Tubular Epithelial Cell Dedifferentiation Is Driven by the Helix-Loop-Helix Transcriptional Inhibitor Id1

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In the fibrotic kidney, tubular cells undergo epithelial-to-mesenchymal transition (EMT), a phenotypic conversion that is characterized by sequential loss of epithelial markers and gain of mesenchymal features. For understanding of the molecular mechanism that governs this process, a high-throughput gene expression microarray analysis was used to identify the critical genes in the initial phase of the TGF-β1–mediated EMT. Inhibitor of differentiation-1 (Id1), a dominant negative antagonist of the basic helix-loop-helix transcription factors, was found to be induced rapidly in human proximal tubular epithelial cells after TGF-β1 treatment. This induction of Id1 depended on intracellular Smad signaling. Ectopic expression of Id1 suppressed epithelial E-cadherin and zonula occludens-1 expression. Id1 physically formed complex with basic helix-loop-helix transcription factor HEB (Hela E-box binding factor), sequestered its ability to bind to E-box, and repressed the *trans*-activation of E-cadherin promoter. However, overexpression of Id1 failed to induce α-smooth muscle actin, matrix metalloproteinase-2, fibronectin, and integrin-linked kinase (ILK), indicating its inability to confer a complete EMT. Overexpression of ILK or inhibition of ILK activity had no effect on Id1 induction by TGF-β1, suggesting that Id1 and ILK have independent roles in epithelial dedifferentiation and EMT. *In vivo*, Id1 was induced exclusively in the degenerated, dilated renal tubular epithelium after unilateral ureteral obstruction. These studies identify Id1 transcriptional inhibitor as a crucial player in mediating cell dedifferentiation of renal tubular epithelium and suggest that EMT is a multistep process in which loss of epithelial adhesion does not necessarily lead to an autonomous mesenchymal transition.

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After sustained injury, tubular epithelial cells in adult kidney can undergo epithelial-to-mesenchymal transition (EMT), a phenotypic conversion that is recognized increasingly to play a critical role in the evolution of renal interstitial fibrosis (1–3). Extensive studies suggest that TGF-β1, a fibrogenic cytokine whose expression is increased in almost every type of chronic kidney disease (CKD), is the major driving force behind tubular EMT under pathologic conditions (4–7). Tubular EMT is characterized by sequential loss of epithelial markers and gain of mesenchymal features. Earlier studies from our laboratory established that the entire EMT course consists of four key steps, in which loss of epithelial cell-to-cell adhesion receptor E-cadherin is an early event (6). This notion is supported by many studies demonstrating an essential role of E-cadherin in the maintenance of the structural integrity and cell polarity of normal tubular epithelium (8,9). However, whether loss of E-cadherin is sufficient for triggering an autonomous progression to mesenchymal transition remains to be determined. Furthermore, little is known about the underlying molecular mechanism of the suppression of E-cadherin in the early phase of EMT.

Given the complexities of tubular EMT, it is conceivable that many genes may be involved intimately in this process. Accordingly, we previously demonstrated that the integrin-linked kinase (ILK), an intracellular serine/threonine protein kinase that interacts with the cytoplasmic domains of β integrins and mediates the integrin signaling, is a key mediator in TGF-β1–induced EMT (10). The ability to identify ILK as a pivotal EMT regulator has prompted us to search for other critical genes that are functionally important in mediating tubular cell phenotypic conversion. Toward this end, we recently used a high-throughput gene expression microarray approach to analyze genes with an altered expression in the initial phase of tubular EMT that is induced by TGF-β1. One such gene is the inhibitor of differentiation-1 (Id1), also known as inhibitor of DNA binding, a helix-loop-helix (HLH) transcriptional inhibitor.

Id1 is the prototype of a family of HLH transcriptional inhibitors that consists of four members (Id1 through Id4), each of them with distinct expression patterns (11–13). Structurally, Id1 represents a truncated form of the basic HLH (bHLH) family of transcription factors that regulate tissue-specific gene expression. Because it lacks the basic DNA binding domain but retains the ability to form heterodimeric complexes with other bHLH proteins, Id1 acts as a transcriptional antagonist to inhibit

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bHLH protein–mediated gene transcription in a dominant negative manner. Id1 has been implicated as an inhibitor in myoblast and neuronal differentiation (14,15), and its expression often is associated with cell dedifferentiation in numerous cell lineages (16,17). Because bHLH proteins control the *trans*-activation of a host of genes that are required for the maintenance of cell differentiated status, the early induction of Id1 in tubular epithelial cells by TGF-β1 likely suggests its potential involvement in the initiation of a cascade of events in EMT program.

In this study, we showed that TGF-β1 rapidly induced Id1 expression in tubular epithelial cells by a Smad-dependent mechanism. We found that Id1 was responsible for tubular epithelial cell dedifferentiation by suppressing E-cadherin and zonula occludens-1 (ZO-1) expression but not for its mesenchymal transition. Our studies suggest that EMT is a multistep process in which tubular epithelial cell dedifferentiation can be uncoupled from mesenchymal transition.

Materials and Methods

Cell Culture and Treatment

Human proximal tubular epithelial cell line (HKC clone 8) was maintained as described previously (18). Serum-starved HKC-8 cells were treated with recombinant TGF-β1 (R&D Systems, Minneapolis, MN) for various periods of time at the concentration of 2 ng/ml except otherwise indicated. The cells then were collected for Northern and Western blot analyses and immunofluorescence staining. In some experiments, cells were pretreated for 30 min with various chemical inhibitors at the concentrations specified, followed by incubation with TGF-β1. PD98059, wortmannin, myristoylated protein kinase A inhibitor, and SC 68376 were purchased from Calbiochem (La Jolla, CA). Small-molecule ILK inhibitor KP392 was described elsewhere (19,20).

Affymetrix Microarray Analysis

A high-throughput gene expression profiling was carried out by using Affymetrix chip analysis (Affymetrix, Santa Clara, CA), as described previously (21,22). Preparation of cRNA, hybridization, and scanning of the arrays were performed essentially according to the protocols specified by the manufacturer. Briefly, double-stranded cDNA was generated from total RNA that were isolated from the HKC-8 cells by using a reverse transcription kit (Invitrogen, Carlsbad, CA) and T7-d$(T)_{24}$ primer. Biotin-labeled cRNA was prepared from the double-stranded cDNA by *in vitro* transcription using MEGAscript system (Ambion, Austin, TX) and fragmented at 95°C for 35 min. The fragmented cRNA then were hybridized with an Affymetrix human Genome U95Av2 Array chip (HG-95Av2) set that was composed of approximately 12,000 fully sequenced and characterized genes. After the hybridization cocktails were removed, the chips then were washed, stained with streptavidin phycoerythrin (Molecular Probe, Eugene, OR), incubated with biotinylated mouse anti-streptavidin IgG, and re-stained with streptavidin phycoerythrin. The chips were scanned in an HP ChipScanner (Affymetrix) to detect hybridization signals. Data were analyzed using GeneChip software (Affymetrix).

Northern Blot Analysis and Reverse Transcriptase–PCR

Total RNA was extracted from the cultured cells and kidney tissue by using TRIzol reagent (Invitrogen). Northern blot analysis for gene expression was carried out by the procedures described previously (23). Reverse transcriptase–PCR (RT-PCR) was performed by standard protocol as described elsewhere (24). The primer sequences for mouse Id1 were as follow: 5′-CATGAACGGCTGCTACTCAC (sense) and 5′-GTGGTCCCGACTTCAGACTC (antisense). The primers for mouse β-actin were described previously (24).

Western Blot Analysis

Western blot analysis was performed essentially according to an established procedure (18). The primary antibodies used were as follows: Anti-Id1 (sc-488), anti-HEB (Hela E-box binding factor; sc-357), and anti-actin (sc-1616; Santa Cruz Biotechnology, Santa Cruz, CA); anti-α-smooth muscle actin (α-SMA; clone 1A4; Sigma, St. Louis, MO), anti–E-cadherin (clone 36), and anti-fibronectin (clone 10; BD Transduction Laboratories, Lexington, KY); anti-ILK (05–575; Upstate Biotechnology, Charlottesville, VA); anti-Myc (2276; Cell Signaling Technology, Danvers, MA); and anti–glyceraldehyde-3-phosphate dehydrogenase (Ambion). Quantification was performed by measurement of the intensity of the bands with the use of NIH Image analysis software.

Construction of Id1 Expression Vector and Establishment of Stable Cell Line

Human full-length coding sequence of Id1 cDNA was cloned by using a standard RT-PCR protocol (25). The PCR product was cloned into the mammalian expression vector pcDNA3 (Invitrogen) and confirmed by DNA sequencing at the University of Pittsburgh Biomedical Research Support Facilities. Flag- or Myc-tagged Id1 expression vectors also were constructed by standard subcloning techniques and confirmed by sequencing. HKC-8 cells were transfected with Id1 expression vector using the Lipofectamine 2000 (Invitrogen). The empty vector pcDNA3 was used as a mock-transfection control. Neomycin-resistant clones were selected and expanded individually, as described previously (10), and ectopic expression of Id1 was confirmed by Western blot analysis. The stable cell lines that overexpressed SnoN (Ski-related novel gene, non–Alu-containing), Smad7, or ILK were established previously and described elsewhere (10,26).

Immunofluorescence Staining

Indirect immunofluorescence staining was performed using an established procedure (18). Briefly, cells that were cultured on coverslips and kidney cryosections were fixed with cold methanol:acetone (1:1) for 10 min at −20°C. After blocking with 20% normal donkey serum in PBS buffer, the slides were incubated with the primary antibodies against E-cadherin, fibronectin, Id1, and ZO-1 (61–7300; Invitrogen). For visualization of the primary antibodies, slides were stained with cyanine Cy2- or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Kidney cryosections also were stained with renal proximal tubular marker fluorescein-conjugated lectin from *Tetragonolobus purpureas* (Sigma). Stained slides were viewed with a Nikon Eclipse E600 Epi-fluorescence microscope equipped with a digital camera (Melville, NY).

Plasmid Construction, Transfection, and Reporter Gene Assay

The E-cadherin promoter-luciferase reporter plasmid was provided by Dr. A. Garcia de Herreros (Universitat Pompeu Fabra, Barcelona, Spain) (27). The E-box SV-promoter reporters were constructed by insertion of oligonucleotides that contained either wild-type or mutant E-boxes in the front of SV-40 promoter (pGL3-promoter vector; Promega, Madison, WI). HKC-8 cells were co-transfected with reporter plasmids and an internal control reporter (pRL-TK; Promega). Luciferase assay was performed using the Dual Luciferase Assay System kit (Promega) (25). Relative luciferase activity (arbitrary unit) was reported after normalizing for transfection efficiency.

Immunoprecipitation

Immunoprecipitation experiments were performed using similar methods as described previously (28). Briefly, cell lysates were centrifuged at 12,000 × *g* for 10 min at 4°C. The resulting supernatants were collected for immunoprecipitation. After preclearing with normal host IgG, the lysates were immunoprecipitated overnight at 4°C with 2 μg of anti-Id1 or anti-HEB antibodies, followed by precipitation with 30 μl of protein A/G Plus-Agarose (Santa Cruz Biotechnology) for 3 h at 4°C. The precipitated complexes were immunoblotted with antibodies against HEB and Myc, respectively.

DNA Affinity Precipitation Assay

DNA–protein interaction was examined by a simple DNA affinity precipitation assay (25). Briefly, the 5′-biotinylated, double-stranded oligonucleotide (0.2 nM) that contained two tandem copies of E-box was mixed with 200 μg of whole-cell extracts for 3 h at 4°C. In some experiments, 100-fold excess of unlabeled oligonucleotides that contained either E-box or mutant E-box was included as competitor DNA in the reaction mixture. After streptavidin-agarose (10 μl of packed beads) was added, the incubation was continued for an additional 3 h at 4°C. The beads were washed, and the eluted proteins were analyzed by Western blotting with anti-HEB antibody.

Statistical Analyses

Unilateral ureteral obstruction (UUO) was performed in male CD-1 mice using an established procedure, as described previously (18). Statistical analysis of the data was performed using SigmaStat software (Jandel Scientific Software, San Rafael, CA).

Results

Id1 Is Induced Rapidly during TGF-β1–Mediated EMT

We used a high-throughput gene expression microarray approach to identify the genes whose expression was altered in the initial stage of tubular EMT that was induced by TGF-β1. As shown in Figure 1A, there were 94 genes whose expression was altered by more than two-fold at 0.5 h in HKC-8 cells after TGF-β1 treatment. Of particular interest, Id1, a transcriptional antagonist that belongs to the HLH protein family (29,30), was induced markedly. RT-PCR studies confirmed the induction of Id1 mRNA in HKC-8 cells that were treated with TGF-β1 (data not shown).

We then cloned and sequenced the entire coding region of human Id1 cDNA from HKC-8 cells. Figure 1B shows a Northern blot analysis of Id1 expression in tubular epithelial cells after TGF-β1 treatment. The steady-state level of Id1 mRNA in HKC-8 cells began to increase as early as 0.5 h, reached the peak between 1 and 3 h, and declined rapidly to baseline level at 6 h. Western blot analysis also revealed a rapid induction of Id1 protein expression after TGF-β1 stimulation. As shown in Figure 1, C and D, Id1 protein peaked at 1 h and declined at 6 h. The induction of Id1 protein by TGF-β1 also was dosage dependent (Figure 1, E and F).

Induction of Id1 Expression by TGF-β1 Depends on Smad Signaling

We next investigated the potential signaling pathways that lead to Id1 induction by TGF-β1 in HKC-8 cells. Previous studies revealed that TGF-β1 activates numerous signaling pathways, such as Smad2/3, p38 mitogen-activated protein kinase (MAPK), and Akt kinase (28). However, pharmacologic inhibition of p38 MAPK and Akt kinase by specific inhibitors did not affect Id1 induction by TGF-β1 (Figure 2A). Likewise, inhibition of extracellular signal–regulated kinase–MAPK or protein kinase A or C also did not influence TGF-β1–initiated Id1 induction (Figure 2A). To study the potential involvement of Smad signaling, we examined the Id1 inducibility in the tubular epithelial cells that overexpressed Smad co-repressor SnoN. As shown in Figure 2B, forced expression of SnoN largely abrogated Id1 induction by TGF-β1. Similarly, overexpression of inhibitory Smad7 significantly inhibited the TGF-β1–mediated Id1 induction (Figure 2, C and D). Therefore, Id1 induction by TGF-β1 depends on intracellular Smad signaling.

Forced Expression of Id1 Suppresses E-Cadherin and ZO-1 Expression

To investigate the functionality of Id1 induction in tubular epithelial cells, we examined the phenotypic changes after ectopic expression of exogenous Id1 by stable transfection. As shown in Figure 3A, four stable cell lines exhibited different levels of Id1 protein expression. Of note, the cell line with mock transfection of pcDNA3 vector displayed a similar level of Id1 as in the parental HKC-8 cells. It is interesting that ectopic expression of Id1 markedly suppressed epithelial E-cadherin. It seemed that the E-cadherin levels were correlated inversely with the abundance of Id1, suggesting a dosage-dependent effect of Id1 on E-cadherin suppression. Figure 3B shows a linear regression plot between Id1 and E-cadherin levels, with the correlation coefficient being 0.91.

We further examined E-cadherin expression in HKC-8 cells by an indirect immunofluorescence staining. As illustrated in Figure 3, C and D, compared with the controls, tubular epithelial cells with overexpression of Id1 lost E-cadherin staining in the plasma membrane. Similarly, ectopic expression of Id1 suppressed ZO-1, a tight junction-associated protein, in tubular epithelial cells (Figure 3, E and F).

Downregulation of Id1 Blocks TGF-β1–Initiated E-Cadherin Suppression

To assess whether Id1 induction is necessary for TGF-β1–mediated E-cadherin suppression, we examined the E-cadherin expression after downregulation of Id1 by antisense strategy in HKC-8 cells. As shown in Figure 4A, antisense strategy resulted in a considerable inhibition of Id1 expression in the absence or presence of TGF-β1. Figure 4B shows the E-cadherin levels in the controls or TGF-β1–treated HKC-8 cells after transient transfection with either sense or antisense Id1 vectors. Transfection of antisense Id1 partially restored E-cadherin expression, which was repressed by TGF-β1 (Figure 4B, lane 4 *versus* 6). As expected, transfection of Id1 in sense orientation or incubation with TGF-β1 suppressed E-cadherin expression in HKC-8 cells (Figure 4B, lane 1 *versus* lane 2 or 4). Hence, Id1 seems to be required for mediating TGF-β1–initiated E-cadherin suppression in tubular epithelial cells.

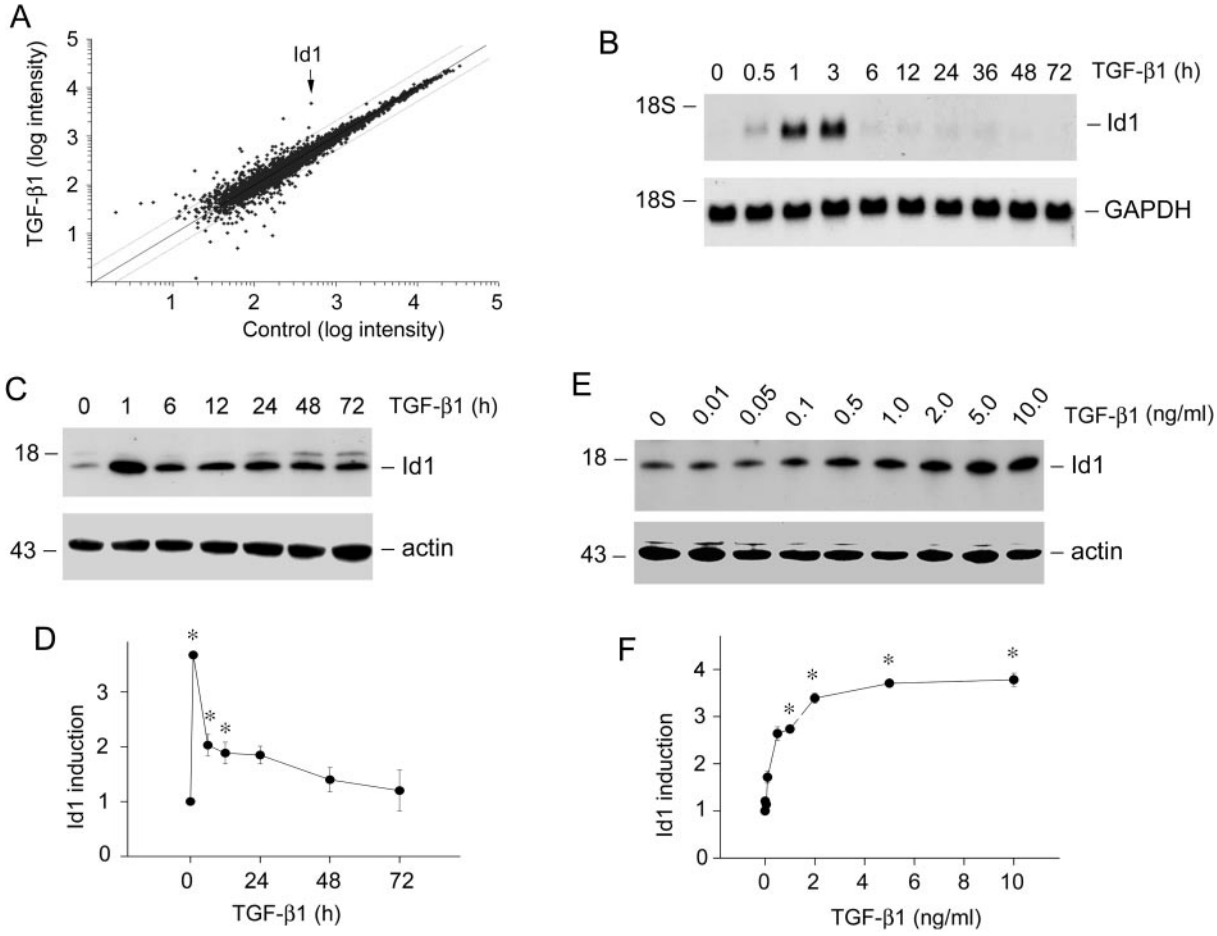

Figure 1. The helix-loop-helix (HLH) transcriptional inhibitor of differentiation-1 (Id1) is induced rapidly during TGF-β1–mediated tubular epithelial-to-mesenchymal transition (EMT). (A) Microarray analysis revealed an early induction of Id1 in renal epithelial cells after TGF-β1 treatment. Human tubular epithelial cells (HKC-8) were incubated without or with 2 ng/ml TGF-β1 for 0.5 h, and total RNA was subjected to Affymetrix microarray analysis. (B) TGF-β1 induced Id1 mRNA expression in tubular epithelial cells. HKC-8 cells were treated with 2 ng/ml TGF-β1 for various periods of time as indicated. Total RNA was analyzed by Northern blot for Id1 expression. The same blot was stripped and reprobed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for ensuring equal loading of each lane. (C through F) TGF-β1 induced Id1 protein expression in a time- and dosage-dependent manner. HKC-8 cells were treated either with 2 ng/ml TGF-β1 for various periods of time (C and D) or various amounts of TGF-β1 for 1 h (E and F) as indicated. Whole-cell lysates were immunoblotted with antibodies against Id1 and actin, respectively. Graphic presentation of the relative abundances of Id1 (fold induction over the controls) after various treatments is shown (D and F). *$P < 0.05$ *versus* control; $n = 3$.

Id1 Suppresses E-Cadherin Promoter by Repressing E-Box–Mediated Gene Transcription

To understand how Id1 mediates the suppression of E-cadherin, we studied its effect on E-cadherin promoter activity. As shown in Figure 5A, Id1 suppressed E-cadherin promoter activity in a dosage-dependent manner. Because E-cadherin promoter harbors three E-boxes, the cis-acting element that is capable of binding bHLH proteins and activating gene transcription, we reasoned that the E-boxes may mediate the transcriptional repression by Id1. To test it, we constructed two copies of E-boxes into the heterologous SV-40 promoter (Figure 5B). After transfection of this construct into HKC-8 cells, luciferase activity was suppressed by Id1 in a dosage-dependent manner (Figure 5C). However, Id1 did not affect luciferase activity when the E-box was mutated. These data suggest that the E-cadherin suppression by Id1 likely is mediated through modulating the E-box–controlled gene transcription.

Id1 Antagonizes the Trans*-activating Action of HEB by Forming HEB/Id1 Heterodimeric Complex*

To delineate further the underlying mechanism of Id1 suppression of E-cadherin, we examined the interaction between Id1 and the E-proteins, members of the bHLH protein family. Preliminary studies showed that the predominant form of bHLH proteins in tubular epithelial cells was HEB (data not shown). However, incubation of HKC-8 cells with TGF-β1 did not alter HEB expression (Figure 6A).

We investigated the potential interaction between HEB and Id1 by co-immunoprecipitation. As shown in Figure 6B, HEB protein was detectable in the complexes that were precipitated

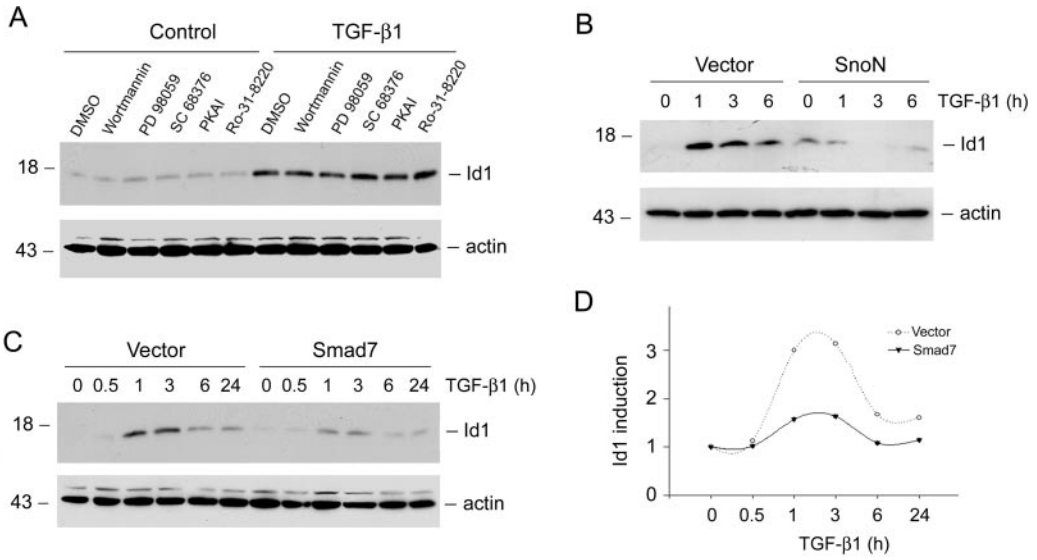

Figure 2. Id1 induction by TGF-β1 in renal epithelial cells depends on Smad signaling. (A) Pharmacologic inhibition of various signal transduction pathways did not affect Id1 induction by TGF-β1. HKC-8 cells were pretreated with various chemical inhibitors or vehicle (DMSO) for 30 min, followed by incubation in the absence or presence of 2 ng/ml TGF-β1 for 1 h. Specific inhibitors for phosphatidylinositol-3-kinase (10 nM wortmannin), Mek1 (10 μM PD98059), p38 mitogen-activated protein kinase (MAPK; 20 μM SC 68376), protein kinase A (PKA; 0.3 μM PKA inhibitor [PKAI]), and PKC (50 nM Ro-31-8220) were used. (B and C) Overexpression of either Smad transcriptional co-repressor SnoN (Ski-related novel gene, non–Alu-containing) or inhibitory Smad-7 inhibited Id1 induction by TGF-β1. Stable cell lines that overexpressed either SnoN (B) or inhibitory Smad-7 (C) were established by transfection of SnoN or Smad-7 expression vectors. Cell lines with mock transfection of empty pcDNA3 vector were used as controls. Cells were treated with 2 ng/ml TGF-β1 for various periods of time as indicated, followed by immunoblotting with antibodies against Id1 and actin, respectively. (D) Graphic presentation of relative Id1 abundance normalized to actin after TGF-β1 treatment in the Smad-7–overexpressing cells.

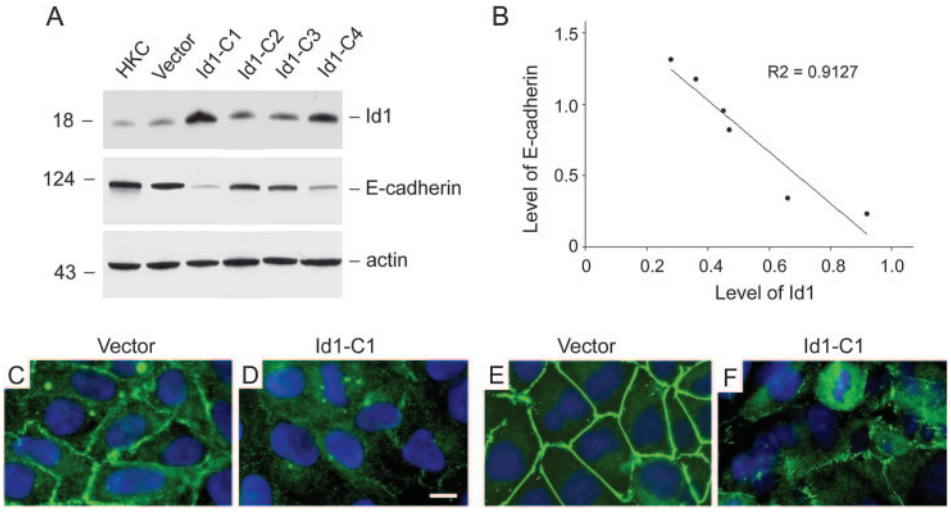

Figure 3. Forced expression of Id1 suppresses E-cadherin and zonula occludens-1 (ZO-1) expression in tubular epithelial cells. Stable cell lines were established by transfection of Id1 expression vectors. Cell line with mock transfection of empty vector pcDNA3 (vector) was used as control. (A) Cell lysates were immunoblotted with antibodies against Id1, E-cadherin, and actin. Numbers (C1 through C4) indicate four individual clones that express various levels of Id1. (B) Linear regression showed a closely inverse relationship between Id1 and E-cadherin in tubular epithelial cells. The correlation coefficient (R^2) is shown. (C and D) Immunofluorescence staining showed E-cadherin localization in HKC-8 cells that were transfected with empty vector (C) or pCMV-Id1 (D). (E and F) Forced expression of Id1 also suppressed ZO-1 expression in tubular epithelial cells. Immunofluorescence staining showed ZO-1 localization at the junctions of HKC-8 cells that were transfected with empty vector (E) or pCMV-Id1 (F). Bar = 5 μm.

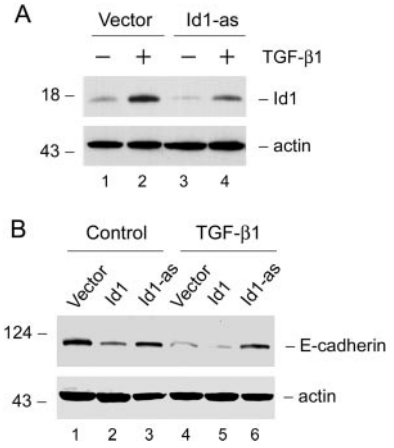

Figure 4. Expression of antisense Id1 largely blocks TGF-β1–mediated E-cadherin suppression. HKC-8 cells that were transfected with empty vector (vector), Id1 (pCMV-Id1), or antisense Id1 (pCMV-Id1-as) were incubated without or with 2 ng/ml TGF-β1. (A) Antisense inhibition of Id1 expression in the absence or presence of TGF-β1 was confirmed by Western blot analyses. (B) Ectopic expression of antisense Id1 largely abolished TGF-β1–initiated E-cadherin suppression in tubular epithelial cells.

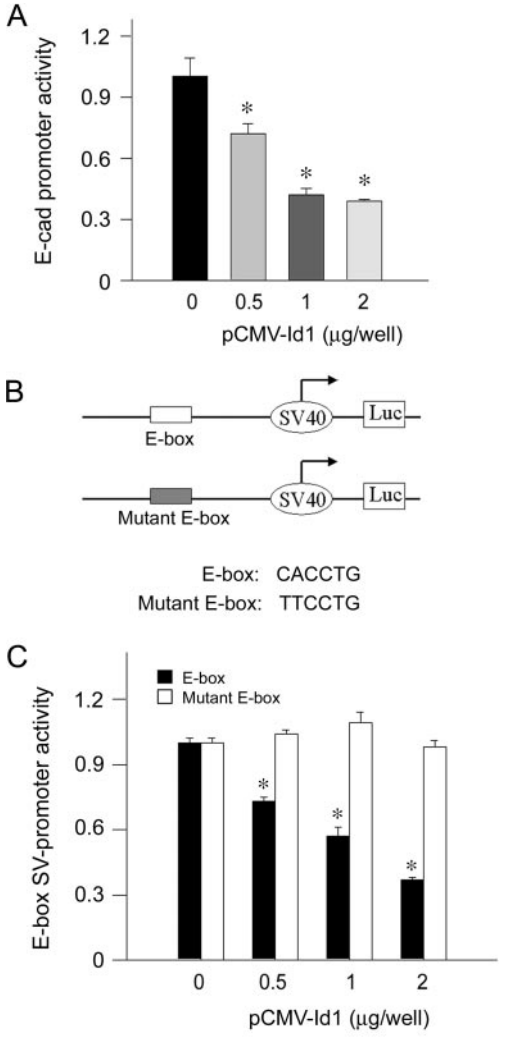

Figure 5. Id1 suppresses E-cadherin promoter activity by repressing E-boxes–mediated gene transcription in tubular epithelial cells. (A) HKC-8 cells were transiently co-transfected with the reporter construct (pE-cad-luc) that contained E-cadherin promoter region that was linked to firefly luciferase reporter gene and increasing amounts of pCMV-Id1 as indicated. The relative luciferase activity (with control group = 1.0) was presented after normalization of transfection efficiency. Data are means ± SEM from three independent experiments. *$P < 0.01$ *versus* controls. (B and C) Id1 suppressed the transcriptional activity of the heterogeneous promoter containing E-box in tubular epithelial cells. Diagram depicts the construction of reporter vectors in which wild-type or mutant E-boxes were cloned into the SV-40 promoter luciferase reporter plasmid (B). The relative luciferase activity (with control group = 1.0) was presented after transfection with various reporter vectors. Data are means ± SEM from three independent experiments. *$P < 0.01$ *versus* controls.

by anti-Id1 antibody, suggesting a physical interaction between HEB and Id1. This interaction was increased after TGF-β1 treatment (Figure 6B). For confirmation of the authenticity of this interaction, Myc-tagged Id1 was expressed in HKC-8 cells by transient transfection, followed by immunoprecipitation with anti-Id1, anti-HEB, or normal IgG, respectively. As shown in Figure 6C, HEB was detected only in the complexes that were precipitated by anti-Id1 but not in that by control IgG. Likewise, Myc was found in the complexes that were precipitated by anti-HEB (Figure 6D). Similar results were obtained when Flag-tagged Id1 expression vector was used (data not shown). Figure 6E shows that HEB protein constitutively bound to the E-boxes in HKC-8 cells under basal condition, as demonstrated by DNA affinity precipitation assay. Addition of wild-type but not mutant E-box DNA completely abolished the binding (Figure 6E, lane 3), suggesting the specificity of this DNA–protein interaction. However, the binding of HEB to the E-box was reduced dramatically after TGF-β1 treatment (Figure 6F). Because TGF-β1 did not affect the abundance of HEB (Figure 6A), this suggests that increased Id1 binding to HEB protein would diminish the availability of HEB to the E-box, thereby sequestering its ability to *trans*-activate gene transcription (Figure 6G).

Overexpression of Id1 Is Not Sufficient to Confer a Complete EMT

Because Id1 overexpression leads to E-cadherin suppression, an early event in tubular EMT (6), we next examined whether increased Id1 results in mesenchymal transition of tubular epithelial cells. As shown in Figure 7, overexpression of Id1 failed to induce the *de novo* expression of α-SMA. Likewise, Id1 did not induce matrix metalloproteinase-2 (Figure 7B), a matrix-degrading enzyme that is of importance in disrupting tubular basement membrane. Finally, Id1 also failed to induce fibronectin expression (Figure 7C). Similar results were obtained when the cells were stained with anti-fibronectin antibody (Figure 7, D through G). As a positive control, TGF-β1 was capable of

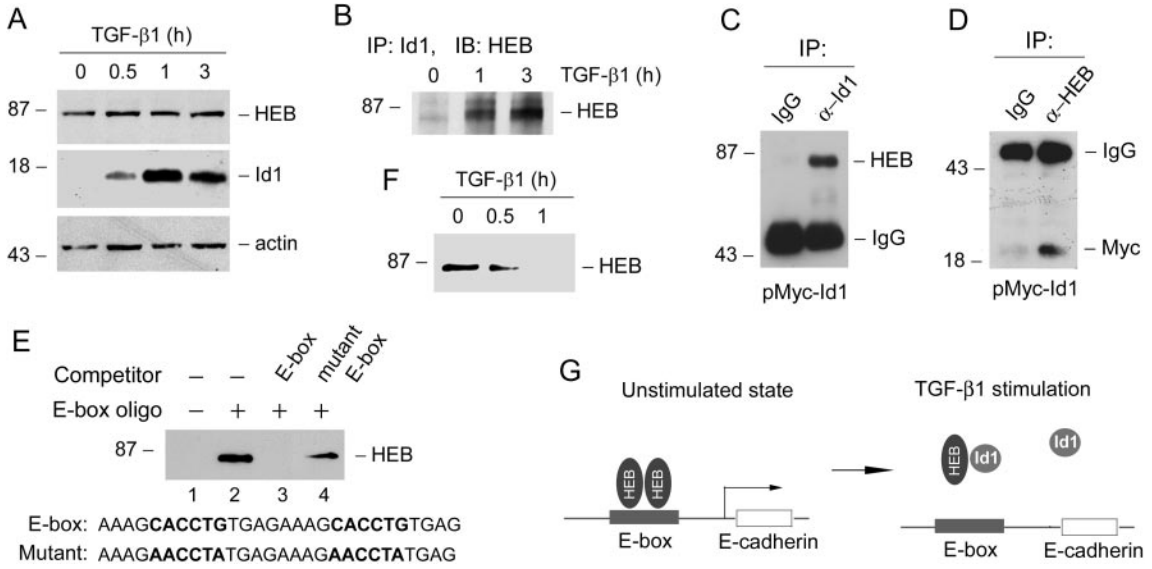

Figure 6. Id1 prevents basic HLH transcription factor HEB binding to the E-box by sequestrating its activity *via* physical interaction. (A) TGF-β1 did not affect HEB protein abundance in tubular epithelial cells. HKC-8 cells were treated with 2 ng/ml TGF-β1 for various periods of time as indicated. Cell lysates were immunoblotted with antibodies against HEB, Id1, and actin. (B) Co-immunoprecipitation revealed a physical association between HEB and Id1 in tubular epithelial cells. Cell lysates that were prepared from HKC-8 cells that were incubated with 2 ng/ml TGF-β1 were immunoprecipitated with antibody against Id1, followed by immunoblotting with anti-HEB as indicated. (C and D) Physical interaction of exogenous Id1 and HEB in tubular epithelial cells. HKC-8 cells were transfected with Myc-tagged Id1 expression vector, and cell lysates were immunoprecipitated with anti-Id1 (C) or anti-HEB (D). Normal IgG was used as a negative control. The precipitates then were immunoblotted with anti-HEB (C) or anti-Myc antibodies. (E) Biotinylated DNA precipitation assay showed a specific binding of HEB to the E-box in the E-cadherin promoter. HEB protein was detectable in the complexes that were precipitated by double-stranded biotinylated DNA that corresponded to the E-box in E-cadherin promoter. For competition experiments, a 100-fold E-box or mutant E-box also was included in the reaction mixture. The sequences of E-box and mutant E-box are shown at the bottom. (F) TGF-β1 reduced the binding of HEB to the E-box in tubular epithelial cells. HKC-8 cells were treated with 2 ng/ml TGF-β1 for various periods of time as indicated. Whole-cell lysates were subjected to biotinylated DNA precipitation assay. (G) Diagram shows the potential mechanism by which Id1 prevents E-protein from binding to the E-box *via* forming a heterodimeric, transcriptionally inactive complex.

inducing all mesenchymal markers in HKC-8 cells, as previously reported (6). These results indicate that Id1 is unable to confer a mesenchymal transition of tubular epithelial cells.

Id1 and ILK Are Independent Downstream Effectors of TGF-β1/Smad Signaling

Because overexpression of ILK also suppresses E-cadherin (10), we sought to investigate any potential relationship between ILK and Id1. As shown in Figure 8, TGF-β1 induced both ILK and Id1 expression in HKC-8 cells. However, overexpression of Id1 did not affect ILK expression (Figure 8A). Conversely, forced expression of ILK did not induce Id1 (Figure 8B). We found that selective inhibition of ILK activity by small-molecule inhibitor KP392 blocked TGF-β1–mediated fibronectin and α-SMA expression but failed to restore E-cadherin expression (Figure 8C). Further studies revealed that inhibition of ILK by KP392 did not abolish TGF-β1–induced Id1 expression (Figure 8D), which may be sufficient for E-cadherin suppression. Together, these data suggest that the loss of E-cadherin after TGF-β1 treatment is mediated by both Id1 and ILK. Because ILK but not Id1 induces mesenchymal transition (10), it seems that Id1 and ILK are two separate and parallel signaling mediators of TGF-β1/Smad that play divergent roles in tubular EMT (Figure 8E).

Tubule-Specific Induction of Id1 in the Fibrotic Kidney

In an effort to address the relevance of Id1 induction to tubular epithelial dedifferentiation *in vivo*, we investigated the expression of Id1 in the fibrotic kidney that was induced by UUO. Similar to *in vitro* situation, Id1 mRNA was induced rapidly and transiently in the obstructed kidney as early as day 1 after UUO (Figure 9, A and B). Id1 protein was increased in the obstructed kidney at day 1 after surgery and sustained to day 14 (Figure 9, C and D). We also examined the localization of Id1 protein in the obstructed kidney by immunofluorescence staining. As shown in Figure 9E, little or no Id1 staining was observed in sham-operated, normal kidney. However, strong Id1 staining was visible in the tubular epithelium of the obstructed kidneys (Figure 9H). Double staining for Id1 (red) and proximal tubular marker (green) revealed that Id1 was localized primarily in the proximal tubules in the obstructed kidney (Figure 9J). Of particular interest, Id1 was increased specifically in the degenerated, dilated tubules with an enlarged lumen (Figure 9J, asterisk). In morphologically intact proximal tu-

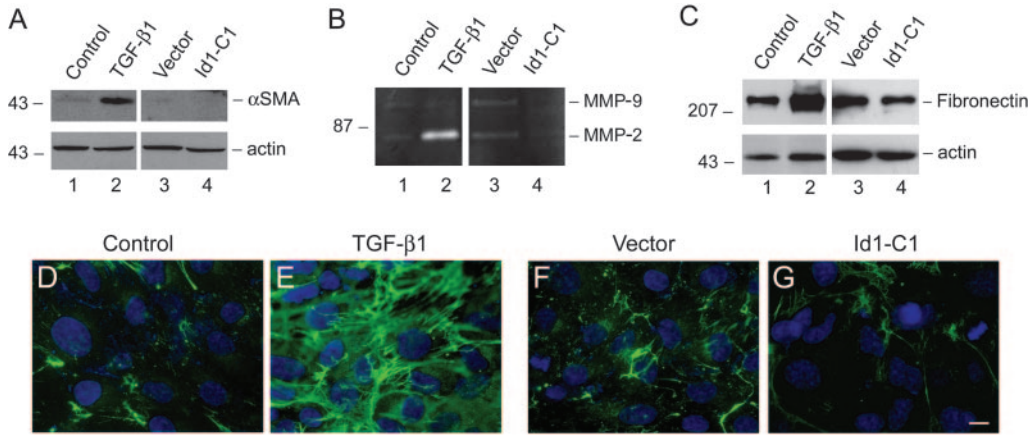

Figure 7. Id1 overexpression does not confer mesenchymal transition of tubular epithelial cells. (A) Overexpression of Id1 did not induce α-smooth muscle actin (α-SMA) expression. Cell lysates that were prepared from the stable cell line that overexpressed Id1 (Id1-C1) or vector control were immunoblotted with antibodies against α-SMA and actin, respectively. HKC-8 cells that were treated without or with 2 ng/ml TGF-β1 for 48 h were used as controls. (B) Zymographic analyses showed that overexpression of Id1 did not induce matrix metalloproteinase-2 expression. (C) Overexpression of Id1 did not induce fibronectin expression. Cell lysates that were prepared from the stable cell line that overexpressed Id1 (Id1-C1) or vector control were immunoblotted with antibodies against fibronectin and actin, respectively. (D through G) Immunofluorescence staining showed fibronectin expression and localization after various treatments. (D) Control HKC-8 cells. (E) HKC-8 cells that were treated with TGF-β1. (F) Stable cell line with empty vector. (G) Stable cell line with Id1 overexpression (Id1-C1). Bar = 5 μm.

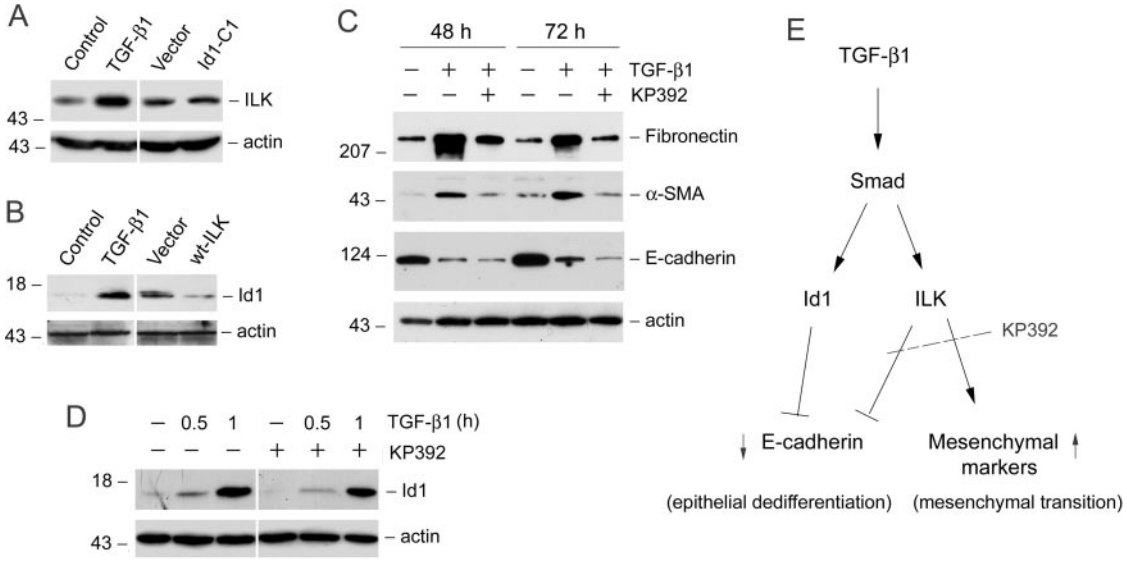

Figure 8. Id1 and integrin-linked kinase (ILK) are independent downstream effectors of TGF-β1/Smad signaling. (A) Id1 did not affect ILK expression in tubular epithelial cells. Cell lysates prepared from the stable cell line that overexpressed Id1 (Id1-C1) or vector control were immunoblotted with antibodies against ILK and actin, respectively. HKC-8 cells treated without or with 2 ng/ml TGF-β1 for 48 h were used as controls. (B) ILK did not induce Id1 expression in tubular epithelial cells. Cell lysates prepared from the stable cell line that overexpressed ILK (wt-ILK) or vector control were immunoblotted with antibodies against Id1 and actin, respectively. (C) Inhibition of ILK activity by small-molecule inhibitor KP392 blocked TGF-β1–mediated fibronectin and α-SMA expression but did not restore E-cadherin expression. HKC-8 cells were treated with TGF-β1 in the absence or presence of KP392 (50 μM) for various periods of time as indicated, and cell lysates were immunoblotted with antibodies against fibronectin, α-SMA, E-cadherin, and actin, respectively. (D) Inhibition of ILK did not abolish TGF-β1–induced Id1 expression. HKC-8 cells were pretreated with KP392 (50 μM) for 30 min, followed by incubation with or without TGF-β1 (2 ng/ml) for 1 h. Cell lysates were immunoblotted with antibodies against Id1 and actin, respectively. (E) Diagram depicts that Id1 and ILK are two independent, parallel signaling mediators of TGF-β1/Smad that play divergent roles in tubular EMT.

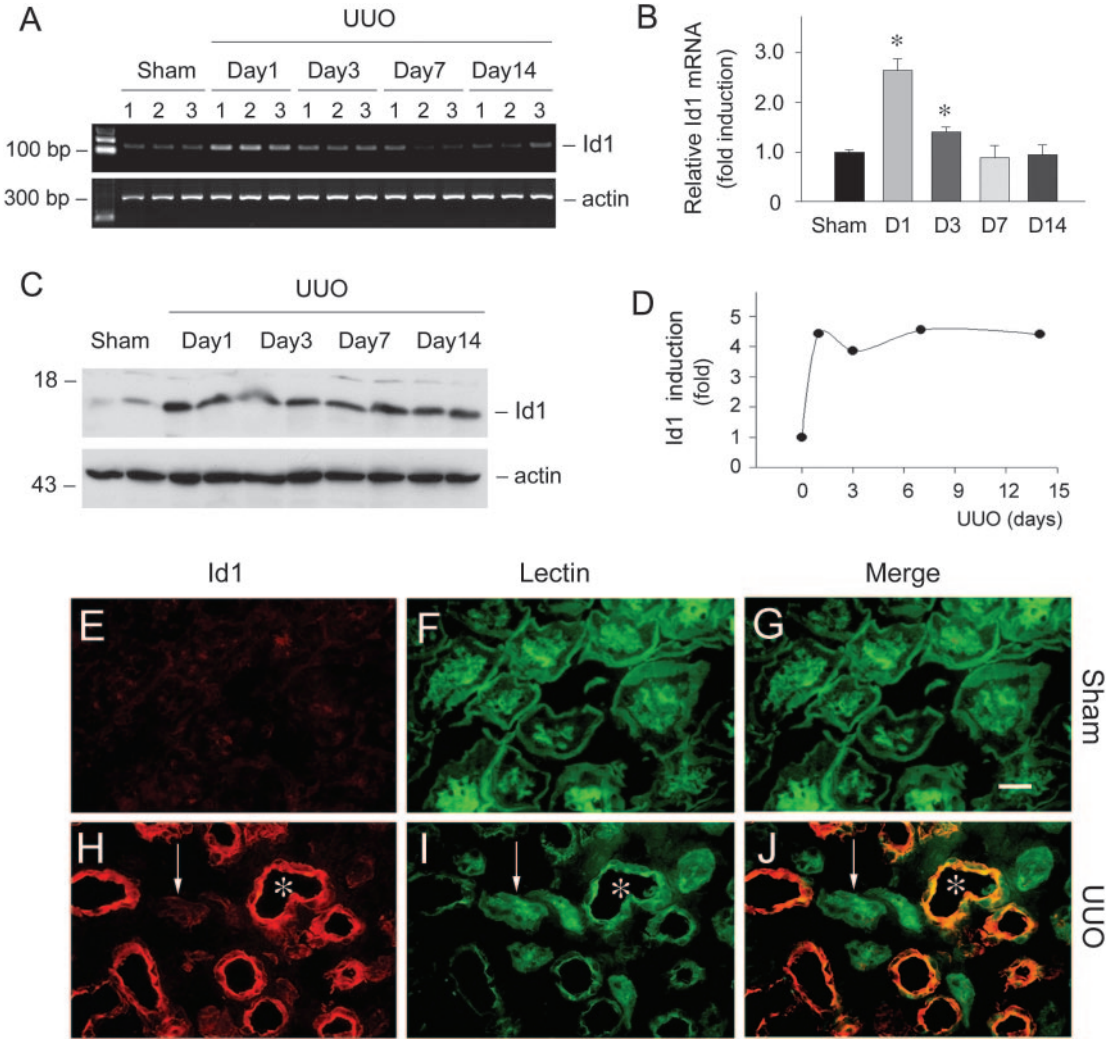

Figure 9. Id1 is induced specifically in the degenerated tubular epithelia of the fibrotic kidney. (A and B) RT-PCR analyses showed an early induction of Id1 mRNA in the fibrotic kidney that was induced by unilateral ureteral obstruction (UUO). RNA samples from three individual animals per time point ($n = 3$) were assessed for the mRNA expression of Id1 and β-actin, respectively. Relative Id1 mRNA levels (fold induction over the sham control) after correction with β-actin are presented (B). *$P < 0.05$ *versus* sham control. (C and D) Western blot analyses revealed an early and sustained induction of Id1 protein in the fibrotic kidney that was induced by UUO. Kidney homogenates were immunoblotted with antibodies against Id1 and actin, respectively. Samples from two representative animals were used at each time point. Relative Id1 protein levels (fold induction over the sham control) after correction with actin are presented (D). (E through J) Immunofluorescence staining displayed Id1 (red) and tubular cell marker lectin (green) localization in the sham kidney (E through G) and obstructed kidneys for 7 d (H through J), respectively. *Id1-positive tubules; arrows indicate renal tubules with negative Id1 staining. Bar = 15 μm.

bules, Id1 essentially was absent in the obstructed kidney (Figure 9J, arrow). These observations strongly suggest that Id1 induction is correlated closely with the dedifferentiation and degeneration of the tubular epithelium *in vivo*.

Discussion

Tubular EMT is an utterly complex process in which many genes could be involved intimately to orchestrate the phenotypic conversion (31,32). Epithelial cell dedifferentiation, manifested by the loss of E-cadherin and ZO-1, is an initial, early step that may be a prerequisite for the tubular cells to endure mesenchymal transition (6). However, the molecular mechanism that governs epithelial dedifferentiation remains poorly understood. Using a microarray gene expression profiling approach, we demonstrated here that Id1 plays a crucial role in E-cadherin and ZO-1 suppression in the early phase of the TGF-β1–mediated EMT. Id1 was induced rapidly and markedly in the tubular epithelial cells after TGF-β1 stimulation *in vitro* (Figure 1) and in the degenerated renal tubules after UUO *in vivo* (Figure 9). Ectopic expression of Id1 suppressed E-cadherin and ZO-1 expression (Figure 3) but did not induce α-SMA, matrix metalloproteinase-2, and fibronectin, markers of mesenchymal transition, in tubular epithelial cells (Figure 7). Therefore, Id1 seems to be responsible merely for tubular epithelial dedifferentiation but not for mesenchymal transition. These findings establish that EMT can be defined into several

phases in which epithelial cell dedifferentiation precedes the acquisition of new mesenchymal features, consistent with a step-wise model of EMT in which various events may be controlled by diverse mechanisms (6).

The induction of Id1 occurs in an immediately early manner, with the mRNA levels peaking at 1 to 3 h after TGF-β1 treatment *in vitro* (Figure 1) and 1 d after UUO *in vivo* (Figure 9). Despite a seemingly transient induction in mRNA level, Id1 protein abundance was sustained for a long period of time *in vivo*, suggesting a prolonged functional impact of Id1 induction on tubular epithelium. The reason behind the discrepancy between the kinetics of Id1 mRNA and protein induction remains unknown at this stage, but it could be related to an altered stability of Id1 protein. It should be noted that several reports demonstrate a repression of Id1 expression by TGF-β1 in different cells (33,34), suggesting that the response of Id1 expression to TGF-β1 is dictated by the particular contents of the cells. Consistent with this notion, recent studies also indicate that TGF-β1 induces Id1 expression in rat hepatic stellate cells and human fetal lung fibroblasts (35,36).

By virtue of suppressing E-cadherin and ZO-1, Id1 functions as a critical player in mediating tubular epithelial dedifferentiation, a common feature in many types of CKD. E-cadherin is an epithelial cell–cell adhesion receptor that plays an indispensable role in epitheliogenesis during embryonic development and in the maintenance of the differentiated state of mature epithelium (8,37). ZO-1, a tight junction-associated protein, also is regarded widely as a caretaker for the epithelial integrity. Therefore, loss of E-cadherin and ZO-1 inevitably would lead to the destabilization of renal epithelium and make tubular cells dissociate from their neighbors and lose polarity. We previously proposed that tubular EMT is a highly regulated, orchestrated process that consists of four key steps: (*1*) loss of epithelial adhesion, followed by (*2*) α-SMA induction and actin cytoskeleton reorganization, (*3*) tubular basement membrane destruction, and (*4*) enhanced migration and invasion (1,6). In support of that notion, our study shows an immediately early induction of Id1 in tubular epithelial cells. This temporal association between early Id1 induction and subsequent E-cadherin suppression accentuates the importance of Id1 in mediating tubular epithelial dedifferentiation. Mechanistically, the suppression of E-cadherin by Id1 is operated by forming a heterodimeric HEB/Id1 complex, thereby preventing HEB from binding to the E-box and sequestering its ability to *trans*-activate E-cadherin gene transcription (Figure 6).

It is of importance to point out that besides Id1, ILK has been demonstrated to play an imperative role in mediating the E-cadherin suppression after TGF-β1 stimulation. Given that ILK induction by TGF-β1 lags behind Id1 expression and both ILK and Id1 induction depend on Smad signaling (10), we initially speculated that Id1 might be involved in mediating ILK expression. However, forced expression of Id1 did not affect ILK expression; *vice versa*, neither ILK overexpression nor inhibition of ILK activity affected Id1 induction by TGF-β1 (Figure 8). These observations clearly establish that Id1 and ILK are two independent, parallel downstream mediators of the TGF-β1/Smad signaling. Nevertheless, we cannot exclude the possibility that Id1 and ILK may be linked at some points along their respective signaling pathways *via* indirect and/or feedback mechanisms. Although the mechanism by which ILK suppresses E-cadherin remains incompletely understood, it is likely to be related to transcription factor Snail. ILK can induce Snail expression (38) and inhibits Snail protein degradation (19,39). Snail possesses the sequence-specific DNA binding ability and competes with bHLH proteins for binding to E-box in the regulatory region of many genes, including E-cadherin (27). Binding of Snail to E-box displaces bHLH proteins and silences E-box–mediated gene transcription. Therefore, both Id1 and ILK/Snail suppress E-cadherin by repressing E-box–mediated gene transcription, but they seem to act *via* distinctive mechanisms.

That both Id1 and ILK suppress E-cadherin suggests that multiple pathways downstream of TGF-β1/Smad could lead to epithelial cell dedifferentiation. However, Id1 and ILK clearly have distinct roles in the process of tubular EMT. After TGF-β1 stimulation, Id1 induction occurs in an immediate-early manner, whereas ILK upregulation requires a prolonged incubation of TGF-β1 (10). More important, unlike Id1, ILK not only suppresses E-cadherin but also induces mesenchymal transition of tubular epithelial cells (10). On the basis of these observations, it is conceivable to propose that Id1 likely plays a leading role in the early phase of E-cadherin suppression, causing epithelial dedifferentiation; however, a complete EMT may depend on the activation and induction of ILK. Along this line, epithelial dedifferentiation may be necessary but certainly is not sufficient for tubular EMT. This notion also is corroborated by many observations in acute renal injury, in which tubular epithelial cells often undergo dedifferentiation but seldom proceed toward EMT and renal fibrotic lesions.

One of the novel findings in this study is that Id1 seems to be induced exclusively in the degenerated and dilated renal tubules in the obstructed kidney (Figure 9). This not only highlights a close correlation between Id1 abundance and epithelial dedifferentiation but also may suggest a potential role of Id1 in the evolution of tubular dilation and perhaps cystic formation. Tubular dilation, characterized by an enlarged lumen with degenerated epithelium, is a common morphologic feature in the tubulointerstitium of many types of CKD. The connection between Id1 induction and tubular degeneration provides a potential explanation for the genesis of tubular dilation under pathologic conditions. In that regard, a recent study indicated that Id2, a member of Id family proteins, binds to polycystin-2 (40). However, whether Id1 also binds to polycystin-2 remains to be determined. Clearly, more studies are needed in this area to characterize further the role of Id1 in the pathogenesis of tubular degeneration and dilation and to delineate better the molecular pathways that lead to tubular epithelial dedifferentiation and mesenchymal transition after chronic injury.

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Disclosures

None.

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