

# Angiotensin II stimulates Pax-2 in rat kidney proximal tubular cells: Impact on proliferation and apoptosis

SHAO-LING ZHANG, JUN GUO, BABAK MOINI, and JULIE R. INGELFINGER

*Pediatric Nephrology Unit, Harvard Medical School, Massachusetts General Hospital, Boston, Massachusetts; and Endocrinology Unit, Harvard Medical School, Massachusetts General Hospital, Boston, Massachusetts*

## Angiotensin II stimulates Pax-2 in rat kidney proximal tubular cells: Impact on proliferation and apoptosis.

**Background.** The intrarenal renin-angiotensin system (RAS) is intimately involved in the tubular cell proliferation, apoptosis and regeneration that occur following renal injury. Though tubular angiotensin II (Ang II) type 2 receptors (AT<sub>2</sub>R) decrease greatly after birth, their number increases after injury. Notably, during recovery from injury, renal tubular cells display a relatively immature phenotype expressing genes that are involved in nephron development, for example, the paired homeobox-2 gene (Pax-2). The present investigation hypothesized that AT<sub>2</sub>R activation would stimulate Pax-2 gene expression in immortalized rat renal proximal tubular cells (IRPTC), as we have found in fetal cells.

**Methods.** Pax-2 gene expression in IRPTC was evaluated by immunofluorescence, Western blot, reverse transcription-polymerase chain reaction (RT-PCR) with or without Ang II treatment; apoptosis and proliferation were analyzed by terminal transferase-mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL) assay and bromodeoxyuridine (BrdU) incorporation in stable IRPTC transformants with Pax-2 sense and antisense orientation, respectively.

**Results.** Ang II up-regulated Pax-2 gene expression via AT<sub>2</sub>R in IRPTC. The stimulatory effect of both Ang II on Pax-2 gene expression was blocked by PD123319 (AT<sub>2</sub>R inhibitor), AG 490 (specific Janus kinase 2 (JAK2) inhibitor) and genistein (tyrosine kinase inhibitor), but not by losartan (AT<sub>1</sub>R inhibitor). Stable transfection of sense Pax-2 cDNA increased, whereas antisense Pax-2 cDNA down-regulated Pax-2 expression.

**Conclusion.** Our studies suggest that Ang II stimulates Pax-2 gene expression in IRPTC via AT<sub>2</sub>R and the JAK2/signal transducers and activators of transcription (STAT) signaling transduction pathway, which may be important in renal repair following injury. Cells lacking Pax-2 gene expression appear to be prone toward apoptosis rather than proliferation.

Renal proximal tubular cells (RPTC) are known to proliferate actively and differentiate in response to kid-

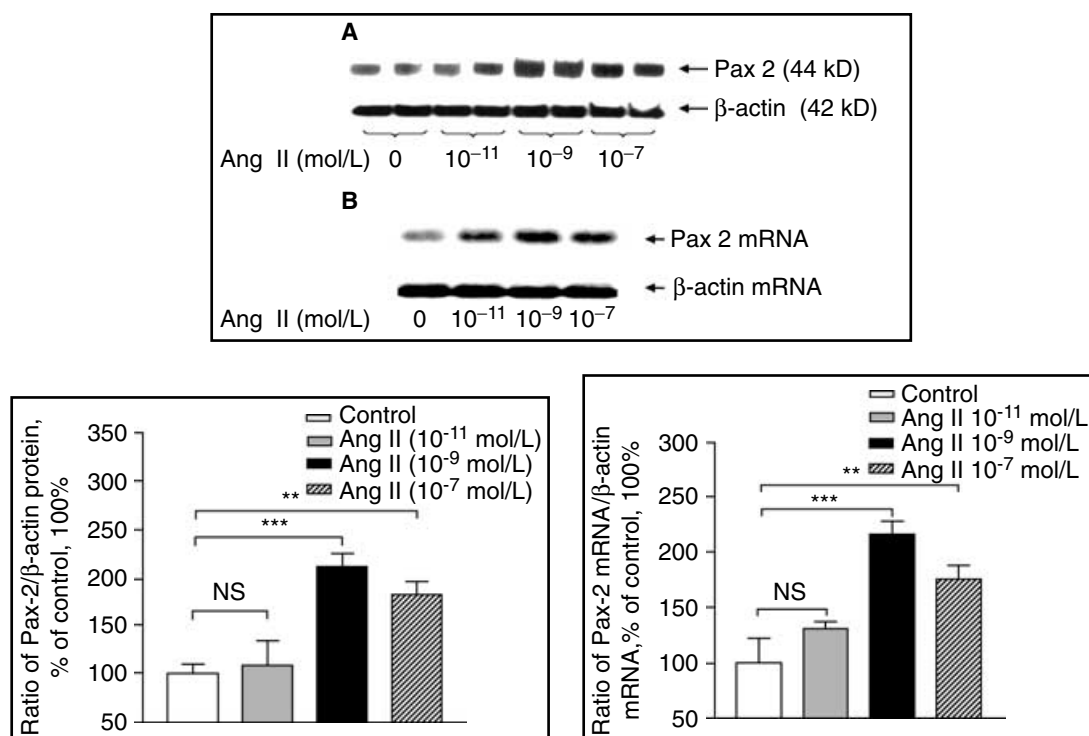
ney injury. Extensive studies indicated that the intrarenal renin-angiotensin system (RAS) has a major impact on tubular cell proliferation, apoptosis and regeneration following kidney injury. As the most active peptide of RAS family, angiotensin II (Ang II) has at least two types of receptors that have been identified by techniques of both pharmacology and molecular biology: the Ang II (type I) receptor (AT<sub>1</sub>R) and the Ang II (type II) receptor (AT<sub>2</sub>R) [1]. Within the kidney AT<sub>1</sub>R is the predominant receptor postnatally, and its functions in the adult kidney have been extensively studied. For example, AT<sub>1</sub>R mediates the effect of Ang II on vasoconstriction [2, 3] and stimulation of sodium and water reabsorption, cell hypertrophy in the proximal tubules [4–6]. The functions of AT<sub>2</sub>R, however, are less well defined. The AT<sub>2</sub>R is mainly expressed in fetal tissues and neonatal kidney [7–9], suggesting that AT<sub>2</sub>R might play an important role in renal organogenesis. AT<sub>2</sub>R expression in the kidney decreases after birth [7, 8] and is expressed at only low levels in the normal adult rat kidney [9–11]. However, AT<sub>2</sub>R is reexpressed in proximal tubules after ischemia [12], as it is in other tissues after injury [13–15], implicating potential role(s) for AT<sub>2</sub>R in modulating tissue repair. Indeed, studies by Zimpelmann and Burns [16] reported that stable transfection of AT<sub>2</sub>R in porcine kidney proximal tubular cells (LLC-PK1) attenuates the mitogenic effect of epidermal growth factor (EGF) and enhances apoptosis. Furthermore, Cao et al [17] have reported that AT<sub>2</sub>R is expressed in adult rat kidney proximal tubular cells and has a role in mediating Ang II-induced proliferation and apoptosis in proximal tubular cells [17]. Hashimoto et al [18] recently demonstrated that overexpression of AT<sub>2</sub>R in the glomerulus is protective, ameliorating glomerular hypercellularity and glomerulosclerosis in a mouse remnant kidney model. Taken together, these studies suggest that AT<sub>2</sub>R signaling plays an important role in proximal tubular cell remodeling during renal injury.

Pax-2 belongs to a family of homeobox genes that include Pax-1 to Pax-9 [19, 20] and contain a paired domain, which binds to the DNA to initiate transcription of specific genes. Pax-2 is the first known kidney-specific gene

**Key words:** Pax-2, angiotensin II, apoptosis, proximal tubular cells.

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**Fig. 1. Angiotensin II (Ang II) leads to a biphasic up-regulation of Pax-2 protein and mRNA expression in immortalized renal proximal tubular cells (IRPTC) as determined by Western blot (A) and reverse transcription-polymerase chain reaction (RT-PCR) (B).** The upper panel shows representative blots; the lower panel presents quantification by densitometry. Cells were incubated in 5 mmol/L D-glucose Dulbecco's modified Eagle's medium (DMEM) containing 1% depleted fetal bovine serum (dFBS) with or without Ang II ( $10^{-11}$  mol/L to  $10^{-7}$  mol/L) for 15 minutes or 30 minutes. Pax-2 expression was assessed by either Western blot or RT-PCR. Membranes were reblotted for  $\beta$ -actin, and relative densities of Pax-2 were normalized to  $\beta$ -actin. The normalized Pax-2 level in cells incubated in 5 mmol/L glucose was considered the control (100%). Each point represents the mean  $\pm$  SD of three independent experiments. \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.005$ .

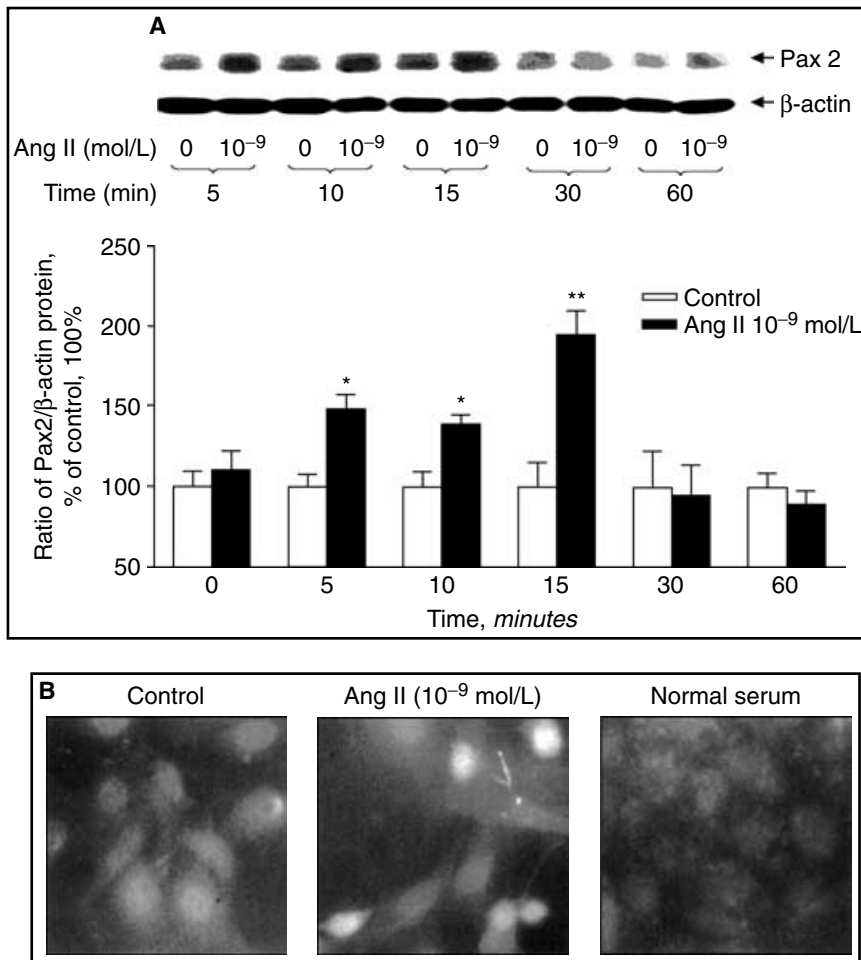
to be expressed in the pronephros of the mouse embryo, and Pax-2 is indispensable for ureteric bud development [21, 22]. Although Pax-2 is not detected in mature kidneys at the completion of nephrogenesis [23], it has been reported that Pax-2 is reexpressed in mouse proximal tubular cells that have been damaged by renal toxins [24]. Recent studies by Maeshima et al [25] also reported that while Pax-2 is not expressed in normal proximal tubular cells, its expression is up-regulated in proximal tubular cells during regeneration after renal ischemia. Such studies suggest that Pax-2 may participate in cell proliferation and differentiation as well as in tubular regeneration and repair after injury in adult kidneys.

We have recently reported that Ang II up-regulates Pax-2 expression in mouse late embryonic mesenchymal epithelial cells (MK4) via  $AT_2R$  but not in mouse early embryonic mesenchymal fibroblasts (MK3) [26], suggesting that there is a functional relationship between  $AT_2R$  and Pax-2 expression during kidney organogenesis. In the present study, we asked whether a link might also exist between the RAS and Pax-2 in adult renal proximal tubular cells, which, if present, might potentially influence renal regeneration. We employed an in vitro approach using adult rat immortalized renal proximal tubular cells

(IRPTC) in culture. Our results, as in fetal cells and metanephroi, indicate that Ang II up-regulates Pax-2 gene expression in IRPTC via the  $AT_2R$ , an effect that appears, at least in part, to take place via the Janus kinase 2 (JAK2)/signal transducers and activators of transcription (STAT) signaling transduction pathway. Our data suggest that cells lacking Pax-2 gene expression are more likely to enter apoptosis than proliferation. We speculate that the observed up-regulation of Pax-2 gene expression by Ang II via  $AT_2R$  might play an important role in renal regeneration.

## METHODS

AG490 (a specific inhibitor of JAK2) and genistein (an inhibitor of tyrosine kinase) all were purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, USA). Oligonucleotides were synthesized by the DNA Synthesis Core Facility at Massachusetts General Hospital, Boston, Massachusetts. The In Situ Cell Death Detection kit was purchased from Roche (Indianapolis, IN, USA). Bromodeoxyuridine (BrdU) (Sigma Chemical Co., St. Louis, MO, USA) labeling was detected immunohistochemically by the Zymed<sup>®</sup> BrdU staining kit



**Fig. 2. Angiotensin II (Ang II) up-regulates Pax-2 expression in a time-dependent manner.** (A) As analyzed by Western blot, cells were incubated in 5 mmol/L D-glucose Dulbecco's modified Eagle's medium (DMEM) containing 1% depleted fetal bovine serum (dFBS) with or without Ang II at concentration of  $10^{-9}$  mol/L for periods of 5 minutes to 60 minutes. The relative densities of Pax-2 were compared with  $\beta$ -actin. The normalized Pax-2 level in cells incubated in 5 mmol/L glucose was considered the control (100%). Each point represents the mean  $\pm$  SD of three independent experiments. \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.005$ . (B) The immunofluorescence staining of Pax-2 in immortalized renal proximal tubular cells (IRPTC) (original magnification  $\times 400$ ). Cells were incubated in 5 mmol/L D-glucose DMEM containing 1% dFBS for 15 minutes without or with Ang II stimulation at concentration  $10^{-9}$  mol/L. As a control, cells were incubated with 5% normal rabbit serum-phosphate-buffered saline (PBS).

(San Francisco, CA, USA). Mouse anti- $\beta$ -actin monoclonal antibody (clone AC-15) was purchased from Sigma Chemical Co. A polyclonal anti-Pax-2 antibody was purchased from Covance (Richmond, CA, USA). Polyclonal antiphospho-JAK2 and anti-JAK2 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY, USA). The plasmid, pcDNA 3.1/Pax-2 was the kind gift provided by Dr. Paul Goodyer (McGill University, Montreal, Canada).

#### Culture of IRPTC

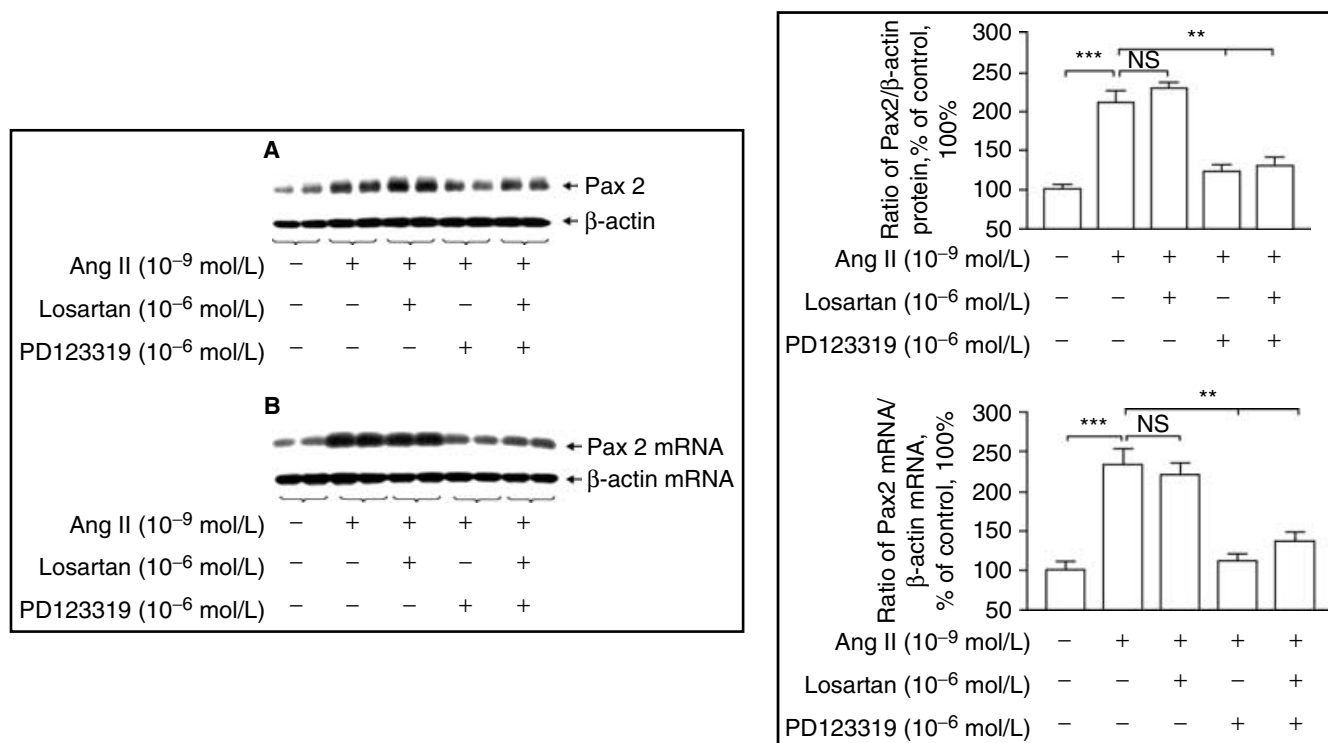
IRPTC, line 93-p-2-1, passages 12 to 18 were used in the present studies [27]. Characteristics of two lines of IRPTC developed in our laboratory have been previously described [27, 28]. Line 93-p-2-1 is highly differentiated, expressing RPTC characteristics as defined by the presence of proximal tubular antigens [27]. Thus, IRPTC resemble primary cultures of RPTCs in vivo. However, in contrast to primary cultures of rat RPTCs, which cannot be passaged more than two to three times, IRPTC grow continuously, with a population doubling time of 16 to 18 hours. By Northern blot [27] and Western blot, both  $AT_1R$  and  $AT_2R$  are present in IRPTC.

IRPTC were grown in  $100 \times 20$  mm plastic Petri dishes (Fisher, Inc., Pittsburgh, PA, USA) in normal glucose (5 mmol/L) Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Inc., Carlsbad, CA, USA), supplemented with 5% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100  $\mu$ g/mL of streptomycin. Prior to experiments, cells were synchronized overnight in serum-free medium. Then, cells were incubated in media containing normal glucose DMEM plus 1% depleted FBS (dFBS) in the absence or presence of various concentrations of Ang II ( $10^{-11}$  mol/L to  $10^{-7}$  mol/L) and in different incubation period.

dFBS, depleted of endogenous steroid and thyroid hormones, was prepared by incubation with 1% activated charcoal and 1% AG 1  $\times$  8 ion-exchange resin (Bio-Rad Laboratories, Inc., Richmond, CA, USA) for 16 to 24 hours at room temperature, as described by Samuels, Standby, and Shapiro [29].

#### Stable gene transfection in IRPTC

IRPTC were plated in 6-well plates at a density of  $1-2 \times 10^5$  cells/well and incubated overnight in normal glucose (5 mmol/L) DMEM containing 5% FBS.



**Fig. 3. The effect of losartan and PD123319 on Pax-2 expression as stimulated by angiotensin II (Ang II) in immortalized renal proximal tubular cells (IRPTC) analyzed by Western blot (A) and reverse transcription-polymerase chain reaction (RT-PCR) (B).** The left panel shows representative blots, and the right panel shows densitometry. Cells were preincubated in 5 mmol/L D-glucose Dulbecco's modified Eagle's medium (DMEM) containing 1% depleted fetal bovine serum (dFBS) for 10 minutes in the absence or presence of losartan ( $1 \times 10^{-6}$  mol/L) and PD123319 ( $1 \times 10^{-6}$  mol/L), then incubated with or without Ang II ( $10^{-9}$  mol/L) for 15 minutes or 30 minutes for Pax-2 expression analyzed by Western blot and RT-PCR, respectively. The relative densities of the Pax-2 were normalized to the  $\beta$ -actin on the same membrane. The normalized Pax-2 level in cells incubated in 5 mmol/L glucose was considered the control (100%). Each point represents the mean  $\pm$  SD of three independent experiments. \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.005$ .

The sense pcDNA 3.1/Pax-2 (+) was kind gift from Dr. Paul Goodyer. We constructed the antisense Pax-2 cDNA plasmid ourselves. In brief, the antisense Pax-2 cDNA plasmid was obtained by recloning the human Pax-2 cDNA from the sense pcDNA3.1(+)/(*KpnI/XbaI*) site to pcDNA(-) 3.1/(*XbaI/KpnI*) site. Those plasmids were stably transfected into IRPTC using lipofectamine reagents (Invitrogen, Inc.). The stable transformants were selected in the presence of geneticin [G418 (500  $\mu$ g/mL)] (Invitrogen, Inc.) as described previously [30].

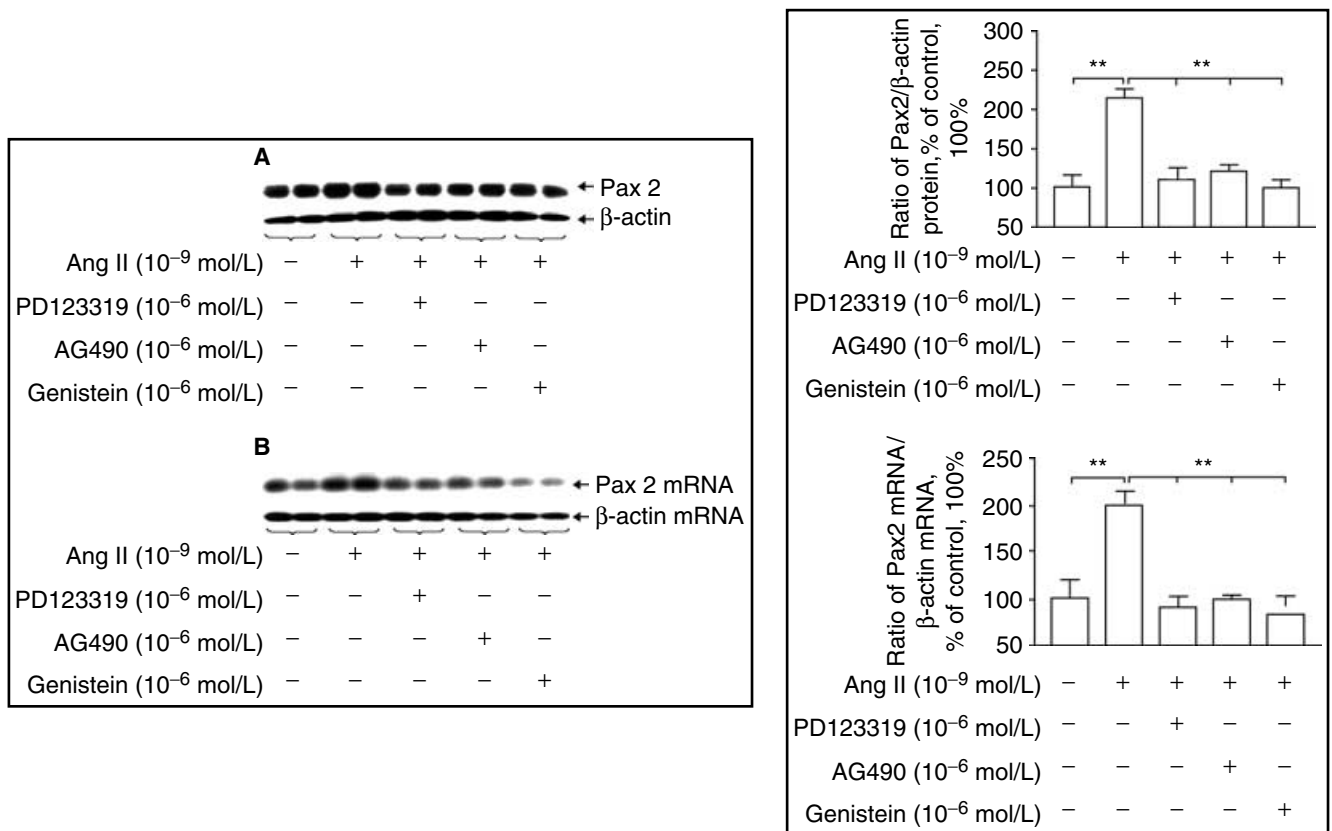
### Immunofluorescence studies

IRPTC were grown on two-chamber cover slides to 70% to 80% confluence and synchronized with serum-free medium overnight. Then, cells were incubated with or without Ang II ( $10^{-9}$  mol/L) for 15 minutes. Cells were washed, fixed with 4% paraformaldehyde/phosphate-buffered saline (PBS) for 20 minutes, followed by gentle washing three times with PBS containing 0.5% bovine serum albumin (BSA). Then, the cells were permeabilized with PBS containing 0.1% (vol/vol) Triton X-100 and 0.5% BSA for 15 minutes followed by gentle wash-

ing three times as before. Cells were then incubated with a primary antibody at 4°C overnight. A fluorescein isothiocyanate (FITC)-labeled goat antirabbit IgG as the secondary antibody was employed (1 hour incubation at room temperature). Immunofluorescence images were recorded with a Wild MPS 46/52 Photoautomat (Heerbrugg, Wild Leitz Ltd., Switzerland). For immunohistochemical controls, the primary antibody was replaced with 5% normal rabbit serum-PBS, which did not show positive staining, confirming specificity.

### Western blotting

Western blot was performed as in previous studies [6, 30]. Briefly, cells were lysed with 700  $\mu$ L of lysis buffer [50 mmol/L Tris-HCl, pH 8.0, containing 1% NP-40, 250 mmol/L NaCl, 2 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 10  $\mu$ g/mL leupeptin, 10  $\mu$ g/mL aprotinin, 1 mmol/L sodium orthovanadate, and 50 mmol/L NaF]. Then, the lysates were sonicated for 30 seconds, heated at 95°C for 5 minutes, and finally centrifuged at 12,000g for 10 minutes at 4°C. The protein



**Fig. 4. The effect of PD123319, AG490, and genistein on Pax-2 expression as stimulated by angiotensin II (Ang II) in mouse late embryonic mesenchymal epithelial cells (MK4) analyzed by Western Blot (A) and reverse transcription-polymerase chain reaction (RT-PCR) (B), respectively.** The left panel shows representative blots, while the right panel shows quantitation of all experiments. Cells were preincubated in 5 mmol/L D-glucose Dulbecco's modified Eagle's medium (DMEM) containing 1% depleted fetal bovine serum (dFBS) for 10 minutes in the absence or presence of PD123319 (10<sup>-6</sup> mol/L), AG490 (10<sup>-6</sup> mol/L), and genistein (10<sup>-6</sup> mol/L), then incubated with or without Ang II (10<sup>-9</sup> mol/L) for 15 minutes or 30 minutes, subsequently lysed and assayed by Western blot or RT-PCR for Pax-2, respectively. Each membrane was reblotted for  $\beta$ -actin. The relative densities of Pax-2 were compared with  $\beta$ -actin. The normalized Pax-2 level in cells incubated in 5 mmol/L glucose was considered the control (100%). Each point represents the mean  $\pm$  SD of three independent experiments. \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.005$ .

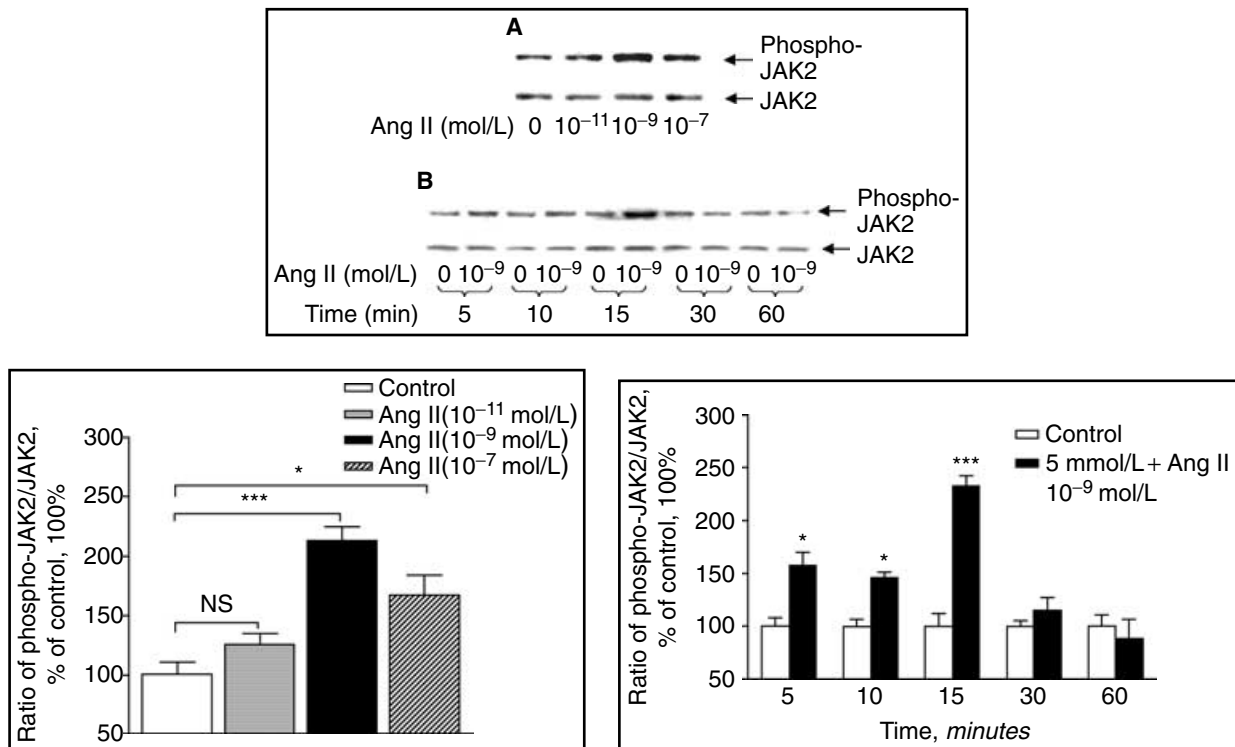
concentrations were measured by the Bradford method (Bio-Rad). Small aliquots (20 to 50  $\mu$ L) of supernatant were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Hybond-P) (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The membrane was first blotted for anti-Pax-2, phospho-JAK2 antibody and then reblotted for  $\beta$ -actin and JAK2 antibody, respectively. The relative densities of the Pax-2 and  $\beta$ -actin bands were measured by computerized laser densitometry.

#### Reverse transcription-polymerase chain reaction (RT-PCR) for Pax-2 mRNA

Total RNA was prepared from cultured cells according to the manufacturer's protocol using Trizol (Invitrogen). First-strand cDNA was synthesized with the Super-Script preamplification system (Invitrogen). The forward primer 5'-TTTGTGAACGGCCGCCCCCTA-

3', and the reverse primer 5'-CATTGTCACAGATGCCCTCGG-3', corresponding to the nucleotide sequences N+622 to N+642 and N+902 to N+922 of Pax-2 cDNA [31] were employed for PCR. Primers specific for rat  $\beta$ -actin [32] (forward and reverse primers 5'-ATG CCA TCC TGC GTC TGG ACC TGG C-3' and 5'-AGC ATT TGC GGT GCA CGA TGG AGG G-3', corresponding to the nucleotide sequences N+155 to N+179 of exon 3 and N+115 to N+139 of exon 5 of rat  $\beta$ -actin), were used for PCR as internal controls. The amplification cycles were 20 seconds at 94°C, 20 seconds at 58°C, and 60 seconds at 72°C for 35 cycles using Rapid Cycler (Idaho Technology, Salt Lake City, UT, USA). The plasmid, pcDNA 3.1/Pax-2 cDNA, was used as positive control.

To identify Pax-2 and  $\beta$ -actin cDNA fragments, 15  $\mu$ L of the PCR product was subjected to electrophoresis on 1.5% agarose gel and transferred to a Hybond XL nylon membrane (Amersham Pharmacia Biotech). Digoxigenin (DIG)-labeled oligonucleotides, 5'-CCTGGGCA



**Fig. 5. Angiotensin II (Ang II) stimulates the phosphorylation of JAK2 in a dose-dependent (A) and time-dependent (B) manner in immortalized renal proximal tubular cells (IRPTC) as analyzed by Western blot.** Representative blots are shown in the upper panel, while corresponding quantitation is shown in the lower panels. (A) Cells were incubated in 5 mmol/L D-glucose Dulbecco's modified Eagle's medium (DMEM) containing 1% depleted fetal bovine serum (dFBS) for 15 minutes with or without Ang II at a concentration range of  $10^{-11}$  mol/L to  $10^{-7}$  mol/L. (B) Cells were incubated in 5 mmol/L D-glucose DMEM containing 1% dFBS for various time period (5 minutes to 60 minutes) in the presence of Ang II ( $10^{-9}$  mol/L). Cells were then lysed and assayed by Western blot for the phosphorylation of JAK2. The same membrane was reblotted for total JAK2. Cells incubated in 5 mmol/L glucose medium were considered controls (100%). Each point represents the mean  $\pm$  SD of three independent experiments. \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.005$ .

GGTACTACGAGACCGG-3', and 5'-TCCTGTGGCA TCCATGAAACTACATTC-3' corresponding to nucleotide N+747 to N+760 of Pax-2 cDNA, and nucleotide N+9 to N+35 of exon 4 of rat  $\beta$ -actin cDNA [32], respectively, (La Roche Biochemicals, Inc., Indianapolis, IN, USA), were used to hybridize the PCR products on the membrane. After stringent washing, specific bands were detected with a DIG luminescent detection kit (La Roche Biochemicals, Inc.) and exposed to Kodak BMR film (Amersham Pharmacia Biotech). Pax-2 mRNA levels were normalized by corresponding  $\beta$ -actin mRNA levels.

#### Terminal transferase-mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL) assay

Apoptosis was determined with the in situ TUNEL assay, using the In Situ Cell Death Detection kit (La Roche Biochemicals, Inc.). Cells grown on 12 mm glass chamber slides and rendered quiescent for 24 hours were incubated in serum-free DMEM for an additional 48 hours without changing medium until assay. For quantitation of apoptosis, the total number of cells was counted and presented

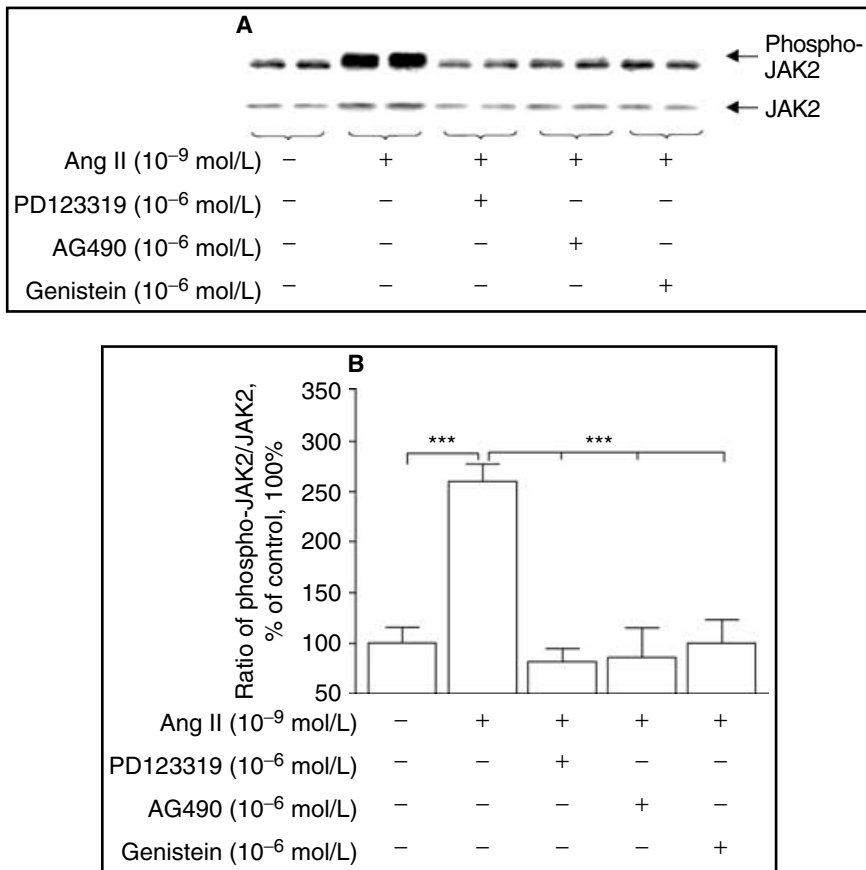
in 10 separate microscopic fields at both  $10\times$  and  $20\times$  magnification, with the scorer blinded to the origin of the slides.

#### BrdU labeling

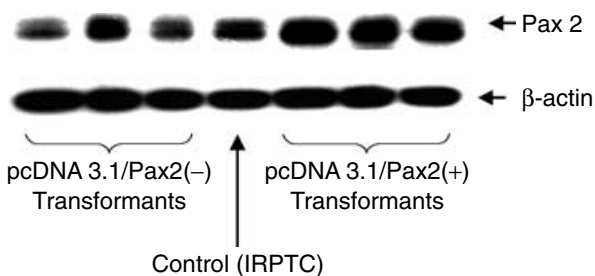
Cells grown on 12 mm glass chamber slides and rendered quiescent for 24 hours were incubated in serum-free DMEM for an additional 48 hours without changing medium. During the last  $2\frac{1}{2}$  hours in culture, BrdU (1 mg/mL) was added, and incorporated label was detected immunohistochemically by commercial kit (Zymed<sup>®</sup> BrdU staining kit). The percentages of BrdU-positive nuclei and the proliferation location were calculated and analyzed in 10 separate microscopic fields at both  $10\times$  and  $20\times$  magnification, with the scorer blinded to the origin of the slides.

#### Statistical analysis

Three to four separate experiments were performed for each protocol. The data were subjected to *t* test or analysis of variance (ANOVA) followed by Bonferroni correction to compare the control and treatment groups



**Fig. 6. The effect of PD123319, AG490, and genistein on phosphorylation of JAK2 stimulation by angiotensin II (Ang II) in immortalized renal proximal tubular cells (IRPTC) as analyzed by Western blot. (A)** A representative blot. **(B)** Quantitation of all experiments. Cells were preincubated in 5 mmol/L D-glucose Dulbecco's modified Eagle's medium (DMEM) containing 1% depleted fetal bovine serum (dFBS) for 10 minutes in the absence or presence of PD123319 (10<sup>-6</sup> mol/L), AG490 (10<sup>-6</sup> mol/L), and genistein (10<sup>-6</sup> mol/L), then stimulated with or without Ang II (10<sup>-9</sup> mol/L) for 15 minutes and subsequently lysed and assayed by Western blot for the phosphorylation of JAK2. The same membrane was reblotted for total JAK2. Cells incubated in 5 mmol/L glucose medium were considered controls (100%). Each point represents the mean  $\pm$  SD of three independent experiments. \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.005$ .



**Fig. 7. Pax-2 expression in stable transformants with sense or anti-sense human Pax-2 cDNA, respectively, analyzed by Western blot.** The plasmids, pcDNA 3.1/Pax-2 (+) and pcDNA 3.1/Pax-2 (-) containing the human Pax-2 cDNA in sense and antisense orientation were stably transfected into immortalized renal proximal tubular cells (IRPTC) using lipofectamine reagents, respectively, selected clones by G418. Non-transfected IRPTC cells were considered controls.

in the same experiment. A probability level of  $P \leq 0.05$  was considered statistically significant.

## RESULTS

### Ang II stimulates Pax-2 expression in IRPTC in a dose- and time-dependent manner

Ang II (10<sup>-11</sup> mol/L to 10<sup>-7</sup> mol/L) stimulated Pax-2 gene expression in a dose-dependent manner in IRPTC,

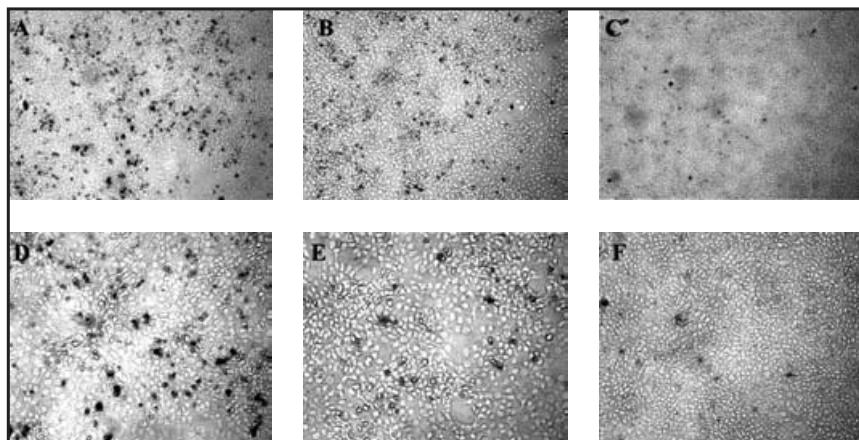
as shown by Western blot (Fig. 1A) and RT-PCR (Fig. 1B). A maximal response to Ang II occurred at 10<sup>-9</sup> mol/L; accordingly, we used Ang II 10<sup>-9</sup> mol/L for the remainder of our experiments. Furthermore, Ang II at 10<sup>-9</sup> mol/L stimulated Pax-2 expression in a time-dependent manner starting from 5 minutes post-exposure, reaching a plateau at 15 minutes, this effect then gradually disappearing, as shown in Figure 2A. We used the 15-minute time point for all subsequent studies.

### Pax-2 immunostaining in IRPTC

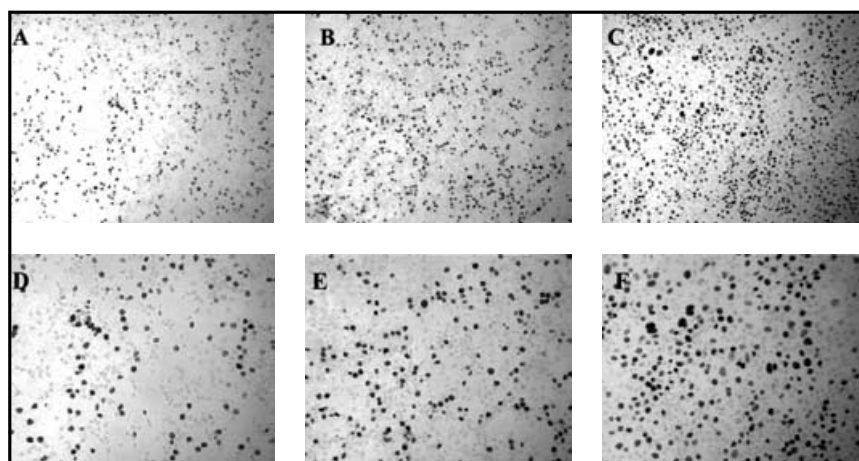
Immunofluorescence data indicates a low basal level of Pax-2 in IRPTC, which was upregulated by Ang II (10<sup>-9</sup> mol/L), as shown in Figure 2B. Immunostaining appeared intranuclear, consistent with the fact that Pax-2 is a nuclear transcription factor [31, 33].

### PD123319 blocks the stimulatory effect of Ang II on Pax-2 gene expression in IRPTC

Results with Western blot (Fig. 3A) and RT-PCR (Fig. 3B) both indicate that PD123319 [an AT<sub>2</sub>R antagonist (10<sup>-6</sup> mol/L)] blocks the stimulatory effect of Ang II on Pax-2 expression in IRPTC, whereas losartan [an AT<sub>1</sub>R antagonist (10<sup>-6</sup> mol/L)] has no effect. No additional inhibitory effects occur when both AT<sub>2</sub>R and



**Fig. 8. Apoptosis in immortalized renal proximal tubular cells (IRPTC) stable transformants analyzed by terminal transferase-mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL) staining.** (A and D) pcDNA3.1/hPax-2 (-) sense transformants. (B and E) Nontransfected IRPTC. (C and F) pcDNA3.1/hPax-2 (+) antisense transformants. Cells grown on 12 mm glass coverslips and rendered quiescent for 24 hours were incubated in serum-free Dulbecco's modified Eagle's medium (DMEM) for an additional 48 hours without change medium and ready for assay. For quantitation of apoptosis, the total number of cells was counted and presented in 10 separate microscopic fields at both  $\times 10$  (A, B, and C) and  $\times 20$  (D, E, and F) magnification, with the viewer blinded to the origin of the slides.



**Fig. 9. Bromodeoxyuridine (BrdU) labeling in immortalized renal proximal tubular cells (IRPTC) stable transformants.** (A and D) pcDNA3.1/hPax-2 (-) sense transformants. (B and E) Nontransfected IRPTC. (C and F) pcDNA3.1/hPax-2 (+) antisense transformants. Cells grown on 12 mm glass coverslips and rendered quiescent for 24 hours were incubated in serum-free Dulbecco's modified Eagle's medium (DMEM) for an additional 48 hours without change medium. During the last 2 $\frac{1}{2}$  hours culture period, BrdU (1 mg/mL) was added and incorporated label was detected immunohistochemically by commercial kit. The percentages of BrdU-positive nuclei and the proliferation location will be calculated and analyzed in 10 separate microscopic fields at both  $\times 10$  (A, B, and C) and  $\times 20$  (D, E, and F) magnification, with the viewer blinded to the origin of the slides.

AT<sub>1</sub>R blockers were used simultaneously, as compared to AT<sub>2</sub>R blocker alone. These data indicate that the Ang II effect on Pax-2 expression is mediated via the AT<sub>2</sub>R, not the AT<sub>1</sub>R in IRPTC.

#### **PD123319, AG490, and genistein block the stimulatory effect of Ang II and on Pax-2 gene expression in IRPTC**

We also observed, in addition to blockade by PD123319, that AG490 (a specific inhibitor of JAK2) and genistein (an inhibitor of tyrosine kinase) could block the stimulatory effect of Ang II on Pax-2 gene expression, as shown by Western blot (Fig. 4A) and RT-PCR (Fig. 4B) in IRPTC. Moreover, these blockers, by themselves, had no effect on Pax-2 expression (data not shown).

#### **Ang II stimulates JAK2/STAT phosphorylation in IRPTC**

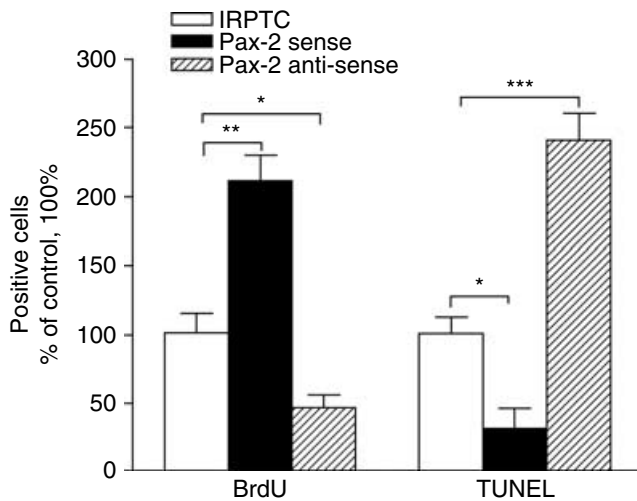
Western blot analysis indicates that Ang II stimulates the phosphorylation of JAK2 in dose-dependent (Fig. 5A) and time-dependent manner (Fig. 5B) in IRPTC. This stimulatory effect of Ang II on JAK2 phos-

phorylation could be blocked by PD123319, AG 490 and genistein (Fig. 6). These data demonstrated that Ang II up-regulates Pax-2 gene expression via the JAK2/STAT signal transduction pathway.

Studies blocking the p38 mitogen-activated protein kinase (MAPK), p44/42 MAPK and reactive oxygen species (ROS) pathways revealed did not prevent up-regulation of Pax-2 by Ang II (data not shown).

#### **Pax-2 protein expression in Pax-2 stable transformants**

Figure 7 shows that Pax-2 expression is up-regulated or down-regulated in IRPTC that have been stably transfected with Pax-2 sense and antisense cDNA, respectively, as compared to controls (nontransfected cells). As expected, cells stably transfected with Pax-2 sense transformants overexpress Pax-2, whereas stably transfected with Pax-2 antisense transformants express less Pax-2 as compared with control. These transformed cells were used for further studies that included cell proliferation and apoptosis.



**Fig. 10. Quantitation of bromodeoxyuridine (BrdU) and terminal transferase-mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL) staining in Pax-2 transformants.** The y-axis shows the percentage of BrdU-, TUNEL-positive cells comparing to control (100%). (□) control; (■) Pax-2 sense transformants; (▨) Pax-2 antisense transformants. \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.005$ .

### Cell proliferation and apoptosis in IRPTC stable transformants

BrdU labeling and in situ TUNEL assay indicated that more cell proliferation and less apoptosis occur in Pax-2 sense transformants as compared to Pax-2 antisense transformants and controls (Figs. 8 and 9). Figure 10 shows quantitation of these studies. In Pax-2 sense transformants, the percentage of BrdU-positive cells was more than twofold higher than control ( $210.8 \pm 19.8\%$  vs.  $100 \pm 15.3\%$ ) ( $P \leq 0.01$ ), and the percentage of apoptotic cells was decreased as compared to controls ( $30.6 \pm 15.3\%$  vs.  $100 \pm 12.3\%$ ) ( $P \leq 0.05$ ), respectively. In contrast, Pax-2 antisense transformants exhibited a decreased the percentage of BrdU-positive ( $45.6 \pm 10.3\%$  vs.  $100 \pm 15.3\%$ ) ( $P \leq 0.05$ ) and an increased percentage of apoptotic cells comparing to control ( $240.6 \pm 20.3\%$  vs.  $100 \pm 12.3\%$ ) ( $P \leq 0.005$ ), respectively. These data indicated that cells lacking Pax-2 gene expression are prone toward apoptosis rather than proliferation.

### DISCUSSION

The present in vitro studies indicate that Ang II, a major effector of the RAS, regulates Pax-2 gene expression in young adult rat RPTCs via  $AT_2R$ . These findings may have substantial implications for cell regeneration following kidney injury, as Pax genes and the RAS both are proven to be critically important for renal cell proliferation and apoptosis.

Pax-2, a transcription factor belonging to the Pax family, plays a key regulatory role during renal organogenesis [34]. During renal development Pax-2 is detected specif-

ically in the caudal mesonephric duct, ureteric bud, and later in mesenchymal condensates induced by the ureteric bud [35–37]. Later during the process of nephrogenesis, Pax-2 is restricted to the distal part of the S-shaped body, and its expression is extinguished as cells differentiate [20]. Pax-2 is likely a master gene essential for controlling tubular cell proliferation and differentiation [25], mediating mesenchymal-to-epithelial transformation as well as apoptosis [38].

Without a functional Pax-2 gene, renal agenesis occurs [34, 39–42]. Dysregulated Pax-2 expression has been associated with developmental renal abnormalities in mice [35, 43] and humans [40, 44]. Pax-2 is the first known kidney-specific gene to be expressed in the pronephros of the mouse embryo, and Pax-2 is indispensable for ureteric bud development [21, 22]. Homozygous null Pax-2 mice fail to form any kidneys, ureters and genital tracts [35]. In humans and mice, heterozygous Pax-2 mutations cause kidney, eye, and central nervous system abnormalities, constituting a syndrome called renal-coloboma syndrome (RCS) [43, 45]. Once nephrogenesis is completed, Pax-2 is down-regulated and not normally detected in the mature mammalian kidney [23]. However, Pax-2 is expressed in response to renal injury, at a point at which numerous tubular cells also display immature phenotype and express vimentin (a mesenchymal marker) [24, 25, 35, 46]. Recent studies revealed that Pax-2 is reexpressed in tubular cells damaged by renal toxins [24] and ischemia [25]. Pax-2 is expressed during tubular regeneration, along with the mesenchymal marker, vimentin, suggesting that the regenerating cells have characteristics of immature progenitor-like tubular cells [24–25]. Pax-2 has been shown to act as a transcriptional factor, inhibiting cell apoptosis or promoting cell survival in a mouse inner medullary collecting duct cell line (mIMCD) [47]. Taken together, these studies raise the possibility that Pax-2 is a critical gene in controlling cell proliferation and differentiation during post-injury kidney regeneration.

The signaling molecule(s) that regulate(s) Pax-2 expression in the kidney is relatively unknown. It has been reported that EGF increased the half-life of Pax-2 protein in a rat kidney epithelial cell line (NRK-52E) [48], whereas transforming growth factor- $\beta$  (TGF- $\beta$ 1) [49] and activin A [25], a member of the TGF- $\beta$  superfamily down-regulate Pax-2 expression in proximal tubular cells. We recently reported that Ang II stimulates Pax-2 protein and Pax-2 mRNA expression in mouse late mesenchymal epithelial cells (MK4) but not in early mesenchymal fibroblasts (MK3) [26], suggesting that RAS and Pax-2 interactions may be important in renal development. We therefore hypothesized that Ang II might be capable of influencing Pax-2 expression in adult kidney proximal tubular cells, which, if true, might have important implications for further understanding of tubular regeneration after injury.

We observed that Ang II at physiological concentration ( $10^{-9}$  mol/L) [50] stimulated Pax-2 gene expression in IRPTC by more than twofold as compared to control (Figs. 1 and 2). This effect occurred rapidly. It is possible that the stimulatory effect of Ang II on Pax-2 protein seen within 5 minutes might be mediated via phosphorylation. Such a phenomenon would not need to change the pattern of Pax-2 nuclear staining as shown in our Figure 2B. Indeed, Cai Y et al [51] demonstrated that Pax2 is phosphorylated by the c-Jun N-terminal kinase (JNK) to enhance Pax2-dependent transcription, but not the ERK1/2 or p38 MAPKs in 293 cells. Unfortunately, due to the lack of commercially available specific antibodies against phosphorylated Pax-2 protein, we could not perform experiments to look more directly at this possibility.

Several studies have demonstrated that AT<sub>2</sub>R is not only present in fetal tissue [7] but also in the adult kidney [17, 52]. AT<sub>2</sub>R has a range of effects related to kidney disease, such as regulating the chemokine RANTES [53] and the matrix protein osteopontin [17], and mediating effects of the vascular kinin system [54], nitric oxide release [55], and prostaglandin E<sub>2</sub> production [56]. Therefore, the AT<sub>2</sub> receptor could have effects on a range of pathophysiologic processes implicated in progressive renal injury. However, the specific role of AT<sub>2</sub>R in the evolution and repair of kidney injury is largely unknown. In our present studies, an AT<sub>2</sub>R antagonist (PD123319), but not an AT<sub>1</sub>R antagonist (losartan), blocked the stimulatory effect of Ang II on Pax-2 and Pax-2 mRNA expression in IRPTC (Fig. 3), suggesting that Ang II up-regulates Pax-2 expression via AT<sub>2</sub>R and not AT<sub>1</sub>R. Furthermore, studies we performed to test whether p38 MAPK, p44/42 MAPK and ROS generation were involved in Ang II up-regulation of Pax-2 in IRPTC were negative.

Recent reports suggest that modulation or activation of JAK/STAT pathway by Ang II has pathologic consequences for cardiovascular [57] and renal [58] function. JAK/STAT pathways are activated by multistep phosphorylation cascades after ligand-cell surface receptor binding which lead directly to target gene promoters in the nucleus, providing mechanisms for transcriptional regulation without second messengers. JAK kinases, once activated, selectively phosphorylate STATs (STAT1, STAT2, STAT3, STAT4, STAT5A/B, and STAT6), that form homo- and heterodimers, rapidly translocating to the nucleus and bind to the "regulatory element" in the promoter of relevant gene [59]. The apparent interaction of Ang II and Pax-2 in our present studies may suggest that Ang II phosphorylates components of the JAK/STAT pathway, which in turn affects Pax-2 gene expression *in vitro*. Our data indicate that AG490 and genistein blocked the Ang II up-regulation of Pax-2 gene expression in IRPTC (Fig. 4). Furthermore, Ang II stimulated the phos-

phorylation of JAK2 in IRPTC (Fig. 5). Since this stimulatory effect of Ang II could be blocked by PD123319, AG490 and genistein in IRPTC (Fig. 6), it appears that Ang II likely upregulates Pax-2 gene expression via the JAK2/STAT signal transduction pathway.

We examined stable transformants of Pax-2 cDNA in sense and antisense orientation to enhance or attenuate the basal Pax-2 expression in IRPTC (Fig. 7). More apoptosis and less proliferation were observed in IRPTC antisense Pax-2 cDNA transformants (Figs. 8, 9, and 10). Thus, IRPTC lacking Pax-2 gene expression are prone to apoptosis as opposed to proliferation. These observations agree with those of Gnarr and Dressler [60], who found that Pax-2 gene function is required for proliferation, as well as differentiation in a renal cell carcinoma (RCC) cell line generally thought to arise from the epithelium of the proximal tubules. These observations agree in part with Torban et al [36, 47] who reported that lacking of Pax-2 gene expression greatly increases cell apoptosis without effect on proliferation rate in cultured murine collecting duct cells (mIMCD-3) and human embryonic kidney cells (HEK-293). Thus, Pax-2 appears to be associated with cell survival, protects against cell apoptosis, but increases cell proliferation, depending on the different cell type response.

While the mechanism by which Pax-2 influences the apoptotic cascade in IRPTC cells has not been delineated, it has been suggested that Pax-2 has a protective effect on caspase-2-induced apoptosis in HEK-293 cells [36, 47]. Pax-2 inhibits transcription of *p53*, a pivotal molecule regulating entry into the final common apoptotic pathway [61, 62]. Moreover, AT<sub>2</sub>R promotes cellular proliferation and apoptosis in proximal tubular cells, perhaps through generation of TGF- $\beta$ , and then triggering the expression of proapoptotic genes such as Fas, FasL or Bax [63]; modulation of heme oxygenase (HO-1) expression [63–64]; formation of nitric oxide [49]; inactivation of Egr-1 activity [18]; or activation of the osteopontin gene [17].

Our studies would suggest that the stimulatory effect of Ang II on Pax-2 gene expression in rat RPTCs is mediated, at least in part, via the AT<sub>2</sub>R and JAK2/STAT signaling transduction pathway; cells lacking Pax-2 gene expression are prone toward apoptosis rather than proliferation in IRPTC. Take together, our data suggest that RAS and Pax-2 interactions that may be important in renal repair following injury.

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Reprint requests to Julie R. Ingelfinger, M.D., Pediatric Nephrology Unit, Harvard Medical School, Massachusetts General Hospital, 55 Fruit Street, BHX-411, Boston, MA 02114-3117.  
E-mail: jingelfinger@partners.org

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