Involvement of Pax-2 in the Action of Activin A on Tubular Cell Regeneration

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Abstract. It has been recently shown that in ischemic rat kidneys activin A is induced in tubular cells and inhibits their regeneration. The present study was conducted to further investigate the action of activin A in tubular cells during regeneration. Among genes thought to be critical for kidney development, Pax-2 was upregulated in tubular cells during regeneration after renal ischemia. Pax-2 protein was localized in nuclei of tubular and interstitial cells, some of which co-expressed a mesenchymal cell marker, vimentin, suggesting that a population of Pax-2–positive cells have properties of immature progenitor-like tubular cells. The Pax-2–expressing cells co-expressed a cell proliferation marker, BrdU, activin A, and the type II activin receptor. Activin A modulated growth of BrdU/Pax-2 double-positive cells since an administration of follistatin increased; conversely, exogenous activin A decreased the number of BrdU/Pax-2 double-positive cells after renal ischemia. Activin A also reduced the expression of Pax-2 in cultured metanephiroi. A proximal tubular cell line, LLC-PK1 cells, was used to further study the mode of action of activin A. The expression of Pax-2 was not detected in quiescent LLC-PK1 cells, but it was markedly increased when growth was stimulated. Under this condition, activin A significantly inhibited DNA synthesis and reduced the expression of Pax-2 in LLC-PK1 cells. In contrast, blockade of the activin signaling by overexpressing dominantly negative mutant receptor enhanced the expression level of Pax-2 in LLC-PK1 cells and induced an immature phenotype. These results suggest that activin A regulates tubular cell growth and differentiation by modulating the expression of Pax-2 during regeneration.

In the kidney, regeneration, reconstruction, and maturation of tubular cells after renal injury have many parallels to the growth and differentiation that take place during kidney organogenesis (1). The adult tubular epithelium has a potential for regeneration after damage. During acute tubular necrosis induced by renal ischemia or renal toxins, normally quiescent cells undergo dedifferentiation and reobtain their potential to divide after enhancing their DNA synthesis. After proliferation, the new cells then differentiate to restore the functional integrity of the nephron (2). Several growth factors, including hepatocyte growth factor (HGF), epidermal growth factor (EGF), insulin-like growth factor-I (IGF-I), and bone morphogenetic protein-7 (BMP-7) have been shown to be involved in tubular regeneration of the kidney (3). These factors are potent regulators of kidney organogenesis (4,5). It has been reported that administration of these growth factors promotes tubular regeneration after a variety of insults (3). This suggests that regeneration processes may be at least partially controlled by the similar mechanism operating during development. However, little is known about the mechanism by which these factors modulate tubular regeneration, although it is considered that these factors play important roles in regeneration processes of the kidney as mitogen, motogen, and morphogen (3).

Activin A, a member of the TGF-β superfamily, modulates cell growth and differentiation in many types of cells (6,7). Activins are dimeric proteins, and subunits of activin are expressed in various organs (8). An important modulator of activin is follistatin (9). This protein specifically binds to activins and related ligands with high affinity and blocks their actions (10,11). Follistatin is synthesized in the target cells of activins and remains in the extracellular matrix. Furthermore, the production of follistatin is regulated by activins. Hence, activin and follistatin modulate cellular function in a complex manner. Activin A and follistatin are expressed in a developing kidney (12). Activin A disrupts ureteric bud branching in an embryonic kidney in organ culture (13) and also inhibits branching tubulogenesis in an in vitro tubulogenesis model (14). The number of glomeruli is increased in the kidney of transgenic mice expressing dominantly negative activin receptors (15). Collectively, activin A is a negative regulator of tubulogenesis during kidney development (16). We recently demonstrated the involvement of the activin-follistatin system in tubular regeneration after renal ischemia (17). Activin A, which was not detected in normal kidney, was upregulated in tubular cells after renal ischemia. Exogenous follistatin accelerated renal regeneration by enhancing DNA synthesis and preventing apoptosis in tubular cells. Presumably, exogenous
follistatin enhanced tubular regeneration by blocking the action of endogenous activin A. However, the mechanism by which endogenous activin A inhibits tubular regeneration still remains unknown. Considering that regeneration processes may recapitulate developmental paradigms to restore organ or tissue function (1), it is quite possible that activin modulates the expression of a set of developmental genes during tubular regeneration.

In the present study, we examined whether or not activin A modulated the expression of developmental genes during tubular regeneration. The results indicate that Pax-2, a transcription factor critical for kidney development (18), was re-expressed in tubular cells after renal ischemia. Exogenous follistatin increased; conversely, exogenous activin A decreased the number of BrdU/Pax-2 double-positive cells after follistatin increased; conversely, exogenous activin A decreased the number of BrdU/Pax-2 double-positive cells after renal ischemia in vitro. Activin A reduced the expression of Pax-2 in embryonic kidney as well as in proximal tubular cell line in vitro. Furthermore, blockade of activin signaling enhanced the expression of Pax-2 and induced an immature phenotype in tubular cells. These results suggest that activin A regulates tubular regeneration by modulating the expression of Pax-2.

Materials and Methods

Materials

Recombinant human activin A, follistatin, and polyclonal rabbit anti-human activin A antibody were provided by Dr. Y. Eto of the central Research Laboratory, Ajinomoto Inc. (Kawasaki, Japan). Polyclonal antibody against Pax-2 was purchased from Berkeley Antibody Company (Berkeley, CA), and polyclonal anti-activin type II receptor antibody was a generous gift from Dr. K. Miyazono (University of Tokyo, Japan). Polyclonal anti-E-cadherin antibody was purchased from NeoMarkers (Fremont, CA). Cy3 (indocarbocyanine)-conjugated goat anti-mouse IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). [3 H]Thymidine was from Dupont-New England Nuclear (Boston, MA).

Experimental Protocol

Male Wistar rats weighing 200 to 300 g were obtained from the Imai Animal Company (Saitama, Japan). Ischemia/reperfusion injury was performed as described previously (17). Briefly, under anesthesia with pentobarbital sodium (30 mg/kg body wt), renal ischemia was induced by clamping both renal arteries for 45 min using a nontraumatic vascular clamp. Core body temperature was maintained 37°C by placing the animal on a homeothermic table and was monitored with a temperature-sensing rectal probe. After removal of the clamp to allow reperfusion for the indicated periods, rats were sacrificed. Then, the kidneys were removed and fresh frozen for RNA extraction and histologic analyses. Sham-operations were performed in a similar manner, except for clamping the renal arteries. To analyze the efficacy of exogenous follistatin or activin A after ischemic renal injury, the indicated dose of recombinant human (rh) follistatin or activin A dissolved in 0.5 ml of physiologic saline was administered via the tail vein at the time of reperfusion. Control animals received the same volume of saline alone.

Reverse Transcription–PCR

Total RNA was isolated with the TRizol Reagent (Life Technologies/BRL, Grand Island, NY) from whole kidneys or cultured metanephroi. First-strand cDNA was made from total RNA using a Superscript Preamplification System (Life Technologies/BRL) as described previously (17) according to the manufacturer’s instructions. Contaminated genomic DNA was removed with RNase-free deoxyribonuclease (DNase). Five micrograms of DNase-treated RNA were incubated with 1 μl of oligo(dT) at 70°C for 10 min. Two microliters 10× PCR buffer, 1 μl of DTT (0.1 M), 2 μl of dNTP mix (10 mM), and 2 μl of MgCl 2 (25 mM) were added to each reaction. After incubation for 5 min at 42°C, 1 μl of reverse transcriptase was added. Samples were incubated at 42°C for 50 min, then at 70°C for 15 min. RNase H (1 μl) was added to each reaction, and samples were incubated at 37°C for 20 min. PCR was performed as indicated by the manufacturer (Perkin-Elmer) with the primers shown in Table 1. Reactions contained 5 μl of a 10× PCR buffer, 2 μl of MgCl 2 (50 mM), 1 μl of dNTP mix, 1 μl of 3′-primer, 1 μl of 5′ primer, 0.5 μl of Taq polymerase, and 1 μl of cDNA. Samples were incubated at 95°C for 5 min, followed by the indicated cycles of 30 s at 94°C, 30 s at 58°C, 90 s at 72°C, and final extension at 72°C for 10 min in a Perkin-Elmer DNA Thermal Cycler. There were 30 cycles of PCR for

<table>
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<tr>
<th>Gene</th>
<th>Primers</th>
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<tbody>
<tr>
<td>Pax-2</td>
<td>5′-TGCTGAAATACAAACGACAGAACC-3′, 5′-GCAAGTGCTTCCGGACAACGT-3′</td>
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<tr>
<td>Pax-8</td>
<td>5′-GCTTTATGGCGTGGGTAGAATG-3′, 5′-TGATGTAGTAGTGGACAGACAG-3′</td>
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<tr>
<td>WT1</td>
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<tr>
<td>Wnt4</td>
<td>5′-GCGTACGGTCTGACAGTCTCTG-3′, 5′-ACACCAATCTTCTCCTACATCGATG-3′</td>
<td>330</td>
</tr>
<tr>
<td>BF-2</td>
<td>5′-AACATTGGTGTTGGTCCGTG-3′, 5′-TTTGACAGACAGAAGAGAAGCAG-3′</td>
<td>389</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5′-CATGACACAGTTCATGCCCATC-3′, 5′-CACCCCTGTCGTAGCCATATTC-3′</td>
<td>451</td>
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Table 1. Sequences of PCR primers used in this study
the Pax-2, WT-1, Wnt-4, Pax-8, BF-2, and 18 cycles for GAPDH. Reactions without cDNA were used as a negative control. Rat embryonic kidney (embryonic day 14) cDNA was used as a positive control in each experiment. Reactions were repeated at least twice.

**Immunohistochemical Analyses**

Kidneys were removed and embedded in a Tissue-Tek OCT compound (Miles, Inc., Elkhart, IN) and frozen in liquid nitrogen. Frozen sections (4 μm) were cut with a Jung CM 3000 cryostat (Leica, Wien, Austria), mounted on poly-L-lysine–coated slides, and fixed in 4% paraformaldehyde for 15 min at room temperature. Sections were then washed in PBS, pretreated with 5% normal goat serum-PBS for 1 h, and covered with a primary antibody at room temperature for 1 h. After washing in PBS, the sections were covered with a mixture of a Cy3-labeled goat anti-rabbit IgG antibody or FITC-labeled rabbit anti-mouse IgG antibody and 4'-diamidino-2-phenylindole (DAPI: Boehringer Mannheim). Immunofluorescence images were recorded with an Olympus AX70 epifluorescence microscope (Olympus, Tokyo, Japan) equipped with a PXL 1400 cooled-CCD camera system (Photometrics, Tucson, AR), which was operated with IP Lab Spectrum software (Signal Analysis, Vienna, VA). For immunohistochemical controls, the primary antibody was replaced with 5% normal goat serum-PBS, which did not show positive staining, confirming specificity.

In separate experiments, kidneys were removed and fixed with 4% formaldehyde. An avidin–biotin coupling (ABC) immunoperoxidase technique using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) was performed according to the manufacturer’s instruction as described previously (17).

**Bromodeoxyuridine Labeling**

DNA synthesis in renal tubular cells was measured using bromodeoxyuridine (BrdU). At the indicated times after reperfusion, BrdU (100 mg/kg), an analogue of thymidine, was injected intraperitoneally into rats. After 1 h, rats were sacrificed and the kidneys were removed and embedded in a Tissue-Tek OCT compound (Miles, Inc., Elkhart, IN) and frozen in liquid nitrogen. The frozen sections were immunostained using a mouse anti-BrdU antibody (Amersham) as described above.

**Quantification of BrdU, Pax-2, and BrdU/Pax-2 Double-Positive Cells**

Quantification of BrdU-positive, Pax-2-positive, and BrdU/Pax-2 double-positive cells in the kidney after renal ischemia was performed by counting the positive nuclei in tubular cells from five randomly selected fields of the outer medulla with epifluorescence microscope at ×400 magnification. The results were expressed as a percentage of total tubular cells in five sections per rat kidney. The average of the five determinations was calculated.

**Cell Culture**

LLC-PK1 cells obtained from American Type Culture Collection (ATCC RL-1392) were cultured in complete medium consisting of Medium 199 (ICN Biomedicals, Inc.) with 5% fetal bovine serum (FBS; Life Technologies/BRL), penicillin, and streptomycin in an atmosphere of 5% CO2 and 95% air at 37°C. The medium was changed every 3 to 4 d. To obtain quiescent cells, cells were incubated in a serum-free medium for 48 h. LLC-PK1 cells expressing truncated activin type II receptor cDNA (LLC-PK1-tARII) and LLC-PK1 cells expressing PCXN2 plasmid vector (LLC-PK1-mock) were generated and cultured as described previously (19).

**Measurement of DNA Synthesis**

DNA synthesis was assessed by measuring [3H]thymidine incorporation into TCA precipitable materials. Serum-starved cells cultured in a 24-well plate were incubated in complete medium containing 5% FBS with or without 10 nM activin A for indicated times. Then, the cells were pulse-labeled with 1 μCi/ml [3H] thymidine for an additional 4 h. [3H]Thymidine incorporation was measured as described previously (14).

**Immunocytochemical Analyses**

Cells grown on coverslips were washed, fixed with 4% paraformaldehyde, permeabilized with PBS containing 0.1% (vol/vol) Triton X-100, and incubated sequentially with 3% bovine serum albumin (BSA) in PBS. Cells were then incubated with a primary antibody at room temperature for 1 h. After washing in PBS, cells were covered with a mixture of a Cy3-labeled goat anti-rabbit IgG antibody and DAPI. Immunofluorescence images were recorded as described above.

**Western Blot Analyses**

Cells were washed three times with PBS, suspended in Laemmli buffer, and heated to 100°C for 10 min. After centrifugation, supernatant was collected and the protein concentration was determined by using a protein assay kit (Bio-Rad Laboratories). Twenty micrograms of protein from each sample were separated by SDS-PAGE under reducing condition and transferred to a PVDF membrane (Nihon Millipore Ltd., Yonezawa, Japan) by electroblotting. To reduce non-specific antibody binding, the membrane was blocked with 5% BSA and 0.1% Na2SO4 dissolved in Tris-saline (TS) for 1 h at 37°C and then incubated overnight with a primary antibody and washed with Tris-PBS (PBST). After incubation with peroxidase-labeled secondary antibody for 1 h at room temperature, the membrane was washed with PBST and analyzed by exposure to x-ray film using ECL Western blotting detection reagent (Amersham Life Science).

**Organ Culture of Metanephros**

Embryos were removed from anesthetized pregnant Wistar rats (Nihon SLC, Inc., Hamamatsu, Japan) on day 14 of the pregnancy. Metanephric rudiments were surgically removed from embryos. Metanephrbi were explanted onto Transwell-clear (pore size, 0.4 μm; Corning Incorporated, Corning, NY) at the interface between air/5% CO2 atmosphere and medium and cultured at 37°C in DMEM containing 5% FBS and antibiotics.

**Statistical Analyses**

The significance of differences between the means was compared by t test; P values < 0.05 considered significant.

**Results**

**Changes in the mRNA Expression of Developmental Genes in the Kidney after Renal Ischemia**

To identify the possible regulator of tubular regeneration, we analyzed the expression of developmental genes critical for kidney organogenesis (4), including Pax-2 (18), Wnt-4 (20,21), Pax-8 (22,23), WT-1 (24), and BF-2 (25), in the kidney after renal ischemia by RT-PCR. As shown in Figure 1, the expression of Pax-2 was not detected in normal and
sham-operated kidney. In contrast, a strong induction of Pax-2 expression was observed in the kidney after renal ischemia. The expression of Wnt-4 was undetectable in either normal or ischemic kidney. The expression of WT-1, Pax-8, and BF-2 was detected in normal and sham-operated kidney, but the expression levels of these factors were not altered after renal ischemia.

Colocalization of the Pax-2 Protein and Vimentin in the Kidney after Renal Ischemia

We next examined the localization of Pax-2 protein in the kidney after renal ischemia by immunohistochemistry. In normal kidney, Pax-2 protein was not detected in either tubular cells or glomeruli in the cortex (data not shown). In contrast, Pax-2 protein was detected in the nuclei of tubular cells (Figure 2A) and interstitial cells (Figure 2D) in the outer medulla after ischemic injury, where proliferating tubular cells are mainly localized. To characterize the Pax-2–positive cells, we examined the expression of vimentin, a mesenchymal cell marker, in the kidney after renal ischemia. In normal kidney, vimentin was expressed in mesangial cells in glomeruli or interstitial fibroblasts, which are derived from mesenchymal cells in the metanephros but not in tubular epithelial cells (data not shown). In contrast, vimentin was observed in the interstitial Pax-2–positive cells (Figure 2E) but was not in tubular cells (Figure 2B) in the outer medulla of ischemic kidney.

Changes in the Number of BrdU-Positive Cells and Pax-2–Positive Cells in the Kidney after Renal Ischemia

To examine the relationship between Pax-2 expression and tubular cell growth, we first analyzed the change in the number of BrdU-positive cells in the kidney after renal ischemia. As shown in Figure 3A, BrdU-positive cells were slightly detected in ischemic kidneys at 18 h after reperfusion. The number of BrdU-positive cells peaked maximum at 24 h after reperfusion and decreased thereafter. On the other hand, Pax-2–positive cells were significantly observed in ischemic kidneys at 12 h after reperfusion. The number of Pax-2–positive cells peaked at 18 h after reperfusion and decreased thereafter, indicating
that Pax-2 expression precedes tubular cell growth in the kidney after renal ischemia.

Changes in the Number of BrdU/Pax-2 Double-Positive Cells in the Kidney after Renal Ischemia

To further examine whether Pax-2–positive cells were growing, we analyzed the localization of BrdU and Pax-2 in the kidney after renal ischemia. Indirect immunofluorescence staining demonstrated that Pax-2–positive tubular cells (Figure 4A) were overlapped with BrdU-positive cells (Figure 4B), suggesting that Pax-2–positive cells are actively engaged in cell proliferation (Figure 4D). Quantitative analysis showed that the number of BrdU/Pax-2 double-positive cells peaked at 24 h in ischemic kidneys after reperfusion (Figure 4E).
Co-localization of Pax-2, Activin A, and the Type II Activin Receptor in the Tubular Cells after Renal Ischemia

We demonstrated previously that activin A was upregulated in tubular cells after renal ischemia and that activin receptors were ubiquitously expressed in tubular cells (17). To examine whether activin A produced in tubular cells was involved in the expression of Pax-2, we analyzed the localization of Pax-2, activin A, and the type II activin receptor in the kidney after renal ischemia using serial sections. Type II activin receptor was localized in tubular cells (Figure 5B). The distribution pattern was not altered by ischemia/reperfusion injury (data not shown). As shown in Figure 5A and 5B, Pax-2-positive cells co-expressed the type II activin receptor. Furthermore, activin A was also expressed in these cells (Figure 5C).

Effect of rh-Activin and rh-Follistatin on the Number of BrdU/Pax-2 Double-Positive Cells in the Kidney after Renal Ischemia

To examine whether or not endogenous activin A is involved in the regulation of BrdU/Pax-2 double-positive cell growth during regeneration process of the kidney, we analyzed the number of BrdU/Pax-2 double-positive cells in an ischemic kidney treated with rh-activin A or rh-follistatin. As shown in Figure 5D, BrdU/Pax-2 double-positive cells were observed in ischemic kidneys but not in sham-operated kidneys. Interestingly, rh-follistatin increased; conversely, rh-activin A reduced the number of BrdU/Pax-2 double-positive cells in the kidneys after renal ischemia. rh-Follistatin did not increase BrdU/Pax-2 double-positive cells in normal kidneys.
Expression of Pax-2 in Embryonic Kidney in Organ Culture

It was reported that activin A inhibited branching morphogenesis of the ureteric bud in organ culture (13). To examine whether activin A also modulated the expression of Pax-2 during development, we used metanephric organ culture system. As shown in Figure 6A, the expression level of Pax-2 was significantly decreased in cultured metanephroi treated with activin A compared with that in cultured metanephroi treated without activin A. Immunohistochemical analyses also demonstrated that Pax-2–positive cells were rarely observed in cultured metanephroi treated with activin A (Figure 6B).

Expression of Pax-2 in Cultured Proximal Tubular Epithelial Cells

To further clarify the role of activin A in the regulation of the expression of Pax-2 in tubular cells, we also used a proximal tubular cell line, LLC-PK₁ cells. First, we examined the relationship between DNA synthesis and the expression of Pax-2 in LLC-PK₁ cells. Serum-starved LLC-PK₁ cells were cultured in complete medium containing 5% FBS with or without 10 nM activin A for the indicated times. As shown in Figure 7A, initiation of DNA synthesis was observed 12 h after growth stimulation. The expression level of Pax-2 was very low in quiescent cells, but it was significantly increased at 6 h and thereafter (Figure 7B), indicating that upregulation of the Pax-2 expression preceded initiation of DNA synthesis. Immunocytochemical staining also showed the presence of Pax-2 protein in growing cells (Figure 7C-b) but not in quiescent cells (Figure 7C-a). These results suggest that induction of Pax-2 is associated with cell growth in LLC-PK₁ cells, and it at least partly mimics in vivo events during tubular regeneration.

We next examined the effect of activin A on the expression of Pax-2 in LLC-PK₁ cells. Consistent with results obtained by in vivo experiments (Figure 5), activin A inhibited DNA synthesis (Figure 7A) and reduced the expression level of Pax-2 (Figure 7B). Activin A also decreased the number of Pax-2–positive nuclei in LLC-PK₁ cells (Figure 7C-c).

Upregulated Expression of Pax-2 Protein in LLC-PK₁ Cells Expressing Dominantly Negative Mutant Receptor

To further investigate the relationship between activin A and Pax-2 expression in tubular cells, we used LLC-PK₁-tARII cells (19). LLC-PK₁-tARII is a stable cell line expressing truncated type II activin receptor that lacks intracellular kinase domain, in which activin signaling pathway was completely blocked (19). Serum-starved LLC-PK₁-mock cells or LLC-PK₁-tARII cells were cultured in complete medium containing 5% FBS, and the expression of Pax-2 was examined at indicated times. As shown in Figure 8, the expression level of Pax-2 was transiently enhanced in LLC-PK₁-mock cells, but it was decreased 48 h after growth stimulation. In LLC-PK₁-tARII cells, the expression level of Pax-2 was also enhanced after growth stimulation. However, the increase continued for 48 h. Regarding that the expression of activin A was upregulated in tubular cells after growth stimulation (19), this result suggest that the expression of Pax-2 in tubular cells was tonically inhibited by endogenous activin A.
Blockade of the Activin Signaling Induced an Immature Cell Phenotype in LLC-PK₁ Cells

We further assessed cell phenotype in LLC-PK₁-tARII cells. We examined the expression of E-cadherin, one of the cell adhesion molecule expressed in differentiated epithelial tubular cells, in LLC-PK₁-mock cells and LLC-PK₁-tARII cells. Indirect immunofluorescence staining showed that the expression of E-cadherin was observed and was localized to the lateral portion of the plasma membrane in a linear staining pattern in LLC-PK₁-mock cells (Figure 9A). In contrast, the expression of E-cadherin was almost absent in LLC-PK₁-tARII cells (Figure 9B). Western blot analyses also demonstrated the decrease of E-cadherin protein in LLC-PK₁-tARII cells compared with that in LLC-PK₁-mock cells (Figure 9C).

Discussion

In the present study, we examined the involvement of developmental genes critical for kidney organogenesis in tubular regeneration. We demonstrated that the expression of Pax-2 was upregulated in tubular cells after ischemic injury (Figures 1 through 3). However, the expression of other transcription factors, such as Pax-8, WT-1, Wnt-4, and BF-2, was not significantly changed in the kidneys after renal ischemia (Figure 1). These results suggest that, among transcription factors involved in renal development, developmental cascade controlled by Pax-2 is reactivated and is potentially involved in tubular regeneration.

Pax-2 (18), a transcription factor belonging to the Pax family, which contains the DNA-binding paired domain, plays a key regulatory role during renal organogenesis (26). Proper temporal and spatial expression of Pax-2 is tightly regulated during normal kidney development (27). Deregulated expression of Pax-2 was shown to be associated with the abnormality of the kidney in mice (28,29) and human (30,31). In renal
MATERIALS AND METHODS. Magnification, ×400. (C) Production of E-cadherin protein in serum-starved LLC-PK₁-mock cells and LLC-PK₁-tARII cells was examined by Western blotting as described in Materials and Methods.

Figure 9. Expression of E-cadherin in LLC-PK₁-mock cells and LLC-PK₁-tARII cells. The expression of E-cadherin in serum-starved LLC-PK₁-mock cells (A) and LLC-PK₁-tARII cells (B) was examined by indirect fluorescence immunostaining as described in Materials and Methods. Magnification, ×400. (C) Production of E-cadherin protein in serum-starved LLC-PK₁-mock cells and LLC-PK₁-tARII cells was examined by Western blotting as described in Materials and Methods.

Although it is unclear at present by which mechanism activin A suppresses the expression of Pax-2 in tubular cells, it is possible that activin A directly modulates the transcription of Pax-2. As an intracellular mediator of activin signaling, Smad proteins have been identified (34). Among the eight cloned Smad genes, Smad-2 and Smad-3 mediate the activin signals. Upon direct phosphorylation by the type I activin receptor, Smad-2 or Smad-3 binds to its partner Smad-4 to form a heteromeric complex and translocates into the nucleus, where it can potentially regulate the transcription of target genes. We found that the proposed Smad binding element (SBE) of CA-GAC was present on the upstream of the transcription start site in the sequence of the human Pax-2 promoter (35). It is also known that binding to the SBE is not sufficient for Smad-dependent transcriptional activation, and additional DNA contacts seem to be necessary for specific, high-affinity binding of a Smad complex to the target gene in many biologic systems (34). In this regard, we also observed the presence of a binding site for transcriptional co-factors of Smad protein, such as Sp1 (36) or NF-κB (37). Therefore, it is quite possible that Smads bind to the Pax-2 promoter and repress its transcription rates with transcriptional co-factors. Further study is necessary to address this issue.

In summary, we demonstrate here that Pax-2 was upregulated in tubular cells after renal ischemia. Pax-2 protein was co-localized with activin A and the type II activin receptor. Furthermore, we show the inhibitory effect of activin A on the expression of Pax-2 in tubular cells both in vivo and in vitro. Activin A may be a critical regulator of tubular regeneration that modulates cell growth and differentiation of Pax-2-positive progenitor-like tubular cells.

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References


