Transforming Growth Factor- β 1 Induces Smad3-Dependent β_1 Integrin Gene Expression in Epithelial-to-Mesenchymal Transition during Chronic Tubulointerstitial Fibrosis

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Transforming growth factor- β 1 (TGF- β 1)-induced epithelial-to-mesenchymal transition (EMT) contributes to the pathophysiological development of kidney fibrosis. Although it was reported that TGF- β 1 enhances β_1 integrin levels in NMuMG cells, the detailed molecular mechanisms underlying TGF- β 1-induced β_1 integrin gene expression and the role of β_1 integrin during EMT in the renal system are still unclear. In this study, we examined the role of β_1 integrin in TGF- β 1-induced EMT both *in vitro* and *in vivo*. TGF- β 1-induced augmentation of β_1 integrin expression was required for EMT in several epithelial cell lines, and knockdown of Smad3 inhibited TGF- β 1-induced augmentation of β_1 integrin. TGF- β 1 triggered β_1 integrin gene promoter activity as assessed by luciferase activity assay. Both knockdown of Smad3 and mutation of the Smad-binding element to block binding to the β_1 integrin promoter markedly reduced TGF- β 1-induced β_1 integrin promoter activity. Chromatin immunoprecipitation assay showed that TGF- β 1 enhanced Smad3 binding to the β_1 integrin promoter. Furthermore, induction of unilateral ureteral obstruction triggered increases of β_1 integrin in both renal epithelial and interstitial cells. In human kidney with chronic tubulointerstitial fibrosis, we also found a concomitant increase of β_1 integrin and α -smooth muscle actin in tubule epithelia. Blockade of β_1 integrin signaling dampened the progression of fibrosis. Taken to-

gether, β_1 integrin mediates EMT and subsequent tubulointerstitial fibrosis, suggesting that inhibition of β_1 integrin is a possible therapeutic target for prevention of renal fibrosis. (Am J Pathol 2010; 177:1743–1754; DOI: 10.2353/ajpath.2010.091183)

Tubulointerstitial fibrosis is a critical event in chronic renal failure.^{1,2} These processes are characterized by accumulation of interstitial fibroblasts, deposition of extracellular matrix (ECM), and loss of renal tubule epithelial cells, which collectively lead to end-stage renal failure.³ Previous studies indicate that the tubular epithelial cells undergoing epithelial-to-mesenchymal transition (EMT) contribute to one-third of myofibroblast during kidney fibrosis.^{4,5} Myofibroblasts are fully differentiated fibroblasts which are α -smooth muscle actin (α -SMA)-positive cells and the main source for ECM. Thus, myofibroblasts can be used as prognostic indicators for disease progression.⁶ In contrast, several studies indicate that such event of EMT in kidney fibrosis could be reversed by growth factors, such as hepatocyte growth factor and bone morphogenetic protein-7.^{7,8}

In addition to organ fibrosis, recent studies indicated the pivotal role of EMT in pathological progression, including cancer metastasis and wound healing.^{9–11} There are two major cellular processes in EMT; one is the loss of epithelial cell polarity. This phenomenon can be manifested by membrane proteins involved in cell polarization and the reduction of cohesive interactions, which mediated by expression of various adherent molecules, such as E-cadherin. The other process is the acquisition of

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mesenchymal cell characteristics, such as enhanced cell motility, contractility or even invasive ability, and deposition of ECM.^{12,13} Many cytokines and growth factors are involved in EMT. Nevertheless, transforming growth factor- β 1 (TGF- β 1) and its downstream Smad-dependent signaling are the major pathways that trigger EMT both *in vitro* and *in vivo*.^{14,15} On binding of TGF- β 1 to its receptor, TGF- β 1 induces receptor autophosphorylation, which in turns activates the downstream R-Smad, Smad2, and Smad3. The activated R-Smads form complex with Smad4, and this complex translocates into nucleus and binds to specific promoter sequence.¹⁶ Smad2 and Smad3 control different target gene expression in response to TGF- β 1. It has been documented that Smad3 mediates most of the gene induction related to TGF- β 1-induced EMT, particularly during kidney fibrosis.^{17,18}

ECM signaling mediated by integrins is important in regulating growth factors signaling, including epidermal growth factor, hepatocyte growth factor, and vascular endothelial growth factor.¹⁹ Among these integrin family members, β ₁ integrin is the most critical one, given that β ₁ integrin can pair with different α subunits that make it become a receptor for many types of ECM component. The expression of β ₁ integrin is ubiquitous in different tissues.²⁰ Knockout of β ₁ integrin in mice resulted in embryonic lethal because of the defect in gastrulation and organ formation.^{21,22} Recent studies have demonstrated that inhibition of β ₁ integrin signal impairs TGF- β 1 downstream signaling and epithelial plasticity in mammary gland epithelial cells.²³ Blocking of integrin downstream signaling including integrin-link kinase (ILK) and focal adhesion kinase (FAK) can also reduce TGF- β 1-induced up-regulation of mesenchymal markers.^{24,25} However, the role of β ₁ integrin in renal fibrosis has not been studied. In addition, although studies have shown that TGF- β 1 induces the gene expression of β ₁ integrin in both mammary and mesangial cells,^{26,27} the molecular mechanism whereby TGF- β 1 up-regulates gene expression of β ₁ integrin is still unclear.

In this study, we demonstrate that TGF- β 1 up-regulates β ₁ integrin gene expression in different types of epithelial cell through transcriptional regulation, and β ₁ integrin gene expression is critical for TGF- β 1-induced EMT. Furthermore, Smad3 and the Smad-binding element on β ₁ integrin promoter sequence are responsible for TGF- β 1-induced β ₁ integrin gene expression. The *in vivo* study also shows the up-regulation of β ₁ integrin during kidney fibrosis. β ₁ integrin and coexpressed with α -SMA in both mouse model and human samples. Furthermore, blocking of β ₁ integrin signal alleviates unilateral ureteral obstruction (UUO)-induced renal fibrosis.

Materials and Methods

Cell Culture and Treatment

LLC-PK1, Madin-Darby canine kidney, HaCaT, and NMuMG cells were cultured in Dulbecco's modified minimal essential medium supplemented with 10% FBS. For experiments, 8×10^5 cells per 10-cm dish were plated

and cultured in medium containing 10% FBS for 24 hours. After that, cells were treated recombinant TGF- β 1 (Pepro-Tech, London, UK) in serum free medium at the indicated time point. For β ₁ integrin blocking experiments, cells were pretreated with blocking antibody 4B4 (Beckman Coulter, Fullerton, CA) at the dosage of 10 μ g/ml for 30 minutes, followed by incubating with 10 ng/ml TGF- β 1 for another 48 hours.

Western Blotting

Western blot was performed according to the established procedure.^{28,29} The monoclonal antibodies against β ₁ integrin, E-cadherin, and fibronectin were purchased from BD Biosciences (San Jose, CA). Antibodies against integrins α ₁, α ₂, α ₅, and α _v were all purchased from Millipore (Billerica, MA). Polyclonal antibody against p-Smad3 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against α -SMA and Smad3 were purchased from Sigma-Aldrich (St. Louis, MO) and Zymed Laboratories (South San Francisco, CA), respectively.

RT-PCR

Total RNA from cultured cells was extracted with the RNeasy Mini kit (Qiagen; Hilden, Germany). Total RNA from each mouse kidney was isolated by using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA). For RT-PCR, first-strand cDNA was synthesized from 0.2 μ g of total RNA with an oligo-dT primer and the Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). The sequences of PCR primers were as follows: forward primer (5'-CGGGAGAAAATGCTC-CAATA-3') and reverse primer (5'-CACACTCAAACGTC-CCATTG-3') were designed from *Sus scrofa* β ₁ integrin (CD29) (NCBI accession number NM_213968), and the resulting PCR product was 227 bp. Forward primer (5'-GCCAGGGCTGGTTATACAGA-3') and reverse primer (5'-TCACAATGGCACACAGGTTT-3') were designed from *Mus musculus* β ₁ integrin (CD29) (NCBI accession number NM_010578), and the resulting PCR product was 226 bp. Forward primer (5'-ATACGCCTGAGTGGCT-GTCT-3') and reverse primer (5'-TCTCTGTGGAGCT-GAAGCAA-3') were designed from *Mus musculus* TGF- β 1 (Tgfb1) (NCBI accession number NM_011577), and the resulting PCR product was 300 bp.

Immunofluorescence

For immunofluorescence studies, cells were fixed with 4% paraformaldehyde in PBS, and cell membrane was permeabilized using 0.5% Triton X-100. These samples were blocked with commercial blocking solution (Thermal Scientific, Rockford, IL) at room temperature and then incubated with primary antibody, followed by incubation with fluorescence-conjugated secondary antibody (Invitrogen-Molecular Probes, Carlsbad, CA).³⁰ Antibodies against fibronectin and E-cadherin were purchased from BD Biosciences. Phalloidin conjugated with tetramethyl-

rhodamine isothiocyanate was purchased from Fluka (Steinheim, Germany). For tissue sections, 4- μ m tissue sections from paraffin or Tissue-Tek OCT compound-embedded were used for analyses. Tissue samples were fixed by acetone for 2 minutes followed by antigen retrieval with citrate acid buffer by microwave. Antibodies against β_1 integrin (BioLegend, San Diego, CA; clone HM β 1-1, Alexa 488), E-cadherin (BD Biosciences), TGF- β 1 (Santa Cruz Biotechnology), p-Smad3 (Santa Cruz Biotechnology), and α -SMA (Sigma-Aldrich) were used. Finally, images were taken by using the confocal microscope (FV-1000; Olympus, Melville, NY).

Short Hairpin RNA Inhibition

To knockdown β_1 integrin expression in LLC-PK1 epithelial cells, 19-mer short hairpin RNA (shRNA) against *Sus scrofa* β_1 integrin expressed in pSUPER vector was synthesized. Sequences for β_1 integrin are 5'-GTGCTCAGCCTTACT-GATA-3' and 5'-TATCAGTAAGGCTGAGCAC-3'. Two mouse Smad3 shRNAs purchased from GenDiscovery Biotechnology (Taipei, Taiwan) (catalog number RMM4431-98765463 and RMM4431-99202964, labeled as numbers 1 and 2, respectively) were used. These clones were expressed in GPLz expression vector (Open Biosystems, Huntsville, AL). One microgram of specific or control shRNA duplexes were transiently transfected into LLC-PK1 cells by using the Arrest-In transfection reagent (Open Biosystems). After transfection, cells were trypsinized and subjected to various experiments.

Plasmid Construction, Transfection, and Reporter Assay

The β_1 integrin promoter (nt -1057 to + 77) was cloned from mouse kidney using PCR amplification; the primer sequence for cloning was 5'-TCCCTCCTCAAGTCA-CACG-3' and 5'-GCTTCTCGGTTGGTCTCG-3'. The promoter sequence was conjugated into pGL3-basic vector by using the NheI and BglII restriction enzyme sites. The identification of the transcriptional factor binding site of β_1 integrin promoter sequence was made by the online software PROMO 3.0 (http://algggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3). The GACA core binding element on Smad-binding element (SBE) in β_1 integrin promoter from -172 to -181 was mutated using PCR amplification with the following primer: 5'-AAAGAC-CGAGCTCCTTTGAAAACACCCTGCCCCACCTTCC-3' and 5'-GGAAGGTGGGGCAGGGTGTTTCAAAGGA-GCTCGGTCGTT-3'. The promoter activity assay was previously described³¹ with modification. The underlined nucleotide sequences were made for generating point mutation in SBE. Briefly, NMuMG cells were cotransfected with reporter plasmids and the β -galactosidase plasmid, used as internal control reporter (provided by Dr. Shaw-Jenq Tsai, National Cheng Kung University, Taiwan, People's Republic of China). Luciferase assay was performed using the Dual Luciferase Assay System kit (Promega) per manufacture's protocol. Relative luciferase activity (arbitrary

unit) was reported after normalizing with transfection efficiency.

Chromatin Immunoprecipitation

The DNA-protein complexes were fixed using 1% formaldehyde at room temperature for 10 minutes at indicating time period. The ice-cold glycine was performed to block the cross-link reaction. Cell lysates were collected and the chromatin was sheared to 200 to 1000 bp by using sonication. Same amount of DNA was used for immunoprecipitation by incubating with anti-Smad3 antibody (Santa Cruz Biotechnology). After reversion of the cross-linking of DNA and protein, the DNA was subject to PCR amplification using primers specific for amplifying regions corresponding to SBE (nt -172 to -181). The DNA was subject to a first round of PCR amplification using the outer primers (5'-GACTGCGGCTGGGCTTTC-3' and 5'-AGCGAGT-GGGCGGAGACT-3') for 20 cycles. The cDNA was then diluted (1/1000) with water and subject to second round of amplification using nested primers (5'-GACTGCG-GCTGGGCTTTC-3' and 5'-AGGCGGCAGGAAGA-AGGA-3') for 30 cycles.

UUO Animals and β_1 Integrin Antibody Treatment

Procedures involving animal subjects have been proved by the Institutional Animal Care and Use Committee at the National Cheng-Kung University (Tainan, Taiwan). One-month-old male C57BL/6 mice, weighing ~20 to 25 g, were obtained from the animal center in National Cheng-Kung University. UUO was performed with an established procedure, as described previously.³² After UUO surgery, mice were sacrificed at various time points, and kidneys were removed. Part of kidney was fix by 4% paraformaldehyde and embedded either in paraffin or in Tissue-Tek OCT compound, respectively. The rest parts of kidney were divided and lysed for either protein or RNA analysis.

For α_5 or β_1 integrin functional blocking experiments, 60 μ l of α_5 integrin blocking antibody (clone eBioHMa5-1; eBioscience, San Diego, CA) or β_1 integrin blocking antibody (3 mg/kg body weight) (clone HM β 1-1, LEAF; BioLegend) was injected into the parenchyma of UUO kidney immediately right after the ureteral obstruction. The same amount of PBS was injected as a control. After 7 days, mice were scarified, and the kidneys were harvested for further analysis.

Human Kidney Specimens

Three delinked human kidney specimens obtained from tissue bank of National Cheng Kung University Hospital were immunohistochemically investigated for the presence of marker for interstitial fibrosis and β_1 integrin. All three patients were diagnosed clinically as chronic kidney diseases (their urine protein/creatinine ratios were all <1, and serum creatinine levels were 4.1, 3.5, and 2.4 mg/dl, re-

Figure 1. TGF-β1 up-regulates β₁ integrin expression. **A:** TGF-β1 induced changes of EMT proteins in LLC-PK1 cells. LLC-PK1 cells were cultured with or without 10 ng/ml TGF-β1 at the indicated times, and protein levels were assessed by Western blot analysis. **B:** LLC-PK1 cells were treated with or without 10 ng/ml TGF-β1 at the indicated times. Cells were harvested, and the levels of β₁ integrin mRNA were analyzed by RT-PCR. **C:** LLC-PK1 cells were treated with or without 10 ng/ml TGF-β1 in the presence or absence of different doses (0.5 or 1 μg/ml) of actinomycin D (Act. D) for 16 hours. Cells were harvested, and their levels of β₁ integrin RNA levels were assessed by RT-PCR. **D:** LLC-PK1 cells treated with 10 ng/ml TGF-β1 for 24 hours were harvested. Protein levels, including α₁, α₂, α₅, α_v, and β₁ integrin, were subjected to Western blot analysis. **E and F:** Epithelial cells, including LLC-PK1, Madin-Darby canine kidney (MDCK), and NMuMG (**E**), and renal fibroblast cells, NRK49F (**F**), were incubated with 10 ng/ml TGF-β1 for 24 hours, and protein abundance of β₁ integrin was assessed by Western blot.

spectively), and renal biopsies were performed for the definite pathological diagnosis. Two patients were diagnosed as chronic and moderate tubulointerstitial fibrosis where the other patient was diagnosed as moderate tubulointerstitial fibrosis and mild mesangial proliferation with small amount of IgA deposition. The distributions of β₁ integrin were stained by anti-human β₁ integrin antibody, 4B4, followed by secondary antibody conjugated with Alexa 488.

Statistical analyses

All results were expressed as mean ± SEM and were analyzed by using one-way analysis of variance (analysis of variance) using GraphPad Prism version 3.0 (GraphPad, San Diego, CA). Tukey's procedure was used to test the differences between individual treatment groups. Differences in comparison were considered as statistically significant when $P < 0.05$.

Results

TGF-β1 Induced Up-Regulation of β₁ Integrin before EMT

To explore the mechanism of TGF-β1-induced EMT, we first examined the changes of β₁ integrin, fibronectin and

E-cadherin in TGF-β1-treated LLC-PK1 cells. On TGF-β1 stimulation, β₁ integrin was up-regulated within 4 hours, whereas fibronectin and α-SMA levels were up-regulated and E-cadherin was down-regulated within 24 hours (Figure 1A). TGF-β1 treatment induced an increase not only in premature β₁ integrin (~110 kDa), but also glycosylated β₁ integrin (~130 kDa), consistent with the previous report.³³ The quantitative results showed that TGF-β1 markedly augmented β₁ integrin levels from 4 to 16 hours and the increase was maintained at 2.5- to 3-fold of control between 24 and 72 hours. In contrast, TGF-β1-triggered alterations of fibronectin, E-cadherin and α-SMA protein levels started at 24 hours (Supplemental Figure 1A, see <http://ajp.amjpathol.org>). To understand the mechanism of TGF-β1-induced up-regulation of β₁ integrin, levels of β₁ integrin mRNA were assessed by RT-PCR. The mRNA levels of β₁ integrin increased within 4 hours, consistent with protein level changes on TGF-β1 stimulation (Figure 1B and Supplemental Figure 1B, see <http://ajp.amjpathol.org>). This result suggests that transcriptional regulation could be involved. To test this possibility, LLC-PK1 cells were treated with TGF-β1 and different concentrations of actinomycin D, 0.5 or 1 μg/ml, for 16 hours. As shown in Figure 1C, TGF-β1-induced augmentation of β₁ integrin mRNA levels was inhibited by actinomycin D in a dose-dependent manner. These data

indicate that TGF- β 1 up-regulates β ₁ integrin through transcriptional regulation.

To examine whether other integrins were regulated by TGF- β 1, the protein levels of α ₁, α ₂, α ₅, and α _v integrin in LLC-PK1 cells treated with TGF- β 1 for 24 hours were assessed. TGF- β 1 up-regulated α ₂ and α _v integrins moderately but not α ₁ and α ₅ integrin protein levels (Figure 1D). Because the protein level changes of β ₁ integrin was more significant on TGF- β 1 stimulation, we then focused on the regulation and function of β ₁ integrin. Next, we examined whether TGF- β 1-induced up-regulation of β ₁ integrin could be observed in different epithelial cell lines, including LLC-PK1, Madin-Darby canine kidney, and NMuMG cells, as well as in renal fibroblast cells (NRK49F). The results show that TGF- β 1-induced up-regulation of β ₁ integrin is a general phenomenon in these epithelial and renal fibroblast cells examined (Figure 1, E and F).

β ₁ Integrin Signal Is Required for TGF- β 1-Induced EMT

Although it had been shown that β ₁ integrin signal is required for TGF- β 1-triggered loss of epithelial plasticity in mammary gland epithelial cells, the functions of β ₁ integrin in renal epithelial cells during EMT has never been reported. Here, we used β ₁ integrin specific blocking antibody, 4B4, to examine the role of β ₁ integrin in TGF- β 1-triggered EMT in renal epithelial cells. 4B4 treatment alone did not affect the protein abundances of fibronectin, E-cadherin, or α -SMA. However, 4B4 treatment significantly blocked TGF- β 1-induced up-regulation of fibronectin and α -SMA and down-regulation of E-cadherin (Figure 2, A and B). Consistent with the results shown in Western blot analysis, 4B4 treatment attenuated TGF- β 1-triggered decrease in E-cadherin and increase in fibronectin and α -SMA staining (Figure 2C). Moreover, 4B4 treatment blocked TGF- β 1-induced spindle shape and stress fiber formation, which are the characteristics of mesenchymal cells.

Usually, fibrosis is accompanied with fibril collagen deposition and α ₂ integrin is the major mediator for collagen signaling. To examine whether α ₂ β ₁ integrin heterodimer was required in TGF- β 1-induced EMT, we used the blocking antibody, 5E8.³⁴ The result showed that blocking of α ₂ β ₁ integrin prevented TGF- β 1-induced up-regulation of fibronectin and α -SMA and down-regulation of E-cadherin (Supplemental Figure 2, see <http://ajp.amjpathol.org>). These results indicate α ₂ β ₁ integrin signal is critical for TGF- β 1-triggered EMT in renal epithelial cells.

Knockdown of β ₁ Integrin Prevents TGF- β 1-Induced EMT

To examine whether protein abundance of β ₁ integrin was important in TGF- β 1-induced EMT, shRNA against *Sus scrofa* β ₁ integrin was performed. RT-PCR result

showed that β ₁ integrin shRNA reduced both mRNA and protein levels of β ₁ integrin compared with scramble shRNA transfection group. Knockdown of β ₁ integrin blocked TGF- β 1-induced up-regulation of fibronectin and α -SMA and down-regulation of E-cadherin (Figure 3A). The data suggest that induction of β ₁ integrin is required for TGF- β 1-triggered EMT.

Next, we examined whether the overexpression of β ₁ integrin alone triggered EMT. HaCaT cells stably expressed with β ₁ integrin were used. On TGF- β 1 stimulation, β ₁ integrin protein level increased within 48 hours (Figure 3B), further suggesting that TGF- β 1-up-regulated β ₁ integrin expression is a general phenomenon in epithelial cells. In addition, TGF- β 1 treatment resulted in the up-regulation of fibronectin and down-regulation of E-cadherin in HaCaT cells, which could also be observed in cells transfected with constitutively active β ₁ integrin (Ca β), clone numbers 1 and 2 (Figure 3B). Immunofluorescence study showed that both TGF- β 1 treatment and overexpression of Ca β induced mesenchymal cell morphology, enhanced fibronectin expression, and reduced E-cadherin levels (Figure 3C). These results indicate that forced increase in β ₁ integrin signal alone accelerates EMT progression.

TGF- β 1 Induces β ₁ Integrin Up-Regulation through Smad3-Dependent Pathway

To identify which signaling pathway was involved in TGF- β 1-induced β ₁ integrin up-regulation, phosphorylation levels of Smad3, Erk, Akt, and p38 were assessed on TGF- β 1 stimulation. TGF- β 1 treatment induced a significant increase of phosphorylation in Smad3, but not Erk, Akt, or p38, within 30 minutes and sustained to 4 hours in both LLC-PK1 and NMuMG cells (data not shown). To further verify the role of Smad3 in TGF- β 1-induced β ₁ integrin gene expression, two different shRNAs against *mus musculus* Smad3 were used, numbers 1 and 2 (Figure 4A). Knockdown of Smad3 suppressed TGF- β 1-induced β ₁ integrin protein and mRNA up-regulation in a dose-dependent manner (Figure 4, A and B). These data indicate that Smad3 is required for TGF- β 1-induced β ₁ integrin up-regulation.

TGF- β 1-Triggered β ₁ Integrin Promoter Activity Is Smad3 Dependent

Because Smad3 is responsible for most of the genes triggered by TGF- β 1,^{17,18} we were wondering whether there was SBE on β ₁ integrin promoter. Using bioinformatics analysis, we have discovered one Smad3 and Smad4 binding element at 182 bp upstream of the transcription start site on mouse β ₁ integrin promoter (Figure 5A). We also analyzed the promoter sequence among different species and found that SBE on the β ₁ integrin promoter within 1 kbp in several species, including mouse, rat, dog, and porcine. We then cloned β ₁ integrin promoter sequence of 1134 bp from mouse kidney genomic DNA into pGL3-Luc plasmid and tested

Figure 2. Blocking of β_1 integrin signal inhibits TGF- β 1-induced EMT. **A:** LLC-PK1 cells were pretreated with or without 10 μ g/ml 4B4, β_1 integrin-specific blocking antibody, for 30 minutes. Cells were cultured with or without 10 ng/ml TGF- β 1 in the presence or absence of 4B4 for another 48 hours, and protein levels were assessed by Western blot. **B:** Quantitative results show the effects of 4B4 on the inhibition of TGF- β 1-induced changes in EMT marker protein abundance, including E-cadherin, α -SMA, and fibronectin. Each bar represents the mean $\pm$ SE from three independent experiments. * P < 0.05; ** P < 0.01; and *** P < 0.001. **C:** LLC-PK1 cells treated with TGF- β 1 in the presence or absence of 4B4 for 48 hours were fixed and immunostained with mouse monoclonal antibodies against E-cadherin, fibronectin, or α -SMA, and the immunocomplex was detected by anti-mouse Alexa-488 (green). F-actin was stained by phalloidin conjugated with tetramethylrhodamine isothiocyanate (red), and nuclei were stained with Hoechst 33258 (blue).

Figure 3. β_1 integrin is required for TGF- β 1-induced EMT. **A:** LLC-PK1 cells transiently transfected with scramble or β_1 integrin shRNA were incubated with or without 10 ng/ml TGF- β 1 for 48 hours. Cells lysates and RNA were harvested and analyzed by Western blot or RT-PCR, respectively. **B, left:** HaCaT cells were treated with or without 10 ng/ml TGF- β 1 for 48 hours, and protein levels were assessed by Western blot analysis. **B, right:** HaCaT cells were stably transfected with constitutively active β_1 integrin (Ca β), and protein levels in vector control cells (mock) and Ca β -overexpressing clones, numbers 1 and 2, were assessed. **C:** Expression of E-cadherin, fibronectin, and actin cytoskeleton was examined in HaCaT cells treated with or without TGF- β 1 for 48 hours or overexpressed with Ca β integrin. Mouse monoclonal antibodies against E-cadherin (upper panels) and fibronectin (lower panels) were detected by anti-mouse Alexa-488 (green). F-actin was stained with phalloidin conjugated with tetramethylrhodamine isothiocyanate (red), and nuclei were stained with Hoechst 33258 (blue).

Figure 4. Knockdown of Smad3 reduces β_1 integrin expression induced by TGF- β 1. NMuMG cells transiently transfected with scramble or two different Smad3 shRNAs (numbers 1 and 2) were cultured in the presence or absence of 10 ng/ml TGF- β 1 for 24 hours. Cells transfected with scramble shRNA were used as a control. **A:** Protein levels of Smad3 and β_1 integrin were assessed by Western blot analysis. **B:** Cells subjected to RNA isolation were used for β_1 integrin mRNA levels analyzed by RT-PCR.

whether TGF- β 1 up-regulated β_1 integrin promoter activity. NMuMG cells transfected with β_1 integrin promoter-driven luciferase showed basal activation levels, whereas TGF- β 1 treatment increased promoter activity in a dose-dependent manner (Figure 5B). To examine the role of Smad3 in TGF- β 1-induced up-regulation of β_1 integrin promoter activity, NMuMG cells were cotransfected Smad3 shRNAs, numbers 1 or 2, and β_1 integrin promoter. The results showed that knockdown of Smad3 decreased TGF- β 1-induced β_1 integrin promoter activity (Figure 5C). These results indicate that Smad3 is required for TGF- β 1-induced β_1 integrin gene expression.

Although bioinformatics analysis and above results suggest that SBE is present on β_1 integrin promoter sequence, the direct evidence of binding of Smad3 to β_1 integrin promoter remains unclear. To delineate whether SBE is responsible for TGF- β 1-triggered β_1 integrin promoter activity, mutation of SBE by site-directed mutagenesis was made. The results showed that SBE mutant markedly reduced TGF- β 1-triggered β_1 integrin promoter activity from eight- to threefold of control (Figure 5D), suggesting that the predicted SBE on β_1 integrin promoter is critical for TGF- β 1-triggered β_1 integrin gene regulation. To further examine whether Smad3 directly binds to β_1 integrin promoter on TGF- β 1 stimulation, we used chromatin immunoprecipitation assay. The results showed that TGF- β 1 induced Smad3 binding to the SBE region on β_1 integrin promoter within 1 to 8 hours (Figure 5E). Quantitative analysis demonstrated that TGF- β 1 augmented the interactions of Smad3 with β_1 integrin promoter (Figure 5F). Taken together, we conclude that TGF- β 1 stimulation triggers physical interaction of Smad3 with SBE of β_1 integrin promoter to enhance gene regulation.

UUO Induces Up-Regulation of β_1 Integrin in Renal Tubular Epithelial Cells during Kidney Fibrosis

Although *in vitro* studies indicate the important role of β_1 integrin in TGF- β 1-induced EMT, little is known about the role of β_1 integrin in the pathogenesis of renal fibrosis. To examine the role of β_1 integrin in the development of kidney fibrosis, we used UUO. Results of Western blot analysis showed that β_1 integrin protein levels increased

Figure 5. TGF- β 1-induced increase of β_1 integrin promoter activity is Smad3 dependent. **A:** Schematic representation of the β_1 integrin promoter sequence shows predicted binding sites for Smad3 and Smad4. The 1134-bp β_1 integrin promoter sequence was cloned from normal mouse kidney. The Smad-binding element mutant (Mut) with core binding element CAGA mutated to TTTT was generated using a point mutagenesis kit. **B:** NMuMG cells transfected with control vector (p-GL3 basic) or wild-type β_1 integrin promoter (β_1 WT) along with a β -galactosidase plasmid were cultured in serum-free media in the presence or absence of different doses of TGF- β 1 (1, 5, or 10 ng/ml) for 24 hours. The β_1 integrin promoter activities were assessed by luciferase assay. **C:** NMuMG cells co-transfected with WT- β_1 integrin promoter (β_1 WT) and different Smad3 shRNAs, number 1 or 2, or scramble shRNA were treated with or without 10 ng/ml TGF- β 1 for 24 hours. **D:** NMuMG cells transfected with wild-type β_1 integrin promoter (β_1 WT) or the mutant form of the β_1 integrin promoter (β_1 Mut) were cultured with or without 10 ng/ml TGF- β 1 for 24 hours and then subjected to promoter activity assay. The β_1 integrin promoter activities were assessed by luciferase assay, and the results were normalized by β -galactosidase activity levels (Luc./ β -gal). *** P < 0.001. **E:** Representative results of chromatin immunoprecipitation (ChIP) assay show that TGF- β 1 enhanced the interactions between Smad3 and the β_1 integrin promoter sequence in NMuMG cells. NMuMG cells were cultured in the presence or absence of 10 ng/ml TGF- β 1 for the indicated times. Cell lysates were immunoprecipitated with monoclonal anti-Smad3 antibody. The immunoprecipitated DNA was subjected to PCR amplification using primers specific for SBE on the β_1 integrin promoter sequence. **F:** Quantitative results of the relative intensity of the PCR product of the β_1 integrin promoter as assessed by ChIP in NMuMG cells treated with TGF- β 1 for the indicated times. Each bar represents the mean $\pm$ SE of three independent experiments. * P < 0.05; ** P < 0.01.

by sixfold in UUO kidney within 3 days, accompanied with the up-regulation of α -SMA and down-regulation of E-cadherin at the same time (Figure 6A). Since up-regulation of TGF- β 1 plays an important role in initiation of renal fibrosis, we assessed the time course of mRNA

Figure 6. β_1 integrin is enhanced in UUO-induced fibrotic kidney. **A:** Mice with UUO were sacrificed at 3, 7, 14, or 21 days, and the sham group was used as a surgery control. The protein levels of β_1 integrin, E-cadherin, and α -SMA were assessed by Western blot analysis of the contralateral kidney (C) or UUO kidney (U). **B:** Kidney samples from mice with UUO for 1, 3, and 7 days were subjected to RT-PCR analysis. The mRNA levels of TGF- β 1 and β_1 integrin were analyzed by specific primers. To rule out contamination by genomic DNA, four samples subjected to RT-PCR without addition of reverse transcriptase (MMLV) enzyme (-RT) were used as a negative control. **C** and **D:** Kidney samples from mice with UUO for 7 days were subjected to double-staining with p-Smad3 and β_1 integrin (**C**) or α -SMA and β_1 integrin (**D**). Tissue sections were incubated with β_1 integrin monoclonal antibody conjugated with Alexa-488 (green) and anti-p-Smad3 antibody, followed by anti-rabbit Alexa-594 (red). Nuclei were stained with Hoechst 33258 (blue). Monoclonal antibody against α -SMA was used followed by anti-mouse Alexa-594 (red). Stars indicated renal tubules with positive β_1 integrin and α -SMA staining.

levels of TGF- β 1 and β_1 integrin. We found that there was an increase in both TGF- β 1 mRNA and β_1 integrin mRNA levels within 1 day of UUO (Figure 6B). Immunofluorescence study was used to detect the alterations of TGF- β 1 and β_1 integrin in UUO for 7 days. In control kidney, the intensity of TGF- β 1 and β_1 integrin in tubule epithelium was relatively weak, whereas the staining of β_1 integrin was high in glomeruli. After UUO, the intensity of TGF- β 1 was markedly enhanced in both glomerulus and tubular epithelial cells, consistent with the previous report.³² The β_1 integrin staining was significantly augmented in renal tubular epithelial cells as well as cortical interstitium (Supplemental Figure 3, see <http://ajp.amjpathol.org>). These results highlight the spatial correlations of TGF- β 1 and β_1 integrin during kidney fibrosis.

To examine the signaling events triggered by TGF- β 1 in UUO, we analyzed the activation levels of Smad3. Interestingly, the up-regulation of both Smad3 protein and p-Smad3 was present in UUO kidney within 1 day, which was associated with the up-regulation of β_1 integrin protein levels (Supplemental Figure 4, A and B, see <http://ajp.amjpathol.org>). To further confirm the signaling cascade of TGF- β 1/p-Smad3/ β_1 integrin gene expression, double-staining of p-Smad3 and β_1 integrin was performed. The results indicated in UUO kidney that the

fluorescence intensity of p-Smad3 in nuclei was increased and associated with the up-regulation of β_1 integrin staining (Figure 6C). These results highlight the importance of Smad3-dependent pathway in TGF- β 1-induced up-regulation of β_1 integrin during kidney fibrosis.

As shown in Figure 6D, normal renal tubule epithelial cells exhibited cuboid cell shape and little immunostaining of α -SMA, whereas UUO triggered these cells to exhibit mesenchymal cell shape with markedly increased α -SMA staining. In addition, expansion of the interstitium with enhanced α -SMA staining could also be observed in UUO kidney (Figure 6D). Interestingly, the tubule epithelial cells exhibiting α -SMA staining also showed strong staining of β_1 integrin, supporting the correlation of β_1 integrin with EMT in renal epithelium during renal fibrosis.

Correlation of α -SMA Expression with β_1 Integrin Expression in Human Kidney with Chronic Tubulointerstitial Fibrosis

It is well established that there is enhanced expression of α -SMA in patients with chronic tubulointerstitial fibrosis.⁶ However, the expression pattern of β_1 integrin in kidney

of chronic tubulointerstitial fibrosis was not clear. Here we examined the expression of β_1 integrin and α -SMA in human kidney sample from patients with chronic tubulointerstitial fibrosis. The results showed that in regions with severe interstitial fibrosis, both α -SMA and β_1 integrin were highly expressed in interstitial cells, particularly α -SMA. More importantly, dilated tubular epithelial cells also showed strong staining of α -SMA and β_1 integrin (Figure 7A). Statistical analysis revealed that there was a significant correlation between expression levels of β_1 integrin and α -SMA. This result suggests the correlation of β_1 integrin with EMT during renal fibrosis (Figure 7B).

Blocking of β_1 Integrin Alleviates Renal Fibrosis

To understand the role of β_1 integrin in the pathophysiology of renal fibrosis, we used β_1 integrin blocking antibody in UUO mice. After the injection of β_1 integrin blocking antibody and concomitant with UUO treatment for 7 days, the protein levels of E-cadherin and α -SMA were assessed. Western blot analysis showed that blocking of β_1 integrin signal alleviated down-regulation of E-cadherin and up-regulation of α -SMA induced by UUO within 7 days (Figure 8, A and B). Histological studies showed that the β_1 integrin blocking antibody reduced interstitial expansion and tubular dilation (Figure 8C, upper panel). In addition, the Sirius red staining showed that blocking of β_1 integrin signal also reduced UUO-induced collagen deposition (Figure 8C, lower panel). These studies demonstrate the critical role of β_1 integrin signal in the development of renal fibrosis.

The immunofluorescence staining was used to further examine the effects of β_1 integrin blocking antibody on the prevention of EMT during renal fibrosis. As shown in Figure 8D, E-cadherin staining, localized in the basal-lateral site of tubular epithelial cells in control kidney, was markedly reduced in UUO kidney. Blocking of β_1 integrin reverted UUO-triggered down-regulation of E-cadherin in tubule epithelial cells (Figure 8D) and alleviated UUO-triggered augmentation of α -SMA staining in both renal epithelium and interstitium (Figure 8E). To further examine the specificity of β_1 integrin blocking antibody, we also tested the effects of α_5 integrin blocking antibody, namely CD49e, on UUO kidney. The Western blotting results showed that injection of CD49e did not rescue UUO-induced down-regulation of E-cadherin and up-regulation of α -SMA (Supplemental Figure 5, A and B, see <http://ajp.amjpathol.org>). The immunofluorescence staining also demonstrated that blocking of α_5 integrin signal did not affect UUO-induced interstitial fibrosis, tubular atrophy, down-regulation of E-cadherin staining (Supplemental Figure 5C, see <http://ajp.amjpathol.org>), or up-regulation of α -SMA staining (Supplemental Figure 5D, see <http://ajp.amjpathol.org>). In summary, although previous study has implicated the involvement of α_5 integrin in TGF- β 1 and UUO-induced EMT,³⁵ our studies reported here show that blocking of α_5 integrin does not prevent UUO-induced renal fibrosis. Taken these results together, β_1 integrin is an important target for alleviation of chronic interstitial fibrosis.

Discussion

TGF- β 1-triggered EMT has been a key issue in pathogenesis of tissue or organ fibrosis. In kidney, TGF- β 1-induced EMT not only results in accumulation of interstitial fibroblasts but also leads to kidney function impairment. However, the molecular mechanisms whereby TGF- β 1 induces EMT have not been fully understood. In this study, we demonstrate that augmented expression of β_1 integrin, a very critical ECM receptor, plays important roles in TGF- β 1-induced EMT both in *in vitro* and *in vivo*. In addition, we also elucidate the molecular mechanisms whereby TGF- β 1 up-regulates β_1 integrin gene expression. Blocking of β_1 integrin signal prevented TGF- β 1-induced down-regulation of E-cadherin and up-regulation of fibronectin and α -SMA in renal tubular epithelial cells. Results from bioinformatics search showed a Smad3/4 binding element on β_1 integrin promoter sequence and knockdown of Smad3 suppressed TGF- β 1-induced β_1 integrin up-regulation. This is the first evidence to demonstrate that the Smad-dependent pathway is responsible for TGF- β 1-induced β_1 integrin gene regulation. These observations indicate that β_1 integrin not only acts as a pivotal mediator of TGF- β 1-induced EMT but also serves as an accelerator for mesenchymal transition. Results from our *in vivo* studies support the hypothesis that β_1 integrin plays essential role in the development of renal fibrosis. First, the increases of β_1 integrin mRNA in kidney appeared as early as 1 day after UUO. Second, β_1 integrin levels were enhanced not only in interstitium but also in tubular epithelium along with the augmentation of α -SMA. Finally, the injection of β_1 integrin blocking antibody significantly reduced renal fibrosis and EMT, as manifested by interstitial expansion, tubular dilation, collagen deposition, E-cadherin down-regulation, and α -SMA up-regulation.

Recently, a series of studies identified the important role of ILK and FAK in TGF- β 1-induced development of EMT. However, the blocking of both ILK and FAK activation could not completely inhibit TGF- β 1-induced EMT,^{24,25} suggesting that signals other than ILK and FAK were involved in TGF- β 1-induced EMT. In our study, inhibition of β_1 integrin either by blocking antibody or shRNA significantly blocked TGF- β 1-induced fibronectin and α -SMA expression and down-regulation and delocalization of E-cadherin. Overexpression of active β_1 integrin in epithelial cells not only lost epithelial phenotype but also resulted in higher fibronectin levels even in the absence of TGF- β 1. Because β_1 integrin blockade exerts a potent effects on inhibition of TGF- β 1-induced EMT, our data support the notion that β_1 integrin is one of the pivotal membrane proteins to orchestra downstream proteins involved in TGF- β 1-induced EMT. There are several downstream proteins mediating β_1 integrin signaling other than focal adhesion complex proteins, such as RhoA, and its downstream proteins, Rho kinase (ROCK) and myosin light chain (MLC).³⁶ It has been documented that Rho/ROCK/MLC proteins are important mediators in TGF- β 1-induced α -SMA expression in renal tubule epithelial cells.^{37,38} TGF- β 1 triggers activation of Rho/ROCK/MLC within 30 minutes, and this activation of MLC induces nuclear translocation of MRTF, a potent transcriptional coactivator for α -SMA gene regulation, to accelerate TGF- β 1-induced α -SMA expression within 24 hours.³⁹ In our study,

Figure 7. Expression of β₁ integrin and α-SMA in human fibrotic kidney. Three human kidney samples with chronic tubulointerstitial fibrosis were coimmunostained with anti-α-SMA antibody conjugated with Cy3 (red) and β₁ integrin antibody, followed by secondary antibody conjugated with Alexa-488 (green). Nuclei were stained with Hoechst 33258 (blue). **A:** Representative immunofluorescence images from three diseased kidneys. **B:** The fluorescence intensity of β₁ integrin and α-SMA was assessed by Olympus FV-1000 software. Five images of each section from each patient were taken for analysis. The correlation coefficient was calculated, $R^2 = 0.7733$ and these data showed significant correlation by using Pearson correlation analysis.

Figure 8. Blocking of β₁ integrin signal alleviates renal fibrosis. **A:** Western blot analysis shows the protein levels of E-cadherin and α-SMA in 7-day UO kidney in mice treated with or without β₁ integrin-blocking antibody (β₁ Ab). The contralateral kidneys were used as a control. **B:** Quantitative results of protein levels of E-cadherin (left) and α-SMA (right) in 7-day UO kidney with or without β₁ Ab are shown. Each bar represents the mean ± SE of four mice. * indicates $P < 0.05$ and *** indicates $P < 0.001$, compared with the control group. **C:** Hematoxylin and eosin (HE) staining (upper panel) and Sirius red staining (lower panel) of 7-day control and UO kidney treated with or without β₁ Ab. Application of β₁ Ab significantly reduced collagen deposition in the UO kidney. Representative immunofluorescence images show the localization of β₁ integrin, E-cadherin (**D**), and α-SMA (**E**) in 7-day control and UO kidney treated with or without β₁ Ab. Kidney specimens were immunostained with E-cadherin or α-SMA monoclonal antibody, followed by anti-mouse Alexa-594 (red), shown in **A** and **B**, respectively, and with β₁ Ab conjugated with Alexa-488 (green). Nuclei were stained with Hoechst 33258 (blue).

we found that ROCK and MLC inhibitors decreased not only α -SMA protein levels but also morphological changes induced by TGF- β 1. However, these inhibitors did not affect TGF- β 1-induced β ₁ integrin (data not shown). Since ROCK and MLC act at the downstream pathways of β ₁ integrin in TGF- β 1-induced EMT, they may not be better therapeutic targets for the treatment of EMT or renal fibrosis.

Studies reported here showed the first evidence on TGF- β 1-induced β ₁ integrin gene expression in renal epithelial cells, which played important roles in pathogenesis of chronic tubulointerstitial fibrosis. Although TGF- β 1 binds to its receptor, the binding complex recruits and activates Smad3, which in turn forms complex with Smad4. The complex translocates to the nucleus to control the target gene expression via binding to specific promoter sequence, SBE. In this study, we identified the presence of CAGA box on putative SBE of β ₁ integrin promoter. Studies on promoter activity by mutation of SBE, located between -181 to approximately -172 bp, showed that this mutant markedly decreased TGF- β 1-triggered promoter activity. These results indicate that SBE core binding element plays important roles in TGF- β 1-induced β ₁ integrin gene up-regulation. More importantly, chromatin immunoprecipitation assay demonstrated that TGF- β 1 treatment enhanced the interactions between Smad3 and β ₁ integrin promoter. Taken together, we conclude that Smad3 mediates β ₁ integrin promoter activity in response to TGF- β 1 stimulation.

It has been shown that the ECM signaling mediated by integrins is required for growth factor-regulated cellular response.^{40,41} In this study, we show the blocking of β ₁ integrin signal decreased TGF- β 1-induced EMT particularly in kidney epithelial cells; however, which α subunit that paired with β ₁ integrin to mediate TGF- β 1-induced EMT is still unclear. We found that TGF- β 1 treatment induced levels of α 2 subunit and blocking of α 2 β ₁ integrin signal completely blocked TGF- β 1-induced down-regulation of E-cadherin and up-regulation of fibronectin and α -SMA, which suggests the importance of α 2 β ₁ integrin. Previous studies showed that α 3 β ₁ integrin was required for TGF- β 1-regulated p-Smad2/p- β -catenin complexes formation through the interactions with TGF- β RI and E-cadherin in both lung and kidney epithelial cells.^{42,43} In α 3-null kidney epithelial cells and cells expressing mutant α 3 integrin, which was unable to interact with E-cadherin, failed to up-regulate mesenchymal marker proteins such as collagen type I, vimentin, and α -SMA, after TGF- β 1 stimulation. They also found the decrease of p-Smad2/p- β -catenin complex in these cells, suggesting the involvement of α 3 integrin in the formation of p-Smad2/p- β -catenin complex. Since the blocking of Smad3 or mutant SBE on β ₁ integrin promoter sequence could not completely block TGF- β 1-triggered promoter activity, it is possible that p-Smad2/p- β -catenin may be involved in TGF- β 1-regulated β ₁ integrin gene expression. Previous study also showed that E-cadherin-mediated cell-cell contact regulated integrins expression, including α 5 and β ₁.⁴⁴ These studies suggest that the cross talk between integrin and E-cadherin-mediated adherent junction is important in regulating TGF- β 1-induced EMT.

β ₁ integrin was shown to be involved in various cellular functions, including adhesion, proliferation, differentiation, and survival.⁴⁵ The role of β ₁ integrin in fibroblast migration, proliferation, and monocyte infiltration had also been reported.^{46–49} Although this study elucidated the mechanism in β ₁ integrin gene regulation triggered by TGF- β 1 in normal epithelial cells, it also implied the role of β ₁ integrin in fibroblast differentiation from both *in vitro* and *in vivo* evidence. Because we not only observed the up-regulation of β ₁ integrin in response to TGF- β 1 stimulation in NRK49F cells, the β ₁ integrin staining also increased in interstitial cells in both human sample and UUO model. Since the expansion of interstitium and development of EMT in renal tubule are two major events in the progression of renal fibrosis, the role of β ₁ integrin in renal fibroblasts could be comprehensive which highlight the possibility that β ₁ integrin could be considered as a therapeutic target for preventing renal fibrosis.

The signaling balance between different cytokines, such as TGF- β 1 and bone morphogenetic protein-7, in maintenance of tubule epithelial cell differentiation or development of EMT, plays very important role of pathogenesis of renal fibrosis. Under physiological conditions, the expression of integrins and adherens junctions is important for maintaining epithelium properties. The basal levels of integrin are critical for cell attachment, survival, and proliferation. From our data, the overproduction of cytokines, such as TGF- β 1, result in abnormal increase in β ₁ integrin induction, which triggers expression of its ligands, ie, fibronectin and collagen. The increase in fibronectin and collagen matrix may subsequently trigger β ₁ integrin activation and results in a positive loop of augmentation of matrix deposition and EMT. In addition, TGF- β 1 induces the increase of ILK and PINCH-1, and activation of FAK and Rho/ROCK/MLC pathways, that are all considered the downstream signal proteins of β ₁ integrin. Thus, TGF- β 1-induced increase in β ₁ integrin is considered the most critical step for TGF- β 1-induced EMT. Our studies both *in vitro* and *in vivo* strongly support the role of β ₁ integrin in the pathogenesis of renal fibrosis progression.

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References

1. Kalluri R, Neilson EG: Epithelial-mesenchymal transition and its implications for fibrosis. *J Clin Invest* 2003, 112:1776–1784
2. Zeisberg M, Kalluri R: The role of epithelial-to-mesenchymal transition in renal fibrosis. *J Mol Med* 2004, 82:175–181
3. Liu Y: Epithelial to mesenchymal transition in renal fibrogenesis: pathologic significance, molecular mechanism, and therapeutic intervention. *J Am Soc Nephrol* 2004, 15:1–12
4. Iwano M, Plieth D, Danoff TM, Xue C, Okada H, Neilson EG: Evidence

- that fibroblasts derive from epithelium during tissue fibrosis. *J Clin Invest* 2002, 110:341–350
5. Strutz F, Okada H, Lo CW, Danoff T, Carone RL, Tomaszewski JE, Neilson EG: Identification and characterization of a fibroblast marker: FSP1. *J Cell Biol* 1995, 130:393–405
 6. Roberts IS, Burrows C, Shanks JH, Venning M, McWilliam LJ: Interstitial myofibroblasts: predictors of progression in membranous nephropathy. *J Clin Pathol* 1997, 50:123–127
 7. Yang J, Lui Y: Blockage of tubular epithelial to myofibroblast transition by hepatocyte growth factor prevents renal interstitial fibrosis. *J Am Soc Nephrol* 2002, 13:96–107
 8. Zeisberg M, Hanai J, Sugimoto H, Mammoto T, Charytan D, Strutz F, Kalluri R: BMP-7 counteracts TGF- β -induced epithelial-to-mesenchymal transition and reverses chronic renal injury. *Nat Med* 2003, 9:964–968
 9. Thiery JP: Epithelial-mesenchymal transition in development and pathologies. *Curr Opin Cell Biol* 2003, 15:740–746
 10. Thiery JP: Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer* 2002, 2:442–454
 11. Radisky DC, Kenny PA, Bissell MJ: Fibrosis and cancer: do myofibroblasts come also from epithelial cells via EMT? *J Cell Biochem* 2007, 101:830–839
 12. Zavadil J, Bottinger EP: TGF- β and epithelial-to-mesenchymal transitions. *Oncogene* 2005, 24:5764–5774
 13. Fan JM, Ng YY, Hill PA, Paterson-Nikolic DJ, Mu W, Atkins RC, Lan HY: Transforming growth factor- β regulates tubular epithelial-myofibroblast transdifferentiation in vitro. *Kidney Int* 1999, 56:1455–1467
 14. Bottinger EP, Bitzer M: TGF- β signaling in renal disease. *J Am Soc Nephrol* 2002, 13:2600–2610
 15. Border WA, Noble NA: Transforming growth factor β in tissue fibrosis. *N Engl J Med* 1994, 331:1286–1292
 16. Feng XH, Derynck R: Specificity and versatility in TGF- β signaling through Smads. *Annu Rev Cell Dev Biol* 2005, 21:659–693
 17. Flanders KC: Smad3 as a mediator of the fibrotic response. *Int J Exp Pathol* 2004, 85:47–64
 18. Roberts AB, Tian F, Byfield SD, Stuelten C, Ooshima A, Saika S, Flanders KC: Smad3 is key to TGF- β -mediated epithelial-to-mesenchymal transition, fibrosis, tumor suppression and metastasis. *Cytokine Growth Factor Rev* 2006, 17:19–27
 19. Streuli CH, Akhtar N: Signal co-operation between integrins and other receptor systems. *Biochem J* 2009, 418:491–506
 20. Al-Jamal R, Harrison DJ: β_1 integrin in tissue remodelling and repair: from phenomena to concepts. *Pharmacol Ther* 2008, 120:81–101
 21. Stephens LE, Sutherland AE, Klimanskaya IV, Andrieux A, Meneses J, Pedersen RA, Damsky CH: Deletion of β_1 integrins in mice results in inner cell mass failure and peri-implantation lethality. *Genes Dev* 1995, 9:1883–1895
 22. Fässler R, Meyer M: Consequences of lack of β_1 integrin gene expression in mice. *Genes Dev* 1995, 9:1896–1908
 23. Bhowmick NA, Zent R, Ghiassi M, McDonnell M, Moses HL: Integrin β_1 signaling is necessary for transforming growth factor- β activation of p38MAPK and epithelial plasticity. *J Biol Chem* 2001, 276:46707–46713
 24. Li Y, Yang J, Dai C, Wu C, Liu Y: Role for integrin-linked kinase in mediating tubular epithelial to mesenchymal transition and renal interstitial fibrogenesis. *J Clin Invest* 2003, 112:503–516
 25. Cicchini C, Laudadio I, Citarella F, Corazzari M, Steindler C, Conigliaro A, Fantoni A, Amicone L, Tripodi M: TGF β -induced EMT requires focal adhesion kinase (FAK) signaling. *Exp Cell Res* 2008, 314:143–152
 26. Xie L, Law BK, Aakre ME, Edgerton M, Shyr Y, Bhowmick NA, Moses HL: Transforming growth factor β -regulated gene expression in a mouse mammary gland epithelial cell line. *Breast Cancer Res* 2003, 5:187–198
 27. Kagami S, Kuhara T, Yasuromo K, Okada K, Loster K, Reutter W, Kuroda Y: Transforming growth factor- β (TGF- β) stimulates the expression of β_1 integrins and adhesion by rat mesangial cells. *Exp Cell Res* 1996, 229:1–6
 28. Yeh YC, Wang CZ, Tang MJ: Discoidin domain receptor 1 activation suppresses $\alpha_2\beta_1$ integrin-dependent cell spreading through inhibition of Cdc42 activity. *J Cell Physiol* 2009, 218:146–156
 29. Wei WC, Lin HH, Shen MR, Tang MJ: Mechanosensing machinery for cells under low substratum rigidity. *Am J Physiol Cell Physiol* 2008, 295:1579–1589
 30. Wang CZ, Yeh YC, Tang MJ: DDR1/E-cadherin complex regulates the activation of DDR1 and cell spreading. *Am J Physiol Cell Physiol* 2009, 297:419–429
 31. Su HW, Yeh HH, Wang SW, Shen MR, Chen TL, Kiela PR, Ghishan FK, Tang MJ: Cell confluence-induced activation of signal transducer and activator of transcription-3 (Stat3) triggers epithelial dome formation via augmentation of sodium hydrogen exchanger-3 (NHE3) expression. *J Biol Chem* 2007, 282:9883–9894
 32. Wu MJ, Wen MC, Chiu YT, Chiou YY, Shu KH, Tang MJ: Rapamycin attenuates unilateral ureteral obstruction-induced renal fibrosis. *Kidney Int* 2006, 69:2029–2036
 33. Bellis SL, Newman E, Friedman EA: Steps in integrin β_1 -chain glycosylation mediated by TGF β 1 signaling through Ras. *J Cell Physiol* 1999, 181:33–44
 34. Chen FA, Repasky EA, Bankert RB: Human lung tumor-associated antigen identified as an extracellular matrix adhesion molecule. *J Exp Med* 1991, 173:1111–1119
 35. White LR, Blanchette JB, Ren L, Awn A, Trpkov K, Muruve DA: The characterization of α_5 -integrin expression on tubular epithelium during renal injury. *Am J Physiol Renal Physiol* 2007, 292:567–576
 36. Giannone G, Dubin-Thaler BJ, Dobereiner HG, Kieffer N, Bresnick AR, Sheetz MP: Periodic lamellipodial contractions correlate with rearward actin waves. *Cell* 2004, 116:431–443
 37. Masszi A, Ciano CD, Sirokmany G, Arthur WT, Rotstein OD, Wang J, McCulloch CA, Rosivall L, Mucci I, Kapus A: Central role for Rho in TGF- β 1-induced α -smooth muscle actin expression during epithelial-mesenchymal transition. *Am J Physiol Renal Physiol* 2003, 284:911–924
 38. Szaszi K, Sirokmany G, Ciano-Oliveira CD, Rotstein OD, Kapus A: Depolarization induces Rho-Rho kinase-mediated myosin light chain phosphorylation in kidney tubular cells. *Am J Physiol Cell Physiol* 2005, 289:673–685
 39. Fan L, Sebe A, Peterfi Z, Masszi A, Thirone AC, Rotstein OD, Nakano H, McCulloch CA, Szaszi K, Mucci I, Kapus A: Cell contact-dependent regulation of epithelial-myofibroblast transition via the Rho-Rho kinase-phospho-myosin pathway. *Mol Biol Cell* 2007, 18:1083–1097
 40. Wang CZ, Su HW, Hsu YC, Shen MR, Tang MJ: A discoidin domain receptor 1/SHP-2 signaling complex inhibits $\alpha_2\beta_1$ -integrin-mediated signal transducers and activators of transcription 1/3 activation and cell migration. *Mol Biol Cell* 2006, 17:2839–2852
 41. Edick MJ, Tesfay L, Lamb LE, Knudsen BS, Miranti CK: Inhibition of integrin-mediated crosstalk with epidermal growth factor receptor/Erk or Src signaling pathways in autophagic prostate epithelial cells induces caspase-independent death. *Mol Biol Cell* 2007, 18:2481–2490
 42. Kim KK, Wei Y, Szekeres C, Kugler MC, Wolters PJ, Hill ML, Frank JA, Brumwell AN, Wheeler SE, Kreidberg JA, Chapman HA: Epithelial cell $\alpha_3\beta_1$ integrin links β -catenin and Smad signaling to promote myofibroblast formation and pulmonary fibrosis. *J Clin Invest* 2009, 119:213–224
 43. Kim Y, Kugler MC, Wei Y, Kim KK, Li X, Brumwell AN, Chapman HA: Integrin $\alpha_3\beta_1$ -dependent β -catenin phosphorylation links epithelial Smad signaling to cell contacts. *J Cell Biol* 2009, 184:309–322
 44. Wu H, Liang YL, Li Z, Jin J, Zhang W, Duan L, Zha X: Positive expression of E-cadherin suppresses cell adhesion to fibronectin via reduction of $\alpha_5\beta_1$ integrin in human breast carcinoma cells. *J Cancer Res Clin Oncol* 2006, 132:795–803
 45. Gilcrease MZ: Integrin signaling in epithelial cells. *Cancer Lett* 2007, 247:1–25
 46. Norman JT, Fine LG: Progressive renal disease: fibroblasts, extracellular matrix, and integrins. *Exp Nephrol* 1999, 7:167–177
 47. Lundberg S, Lindholm J, Lindbom L, Hellstrom PM, Werr J: Integrin $\alpha_2\beta_1$ regulates neutrophil recruitment and inflammatory activity in experimental colitis in mice. *Inflamm Bowel Dis* 2006, 12:172–177
 48. Issekutz TB: In vivo blood monocyte migration to acute inflammatory reactions. IL-1 α , TNF- α , IFN- γ , and C5a utilizes LFA-1, Mac-1, and VLA-4. *J Immunol* 1995, 154:6533–6540
 49. Hu K, Wu C, Mars WM, Liu Y: Tissue-type plasminogen activator promotes murine myofibroblast activation through LDL receptor-related protein 1-mediated integrin signaling. *J Clin Invest* 2007, 117:3821–3832