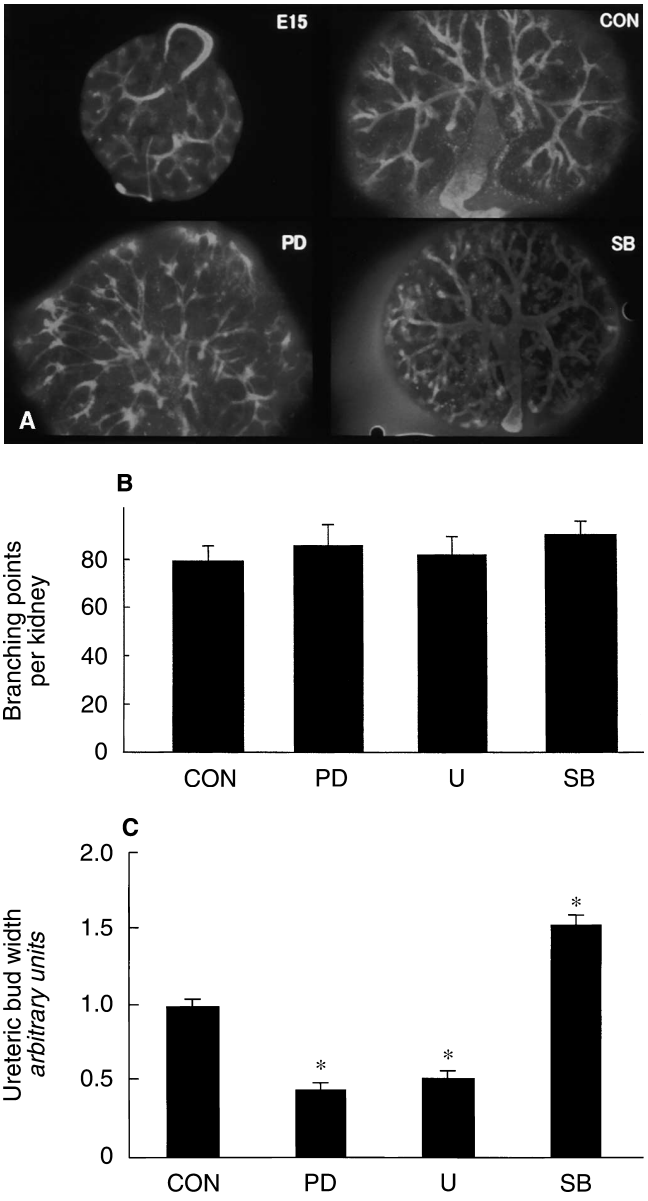

Fig. 2. Effects of ERK or p38 inhibition on ureteric bud growth. Metanephroi were freshly dissected from 15-day-old embryos (E15), cultured for 24 hours, and exposed to vehicle (CON), 300 μmol/L PD98059 (PD), 10 μmol/L U0126 (U), or 30 μmol/L SB203580 (SB) for 96 hours. (*A*) Ureteric buds were visualized with DBA-staining of whole mount of organ culture. (*B*) Quantitative analysis of branching points. (*C*) Quantitative analysis of ureteric bud width measured in the third branch. Values are mean ± SEM. $*P < 0.05$ vs. CON, $N = 6$.

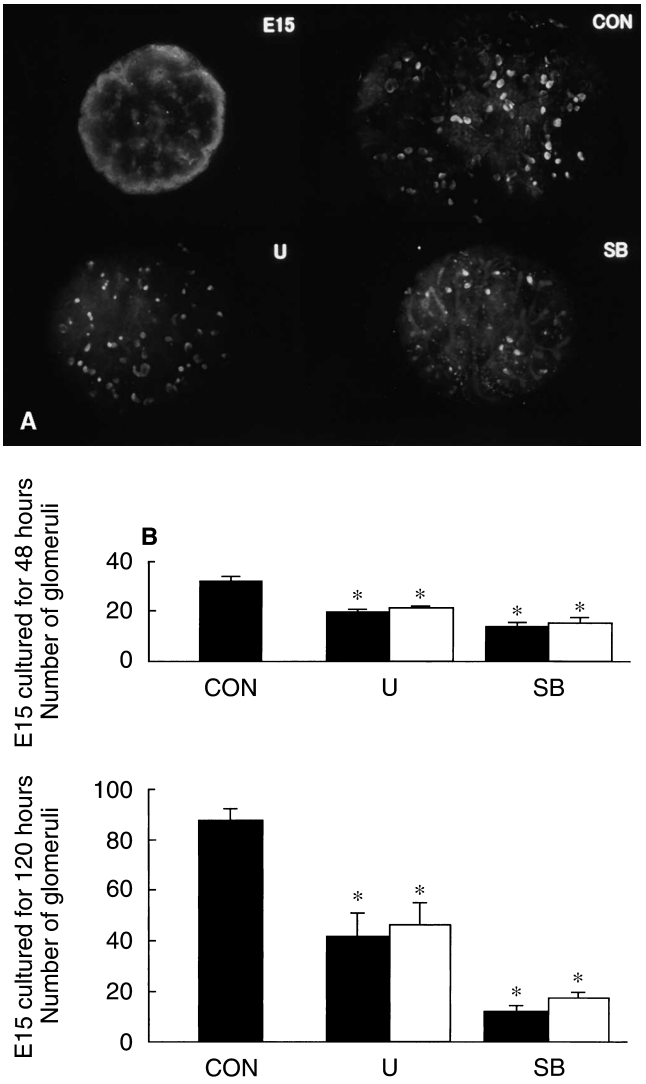

Fig. 3. Effects of ERK or p38 inhibition on glomerular number. Metanephroi were freshly dissected from 15-day-old embryos (E15), cultured for 24 hours, and exposed to vehicle (CON), 10 μmol/L U0126 (U), or 30 μmol/L SB203580 (SB) for 24 or 96 hours. (*A*) Glomerulotubular structures visualized with PNA-staining of whole mount of organ culture at 120 hours. (*B*) Quantitative analysis of glomerular number/kidney (■), and glomerular number/kidney surface area (□). Values are mean ± SEM. $*P < 0.05$ vs. CON, $N = 3$.

cells was increased slightly in PD98059-treated kidneys, and markedly in SB203580-treated cultures (Fig. 6 b, c). Intense staining was observed in the interstitium of the medulla, as well as in uninduced mesenchymal cells in the cortical parts of metanephroi, where mesenchymal differentiation takes place under normal conditions. Similar results were obtained by ssDNA staining (Fig. 7). Furthermore, characteristic apoptotic changes in nuclear morphology was observed in SB203580-treated cultures and occasionally in PD98059-treated kidneys (Fig. 8). Finally, apoptosis was confirmed by DNA ladder (Fig. 9). In accord with the results of TUNEL assay, ssDNA staining, and nuclear morphology, DNA ladder formation was most obvious in SB203580-treated kidneys, and also detected in cultures treated with MEK inhibitors. No DNA fragmentation was observed in control kidneys.

WT1

The WT1 Wilms' tumor suppressor gene expression is low in uninduced mesenchyme, and increases during

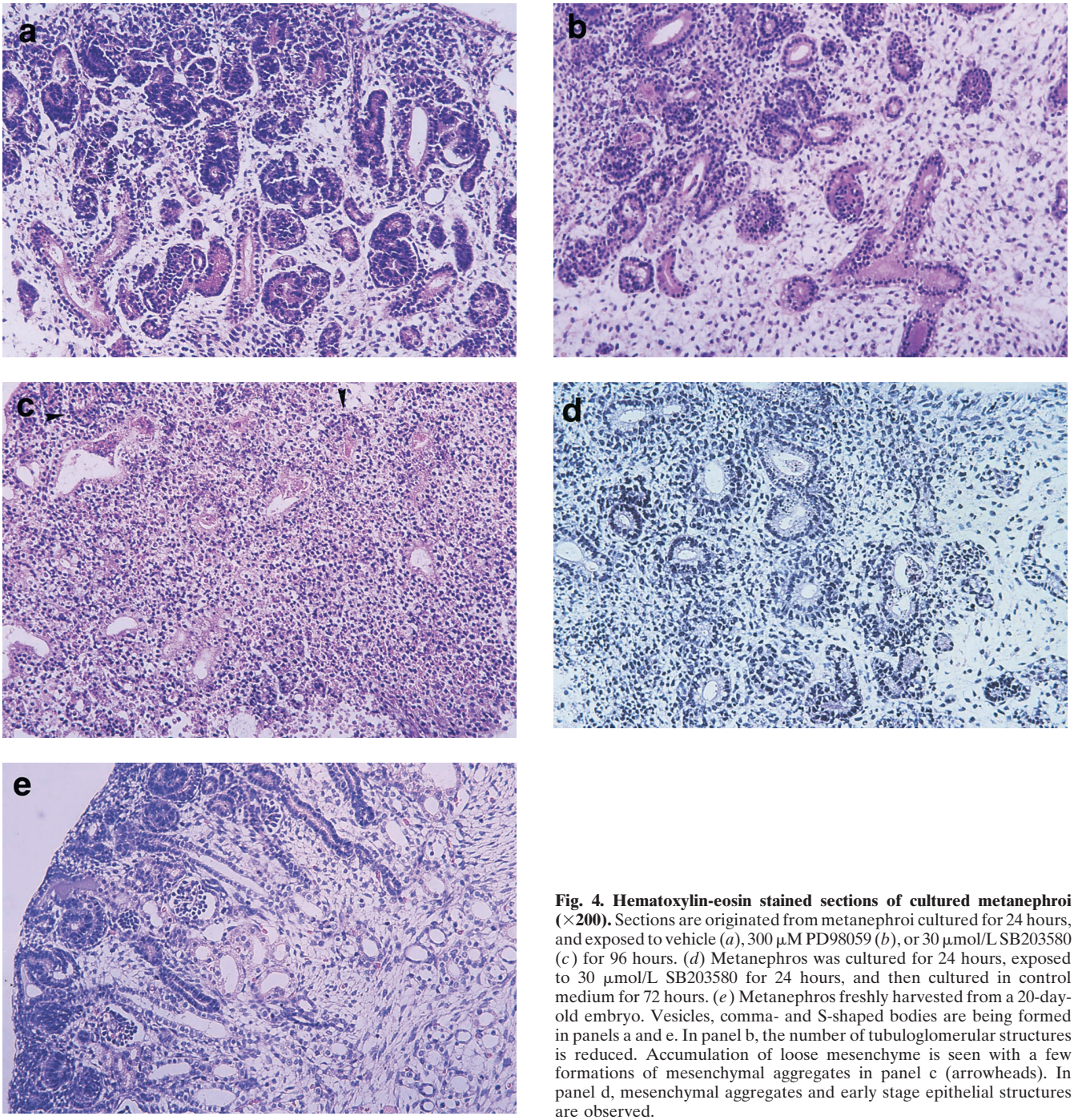

Fig. 4. Hematoxylin-eosin stained sections of cultured metanephroi (×200). Sections are originated from metanephroi cultured for 24 hours, and exposed to vehicle (*a*), 300 μM PD98059 (*b*), or 30 μmol/L SB203580 (*c*) for 96 hours. (*d*) Metanephros was cultured for 24 hours, exposed to 30 μmol/L SB203580 for 24 hours, and then cultured in control medium for 72 hours. (*e*) Metanephros freshly harvested from a 20-day-old embryo. Vesicles, comma- and S-shaped bodies are being formed in panels a and e. In panel b, the number of tubuloglomerular structures is reduced. Accumulation of loose mesenchyme is seen with a few formations of mesenchymal aggregates in panel c (arrowheads). In panel d, mesenchymal aggregates and early stage epithelial structures are observed.

mesenchyme-epithelium conversion. In control explants, WT1 expression was observed in condensing mesenchyme, vesicles, and prepodocytes of comma- and S-shaped bodies (Fig. 10a). In PD98059-treated kidneys, distribution of WT1-positive cells was similar to that in controls (Fig. 10b). In SB203580-treated cultures, on the other hand, WT1-positive cells were distributed loosely in mesenchyme. Cells around ureteric buds, which showed some signs of condensation or compaction of mesenchyme, tended to have stronger expression (Fig. 10c).

DISCUSSION

The present study using rat metanephric organ culture demonstrates that both ERK and p38 are required for normal kidney development. Kidney growth and induc-

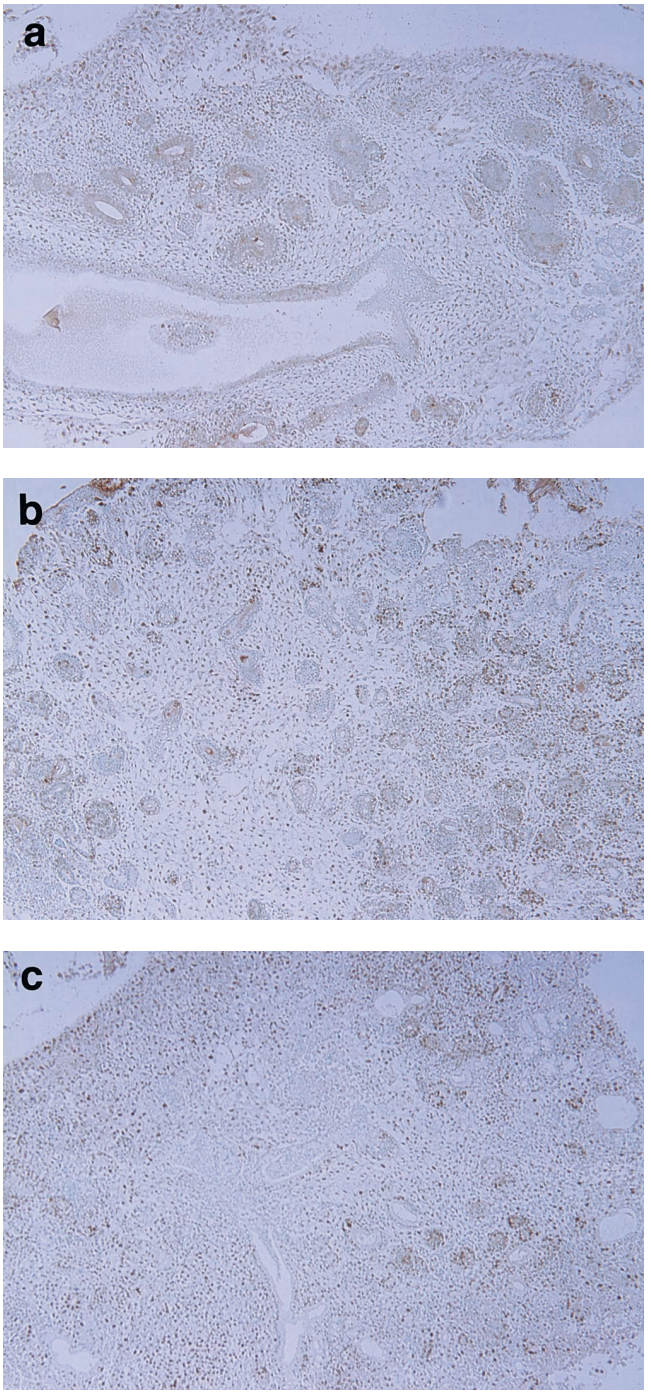

Fig. 5. Immunohistochemical localization of PCNA staining (×100). Sections are originated from metanephroi cultured for 24 hours, and exposed to vehicle (*a*), 300 μmol/L PD98059 (*b*), or 30 μmol/L SB203580 (*c*) for 96 hours. PCNA-positive cells were distributed throughout the kidney in panels a and b. In panel c, PCNA-staining was reduced and restricted to the periphery.

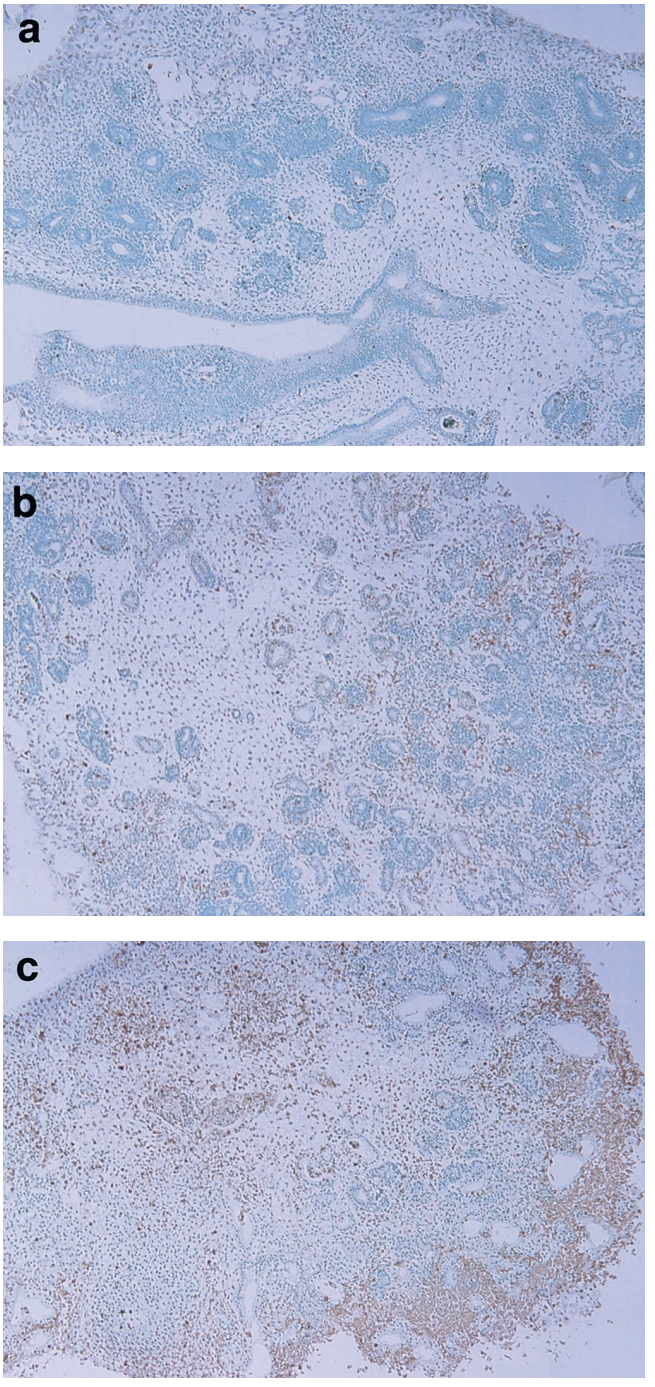

Fig. 6. TUNEL staining of cultured metanephroi (×100). Labeling of apoptotic cells using TUNEL method. Sections are originated from metanephroi cultured for 24 hours, and exposed to vehicle (*a*), 300 μmol/L PD98059 (*b*), or 30 μmol/L SB203580 (*c*) for 96 hours. TUNEL-positive cells were observed weakly in the cortex and occasionally in ureteric bud epithelial cells in a. Apoptotic cells were markedly increased in cortical area, and also were observed in the interstitium of the medulla in panel c, and to a lesser extent in panel b.

tion of nephrons are markedly suppressed by p38 inhibition. On the other hand, ERK inhibition attenuated nephron formation with minimal effect on kidney size. Ureteric bud branching was not affected by either p38 or ERK inhibition.

Mitogen-activated protein kinases have been reported to be essential in embryogenesis. Overexpression of active ERK induced mesoderm in *Xenopus* [14]. In contrast, inactivation of ERK by overexpression of MAPK

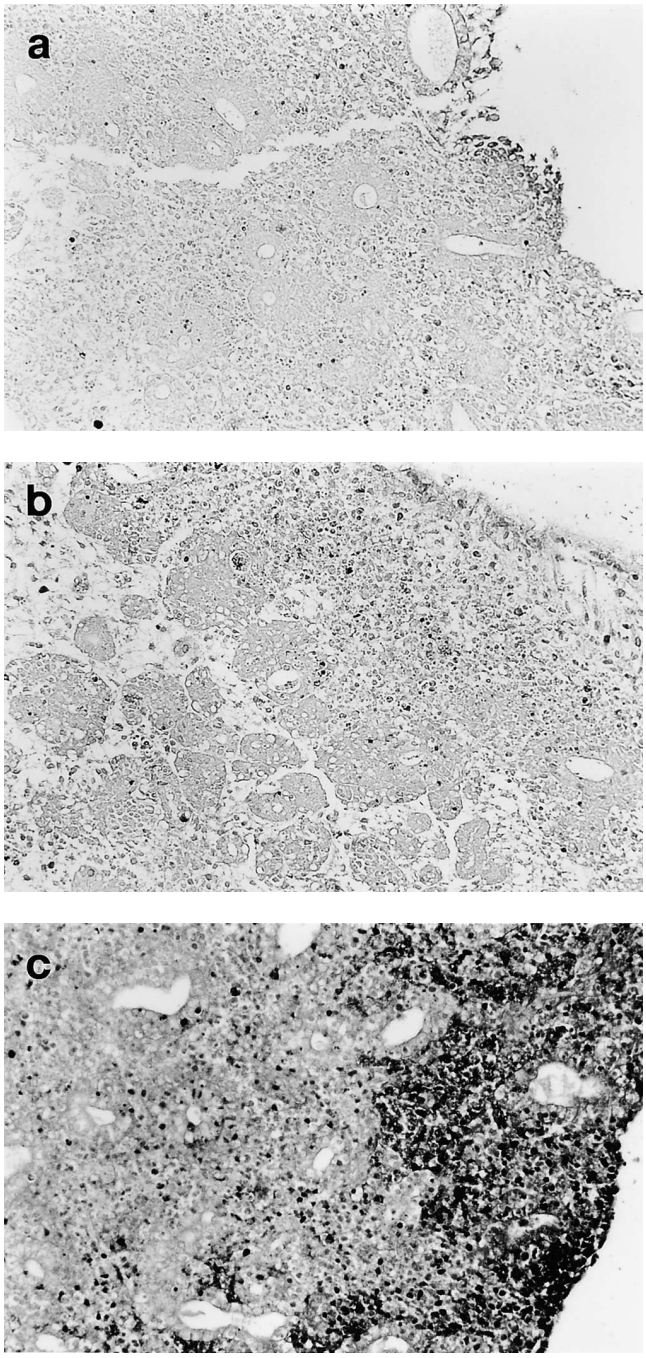

Fig. 7. Single strand DNA (ssDNA) staining of cultured metanephroi (×200). Labeling of apoptotic cells using immunohistochemical staining for ssDNA. Sections are originated from metanephroi cultured for 24 hours, and exposed to vehicle (*a*), 300 μmol/L PD98059 (*b*), or 30 μmol/L SB203580 (*c*) for 96 hours. ssDNA-positive cells were observed weakly in the cortex in panel a. Positive cells were markedly increased in cortical area, and also were observed in the interstitium of the medulla in panel c, and to a lesser extent in panel b.

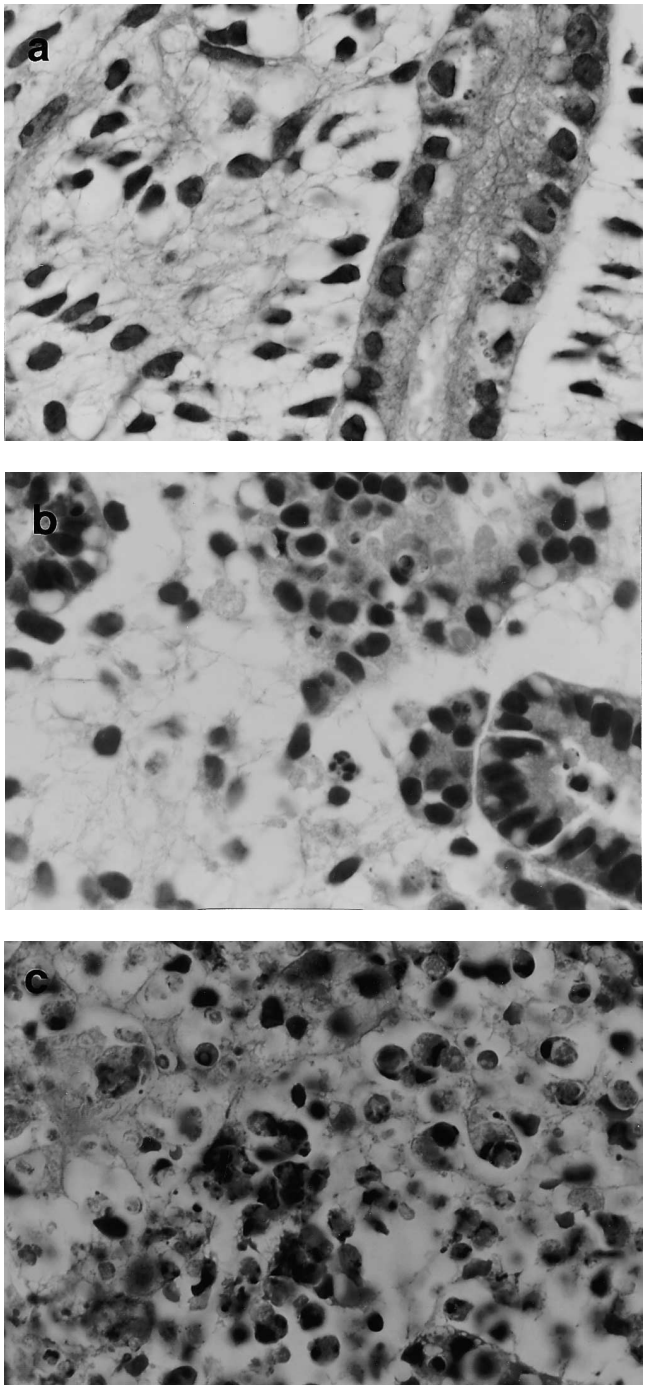

Fig. 8. Nuclear morphology (×1000). H&E staining of sections originated from metanephroi cultured for 24 hours, and exposed to vehicle (*a*), 300 μmol/L PD98059 (*b*), or 30 μmol/L SB203580 (*c*) for 96 hours. Most nuclei in c are small, dense, and fragmented compared to those in panel a. Several apoptotic nuclei are also observed in panel b.

phosphatase-1 disrupted normal mesoderm induction [15]. Mice lacking MEK1, an upstream activator of ERK, have been reported to die at 10.5 days of gestation [16]. p38 also has been suggested to be important in organogenesis. Targeted disruption of SEK1, an activator of p38, caused increased apoptosis of hepatocytes and resulted in embryonic death before 14 days of gestation [17]. On the other hand, targeting only one isozyme of ERK or p38 did not have obvious effects on kidney organogenesis, probably because of the existence of redundant sig-

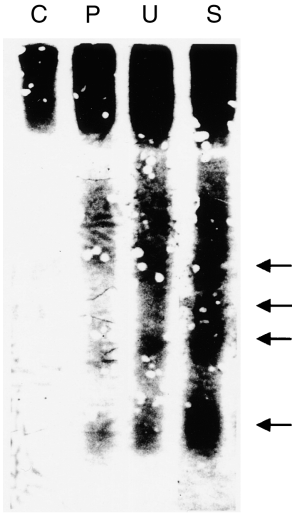

Fig. 9. DNA ladder formation. Metanephroi were freshly dissected from 15-day-old embryos (E15), cultured for 24 hours, and exposed to vehicle (C), 300 μmol/L PD98059 (P), 10 μmol/L U0126 (U), or 30 μmol/L SB203580 (SB) for 96 hours. Kidneys were then processed for DNA fragmentation assay as described in the **Methods** section. Control metanephroi exhibited no DNA fragmentation. Oligonucleosome-sized DNA fragments were observed in SB203580-treated, and to a lesser degree, PD98059- or U0126-treated cultures as indicated by arrows.

naling pathway [18, 19]. Thus, one needs another method to investigate the role of ERK and p38 in kidney development. We used metanephric organ culture system in the present study.

The size and thymidine incorporation was significantly less in metanephroi treated with SB203580 compared with controls. Reduced proliferation was confirmed by PCNA staining. Furthermore, SB203580-treated kidneys were characterized by increased apoptosis demonstrated by TUNEL assay and DNA fragmentation. These results suggest that p38 is important in cell proliferation as well as rescue of metanephric mesenchyme from apoptosis. During kidney development, large-scale apoptosis has been reported to occur [20]. It is hypothesized that the default pathway for a mesenchymal cell in the absence of survival factors is to die. Although p38 has been suggested to inhibit proliferation and to induce apoptosis, we previously showed that p38 expression and activation correlated with proliferation rather than apoptosis in rat embryonic kidney [3]. The present study further supports the role of p38 in rescue of apoptosis and cell proliferation during kidney development. In contrast, MEK inhibitors had minor effects on kidney growth and cell proliferation. Apoptotic cells were only slightly increased in PD98059-treated metanephroi compared to controls. Of note, the spatial expression of p38 and ERK were different in the embryonic kidney [3]. Thus, p38 was uniformly expressed in the embryonic kidney, whereas the location of ERK changed during kidney development from the periphery toward the medulla, correlating with nephrogenesis and tubule maturation. Together with the present results, ERK may be involved in differentiation, whereas p38 may have a role in both proliferation and differentiation of mesenchymal cells.

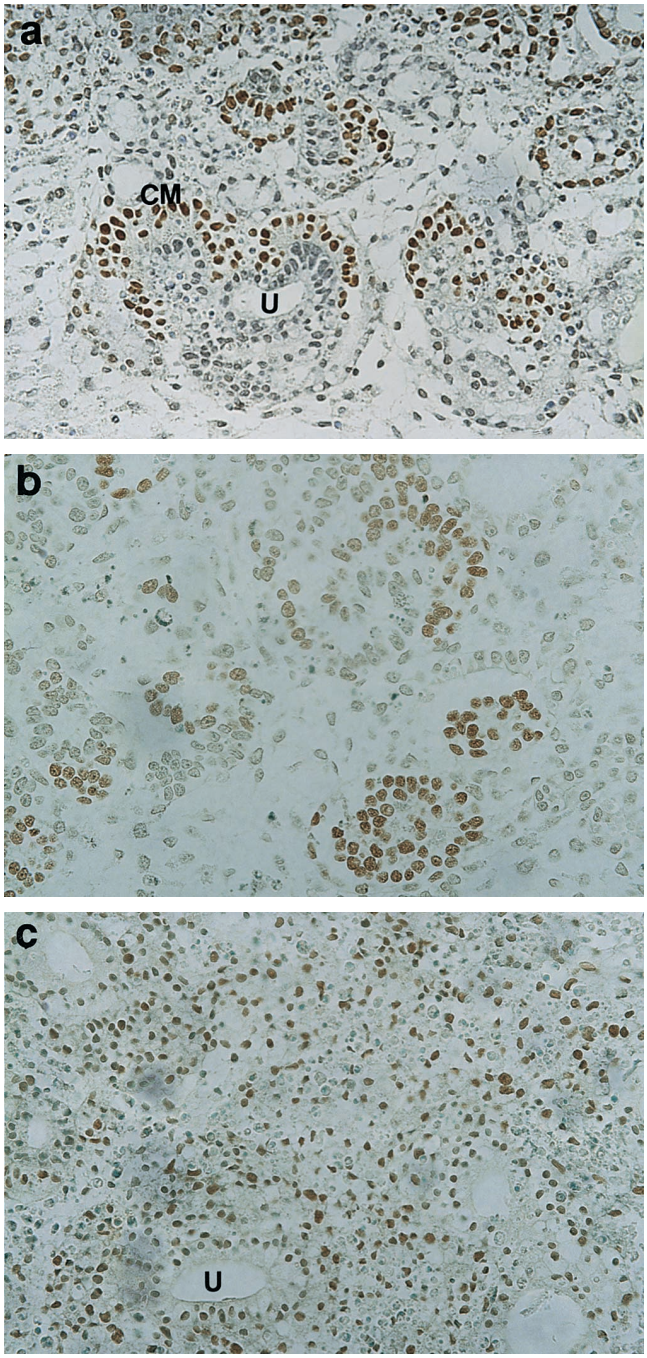

Fig. 10. Immunohistochemical localization of WT1 in cultured metanephroi. Sections are originated from metanephroi cultured for 24 hours, and exposed to vehicle (*a*), 300 μmol/L PD98059 (*b*), or 30 μmol/L SB203580 (*c*) for 96 hours (×400). Abbreviations are: U, ureteric bud; CM, condensing mesenchyme. In panels a and b, WT1 was expressed in mesenchymal cells surrounding ureteric buds and in epithelial cells destined to become podocytes of comma- and S-shaped bodies. In panel c, weak staining was observed diffusely in mesenchymal cells. Cells around ureteric buds tended to have stronger expression.

Induction of the metanephric mesenchyme and epithelialization were suppressed by the inhibition of p38 or ERK. Glomerular number as detected by PNA binding was markedly reduced by SB203580 and to a lesser extent by U0126. On histological examination, condensing mesenchyme, comma- and S-shaped bodies were present, although reduced in number, in MEK inhibitor-treated kidneys. In SB203580-treated cultures, very few mesenchymal condensates were observed. Of note, the expression of WT1, the earliest events during induction, was preserved in PD98059-treated metanephroi, but markedly altered in SB203580-treated cultures. Thus, WT1 expression was weak, and loosely distributed in mesenchyme of explants incubated with SB203580. Along with the results of PNA binding, p38 may have a more important role in mesenchymal-to-epithelial transformation than ERK. Whether p38 suppression directly inhibits WT1 expression or via suppression of mesenchymal condensation remains to be clarified.

Mesenchymal-to-epithelial transformation requires rescue from apoptosis, mitogenesis, and differentiation [4]. Factors produced by ureteric buds are thought to mediate these events [21]. Importantly, SB203580 and MEK inhibitors suppressed nephron formation despite normal ureteric bud branching. Thus, p38 and ERK may mediate the signal of molecules secreted from ureteric buds. FGF2 is one of factors implicated in metanephric mesenchyme induction. It can promote condensation of mesenchyme and mediate the transcriptional activation of WT1, the earliest events during induction, although it cannot promote the epithelial conversion [22]. Other factors implicated in mesenchymal-to-epithelial conversion are Wnt-4 and BMP-7 [23, 24]. Wnt-4 deletion in mice results in a lack of mesenchymal condensation despite normal ureteric bud branching, and exogenous Wnt-4 into Wnt-4 deficient mesenchyme induced epithelialization [23, 25]. BMP-7 has been shown to induce epithelial glomerulus-like structure in metanephric mesenchyme [24]. BMP-7-deletion results in hypocellular kidneys with few nephrons and accumulation of loose interstitial mesenchyme, which are reminiscent of the kidneys treated with SB203580 [26]. More recently, conditioned medium from ureteric bud cells has been shown to induce nephron differentiation in combination with FGF2 [27]. Purification and sequencing of one of factors secreted by ureteric bud cells identified it as leukemia inhibitory factor (LIF), a molecule of the interleukin-6 (IL-6) family [28]. Cellular signaling of FGF2, Wnt-4, BMP-7, and LIF in metanephric mesenchymal cells are largely unknown. FGF2 has been reported to stimulate proliferation in mouse kidney mesenchymal cells. Since FGF2 activates p38 and ERK in other cells [29, 30], FGF2-induced proliferation of mesenchymal cells may involve p38 or ERK. The role of p38 and ERK in the signaling of other molecules in metanephric mesenchymal cells remains to be determined.

While nephron formation was suppressed by the inhibition of p38 or ERK, neither SB203580 nor PD98059 affected the branching of ureteric buds. In rats, E13 kidney represents the earliest developmental state of the metanephric kidney. Since we used E15 kidneys, we cannot exclude the possibility that p38 or ERK inhibition might have affected ureteric bud branching at an earlier stage. The significance and mechanism of the effects of MEK inhibitors or SB203580 on ureteric bud width remain to be clarified.

In conclusion, the present study points toward important roles for ERK and p38 in embryonic kidney growth and development. Further studies examining the role of ERK and p38 in specific cell types and their interactions may provide a better understanding of the mechanisms that lead to kidney malformation. Renal developmental abnormality is thought to be caused by teratogens, maternal nutrition, obstruction, and mutations [31]. Various physical and chemical factors have been shown to affect MAPK activity [1]. It is therefore conceivable that extracellular stimuli may perturb ERK or p38 signaling necessary for normal renal development, resulting in kidney malformation.

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REFERENCES

1. Bokemeyer D, Sorokin A, Dunn MJ: Multiple intracellular MAP kinase signaling cascades. *Kidney Int* 49:1187–1198, 1996
2. Xia Z, Dickens M, Raingeaud J, *et al*: Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* 270:1326–1331, 1995
3. Omori S, Hida M, Ishikura K, *et al*: Expression of mitogen-activated protein kinase family in rat renal development. *Kidney Int* 58:27–37, 2000
4. Stuart RO, Nigam SK: Developmental biology of the kidney, in *The Kidney* (6th ed), edited by Brenner BM, Philadelphia, WB Saunders, 2000, pp 68–92
5. Clark AT, Bertram JF: Molecular regulation of nephron endowment. *Am J Physiol* 276:F485–F497, 1999
6. Saxn L, Lehtonen E: Embryonic kidney in organ culture. *Differentiation* 36:2–11, 1987
7. Alessi DR, Cuenda A, Cohen P, *et al*: PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. *J Biol Chem* 270:27489–27494, 1995
8. Favata MF, Horiuchi KY, Manos EJ, *et al*: Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J Biol Chem* 273:18623–18632, 1998
9. Cuenda A, Rouse J, Doza YN, *et al*: SB 203580 is a specific in-

hibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett* 364:229–233, 1995
10. Goedert M, Cuenda A, Craxton M, *et al*: Activation of the novel stress-activated protein kinase SAPK4 by cytokines and cellular stresses is mediated by SKK3 (MKK6); comparison of its substrate specificity with that of other SAP kinases. *EMBO J* 16:3563–3571, 1997
11. Quaggin SE, Yeger H, Igarashi P: Antisense oligonucleotides to Cux-1, a Cut-related homeobox gene, cause increased apoptosis in mouse embryonic kidney culture. *J Clin Invest* 99:718–724, 1997
12. Holthofer H: Lectin binding sites in kidney. *J Histochem Cytochem* 31:531–537, 1983
13. Gilbert T, Gaonach S, Moreau E, Merlet-Benichou C: Defect of nephrogenesis induced by gentamicin in rat metanephric organ culture. *Lab Invest* 70:656–666, 1994
14. Umbhauer M, Marshall CJ, Mason DS, *et al*: Mesoderm induction in xenopus caused by activation of MAP kinase. *Nature* 376:58–62, 1995
15. LaBonne C, Burke B, Whitman M: Role of MAP kinase in mesoderm induction and axial patterning during *Xenopus* development. *Development* 121:1475–1486, 1995
16. Giroux S, Tremblay M, Bernard D, *et al*: Embryonic death of Mek-deficient mice reveals a role for this kinase in angiogenesis in the labyrinthine region of the placenta. *Curr Biol* 9:369–372, 1999
17. Ganiatsas S, Kwee L, Fujiwara Y, *et al*: SEK1 deficiency reveals mitogen-activated protein kinase cascade crossregulation and leads to abnormal hepatogenesis. *Proc Natl Acad Sci USA* 95:6881–6886, 1998
18. Pages G, Guerin S, Grall D, *et al*: Defective thymocyte maturation in p44 MAP kinase (Erk 1) knockout mice. *Science* 286:1374–1377, 1999
19. Adams RH, Porras A, Alonso G, *et al*: Essential role of p38α MAP kinase in placental but not embryonic cardiovascular development. *Mol Cell* 6:109–116, 2000
20. Coles HS, Burne JF, Raff MC: Large-scale normal cell death in the developing rat kidney and its reduction by epidermal growth factor. *Development* 118:777–784, 1993
21. Barasch J, Qiao J, McWilliams G, *et al*: Ureteric bud cells secrete multiple factors, including bFGF, which rescue renal progenitors from apoptosis. *Am J Physiol* 273:F757–F767, 1997
22. Perantoni AO, Dove LF, Karavanova I: Basic fibroblast growth factor can mediate the early inductive events in renal development. *Proc Natl Acad Sci USA* 92:4696–4700, 1995
23. Stark K, Vainio S, Vassileva G, McMahon AP: Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by Wnt-4. *Nature* 372:679–683, 1994
24. Vukicevic S, Kopp JB, Luyten FP, Sampath TK: Induction of nephrogenic mesenchyme by osteogenic protein 1 (bone morphogenetic protein 7). *Proc Natl Acad Sci USA* 93:9021–9026, 1996
25. Kispert A, Vainio S, McMahon AP: Wnt-4 is a mesenchymal signal for epithelial transformation of metanephric mesenchyme in the developing kidney. *Development* 125:4225–4234, 1998
26. Dudley AT, Lyons KM, Robertson EJ: A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye. *Genes Dev* 9:2795–27807, 1995
27. Karavanova ID, Dove LF, Resau JH, Perantoni AO: Conditioned medium from a rat ureteric bud cell line in combination with bFGF induces complete differentiation of isolated metanephric mesenchyme. *Development* 122:4159–4167, 1996
28. Barasch J, Yang J, Ware CB, *et al*: Mesenchymal to epithelial conversion in rat metanephros is induced by LIF. *Cell* 99:377–386, 1999
29. Drummond IA, Mukhopadhyay D, Sukhatme VP: Expression of fetal kidney growth factors in a kidney tumor line: Role of FGF2 in kidney development. *Exp Nephrol* 6:522–533, 1998
30. Maher P: p38 mitogen-activated protein kinase activation is required for fibroblast growth factor-2-stimulated cell proliferation but not differentiation. *J Biol Chem* 274:17491–17498, 1999
31. Woolf AS, Winyard PJ: Advances in the cell biology and genetics of human kidney malformations. *J Am Soc Nephrol* 9:1114–1125, 1998