Inhibition of Autoregulated TGF β Signaling Simultaneously Enhances Proliferation and Differentiation of Kidney Epithelium and Promotes Repair Following Renal Ischemia

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We studied autocrine transforming growth factor (TGF) β signaling in kidney epithelium. Cultured proximal tubule cells showed regulated signaling that was high during log-phase growth, low during contact-inhibited differentiation, and rapidly increased during regeneration of wounded epithelium. Autoregulation of signaling correlated with TGF β receptor and Smad7 levels, but not with active TGF β , which was barely measurable in the growth medium. Confluent differentiated cells with low receptor and high Smad7 levels exhibited blunted responses to saturating concentrations of exogenously provided active TGF β , suggesting that TGF β signaling homeostasis was achieved by cell density-dependent modulation of signaling intermediates. Antagonism of Alk5 kinase, the TGF β type I receptor, dramatically accelerated the induction of differentiation in sparse, proliferating cultures and permitted better retention of differentiated features in regenerating cells of wounded, confluent cultures. Alk5 antagonism accelerated the differentiation of cells in proximal tubule primary cultures while simultaneously increasing their proliferation. Consequently, Alk5-inhibited primary cultures formed confluent, differentiated monolayers faster than untreated cultures. Furthermore, treatment with an Alk5 antagonist promoted kidney repair reflected by increased tubule differentiation

and decreased tubulo-interstitial pathology during the recovery phase following ischemic injury *in vivo*. Our results show that autocrine TGF β signaling in proliferating proximal tubule cells exceeds the levels that are necessary for physiological regeneration. To that end, TGF β signaling is redundant and maladaptive during tubule repair by epithelial regeneration. (Am J Pathol 2009, 174:1291–1308; DOI: 10.2353/ajpath.2009.080295)

Regeneration of an adult epithelium such as those lining the kidney tubules involves not only proliferation but also de-differentiation, followed by growth arrest and re-differentiation.^{1–3} The signaling cues that coordinate these processes are largely unknown. Endocrine and paracrine factors affect epithelial repair following injury *in vivo*. Nevertheless, epithelial homeostasis is also regulated by density-dependent contact-inhibition and attendant differentiation. The mechanisms that mediate these processes are poorly understood, but likely involve transforming growth factor (TGF) β , in addition to signals generated by the phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways. These considerations prompted us to investigate how endogenously generated signals might control epithelial regeneration in terms of cell proliferation and differentiation.

Increased TGF β signaling can lead to apoptosis, growth inhibition, or epithelial-mesenchymal transitions (EMT) of epithelial cells, including kidney epithelial cells.^{4–9} Prolonged exposure to high concentrations of

Supported by grants from the National Institutes of Health DK37139 (M.A.V.), DK54472 (P.S.), DK34275 (J.M.W.), AR050024 (M.C.N.), NIEHS-ES005022 (A.I.B.), and VA Merit Review (J.L.B.).

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Accepted for publication January 6, 2009.

Supplemental material for this article can be found on <http://ajp.amjpathol.org>.

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active TGF β is frequently used to model these alterations. While these effects of sustained high intensity TGF β signaling are well studied, much less is known about physiologically regulated TGF β signals and how they become increased by epithelial injury and subsequent regeneration. Autocrine TGF β signals are antiproliferative for epithelial cells and cultures from TGF β_1 null kidney tubules display enhanced proliferative rates.¹⁰ Nevertheless, TGF β signaling was found to be increased rather than decreased during the proliferation of surviving kidney epithelium following cell loss by ischemia, and this was accompanied by increased expression of TGF β and its receptors in regenerating cells.¹¹ Similarly, proliferating keratinocytes in skin wounds show enhanced TGF β signaling.¹² It has been puzzling why antiproliferative TGF β signaling becomes enhanced in rapidly growing cells under pathological conditions.

In this study, we have investigated the functional relevance of cell-autonomous, ie, endogenously generated, TGF β signals for regenerating kidney epithelial cells in culture and *in vivo*. Fully differentiated proximal tubule (PT) cells retain the ability to undergo mitotic division,^{13–15} and, following cell loss by injury, survivors dedifferentiate, proliferate, and then redifferentiate to reconstitute the lost cell mass.^{1–3,13,16} We found that cell-autonomous TGF β signals are tightly autoregulated during repeated cycles of proliferation and contact-inhibition in PT cultures. Signaling was high during log phase growth and became progressively suppressed as cultures became contact-inhibited and differentiated. It was decreased in growing subconfluent cultures by neutralizing TGF β antibodies, indicating a requirement for extracellular ligand. However, in the absence of TGF β antibodies, increases and decreases of signaling were determined solely by cell density, and occurred independently of the concentrations of barely measurable active TGF β in growth medium. Instead, the signaling fluctuations were associated with increased and decreased expression of TGF β receptor and reciprocal alterations of inhibitory Smad7. Moreover, saturating concentrations of exogenous TGF β were found to elicit blunted signaling responses from contact-inhibited differentiated cells relative to growing undifferentiated cells. These observations suggested that: (1) extracellular TGF β ligand played a permissive role but did not, by itself, determine the intensity of signaling fluctuations during the epithelial growth cycle; and (2) signaling homeostasis during growth and quiescence was related to the modulation of TGF β receptors and Smad7. Functionally, we found that inhibition of cell-autonomous TGF β signals resulted in remarkably accelerated differentiation and concurrent stimulation of proliferation in growing PT cultures. Importantly, we extended our observations to demonstrate that treatment with small molecule Alk5 inhibitors not only promoted differentiation in regenerating PT epithelium during wound healing *in vitro*, but also improved the repair of kidney damage with greater restoration of epithelial differentiation and tubule integrity following ischemia *in vivo*. These unprecedented findings have direct relevance to the development of treatments that might promote repair and recovery following loss of epithelium by acute kidney injury (AKI).

Materials and Methods

Cell Culture, Plasmids, and Adenoviral Vectors

Boston University mouse proximal tubule cells (BUMPT-Clone 306; from Drs. W. Lieberthal and J. Schwartz) were grown at 37° C in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum or in serum-free medium supplemented with insulin (10 μ g/ml), epidermal growth factor (10 ng/ml), transferrin (5 μ g/ml), Na selenite (6.7 μ g/ml), and dexamethasone (4 μ g/ml). BUMPT cells were derived from primary cultures of kidney proximal tubules of F1 hybrid mice with single copies of the H-2Kb-tsA58 transgene.^{17,18} Expression of large T antigen by the transgene at 39.5° C without γ -interferon is inhibited >95% relative to cells at 33° C with the cytokine.¹⁸ Confluent BUMPT cells display proximal tubule characteristics and develop transepithelial resistance of $\sim$ 300 Ω /cm² when grown at 37° C.¹⁷ In addition, they also express meprin, a proximal tubule brush border marker enzyme (data not shown). We found that SV40 large T antigen was equally suppressed at 39.5° C and 37° C, as compared with 33° C (data not shown). BUMPT cells were transfected with the p3TP-Lux reporter¹⁹ and 0.2 μ g of pCV-neo. Stably transfected cells were selected with G418 and clones assayed for luciferase activity after treatment with and without TGF- β (2 ng/ml). Mouse proximal tubule cells were grown in primary culture as described.²⁰ The growth medium was modified from published formulations^{20,21} and contained insulin (10 μ g/ml), epidermal growth factor (10 ng/ml), transferrin (5 μ g/ml), Na selenite (6.7 μ g/ml), dexamethasone (4 μ g/ml), and L-ascorbic acid-2-phosphate (50 μ mol/L). Cells were used at first passage.

Type 5 E1/E3 deleted recombinant human adenoviral vectors with HA tagged Alk4 KR and Alk5KR, and FLAG-tagged wild type smad7 were from M. Fujii.²² AdCMV.dIE3 empty virus without transgene was from University of Michigan Vector Core Laboratory.

Antibodies and Immunological Detection

Antibodies were obtained from the following sources: Akt, p-Akt S473, c-Myc, p-Smad2 (S465/467), p-Smad3 (S433/435)/p-Smad1 (S463/465), p15^{ink4}, phospho-retinoblastoma protein (Rb, S608), and phospho-Rb (S807/811) (Cell Signaling, Danvers, MA); cyclin D1/2, TGF β receptor type II, E-cadherin, Smad2/3, (BD-Transduction, San Diego, CA); Na⁺, K⁺-ATPase α -subunit, 5-bromo-2'-deoxyuridine (BrdU, DSHB, Iowa City, IA); CD26/dipeptidylpeptidase IV (DPP IV), p27^{Kip1}, α -smooth muscle actin (SMA, Lab Vision, Fremont, CA); CD10/Nephrilysin/neutral endopeptidase (NEP, NovoCastra, Newcastle upon Tyne, UK); β -catenin, N-Myc downstream regulated gene 1 (NDRG1, Santa Cruz); Smad7 (Imgenex, San Diego, CA); TGF β receptor type I, pan TGF β (R&D systems, Minneapolis, MN); ZO-1, Ksp cadherin (Zymed, San Francisco, CA); HA (Sigma, St. Louis, MO); FLAG (Eastman Kodak, Rochester, NY); pan-actin Clone C4 (Chemicon, Billerica, MA); and glyceraldehyde-3-phosphate dehydrogenase (Rockland, Gilbertsville, PA). Me-

prin HMC14 antibody was from Judith Bond and John Bylander. Rabbit antibody to megalin was from Marilyn Farquhar. Antibody to mouse Rb (Clone 245) was from Wen-Hwa Lee. Epithelium aminopeptidase P (rat) monoclonal antibody (JG12) was from Dontscho Kerjaschki. For immunoblotting, cells were washed twice with ice-cold PBS and collected in Laemmli buffer, reduced, and boiled. Proteins were separated by SDS-polyacrylamide gel electrophoresis on 10% bis-Tris or 8% Tris-glycine gels (Invitrogen) and transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Membranes blocked with 5% nonfat milk or bovine serum albumin in PBS-0.2% Tween-20 (PBST) were incubated with primary antibodies in blocking buffer or in 5% bovine serum albumin-PBST overnight at 4° C. After incubation with affinity purified secondary antibodies conjugated with horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA), IRDye680 or IRDye 800 (Rockland immunochemicals, Gilbertsville, PA) at a dilution of 1:2500, proteins were visualized by electrochemiluminescence or infrared fluorescence (Odyssey Infrared Imaging System, LI-COR Biosciences, Lincoln, NE). For immunofluorescence, coverslips with cells were fixed with 4% paraformaldehyde for 30 minutes, and exposed to primary antibodies followed by secondary antibodies labeled with Alexa488 (Molecular Probes, Eugene, OR) or Cy3 (Jackson ImmunoResearch). Samples were examined by epifluorescence with an Olympus AX70 microscope or an Olympus FV-500 Laser Scanning Confocal Microscope.

Wounding of Cell Monolayers

BUMPT monolayers in 21 cm² culture dishes were wounded using a device fabricated at the University of Texas Health Science Center Instrumentation facility. The device consisted of a flat ~21 cm² etched rubber disk with a series of alternating 0.2-mm wide concentric grooves and 0.8-mm wide unetched ridges. A spring-loaded piston was used to lower the disk to clamped culture dishes. The disk was rotated to wound the monolayers in concentric circular swaths with preserved strips of cells in between. For immunofluorescence microscopy, contact inhibited confluent cultures on glass coverslips were wounded using a rectangular piece of rubber which had been machine etched with parallel alternating 0.2-mm grooves and 0.8-mm unetched ridges.

Electron Microscopy

Cell monolayers were fixed with 2.5% glutaraldehyde in 0.1 M/L Na cacodylate buffer, pH 7.4 for 3 hours, post-fixed in cold 1% OsO₄ in water, stained "en bloc" with 2% uranyl acetate for 1 hour and dehydrated in cold acetone. Cells were then embedded in epoxy resin, and thin sectioned for electron microscopy.

BrdU Labeling

BUMPT cells seeded on coverslips were exposed to 10 μ mol/L BrdU (Sigma) during the last 1 hour before fixation.

After paraformaldehyde fixation and three washes with PBS/0.1 M/L glycine-PBS, cells were exposed to 1% SDS in PBS for 5 minutes, and washed with PBS three times. DNA was denatured at 70°C with 9:1 formamide: 0.1 M/L NaPi, pH 7.4 for 40 minutes. The coverslips were then incubated sequentially with BrdU antibody and Cy3 labeled secondary antibody, and mounted in ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole (Molecular Probes). BrdU label (Cy3) and nuclear marker (4',6-diamidino-2-phenylindole) in at least 500 cells per coverslip were digitally recorded and BrdU labeled nuclei were expressed as a percentage of total 4',6-diamidino-2-phenylindole-stained nuclei. Primary cultures of proximal tubules were assayed similarly for BrdU uptake after 1 hour incubation with 20 μ mol/L BrdU.

Ischemia-Reperfusion of Rat Kidneys

Male Sprague-Dawley rats ~250 g body weight were subjected to ischemia-reperfusion of the left kidney with contralateral nephrectomy as described.²³ Laparotomy was done on a heated table under isoflurane anesthesia. Body temperature was maintained at 37° C. The left renal vascular pedicle was clamped with aneurysm clips (Roboz instruments); in a control group, the pedicle was dissected, but not clamped. The right kidney was removed in both groups. After 45 minutes, the clamp was removed, the abdominal incision closed and animals returned to their cages. Blood samples were obtained before surgery and daily to measure serum creatinine by high-performance liquid chromatography.²⁴ Body weights were recorded daily. Four hours after surgery, ischemia and control groups of rats were given by gavage either 60 mg/kg of SD-208 (2-[5-Chloro-2-fluorophenyl]piperidin-4-yl)pyridin-4-yl amine (SCIOS, Inc.), a highly specific small molecule antagonist of Alk5 kinase²⁵ suspended in 1% methylcellulose, or 1% methylcellulose alone. The treatments were repeated at 12 hourly intervals for 4 days.

Analysis of Kidney Tissue

For morphology and immunohistochemistry, kidneys were fixed by vascular perfusion²⁶ with periodic acid-lysine-paraformaldehyde.²⁷ Following overnight fixation at 4° C, kidney slices were washed twice in PBS and once in PBS-100 mmol/L glycine and either cryoprotected with 30% sucrose in PBS or transferred to 70% ethanol for dehydration in increasing concentrations of ethanol and embedding in paraffin. Cryosections were permeabilized with 1% SDS before blocking and exposure to antibodies for indirect immunofluorescence by single or double labeling using Cy3 and Alexa488 labeled secondary antibodies. For immunohistochemistry, ~4-micron deparaffinized sections were exposed for 30 minutes to Tris-EDTA pH 8.0 at ~100° C for antigen retrieval, blocked with 2.5% horse serum and exposed to primary antibodies at 4°C overnight with rocking. After washing and second blocking step, sections were exposed to ImmPRESS secondary antibodies (Vector Labs) and color developed with ImmPACT DAB reagent (Vector

Labs) according to manufacturer's instructions. After exposure to 0.2% OsO₄ for 1 minute to form oxidized DAB-Osmium Black polymer, coverslips were applied with Crystal Mount. Deparaffinized sections were also stained with H&E and PAS-hematoxylin. For morphometric analysis, the outer stripe of the outer medulla was photographed at $\times 200$ and printed on 8.5 $\times$ 11" paper either directly or overlaid on a cross-hatched grid with 154 points. All tubule profiles in five photographs from each rat were graded to assess tubule differentiation on a 1 to 5 scale by one of the authors (M.V.). Scores were determined by these criteria: 1. proximal tubule profiles that were normal/indistinguishable from normal; 2. profiles identifiable as proximal tubules with thin cytoplasm; 3. profiles identifiable as proximal tubules with severely thinned cytoplasm, all distal nephron segments and atrophic tubules of unknown identity indistinguishable from distal nephron profiles; 4. abnormally dilated profiles of unknown identity with thin cytoplasm; 5. severely dilated profiles of unknown identity with thin cytoplasm. The total number of tubule profiles graded per rat averaged 375. The total of weighted tubule scores divided by total tubule number gave the tubule differentiation index for each rat. Normal distal nephron profiles could not be distinguished from non-dilated undifferentiated or atrophic tubule profiles and both types of profiles received a score of 3. Consequently, the scoring system overestimated the number of less differentiated tubules in control and SD-208 treated ischemic groups relative to vehicle treated rats, making the discrimination tests more stringent. The tubulo-interstitial index was calculated by one of the authors (H.G.) and expressed as a ratio of all points on the cross-hatched grid from the five photographs that fell on the interstitium (blood vessels excluded) and interstitium plus tubule cells (tubule lumina excluded) by a method we have described.²⁶ For biochemical analysis, kidney cortex and outer stripe of outer medulla were dissected separately on a cold plate and flash frozen in liquid nitrogen. Tissue was ground to fine powder under liquid nitrogen with mortar and pestle and extracted with 4% SDS sample buffer with Benzon endonuclease, and protease/phosphatase inhibitors to get an SDS:protein ratio of at least 3:1. Proteins in reduced, boiled extracts were separated by SDS-polyacrylamide gel electrophoresis for immunoblotting.

Other Assays

Neutralizing antibodies to TGF β 1, 2, and 3 (R&D Systems) or non-immune rabbit IgG were included in the culture medium of growing subconfluent BUMPT cells given in two divided doses of 15 μ g/ml over a duration of 36 hours after which the cells were extracted with Laemmli sample buffer for immunoblotting studies. A single dose of 15 μ g/ml of neutralizing antibodies or non-immune IgG was included in the growth medium after BM-Lux cells were wounded. The cells were lysed 6 hours after wounding to measure TGF β reporter activity by luciferase assay. Luciferase was assayed in cell lysates by using the Luciferase Reporter Assay System (Pro-

mega). To measure active TGF β in growth medium and conditioned media, we used mink lung epithelial cells stably transfected with the PAI-I promoter linked to luciferase.²⁸ Using a standard curve for added recombinant human TGF β ₁ between 2 and 100 pg/ml, we performed the bioassay as described.²⁸ To measure Na⁺ dependent glucose transport, primary cultures of proximal tubules were incubated with 2 μ Ci ¹⁴C-methyl- α -D-glucopyranoside (Amersham) and 0.5 mmol/L unlabeled methyl- α -D-glucopyranoside (Sigma) in Tris-buffered physiological solution for 30 minutes at room temperature. Parallel dishes were incubated with sucrose instead of sodium chloride or 0.5 mmol/L phloridzin (Sigma) as described.²⁹

Other Chemicals

SB431542 was from Sigma. "TGF- β RI Kinase Inhibitor" ("Alk5 Inhibitor I") was from CALBIOCHEM. Recombinant human TGF β ₁ was from R&D Systems.

Statistical Analysis

Log-transformed values for serum creatinine, tubule differentiation index and tubulo-interstitial index were subjected to analysis of variance and pair-wise multiple comparison procedure using Sigma Stat software. All other statistical tests were done by paired Student's *t*-test.

Results

TGF β Signaling Is High during Log Phase Growth and Becomes Suppressed during Contact-Inhibited Growth Arrest and Differentiation of BUMPT Cells

With each subculture, BUMPT cells underwent cycles of proliferation and de-differentiation after seeding at sub-confluent density, followed by confluent growth arrest and redifferentiation (Figure 1). Seeded at 13,000/cm² and cultured at 37° C, the cells showed growth arrest at confluence by 4 days (Figure 1A) with decreased proliferation markers cyclin D and c-Myc, and increase of cyclin dependent kinase (cdk) inhibitor p27^{kip1} (Figure 1B). Progression to growth arrest was associated with the induction of differentiation evidenced by increased expression of NDRG1, Na⁺/K⁺-ATPase, DPP IV, and NEP proteins and the formation of intercellular junctions exhibiting E-cadherin and ZO-1 (Figure 1, C and D). NDRG1, which is repressed by N-Myc and c-Myc, marks differentiation in urogenital epithelia.³⁰ The sodium pump Na⁺/K⁺-ATPase and brush border peptidases DPP IV and NEP are known to increase when proximal tubules differentiate.^{31,32} These alterations of cell cycle and differentiation markers were accompanied by corresponding changes of mRNA (data not shown).

We also found cell density-dependent increases of E-cadherin in cells (Figure 1C). Since E-cadherin is transcriptionally repressed by TGF β ,^{4,6} we asked if TGF β signaling is diminished at confluence. As cells increased

Figure 1. BUMPT cells undergo density-dependent growth arrest and differentiation. Cells were plated at 13,000/cm² and examined after 1, 2, 3, or 4 days. **A:** Cells were counted ($n = 4$). **B:** SDS extracts were immunoblotted for cyclin D, c-Myc, and p27^{kip1}. **C:** SDS extracts were immunoblotted for differentiation markers NDRG1, Na⁺/K⁺ ATPase, DPP IV, NEP, and E-cadherin. **D:** Fixed cells were examined by phase contrast (**top** panels) and immunofluorescence for E-cadherin and ZO-1 (**middle** and **bottom** panels). Magnification: phase contrast, $\times 200$; immunofluorescence, $\times 400$. SDS extracts from the same experiment were used for Figures 1 (**B**) and (**C**); glyceraldehyde-3-phosphate dehydrogenase loading controls from the same extracts are shown in (**C**) only.

in number, became growth arrested and differentiated, TGF β receptors types I and II (T β RI, T β RII) and cell-associated TGF β decreased, accompanied by increase of Smad7, an inhibitor of Smad2/3 phosphorylation by T β RI^{4,5,7}; correspondingly, C-terminal S465/467 phosphorylation of Smad2 was suppressed (Figure 2A). Phosphorylation of Smad3, which was less abundant than Smad2, was not detectable unless exogenous TGF β was added to the medium (Figure 2F). We monitored TGF β dependent transcriptional activity in BUMPT cells stably transfected with p3TP-Lux, an activin/TGF β -responsive reporter for signaling by Smad2 and Smad3.^{19,33,34} Five clones expressing p3TP-Lux were isolated (BM-Lux cells) and all five exhibited increased and decreased luciferase activity respectively in response to TGF β and SB431542, an Alk5 kinase antagonist³⁵ (see supplementary Figure S1, A and B at <http://ajp.amjpathol.org/>). With increasing cell density and growth arrest, all five clones showed spontaneous and progressive suppression of luciferase activity (Figure 2B). Successive cycles of subculture and growth were repetitively punctuated by these cell density-dependent signaling fluctuations. Therefore, these findings firmly established that cell autonomous TGF β signals were tightly autoregulated during the growth cycle of PT epithelium. They also raised the possibility that autoregulated fluctuations of TGF β signaling were related to variations in the expression of TGF β receptor and Smad7 and that these fluctuations were affecting the growth and differentiation status of cells.

TGF β Signals in BUMPT Cells Require Extracellular Ligand, but Cell Density Dependent Signaling Fluctuations Occur Independently of Active TGF β Concentrations in Growth Medium

To determine whether extracellular ligand was required for cell-autonomous TGF β signaling in BUMPT cells, we included neutralizing TGF β antibodies in the growth medium. TGF β antibodies but not non-immune IgG decreased the phosphorylation of Smad2 C-terminal phospho-sites when cells were cultured either in serum replete medium (Figure 2C) or in serum free medium (see supplementary Figure S2A at <http://ajp.amjpathol.org/>). Next, we examined whether the signaling fluctuations that took place during the growth cycle of BUMPT cells could have been caused by corresponding changes in the concentrations of active TGF β in culture medium. Measurements using a sensitive bioassay²⁸ showed that active TGF β concentrations in the medium did not exceed 5 pg/ml regardless of whether the cells were subconfluent and proliferating, or confluent, growth arrested, and differentiated. The individually measured values could not be correlated with the growth status of cultures. In support of these observations, cross-transfer of conditioned medium from subconfluent and confluent cultures of BM-Lux cells elicited only trivial decreases or increases of p3TP-Lux reporter activity (Figure 2D) and frequent replacement of growth medium did not significantly decrease or

Figure 2. TGF β signaling is suppressed in BUMPT cells undergoing density-dependent growth arrest and differentiation. Cell-autonomous TGF β signaling requires extracellular TGF β ligand, but autoregulated signaling does not depend on variations in the composition of growth medium. The signaling pathway becomes refractory in dense, growth-arrested cultures. BUMPT or BM-Lux cells were plated at 13,000/cm² and examined after 1, 2, 3, or 4 days. Cells were growth arrested and differentiated by days 3 to 4 as shown in Figure 1. **A:** SDS extracts of cells were immunoblotted for TGF β receptors (T β RI and T β RII), cell-associated TGF β , Smad7, phospho-Smad2 (S465/467), and Smad2. **B:** Luciferase activity was measured after 1, 2, 3, and 4 days of seeding in each of five clones of BUMPT cells carrying stably incorporated p3TP-Lux TGF β reporter (BM-Lux cells). **C:** Neutralizing TGF β antibodies or non-immune IgG were added to the culture medium of subconfluent (Day 1 density) cells in two divided doses of 15 μ g/ml each over 36 hours, after which SDS extracts were immunoblotted for Smad2 and phospho-Smad2 (S465/467). **D:** BM-Lux cells (Clone 5) were grown to subconfluent (day 1) or contact-inhibited (day 4) density and luciferase activity was measured in cells that had been in medium with serum (FCS+) or without serum (FCS-) for 16 hours. The **middle** pair of bars shows luciferase activity following cross-transfer of conditioned medium from subconfluent cells to confluent cells and vice versa for 6 hours (FCS+/FCS- media switch; mean $\pm$ SE, $n = 3$). **E:** Subconfluent (day 1) and contact-inhibited (day 4) BM-Lux cells were incubated without or with 0.05 to 2.0 ng/ml TGF β 1 in growth medium, and luciferase activity was measured 6 hours later; mean $\pm$ SE, $n = 3$. **F:** Subconfluent (day 1) and growth-arrested (day 4) cells were exposed to 2 ng/ml TGF β 1 in growth medium for 6 hours. SDS extracts were immunoblotted for Smad2/3, phospho-Smad2 (S465/467), and phospho-Smad3 (S423/425). (Phospho-Smad2 signals in cells without TGF β are not evident in panel 2F for technical reasons; the times of exposure to the electrochemiluminescence reagent needed to visualize them would have resulted in unacceptably large signals in the TGF β -stimulated lanes).

increase the elevated TGF β signals in subconfluent growing cells or reverse the decrease of TGF β signaling in confluent growth arrested cells. Furthermore, we found that TGF β signaling was autoregulated under serum-free conditions also. As they became confluent and growth arrested in serum free medium, BUMPT cells showed decreased T β RII, increased Smad7 and decreased Smad2 phosphorylation at C-termini (see supplementary Figure S2B at <http://ajp.amjpathol.org/>) and BM-Lux cells showed diminished p3TP-Lux reporter activity (Figure 2D). Similar to the observations made on cells grown with serum containing growth medium, cell density-dependent decreases of TGF β signaling in serum-free medium were also associated with increased expression of differentiation markers (see supplementary Figure S2B at <http://ajp.amjpathol.org/>). Taken together, our observations showed that extracellular ligand was required for signaling. However, they also excluded the possibility that differences in signaling between subconfluent and

confluent cells were caused by accumulation of secreted TGF β or depletion of latent TGF β or other growth factors and nutrients in the growth medium. They were also consistent with prior studies showing that cells can generate active TGF β at the cell surface not only from inactive blood-derived precursor, but also from secreted latent peptide bound to the extracellular matrix.^{36–38}

The TGF β Signaling Pathway Becomes Refractory to Exogenous Active TGF β Ligand in Confluent Growth-Arrested Cells

Our data showed a high degree of signaling suppression by cell density, despite the fact that active TGF β concentrations in growth medium were barely measurable and did not vary. One explanation for the low level of signaling in contact-inhibited cells could have been decreased availability of TGF β released from the extracellular matrix.

Indeed, immunoblotting of SDS extracts showed that there was less TGF β associated with contact-inhibited cells than with growing subconfluent cells (Figure 2A; and supplementary Figure S2B at <http://ajp.amjpathol.org/>). This peptide represents latent TGF β bound to the extracellular matrix.^{36–38} However, it seemed unlikely that availability of TGF β precursor was the limiting factor underlying the signaling differences between growing and contact-inhibited cells, since fetal calf serum (FCS) in the growth medium contains abundant inactive TGF β sufficient to generate active ligand. Therefore, we investigated the possibility that signaling refractoriness, rather than decreased availability of TGF β , was the cause of diminished signaling in contact-inhibited cells. First, we showed in our culture model that signaling responses to exogenous active TGF β (0.01 to 10.0 ng/ml) became saturated at 1 ng/ml. (data not shown). Then, we compared the responses of subconfluent and confluent cells to exogenous ligand. Stimulation by active TGF β_1 at concentrations ranging from 0.05 to 2.0 ng/ml led to large increases of p3TP-Lux activity in subconfluent BM-Lux cells relative to basal levels. The corresponding signals in confluent cells were far lower (Figure 2E). After exposure to 2 ng/ml of active TGF β_1 , subconfluent BUMPT cells showed far more intense phosphorylation of Smad2 and Smad3 in their C-termini than in their basal state (Figure 2F). However, the corresponding signaling responses in confluent contact inhibited cells were much less intense (Figure 2F). When experiments were done with cells grown in serum-free medium, the results were similar (see supplementary Figure S2C at <http://ajp.amjpathol.org/>). On the one hand, low levels of cell-autonomous signaling in confluent cells could not be explained by depletion of serum derived latent TGF β precursor or by variations of active TGF β . On the other hand, even in the presence of saturating concentrations of active TGF β , confluent cells displayed blunted responses. Collectively, these observations showed that BUMPT and BM-Lux cells not only were able to autoregulate their TGF β signaling in a pattern that was independent of active or latent TGF β concentrations in the medium, but also responded with differential sensitivity to saturating concentrations of exogenous active TGF β added to the medium. The results indicated that the signaling pathway became refractory in contact-inhibited cultures. Although other modifications and rearrangements of signaling intermediates could have played additional roles, it seemed likely to us that the cell density-dependent fluctuations of TGF β signaling that took place during the epithelial growth cycle were related to corresponding alterations in the expression of TGF β receptor and inhibitory Smad7.

TGF β Signals Are High during the Growth Phase and Become Suppressed during Contact-Inhibited Growth Arrest and Differentiation of PT Primary Cultures

BUMPT cells carry a temperature-sensitive SV40 T-antigen transgene. We found that BUMPT cells show some

expression of T-antigen at the nonpermissive temperature of 37° C used for our studies (data not shown). Because T-antigen can bind the Rb protein and thereby abrogate TGF β -mediated growth suppression,^{39,40} we extended our observations to untransformed primary cultures of mouse PT cells. Seeded at first passage after explant culture, PT cells proliferated at rates slower than BUMPT cells. At confluence, they formed “domes” indicative of vectorial transport (see supplementary Figure S3A at <http://ajp.amjpathol.org/>) and became growth-arrested; this was accompanied by decrease of cyclin D and increase of cdk inhibitor p27^{kip1} proteins and decreased phosphorylation of Rb (see supplementary Figure S3B at <http://ajp.amjpathol.org/>).

Confluent growth-arrested cells exhibited differentiated features: increased expression of Na⁺/K⁺-ATPase, brush border proteases DPPIV and NEP, and cadherin-16, the kidney-specific (Ksp) cadherin⁴¹ (see supplementary Figure S3C at <http://ajp.amjpathol.org/>). Moreover, as shown in a subsequent section, they exhibited phloridzin-inhibited, sodium-dependent, glucose transport, a differentiated PT function.²⁹ Concurrently, there were decreases of the phosphorylation of Smad2 (S465/467) (supplementary Figure S3D at <http://ajp.amjpathol.org/>). Thus, TGF β signaling in PT primary cultures was also autoregulated; growing undifferentiated primary PT cultures displayed higher signaling levels than in contact-inhibited, differentiated cultures, exactly as in BUMPT cells.

Alk5 Antagonism by Mutant Alk5KR or Inhibitory Smad7 Induces Accelerated Epithelial Clustering and Differentiation of Subconfluent BUMPT Cells

The findings reported thus far raised important questions. What are the functions of high and low TGF β signaling in the physiological context of proliferation and growth arrest? Is spontaneous suppression of TGF β signaling in dense cultures related to the induction of differentiation? Is TGF β signaling necessary for appropriate regeneration? Based on our results, we surmised that growing cells use autocrine TGF β signals to decrease differentiation. To investigate the specific role that TGF β signaling might have played in suppressing differentiation and promoting cell migration and proliferation, we used adenoviral vectors to express either a dominant negative T β RI construct (Alk5KR) or wild type Smad7 to inhibit the kinase activity of T β RI (Alk5).²² Sparse cultures of subconfluent BUMPT cells were infected with adenoviral vectors. Infection with either Alk5KR or Smad7 adenovirus resulted in suppressed p3TP-Lux reporter activity in BM-Lux cells (data not shown) and premature formation of epithelial islands (Figure 3A). By immunoblotting, expression of Alk5KR or Smad7 resulted in decreased phosphorylation of Smad2 (S465/467), increase of E-cadherin and increased expression of the differentiation marker NEP (Figure 3, B and C). Despite accelerated induction of clustering and differentiation, Alk5-inhibited cultures

Figure 3. TGF β Receptor I kinase antagonism by mutant receptor Alk5KR or inhibitory Smad7 induces epithelial clustering and accelerated differentiation of subconfluent cells. Subconfluent BUMPT cells were infected with adenoviral vectors: 10 MOI of AdAlk5KR or empty AdCMV; 5 MOI of AdSmad7 or empty AdCMV. **A:** After 48 hours, cultures were photographed (original magnification $\times 200$) and then extracted with SDS for electrophoresis. **B, C:** SDS extracts were immunoblotted with antibodies against HA (for Alk5KR), FLAG (for Smad7), phospho-Smad2 (S465/467), and Smad2, NEP, and E-cadherin.

continued to proliferate and reached confluence. In contrast, a control adenovirus had no effects on Smad phosphorylation, cell clustering, or differentiation (Figure 3). The specificity of Alk5 inhibition by adenoviral vectors was demonstrated further. Adenovirus-mediated expression of Alk4KR, a dominant negative antagonist of the closely related Type I Activin receptor,²² failed to induce epithelial clustering or differentiation (data not shown).

Chemical Antagonism of Alk5 Kinase Induces Accelerated Epithelial Clustering and Differentiation of Subconfluent BUMPT Cells without Inhibiting their Proliferation

The striking promotion of differentiation by Alk5KR and Smad7 led us to examine whether chemical antagonism of TGF β signaling would produce similar effects. We used Alk5 antagonists for this purpose because high affinity binding by cell permeable inhibitors would result in rapid inhibition of receptor kinase activity and permit the discrimination of early TGF β -specific changes from late effects caused by cell crowding and contact inhibition. We used two structurally different inhibitors of T β RI kinase: SB431542 (1 or 2 μ mol/L)³⁵ and Alk5 inhibitor I (100 nmol/L to 1 μ mol/L)⁴² on BUMPT and BM-Lux cells. Figures 4 and 5 show the data using SB431542. Results using Alk5 inhibitor I were similar (see supplementary Figure S4 at <http://ajp.amjpathol.org/>). For the experiments shown in Figures 4 and 5, cells were deliberately seeded at very low density, at 830/cm², which is 16-fold less than in Figures 1 and 2. Treated without or with SB431542, these sparsely seeded cells remained subconfluent during 4

days of growth (Figure 4). Treatment with SB431542 dramatically accelerated the formation of epithelial islands containing E-cadherin, ZO-1, actin, and β -catenin along circumferential zones of intercellular contact (Figure 4, A and B). Cells without SB431542 showed a distribution of actin along stress fibers (Figure 4B), and did not express α -SMA, vimentin, or S100A4, antigens that have been reported to be expressed by cells with TGF β -induced EMT (data not shown). By electron microscopy, SB431542-treated cells showed cuboidal morphology with apical microvilli, whereas untreated cells were flatter with fewer microvilli (Figure 4C). By video microscopy, motile dimethyl sulfoxide (DMSO)-treated cells in sparse cultures made random contacts with neighbors, but did not make stable adhesions, whereas SB431542-treated cells remained adherent after contact. Moreover, without inhibitor, daughter cells migrated away following mitosis whereas SB431542-treated cells remained in place, forming clusters (see supplementary Movie 1 at <http://ajp.amjpathol.org/>). Notwithstanding the accelerated development of epithelial phenotype, SB431542-treated cells proliferated equally well as controls up to 48 hours; even later, as cells became crowded within epithelial islands (Figure 4A, 96 hours), they continued to proliferate, albeit at slightly decreased rates (Figure 4D). SB431542 did not decrease BrdU labeling of nuclei after 24 hours of treatment, and there were only modest decreases by 48 hours and thereafter (see supplementary Table S1 at <http://ajp.amjpathol.org/>).

In studies parallel to those depicted in Figure 4, cells seeded at 830/cm² were grown without or with SB431542 and examined by immunoblotting at 24-hour intervals. As in Figure 4 and unlike cells plated at higher density (Figure 1), these cultures remained subconfluent and were in growth phase throughout the experiment (Figure 5A). Cells treated with SB431542 showed decreased phosphorylation of Smad2 (S465/467), and progressive increases of E-cadherin and the differentiation markers NDRG1, Na⁺/K⁺-ATPase, DPP IV, and NEP (Figure 5B). Consistent with undiminished proliferation in the presence of SB431542 (Figure 4D), these cells with inhibited TGF β signaling did not exhibit decreases of the proliferation markers cyclin D and c-Myc or increase of the cell cycle inhibitor p27^{kip1} for 2 days. These alterations occurred only late, after 3 days of SB431542 treatment (Figure 5C), when cells became crowded within the SB431542 induced epithelial islands (Figures 4, A and B, and 5A). We also investigated the possibility that TGF β signaling inhibition caused by TGF β neutralizing antibodies might increase the expression of differentiation markers and mimic the effects of Alk5 inhibitors. To optimize the effects of neutralizing antibodies, cells were grown in serum-free medium. Consequently, the cells did not have access to serum derived latent TGF β . Neutralizing antibodies reduced the intensity of TGF β signaling reflected by Smad2 C-terminal phosphorylation, and increased the expression of differentiation marker NEP and adherens junction protein E-cadherin (see supplementary Figure S2A at <http://ajp.amjpathol.org/>), but the effects were only modest, in contrast to the dramatic effects of Alk5 inhibition (Figures 3, B and C, and 5 B).

Figure 4. TGF β receptor I kinase antagonism by a chemical inhibitor accelerates the development of epithelial phenotype in subconfluent BUMPT cells. To ensure that the cells continued to be subconfluent and in log phase growth over the 4-day duration of the experiment, they were plated at 830/cm², or 16-fold lower than in Figure 1 (except that the cells shown in Figure 5C which were plated at 13,000/cm²). After 2 hours to allow attachment, SB431542 or DMSO vehicle was added and cells were examined after 48, 72, or 96 hours. **A:** Cells were examined by immunofluorescence for E-cadherin (**top** panel) and ZO-1 (**bottom** panel). **B:** Cells were examined by immunofluorescence for actin (**top** panel) and β -catenin (**bottom** panel); Original magnification $\times 400$. **C:** After 48 hours of treatment with SB431542 or DMSO, subconfluent cells (day 2 density – Figure 1) were fixed and examined by electron microscopy; (original magnification $\times 5000$ in left and right panels). **D:** Cells were counted after 48 and 96 hours of treatment with SB431542 or vehicle (mean $\pm$ SE, $n = 3$).

As cited in an earlier section, the presence of T-antigen in the SV40 T-antigen-transformed BUMPT cells could interfere with Rb protein-mediated inhibition of the cell cycle by TGF β .^{39,40} Therefore, we examined the effects of Alk5 inhibition on growth and differentiation of PT cells in primary culture.

Alk5 Antagonism by a Chemical Inhibitor Increases DNA Synthesis and Proliferation of Subconfluent PT Primary Cultures, but Concurrently Increases the Formation of Epithelial Clusters and Expression of Ksp-Cadherin

Subconfluent primary cultures of PTs in first passage were exposed to 2 μ mol/L SB431542 or DMSO vehicle for 2, 4, or 6 days. DNA synthesis was monitored by BrdU uptake. Cells with SB431542 showed more BrdU-labeled nuclei than controls throughout the 6-day experimental period, although the differences became narrower in both groups as cells became more crowded (Figure 6A). Enhanced DNA synthesis was accompanied by increased proliferation; after 6 days, SB431542-treated cells were $\sim$ threefold more numerous than untreated controls (Figure 6B). Control cells displayed flat and/or elongated irregular morphology and tended to remain in isolation or loose clusters; in contrast, cells with SB431542 were more numerous and exhibited a cuboidal and epithelial morphology and formed tight

clusters of cells with increased expression of Ksp-cadherin in cell junctions (Figure 6, C and D).

Alk5 Antagonism by a Chemical Inhibitor Induces Rb Phosphorylation in PT Primary Cultures

Subconfluent primary cultures of PTs were exposed to 2 μ mol/L SB431542, which led to diminished Smad2 phosphorylation at S465/467 (Figure 6E). By 12 hours, there were increases of the slow migrating (hyperphosphorylated) form of Rb and enhanced phosphorylation of cdk phospho-sites S601 and S800/804 of mouse Rb (Figure 6E). In contrast, there were no alterations in the expression of cyclins and cdk inhibitors p15^{ink4}, p21^{waf1}, and p27^{kip1} (data not shown). The effects of Alk5 inhibition on Rb phosphorylation and cell proliferation probably involved the activation of cdk by altered cyclin, cdk, and cdk-inhibitor interactions, since treatment with exogenous TGF β has been shown to interfere with the formation stable cyclin-cdk complexes and thereby inhibit cdk activity.^{43,44}

Alk5 Kinase Antagonism by Chemical Inhibitor or Mutant AIK5KR Promotes Differentiation in PT Primary Cultures Proliferating at Increased Rates

First-passage primary cultures of PTs were seeded at subconfluent density and treated with 2 μ mol/L

Figure 5. TGF β receptor I kinase antagonism by a chemical inhibitor promotes differentiation of subconfluent BUMPT cells. To ensure that the cells continued to be subconfluent and in log-phase growth over the duration of the experiment, they were plated at 830/cm², or 16-fold lower than in Figure 1. Cells were treated with 1 μ mol/L SB431542 or vehicle and examined after 24, 48, and 72 hours. **A:** Formaldehyde-fixed cells were stained with toluidine blue/basic fuchsin. The cell densities correspond to cultures extracted for protein analysis in **(B)** and **(C)**; magnification = original $\times$ 200. **B:** SDS extracts were immunoblotted for phospho-Smad2 (S465/467) and Smad2, E-cadherin, NDRG1, Na⁺/K⁺ ATPase, DPP IV, and NEP. **C:** SDS extracts were immunoblotted for cyclin D, c-Myc, and p27^{kip1}.

SB431542 or vehicle for 2 or 4 days, intervals during which they were proliferating at enhanced rates (Figure 6, A and B). Inhibitor-treated cells showed decreased Smad2 (S465/467) phosphorylation and increases in the protein content of the differentiation markers Na⁺/K⁺ ATPase, DPP IV and NEP, and Ksp-cadherin (Figure 7A). Inhibition of TGF β signaling produced by adenoviral expression of the dominant-negative TGF β receptor Alk5KR yielded similar results (Figure 7B). In addition, primary cultures grown to confluence in the presence of SB431542 showed greater phloridzin-inhibitable, sodium-dependent glucose transport than controls exposed to vehicle only (Figure 7C). Consistent with these observations, Alk5 inhibited primary cultures formed differentiated and contact-inhibited confluent

monolayers much faster than untreated cultures (data not shown).

Thus, remarkably, diverse molecular and chemical inhibitors of T β RI kinase powerfully induced the differentiation of PT cells growing in log-phase, substantially reproducing in subconfluent cultures the phenotype normally displayed by confluent growth-arrested cells. Notably, in primary cultures of PT in which the growth inhibitory function of Rb protein is not compromised by T-antigen, inhibition of TGF β signaling not only induced accelerated differentiation as in BUMPT cells, but also simultaneously and substantially increased their proliferative rate.

Wound-Induced Migration and Proliferation of Confluent BUMPT Cells Is Accompanied by Increased TGF β Signaling, Loss of E-Cadherin, and Decreased Differentiation

The spontaneous suppression TGF β signaling that took place during the transition of proliferating PT cells to the contact-inhibited differentiated state suggested that rapid release from contact inhibition would stimulate TGF β signaling. We tested this possibility in a wound healing model.

Following mechanical removal of $\sim$ 80% of a contact-inhibited BUMPT monolayer, $\sim$ 200 μ m wide concentric strips of cells remained, alternating with $\sim$ 800 μ m wide "wounds" (Figure 8A). With time, several rows of cells at wound edges became activated and polarized, migrated into wounds and proliferated (Figure 8 A; and supplementary Movie 2, DMSO at <http://ajp.amjpathol.org/>). Wounded cells exhibited altered expression of TGF β receptors and Smad7. T β RII protein increased in wounded cells by 6 hours, and increased further by 12 hours (Figure 8B). T β RI protein increased modestly, by 12 hours; in contrast, Smad7 protein decreased (Figure 8B). Wounding led to increased TGF β signaling as shown by phosphorylation of Smad2 at C-terminal S465/467 and enhanced p3TP-Lux reporter activity (Figure 8, B and C). Wound-stimulated increase of TGF β signaling required extracellular TGF β ligand; neutralizing TGF β antibodies in the medium blunted the enhanced p3TP-Lux reporter activity caused by wounding (Figure 8D). However, measurement of active TGF β using a sensitive bioassay showed that active TGF β levels in the medium were not only extremely low (<5 pg/ml), but did not increase after wounding. Wound-stimulated TGF β signaling was accompanied by decreased E-cadherin and differentiation marker NEP (Figure 8E).

Alk5 Antagonism Promotes Intercellular Adhesion and Permits Increased Retention of E-Cadherin and Differentiation Markers in Wounded Cultures of BUMPT Cells without Compromising Migration and Proliferation

The differentiation-promoting effects of Alk5 antagonists in subconfluent PT cells prompted us to examine whether

Figure 6. TGF β receptor I kinase antagonism by a chemical inhibitor increases DNA synthesis, cell proliferation, and Rb phosphorylation but concurrently accelerates the development of epithelial phenotype in subconfluent primary cultures of PT cells. Primary cultures of PT cells were seeded at low density. Following cell attachment, 2 $\mu\text{mol/L}$ SB431542 or vehicle were added and the cells were examined after durations of up to 6 days. **A:** Cells were incubated with BrdU for 1 hour before fixation and the percentage of BrdU-labeled nuclei determined by immunofluorescence ($n = 5$, mean $\pm$ SE $P < 0.05$ for differences at 2 and 4 days). **B:** Cells were counted using a hemacytometer ($n = 4$, mean $\pm$ SE $P < 0.05$ for differences at 4 and 6 days). **C:** Phase contrast micrographs after 4 days of treatment (original magnification $\times 200$). **D:** Cells were fixed after 3 days of treatment and examined by immunofluorescence for Ksp-cadherin (magnification = original $\times 400$). **E:** After 12 and 24 hours of treatment, SDS extracts were immunoblotted for phospho-Smad2 (S465/467), Smad2, phospho-Rb (S608 - S601 of mouse Rb), phospho-Rb (S807/811 - S800/804 of mouse Rb), and Rb.

inhibition of TGF β signaling would modify the regenerative response of survivor cells following wounding of confluent BUMPT monolayers. Treatment with SB431542 blocked the wound-stimulated p3TP-Lux reporter activity and phosphorylation of Smad2 at C-terminal S465/467 (Figure 8, C and F) and partially prevented the wound-induced decrease of E-cadherin and differentiation marker NEP (Figure 8F). In wounded cultures treated with vehicle only, cells at wound edges exhibited little E-cadherin and migrated individually; in contrast, SB431542 treatment promoted increased cellular cohesion at wound edges with more abundant intercellular E-cadherin as revealed by immunofluorescence staining (Figure 8G). Wounded cells without or with SB431542 treatment migrated at the same rate (see supplementary Movie 2 at <http://ajp.amjpathol.org/>) and proliferated equally well, monitored as BrdU uptake (see supplementary Table S2 at <http://ajp.amjpathol.org/>).

Alk5 Antagonism Promotes Tubulo-Interstitial Repair Following Kidney Ischemia in Vivo

Structural repair following episodes of AKI is often incomplete despite the abatement of azotemia.^{3,11,23,45,46}

Weeks to months after apparent recovery of renal function following ischemic injury, kidneys may display chronic disease in the form of tubule atrophy, interstitial fibrosis, and diminished vascular density.^{3,11,23,45,46} It seemed possible to us that tubulo-interstitial pathology may be caused by the failed differentiation of regenerating tubules expressing increased TGF β and TGF β receptors.¹¹ We surmised that Alk5 antagonists might have the potential to facilitate repair of tubules following AKI. Conceivably, Alk5 inhibitors could promote the differentiation of regenerating epithelium *in vivo* as they did in culture, and thereby enhance the recovery of normal structure. To test this possibility, we used the rat model of left kidney ischemia-reperfusion with contralateral nephrectomy²³ used to simulate AKI in the transplanted kidney.⁴⁶ After reperfusion was established for 4 hours, rats received SD-208 or vehicle alone for 4 days. SD-208 is known to inhibit C-terminal phosphorylation of Smad2 in cultured cells and in experimental animals *in vivo*.²⁵ Furthermore, we confirmed that the effects of SD-208 on cultured PT cells were similar to those of SB431542 and Alk5 inhibitor I (data not shown). Reperfusion of ischemic kidneys caused PT necrosis, predominant in the outer stripe of the outer medulla as reported^{3,16} (data not shown). Se-

Figure 7. TGF β receptor I kinase antagonism by chemical inhibitor or mutant receptor Alk5KR promotes differentiation in primary cultures of PTs. **A** and **B**: Primary cultures of PTs were seeded at low density. After attachment, 2 μ mol/L SB431542 or vehicle only was added (**A**) or, after 24 hours of seeding, cells were infected with 5 MOI of either empty adenovirus (AdCMV) or adenovirus carrying Alk5KR (**B**). After 4 days (**A**) or 2 days (**B**) SDS extracts were immunoblotted for phospho-Smad2 (S465/467), Smad2, Na⁺K⁺ATPase, DPP IV, NEP, and Ksp-cadherin. **C**: Primary cultures of PTs were seeded at subconfluent density. After attachment, 2 μ mol/L SB431542 or vehicle was added and the cells were allowed to reach confluent growth arrest. Phloridzin-sensitive, sodium-dependent glucose transport was then measured ($n = 5$, mean $\pm$ SE, $P < 0.05$ for difference between SB431542 and DMSO).

rum creatinine increased during reperfusion, peaked at 24 hours, and declined gradually thereafter as reported for this model of AKI²³ (Figure 9A). SDS extracts of the outer stripe of the outer medulla from reperfused kidneys showed increased C-terminal phosphorylation of Smad2 that was ameliorated by treatment with SD-208 (Figure 9B). There were corresponding alterations of T β RI and T β RII (Figure 9B) paralleling the observations made on wounded BUMPT cells (Figure 8B). Treatment with SD-208 did not modify either the development or subsequent remission from azotemia (Figure 9A). To assess the effects of SD-208, we performed a detailed histological, morphometric, and immunocytochemical analysis of kidneys 14 days after ischemia. Consistent with the observation that early ischemic necrosis occurred largely in PTs and was predominant in the outer stripe of outer medulla, we observed that tubulo-interstitial pathology in 14-day vehicle-treated kidneys occurred mainly in the outer stripe (Figure 9C) with only focal lesions in the cortex (not shown). Normal PT profiles were decreased in number in the outer stripe (Figure 9, C and D). Many tubules were variably dilated and lined by flat undifferentiated epithelium or poorly differentiated PT cells with

attenuated brush borders (Figure 9, C and D). The interstitium was widened with infiltrating cells (Figure 9, C and D). The pathology was ameliorated by treatment with SD-208 (Figure 9, C and D). Evaluation by a morphometric method and semiquantitative analysis of tubule differentiation by a grading system confirmed that SD-208 had major effects on the postischemic kidney—tubules from treated kidneys were more differentiated, and interstitial pathology was attenuated (Figure 9, E and F). To evaluate the effects of SD-208 on PT differentiation and interstitial pathology, we performed immunocytochemical studies. The intensity of staining for differentiation markers—Ksp-cadherin for a nephron specific adherens junction protein, meprin for brush border microvilli, and Na⁺K⁺-ATPase α -subunit for the basolateral sodium pump—was variably decreased or absent in tubule profiles of vehicle treated kidneys, particularly in dilated tubules with flattened epithelium (Figure 10). These changes were reversed by SD-208 (Figure 10). Thus, Alk5 inhibition reproduced *in vivo* the same differentiation promoting effects that it had on cultured cells. The beneficial effects of SD-208 were far-reaching. Kidneys from vehicle treated rats showed infiltrates of myofibroblasts around atrophic and dilated tubules with abnormally thick basement membranes staining for Type IV collagen; furthermore, there was increased deposition of Type I collagen and reduction of capillary density in the interstitium (Figure 11). In kidneys from SD-208 treated rats, these pathological alterations were largely attenuated (Figure 11).

Discussion

The major findings of this study were: 1. Cell autonomous TGF β signaling fluctuated with and controlled the growth and differentiation status of PT cells; 2. The signaling fluctuations were associated with reciprocal alterations of TGF β receptor and Smad7 expression; 3. Inhibition of TGF β signaling by Alk5 antagonism accelerated the differentiation of PT cells while simultaneously stimulating their proliferation. Despite the increased proliferative rates, Alk5 inhibited cells became normally contact inhibited in a density dependent manner; 4. In a wound induced model of PT cell regeneration, inhibition of auto-crine TGF β signaling permitted vigorous and unmitigated epithelial migration and proliferation, but concurrently enabled the retention of a stronger epithelial phenotype with better differentiation; and 5. Treatment with a small molecule inhibitor of Alk5 resulted in enhanced tubule differentiation and decreased tubulo-interstitial pathology during the recovery phase following ischemic injury. Paradoxically, our results using cultured cells suggest that endogenously generated TGF β signals in proliferating PT cells become more elevated than is necessary to modulate a physiological regenerative process. To that extent, the signaling increases may be redundant and possibly maladaptive. This inference is supported by the effects of pharmacological TGF β signaling antagonism on the regeneration of kidney epithelium after injury *in vivo*.

Both in a cell line and primary cultures, PT cells displayed tight autoregulation of TGF β signals. Contrary to

Figure 8. **A:** Contact-inhibited (day 4) cells were wounded, after which $\sim 200\text{-}\mu\text{m}$ wide concentric strips of epithelium remained, alternating with $\sim 800\text{-}\mu\text{m}$ wide wounds (left panel). Migration of “activated” and polarized surviving cells into the wounds is shown in the right panels. Formaldehyde-fixed cells were stained with toluidine blue/basic fuchsin. **B:** After 6 and 12 hours of wounding, SDS extracts of wounded cells, “0 time” controls and non-wounded “time controls” were immunoblotted for phospho-Smad2 (S465/467), Smad2, T β RII, T β RI, and Smad7. **C:** Contact-inhibited (day 4) BM-Lux cells were wounded and incubated with $2\text{ }\mu\text{mol/L}$ SB431542 or vehicle for 6 hours before measurement of luciferase activity; mean $\pm$ SE, $n = 3$. $P < 0.005$ for all differences. **D:** Contact-inhibited (day 4) BM-Lux cells were wounded and $15\text{ }\mu\text{g/ml}$ of neutralizing TGF β antibodies or non-immune IgG were added to the incubation medium, 6 hours before measurement of luciferase activity; mean $\pm$ SE, $n = 3$. $P < 0.05$. **E:** After 6 and 12 hours of wounding, SDS extracts of wounded cells and unwounded controls were immunoblotted for the differentiation marker NEP and E-cadherin. **F:** After 12 hours of wounding and treatment with SB431542 or vehicle, SDS extracts of wounded cells and unwounded controls were immunoblotted for phospho-Smad2 (S465/467), and Smad2, or the differentiation marker NEP and E-cadherin. **G:** Unwounded controls and wounded cells incubated for 12 hours with SB431542 or vehicle only were fixed with formaldehyde and assessed by immunofluorescence for E-cadherin.

what might be expected for a cytokine that induces growth arrest in epithelial cells, endogenously generated TGF β signals were high during log phase growth and low during contact-inhibited growth arrest. TGF β signals fluctuated not only during regeneration induced by subculturing confluent cells, but also following wound-induced release from contact inhibition. The apparent paradox of high TGF β signaling in rapidly dividing epithelial cells can be resolved by considering that the cell cycle is controlled by several signaling pathways. Therefore, if signals that positively regulate the cell cycle are increased when TGF β signaling is also high, ie, during the early phase of log-phase growth following subculture or wound healing, the cytostatic effects of TGF β will be overwhelmed by mitogenic signals. The mitogenic signals that oppose TGF β are likely derived from the MAPK and PI3K pathways operating independently. Indeed, we

found that MAPK and PI3K signals were high in growing cells and low in dense, confluent cultures; and inhibition of MAPK and/or PI3K pathways led to decreased proliferation (data not shown). Moreover, selective TGF β antagonism had little or no effect on high MAPK and PI3K signals in cells induced to proliferate by subculture or wounding (data not shown). As reviewed recently, mitogenic signaling through the MAPK and PI3K pathways and antiproliferative signaling by TGF β have opposing effects on Rb phosphorylation, the key step in cell cycle progression.⁴⁴ Whereas MAPK and PI3K signals promote Rb phosphorylation, TGF β decreases Rb phosphorylation through effects on cyclin, cdk, and cdk inhibitor interactions that compromise the formation of stable cyclin-cdk complexes. Therefore, cell cycle entry is determined by the sum of mitogenic signals such as those generated by MAPK and PI3K and inhibitory signals from

Figure 9. Treatment with Alk5 inhibitor SD-208 does not modify azotemia or its remission after ischemic kidney injury but ameliorates tubulo-interstitial pathology. **A:** Serum creatinine in control rats (vehicle and SD-208 treated rats pooled), and rats with ischemia-reperfusion (IR) treated with vehicle alone or with SD-208. **B:** SDS extracts of the outer stripe of outer medulla from normal rats without surgery (NK), nephrectomized controls (C), rats with ischemia-reperfusion (IR) (3, 5, and 7 days after surgery) and rats with IR treated with SD-208 (SD) 3 days after surgery were immunoblotted for phospho-Smad2 (S465/467), Smad2, TβRI, and TβRII. **C:** Kidney sections stained with H&E from control rat, and rats with ischemia-reperfusion treated with vehicle only or with SD-208, 14 days after surgery. Magnification = original ×100. **D:** Kidney sections stained with Periodic Acid Schiff stain from rats with ischemia-reperfusion treated with vehicle only or with SD-208, 14 days after surgery. The areas shown are from the outer stripe of outer medulla and were selected to highlight the type of tubulo-interstitial pathology and tubule morphology that was analyzed by morphometry and grading. Magnification = original ×200. **E, F:** Tubulo-interstitial index and tubule differentiation index calculated from representative photographs of the outer stripe of outer medulla. All relevant difference were significant ($n = 7$ for controls and vehicle treated IR; $n = 6$ for SD-208 treated IR); vehicle treatment versus nephrectomized control, * $P < 0.001$; SD-208 treatment versus vehicle treatment, ** $P < 0.001$).

the TGFβ pathway. Consistent with the operation of this mechanism, we found that TGFβ signaling antagonism by SB431542 enhanced Rb phosphorylation above basal levels in proliferating subconfluent PT primary cultures (Figure 6E), promoted BrdU uptake by nuclei (Figure 6A) and enhanced the rate of cell proliferation (Figure 6B). We infer from these results that the antiproliferative actions of high TGFβ signaling during log phase growth are normally offset by mitogenic MAPK and PI3K signals, and that TGFβ antagonism by SB431542 augments proliferation by further increasing the phosphorylation of Rb. While the proliferative effects of Alk5 antagonism are not surprising in view of the known cell cycle inhibitory actions of TGFβ, our findings raised questions regarding the significance of TGFβ signaling autoregulation during PT cell growth and contact inhibition.

What then are the functions of high autocrine TGFβ signaling in cells that are proliferating as a result of stimulatory cues from other pathways? Does TGFβ signaling serve only a homeostatic function with respect to cell growth, ie, it tempers excessive proliferation by its inhibitory actions, or does it have another role? Clues to answering this question were provided by two observations: 1. without or with Alk5 antagonism, proliferating BUMPT cells, as well as PT cells in primary culture became growth arrested by contact inhibition; and 2. the differentiation status of proliferating PT cells was coupled to TGFβ signaling activity. The first observation suggested that the antiprolif-

erative functions of TGFβ were redundant—cells proliferated regardless of Alk5 antagonism and growth arrest occurred independently of TGFβ signaling activity in a density-dependent manner. The second observation suggested that the function of high TGFβ signaling in proliferating PT cells is to decrease differentiation and that the induction of differentiation by increased cell density is mediated by the suppression of TGFβ signals.

As in the case of the anti-proliferative effects of TGFβ, we questioned whether the differentiation-decreasing effects of endogenous TGFβ signaling were necessary or redundant. We considered the possibility that optimal migration and proliferation of regenerating PT cells required them to be undifferentiated. The effects of Alk5 antagonism show that this was not the case. Consistent with the notion that spontaneous suppression of TGFβ signaling was responsible for inducing density-dependent differentiation in confluent cultures, Alk5 antagonism dramatically accelerated the development of epithelial characteristics and differentiated features in rapidly proliferating subconfluent cultures. Although the random movements of growing BUMPT cells were decreased by SB431542 treatment, this occurred as a result of increased intercellular adhesion and the formation of cell clusters (as shown by supplemental Movie 1 at <http://ajp.amjpathol.org/>). Lamellipodial extensions formed freely from the edges of differentiating epithelial clusters in which cells continued to proliferate and peripherally

Figure 10. Immunohistochemistry for Ksp-cadherin, meprin, and Na⁺/K⁺ATPase in kidney sections from control rats and rats with ischemia-reperfusion of the kidney treated with vehicle only or with SD-208, 14 days after surgery. Photographs are from outer stripe of outer medulla. Magnification = original $\times 200$.

located cells migrated centrifugally to fill the available culture substratum. The effects of Alk5 antagonism on migration and proliferation of wounded BUMPT cells are particularly illustrative of this point. When they were treated with SB431542, wounded cells displayed better retention of epithelial phenotype and intercellular adhesion with partial retention of differentiation markers, but nevertheless migrated and proliferated equally as well as wounded controls not exposed to the Alk5 inhibitor (as shown in supplemental Movie 2 at <http://ajp.amjpathol.org/>). These observations and considerations imply that enhanced TGF β signaling in proliferating subconfluent cells and in regenerating wounded cultures did not serve a necessary function. PT cultures migrated, proliferated, and became contact-inhibited, regardless of TGF β signaling activity. Indeed, Alk5 inhibited cultures displayed properties that can be considered to be favorable for optimal regeneration—uninhibited migration and proliferation and speedier differentiation.

To our knowledge, the induction of differentiated properties in adult epithelial cells stimulated to proliferate faster by TGF β signaling antagonism is without precedent. As such, our findings have implications to the understanding of the role played by TGF β signaling in epithelial regeneration following injury. When epithelial integrity is compromised, surviving cells undergo de-differentiation, migrate into denuded areas and proliferate; this is followed by density-dependent growth arrest and re-differentiation. The healing process is under con-

Figure 11. Immunofluorescence for α -SMA, collagen I, collagen IV, and endothelial marker aminopeptidase P (JG12) in cryosections from rats with ischemia-reperfusion of the kidney treated with vehicle only or with SD-208, 14 days after surgery. In the third panel from top, sections were doubly stained for type IV collagen (Green) and monocyte marker, ED1 (Red). Photographs are from outer stripe of outer medulla. Magnification = original $\times 200$.

trol of a multitude of signaling cues related to the disruption and restoration of cell-cell contact, remodeling of cell-extracellular matrix adhesion and activation of growth factor receptors.^{1–3,12,47} Disturbed orchestration of these stimuli can lead to poor healing, stromal overgrowth and fibrosis; overactive TGF β signaling can underlie this disorder.¹² As alluded to earlier, TGF β signaling was reported to be enhanced in wounded skin and regenerating kidney epithelium following ischemic damage *in vivo*.^{11,12} Wounds heal faster in mice with gene deletion of Smad3, transgenic expression of dominant negative T β RII or adenoviral transduction of Smad7.^{48–50} Conceivably, these findings can be explained by diminished inflammation, decreased production of scar tissue or increased proliferation of cells at wound edges. By extrapolation, our data would suggest that speedier differentiation, as well as increased proliferation, could have been important factors that accounted for the wound healing benefits that accrued from these interventions. The data reported here strongly support this notion.

Antagonism of TGF β signaling by a small molecule inhibitor SD-208 enhanced the return of normal structure, improved the differentiation status of tubules and dimin-

ished the extent of tubulo-interstitial pathology in kidneys during the prolonged phase of recovery from ischemic damage. Our studies confirm the findings of Spurgeon et al¹¹ and significantly extend their observations. These investigators used neutralizing TGF β antibodies to diminish TGF β signaling in kidneys recovering from ischemic injury. They found that antibody treatment increased the proliferation of tubule epithelium by the third day and protected against the development of interstitial fibrosis and diminished vascular density by 35 days after ischemia.¹¹ Comparing our results to those of Spurgeon et al,¹¹ SD-208 appeared much more effective than TGF β neutralizing antibodies in promoting tubulo-interstitial repair. Furthermore, SD-208 dramatically improved the differentiation status of tubules, an aspect of tubule structure that Spurgeon et al did not comment on.¹¹

It has been unclear why post-ischemic kidneys develop atrophic tubules with poorly differentiated epithelium, interstitial inflammation, and fibrosis.^{3,23,45,46} Conceivably, these long-term changes occur as a result of redundant and possibly maladaptive TGF β signaling during the early stages of tubule regeneration and are prevented by inhibiting the TGF β signaling response. Because regeneration *in vivo* is associated with inflammation, study of signaling homeostasis is complicated, owing to epithelial stimulation by growth factors, cytokines, and hormones derived from leukocytes and reactive stromal cells. Our findings obtained in isolation from cultured PT cells raise the possibility that maladaptive cell autonomous TGF β signaling plays an important role in delaying the differentiation of regenerating epithelium and contributes to improper repair. However, in addition to maladaptive epithelial autocrine signaling, TGF β derived from inflammatory cells is also likely to play a role. Our findings, together with those of Spurgeon et al¹¹ show that small molecule inhibitors of TGF β signaling already developed for cancer therapeutics have the potential to promote faster and more optimal regeneration of differentiated epithelium following kidney injuries.

Endogenously generated TGF β signals in cultured PT cells required the presence of extracellular TGF β ligand, as shown in our experiments by the effects of neutralizing antibodies when they were added to the culture medium. However, paradoxically, concentrations of active TGF β were vanishingly low in the medium as measured by a sensitive bioassay using mink lung epithelial cells stably transfected with a PAI-I luciferase reporter, and the measured concentrations of active TGF β did not correlate with signaling intensities. Moreover, neither the frequent change of culture medium nor cross-transfer of medium affected the TGF β signals. These apparently conflicting observations can be explained by well-documented studies showing that conversion of latent TGF β to active TGF β is a highly regulated process that takes place at the cell-extracellular matrix interface. Nascent ligand generated from inactive complexes of latent TGF β and latent TGF β binding proteins becomes receptor bound and endocytosed while unused active TGF β becomes protein bound and inactivated again.^{36–38} Therefore, our failure to find measurable differences in the concentrations of active TGF β in the culture medium between groups of cells with large differences in

cell-autonomous TGF β signaling activity is not inconsistent with the requirement for extracellular ligand.

Our results show that PT cells growing in sparse cultures and displaying high autocrine TGF β signaling exhibit “fibroblastoid” morphology with actin stress fibers, an appearance similar to that of EMT induced in cultured cells by TGF β . However, TGF β -induced EMT of cultured cells occurs in the context of sustained signaling by abnormally high concentrations of the cytokine well above those required to saturate the TGF β receptors, and cells with EMT express the mesenchymal antigen α -SMA.^{6,8,9} Subconfluent PT cells did not express α -SMA and spontaneously developed epithelial features as cell density increased. Of note, the conversion of fibroblast-like epithelial cells with actin stress fibers to differentiated epithelium with peripheral distribution of actin was accelerated by Alk5 antagonism. This phenotypic change bears some resemblance to the mesenchymal epithelial transition, during which cells of mesenchymal lineage become epithelial cells.⁵¹ We do not know if the processes that convert proliferating undifferentiated PT cells to a growth-arrested differentiated epithelium overlap with genetic programs that give rise to EMT and mesenchymal epithelial transition.

Finally, our studies of signaling autoregulation during contact inhibition of an epithelium emphasize the lack of information regarding the origin and termination of signals that vary predictably in a manner related to cell density but unrelated to factors in the growth medium. There is pressing need to study how signaling becomes stimulated in cells released from contact inhibition and how they become suppressed once more by increased cell density. Our current observations could provide the basis for further investigations of the signaling underpinnings of epithelial contact inhibition.

Acknowledgments

We are grateful to the following people for their generous gifts: Makiko Fujii for adenoviral vectors with Alk4KR, Alk5KR, and Smad7; Daniel Rifkin for MLEC-PAI-I reporter cells; Judith Bond and John Bylander for meprin antibody; Wen-Hwa Lee for anti-mouse Rb monoclonal antibody; Dentscho Keraschki for epithelium aminopeptidase P (rat) monoclonal antibody (*JG12*); and John Schwartz and Wilfred Lieberthal for BUMPT cells. We thank Yogendra Patel for creatinine measurements and Xueying Li for statistical help. SD-208 was generously provided by Drs. George Schreiner, Linda Higgins and Satya Medicherla, formerly of SCIOS Inc. now a subsidiary of Johnson and Johnson; SD-208 is now available from Calbiochem. We are indebted to Goutam Ghosh Choudhury, Susan Weintraub, and Zheng Dong for their criticism and review of the manuscript.

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