Activin A: An autocrine regulator of cell growth and differentiation in renal proximal tubular cells

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**Background.** Activin A is involved in tubular regeneration after ischemia/reperfusion injury. The present study was conducted to examine the role of activin A in cell growth, apoptosis and differentiation of tubular cells.

**Methods.** We performed cell proliferation assays (MTT assay, [3H]-thymidine incorporation) and apoptosis detection assays (nuclear staining, DNA ladder formation, TUNEL staining) using LLC-PK1 cells. Expression of activin and activin receptor in LLC-PK1 cells also were examined by real-time polymerase chain reaction (PCR) and immunostaining. Stable cell lines expressing the truncated type II activin receptor were generated and the phenotype of these cells was analyzed.

**Results.** Activin A inhibited DNA synthesis and cell growth in a dose-dependent manner and induced apoptosis in LLC-PK1 cells. The expression level of mRNA for the activin βA subunit was markedly increased when the growth was stimulated. The expression of the type II activin receptor was observed in LLC-PK1 cells. The growth rate of cells expressing dominantly negative activin receptor was significantly faster than that of non-transfected cells. The expression level and pattern of cytokeratin and vimentin in these cells were quite different compared to non-transfected cells. When cultured in collagen gel, these cells formed multiple processes, which was not observed in non-transfected cells. Finally, the expression of Pax-2 was markedly elevated in these cells.

**Conclusions.** Activin A acts as an autocrine inhibitor of cell growth, an inducer of apoptosis, and an important modulator of differentiation in cultured proximal tubular cells.

**Activins** are multifunctional cytokines belonging to the transforming growth factor-β (TGF-β) superfamily, which regulate cell growth and differentiation in many biological systems [1, 2]. Activins are dimeric proteins and subunits of activin are expressed in various organs [3]. In the kidney, activin A is expressed in the developing stage [4] and has been shown to inhibit branching morphogenesis of ureteric buds in organ culture [5]. In an in vitro tubulogenesis model, activin A produced in Madin-Darby canine kidney (MDCK) cells tonically inhibited branching tubulogenesis [6]. In addition, hepatocyte growth factor (HGF) induced branching tubulogenesis mainly by blocking the production of activin A [6]. This raises the possibility that activin A negatively regulates tubulogenesis during the kidney development [7]. Although activin A may play an important role for tubulogenesis in kidney development, its biological effects in this organ after completion of development is still unknown.

Recently, we demonstrated that the expression of activin A was up-regulated in tubular cells after ischemia/reperfusion injury [8]. Furthermore, intravenously administered follistatin, which specifically binds to activins and blocks their actions [9–11], improved renal dysfunction and histological changes after renal ischemia [8]. Follistatin also accelerated renal tubular regeneration by enhancing DNA synthesis and preventing apoptosis in tubular cells [8]. Based on these results, we hypothesized that endogenous activin A tonically inhibits cell growth, induces apoptosis and modulates differentiation in tubular cells. To further address this issue, we examined the role of activin A in cell growth, apoptosis and differentiation using LLC-PK1 cells, a proximal tubular cell line, as a model system. The results showed that activin A inhibited cell proliferation and induced apoptosis in LLC-PK1 cells. The expression of activin and activin receptor was observed in these cells. Furthermore, the blockade of activin signaling by overexpression of the dominantly negative activin receptor promoted tubular cell growth and induced a phenotype resembling that of progenitor-like tubular cells observed during regeneration. We conclude that activin A acts as an autocrine inhibitor of cell growth as well as an inducer of apoptosis in tubular cells, and also that it is necessary for tubular cell differentiation.

**METHODS**

**Materials**

Recombinant human activin A and polyclonal anti-human activin A antibody were provided by Dr. Y. Eto.
of the Central Research Laboratory, Ajinomoto Inc. (Kawasaki, Japan). Recombinant human HGF was obtained from Funakoshi (Tokyo, Japan). Epidermal growth factor (EGF) was purchased from Collaborative Research (Lexington, MA, USA). Polyclonal antibody against the activin type II receptor was generously provided by Dr. K. Miyazono (University of Tokyo, Tokyo, Japan). Polyclonal anti-vimentin antibody and polyclonal anti-cytokeratin antibody were obtained from Neo Markers (Fremont, CA, USA) and Dako (Glostrup, Denmark), respectively. Polyclonal antibody against Pax-2 was obtained from Berkeley Antibody Company (Richmond, CA, USA). Polyclonal goat anti-human Smad-2 antibody, and polyclonal rabbit anti-dipeptidyl peptidase (DPP) IV antibody were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). [3H] Thymidine and [32P] deoxyctydine triphosphate were from Dupont-New England Nuclear (Boston, MA, USA).

Cell culture

LLC-PK₁ cells were obtained from the American Type Culture Collection (ATCC CRL-1392; Rockville, MD, USA) and were cultured in complete medium consisting of Medium 199 (ICN Biomedicals, Inc.) with 5% fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA), penicillin, streptomycin in an atmosphere of 5% CO₂-95% air at 37°C. Total RNA was extracted using TRIzol reagent (Gibco BRL) according to the manufacturer’s instructions. First-strand cDNA was made from total RNA using the Superscript Preamplification System (Gibco BRL) and reverse-transcription-polymerase chain reaction (RT-PCR) was performed as described previously [8]. The expression of Pax-2 was examined using the following primers: sense and anti-sense were 5'-TGCTGAATACAAACGACAGAAACC-3' and 5'-GCAAGTGTTCCGCAAACGTG-3', respectively.

Culture in collagen gel

Cells were suspended at 5 × 10⁶ cells/ml in a neutralized collagen solution (Koken, Tokyo, Japan), dispensed into 24-well plates and incubated at 37°C. After the collagen solution had gelled, the complete culture medium was added and renewed every two to three days. The cultures were photographed at 5 days under phase contrast using a Nikon Diaphot TMD inverted microscope (Tokyo, Japan).

Measurement of DNA synthesis and cell viability

DNA synthesis was assessed by measuring [³H] thymidine incorporation into trichloroacetic acid precipitable materials as described previously [12]. Cell viability was assessed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay [6].

Nuclear DNA staining with DAPI

Cells grown on cover slips were cultured in the serum-free medium with or without 10 nmol/L activin A for 48 hours. After washing with phosphate-buffered saline (PBS), the cells were incubated with DAPI (4',6-diamidino-2'-phenylindole dihydrochloride) (Boehringer Mannheim, Mannheim, Germany) for 30 minutes at 37°C. Cells were washed with PBS and mounted. The fluorescent dye DAPI stains nuclear DNA so that condensation and division of chromatins can be readily detected.

DNA electrophoresis

Cells cultured in the serum-free medium with or without activin A for 48 hours were detached with trypsin in PBS and centrifuged together with the cells present in the culture medium. The oligonucleosomal fragmentation of DNA associated with apoptosis [13] was evaluated by DNA electrophoresis using an Apoptosis Ladder Detection Kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan) according to the manufacturer’s instructions.

TdT-mediated dUTP nick end labeling staining

To identify nuclei with DNA strand breaks at the cellular level, the TdT-mediated nick end labeling (TUNEL) method [14] was done using an Apoptosis in situ Detection Kit (Wako) as described previously [8]. To calculate the percentage of TUNEL positive cells, we counted all cells from four random microscopic fields at ×100 magnification.

Real-time reverse-transcription polymerase chain reaction

Total RNA was extracted using TRIzol reagent (Gibco BRL) according to the manufacturer’s instructions. First-strand cDNA was made from total RNA using the SuperScript Preamplification System (Gibco BRL) and reverse-transcription-polymerase chain reaction (RT-PCR) was performed as described previously [8]. The expression of Pax-2 was examined using the following primers: sense and anti-sense were 5'-TTCGCACCTCA AGTCTG-3' and 5'-ATGATGTTCCTGGCGTGCTGAG-3', respectively.

Real-time PCR was performed by the ABI Prism 7700 Sequence Detection System using the SYBR Green PCR Core Reagent Kit (Perkin Elmer Applied Biosystem, Foster City, CA, USA). Reactions included 5 µL of a 10 × SYBR PCR buffer, 2 µL MgCl₂ (50 mmol/L), 1 µL dNTP mix, 1 µL 3' primer, 1 µL 5' primer, 0.5 µL AmpliTaq Gold and 1 µL cDNA. Samples were incubated at 94°C for five minutes, followed by 40 cycles of 30 seconds at 94°C, 30 seconds at 58°C, 60 seconds at 72°C, and a final extension at 72°C for 10 minutes. The expression of the β₅, subunit and GAPDH in each sample was quantified in separate tubes with the following primers: porcine β₅ subunit (sense and antisense were 5'-TTCGCAAACTCTCA AGTCTG-3' and 5'-ATGATGTTCCTGGCGTGCTGAG-3', respectively), porcine GAPDH (sense and antisense were 5'-ATGCTGTGTGTCTGGAGTATGTC GTGG-3' and 5'-AGATGATGACCCCTTTTG GCTCC-3', respectively). No PCR products were detected in the real-time PCR procedure without reverse transcription, indicating that the contamination of geno-
mic DNA was negligible. Gels of the PCR products after quantification of the indicated genes by real-time PCR showed single bands (βA subunit: 83 base pair, and GAPDH, 99 base pair, respectively) in which the size was the same as expected (data not shown).

**Immunocytochemistry**

Cells cultured on cover slips were washed with PBS, 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 primary antibodies at room temperature for one hour. After washing in PBS, cells were covered with a mixture of a Cy3 (indocarbocyanine)-labeled goat anti-rabbit IgG antibody or FITC-labeled rabbit anti-mouse IgG antibody or Rhodamine-labeled rabbit anti-goat IgG antibody and DAPI. 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, USA), which was operated with IP Lab Spectrum software (Signal Analysis, Vienna, VA, USA).

**Transfection and establishment of stable cell line**

LLC-PK1 cells were transfected by Lipofectamine Plus™ Reagent (Life Technologies, Paisley, UK) with PCXN2 plasmid containing truncated activin type II receptor (tARII) and selected by continuous growth in G418 (800 μg/mL geneticin; Life Technologies). After five passages, 10 individual clones were isolated and screened for tARII expression by Northern blot analysis. Three clones expressing high levels of message were identified and routinely utilized in this study. No significant differences were observed among these three clones in all of our experiments. As a control, LLC-PK1 cells also were transfected with PCXN2 plasmid alone and grown and maintained in G418.

**RNA extraction and Northern blot analysis**

Total RNA (20 μg) was extracted from cells using TRIzol Reagent. Northern blotting was done as described previously [6]. The membrane was subjected to autoradiography and analyzed using a Fujix BAS 2000 (Fuji Photo Film, Tokyo, Japan).

**Western blot analysis**

Cells were washed three times with PBS, suspended in a buffer containing 50 mmol/L Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol, 2-mercaptoethanol (2-ME), and heated to 100°C for 10 minutes. After centrifugation, the supernatant was collected, and the protein concentration was determined by using a protein assay kit (Bio-Rad Laboratories, Munich, Germany). Twenty micrograms of protein from each sample were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing condition and transferred to a polyvinylidene difluoride (PVDF) membrane (Nihon Millipore Ltd., Yonezawa, Japan) by electroblotting. To reduce nonspecific antibody binding, the membrane was blocked with 5% BSA, 0.1% NaN3 dissolved in Tris-saline (TS) for one hour at 37°C, incubated overnight at 4°C with goat polyclonal anti-human activin type II receptor antibody or rabbit polyclonal anti-DPP IV antibody, and washed with Tris-PBS (PBST). After incubation with peroxidase labeled anti-rabbit or anti-goat IgG antibody for one hour 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, Tokyo, Japan).

**Statistical analysis**

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

**RESULTS**

**Effects of activin A on DNA synthesis in LLC-PK1 cells**

The effects of activin A on DNA synthesis were examined in LLC-PK1 cells. Serum-starved LLC-PK1 cells were incubated in complete medium containing 2% FBS with various concentrations of activin A. As shown in Figure 1A, recombinant human activin A induced dose-dependent inhibition of FBS-induced DNA synthesis. Significant inhibitory effects of activin A on DNA synthesis were observed at doses higher than 1 nmol/L (P < 0.05). No further inhibition was obtained even at a dose as high as 20 nmol/L (data not shown). Activin A also inhibited DNA synthesis induced by growth factors such as HGF and EGF, potent mitogens for LLC-PK1 cells (data not shown).

**Effect of activin A on cell growth in LLC-PK1 cells**

To confirm that activin A inhibits cell growth, the effect of activin A on LLC-PK1 cell growth was assessed by MTT assay. Cells were cultured for four days in complete medium containing 2% FBS in the presence of activin A at the indicated concentrations. Consistent with the inhibition of DNA synthesis, recombinant human activin A reduced the cell number in a dose-dependent manner (Fig. 1B). The effect of activin A was significant (P < 0.05) at doses higher than 1 nmol/L.

**Induction of apoptosis by activin A in LLC-PK1 cells**

To determine whether activin A induced apoptosis in renal tubular cells, LLC-PK1 cells were incubated with various concentrations of recombinant human activin A in the serum-free medium for two days. Compared with con-
trol cells (without activin A), activin-treated cells showed the classical morphological features of apoptosis, including chromosomal condensation (Fig. 2A) and nuclear DNA fragmentation (Fig. 2B). These cells become TUNEL positive (Figs. 2C). Quantification of TUNEL assays revealed that activin A increased the number of apoptotic cells at concentrations higher than 10 nmol/L (Fig. 2D).

**Induction of cell differentiation by activin A in LLC-PK1 cells**

To examine whether activin A is involved in cell differentiation, we studied the expression of DPP IV in LLC-PK1 cells treated with or without activin A by Western blotting. DPP IV is a 110 to 120 kD glycoprotein presented in the brush border of proximal tubules and functions as a hydrolase [15]. As shown in Figure 3, this apical marker of proximal tubules was detected slightly in LLC-PK1 cells. In contrast, the expression level of DPP IV significantly increased in LLC-PK1 cells treated with activin A.

**Expression of activin A in LLC-PK1 cells**

Next, the mRNA expression of the \( \beta_{A} \) subunit of activin in LLC-PK1 cells was examined by real-time PCR. As shown in Figure 4A, the activin \( \beta_{A} \) subunit expression was very low in quiescent LLC-PK1 cells, but was strongly induced when growth was stimulated with FBS. Consistent with the results from real-time PCR, immunoreactive activin A was observed in growing LLC-PK1 cells after growth stimulation, which revealed granular cytoplasmic staining of cells, but not in quiescent LLC-PK1 cells (Fig. 4B).

**Expression of the type II activin receptor in LLC-PK1 cells**

The biological activity of activin is mediated by two different types of receptors, type I and type II [2]. Activin first binds to the type II receptor. Immunocytochemistry was used to examine the expression of the type II activin receptor in LLC-PK1 cells. Figure 5 shows that the type II activin receptor was located within the cytoplasm of LLC-PK1 cells. The reason for an apparent detection of the receptor in the cytoplasm was that we did not use confocal microscopy. The expression level and localization of the type II activin receptor in LLC-PK1 cells were not altered when the growth was stimulated (data not shown). Two bands (62 and 75 kD) were detected for the type II activin receptor by Western blot analysis (Fig. 5C), which is consistent with an earlier study [16].

**Generation of a stable cell line expressing truncated type II activin receptor**

To examine the function of endogenous activin A in LLC-PK1 cell growth and differentiation, stable cell lines expressing the truncated type II activin receptor (LLC-PK1-tARII) were generated that lacked the intracellular kinase domain. Overexpression of this mutant receptor blocked the activin signaling in a dominant negative fashion [17]. First, we examined the expression level of the truncated activin type II receptor in LLC-PK1-tARII cells by Northern blotting (Fig. 6A). Expression of its cDNA was visible only in the lanes of quiescent LLC-PK1-tARII, but not in wild-type or vector transfected LLC-PK1 cells. As shown in Figure 6B, the inhibitory effect of activin A on DNA synthesis was completely abolished in LLC-PK1-tARII cells. Furthermore, translocation of Smad protein into the nucleus after activin treatment was not observed in LLC-PK1-tARII cells (Fig. 6C), confirming the blockade of activin signaling. Morphologically, there was no change in LLC-PK1-tARII cells compared to wild-type or vector-transfected LLC-PK1 cells in monolayer culture (data not shown).
Changes in tubular cell phenotype induced by blockade of the activin signaling

To assess the phenotype of cells expressing tARII, we examined the expression of cytokeratin and vimentin, which are known to be epithelial and mesenchymal cell markers, respectively, in wild-type LLC-PK₁ and LLC-PK₁-tARII cells. Indirect immunofluorescence staining showed that the expression of cytokeratin was observed and located in the cytoplasm in wild-type LLC-PK₁ cells (Fig. 7B, panel e). In contrast, vimentin distributed diffusely throughout the cytoplasm in wild-type LLC-PK₁ cells (Fig. 7B, panel g).

Changes in the morphology by blockade of the activin signaling in LLC-PK₁ cells cultured in collagen gel

To examine the role of endogenous activin in cell differentiation, a collagen gel culture system was used. Morphological changes in LLC-PK₁-tARII cells cultured in collagen gel were observed for five days. When wild-type LLC-PK₁ cells were cultured in collagen gel, these cells formed a spherical cluster (Fig. 7C, panel a). In contrast, LLC-PK₁-tARII cells cultured in collagen gel formed multiple elongated processes (Fig. 7C, panel b).

Up-regulation of Pax-2 expression by the blockade of activin signaling

The expression of Pax-2 in cells expressing tARII was examined, as Pax-2 is a transcription factor thought to be critical in organogenesis of the kidneys [18]. Pax-2, which is expressed in embryonic kidneys, is re-expressed significantly faster than that of either wild-type or vectortransfected LLC-PK₁ cells (Fig. 7A).
Fig. 4. Expression of activin A in LLC-PK₁ cells. (A) Time course of β₃ subunit mRNA expression in LLC-PK₁ cells after growth stimulation. Serum-starved cells were cultured in complete medium containing 5% FBS. Total RNA was extracted at indicated times. Real-time RT-PCR was performed as described in the Methods section. Results are presented as the mean ± SE of three independent experiments. (B) Representative data of immunoreactive activin A in LLC-PK₁ cells. Serum-starved cells (panels a, b) were cultured in complete medium containing 5% FBS for 2 days (panels c, d). Immunocytochemistry was performed as described in the Methods section (magnification ×400; a and c, activin A (red), nuclei (blue); b and d, Nomarski image).

in tubular cells of the regenerating kidney in adults [19]. As shown in Figure 8A, the expression of Pax-2 was markedly increased in LLC-PK₁-tARII cells, but not in either the wild-type or mock-transfected cells. Likewise, immunoreactive Pax-2 was detected in the nuclei of LLC-PK₁-tARII but not in wild-type LLC-PK₁ cells (Fig. 8B).

DISCUSSION

Adult tubular epithelium has a potential of regeneration following a variety of insults. During tubular regeneration, quiescent cells undergo dedifferentiation, reobtain their potential to divide, and then differentiate to restore the functional integrity of the nephron [20]. Several growth factors have been reported to be potentially involved in kidney regeneration and play important roles in these processes as a mitogen, motogen, and morphogen [21–25].

The present study demonstrated the expression of activin A and the activin receptor in LLC-PK₁ cells. In addition, the expression of activin A in LLC-PK₁ cells was not detected in a quiescent state, but was markedly up-regulated when growth was stimulated. We previously showed that activin A is up-regulated in tubular cells after renal ischemia [8], whereas the expression of the activin receptor was ubiquitously observed in tubular cells of the kidney [8]. Collectively, activin A acts as an autocrine factor in tubular cells both in vivo and in vitro. LLC-PK₁ is a renal epithelial cell line derived from proximal tubular cells [26], which actively participates in cell growth during tubular regeneration [20]. These cells provide a useful model to study the activin action on tubular cells in detail.

The present results show that activin A inhibited cell growth in a dose-dependent manner and induced apoptosis in LLC-PK₁ cells; furthermore, the blockade of the activin signaling by overexpressing the truncated type II activin receptor promoted cell growth. The growth rate of LLC-PK₁-tARII cells was faster than that of wild-type or vector-transfected cells. Collectively, activin A functions as an autocrine inhibitor of cell growth in LLC-PK₁ cells. The expression of activin A was induced in
LLC-PK₁ cells, regardless of growth stimuli, suggesting that these cells are programmed to express activin A when the growth was stimulated. If this idea could be applied to the regeneration processes of the kidney, it is possible that, during regeneration after renal ischemia, proliferating tubular cells are programmed to express activin A and then stop cell growth or lapse into apoptosis for the prevention of excessive cell growth. On the other hand, activin A enhanced the expression level of DPP IV, an apical marker of proximal tubules. This observation suggests that activin A has the potential to induce, at least in part, maturation of proximal tubular cells. Therefore, it is likely that activin A is a growth inhibitor as well as a regulator of cell differentiation to restore epithelial integrity of the tubules during regeneration.

It should be noted that renal regeneration after renal ischemia is a complex phenomenon regulated by variety of factors. Hence, the present study does not address all of the mechanisms involved in vivo. For example, neutrophils are activated after renal ischemia and resultant release of bioactive substances induce tubular damage [20, 27]. Those aspects were not mentioned in the present study.

The up-regulation of activin expression is significant in the regeneration processes of the kidney [8]. We then asked which mechanism regulates the expression of activin in tubular cells. Possibly the most important mediator of activin A expression is the mitogens for tubular cells. Our present study shows that the expression of activin A was markedly increased in LLC-PK₁ cells when the growth is stimulated by complete medium containing 5% FBS. We also observed an increase in the expression level of activin A when the growth was stimulated by HGF or EGF (data not shown). These factors are local regulators of tubular cell growth after renal injury [23–26]. Hence, these mitogens may induce the expression of activin A during regeneration. Pro-inflammatory cytokines also are possible mediators of activin expression. It was reported that the expression level of activin A was correlated with the degree of inflammation in inflammatory bowel disease [28, 29]. A strong expression of activin A was induced by the pro-inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) in vitro [30]. In an ischemia/reperfusion model, reperfusion induces inflammation such as neutrophil infiltration in the kidney, potentially contributing to the development of postischemic tubular cell death [27]. Nevertheless, no induction of activin expression was observed in LLC-PK₁ cells treated with pro-inflammatory cytokines such as TNF-α or IL-6 (unpublished observation). Thus, pro-inflammatory cytokines may not induce activin expression during regeneration of the kidney.

Progenitor-like tubular cells with high proliferative potential participate in tubular regeneration [21, 22]. These cells express a mesenchymal marker, vimentin, and resemble immature mesenchymal cells observed during kidney development [31]. In the present study, we observed that the expression level of cytokeratin, an epithelial marker, was decreased in LLC-PK₁-tARII cells compared to wild-type LLC-PK₁ cells. The expression level of vimentin was almost similar to that of wild-type LLC-
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Fig. 8. Expression of Pax-2 in LLC-PK1 cells expressing truncated type II activin receptor. (A) Expression of Pax-2 mRNA in wild-type (lane 1), vector-transfected (lane 2), and LLC-PK1-tARII (lane 3) cells was examined by RT-PCR. Data are representative of three independent experiments. *P < 0.01 vs. wild-type. (B) Localization of Pax-2 protein in wild-type (panels a, b) and LLC-PK1-tARII (panels c, d) cells was examined by indirect fluorescence immunostaining. Note that Pax-2 protein (red) was localized in the nuclei (blue) of LLC-PK1-tARII cells, but was not observed in wild-type LLC-PK1 cells. b, d: Nomarski images. Magnification 400.

Fig. 7. Characteristics of LLC-PK1 cells expressing truncated type II activin receptor. (A) Growth rate of LLC-PK1 cells expressing truncated type II activin receptor wild-type, vector-transfected (●), and tARII-transfected (○) LLC-PK1 cells were cultured in complete medium containing 5% FBS for indicated periods. MTT assay was performed as described in the Methods section. Results are presented as the mean ± SE of three independent experiments. *P < 0.01 vs. wild-type. (B) Expression and localization of cytokeratin and vimentin in LLC-PK1 cells expressing truncated type II activin receptor wild-type (panels a, b and c, d) and LLC-PK1-tARII (panels e, f and g, h) cells were cultured on cover slips, washed with PBS and fixed with 4% paraformaldehyde. Localization of cytokeratin (a-d) and vimentin (e-h) was determined by immunocytochemistry as described in the Methods section. (b, d, f, h) Nomarski images, magnification X1000 in a-d and X400 in e-h. (C) Morphological changes in LLC-PK1 cells expressing truncated type II activin receptor cultured in collagen gel. Wild-type (panel a) and LLC-PK1-tARII (panel b) cells were cultured in collagen gel for 5 days as described in the Methods section (magnification X400). Note that multiple processes (arrowheads) were observed in LLC-PK1-tARII cells, but not in wild-type cells.

Pax-2, which was not observed in wild-type or vector-transfected LLC-PK1 cells. Pax-2 is a developmental gene critical for nephron formation in embryonic kidney [18] as well as for cell proliferation or apoptosis in the regenerating kidney [19]. Collectively, LLC-PK1-tARII cells resemble in many respects renal progenitor-like cells observed during tubular regeneration. These results suggest that activin A is necessary for maintaining differentiated properties and maturation of tubular cells in the regeneration processes of the kidney.

In summary, our results show that activin A tonically inhibited cell growth, induced apoptosis and was needed for maintaining differentiation functions in LLC-PK1 cells. Activin A is likely to be up-regulated in tubular cells to prevent abnormal cell growth and to induce differentiation and maturation during tubular regeneration.

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