Regulation of proximal tubular cell differentiation and proliferation in primary culture by matrix stiffness and ECM components

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Chen WC, Lin HH, Tang MJ. Regulation of proximal tubular cell differentiation and proliferation in primary culture by matrix stiffness and ECM components. Am J Physiol Renal Physiol 307: F695–F707, 2014. First published July 23, 2014; doi:10.1152/ajprenal.00684.2013.—To explore whether matrix stiffness affects cell differentiation, proliferation, and transforming growth factor (TGF)-β₁-induced epithelial-mesenchymal transition (EMT) in primary cultures of mouse proximal tubular epithelial cells (mPTECs), we used a soft matrix made from monomer collagen type I-coated polyacrylamide gel or matrigel (MG). Both kinds of soft matrix benefited primary mPTECs to retain tubular-like morphology with differentiation and growth arrest and to evade TGF-β₁-induced EMT. However, the potent effect of MG on mPTEC differentiation was suppressed by glutaraldehyde-induced cross-linking and subsequently stiffening MG or by an increasing ratio of collagen in the soft mixed gel. Culture media supplemented with MG also helped mPTECs to retain tubular-like morphology and a differentiated phenotype on stiff culture dishes as soft MG did. We further found that the protein level and activity of ERK were scaled with the matrix stiffness. U-0126, a MEK inhibitor, abolished the stiff matrix-induced differentiation and proliferation. These data suggest that the ERK signaling pathway plays a vital role in matrix stiffness-regulated cell growth and differentiation. Taken together, both compliant property and specific MG signals from the matrix are required for the regulation of epithelial differentiation and proliferation. This study provides a basic understanding of how physical and chemical cues derived from the extracellular matrix regulate the physiological function of proximal tubules and the pathological development of renal fibrosis.

matrix stiffness; renal proximal tubule cell; proliferation; differentiation; transforming growth factor-β₁-induced epithelial-mesenchymal transition; extracellular matrix

MOST TISSUES are soft, with an elastic modulus scaling from 100 Pa to 100 kPa in tissues ranging from the brain to soft cartilage, respectively (25). Tissue stiffness, contributed mostly by the composition and quantity of the extracellular matrix (ECM), plays a critical role in regulating gene expression, cell growth, apoptosis, and differentiation (12, 28, 35, 42, 49). In an in vitro study (11), matrix stiffness that resembles the elastic property of particular tissue environments potentiates the specific lineage differentiation of mesenchymal stem cells. Physiological tissue stiffness has also been suggested to act as a cell cycle inhibitor, and comparable increases in tissue stiffness facilitate at sites of cell proliferation in vivo and in vitro (22). Moreover, increased tissue stiffness through collagen deposition or cross-linking affects tissue development and disease progression (18). Hardened tissue serves as a disease marker in scleroderma (7), atherosclerosis (34, 48), cancer (2), and fibrosis (14, 40). Therefore, the maintenance of tissue stiffness is significant for the physiological function of organs.

Kidneys, which are essential in the urinary system, regulate fluid and electrolyte homeostasis through the process of filtration, reabsorption, and secretion. Renal fibrosis, the inevitable consequence of an excessive accumulation of the ECM, occurs in virtually every type of chronic kidney disease and eventually contributes to organ failure. Proximal tubular epithelial cells (PTECs), the major part of the nephron, play a central role in the response of kidney to insult. Traditional cell biological approaches have suggested that transforming growth factor (TGF)-β₁-regulated tubular epithelial-mesenchymal transition (EMT) is the major avenues for the generation of matrix-producing cells in fibrogenesis (21). However, these results are based on the exploration of cells cultured on plastic dishes with an elastic modulus of >10⁵ Pa, which have little relation to cells encountered in vivo. Naturally, PTECs are surrounded by a laminin-rich basement membrane (BM), which serves as a barrier to separate the epithelial monolayer from the underlying collagen rich-connective tissues and as the differentiation promoter (23). Matrigel (MG), which is rich in laminin and collagen type IV, resembles the specialized BM and has been well studied for its ability to promote differentiation on several cell types (1, 29, 32). While much research effort has focused on chemical cues from MG in controlling cell behaviors, little is known about its physical cues. In addition, early disruption of BM integrity by matrix metalloproteinases has been shown to trigger fibrotic processes (6, 20, 43). In vitro studies (39, 57) have shown that collagen, a fibrogenic molecule, potentiated TGF-β₁-induced EMT. Whether the exposure of the collagen underlying the discontinuous BM permits the transdifferentiated phenotype of PTECs needs to be further investigated. A primary culture of PTECs provides the appropriate model to study the regulation of cell growth, apoptosis, and differentiation (44). The freshly isolated proximal tubules collapsed and started to spread out accompanied with spontaneous dedifferentiation and proliferation when cultured on plastic culture dishes (45). Growth factors as well as BM proteins have been accepted for the importance of the preservation of the differentiated phenotype in different types of epithelial cells (17, 23). Knowing the importance of matrix stiffness on cell behaviors, we believed that the physical and chemical properties of the matrix should be integrated for their synergistic effects on the differentiation of primary PTECs.

In the present study, we attempted to clarify whether matrix stiffness and/or specific ECM components regulate cell behaviors, including cell spreading, differentiation, proliferation, and TGF-β₁-induced EMT, in primary mouse (m)PTECs. We found that soft MG facilitated mPTECs to preserve tubular-like
morphology with a differentiated phenotype and suppressed TGF-β1-induced EMT. However, hardening MG or collagen-containing soft gel did not elicit the ability to promote differentiation as soft MG did. We further delineated the signaling pathways involved in matrix stiffness-regulated cell growth and differentiation in primary mPTECs. Our results suggest that the synergistic interaction of physical and biochemical signaling is required for the maintenance of differentiated kidney tubules. Furthermore, the essential interplay between physical and biochemical cues is important for kidney morphogenesis.

**METHODS**

**Cell line and cultures.** The pig kidney proximal tubule (LLC-PK1) cell line was purchased from American Type Culture Collection (Manassas, VA) and regularly maintained in low-glucose DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 5% FBS (Invitrogen, Carlsbad, CA), 100 IU/ml penicillin, and 100 µg/ml streptomycin (Sigma-Aldrich) at 37°C in air containing 5% CO2. Cells were seeded on different substrates at a density of 106 cells/100-mm dish.

**Isolation of primary PTECs from mouse kidneys.** Primary PTECs from mouse kidneys were harvested according to the method of Tang and Tannen (46) with minor modifications. All procedures were reviewed and approved through the Institute of Animal Care and Use Committee of the Medical College of National Cheng Kung University. Briefly, kidneys were surgically removed from anesthetized male C57Bl/6 mice (1 mo old). The renal cortices were sliced, minced, and digested in high Krebs-Henseleit-saline (KHS) buffer containing 0.5 mg/ml type collagenase II (Worthington, Lakewood, NJ) and 0.5 mg/ml trypsin inhibitor (Sigma-Aldrich). Highly purified proximal tubules were isolated through a 42% Percoll density gradient centrifugation. Freshly isolated tubules were maintained in DMEM-F-12 medium supplemented with 1× insulin-transferrin-selenium, 1× MEM nonessential amino acids, 0.1 µM hydrocortisone (Invitrogen), 100 IU/ml penicillin, 100 µg/ml streptomycin, and 1% FBS. The culture was maintained in an incubator under 5% humidified CO2 at 37°C, and the medium was changed every 3 days until the experiment was terminated. In several experiments, LLC-PK1 cells and mPTECs were treated with or without 10 ng/ml TGF-β1 (PeproTech, London, UK) for the indicated times and refreshed every 2 days. For some experiments, mPTECs were incubated with or without 10 µM SB-431542, 20 µM U-0126, 20 µM M U-0126, 20 µM 4B4 (Beckman Coulter, Fullerton, CA), and 0.5 µg/ml 4B4 (Beckman Coulter, Fullerton, CA) for the indicated times, as shown in Fig. 7.

**Preparation of the polyacrylamide gel and MG-collagen mixed gels with varying stiffness.** The polyacrylamide (PA) gel was prepared according to the method of Wei et al. with minor modifications (51). In brief, varying concentrations of bisacrylamide were mixed with a mixture of acrylic acid (0.3%, Sigma-Aldrich), ylenediamine and ammonium persulfate to prepare PA gels on glass slides with different stiffness values. The PA gels were washed and soaked in the culture medium for at least 1 h before cells were plated in an incubator under 5% CO2 at 37°C. The elastic modulus of the PA gels was measured using atomic force microscopy (AFM).

For cells embedded in three-dimensional (3-D) MG, freshly isolated mPTECs were mixed well with soluble MG and seeded immediately onto dishes precoated with a thin layer of MG, which prevented cells from the stiff dishes during long-term culture.

**Collection of cell lysates and Western blot analysis.** Cells were harvested and lysed in RIPA buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, and protease inhibitor cocktail (Complete, Roche, Mannheim, Germany). For Western blot analysis, 20–30 µg of protein were resolved using 10% SDS-PAGE gel and electrophoretically blotted onto nitrocellulose paper. The blot was then incubated with specific antibodies against E-cadherin, fibronectin, β1-integrin, Smad2/3 (BD Biosciences PharMingen), α-smooth muscle actin (α-SMA; Sigma-Aldrich), collagen type I, cyclin D1, GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA), active β1-integrin ligand-induced binding site (LIBS; clone B44, Millipore, Temecula, CA), phosphorylated (p-)Smad3, p-ERK, and ERK (Cell Signaling, Boston, MA). The blot was then incubated with a horse-radish peroxidase-conjugated secondary antibody. Immunocomplexes were subsequently visualized using fluorography using an enhanced chemiluminescence reagent (GE Healthcare Life Sciences, Buckinghamshire, UK).

**RNA isolation and RT-PCR.** Total RNA obtained from mPTECs was purified using TRIzol reagent (Invitrogen-Molecular Probes, Carlsbad, CA) according to the manufacturer’s instructions. For RT-PCR, first-strand cDNA was synthesized from 2 µg total RNA using oligo-dT primers and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). PCR was performed with specific primer sets at 94°C for 5 min followed by 27 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 30 s, and a final step at 72°C for 7 min. The primer sequences are shown in Table 2. PCR products were subjected to 1.2% agarose gel electrophoresis, stained with ethidium bromide, and visualized under an ultraviolet transilluminator.

**Immunofluorescence staining.** LLC-PK1 cells and mPTECs were cultured on different substrates for the indicated times. Cells were

| Table 1. Effect of GLA treatment on the elastic modulus of MG, CG, and mixed gel, which was measured using atomic force microscopy |
|------------------|------------------|------------------|
|                  | 0% GLA           | 0.025% GLA       | 0.05% GLA       |
|                  | Pa ± S.E. n       | Pa ± S.E. n       | Pa ± S.E. n       |
| CG (0.9 mg/ml)   | 58.1 ± 2.0 68    | 586.9 ± 16.0 35  | ND               |
| MG (6 mg/ml)     | 66.0 ± 0.3 51    | 516.0 ± 12.6 54  | 1,370.0 ± 28.5 55 |
| Mixed gel (MG-to-CG ratio: 1:1) | 60.2 ± 4.5 51 | 615.9 ± 40.5 68 | 955.9 ± 14.8 54 |

Each value represents the mean ± standard error. GLA, glutaraldehyde; CG, collagen gel; MG, matrigel; ND, not detected.
rinsed with ice-cold PBS and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Fixed cells were permeabilized with 0.5% Triton X-100 in PBS and blocked with SuperBlock buffer (Thermo Scientific, Rockford, IL) for 1 h. Next, cells were incubated with the selected primary antibody at 4°C overnight. After an extensive wash with PBS, cells were incubated with the secondary antibody for anti-mouse or anti-rabbit IgG conjugated with Alexa 488 or 594 (Invitrogen-Molecular Probes) for 1 h. For some experiments, cells were stained with phalloidin-TRITC (Sigma-Aldrich) and 10 μg/ml Hoechst 33258 for 15 min at room temperature. Immunocomplexes were visualized using confocal microscopy (FV-1000, Olympus, Tokyo, Japan). The movies of 3-D images were reconstructed from serial confocal Z-section scanning images using Avizo 3D imaging and analysis software (version 6.0, Mercury computer systems).

Evaluation of cell proliferation with Click-IT Edu kits. Cell proliferation was evaluated by a Click-IT Edu Alexa Fluor 488 Imaging Kit (Invitrogen-Molecular Probes). Briefly, mPTECs were cultured on different substrates, as shown in Fig. 3A, for 3 days and incubated with Edu/ for 6 h before analysis. After fixation and permeabilization, cells were incubated with a Click-IT Edu reaction cocktail for 30 min at room temperature. After Edu detection, cells were washed and blocked with 3% BSA for 1 h. Cells were then incubated with primary mouse antibody against cyclin D1 (Santa Cruz Biotechnology) at 4°C overnight. After an extensive rinse with PBS, cells were incubated with the secondary antibody for anti-mouse IgG conjugated with Alexa 594 (Invitrogen-Molecular Probes) and 10 μg/ml Hoechst 33258 for 1 h. Immunocomplexes were visualized using confocal microscopy (FV-1000, Olympus).

Statistics. All data are expressed as means ± SE of at least three independent experiments. A two-tailed Student’s t-test was used to compare differences between two groups, and one-way ANOVA was used to compare differences when group numbers were more than three. Tukey’s procedure was used to test differences between individual treatment groups. GraphPad Prism (version 3.0, GraphPad Software, San Diego, CA) was used for the statistics. Statistical significance was set at P ≤ 0.05. *P < 0.05, **P < 0.01, ***P < 0.001.

RESULTS

Soft matrix contributes to tubular differentiation, cell growth arrest, and prevention of TGF-β1-induced EMT. To elucidate the effect of matrix stiffness on cell behaviors, LLC-PK1 cells were cultured on monomeric collagen type I-coated PA gels of varying stiffness. LLC-PK1 cells on a stiff matrix (with a stiffness higher than 0.7 kPa) exhibited well spreading and much stiffer than most tissues in our body. When primary mPTECs were cultured on monomeric collagen type I-coated PA gels of varying stiffness. A decrease of matrix stiffness greatly promoted cells toward EMT, as confirmed by the fibroblast-like morphology, the gradually loss of E-cadherin, and highly expressed mesenchymal-related markers (Fig. 2A). β1-Integrin, fibronectin, and collagen type I (mesenchymal-related markers) were slightly increased with culture time (Fig. 2B). TGF-β1 greatly promoted cells toward EMT, as confirmed by the fibroblast-like morphology, the gradually loss of E-cadherin, and highly expressed mesenchymal-related markers (Fig. 2A). Proximal tubular differentiation markers [Na+/H+ exchanger isoform 3 (NHE3)] were downregulated within 3 days regardless of TGF-β1. This result indicated that cells underwent dedifferentiation during culture (Fig. 2D). To examine the effect of matrix stiffness on the proliferation, differentiation, and TGF-β1-induced EMT of primary mPTECs, mPTECs were cultured on monomeric collagen type I-coated PA gels of varying stiffness. A decrease of matrix stiffness suppressed cell spreading and remained the tubular-like phenotype. TGF-β1 induced fibroblast-like morphology with a significant increase in α-SMA only on a stiff matrix (>20 kPa), as shown by phase-contrast images and immunostaining (Fig. 2E). Western blot results showed that the TGF-β1-induced downregulation of E-cadherin and upregulation of α-SMA and β1-integrin in mPTECs were stunted on a soft matrix (Fig. 2F). In terms of differentiation, the soft matrix not only preserved tubular-like cell aggregates but also maintained SGLT2 mRNA expression on day 5 (Fig. 2, E and G). In contrast, cyclin D1, one of the proliferation markers, was greatly upregulated within 1 day on the stiff matrix. However, the upregulation of cyclin D1 was lagged with decreasing matrix stiffness in a time-dependent manner (Fig. 2G). Taken together, these data indicated that matrix stiffness plays an

### Table 2. Sequences of RT-PCR primer sets and predicted product sizes for the mouse genes

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Product Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-cadherin</td>
<td>5′-GGCTTCAGTTCTCCGGGTCTTACA-3′</td>
<td>5′-TACAGGCTGTGTCACCTTCA-3′</td>
<td>529</td>
</tr>
<tr>
<td>α-Smooth muscle actin</td>
<td>5′-TCAAGGGGCTGAACTTCTCCTA-3′</td>
<td>5′-ACGGAGTAAGGTAACACTGAC-3′</td>
<td>69</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>5′-GGACTGCTCACCTGCTTACAG-3′</td>
<td>5′-GGCTGTTAGGGTATGAAAGG-3′</td>
<td>166</td>
</tr>
<tr>
<td>Na+-dependent glucose</td>
<td>5′-AGAGGACCCTGCTGTTGGAGCA-3′</td>
<td>5′-ACGGCGACAGAGAGAGACG-3′</td>
<td>707</td>
</tr>
<tr>
<td>cotransporter type 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na+/H+ exchanger isoform 3</td>
<td>5′-GTCCTGCTTCATGATTGTCTT-3′</td>
<td>5′-GAGGGTTGCTTGCACCTAC-3′</td>
<td>360</td>
</tr>
<tr>
<td>Kidney-specific cadherin</td>
<td>5′-GGACTGCTCCCTGTGACCTACG-3′</td>
<td>5′-GGGATTGCTCATGCTCAAAAGG-3′</td>
<td>153</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>5′-AGAGGACCAGTGAGAGGAGG-3′</td>
<td>5′-CAAGAGGATTGCTCTCTCCTCA-3′</td>
<td>546</td>
</tr>
<tr>
<td>Cyclin A</td>
<td>5′-CTTGCTGCTGAGACACAAATTA-3′</td>
<td>5′-AGATGCTAGAGCCAGCTCTT-3′</td>
<td>212</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5′-AGGGACAGTGTCAGGTCAGG-3′</td>
<td></td>
<td>555</td>
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with significant increases in mesenchymal-related markers (α-SMA, fibronectin, and β1-integrin) and a decrease in epithelium-related markers (E-cadherin) on a stiff matrix. In contrast, TGF-β1 neither changed cell morphology nor significantly enhanced the levels of mesenchymal-related proteins on a soft matrix (Fig. 1, B–D). However, TGF-β1 downregulated E-cadherin regardless of matrix stiffness (Fig. 1, B and C). The confocal immunofluorescence images showed that the TGF-β1-induced stress fiber formation and α-SMA increase were dependent on matrix stiffness. Furthermore, α-SMA was co-localized along with stress fibers only on PA gels with a stiffness value of >2 kPa (Fig. 1E).

Traditionally, most cell lines were regularly cultured on plastic dishes with an elastic modulus of >10⁶ Pa, which are much stiffer than most tissues in our body. When primary mPTECs were cultured on culture dishes, 3-D tubules started to spread and proliferated within 1 day and developed a monolayer with cell-cell contact within 7 days (Fig. 2A). β1-Integrin, fibronectin, and collagen type I (mesenchymal-related markers) were slightly increased with culture time (Fig. 2B). TGF-β1 greatly promoted cells toward EMT, as confirmed by the fibroblast-like morphology, the gradually loss of E-cadherin, and highly expressed mesenchymal-related markers (Fig. 2A). Proximal tubular differentiation markers [Na+/H+ exchanger isoform 3 (NHE3)] were downregulated within 3 days regardless of TGF-β1. This result indicated that cells underwent dedifferentiation during culture (Fig. 2D). To examine the effect of matrix stiffness on the proliferation, differentiation, and TGF-β1-induced EMT of primary mPTECs, mPTECs were cultured on monomeric collagen type I-coated PA gels of varying stiffness. A decrease of matrix stiffness suppressed cell spreading and remained the tubular-like phenotype. TGF-β1 induced fibroblast-like morphology with a significant increase in α-SMA only on a stiff matrix (>20 kPa), as shown by phase-contrast images and immunostaining (Fig. 2E). Western blot results showed that the TGF-β1-induced downregulation of E-cadherin and upregulation of α-SMA and β1-integrin in mPTECs were stunted on a soft matrix (Fig. 2F). In terms of differentiation, the soft matrix not only preserved tubular-like cell aggregates but also maintained SGLT2 mRNA expression on day 5 (Fig. 2, E and G). In contrast, cyclin D1, one of the proliferation markers, was greatly upregulated within 1 day on the stiff matrix. However, the upregulation of cyclin D1 was lagged with decreasing matrix stiffness in a time-dependent manner (Fig. 2G). Taken together, these data indicated that matrix stiffness plays an
Fig. 1. Soft matrix stunts transforming growth factor (TGF)-β1-induced epithelial-mesenchymal transition (EMT) of LLC-PK1 cells. LLC-PK1 cells were plated on collagen type I-coated dishes or on polyacrylamide (PA) gels of various stiffness with or without TGF-β1 (10 ng/ml) for 2 days. A: phase-contrast images of cells under different conditions. B: representative Western blot results of cells under different conditions. Protein levels of EMT-related markers [E-cadherin, α-smooth muscle actin (α-SMA), β1-integrin, and fibronectin] were analyzed. GAPDH was used as an internal control. C: quantitative results of E-cadherin from B. D: quantitative results of α-SMA, fibronectin, and β1-integrin from B. GAPDH-normalized data in each condition were compared with those of cells cultured on dishes. *P < 0.05 and ***P < 0.001, control vs. TGF-β1. G: confocal immunofluorescence images of cells under different conditions. Cells were stained for α-SMA (green), F-actin (red), and nuclei (blue). Bars = 100 μm in A and 40 μm in E.

important role for switching differentiation to proliferation in mPTECs. A soft matrix, which mimics the elasticity of the physiological matrix, tends to preserve primary mPTECs with tubular-like morphologies and differentiated properties. More importantly, the soft matrix acts as a physical barrier to inhibit cell growth and TGF-β1-induced EMT in mPTECs.

Synergistic cooperation between chemical compositions and physical properties of ECM on mPTEC differentiation. Previously results have demonstrated that a soft matrix retained cell differentiation and prevented TGF-β1-induced EMT. All of these findings were based on two-dimensional (2-D) culture. However, the best way to study epithelial organization and function is to culture cells within a 3-D matrix, which could provide a physiologically relevant in vivo microenvironment. These motivated us to question whether cells cultured on a 2-D matrix or in a 3-D matrix, with the same physical properties, displayed a differential response on the proliferation and differentiation of primary mPTECs. First, we chose MG as a supporting matrix for its notable action on differentiation promotion. Freshly isolated mPTECs cultured on MG-coated dishes displayed the spread-out phenotype that was similar to how cells behaved on culture dishes (Fig. 3A). On MG (2-D),
mPTECs aggregated without spreading and grew to form cyst-like structures. In MG (3-D), mPTECs completely retained their tubular morphology for at least 14 days (Fig. 3A and data not shown). RT-PCR results showed that the expression of SGLT2 in mPTECs was preserved on day 1 and totally downregulated on day 3 regardless of culture conditions (Fig. 3B). In contrast to SGLT2, expression of NHE3 was completely repressed on MG-coated dishes and in MG at day 1. However, the downregulation of NHE3 mRNA was partially alleviated when cells were cultured on MG, indicating that cell differentiation is only maintained in 2-D MG (but not 3-D MG). In terms of proliferation, expression of cyclin D1 and cyclin A were upregulated on MG-coated dishes and on MG within 3 days. mPTECs grown in MG expressed neither cyclin D1 nor cyclin A (Fig. 3B). To measure cell proliferation directly, we used an EdU incorporation assay combined with cyclin D1 staining. On MG-coated dishes, cells were highly incorporated by EdU and stained by cyclin D1 in the nuclei. However, these phenomena were almost suppressed on MG or in MG (Fig. 3C). Although cyclin D1 mRNA and protein levels could be detected in cells cultured on MG, the distribution of cyclin D1 was majorly located in the cytosol, indicating cell cycle arrest. Previously, we have demonstrated that a soft matrix prevented TGF-β1-induced EMT in a 2-D PA gel system. We further investigated the effect of 2-D and 3-D MG, both with the same physical properties, on TGF-β1-induced EMT in mPTECs. TGF-β1 induced fibroblast-like morphology with stress fiber formation and significant decreased in E-cadherin in mPTECs cultured on MG-coated dishes. Such alterations were suppressed when cells were cultured on MG or in MG. In this case, E-cadherin was successfully restored at cell-cell junctions, even in the presence of TGF-β1 (Fig. 3, D and E). In summary, both 2-D and 3-D cultures with MG prevented mPTEC proliferation and TGF-β1-induced EMT.
Next, we were curious about how the effects of 2-D and 3-D cultures with MG were linked to their chemical composition or physical properties. To clarify the role of chemical composition in MG, we mixed MG and CG in MG-to-CG ratios from 1:1 to 1:3 to prepare soft gels (60 Pa) of varying compositions (Table 1). The mixed gel in a MG-to-CG ratio of 1:1 induced disorganization of E-cadherin but preserved epithelial cell islands and differentiation markers (NHE3 and SGLT2; Fig. 4, A–C). The mixed gel in MG-to-CG ratio of 1:3 induced stress fiber formation and dedifferentiation, as confirmed by decreases in E-cadherin, NHE3, and SGLT2 expression. CG promoted cells toward EMT, including fibroblast-like morphology, loss of E-cadherin, and upregulation of α-SMA and β1-integrin (Fig. 4, D and E). These data revealed that the chemical components of MG were critical for the maintenance of tubular architecture and differentiation. To clarify the role of physical properties on MG-induced cell differentiation, we applied 0.025% GLA to cross-link the soft gel, which resulted in a 10-fold increase in gel stiffness without changing the gel composition (Table 1). All GLA-stiffened gels promoted cell spreading and F-actin rearrangement. GLA-stiffened gels also suppressed the expression of E-cadherin, NHE3, and SGLT2 regardless of the gel composition (Fig. 4, A–C). Notably, GLA-stiffened CG greatly enhanced the expression of mesenchymal-related markers, including α-SMA and β1-integrin (Fig. 4, D and E). In conclusion, these data suggest that the maintenance of a soft matrix must be required for cell differentiation.

To further clarify the effect of chemical cues derived from MG on the differentiation of mPTECs, cells were cultured on culture dishes and treated with media-containing MG (MCM). Of the three compared concentrations (0.5%, 2%, and 5%), 5% MCM provided the best benefit to maintain tubular-like structures. Lower than 2% MCM led to cell spreading and proliferation (Fig. 5A). Supplement of 2% MCM partially preserved tubular-like structures and inhibited the stiff substrate-induced upregulation of α-SMA, fibronectin, and β1-integrin (Fig. 5B). Furthermore, 2% MCM suppressed TGF-β1-induced fibroblast-like morphology and upregulation of α-SMA, fibronectin, and β1-integrin (Fig. 5, B and C). Similar results were
observed in LLC-PK1 cells (data not shown). We further evaluated the effect of 5% MCM combined with matrix stiffness on the differentiation of mPTECs. Cells grown on stiff MG-coated dishes or soft CG started to spread out and form a monolayer. Supplement of 5% MCM suppressed cell spreading and preserved tubular-like structures. Cells cultured on MG-coated soft PA gels (200 Pa) or soft mixed gels (MG-to-CG ratio of 1:1, 60 Pa) remained aggregated status regardless of 5% MCM. Notably, E-cadherin was restored at cell-cell junctions only in the presence of 5% MCM (Fig. 5D and Supplemental Movies S1 and S2 in the supplemental material).1 In addition, we also found that GLA-stiffened gels induced a loss of NHE3 and SGLT2 mRNA expression that was rescued by 2% MCM, indicating that the presence of MCM facilitated soft matrix-preserved mPTEC differentiation (Fig. 5E). Taken together, these data suggest that both chemical composition and physical properties of the matrix are required to exert the synergistic effect on the differentiation of mPTECs.

Stiff matrix facilitates cell proliferation, dedifferentiation, and TGF-β1-induced EMT through fine tuning ERK expression and activity. We have shown that a stiff matrix permitted TGF-β1-induced EMT in both LLC-PK1 cells and mPTECs. To elucidate the underlying mechanism, we compared the TGF-β1 downstream signaling, which included Smad3 and ERK, in LLC-PK1 cells cultured on substrates of varying stiffness with or without TGF-β1. TGF-β1-induced phosphorylation of Smad3 and ERK was permitted on a stiff matrix and repressed on a soft matrix. In addition, the soft matrix also diminished protein levels of Smad2/3 and ERK (Fig. 6A). We further checked whether this protein downregulation by the soft matrix were also occurred in mPTECs. The results showed that a soft matrix significantly repressed the protein level of ERK in a PA gel system (Fig. 6B). Similar results were observed when mPTECs were cultured on a GLA-stiffened mixed gel system. The soft matrix significantly suppressed not only ERK phosphorylation but also its protein level (Fig. 6C). Our previous studies found that blockage of β1-integrin by neutralized antibody (4B4) suppressed TGF-β1-induced EMT in LLC-PK1 cells. Thus, the involvement of a β1-integrin signal in the EMT event was established (56). In the present

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1 Supplemental material for this article is available at the American Journal of Physiology-Renal Physiology website.
In our study, we found that the protein level of β1-integrin in mPTECs was markedly enhanced on the stiff matrix and repressed on the soft matrix within 1 day (Fig. 6B). Similar results were also found in LLC-PK1 cells (Fig. 1, B and D). Next, we were interested in whether these EMT-related signals were involved in stiff matrix-induced cell proliferation and dedifferentiation of mPTECs. We applied several inhibitors, including SB-431542 (TGF-β1 receptor inhibitor), U0126 (MEK inhibitor), SB203580 (p38 MAPK inhibitor), and 4B4. After being plated on culture dishes, 91–92% of the tubules spread out and grew to small cell islands within 1 day. The inhibitors SB431542, U0126, SB203580, and 4B4 reduced spread tubules to 77%, 21%, 37%, and 8%, respectively (Fig. 7A). During the culture, cells started to proliferate with the increase of cyclin D1 in protein and mRNA levels (Fig. 7, B and C). In contrast, mRNA expression of proximal tubule markers [NHE3 and kidney-specific cadherin (Ksp-cadherin)] was gradually decreased with time (Fig. 7D). These data suggest that cell proliferation was accompanied by dedifferentiation during primary culture of mPTECs. RT-PCR results showed that only U-0126 significantly inhibited the upregulation of cyclin D1 on day 1 (Fig. 7B). However, the inhibitors, including U0126, SB203580, and 4B4, significantly inhibited the upregulation of Cyclin D1 protein on day 3, indicating that the activation of ERK, p38 MAPK, and β1-integrin participated in the regulation of cell growth (Fig. 7D). These data indicate that the ERK signal might play a major role to promote the dedifferentiation of mPTECs compared with β1-integrin. Western blot results showed that U0126 significantly inhibited both ERK activation and β1-integrin activation, but 4B4 only blocked the activation of β1-integrin, as predicted (Fig. 7E). Therefore, ERK might serve as an upstream regulator of β1-integrin activation. Finally, we examined the effect of U-0126 and 4B4 on the distribution of E-cadherin and F-actin in mPTECs. Both U0126 and 4B4 stunted stiff matrix- and TGF-β1-induced tubule spreading of mPTECs. Soluble MG blunts stiff substrate-induced cell spreading, dedifferentiation, and TGF-β1-induced EMT in mPTECs. A: representative phase-contrast images of primary mPTECs cultured on culture dishes and treated with varying amounts of media-containing MG (MCM) for 3 days. B: representative Western blot results of primary mPTECs cultured on culture dishes and treated with or without 2% MCM in the presence or absence of TGF-β1 for the indicated times. Protein levels of EMT-related markers (E-cadherin, α-SMA, fibronectin, and β1-integrin) were analyzed. C: representative phase-contrast images (top) and confocal immunofluorescence images (bottom) of primary mPTECs cultured on culture dishes and treated with or without 2% MCM in the presence or absence of TGF-β1 on day 3. Cells were stained for α-SMA (red) and nuclei (blue). D: representative phase-contrast images (top) and confocal immunofluorescence images (bottom) of primary mPTECs cultured under the indicated matrices and treated with or without 5% MCM on day 5. Cells were stained for E-cadherin (green), F-actin (red), and nuclei (blue). The 3-D movies reconstructed from sequential z-series scanning confocal images are shown in Supplemental Movies S1 and S2, respectively. E: representative RT-PCR results of primary mPTECs cultured on a mixed gel (MG-to-CG ratio: 1:1) or a GLA-stiffened mixed gel in the presence or absence of 2% MCM on day 3. mRNA expression of differentiation-related genes (SGLT2 and NHE3) was analyzed. GAPDH was used as an internal control in Western blot and RT-PCR analyses. Bars = 50 μm. A, C, and D, top, 40 μm in C, and 20 μm in D, bottom.
spreading and stress fiber formation; meanwhile, these inhibitors also suppressed E-cadherin downregulation and partially retained E-cadherin at cell-cell junctions (Fig. 7F). Taken together, these data provide a possible mechanism for how a soft matrix retains cell differentiation and prevents proliferation of mPTECs through the inhibition of ERK activation.

**DISCUSSION**

In the present study, we identified the appropriate culture conditions that advantage primary mPTECs to maintain their differentiated phenotypes ex vivo. We found that both chemical cues and physical cues from the culture matrix were indispensably required to suppress cell growth, dedifferentiation, and TGF-β₁-induced EMT. Primary cultures of PTECs have become a crucial tool to study proximal tubule cell function and transport processes as well as ischemic or cytotoxic injury effects (3, 4, 9, 13, 38). However, dedifferentiation-associated changes in morphology and gene expression in primary culture of renal cells are major drawbacks for these functional analyses. Based on the modification of chemical factors, many protocols were developed to obtain good quality with limited dedifferentiation of tubular cells (5, 15). Now, it is known that the mechanical properties of tissues or ECM also play important roles in the regulation of cellular function. Previous studies (10, 41) have shown that cells grown on flexible PA gels with a stiffness mimicking physiological tissues display in vivo-like morphology and differentiated phenotypes. Moreover, a stiff matrix facilitated cells to proliferate, whereas a soft matrix advantaged cells to differentiate (1, 22). Conventionally, primary renal cells were initiated on stiff and impermeable plastic or glass dishes at ~10,000 kPa, which are extremely stiffer than most tissues in our body (1–50 kPa). Using the Bio-AFM technique, we showed that the elastic modulus of a normal mouse kidney was ~300 Pa compared with that of a fibrotic kidney induced through ureter unilateral obstruction, which was ~1 kPa (unpublished observations). Here, we showed that primary mPTECs exhibited tubular-like morphology and growth arrest when grown on substrates as soft as normal mice kidneys (<1 kPa).

MG, a reconstituted basement membrane isolated from Engelbreth-Holm-Swarm sarcoma tumor, resembles the complex extracellular environment found in many tissues and is notable for its differentiation promoting ability. For example, MG could promote tube formation in human umbilical cord endothelial cells (24). MG has also been shown to stimulate testicular cord formation and germ cell development in vitro (16, 19). Taub et al. (47) demonstrated that both MG and specific chemical growth factors were required for the formation of tubular structures from primary baby mouse kidney epithelial cells. Recent studies (30, 31) have also indicated that 3-D MG was essential for embryonic stem cells to form tubular structures and directly differentiate into functional proximal tubules. However, the mechanism of how MG affects the tubular differentiation of embryonic stem cells or primary mPTECs remains unclear. Our study showed that mPTECs grown on MG-coated dishes or GLA-stiffened MG induced cell spread out and proliferation with a decrease in tubular markers (Figs. 3 and 4). These data support the idea that cells retain in vivo-like morphologies and differentiated properties only when grown within the physiological range of stiffness. Our data might provide an elucidation how MG plays a role in determining proximal tubule lineage in undifferentiated stem cells. However, soft gel is not a panacea. Naturally, the BM separates the cell monolayer from the underlying collagen-rich connective tissue. In vivo studies (6, 20) have indicated that early disruption of the BM integrity induced fibrosis. Here, we found that increasing the collagen ratio in MG-CG mixed gels without changing the elastic properties led to dedifferentiation and proliferation in primary mPTECs (Fig. 4). Taken together, our data highlight the importance of BM integrity for preventing interactions between epithelial and connective tissue layers that advantaged cells to proliferate and dedifferentiate. Early dis-
ruption of BM integrity and increased TGF-β1 expression thus seem to interact in an autocrine, positive feedback loop that drives EMT and fibrosis. Taken together, we suggest both chemical cues (collagen type IV and laminin) and physical cues (high compliance) from MG are necessary for primary mPTECs to maintain their tubular structure and growth arrest. The stiff matrix permitted cells toward EMT in response to TGF-β1 (Figs. 1–3). A study (54) on liver fibrosis revealed a positive correlation between organ stiffness and the degree of fibrosis. In the early stage of liver fibrosis, the local elasticity increase in the cell-surrounding microenvironment was a result of lysyl oxidase-mediated collagen fibril cross-linking. Therefore, β-aminopropionitrile, an inhibitor of lysyl oxidase, not only reduced tissue stiffening but also alleviated myofibroblast activation and reduced the progression of renal fibrosis in vivo (14). In vitro studies (28, 52) have shown that a stiff matrix, but not a soft matrix, permitted portal fibroblasts and hepatic stellate cells to undergo myofibroblast differentiation in response to TGF-β1 treatment. Our preliminary experiments also revealed that β-aminopropionitrile treatment alleviated the deposition of cross-linked collagen and the progression of renal fibrosis (unpublished observations). Disrupted BM, cross-linked collagen, and the TGF-β1 network facilitated the vicious cycle of stiff matrix and TGF-β1 stimulation. Taken together, these data strongly support that cells retain in vivo-like morphologies and differentiated properties when grown within the physiological range of tissue stiffness with well-organized BM architecture.

Fig. 7. Inhibition of the ERK signal dampens stiff substrate-induced cell spreading, proliferation, and dedifferentiation in mPTECs. A, top: representative phase-contrast images of primary mPTECs cultured on culture dishes treated with DMSO (solvent control) or inhibitors (10 μM SB431542, 20 μM U0126, 20 μM SB203580, or 5 μg/ml 4B4) for 1 day. The spread tubule was evaluated by spreading cells in the bottom layer. The percentage of spread tubules in the total tubule population was calculated from five randomly selected views under low power and shown at the bottom. B: representative RT-PCR results of primary mPTECs cultured on culture dishes treated with DMSO or inhibitors for 3 days. The protein level of cyclin D1 was analyzed. C, top: representative Western blot results of primary mPTECs cultured on culture dishes treated with DMSO or inhibitors for 3 days. The protein level of cyclin D1 was analyzed. Quantification results of cyclin D1 are shown at the bottom. β-Actin were used as an internal control. β-Actin-normalized data in each condition were compared with those of cells on day 0 (red dotted line). **P < 0.01. D, top: representative RT-PCR results of primary mPTECs cultured on culture dishes treated with DMSO or inhibitors for 1 day. Protein levels of the ligand-induced binding site of β1-integrin (LIBS), β1-integrin, p-ERK, and ERK were analyzed. E: confocal immunofluorescence images of primary mPTECs cultured on culture dishes treated with DMSO or inhibitors for 1 day. Protein levels of the ligand-induced binding site of β1-integrin (LIBS), β1-integrin, p-ERK, and ERK were analyzed. F: confocal immunofluorescence images of primary mPTECs cultured on culture dishes treated with DMSO or inhibitors for 1 day. Protein levels of the ligand-induced binding site of β1-integrin (LIBS), β1-integrin, p-ERK, and ERK were analyzed. G: confocal immunofluorescence images of primary mPTECs cultured on culture dishes treated with DMSO or inhibitors for 1 day. Protein levels of the ligand-induced binding site of β1-integrin (LIBS), β1-integrin, p-ERK, and ERK were analyzed.
dantly expressed in the apical membrane of the proximal tubule and functions to drive Na\(^+\) reabsorption. Proximal tubule-specific NHE3 knockout mice display reductions in salt and volume reabsorptions (27). Fluid shear stress produced by renal tubular flow has been shown to stimulate both apical NHE3 and basolateral Na\(^+\)K\(^+\)-ATPase expression and trafficking (8). In addition, chronic hyperfiltration induced by unilateral nephrectomy increased NHE3 and Na\(^+\)K\(^+\)-ATPase activities and cell volume (33). All the above studies indicated that fluid shear stress played a significant role to regulate the expression and activity of NHE3. In the present study, we suggested that matrix stiffness and ECM component also contribute to regulate NHE3 expression. Soft MG, but not GLA-stiffened MG or CG, promoted differentiation, as characterized by retaining NHE3 expression (Fig. 4, A–C). Thus, it is easily to explain why the expression of NHE3 was greatly downregulated in stiffened fibrotic kidneys induced by ureter unilateral obstruction, even in the presence of hyperfiltration stimulation (26).

The present study highlights the importance of ERK and \(\beta_1\)-integrin in mediating matrix stiffness-modulated tubular cell responses, including proliferation and differentiation. Previously, Xie et al. (53) demonstrated that U-0126 (MEK inhibitor) inhibited ERK activation and also blocked TGF-\(\beta_1\)-induced EMT. Provenzano et al. (36) further showed that stiff matrix-induced aberrant mammary epithelial phenotypes could be rescued by inhibition of ERK activity. Here, we found similar results in that inhibition of ERK activation by U-0126 mimicked a soft matrix to preserve differentiation and growth arrest in primary mPTECs (Fig. 7, B–F). Notably, the soft matrix downregulated not only ERK activity but also its protein level in both LLC-PK1 cells and primary mPTECs (Fig. 6, A–C). Similar phenomena also could be observed in the regulation of \(\beta_1\)-integrin by matrix stiffness (Figs. 1, B and D, and 2F). \(\beta_1\)-Integrin, the major receptor of the ECM, controls many cell fates, including embryonic development, adhesion, migration, and differentiation (55). As a mechanosensor, \(\beta_1\)-integrin responded and clustered on matrix with a stiffness of 0.4 kPa (51). Both in vivo and in vitro studies have confirmed that suppression of \(\beta_1\)-integrin activation alleviated TGF-\(\beta_1\)-induced EMT and renal fibrosis through Smad3 inactivation (56). Here, we found that block of ERK activation by U-0126 and hindrance of \(\beta_1\)-integrin activation by 4B4 suppressed cell proliferation, dedifferentiation, and the EMT event (Fig. 7, C–F). MAPK/ERK linkage communicates both chemical and physical signals from a receptor to DNA in the nucleus of the cell. We found that inhibition of ERK activation interfered with \(\beta_1\)-integrin activation (Fig. 7E). Thus, these data might explain why U0126 exerts better work than 4B4 to maintain cell differentiation and growth arrest in mPTECs (Fig. 7, C–F). Taken together, we suggest that the soft matrix advantaged cells to preserve their normal epithelial phenotype and growth arrest and to evade TGF-\(\beta_1\)-induced EMT by suppressing the expression and activation of ERK and \(\beta_1\)-integrin. However, not all soft matrix is benefit to the maintenance of epithelial phenotype and growth arrest. Increasing the collagen ratio in MG-CG mixed gels without changing the elastic property led to dedifferentiation and proliferation in primary mPTECs (Fig. 4). Wei et al. (50) demonstrated that soft collagen gel triggered ERK activation. As previously described, ERK activity played an important role in the regulation of cell differentiation and proliferation. It is conceivable that increasing the collagen ratio in soft gel induced cell dedifferentiation and proliferation. Apparently, both chemical composition and physical properties of the matrix are required to exert the synergistic effect on the differentiation of mPTECs through the precise control of ERK activity. However, how ERK activity is modulated by matrix stiffness or ECM components remains unknown. We hypothesis that the soft matrix interfered with ERK activation due to the limited ERK protein level through transcriptional regulation. Moreover, the MAPK/ERK signaling cascade can be activated by a wide variety of receptors involved in growth and differentiation, including receptor tyrosine kinases, integrins, and ion channels. In addition to kinases, specific phosphatases, such as serine-threonine phosphatases, or a dual-specificity phosphatase family are also linked to negatively regulate the phosphorylation status of ERK (37). What kind of kinase or phosphatase is involved in soft matrix-suppressed ERK activation will be examined in the future.

In conclusion, we use the ex vivo model to elucidate how kidney tubular cells act in response to microenvironment changes during renal fibrosis in the early stage (loss of BM integrity) and late stage (increase of tissue stiffness). Our study provides a basic understanding of how physical and chemical cues from the ECM interplay in the control of the transition from physiological to pathological development in the kidney. We suggest that delaying or eliminating the deposition or cross-linking of collagen during renal fibrosis might be useful to preserve tubular differentiation and insensitivity to the growth factor. Although tubular functions remain to be assessed in the future, our study highly supports recent developments in embryonic stem cell differentiation to renal cell types in vitro (30, 31). We also demonstrated that the importance of matrix stiffness-regulated ERK activity in determining tubular cell fates. Further exploring the underlying mechanism will facilitate the progression of regeneration medicine in artificial kidneys.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: W.-C.C., H.-H.L., and M.-J.T. conception and design of research; W.-C.C. performed experiments; W.-C.C. analyzed data; W.-C.C. and H.-H.L. interpreted results of experiments; W.-C.C. and H.-H.L. prepared figures; W.-C.C., H.-H.L., and M.-J.T. drafted manuscript; W.-C.C., H.-H.L., and M.-J.T. edited and revised manuscript; W.-C.C., H.-H.L., and M.-J.T. approved final version of manuscript.

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