EPITHELIAL AND MESENCHYMAL CELL BIOLOGY

$\alpha 3$ Integrin of Cell-Cell Contact Mediates Kidney Fibrosis by Integrin-Linked Kinase in Proximal Tubular E-Cadherin Deficient Mice

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Loss of E-cadherin marks a defect in epithelial integrity and polarity during tissue injury and fibrosis. Whether loss of E-cadherin plays a causal role in fibrosis is uncertain. $\alpha 3\beta 1$ Integrin has been identified to complex with E-cadherin in cell-cell adhesion, but little is known about the details of their cross talk. Herein, E-cadherin gene (*Cdh1*) was selectively deleted from proximal tubules of murine kidney by *Sglt2Cre*. Ablation of E-cadherin up-regulated $\alpha 3\beta 1$ integrin at cell-cell adhesion. E-cadherin-deficient proximal tubular epithelial cell displayed enhanced transforming growth factor- $\beta 1$ -induced α -smooth muscle actin (α -SMA) and vimentin expression, which was suppressed by siRNA silencing of $\alpha 3$ integrin, but not $\beta 1$ integrin. Up-regulation of transforming growth factor- $\beta 1$ -induced α -SMA was mediated by an $\alpha 3$ integrin-dependent increase in integrin-linked kinase (ILK). Src phosphorylation of β -catenin and consequent p- β -catenin-Y654/p-Smad2 transcriptional complex underlies the transcriptional up-regulation of ILK. Kidney fibrosis after unilateral ureteric obstruction or ischemia reperfusion was increased in proximal tubule E-cadherin-deficient mice in comparison to that of E-cadherin intact control mice. The exacerbation of fibrosis was explained by the $\alpha 3$ integrin-dependent increase of ILK, β -catenin nuclear translocation, and α -SMA/proximal tubular-specific Cre double positive staining in proximal tubular epithelial cell. These studies delineate a nonconventional integrin/ILK signaling by $\alpha 3$ integrin-dependent Src/p- β -catenin-Y654/p-Smad2-mediated up-regulation of ILK through which loss of E-cadherin leads to kidney fibrosis. (*Am J Pathol* 2016, 186: 1847–1860; <http://dx.doi.org/10.1016/j.ajpath.2016.03.015>)

Fibrosis is a key component of parenchymal destruction in all chronic diseases leading to organ failure.¹ Loss of E-cadherin marks a defect in epithelial integrity and polarity during tissue injury and fibrosis, but a causative role for E-cadherin in fibrosis has never been clearly defined. Previous *in vitro* studies in cancer all suggest a protective role for E-cadherin against transforming growth factor (TGF)- β

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signaling in epithelial-mesenchymal transition (EMT),^{2–4} whereas EMT-like TGF- β –induced profibrotic changes (type II EMT) in epithelial cells contribute to organ fibrosis.^{5,6} In contrast to previous findings, E-cadherin was recently reported to assist $\alpha 3\beta 1$ integrin–mediated profibrotic changes and fibrosis.⁷ More important, the controversial roles of E-cadherin have not been tested *in vivo* in fibrotic disease in which the EMT differs from that of cancer and development.^{5,8}

E-cadherin is a single-pass transmembrane cell-cell adhesion molecule that participates in the formation of adherens junctions, and other epithelial cell connections, including tight junctions.^{9,10} In addition to its major role in the establishment and maintenance of epithelial cell polarity, E-cadherin has been implicated in transmembrane signal transduction.^{11,12} Integrins, such as $\alpha 3\beta 1$, are primarily responsible for cell-matrix interaction at the basal side of epithelia¹³ and have also been implicated in E-cadherin–mediated cell-cell adhesion.^{14,15} More important, integrin activation imparts growth factor receptor–mediated intracellular signal transduction.^{16,17} $\alpha 3$ Integrin null lung epithelial cells did not undergo profibrotic changes in response to TGF- β ,⁷ whereas mice without lung epithelial $\alpha 3$ integrin were protected against bleomycin-induced fibrosis.^{18,19} In contrast to previous findings, E-cadherin was suggested to assist $\alpha 3\beta 1$ integrin–dependent profibrotic changes.⁷ Nevertheless, the details of the cross talk between E-cadherin and $\alpha 3\beta 1$ integrin in the cell-cell adhesion compartment remain unclear. Although integrin-linked kinase (ILK) has been proved to be a key mediator of TGF- β –induced fibrosis,^{20–22} the reported profibrotic role of $\alpha 3$ integrin has never been linked to ILK. Little is known about how ILK expression is up-regulated by TGF- β in fibrosis. More important, whether E-cadherin or loss of E-cadherin contributes to TGF- β –induced fibrosis remains uncertain.

By using the Cre-loxP system,²³ E-cadherin has been conditionally depleted in several types of epithelial cell. Previous studies mainly focused on roles of E-cadherin in tissue differentiation and maintenance of cell-cell adhesion junctions,²⁴ whereas the impact of loss of E-cadherin on tissue fibrosis has not been examined. Herein, we inactivated the E-cadherin gene (*Cdh1*) specifically in murine kidney proximal tubules using *Sgt2Cre*²⁵ to explore the role of E-cadherin in kidney fibrosis in the context of unilateral ureteral obstruction (UUO). We show that loss of E-cadherin results in the up-regulation of $\alpha 3\beta 1$ integrin in the proximal tubule, whereas kidney morphology, expression of cell adhesion factors, such as N-cadherin and zonular occludens 1 (ZO-1), and kidney function are not changed. However, kidney fibrosis is exacerbated in the proximal tubule-specific E-cadherin–deficient mice after UUO. This is explained by an up-regulation of $\alpha 3\beta 1$ integrin at cell-cell adhesion of E-cadherin–deficient proximal tubules, and an $\alpha 3$ integrin–dependent enhancement of TGF- β –induced profibrotic changes via p- β -catenin-Y654/p-Smad2 up-regulation of ILK.

Materials and Methods

Ethics Statement

All animal experiments described herein were approved by the Animal Ethics Committee of Western Sydney Local Health District and performed in strict accordance with guidelines of the committee.

Generation of Conditional E-Cadherin Knockout Mouse

Mice with conditional knockout of E-cadherin in renal proximal tubular epithelial cells (PTECs) only were generated by crossing B6.129-*Cdh1*^{tm2Kem/J} (*E-cad*^{flox/flox}) mice sequentially with B6.129-*CMV*^{Cre} (*CMV.Cre*), B6.D2-*Sgt2*^{Cre} (*Sgt2.Cre*) mice²⁵ and then backcrossed to the *E-cad*^{flox/flox} mice (Supplemental Figure S1). B6.129-*Cdh1*^{tm2Kem/J} (*E-cad*^{flox/flox}) mice, which have the E-cadherin (*Cdh1*) gene (exon 6 to 10) flanked by two loxP sequence, were purchased from Jackson Laboratories (Bar Harbor, ME). B6.D2-*Sgt2*^{Cre} (*Sgt2.Cre*) mice specifically express *Cre* recombinase in kidney proximal tubule. B6.129-*CMV*^{Cre} (*CMV.Cre*) mice carrying an X chromosome-linked *Cre* driven by human cytomegalovirus minimal promoter were used as a general deleter. *E-cad*^{flox/flox} mice were first crossed with *CMV.Cre* to generate *E-cad*^{-/+}; *CMV.Cre*. The X-linked *CMV.Cre* was then removed by brother-sister crossing between *E-cad*^{-/+}; *CMV.Cre* and *E-cad*^{flox/+} mice to generate *E-cad*^{-/+} mice. *E-cad*^{-/+} mice were then crossed with *Sgt2.Cre* to generate *E-cad*^{-/+}; *Sgt2.Cre* phenotype. The proximal tubule depletion of E-cadherin (*E-cad*^{-flox}; *sgt2.Cre*: mutant, knockout) was achieved by back-crossing the *E-cad*^{-/+}; *Sgt2.Cre* with *E-cad*^{flox/flox} mice. *E-cad*^{+flox}; *Sgt2.Cre* littermates were used as control. By this method, selective depletion of E-cadherin from PTECs was achieved and the chance of a mosaic phenotype was minimized. Conditional E-cadherin–deficient and littermate control animals were on a mixed C57BL/6/129 genetic background.

Genotyping

The genotypes were determined with PCR primer sets: oIMR9338, 5'-GGGTCTCACCGTAGTCCTCA-3', and oIMR9339, 5'-GATCTTTGGGAGAGCAGTCG-3' for detection of *Cdh1*^{flox} allele (Supplemental Figure S1C); *cre* forward primer, 5'-TCCTGGAAAATGCTTCTGTCCG-3', and *cre* reverse primer, 5'-CAGGGTGTTATAAGCAA-TCCC-3', for detection of *Sgt2 cre* allele (Supplemental Figure S1B); del forward primer, 5'-GAATTCTGAACAT-CATTATCAGTATTTA-3', and del reverse primer, 5'-TGACACATGCCTTTACTTTAGT-3', for detection of E-cadherin deletion allele (*E-cad*⁻) (Supplemental Figure S1A), and *CMV cre* forward primer, 5'-CTGACCGTACACCA-AAATTTGCCTG-3', and *CMV cre* reverse primer, 5'-GAT-AATCGCGAACATCTTCAGGTTC-3', for detection of *CMV.cre* allele.

UUO Model

UUO was performed by left proximal ureteral ligation under anesthesia, as described elsewhere.²⁶ UUO mice were sacrificed 2 weeks after UUO. Both obstructed and contralateral unobstructed kidneys from PTEC *E-cad*^{-/-} and littermate control mice were harvested.

IR Model

Unilateral kidney ischemia reperfusion (IR) was induced by clamping left renal pedicles for 45 minutes under anesthesia (2% isoflurane) of both PTEC *E-cad*^{-/-} and control mice. Mice were sacrificed to collect kidney tissue 3 weeks after IR.

Renal Proximal Tubular Epithelial Cell Culture and Treatment

Kidneys were excised, the capsules were removed, and washed in cold Dulbecco's modified Eagle's medium:Ham's F12; 1:1 v/v (Invitrogen, Carlsbad, CA). Primary tubular epithelial cells were obtained from the cortex of mouse kidney using established methods.²⁷ The PTEC was further purified by cell sorting using antimegalin antibody. Isolated primary PTECs were cultured in complete K1 medium [Dulbecco's modified Eagle's medium:F12 containing 25 μ g/mL of epidermal growth factor (Sigma, St. Louis, MO), 25 μ mol/L of HEPES, and hormone mixture] with 5% fetal calf serum (Invitrogen) at 37°C with 5% CO₂ for 24 hours, and washed and cultured in serum-free complete K1 medium for 2 to 3 days before treatment with TGF- β 1. In experiments using TGF- β 1 treatment, subconfluent cultures of PTECs were rinsed with phosphate-buffered saline (PBS), the corresponding serum-supplemented media were replaced with respective serum-free media, and then treated with 5 ng/mL TGF- β 1 (Invitrogen) for 48 hours.

Plasmid DNA and siRNA Transfection

E-cadherin expression construct (wild-type *E-cad*) was made by cutting mouse *E-cadherin* ORF from plasmid moEcad/BIISK1 (BglII) with *EcoR* V/NotI and inserting into the corresponding sites of pIRESneoGL2. Integrin α 3 siRNA (sc-37120), integrin β 1 siRNA (sc-35675), ILK siRNA (sc-35667), and control siRNA (sc-44230) were purchased from Santa Cruz Biotechnology (Dallas, TX). Plasmid DNA or siRNA transfection of 60% to 80% confluent cells was performed using Lipofectamine LTX and Lipofectamine RNAiMAX (Invitrogen), respectively, following the manufacturer's instructions. Medium was changed after 6 to 8 hours. Cells were analyzed 48 to 72 hours after transfection.

Antibodies

Mouse Lrp2/megalin polyclonal antibody, rabbit anti-*E-cadherin* monoclonal antibody, rabbit anti- β -catenin

antibody, rabbit anti-ZO-1 antibody, mouse anti-vimentin, rabbit anti- α -smooth muscle actin (α -SMA) antibody, and rabbit phospho-glycogen synthetase kinase-3 β (Ser9) antibody were purchased from Abcam (Cambridge, MA). Mouse anti-actin polyclonal antibody, rat anti-integrin β 1 antibody, and rabbit anti-integrin α 3 monoclonal antibody were from Millipore (Darmstadt, Germany). Mouse anti-fibronectin was from Santa Cruz Biotechnology. Rabbit anti-ILK antibody, anti-mouse IgG, and anti-rabbit IgG, horseradish peroxidase-linked antibodies, and rabbit Cre monoclonal antibody were from Cell Signaling Technology (Danvers, MA), Alexa Fluor 546 goat anti-mouse IgG (H+L), and Alexa Fluor 488 goat anti-rabbit IgG (H+L) were from Invitrogen.

Coimmunoprecipitation

Nuclear proteins were extracted using a NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific, Waltham, MA), according to the manufacturer's instructions, and then quantified by BCA assay kit (Thermo Scientific). Coimmunoprecipitation (Co-IP) was performed using the Protein-G Immunoprecipitation Kit (Invitrogen). Briefly, 2 μ g of affinity-purified antibody was incubated with 50 μ L Protein-G agarose beads for 10 minutes at room temperature, after three times washes with antibody bind/wash buffer, 800 μ g nuclear proteins were added for incubation at 4°C overnight. The agarose beads were collected by pulse centrifugation, the supernatant was discarded, and beads were washed three times with 200 μ L IP wash buffer. Finally, immunoprecipitated proteins were subjected to SDS-PAGE followed by Western blot with indicated primary antibody. Western blot analyses of nuclear proteins served as input.

Western Blot Analysis

Cells were lysed in Tris glycine SDS sample buffer (Gradipore, Frenchs Forest, Australia), and cell extracts were separated by NuPAGE 4% to 12% Bis-Tris gel (Invitrogen). Proteins were then transferred to polyvinylidene difluoride membranes (Millipore), blocked with 5% skim milk in PBS, and probed with corresponding primary antibodies overnight at 4°C. Membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 40 minutes at room temperature. Proteins were finally visualized using enhanced chemiluminescence reagents (Amersham Bioscience, Piscataway, NJ). Protein expression was measured with Gel-Pro Analyzer image software version 3.1 (Informer Technologies, Madrid, Spain; <http://gel-pro-analyzer.software.informer.com/3.1>) by quantifying the relative expression of target protein versus β -actin.

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation assays were performed using a kit from Cell Signaling Technology, according to

the protocol supplied. In brief, cells were fixed with 1% formaldehyde in PBS to cross-link chromatin. Cell lysates were prepared and digested by micrococcal nuclease to break chromatin DNA, ranging approximately 150 to 900 bp on average. After a preclearing step, IP was performed at 4°C overnight with anti-smad2 antibody or normal rabbit IgG (negative control antibody). Immune complexes were collected with protein-G magnetic beads. After extensive washing, the immunoprecipitated complexes were eluted and protein-DNA cross-links were reversed by incubating at 65°C for 2 hours. DNA was purified using proteinase K digestion and collected using DNA Clean-Up Column. For the smad binding site at position 247 of the mouse ILK promoter, the 219 and 289 region was amplified by real-time PCR with the primer pairs set: 5'-GCTGACACCA-CTCGGGAAC-3' (forward) and 5'-AGAGGTGTCAGT-GTGGGATG-3' (reverse). The amount of ILK promoter that was present in the IP and input fractions was calculated from the standard curve. The input represents 2% of the material used in the IP assay. The results were expressed as the IP/input ratios of the PCR products that were used for comparison.

Histology and Immunohistochemistry

For histology and immunohistochemistry, the kidneys were fixed in 10% neutral buffered formalin and embedded in paraffin. Microtome sections (4 to 5 µm thick) were deparaffinized, rehydrated, and blocked with 3% H₂O₂ in methanol (v/v) for 8 minutes. Antigen retrieval was performed using antigen deloaker (Biocare Medical, Concord, CA) for 10 minutes at 95°C for fibronectin and vimentin staining. For frozen kidney samples, cryosections were fixed for 10 minutes with cold methanol. Kidney tissue sections were blocked with 2% bovine serum albumin in PBS for 1 hour at room temperature and subjected to staining with primary antibodies. The sections were washed three times with PBS with 0.1% Tween-20 before incubation with their respective horseradish peroxidase-conjugated secondary antibodies in 2% bovine serum albumin in PBS for 40 minutes at room temperature or 4°C overnight. Antibody binding was visualized with 3'-diaminobenzidine (Dako) and sections were counterstained with hematoxylin (Sigma). To assess interstitial fibrosis, Gomori trichrome and Sirius Red staining were performed.

For quantitation, stained specimens were scanned and analyzed with ScanScope digital scanner (Aperio, Oakleigh, Australia).

Immunofluorescence Staining

For indirect immunofluorescence staining, cells cultured on coverslips or kidney cryosections were fixed with cold methanol for 10 minutes at -20°C, then blocked with 2% bovine serum albumin in PBS for 1 hour at room temperature, followed by incubation with primary antibodies for 1

hour at room temperature or overnight at 4°C. After washing with PBS, cells or cryosections were incubated with Alexa Fluor-488 or Texas Red conjugated secondary antibodies for 40 minutes at room temperature. They were then washed with PBS and counterstained with DAPI (Invitrogen) for 5 minutes before mounting with fluorescent-mounting medium (Vector Laboratories, Burlingame, CA). Images were visualized and captured by a fluorescence microscope (Olympus BX51) or a Delta Vision microscope. A minimum of 10 consecutive nonoverlapping fields were taken at ×200 magnification for each slide.

Electron Microscopy

Kidney specimens were fixed in modified Karnovsky's fixative buffer and 0.1 mL/L 3-morpholinopropane-1-sulfonic acid buffer and then embedded in epoxy resin. Semithin and ultrathin sections were stained with uranyl acetate and lead citrate. All samples were examined in a Philips CM120 BioTWIN transmission EM (FEI) operated at 80 kV. Images were acquired using an SIS Morada digital camera (Soft Imaging Systems, Berlin, Germany) using the iTEM software suite.

Renal Function Assay

Renal function was assessed by the measurement of serum creatinine and urine albumin, creatinine, total protein, K⁺, Na⁺, pH, and glucose, as described previously.²⁸ All urine and blood specimens were examined by the Institute of Clinical Pathology and Medical Research (Westmead Hospital).

Creatinine clearance (µL/minute) = (urine creatinine/serum creatinine) × urine volume (µL)/[time (hours) × 60].

Real-Time Quantitative RT-PCR

RNA was extracted from primary cultured PTECs using RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was synthesized using total RNA by Superscript First strand synthesis system (Invitrogen). Real-Time Quantitative PCR was performed using following primer pairs: SNAIL 1, 5'-CTTGTGTCTGCACGACCTGT-3' (forward) and 5'-CTTCACATCCGAGTGGGTTT-3' (reverse); integrin α3, 5'-CCTCTTCGGCTACTCGGTC-3' (forward) and 5'-CCGGTTGGTATAGTCATCACCC-3' (reverse); integrin β1, 5'-ATGCCAAATCTTGCGGAGAAT-3' (forward) and 5'-TTTGCTGCGATTGGTGACATT-3' (reverse); β-actin, 5'-GATTACTGCTCTGGCTCCTAGCA-3' (forward) and 5'-GCCACCGATCCACACAGAGT-3' (reverse); collagen I, 5'-GCGAAGGCAACAGTCGCT-3' (forward) and 5'-CTTGGTGGTTTTGTATTTCGATGAC-3' (reverse), and SYBR Green mastermix (Invitrogen) in Corbett Rotorgene 3000 Real-Time Thermo cycler. Housekeeping gene β-actin was used as endogenous control.

Statistical Analysis

Results from at least three independent experiments were expressed as means $\pm$ SD. A *t*-test was used to determine the significance of differences between two groups, whereas the one-way analysis of variance was used for comparison of multiple groups. $P < 0.05$ was considered significant.

Results

Depletion of E-Cadherin by *Sglt2.Cre* Activity in PTECs

PTEC-specific deletion of E-cadherin was generated by crossing *E-cad^{fllox/fllox}* (B6.129-*Cdh1^{tm2Kem/J}*) mice sequentially with general deleter *CMV.Cre* (B6.129-*CMV^{Cre}*) mice and PTEC-specific *Sglt2.Cre* (B6.D2-*Sglt2^{Cre}*) mice and then

backcrossing to *E-cad^{fllox/fllox}* mice (Supplemental Figure S2). To prove ablation of E-cadherin in PTEC in conditional E-cadherin mutant mice (referred to hereafter as PTEC *E-cad^{-/-}* mice), E-cadherin expression was examined by indirect immunofluorescence and Western analysis (Figure 1) in primary cultures of PTECs and histological sections of control (*E-cad^{+/+}*; *Sgt2.Cre*) and PTEC *E-cad^{-/-}* (*E-cad^{fllox}*; *Sgt2.Cre*) mice. E-cadherin expression was not detected in PTECs and kidney proximal tubules (Figure 1A) of PTEC *E-cad^{-/-}* mice. Western blot analysis indicated a significant reduction (>90%) of E-cadherin in PTECs isolated from PTEC *E-cad^{-/-}* mice (Figure 1, B and C).

E-Cadherin Depletion in PTEC Up-Regulates Expression of Integrin $\alpha 3 \beta 1$

Integrin $\alpha 3 \beta 1$ is a critical component of E-cadherin-mediated cell-cell contact.¹⁵ To determine the effect of E-cadherin depletion on integrin $\alpha 3 \beta 1$, confocal microscopy, indirect immunofluorescence, and Western blot were performed. A stronger lateral membrane staining for integrin $\alpha 3$ and $\beta 1$ was shown clearly by XZ sections of their immunofluorescence staining in cultured *E-cad^{-/-}* PTECs compared with control PTECs (Figure 2A). $\alpha 3$ Integrin colocalized with E-cadherin in cultured PTECs of control mice (Figure 2A). The immunofluorescence staining demonstrated that depletion of E-cadherin up-regulated both $\alpha 3$ and $\beta 1$ integrin at the lateral membrane. This may serve to compensate for loss of E-cadherin to maintain cell-cell adhesion. Consistent with these immunofluorescence results, Western blot and real-time RT-PCR analysis demonstrated a significant increase of $\alpha 3$ and $\beta 1$ integrin protein and transcripts in *E-cad^{-/-}* PTECs compared with control

(Figure 2, B–E). To determine whether E-cadherin depletion caused the up-regulation of integrin $\alpha 3 \beta 1$, we forced the expression of E-cadherin (wild-type E-cad) in control and *E-cad^{-/-}* PTECs. After transfection with wild-type E-cad, increased $\alpha 3$ and $\beta 1$ integrin protein levels in *E-cad^{-/-}* PTECs were reduced (Figure 2, F and G), and their protein levels in control PTECs were also reduced. This result confirmed that depletion of E-cadherin in PTECs caused the up-regulation of integrin $\alpha 3 \beta 1$ expression.

Effect of E-Cadherin Depletion on β -Catenin, ZO-1, and N-Cadherin Levels in PTECs

To determine if loss of E-cadherin affects other adhesion molecules of cell-cell junctions, expression of β -catenin, ZO-1, and N-cadherin was examined by Western blot and immunofluorescence staining in cultured control and *E-cad^{-/-}* PTECs (Figure 3). Expression and localization of ZO-1, an epithelial tight junction molecule, were unaffected by loss of E-cadherin (Figure 3, A–C). N-cadherin is normally expressed by PTECs,²⁹ and loss of E-cadherin is often accompanied by a cadherin switch to N-cadherin during EMT.³⁰ We found that N-cadherin expression shown by both immunofluorescence staining (Figure 3A) and Western blot (Figure 3, B and C) was not affected by the loss of E-cadherin, and there was no cadherin switch. β -Catenin is the direct binding partner of E-cadherin intracellular domain anchoring E-cadherin to the actin cytoskeleton. Depletion of E-cadherin did not alter overall protein level of β -catenin (Figure 3, B and C); immunofluorescence showed a membrane staining pattern of β -catenin in cultured *E-cad^{-/-}* PTECs slightly different from strong membrane staining in control PTECs (Figure 3A).

Figure 3 Effect of E-cadherin deletion on β -catenin, zonula occludens 1 (ZO-1), and N-cadherin expression in proximal tubular epithelial cell (PTEC). **A:** Immunofluorescence staining of cultured control and *E-cad^{-/-}* PTEC stained for β -catenin (green), ZO-1 (green), N-cadherin (green), and PTEC marker megalin (red). **B** and **C:** Western blot analysis and quantification of β -catenin, ZO-1, and N-cadherin (N-cad) in lysates of cultured control and *E-cad^{-/-}* PTEC. Data are shown as means $\pm$ SD (**C**). $n = 3$ (**C**). Scale bar = 20 μ m (**A**).

Figure 4 Deletion of E-cadherin does not have significant impact on the ultrastructure or morphology of proximal tubular epithelial cell (PTEC) and kidney function. **A:** Representative images of renal histology of control and PTEC *E-cad*^{-/-} mice aged 3 to 5 months. Gomori trichrome (GT), periodic acid Schiff (PAS), and Sirius Red staining (SR). **B and C:** Electron microscopic images and quantification of tight junction (arrows) width in kidney tissue of control and PTEC *E-cad*^{-/-} mice aged 3 to 5 months. **D:** Kidney function of control and PTEC *E-cad*^{-/-} mice aged 3 to 5 months was determined by creatinine clearance (CrCl), urinary albumin and protein, urinary protein/creatinine, urine pH, and urine Na⁺ and K⁺ excretion. Data are shown as means ± SD (**C** and **D**). *n* = 3 (**C** and **D**). Scale bars: 100 μm (**A**); 1000 nm (**B**).

Depletion of E-Cadherin in PTECs Does Not Have Significant Impact on the Ultrastructure and Morphology of PTECs and Kidney Function *in Vivo*

To examine the potential effects of E-cadherin depletion in PTECs on structure of proximal tubules and kidney histology, electron microscopy and histochemistry were performed. Electron microscopy, Gomori trichrome staining (extracellular matrix proteins), periodic acid-Schiff staining (basement membranes, glycoproteins), and Sirius Red staining (collagen) revealed no obvious changes in ultrastructure of kidney proximal tubules. The electron microscopy images showed no obvious difference in tight

junctions and microvilli between the PTECs of *E-cad*^{-/-} and control mice (Figure 4B), suggesting that loss of PTEC polarity in *E-cad*^{-/-} mice is unlikely.^{31,32} No changes were found in ECM staining of kidney between PTEC *E-cad*^{-/-} and control mice aged 3, 4, or 5 months (Figure 4, A and C). Urine and serum analysis was also performed to examine kidney function of PTEC *E-cad*^{-/-} mice (Figure 4D). No significant changes were observed in creatinine clearance, urinary albumin, total protein, Na⁺ and K⁺ excretion, urinary protein/creatinine ratio, and urine pH in PTEC *E-cad*^{-/-} mice compared with control mice aged 3, 4, or 5 months. Glucose was undetectable in urine samples collected from both PTEC *E-cad*^{-/-} and control mice. Thus,

Figure 5 Deletion of E-cadherin in proximal tubular epithelial cell (PTEC) enhances unilateral ureteral obstruction (UUO)-induced kidney fibrosis. **A:** Kidney histology and immunohistochemical staining for Gomori trichrome (GT), Sirius Red (SR), and fibronectin expression of control and PTEC *Ecad*^{-/-} mice after UUO. Quantitation of the staining data. **B:** Immunohistochemical staining and quantification of α -SMA and vimentin in kidney of control and PTEC *Ecad*^{-/-} mice after UUO. Data are shown as means $\pm$ SD (**A** and **B**). $n = 6$ (**A** and **B**). ** $P < 0.01$. Scale bar = 200 μ m (**A** and **B**).

depletion of E-cadherin by itself did not significantly alter the structure of proximal tubules and the kidney functions examined. No fibrosis was observed after loss of E-cadherin in PTECs.

Depletion of E-Cadherin in PTEC Enhances UUO-Induced Kidney Fibrosis

Lack of structural and functional changes after PTEC deletion of E-cadherin in normal mice led us to investigate the effect of E-cadherin depletion on fibrosis, using a classic murine model of kidney fibrosis, UUO, which is characterized by selective up-regulation of TGF- β 1 and profibrotic changes in the PTEC.^{33,34} UUO was performed in PTEC *Ecad*^{-/-} and control mice. We found that after UUO, kidney fibrosis was much more severe in PTEC *Ecad*^{-/-} mice compared with that in littermate controls (Figure 5). Gomori trichrome staining and Sirius Red staining for extracellular matrix were significantly more intense in kidney cortex of PTEC *Ecad*^{-/-} mice compared with those of control mice after UUO (Figure 5A). Immunohistochemical staining for fibronectin (Figure 5A), α -SMA, and vimentin (Figure 5B) was also significantly more intense in kidney cortex of PTEC *Ecad*^{-/-} mice.

Depletion of E-Cadherin in PTEC Enhances Integrin α 3-Dependent TGF- β 1-Induced Profibrotic Processes

To address the mechanism underlying increased kidney fibrosis in PTEC *Ecad*^{-/-} mice after UUO, we mimicked the *in vivo* environment of kidney fibrosis by treatment of primary cultures of PTECs from control and PTEC *Ecad*^{-/-} mice with TGF- β 1, a key profibrotic cytokine^{35,36} up-regulated in PTECs after UUO.³⁷ Immunofluorescence staining and Western blot analysis showed that the expression of both α -SMA and vimentin was significantly increased after TGF- β 1 treatment in *Ecad*^{-/-} PTECs compared with control PTECs (Figure 6, A–D). Knock-down of integrin α 3 using RNA interference (Supplemental Figure S3A) resulted in less

α -SMA expression in TGF- β 1-treated *Ecad*^{-/-} PTECs (Figure 6E). This result demonstrated that the increased α -SMA expression of *Ecad*^{-/-} PTECs depends on up-regulation of integrin α 3. However, silencing of integrin β 1 (Supplemental Figure S3B) failed to block the increase of α -SMA expression (Figure 6F). Moreover, TGF- β 1 induction of α -SMA in *Ecad*^{-/-} PTECs was further increased by β 1 integrin siRNA (Figure 6F). The contrasting results from selective knock-down of the two subunits of α 3 β 1 integrin demonstrated a dependence on α 3 but not β 1 integrin in TGF- β 1-induced profibrotic changes in *Ecad*^{-/-} PTECs. Taken together, loss of E-cadherin in kidney PTECs increased TGF- β 1-induced profibrotic processes that were dependent on up-regulation of α 3 integrin.

ILK Mediates Integrin α 3-Dependent Enhancement of TGF- β 1-Induced Profibrotic Processes in *Ecad*^{-/-} PTEC

ILK and its downstream signaling have been reported to regulate TGF- β 1-induced profibrotic processes and fibrosis.^{20,22} Moreover, involvement of other major intracellular pathways (including Rho family GTPases, MEK, p38 kinase, c-Jun NH2-terminal kinase, PI3K, and PKC) has been excluded from α 3 β 1 integrin signaling.³⁸ We therefore examined whether ILK and its downstream signaling are involved in the α 3 integrin-dependent profibrotic changes after loss of E-cadherin. Western blot analysis (Figure 7A) showed that expression of ILK was higher in TGF- β 1-treated *Ecad*^{-/-} PTECs than controls. By RT-PCR, the expression of Snail1, a key profibrotic transcription factor targeted by ILK through β -catenin, was also significantly increased in TGF- β 1-treated *Ecad*^{-/-} PTECs (Figure 7B). The increase of Snail1 expression in *Ecad*^{-/-} PTECs was accompanied by an increase of TGF- β 1-stimulated collagen I transcription, showing increased ECM production by *Ecad*^{-/-} PTECs (Figure 7B). Given the evidence of α 3 integrin-dependent

Figure 6 Deletion of E-cadherin in proximal tubular epithelial cell (PTEC) enhances integrin $\alpha 3$ -dependent transforming growth factor (TGF)- β 1-induced epithelial-mesenchymal transition. **A** and **B**: Immunofluorescence staining and quantification of α -smooth muscle actin (α -SMA; green), vimentin (green), and PTEC marker megalin (red) in cultured control and *E-cad*^{-/-} PTEC, treated or not with 5 ng/mL TGF- β 1 for 48 hours. TGF- β 1 induces a significantly greater number of α -SMA or vimentin staining cells in *E-cad*^{-/-} PTEC compared with control. **C** and **D**: Western blot analysis of α -SMA and vimentin in lysates of cultured control and *E-cad*^{-/-} PTEC, treated or not with 5 ng/mL TGF- β 1 for 48 hours. **E**: Western blot analysis and quantification of α -SMA in lysates of cultured control and *E-cad*^{-/-} PTEC transiently transfected with 60 pmol/mL control or 60 pmol/mL integrin $\alpha 3$ siRNA, with or without 5 ng/mL TGF- β 1 treatment for 48 hours. Integrin $\alpha 3$ siRNA significantly reduces TGF- β 1-induced α -SMA in *E-cad*^{-/-} PTEC. **F**: Western blot analysis and quantification of α -SMA in lysates of cultured control and *E-cad*^{-/-} PTEC transiently transfected with 60 pmol/mL control or 30 or 60 pmol/mL integrin $\beta 1$ siRNA, with or without 5 ng/mL TGF- β 1 treatment for 48 hours. Integrin $\beta 1$ siRNA did not reduce but increased TGF- β 1-induced α -SMA in *E-cad*^{-/-} PTEC. Data are shown as means $\pm$ SD (**B** and **D–F**). *n* = 4 (**B**); *n* = 3 (**D–F**). **P* < 0.05, ***P* < 0.01. Scale bar = 20 μ m (**A** and **B**).

increase of TGF- β 1-induced profibrotic processes in *E-cad*^{-/-} PTECs, we next tested whether $\alpha 3$ integrin mediates the profibrotic changes through up-regulation of ILK by siRNA knockdown of $\alpha 3$ and $\beta 1$ integrins. Silencing of $\alpha 3$ but not $\beta 1$ integrin significantly reduced TGF- β 1-induced up-regulation of ILK in control and *E-cad*^{-/-} PTECs (Figure 7C). To confirm $\alpha 3$ integrin-dependent up-regulation of ILK is responsible for the enhanced TGF- β 1-induced profibrotic processes in *E-cad*^{-/-} PTECs, we performed siRNA silencing of ILK to control and *E-cad*^{-/-} PTECs (Supplemental Figure S3C). TGF- β 1-induced α -SMA protein expression in *E-cad*^{-/-} PTECs was

significantly higher than in control PTECs, and ILK siRNA reduced the α -SMA protein in *E-cad*^{-/-} PTECs to the level of control PTECs (Figure 7D), proving that the enhancement of TGF- β 1-induced profibrotic processes in *E-cad*^{-/-} PTECs is ILK dependent.

Src Phosphorylation of β -Catenin and p- β -Catenin-Y654/Smad2 Transcriptional Complex Mediates $\alpha 3$ -Integrin-Dependent Up-Regulation of ILK

To explore an $\alpha 3$ but not $\beta 1$ integrin-dependent up-regulation of ILK, we examined Src kinase activity, which

Figure 7 ILK mediates integrin $\alpha 3$ -dependent enhancement of transforming growth factor (TGF)- $\beta 1$ -induced profibrotic processes in *Ecad*^{-/-} proximal tubular epithelial cell (PTEC). **A:** Western blot analysis and quantification of ILK in lysates of cultured control and *E-cad*^{-/-} PTEC, treated or not with 5 ng/mL TGF- $\beta 1$ for 48 hours. TGF- $\beta 1$ treatment induces significantly higher levels of ILK protein in *E-cad*^{-/-} PTEC compared with control. **B:** Real-time RT-PCR analysis of Snail 1 and collagen I expression in cultured control and *Ecad*^{-/-} PTEC, treated or not with 5 ng/mL TGF- $\beta 1$ for 48 hours. TGF- $\beta 1$ -induced increases of both Snail1 and collagen I mRNA levels are significantly higher in *E-cad*^{-/-} PTEC compared with control. **C:** Western blot analysis and quantification of ILK in lysates of cultured control and *E-cad*^{-/-} PTEC, transfected with integrin $\alpha 3$ or $\beta 1$, or 60 pmol/mL control siRNA with or without TGF- $\beta 1$ treatment for 24 hours. Integrin $\alpha 3$ but not $\beta 1$ siRNA significantly reduces TGF- $\beta 1$ -induced ILK in both control and *E-cad*^{-/-} PTEC. **D:** Western blot analysis and quantification of α -SMA in lysates of cultured control and *E-cad*^{-/-} PTEC, transfected with ILK, or 60 pmol/mL control siRNA with or without TGF- $\beta 1$ treatment. Data are shown as means $\pm$ SD (A–D). *n* = 3 (A–D). **P* < 0.05, ***P* < 0.01.

was reported downstream of $\alpha 3$ integrin independent of $\alpha 3\beta 1$ -mediated cell-matrix adhesion.³⁸ Interestingly, TGF- $\beta 1$ -induced activation of Src (p-Src) was significantly higher in *E-cad*^{-/-} PTECs compared with control PTECs (Figure 8A). Src kinase is known for its dissociation of β -catenin from E-cadherin-mediated cell-cell adhesion by phosphorylation of β -catenin at Y-654, whereas a TGF- β receptor1/E-cadherin/ $\alpha 3\beta 1$ integrin-dependent p- β -catenin-Y654/p-Smad2 complex was induced by TGF- $\beta 1$.⁷ This prompted us to examine whether the $\alpha 3$ integrin-dependent activation of Src kinase underlies the p- β -catenin-Y654/Smad2 complex induction in absence of E-cadherin. Indeed, we found by coimmunoprecipitation assay that p- β -catenin-Y654/p-Smad2 complex formation was significantly higher in TGF- $\beta 1$ -treated *E-cad*^{-/-} PTECs compared with control PTECs (Figure 8B). Given that $\alpha 3$ integrin-dependent up-regulation of ILK is responsible for the EMT of *E-cad*^{-/-} PTECs, we further

examined whether p- β -catenin-Y654/p-Smad2 complex regulates a transcriptional activation of ILK. Sequence analysis by online software Transcriptional Regulatory Element Database (<https://cb.utdallas.edu/cgi-bin/TRED/tred.cgi?process=home>; last accessed February 15, 2016) revealed a Smad binding sequence (GTCT) located at 247 to 250 of mouse ILK promoter (Figure 8C). Chromatin immunoprecipitation showed an increase of p-Smad2 binding at 247 when complexed with p- β -catenin-Y654 (Figure 8C). Thus, our results delineated a novel $\alpha 3$ but not $\beta 1$ integrin-dependent and p- β -catenin-Y654/p-Smad2-mediated up-regulation of ILK in *E-cad*^{-/-} PTECs.

Knockout of E-Cadherin in PTEC Aggravates Kidney Fibrosis in UUO and IR via $\alpha 3$ Integrin/ILK

To further examine the aggravation of kidney fibrosis after UUO in PTEC *E-cad*^{-/-} mice, we investigated the activity of

Figure 8 Src phosphorylation of β -catenin and p- β -catenin-Y654/Smad 2 transcriptional complex mediates $\alpha 3$ -integrin-dependent up-regulation of ILK. **A:** Western blot analysis and quantification of Src kinase in lysates of cultured control and *E-cad*^{-/-} proximal tubular epithelial cell (PTEC), treated or not with 5 ng/mL transforming growth factor (TGF)- $\beta 1$ for 48 hours. TGF- $\beta 1$ treatment significantly activates Src kinase in *E-cad*^{-/-} PTEC compared with control. **B:** Coimmunoprecipitation (IP) of p- β -catenin (Y-654) and P-Smad2 antibody from control and *E-cad*^{-/-} PTEC nuclear proteins with rabbit anti-P-Smad2 antibody. Purified rabbit IgG was used as a negative control. Immunoprecipitates were analyzed by Western blotting with anti-p- β -catenin (Y-654) and anti-P-Smad2 antibodies. Input refers to analysis of nuclear proteins before immunoprecipitation. Quantification of immunoblots (relative intensity) is shown by fold-increase in densitometry intensity (the intensity of the control is arbitrarily defined as 1). TGF- $\beta 1$ treatment induces significant activation of Smad2 in both control and *E-cad*^{-/-} PTEC nuclear proteins, whereas the phosphorylated β -catenin, p- β -catenin (Y-654) binding to Smad2 is significantly increased in *E-cad*^{-/-} PTEC compared to control. **C:** Chromatin immunoprecipitation (ChIP) assay of Smad2 binding site at position 247 of the mouse ILK promoter by amplification of 219 to 289 promoter region precipitated by rabbit anti-P-Smad2 antibody. Purified rabbit IgG was used as a negative control. Binding of Smad2 to ILK promoter at position 247 significantly increases in *E-cad*^{-/-} PTEC compared with control. Data are shown as means $\pm$ SD (A–C). *n* = 3 (A–C). ***p* < 0.01.

ILK and consequent β -catenin nuclear translocation^{20,21} in kidney of control and PTEC *E-cad*^{-/-} mice after UUO. PTEC-specific depletion of E-cadherin caused significant increases in ILK expression (Figure 9A) and nuclear accumulation of β -catenin in PTEC of PTEC *E-cad*^{-/-} mice (Figure 9B) after UUO. Inflammation is known to be an important contributor to fibrosis, including that arising through proximal tubular epithelial cells in UUO kidney.³⁴ We next examined interstitial macrophage infiltration by immunohistochemical staining. There was no significant difference in the extent of macrophage infiltrations in UUO kidney of PTEC *E-cad*^{-/-} and control mice (Figure 9C). These results suggest that depletion of PTEC E-cadherin per se does not cause more inflammation, which could otherwise explain the increased kidney fibrosis of PTEC *E-cad*^{-/-} mice after UUO. However, there was a significant increase of α -SMA expression in the proximal tubules and interstitial cells that, in some cases, overlapped with Cre expression in the PTEC *E-cad*^{-/-} mice (Figure 9D). This proximal tubular specific Cre expression served as genetic labeling analogous to that of lineage-tagged mice. Presence of α -SMA and Cre double staining provides *in vivo* evidence for profibrotic changes of PTEC. Profibrotic processes in E-cadherin depleted PTEC are therefore associated with increased ILK activity and nuclear accumulation of β -catenin in PTEC *E-cad*^{-/-} mice. In addition, the effect of PTEC E-cadherin depletion on kidney fibrosis was also examined in kidney IR model. The kidney fibrosis was much more severe in PTEC *E-cad*^{-/-} mice than control mice, as shown by Gomori trichrome and Sirius Red staining in kidney cortex 3 weeks after IR (Supplemental Figure S4A). α -SMA expression was also significantly higher in PTEC of PTEC *E-cad*^{-/-}

mice compared with that of control mice (Supplemental Figure S4B). Consistent with this, ILK expression was higher in IR kidney of PTEC *E-cad*^{-/-} mice (Supplemental Figure S4C). Taken together, our results demonstrate that up-regulation of ILK and consequent profibrotic genes in E-cadherin-deficient PTEC contributed to the increase of kidney fibrosis in PTEC *E-cad*^{-/-} mice after UUO or IR.

Discussion

In this study, we show for the first time, by conditional depletion of E-cadherin from mouse proximal tubular epithelial cells, that E-cadherin in kidney protects against fibrosis. Ablation of E-cadherin from proximal tubular epithelial cells up-regulated $\alpha 3\beta 1$ integrin, but did not result in a significant alteration of kidney structure or function in otherwise normal mice. However, after UUO, absence of E-cadherin in murine kidney worsened fibrosis through promotion of profibrotic processes by an $\alpha 3$ integrin- and ILK-linked mechanism. Our study provides *in vivo* evidence for a protective role of E-cadherin against fibrosis, and reveals a novel $\alpha 3$ integrin/p- β -catenin-Y654/p-Smad2-mediated up-regulation of ILK in promotion of TGF- β -induced profibrotic changes on loss of E-cadherin.

Cross talk between integrins and cadherins has been investigated in integrin-mediated cell-matrix interaction and E-cadherin-mediated cell-cell adhesion.^{14,39,40} Up-regulation of adherens junction-associated $\alpha 3\beta 1$ integrin on loss of E-cadherin has not been identified previously. Coincidentally, both elevated $\alpha 3\beta 1$ integrin levels and loss of E-cadherin have each separately been associated with

Figure 9 Deletion of E-cadherin in proximal tubular epithelial cell (PTEC) aggravates kidney fibrosis in unilateral ureteral obstruction (UUO) through up-regulation of epithelial-mesenchymal transition (EMT). **A:** Western blot analysis and quantification of ILK. **B:** Immunohistochemical staining and quantification of β -catenin nuclear translocation in kidney of control and PTEC *E-cad*^{-/-} mice after UUO. ILK protein levels are significantly higher in UUO kidney of PTEC *E-cad*^{-/-} mice compared with controls. **C:** Immunohistochemical staining and quantification of macrophage infiltration in kidney of control and PTEC *E-cad*^{-/-} mice with or without UUO. Compared with non-UUO (contralateral kidneys). **D:** Immunofluorescence staining of Cre (green) and α -SMA (red) and merged double positive proximal tubular cells (arrowheads) in kidney of control and PTEC *E-cad*^{-/-} mice after UUO. Significantly higher numbers of α -SMA and Cre double-positive proximal tubule cells are seen in UUO kidney of PTEC *E-cad*^{-/-} mice compared with controls. Data are shown as means $\pm$ SD (**B–D**). $n = 6$ (**B–D**). $^{**}P < 0.01$. Scale bars: 50 μ m (**B** and **D**); 100 μ m (**C**).

invasiveness of malignant carcinomas, mimicking our findings in PTEC.^{41,42} Herein, we provide the first evidence linking loss of E-cadherin with up-regulation of $\alpha 3\beta 1$ integrin within cell-cell adhesion compartment in noncancerous epithelial cells.

$\alpha 3\beta 1$ Integrin has been found to enhance E-cadherin-dependent cell-cell adhesion via interaction with CD151¹⁵ or to down-regulate E-cadherin by binding to uPAR.³⁸ Kim et al^{7,18} demonstrated that mice with specific depletion of lung epithelial $\alpha 3$ integrin failed to develop lung fibrosis. However, the underlying mechanism revealed was that $\alpha 3$ integrin binding to E-cadherin was required for $\alpha 3\beta 1$ integrin–E-cadherin–TGF- β R1 cocluster formation at epithelial cell surface, leading to phosphorylation of E-cadherin bond β -catenin (pY654– β -catenin) and thereby p-Smad2-pY654– β -catenin complexes formation to initiate profibrotic processes.⁷ The $\alpha 3$ integrin-dependent signaling uncovered by our E-cadherin depletion study rules out a requirement of E-cadherin for $\alpha 3\beta 1$ integrin–E-cadherin–TGF- β R1 cocluster-dependent epithelial profibrotic changes. Indeed, Kim et al⁷ also found that E-cadherin was present in TGF- β R1 immunoprecipitates regardless of whether E-cadherin associates with the integrin (wild-type $\alpha 3$ integrin) or not (mutant $\alpha 3$ integrin). In addition, clusters of E-cadherin and TGF- β R1 disappeared from the surface of $\alpha 3$ wild-type cells undergoing TGF- β 1-induced EMT, but persisted on cells expressing mutant $\alpha 3$ integrin not

responsive to TGF- β 1 stimulation. Interestingly, both studies from Kim et al^{7,18} and us identified p-Smad2-pY654– β -catenin complex in mediating TGF- β 1-induced profibrotic changes. Our study revealed, for the first time, that ILK is the downstream target by p-Smad2-pY654– β -catenin complex.

ILK is induced by TGF- β during EMT, whereas elevated ILK expression is known to contribute to EMT and fibrosis.^{20,43,44} However, TGF- β signaling in up-regulation of ILK remains largely unknown. We provided an $\alpha 3$ integrin-dependent mechanism for TGF- β induction of ILK expression via p-Smad2-pY654– β -catenin, thereby linking $\alpha 3$ integrin to up-regulation of ILK rather than the conventional ILK activation through β integrin in cell-matrix interaction.⁴⁵ By using mutant $\alpha 3$ integrin with mutation outside its cell matrix binding domain, $\alpha 3$ integrin binding to nonmatrix ligands has been crucial in TGF- β -induced profibrotic changes.³⁸ A Src activation followed by Slug expression underlies the $\alpha 3$ integrin-dependent profibrotic changes.³⁸ However, a link between Src and up-regulation of Slug is missing, for Src is known to disrupt E-cadherin distribution rather than down-regulate E-cadherin. Our result showed that Src-dependent phosphorylation of β -catenin at Y654, known for Src disruption of E-cadherin distribution, led to p-Smad2-pY654– β -catenin complex formation and the complex-mediated transcription of ILK. Then, the Src

activity is now logically associated with up-regulation of ILK, which up-regulates Slug.⁴⁶ This cell-matrix-independent pathway within cell-cell adhesion compartment, as we found in current study, is less likely to involve conventional $\beta 1$ integrin/ILK or $\beta 1$ integrin/Src found in cell-matrix interaction. We speculate that the conventional $\beta 1$ integrin/ILK signaling in cell-matrix interaction is most likely physiological; podocyte-specific deletion of ILK led to loss of podocyte structure and function.⁴⁷ It is the overexpression of ILK that causes profibrotic changes of tubular epithelial cells and kidney fibrosis.^{20,21} We therefore identify a central role for the $\alpha 3$ (rather than $\beta 1$) subunit of $\alpha 3\beta 1$ integrin in TGF- β -induced profibrotic processes on loss of E-cadherin.

Controversy exists as to whether tubular EMT is a contributor to kidney fibrosis.^{48,49} In our study, targeted deletion of E-cadherin from PTECs provided evidence for contribution of PTEC profibrotic processes to kidney fibrosis, analogous to data from studies using epithelial lineage-tagged mice. E-cadherin depletion in PTECs did not result in a secondary increase in interstitial macrophage infiltration. Endothelial-mesenchymal transition^{50,51} is also a most unlikely explanation in this case where E-cadherin depletion was restricted to tubular cells. We demonstrated that increased profibrotic processes in proximal tubules of PTEC *E-cad*^{-/-} mice underlie the exacerbation of kidney fibrosis after UUO.

In conclusion, the current study provides *in vivo* evidence for the role of E-cadherin in kidney fibrosis, using conditional deletion of E-cadherin in proximal tubular cells. Our results suggest that E-cadherin's role in signal transduction associated with cell-cell adhesion is more dominant than its role in maintenance of adherens junction integrity in terminally differentiated adult tissues, such as kidney proximal tubules. The role of E-cadherin in suppressing $\alpha 3$ integrin/ILK and the definition of specific mechanisms leading to TGF- β -driven fibrosis may provide therapeutic targets for preventing kidney fibrosis and suggest therapeutic avenues in other organs where fibrosis leads to organ failure.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2016.03.015>.

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