Activin A Produced by Ureteric Bud Is a Differentiation Factor For Metanephric Mesenchyme

AKITO MAESHIMA,† SHIN YAMASHITA,* KYOKO MAESHIMA,†
ITARU KOJIMA,† and YOSHIHISA NOJIMA*

*Third Department of Internal Medicine, Gunma University School of Medicine, Maebashi, Japan; †Institute for Molecular & Cellular Regulation, Gunma University, Maebashi, Japan.

Abstract. The present study was conducted to investigate the role of the activin-follistatin system in the development of metanephros. Organ culture system and cultured metanephric mesenchymal cells were used to address this issue. Activin A was localized in ureteric bud. Activin type II receptor was localized in ureteric bud as well as metanephric mesenchyme. In an organ culture system, exogenous activin A reduced the size of cultured metanephroi, delayed ureteric bud branching, and enlarged the tips of ureteric bud. Follistatin, an antagonist of activin A was used to clarify the role of endogenous activin A. Exogenous follistatin enlarged the size of cultured metanephroi, increased ureteric bud branching, and promoted cell growth in ureteric bud. Blockade of activin signaling by adenoviral transfection of dominantly negative activin mutant receptor mimics the effect of follistatin. In cultured metanephric mesenchymal cells, activin A promoted cell growth; conversely, follistatin induced apoptosis. Furthermore, activin A induced the expressions of epithelial differentiation markers in these cells. These results suggest that activin A produced by ureteric bud is not only an important regulator of ureteric bud branching, but also a differentiation factor for metanephric mesenchyme during kidney development.

Kidney organogenesis depends on a series of reciprocal inductive interactions between the epithelial ureteric bud and metanephric mesenchyme (1). The signals from metanephric mesenchyme initiate kidney development by inducing formation of the ureteric bud from the Wolffian duct. Subsequently, the growing ureteric bud also starts to branch as a response to the mesenchymal signals and in return secretes signals that induce mesenchymal cells to condensate and generate pretubular aggregates at the tips of ureteric bud branches. Aggregates undergo tubulogenesis via comma-shaped and S-shaped bodies. These tubular structures will finally fuse to the forming collecting duct. Forming nephrons also attract endothelial cells to make a functional glomerulus (2,3). A large number of soluble factors, extracellular matrix, proteases, and protease inhibitors have been reported to be involved in kidney development by using gene targeting, in vitro tubulogenesis model, and organ culture system (4–6).

Activins are multifunctional cytokines belonging to the transforming growth factor-β (TGF-β) superfamily, which regulate cell growth and differentiation in many biologic systems (7,8). Activins are dimeric proteins, and subunits of activin are expressed in various organs (9). The biologic activity of activin is mediated by heteromeric receptor complexes consisting of two different types of receptor, the type I receptors (ActRI) and type II receptors (ActRII), which are characterized by an intracellular serine/threonine kinase domain (7). Activin first binds to the ActRIII, and then ActRI is recruited into the complex. ActRII phosphorylates ActRI in the GS domain to activate it. Ligand binding triggers the assembly of the receptor complex, but the complex is also stabilized by direct interaction between the cytoplasmic parts of the receptors. As an intracellular mediator of activin signaling, the Smad proteins have been identified (7). Eight Smad genes have been cloned; of these, Smad-2 and Smad-3 mediate the activin signals. Upon direct phosphorylation by ActRI, Smad-2 or Smad-3 binds to its Smad-4 partner to form a heteromer complex and translocates into the nucleus, where they can potentially regulate the transcription of target genes. The important modulator of activin action is follistatin (10). This protein specifically binds to activins and related ligands with high affinity and blocks their action (11,12). Follistatin is synthesized in the target cells of activins and remains in the extracellular matrix (13). Furthermore, the production of follistatin is regulated by activins. Hence, activin and follistatin modulate cellular functions in a complex manner. Many kinds of knockout mice, which lack activin subunit or activin receptors, were developed (14–17). However, abnormality of the kidney was not described in these mutant mice. This is probably due to the redundancy in the ligands and receptor systems. We recently generated transgenic mice expressing truncated type II activin receptor under the control of β-actin promoter. In these mice, the number of glomeruli was significantly increased (18). This increase in the nephron number may result from the enhanced ureteric bud branching during development caused by the inhibition of the activin signal. It was also reported that activin A was expressed...
in the metanephros (19) and inhibited the branching of ureteric bud in the metanephric organ culture system (20). In an in vitro tubulogenesis model, activin A produced by MDCK cells tonically inhibits branching tubulogenesis and HGF-induced branching tubulogenesis mainly by blocking the production of activin A (21). Collectively, activin A may be a negative regulator of ureteric bud branching during kidney development (22,23). However, the precise mechanism of activin A in kidney development still remains unknown. In addition, the action of activin A on metanephric mesenchyme is not clarified at all.

The present study demonstrated that activin A and activin receptors were expressed in ureteric bud. Activin type II receptor was also expressed in metanephric mesenchyme. In an organ culture system, exogenous activin A delayed the branching of ureteric bud and enlarged the tips of ureteric bud. On the other hand, follistatin enlarged the size of cultured metanephroi, promoted the branching of ureteric bud, and increased cell growth in ureteric bud, but it induced mesenchymal cell apoptosis. In cultured metanephric mesenchymal cells, activin A promoted cell proliferation; conversely, follistatin induced apoptosis. Furthermore, activin A induced the expressions of epithelial differentiation markers in these cells. These results suggest that activin A produced by ureteric bud acts as a differentiation factor for metanephric mesenchyme.

Methods and Materials

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 Co. (Kawasaki, Japan). Monoclonal mouse anti-human follistatin antibody was obtained from R&D System Inc. (Minneapolis, MN). Polyclonal rabbit anti-activin type I receptor and anti-activin type II receptor antibodies were provided by Dr. K. Miyazono (Tokyo University, Tokyo, Japan). Polyclonal mouse anti-vimentin antibody and polyclonal mouse anticytokeratin antibody were obtained from NEO MARKERS (Fremont, CA) and DAKO (Denmark), respectively. Polyclonal rabbit antibodies against E-cadherin, WT1, and polyclonal goat antibodies against Smad2/3, proliferating cell nuclear antigen (PCNA), and Wnt4 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). FITC-conjugated anti-Dolichos biflorus lectin (DBA) antibody was obtained from EY Laboratories, Inc. (San Mateo, CA). Polyclonal rabbit antibody against Pax-2 was obtained from Berkeley Antibody Company (Richmond, CA). Dulbecco modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Life Technologies/BRL (Grand Island, NY).

Metanephric Organ Culture

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. Metanephroi were explanted onto Transwell-clear (pore size: 0.4 μm; Corning Incorporated, NY) at the interface between air/5% CO₂ atmosphere and medium and cultured at 37°C in DMEM containing 5% FBS and antibiotics. Cultured metanephroi were examined daily under a phase contrast microscope for up to 3 d.

Morphometry of Cultured Metanephroi

To examine the size of cultured metanephroi, we performed morphometrical analysis using image analysis software (NIH Image, public domain; NIH, Bethesda, MD) (24). Cultured metanephroi (n = 6) in each condition were photographed with a light microscope at ×200 magnification at the indicated periods. The outline of cultured metanephroi was traced and each area was calculated.

Immunohistochemistry

Immunohistochemistry via an avidin-biotin coupling (ABC) immunoperoxidase technique was performed using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instruction. Briefly, rat embryos were fixed with 4% paraformaldehyde and embedded in paraffin. Four-micrometer sections were deparaffinized and rehydrated in a routine manner. After inactivation of endogenous peroxidase with 1% metaperiodate in PBS for 10 min at room temperature (RT), sections were preincubated with normal goat/horse/rabbit serum for 60 min. Sections were then incubated for 2 h with primary antibody as follows: anti-activin A (1:100), anti-follistatin (1:20), anti-activin type I receptor (1:100), anti-activin type II receptor (1:100), anti-Smad2/3 (1:100), and anti-PCNA (1:100) antibodies. Sections were washed with PBS and reacted with a biotinylated secondary antibody for 1 h. After washing with PBS, sections were reacted with a Vectastain Elite ABC Reagent. The antibody was detected with diaminobenzidine tetrahydrochloride (DAB) in PBS, and the sections were counterstained with hematoxylin. For immunohistochemical controls, the primary antibody was replaced with normal serum of the same species as the secondary antibody, which did not show positive staining, confirming specificity. Tissue sections were also stained with hematoxylin and eosin (H&E) in a routine manner.

To examine the morphologic change of ureteric bud, whole-mount indirect immunofluorescence was performed. DBA was used to specifically stain ureteric bud (25). Cultured metanephroi were fixed in 4% paraformaldehyde for 1 h, washed in Tris-buffered saline containing 1 mM CaCl₂ (pH 7.6) (TBS-Ca) for 5 min three times, and then fixed in cold 100% methanol for 20 min at −20°C. After incubation in 2% BSA-TBS for 1 h at 4°C, cultured metanephroi were reacted

Figure 1. Expressions of mRNA for βA subunit, activin receptors, and follistatin in developing, neonatal, and adult kidneys. Total RNA was isolated from developing, neonatal, and adult kidneys. The expressions of mRNA for βA subunit, type I and type II activin receptor, and follistatin were analyzed by reverse transcriptase–PCR (RT-PCR). Product sizes are indicated in the right. Representative result of three separate experiments was shown. E14 to E18, embryonic days 14 to 18; N1, neonatal 1 day; Adult, 6 wk.
with FITC-labeled anti-DBA antibody (1:50) at 4°C overnight. After 8 h washing in TBS, cultured metanephroi were mounted and analyzed 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).

**TUNEL Staining**

To identify nuclei with DNA strand breaks at the cellular level, the TdT-mediated nick end labeling (TUNEL) method was done using an Apoptosis in situ Detection Kit (Wako, Tokyo, Japan).

**Reverse Transcriptase–PCR**

Total RNA was isolated with the TRIzol Reagent (Life Technologies/BRL) from whole kidneys of fetal (E14, \( n = 10 \); E16, \( n = 10 \); E18, \( n = 6 \)), neonatal (1 d, \( n = 1 \)), and adult (6 wk, \( n = 1 \)) rats. First-strand cDNA was made from total RNA using Superscript Preamplification System (Life Technologies/BRL) according to the manufacturer’s instruction. Contaminated genomic DNA was removed with RNase-free deoxyribonuclease (DNase). Five micrograms of DNase-treated RNA was incubated with 1 μl of oligoT at 70°C for 10 min. Two microliter 10 × PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 1 μl of DTT (0.1 M), 2 μl of dNTP mix (10 mM), and 2 μl of MgCl₂ (25 mM) were added to each reaction. After incubation for 5 min at 42°C, 1 μl of reverse transcriptase (RT) was added. Samples were incubated at 42°C for 50 min, and 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 as follows; rat β₅ subunit (sense and antisense: 5'-GGACCTAACTCTCAGCAGATG-3' and 5'-TCTCAAAATGCAGTGTCTTGTGG-3', respectively), rat activin type I receptor (sense and antisense: 5'-GGTCTATGAGCAGGGGAAGATGAC-3' and 5'-ACATTITCGCCTTGCCAGC-3', respectively), rat activin type II receptor (sense and antisense: 5'-AGATGGAAGTCACAGCCAC-3' and 5'-CACAATGGGTGCTTGTTCG-3', respectively), rat follistatin (sense and antisense: 5'-GGTTGGATCAGACCAATAATGCCTAC-3' and 5'-TGGAGTAGTGGCCATCTTCTTCTTG-3', respectively), rat GAPDH (sense and antisense: 5'-CATGACCACAGTCGTACATCAGT-3' and 5'-CACCTTGTTGCTGAGCATATCC-3', respectively). Reactions included 5 μl of 10 × PCR buffer, 2 μl of MgCl₂ (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 94°C for 5 min, followed by the indicated cycles of 30 s at 94°C, 30 s

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**Figure 2.** Localization of activin A, activin receptors, follistatin and Smad2/3 protein in developing kidneys. Localization of activin A (A and D), activin type I receptor (E), activin type II receptor (B and F), follistatin (C and G), and Smad2/3 (H) in the metanephros were examined by immunohistochemistry as described in Materials and Methods. (A through C) embryonic day 13; magnification, ×1000. (D through H) Embryonic day 16; magnification, ×400 in D, E, G, H and ×1000 in F.
at 58°C, 90 s at 72°C, and final extension at 72°C for 10 min in a Perkin-Elmer DNA Thermal Cycler. PCR reactions were 30 cycles for βA subunit, activin type I and type II receptor, and follistatin and 18 cycles for GAPDH. Reactions without cDNA were used as a negative control. Rat hepatocyte cDNA was used as a positive control in each experiment. Reactions were repeated at least twice.

Construction of a Replication-Deficient Recombinant Adenovirus Vector

The recombinant adenovirus, AdexARII, carrying truncated type II activin receptor (tARII) cDNA was generated by homologous recombination between the expression cosmid cassette and the parental virus genome as described previously (21). Briefly, the expression cosmid cassette was constructed by inserting the expression unit into the Swa I site of pAdex1cw (provided by I. Saito, University of Tokyo). The expression unit comprised a CMV (cytomegalovirus) promoter, tARII cDNA, and a poly A sequence. The expression cosmid cassette and adenovirus DNA-terminal protein (DNA-TPC) were cotransfected into 293 cells using a DOTAP Liposomal Transfection Reagent (Boehringer Mannheim GmbH, Mannheim, Germany). The desired recombinant adenovirus was amplified in 293 cells, purified using the CsCl density centrifugation method (27), and stored at −80°C. The titer of viral stocks was determined by plaque formation assay using 293 cells. The recombinant adenovirus, AdexlacZ, carrying the LacZ gene that codes for the Escherichia coli β-galactosidase, was provided by Dr. T. Takeuchi (Gunma University, Maebashi, Japan) and was used as a control for confirming successful transfection.

In Vitro Transfection

Embryonic kidneys removed from rats on day 14 of the pregnancy were incubated in the indicated concentrations of viral solution for 3 h at 37°C, washed with a serum-free medium, and then cultured in the complete medium containing 5% FBS for 3 d.

Detection of β-Galactosidase Activity

Embryonic kidneys were fixed with 0.25% glutaraldehyde for 10 min at 4°C, rinsed with PBS three times, and incubated for 3 h in a staining solution at 37°C. The staining solution comprised 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), 1 mM MgCl₂, 3 mM K₃Fe(CN)₆, 3 mM K₄Fe(CN)₆, 0.1% Triton X-100, and 10 mM KCl in a 100 mM sodium phosphate buffer (pH 7.5). β-Galactosidase expression was detected as the development of blue pigmentation due to the enzymatic cleavage of X-gal.

Isolation of Metanephric Mesenchymal Cells

Metanephric mesenchymal cells were isolated and cultured as described by Arar et al. (28). Fourteen-day gestation embryonic kidney rudiment was isolated, and the ureteric bud was freed surgically. The remaining mesenchyme was incubated in 0.2% collagenase (Sigma) for 5 min. Metanephric mesenchymal cells were then mechanically dissociated by gentle aspiration through repeated pipetting and plated at the density of 10⁴ cells/cm². Cells were grown in DMEM supplemented with 5% FBS, penicillin, streptomycin in an atmosphere of 5% CO₂–95% air at 37°C. Culture medium was changed every 48 h.

Immunocytochemistry

Cells grown on noncoated 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 at RT for 1 h with primary antibody as follows: anti-vimentin (1:100), anti-cytokeratin (1:100), and anti-E-cadherin (1:100) antibodies. After washing in PBS, cells were covered with a mixture of a Cy3-labeled goat anti-rabbit IgG antibody or FITC-labeled rabbit anti-mouse IgG and 4′-diamidino-2-phenylindole (DAPI: Boehringer Mannheim). Immunofluorescence images were recorded as described above.

Western Blot Analysis

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 using a protein assay kit (Bio-Rad Laboratories). Twenty micrograms of protein from each sample was separated by SDS-PAGE under reducing conditions and transferred to a 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 1 h at 37°C and then incubated overnight with primary antibody as follows: anti-βA subunit (1:1000), anti-activin type II receptor (1:100), anti-WT1 (1:200), anti-Pax-2 (1:1000), anti-E-cadherin (1:200), anti-vimentin (1:200), and anti-Wnt4 (1:100) antibodies, and washed with Tris-PBS (PBST). After incubation with peroxidase-labeled anti-goat/rabbit/mouse IgG antibody for 1 h at RT, the membrane was washed with PBST and was analyzed by exposure to x-ray film using ECL Western blotting.
detection reagent (Amersham Life Science). Briefly, the membrane was incubated in equal volumes of detection reagent 1 and detection reagent 2 for 1 min. After draining off detection reagent, the membrane were wrapped in Saran wrap. Hyperfilm ECL (Amersham Pharmacia Biotech) was put on the membrane and expose film for 30 s to 2 min.

Cell Viability
Cell viability was assessed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay as described previously (26).

Statistical Analyses
The significance of differences between means was compared by $t$ test, with $P < 0.05$ considered significant.

Results
Expressions of Activin $\beta_A$ Subunit, Activin Receptors, and Follistatin in the Metanephros
We first examined the expressions of $\beta_A$ subunit, activin type I and type II receptors and follistatin in the metanephros by RT-PCR. As shown in Figure 1, mRNA expression of $\beta_A$ subunit was detected in the metanephros (E14 to E18), but not in neonatal and adult kidneys. In contrast, mRNA expression of follistatin was slightly detected in the metanephros (E14 to N1), and its expression level was markedly increased in adult kidney. mRNA expressions of activin type I and type II receptor were detected in both metanephros and adult kidneys.

Localization of Activin A, Activin Receptors, Follistatin, and Smad2/3 Protein in the Metanephros
We next examined the localization of activin A and activin receptors in the metanephros by immunohistochemistry. In E13 metanephros, activin A was slightly expressed in unin-duced metanephric mesenchyme as well as ureteric bud (Figure 2A). In E16 metanephros, activin A was localized in ureteric bud (Figure 2D), but not in neonatal and adult kidney (data not shown). ActRII was expressed in both metanephric mesenchyme and ureteric bud of E13 (Figure 2B) as well as E16 (Figure 2F) metanephros. ActRI was also localized in ureteric bud of E16 metanephros (Figure 2E). We determined the localization of follistatin and Smad2/3 protein, an intracellular mediator of activin signaling, in the metanephros. As shown in Figure 2C, follistatin was not expressed in E13 metanephros, but weak staining for follistatin was observed in ureteric bud of
E16 metanephros (Figure 2G). Smad2/3 protein was also localized in the epithelial cells of ureteric bud (Figure 2H).

**Morphology of Cultured Metanephroi Treated with Activin A or Follistatin**

To clarify the role of activin A in kidney development, we performed metanephric organ culture system. First, we analyzed the effect of activin A and follistatin on the size of cultured metanephroi. Dissected metanephroi were cultured in complete medium containing 5% FBS with activin A or follistatin for the indicated periods. As shown in Figure 3, the size of activin-treated metanephroi was smaller than that of control metanephroi. In contrast, the size of follistatin-treated metanephroi was significantly larger than that of control metanephroi. We next examined the effect of activin A and follistatin on the morphology of cultured metanephroi. As shown in Figures 4A and 4D, S-shape or comma-shaped bodies were observed in control metanephroi. In contrast, some ureteric buds with enlarged lumen were observed in activin-treated metanephroi (Figures 4B and 4E). On the other hand, many elongated ureteric bud were observed in follistatin-treated metanephroi (Figures 4C and 4F). Whole-mount DBA staining showed that activin A delayed (Figure 4H) and conversely follistatin enhanced ureteric bud branching (Figure 4I).

**Promotion of Cell Growth in Ureteric Bud of Cultured Metanephroi by Follistatin**

We examined the effect of activin A and follistatin on cell growth in cultured metanephroi. Cell proliferation was assessed by PCNA staining. In control and activin-treated metanephroi, PCNA-positive cells were observed in both ureteric bud and metanephric mesenchyme (Figures 5A and 5B). In follistatin-treated metanephroi, PCNA-positive cells were abundantly observed in ureteric bud. On the other hand, PCNA-positive cells were rarely observed in metanephric mesenchyme (Figure 5C). Quantitative analysis showed that follistatin significantly increased the number of PCNA-positive cells in ureteric bud, but decreased that in metanephric mesenchyme (Figure 5D).

**Induction of Apoptosis in Cultured Metanephroi by Follistatin**

We next examined the effect of activin A and follistatin on apoptosis in cultured metanephroi. Apoptotic cells were detected by the TUNEL method. In control metanephroi as well as activin-treated metanephroi, TUNEL-positive cells were rarely observed (Figures 6A and 6B). In contrast, TUNEL-positive cells were abundantly observed in metanephric mesenchyme of follistatin-treated metanephroi (Figures 6C and 6D).

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**Figure 5.** Effect of activin A and follistatin on cell proliferation in cultured metanephroi. Cultured metanephroi were incubated in complete medium containing 5% FBS alone (control) (A) or with activin A (B) or with follistatin (C) for 3 d. Cell proliferation was examined by PCNA staining. Magnification, ×400. (D) Quantitative analysis of the number of PCNA-positive cells in ureteric bud (UB) and metanephric mesenchyme (MM) of control metanephroi (black bars), activin-treated metanephroi (white bars), and follistatin-treated metanephroi (gray bars). Quantification of PCNA-positive cells was performed by counting positive cells from five randomly selected fields per metanephros with a light microscope at ×400 magnification. The average of the five determinations was calculated. Values are means ± SEM (n = 6). * P < 0.01 versus control metanephroi.
Adenoviral Transfection of Truncated Type II Activin Receptor into Cultured Metanephroi

To further examine the role of endogenous activin A, we analyzed the effect of adenoviral transfection of cDNA encoding truncated type II activin receptor, which lacks an intracellular kinase domain (21). To examine the efficiency of transfection, we first infected cultured metanephroi with AdexLacZ at various titers of virus solution and examined β-galactosidase activity by X-gal staining. As shown in Figure 7A, β-galactosidase activity was strongly detected in cultured metanephroi infected with AdexLacZ at a titer of 10^7 pfu/kidney. In these metanephroi, β-galactosidase activity was detected in ureteric bud (Figure 7A, panel f) as well as metanephric mesenchyme (Figure 7A, panel g). Adenoviral transfection did not affect the size (Figure 7B) as well as the morphologic changes (data not shown) of cultured metanephroi, suggesting the successful transfection with no cytotoxicity. We then examined the effect of infection with AdextARII at a titer of 10^7 pfu/kidney on cultured metanephroi. As shown in Figure 7B, the size of cultured metanephroi infected with AdextARII was significantly larger than that of cultured metanephroi infected with AdexLacZ. In these metanephroi, branching of the ureteric bud was significantly increased (data not shown). Furthermore, the number of PCNA-positive cells in ureteric bud of these metanephroi was significantly increased. However, the number of PCNA-positive cells was significantly decreased in metanephric mesenchyme of these metanephroi compared with that of cultured metanephroi infected with AdexLacZ (Figures 7C and 7D).

Effect of Activin A on Cell Growth in Isolated Metanephric Mesenchymal Cells

To examine the effect of activin A on cell growth in metanephric mesenchyme, we isolated metanephric mesenchymal cells from E14 metanephros and then cultured them. These cells were confirmed as mesenchymal cells as described by Arar et al. (28) by expressing a mesenchymal marker, vimentin (Figure 8B), but not an epithelial marker, E-cadherin and cytokeratin (data not shown). Consistent with the result obtained by immunohistochemical analysis (Figure 2), these cells produced activin type II receptor, but not activin A (Figure 8C). Activin A significantly promoted cell growth in cultured metanephric mesenchymal cells (Figure 8D).

Induction of Apoptosis in Cultured Metanephric Mesenchymal Cells by Follistatin

We also examined whether follistatin induces apoptosis in cultured metanephric mesenchymal cells. Follistatin significantly decreased cell growth in a dose-dependent manner (data not shown). In addition, follistatin significantly induced apoptosis in cultured metanephric mesenchymal cells at a concentration of 10 nM and above (Figure 9).

Changes in the Expressions of Metanephric Differentiation Markers by Activin A

To examine the effect of activin A on the differentiation of metanephric mesenchyme, we analyzed the expressions of
metanephric differentiation markers by Western blotting. As shown in Figure 10, activin A increased the production of WT1, Wnt4, and decreased the production of Pax-2 in isolated metanephric mesenchymal cells. Furthermore, activin A increased the production of E-cadherin, but decreased the production of vimentin in these cells. Immunocytochemical analysis also demonstrated that activin A induced the expression of E-cadherin, but reduced the expression of vimentin in these cells (data not shown).

**Discussion**

In the kidney, reciprocal interaction between ureteric bud and metanephric mesenchyme is essential to its development (1). As mesenchymal signals, hepatocyte growth factor (HGF) mediates the signal that induces branching of ureteric bud (29,30). Glial cell-derived neurotrophic factor (GDNF) stimulates ureteric bud outgrowth by activation of RET receptor tyrosine kinase (31–33). On the other hand, as ureteric bud signals, bone morphogenic protein (BMP)-7 is required for the differentiation of metanephric mesenchyme (34–36). Fibroblast growth factor (FGF)-2 (37) as well as Leukemia Inhibitory Factor (LIF) (38) also has a potential to induce the differentiation of metanephric mesenchyme. Considering that such paracrine or juxtacrine interactions between ureteric bud and metanephric mesenchyme are prevalent during metanephric development (6), the expression pattern of activin A and its receptors appears to be unique. Activin type II receptor was localized in metanephric mesenchyme as well as ureteric bud throughout development (Figure 2). In contrast, βA subunit for activin A was expressed in metanephric mesenchyme as well as ureteric bud of E13 metanephros (Figure 2), but its expression was restricted to ureteric bud in E16 (Figure 2) or E18 (data not shown) metanephros. These results suggest that activin A acts as an autocrine factor on ureteric bud at the early phase of metanephric development, and also that at the mid or late phase of nephron differentiation, activin A plays a role on metanephric mesenchyme as a paracrine/juxtacrine factor.

As shown in Figure 1, activin type II receptor was expressed...
in developing, neonatal, and adult kidneys. In adult kidneys, activin type II receptor was ubiquitously localized in tubular cells, including proximal and distal tubules, and collecting ducts (39). In contrast, the expression of βA subunit for activin A was not detected in adult kidneys (Figure 1). The significance of variant pattern of their expressions remains unknown at present. However, we previously demonstrated that the expression of activin A was upregulated in tubular cells in the kidney after renal ischemia (39). Furthermore, activin A has a potential to induce tubular cell differentiation (26). Therefore, it is quite possible that activin A acts as an autocrine factor essential for kidney regeneration even after the completion of development.

In the present study, we demonstrated that activin A and activin receptors were localized in the epithelial cells of ureteric bud (Figure 2). Furthermore, Smad2/3 protein was also localized in ureteric bud (Figure 2), indicating that activin signaling pathway was activated mainly in ureteric bud. Consistent with previous findings (20), we observed that activin A delayed ureteric bud branching and enlarged the tips of ureteric bud (Figure 4). Adenoviral transfection of the gene encoding the truncated type II activin receptor, which inhibits the signaling via activin receptor in a dominant-negative fashion (21), completely reproduced the effect of follistatin (Figure 7), suggesting that endogenous activin A tonically inhibits ureteric bud branching.

By which mechanism activin regulates ureteric bud branching is a question of great interest. One possibility is that activin A controls cell growth in ureteric bud. Follistatin (Figure 5) as well as adenoviral transfection of dominantly negative mutant receptor (Figure 7) significantly promoted cell growth in ureteric bud. However, activin A did not inhibit cell growth in ureteric bud (Figure 5). We previously demonstrated that activin A produced by MDCK cells tonically inhibits branching morphogenesis and blockade of activin signaling induced branching morphogenesis in an in vitro tubulogenesis model (21). Activin A did not inhibit cell growth in MDCK cells (21). Taken together, these results suggest that activin A did not directly inhibit cell cycle progression, but it is needed for ureteric bud cells to remain in the differentiated state. It is possible that blockade of activin action markedly induces cytoskeletal reorganization, immature phenotype, and subsequently cell proliferation.

One of the essential mechanisms of tissue remodeling is the process of apoptosis. During kidney development, metanephric mesenchyme is programmed for apoptosis (40). Conversion of mesenchyme to epithelium requires at least two steps: rescue of

Figure 8. Effect of activin A on cell growth in cultured metanephric mesenchymal cells. Metanephric mesenchymal cells (A) were isolated from metanephros with 0.2% collagenase as described in Materials and Methods. The expression of vimentin (B) was examined by immunocytochemistry. (C) The production of βA subunit for activin A and activin type II receptor were examined by Western blotting. (D) The effect of activin A on cell growth in cultured metanephric mesenchymal cells. Isolated metanephric mesenchymal cells were incubated in complete medium containing 5% FBS with the indicated concentrations of activin A for 3 d. Cell growth was assessed by MTT assay. *P < 0.01 versus control.
the mesenchyme from apoptosis and induction of differentiation (40). In the present study, we showed the production of activin type II receptor in cultured metanephric mesenchymal cells (Figure 8). Activin A promoted cell growth in these cells (Figure 8). In contrast, follistatin induced apoptosis in metanephric mesenchyme of cultured metanephroi (Figure 6) in addition to cultured metanephric mesenchymal cells (Figure 9). These results suggest that endogenous activin A produced by ureteric bud acts as a survival factor for metanephric mesenchymal cells. Considering that BMP-7 is known to be a survival factor for metanephric mesenchyme (34–36), it is possible that blockade of activin action by follistatin resulted in the downregulation of BMP-7. Activin A may potentiate BMP-7 action though the induction of BMP-7 expression or activation of BMP-7 signaling.

Metanephric organ culture is routinely performed using serum-free medium, because serum influences metanephric growth. In our experiments, we cultured metanephric kidneys in DMEM medium containing 5% FBS. Serum includes several factors that modulate mesenchymal cell growth and differentiation, including transferrin (41). Therefore, the possibility cannot be denied that activin A induced morphologic changes of cultured metanephroi in cooperation with other factors in serum.

A large number of genes have been identified and their essential roles in epithelial differentiation of the metanephric mesenchyme (42). The Wilms tumor suppressor gene WT1 is expressed in the metanephric blastema and is required for survival of the mesenchyme surrounding the Wolffian duct (43). WT1 is required for normal renal development and is upregulated during the mesenchymal-epithelial transition. Wnt4, a member of the Wnt gene family of secreted glycoproteins, is also critical for the mesenchyme-to-epithelial transition during kidney development (44). On the other hand, Pax-2 is expressed in undifferentiated mesenchymal cells at the earliest stages of epithelialization of mesenchyme, but it is repressed in mature renal epithelium (45). In the present study, we demonstrated that activin A increased the expression of WT1, Wnt4 and decreased the expression of Pax-2 in cultured metanephric mesenchymal cells (Figure 10), suggesting that activin A promoted mesenchymal-epithelial transition. We also showed that activin A induced the expression of an epithelial marker, E-cadherin; in contrast, it decreased the expression of mesenchymal marker, vimentin, in these cells. These results suggest that activin A acts as differentiation factor for metanephric mesenchyme. WT1 expression can activate expression of a set of genes in mesenchymal cells associated with epithelial differentiation (46). E-cadherin (47), as well as Wnt4 (48), is one of the target genes of WT1. During epithelialization of mesenchyme, a marked increase in WT1 protein levels coincided precisely with downregulation of the Pax-2 gene. Further...
thermore, WT1 directly represses Pax-2 transcription (49). Collectively, activin A may induce differentiation of metanephric mesenchymal cells by transcriptional activation of WT1. Further studies are necessary to address this issue.

In summary, we demonstrated that activin A and activin receptors were expressed in ureteric bud. Activin type II receptor was also expressed in metanephric mesenchyme. In an organ culture system, exogenous activin A delayed branching and enlarged the tips of ureteric bud. In contrast, follistatin increased ureteric bud branching and induced mesenchymal cell apoptosis. In cultured metanephric mesenchymal cells, activin A promoted cell growth; conversely, follistatin induced apoptosis. Activin A induced the expressions of differentiation markers in these cells. The present results suggest that activin A produced by ureteric bud is not only an important regulator of ureteric bud branching, but also a differentiation factor for metanephric mesenchyme during kidney development.

References

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