Renal Fibrosis Is Alleviated through Targeted Inhibition of IL-11—Induced Renal Tubular Epithelial-to-Mesenchymal Transition

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Renal fibrosis is a pathologic process that leads to irreversible renal failure without effective treatment. Epithelial-to-mesenchymal transition (EMT) plays a key role in this process. The current study found that aberrant expression of IL-11 is critically involved in tubular EMT. IL-11 and its receptor subunit alpha-1 (IL-11Rα1) were significantly induced in renal tubular epithelial cells (RTECs) in unilateral ureteral obstruction (UUO) kidneys, co-localized with transforming growth factor-β1. IL-11 knockdown ameliorated UUO-induced renal fibrosis in vivo and transforming growth factor-β1—induced EMT in vitro. IL-11 intervention directly induced the transdifferentiation of RTECs to the mesenchymal phenotype and increased the synthesis of profibrotic mediators. The EMT response induced by IL-11 was dependent on the sequential activation of STAT3 and extracellular signal-regulated kinase 1/2 signaling pathways and the up-regulation of metadherin in RTECs. Micheliolide (MCL) competitively inhibited the binding of IL-11 with IL-11Rα1, suppressing the activation of STAT3 and extracellular signal-regulated kinase 1/2—metadherin pathways, ultimately inhibiting renal tubular EMT and interstitial fibrosis induced by IL-11. In addition, treatment with dimethylaminomicheliolide, a pro-drug of MCL for in vivo use, significantly ameliorated renal fibrosis exacerbated by IL-11 in the UUO model. These findings suggest that IL-11 is a promising target in renal fibrosis and that MCL/dimethylaminomicheliolide exerts its antifibrotic effect by suppressing IL-11/IL-11Rα1 interaction and blocking its downstream effects. (Am J Pathol 2023, 193: 1936–1952; https://doi.org/10.1016/j.ajpath.2023.07.005)

Chronic kidney disease (CKD), with its high morbidity and mortality, is a global public health problem. CKD affects 10% of the world’s population, resulting in 1.2 million deaths and 28.0 million years of life lost annually.1 Renal fibrosis is a common pathologic feature of progressive CKD, which manifests as excessive deposition of the extracellular matrix in the tubulointerstitial area and loss of capillary networks caused by fibroblast activation and inflammatory cell infiltration.2 Although a glomerular injury is specific to the potential etiology of a particular kidney disease, the manifestation of fibrosis is nearly identical in all progressive forms of CKD, suggesting a common final pathway in these conditions.3

Epithelial-to-mesenchymal transition (EMT) is a phenotypic conversion process that endows epithelial cells with migratory and invasive characteristics during development, tissue repair, and malignancies.4,5 During the pathogenesis of renal fibrosis, EMT occurs in renal tubular epithelial cells (RTECs) and primarily manifests as alterations in the cellular phenotype of the epithelia, such as loss of adherence

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junction proteins (eg, E-cadherin) and acquisition of interstitial cellular markers [eg, vimentin and α-smooth muscle actin (α-SMA)]. EMT has a substantial impact on the functional capabilities of RTECs, reducing the expression of proteins responsible for absorption and secretion. EMT induces G2/M cell cycle arrest, thus impairing the regeneration capacity of the damaged tissue. However, as confirmed by lineage tracing, RTECs undergoing EMT are not converted into interstitial fibroblasts but remain attached to the basement membrane of renal tubules, displaying a partial EMT. RTECs undergoing partial EMT drive interstitial fibroblast activation and immune recruitment through a modified profibrotic and proinflammatory secretome profile in a paracrine manner.

Among the many fibrogenic factors regulating renal fibrosis, transforming growth factor-β1 (TGF-β1) is a key mediator that is critical in inducing EMT through the TGF-β1/Smad signaling pathway. However, because of the pleiotropic role of TGF-β1, its inhibition is associated with many side effects. The downstream molecules of TGF-β1 with less versatility may therefore be attractive therapeutic targets. Several studies have recently indicated that IL-11 is a critical profibrotic factor downstream of TGF-β1. IL-11 is a member of the IL-6 cytokine family. It was discovered in 1990 and was initially described as a hematopoietic factor that stimulates megakaryocyte formation. Similar to the mode of action of IL-6, IL-11 initiates signaling by forming a hexameric complex with the subunit alpha-1 of its cognate receptor (IL-11Rα1) and then interacts with gp130 molecules, triggering dimerization and activation of subsequent downstream signaling. Unlike CD126 (IL-6 receptor), which is widely expressed in inflammatory cells, IL-11Rα1 is mainly expressed in fibroblasts, vascular smooth muscle cells, and epithelial cells in some tissues.

Using single-cell RNA sequencing, Shafer et al were the first to show that IL-11 expression is the dominant transcriptional response to TGF-β1 exposure in cardiac fibroblasts. The blockage of IL-11 inhibits fibroblast activation across organs and species in response to a range of important profibrotic stimuli. Therefore, IL-11 is a promising antifibrotic therapeutic target downstream of TGF-β1.

Micheliolide (MCL) is a novel guaianolide sesquiterpene lactone derived from parthenolide that possesses higher chemical stability, lower toxicity, and a longer half-life than parthenolide. The prodrug of MCL, dimethylaminomicheliolide (DMAMCL), which is modified from MCL for oral utilization, has better water solubility and exhibits therapeutic efficacy by slowly and continuously releasing MCL in the plasma and tissues. DMAMCL prevents renal fibrosis induced by unilateral ureteral obstruction (UUO) and ischemia-reperfusion injury; however, the underlying mechanism needs to be further elucidated. MCL recognizes and forms hydrogen bonds with multiple amino acid residues of IL-11 on the binding surface of IL-11/IL-11Rα1 in simulated molecular docking, suggesting that MCL may exert antifibrotic effects by targeting IL-11.

The current study therefore aimed to further explore the role of IL-11 in renal fibrosis and clarify the antifibrotic mechanism of DMAMCL to propose a new possible approach for treating renal fibrosis.

Materials and Methods

Ethics Statement

The animal experimental protocols were approved by the Zhujiang Hospital Ethics Committee of the Southern Medical University (approval no. LAEC-2020-068) and strictly complied with ethical principles.

MCL and DMAMCL

Accendatech Co., Ltd (Tianjin, China) synthesized MCL and DMAMCL. MCL was dissolved in dimethyl sulfoxide (Sigma Aldrich, St. Louis, MO) at 20 mmol/L and stored at −20°C for cell treatment. DMAMCL was dissolved in physiological saline at 2.5 mg/mL concentration for animal experiments. A corresponding intragastric dose was administered according to the daily body weight of each mouse.

Animal Model

C57BL/6 mice (male, 6 weeks old, weighing 18 to 22 g) were purchased from Hunan SJA Laboratory Animal Co., Ltd. (Changsha, China) and housed at the Zhujiang Hospital Laboratory Animal Centre, Southern Medical University (Guangzhou, China). The mice were randomly divided into three groups (n = 6 per group): i) sham + pGV102-shRNA (Ctrl-shR), ii) UUO + Ctrl-shR, and iii) UUO + IL-11-shR after 2 weeks of adaptation to verify the effect of IL-11 knockdown in UUO mice. The UUO model was established by double-ligating the left ureter using a 4-0 silk suture after a midline abdominal incision. The ureters of sham-operated mice were exposed and manipulated but not ligated. In vivo knockdown or expression of IL-11 in mice was achieved by using a hydrodynamic-based gene delivery approach as described previously.

Subsequently, the groups of mice were administered an IL-11–targeted shRNA expression plasmid (pGV102-shIL-11) or control plasmid (pGV102-shCtrl) at 1 μg/g body weight via rapid injection of a large volume of DNA solution (0.1 mL/g body weight) through the tail vein on the third day after UUO surgery. The mice were sacrificed on day 10, and renal tissues were collected under appropriate conditions.
The mice were randomly divided into five groups \((n = 6\) per group): i) sham + vector, ii) UUO + vector, iii) UUO + pIL-11, iv) UUO + vector + DMAMCL, and v) UUO + pIL-11 + DMAMCL to verify if MCL protected the kidney from fibrosis exacerbated by IL-11 over-expression in UUO mice. The methods for UUO model establishment and injection of the IL-11 gene expression plasmid (pIL-11) or control plasmid (pEX-5) were performed as described in the previous two paragraphs. Intragastric administration of DMAMCL (25 mg/kg per day) or normal saline was started on the third day after UUO surgery and continued until the day before sampling. The mice were sacrificed on day 10, and renal tissues were collected under appropriate conditions.

The mice were raised in a specific pathogen−free environment for these animal experiments with a fixed temperature \((23 \pm 3^\circ C)\), constant humidity \((55\% \pm 15\%)\), and a 12-hour light-dark cycle. They were provided standard food and water.

**Histology**

Kidney tissues were fixed with a 4% paraformaldehyde solution (Boster Biological Technology, Wuhan, China), embedded in paraffin, and sectioned into 2-μm-thick slices. Paraffin sections were heated at 65°C for 1.5 hours, de-waxed with xylene for three 10-minute cycles, rehydrated using an alcohol gradient, and washed with phosphate-buffered saline (PBS) with Tween 20. Subsequently, the sections were stained using the Masson trichrome dye kit (Leagene, Beijing, China) according to the manufacturer’s instructions and examined under a light microscope. Paraffin sections were dewaxed, rehydrated, and stained using a commercial kit (Solarbio, Beijing, China) according to the manufacturer’s instructions for periodic acid–Schiff staining.

**In Situ Hybridization**

Kidney tissues were fixed with a 4% paraformaldehyde solution containing 0.1% diethyl pyrocarbonate for 3 to 4 hours, embedded in paraffin, and sectioned into 4-μm-thick slices. Paraffin sections were dewaxed, rehydrated, and treated with a commercial kit (Boster Biological Technology) according to the manufacturer’s instructions. The sections were stained with dianminobenzidine (Boster Biological Technology) and captured using a light microscopy. The forward sequences of IL-11-probe and Ctrl-probe (IL-11) were 5’-CAGACCCCTC-GAGCAGATCTGGAGCAGGCGTGTTCCTCAAC-3’ and 5’-GTAGGAGAAACAGCCGTGTCCAGATCTGCTCGA-3’, respectively; the forward sequences of IL-11Rz1-probe and Ctrl-probe (IL-11Rz1) were 5’-GCC AAGCGGTAGACTATGAAACTTCTCTGACTTGAG-3’ and 5’-CTCCAGTACAGGAAAGTTTTCATAGCTTACCGCTTGCC-3’, respectively.

**Immunohistochemical Staining**

Kidney tissues were fixed with a 4% paraformaldehyde solution, embedded in paraffin, and sectioned into 4-μm-thick slices. Paraffin sections for immunohistochemical staining were dewaxed and rehydrated, blocked with 3% hydrogen peroxide, and treated with Citrate Antigen Retrieval Solution (Beyotime Institute of Biotechnology, Shanghai, China) at 90°C to 95°C for 15 minutes to retrieve the antigen. After blocking with 5% goat serum for 1 hour, the sections were incubated overnight at 4°C with the following primary antibodies: rabbit anti-fibronectin (1:100; Abcam, Cambridge, UK), rabbit anti-collagen type I (1:100; Boster Biological Technology), and rabbit anti-α-SMA (1:50; Affinity Biosciences, Cincinnati, OH). Sections were counterstained with hematoxylin followed by a bluing reagent, and then detected with horseradish peroxidase–conjugated goat anti-rabbit or mouse secondary antibody using a commercial kit (GeneTech, Shanghai, China) according to the manufacturer’s protocol. These sections were captured by using light microscopy.

**Cell Culture and Intervention**

Mouse tubular epithelial cells (mTECs) (a gift from Jeffrey B. Kopp, NIH, Bethesda, MD) were cultured in Dulbecco’s modified Eagle’s medium/F12 (Gibco BRL, Gaithersburg, MD), supplemented with 10% fetal bovine serum (Gibco BRL), penicillin (Gibco BRL; 100 U/mL), and streptomycin (Gibco BRL; 100 mg/mL) at 37°C and 5% carbon dioxide. For the experiments, upon reaching approximately 50% confluence, the medium was replaced with a serum-free medium, and the cells were treated with recombinant human TGF-β1 (R&D Systems, Minneapolis, MN; 5 ng/mL) or recombinant mouse IL-11 (R&D Systems; 50 ng/mL) for 24 hours. To detect the effects of IL-11 on the TGF-β1–induced EMT response, mTECs were transfected with an IL-11 shRNA expression plasmid or control plasmid and treated with TGF-β1 after 6 hours. To detect the activation of downstream IL-11 signaling pathways, mTECs were divided into different groups and then treated with recombinant mouse IL-11 protein at different durations (0, 5, 15, 30 minutes and 1, 2, 6, 12, and 24 hours).

To verify whether IL-11 promoted EMT by activating corresponding pathways, mTECs were transfected with the IL-11 gene expression plasmid or control plasmid and then treated with a specific small-molecule inhibitor (SCH772984 or Statick) for 24 hours. To clarify the role of mediator (MTDH) in IL-11–mediated EMT, mTECs were transfected with an MTDH shRNA expression plasmid or control plasmid for 6 hours and then further transfected with the IL-11 gene expression plasmid or control plasmid; cell culture was continued for 24 hours. To detect the effect of MCL on IL-11–induced EMT, mTECs were pre-incubated with various MCL concentrations (0, 2.5, 5, and 10 μmol/L) for half an hour and then treated with...
recombinant mouse IL-11 for 24 hours. In addition, mTECs were transfected with the IL-11 gene expression plasmid or control plasmid, followed by incubation with MCL for 24 hours.

Plasmid Transfection

For transfection, an IL-11 shRNA expression plasmid was constructed by Shanghai Genechem Co., Ltd. (Shanghai, China); IL-11 gene and MTDH shRNA expression plasmids were constructed by GenePharma (Suzhou, China). Tables 1 and 2 list the shRNA interference sequence of IL-11 and MTDH, respectively. For the experiments, sub-confluent mTECs were transfected with corresponding plasmids using Lipofectamine 2000 (Invitrogen, Waltham, MA) according to the manufacturer’s instructions. After 6 hours, the medium was replaced with a serum-free medium, and other interventions were performed according to experimental requirements. The cells were cultured for an additional 24 hours before harvest.

Immunofluorescence Staining

Kidney tissues were fixed with a 4% paraformaldehyde solution, embedded in paraffin, and sectioned into 4-μm-thick slices. Paraffin sections were de waxed, rehydrated, and treated with citrate-EDTA Antigen Retrieval Solution (Boster Biological Technology) at 90°C to 95°C for 15 minutes. After permeabilization and blocking with 5% goat serum at 37°C for 1 hour, the sections were incubated overnight at 4°C with a primary antibody. For cellular immunofluorescence staining, mTECs were cultured on glass coverslips in 6-well plates. When 80% to 90% confluence was reached, the cells were fixed in a 4% paraformaldehyde solution. The cells were washed with PBS after approximately 20 minutes and then permeabilized with 0.3% Triton for 20 minutes. Subsequently, the coverslips were blocked with 5% bovine serum albumin in PBS with Tween 20 for 30 minutes at room temperature and then incubated overnight at 4°C with a primary antibody. The following primary antibodies were used: rabbit anti-fibronectin antibody (1:100; Abcam), rabbit anti–IL-11 antibody (1:100; Invitrogen, Carlsbad, CA), rabbit anti–IL-11Rα/1 antibody (1:100; Abcam), rabbit anti–α-SMA antibody (1:200; Abcam), Alexa Fluor 594–conjugated anti-ACTA2 (α-SMA) (1:200; Abcam), mouse anti–TGF-β1 antibody (1:200; Santa Cruz Biotechnology, Dallas, TX), and mouse anti–E-cadherin antibody (1:50; BD Biosciences, San Jose, CA). Subsequently, the sections or cell slides were treated with the following secondary antibodies: anti-mouse IgG (H + L), F(ab’)2 Fragment (Alexa Fluor 488 Conjugate) (1:500; Cell Signaling Technology, Danvers, MA), anti-mouse IgG (H + L), F(ab’)2 Fragment (Alexa Fluor 555 Conjugate) (1:500; Cell Signaling Technology), anti-rabbit IgG (H + L), F(ab’)2 Fragment (Alexa Fluor 488 Conjugate) (1:500; Cell Signaling Technology), or anti-rabbit IgG (H + L), F(ab’)2 Fragment (Alexa Fluor 555 Conjugate) (1:500; Cell Signaling Technology) at 37°C for 60 minutes. The sections were then mounted with DAPI (Beyotime Institute of Biotechnology) and captured by using fluorescence microscopy.

RNA Isolation and Quantitative Real-Time PCR for mRNA

The total RNA was extracted by applying the TRIzol reagent (Hunan Accurate Biotechnology Co., Ltd., Hunan, China) according to the manufacturer’s protocol. Immediately after isolation, RNA quantity and quality were determined by using the NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). A total of 500 ng of total RNA were reversely transcribed using the Evo M-MLV RT Mix Kit with gDNA Clean (AG). The quantitative PCR analysis was performed in the CFX Connect Real-Time Detection System (Bio-Rad, Singapore) using the SYBR Green Premix Pro Taq HS qPCR kit (AG). Table 3 lists the mRNA primer sequences.

Cell Cycle Distribution Analysis

According to standard protocols, cells were prepared for propidium iodide staining (KeyGEN BioTECH, Jiangsu, China). Briefly, after treatment, cells were washed twice with ice-cold PBS, harvested, fixed with 70% ethanol at 4°C overnight, and stained with propidium iodide for 30 minutes at 37°C. DNA content was determined by using a CytoFLEX analyzer (Beckman Coulter, Brea, CA).

Western Blot Analysis

Whole-cell lysates of mTECs and renal tissue were prepared by using a radioimmunoprecipitation lysis buffer containing a 1× protease inhibitor and a 1× phosphatase inhibitor (Beijing CWBIO, Beijing, China). The protein concentration was measured by using a bicinchoninic acid protein assay kit.
(Tiangen Biotech, Beijing, China) before denaturation at 100°C for 10 minutes. Samples were resolved via SDS-PAGE on 8%, 10%, or 12% gels, followed by electro-transfer of the separated proteins onto polyvinylidene difluoride membranes (Millipore, Bedford, MA; pore size, 0.45 μm). After blocking with 5% skimmed milk or 5% bovine serum albumin (Merck, Darmstadt, Germany) for 1 hour at room temperature, the membranes were incubated overnight at 4°C with a primary antibody. The following antibodies were used: mouse anti-ACTB (1:5000; EarthOx Life Sciences, Burlingame, CA), mouse anti-GAPDH (1:10,000; Ray Antibody, Beijing, China), mouse anti-α-Tubulin (1:10,000; Ray Antibody), mouse anti-E-cadherin (1:2500; BD Biosciences), rabbit anti-fibronectin (1:5000; Abcam), rabbit anti-collagen type I (1:400; Boster Biological Technology), rabbit anti-α-SMA (1:1000; Abcam), mouse anti-IL-11 (1:1000; R&D Systems), rabbit anti-IL-11Rα (1:1000; Abcam), mouse anti-TGF-β1 (1:1000; Santa Cruz Biotechnology), rabbit anti-MTDH (1:10,000; Abcam), rabbit anti-STAT3 (1:1000; Cell Signaling Technology), rabbit anti-phosphorylated-STAT3 (1:1000; Cell Signaling Technology), rabbit anti-extracellular signal-regulated kinase (ERK) 1/2 (1:1000; Cell Signaling Technology), rabbit anti-phosphorylated-ERK1/2 (1:1000; Cell Signaling Technology), and rabbit anti-Snail (1:1000; Abcam). After washing three times with tris-buffered saline with Tween 20, the membranes were incubated in an horseradish peroxidase–conjugated secondary antibody solution (1:5000; EarthOx Life Sciences) for 1 hour at room temperature. Band signals were recorded by using Image Lab software version 6.0 (Bio-Rad Laboratories, Hercules, CA) and a Pierce ECL substrate kit (Millipore). Quantification was performed by measuring the grayscale intensity of the bands using Image Lab software.

**Pull-Down Experiment**

Whole-cell mTECs were transiently transfected with pflagIL-11 using Lipofectamine 2000; some were treated with MCL, while others were not. Subsequent reagent-specific procedures were performed per the manufacturer’s protocols. Briefly, cells were washed three times with ice-cold PBS solution and lysed in a 1% NP-40 buffer. The samples were precipitated with FLAG antibodies (1 μg; Sigma-Aldrich) and protein A/G—agarose beads (Santa Cruz Biotechnology) by overnight incubation at 4°C. The bound proteins were removed by boiling in an SDS buffer and resolved in 12% polyacrylamide gels for Western blot analysis with IL-11Rα (1:1000; Affinity Biosciences) and FLAG (1:1000; Sigma-Aldrich) antibodies.

**Statistical Analysis**

All data are expressed as means ± SEM from at least three independent experiments. For normally distributed data, a parametric t-test was performed for comparing two groups. One-way analysis of variance followed by Tukey-Kramer multiple comparisons test was performed for comparing three or more groups within the same study. For data that were not distributed normally, the U-test or Kruskal-Wallis test was used to analyze differences between indicated groups. All statistical analysis was performed by using GraphPad Prism version 8.0 (GraphPad Software Inc., La Jolla, CA). Statistical significance was set at P < 0.05.

**Results**

IL-11 and IL-11Rα1 Are Induced in the UUO Kidneys and Expressed in RTECs

UUO is a well-established experimental model for renal fibrosis. This study first determined whether there was a difference in the expression of IL-11 and IL-11Rα1 between the sham and UUO groups. Western blot analysis indicated

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### Table 3  mRNA Primer Sequences

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>AQP-1</td>
<td>5’-GACTCACACTGGCTGCGGTATCAAC-3’</td>
<td>5’-TCGTCAGCACTCCAGGCTCATACTCC-3’</td>
</tr>
<tr>
<td>Twist1</td>
<td>5’-TGAAGCAACAGCAGAGAGAAG-3’</td>
<td>5’-TGAAGCACTCTCTGAGTACGAC-3’</td>
</tr>
<tr>
<td>CtgF</td>
<td>5’-AGCTGCCTACGCAGTGCGAAAGAC-3’</td>
<td>5’-GGTTGTTCTGGGCGGCTGTC-3’</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>5’-ACCGCAACACAGCAGCTCTGGAG-3’</td>
<td>5’-GGTTCAGCCACTGCCTGACAC-3’</td>
</tr>
<tr>
<td>MTDH</td>
<td>5’-AGACTCTTCTCCTCCTGCTACTCTTG-3’</td>
<td>5’-CTTAGTAACATGCGGCAACTTGG-3’</td>
</tr>
<tr>
<td>ACTB</td>
<td>5’-TATGGCTCTCTCTCAGCACCATCC-3’</td>
<td>5’-GTCAGCGACGATTCCCTCAG-3’</td>
</tr>
<tr>
<td>Snai1</td>
<td>5’-AGTGCACATCAGCAAGCCACACG-3’</td>
<td>5’-GGCAGCTGGAATCCTCTCAGC-3’</td>
</tr>
</tbody>
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that IL-11, IL-11Rα1, and fibrotic indicator levels, including TGF-β1, α-SMA, fibronectin, and collagen type I, were all augmented in UUO kidneys (Figure 1, A–G). The expression sites of IL-11 and IL-11Rα1 were then explored in the UUO model. As shown by in situ hybridization staining, the mRNA transcription of IL11 and IL11Rα1 was detected primarily in renal tubules (Figure 1H and Supplemental Figure S1). In addition, immunofluorescence revealed significant co-localization of IL-11 with E-cadherin, an epithelial marker, at the protein level (Figure 1I). The tissue localization of IL-11Rα1 also predominantly overlapped with E-cadherin and barely overlapped with α-SMA in UUO kidneys (Figure 1J and Supplemental Figure S2). These data indicate that IL-11 and IL-11Rα1 are significantly up-regulated in UUO-induced renal fibrosis and that both are primarily expressed by RTECs.

Knockdown of IL-11 Ameliorates UUO-Induced Renal Fibrosis in Vivo and TGF-β1—Induced EMT in Vitro

To establish the role of IL-11 in renal fibrosis, the IL-11-interfering plasmid pGV102-shIL-11 (IL-11-shR) was delivered to mice via a hydrodynamic-based gene delivery, a routinely administered approach for in vivo expression or knockdown of various genes.27 IL-11-shR or Ctrl-shR was administered intravenously 3 days after ureteral ligation (Figure 2A). IL-11 knockdown in UUO kidneys was confirmed by Western blot analysis. Renal expression of IL-11Rα1, α-SMA, TGF-β1, fibronectin, and collagen type I induced by UUO was suppressed after IL-11 knockdown. Correspondingly, E-cadherin expression in UUO mice was restored after IL-11 knockdown (Figure 2, B–I). In addition, the activation of STAT3 and ERK1/2 signaling was detected.

Figure 1  The expression of IL-11 and IL-11 receptor subunit alpha-1 (IL-11Rα1) is induced in the unilateral ureteral obstruction (UUO) kidney and mainly detected in renal tubular epithelial cells. A: Representative Western blots show the renal expression of IL-11, IL-11Rα1, transforming growth factor-β1 (TGF-β1), α-smooth muscle actin (α-SMA), fibronectin, and collagen type I in the sham-operated and UUO groups, as indicated. B–G: Graphical representations of IL-11 (B), IL-11Rα1 (C), TGF-β1 (D), α-SMA (E), fibronectin (F), and collagen type I (G) expression levels in the two groups. H: Representative micrographs of in situ hybridization staining for IL-11 and IL-11Rα1 nucleic acid in kidney tissues from the sham-operated and UUO mice. I: Representative staining pictures show co-localization of IL-11 and E-cadherin in tubules of UUO mice. Paraffin-embedded kidney sections were immunofluorescence stained for IL-11 and E-cadherin. J: Co-localization of IL-11Rα1 and E-cadherin in the tubules of UUO mice. Paraffin-embedded kidney sections were immunofluorescence stained for IL-11Rα1 and E-cadherin. Data are expressed as means ± SEM; statistical significance was determined by using parametric t-test (B–E) and U-test (F and G). *P < 0.05, **P < 0.01 versus sham controls (n = 6) (B–G). Scale bars = 50 μm (H–J). ACTB, actin beta.
in UUO kidneys, and blocked by IL-11 knockdown (Supplemental Figure S3, A–C). Kidney morphology was examined by using periodic acid-Schiff and Masson’s trichrome staining. IL-11 knockdown significantly improved UUO-induced renal tubular injury and interstitial collagen deposition (Figure 2, J–K). IL-11 knockdown markedly reduced the renal expression of key fibrosis biomarkers, including α-SMA, fibronectin, and TGF-β1, as indicated by immunostaining (Figure 2, L–M).

TGF-β is a primary profibrotic factor that drives renal fibrosis. Herein, significant co-localization of IL-11 and IL-11Rα1 with TGF-β1 were observed in the paraffin sections of UUO kidneys (Figure 3, A and B). In vitro, IL-11 and IL-11Rα1 were both up-regulated by TGF-β1 treatment in mTECs, although to different degrees (Figure 3, C and D). Hence, the effects of IL-11 on the TGF-β1–induced EMT response were revalidated through IL-11 knockdown. Western blot analysis indicated that IL-11 knockdown significantly inhibited TGF-β1–induced α-SMA, fibronectin, and collagen type I expression in mTECs, while restoring E-cadherin expression (Figure 3, E–J). These results suggest that IL-11 knockdown relieved UUO-induced renal fibrosis in vivo and TGF-β1–induced EMT in vitro.

IL-11 Induces EMT and G2/M Arrest in Vitro

The direct effect of IL-11 on EMT was investigated in mTECs. Two groups of mTECs treated with or without recombinant mouse IL-11 (50 ng/mL, 24 hours) were compared (Figure 4, A and B). Western blot analysis showed that α-SMA, fibronectin, and collagen type I expression was up-regulated by IL-11 treatment, whereas E-cadherin expression was inhibited. Similar results were observed with immunostaining for α-SMA and E-cadherin (Figure 4C). The effect of IL-11 on cell cycle progression in RTECs was further examined by using flow cytometry. A 24-hour IL-11 (50 ng/mL) treatment markedly increased the proportion of cells in the G2/M phase (Figure 4, D and E). In contrast, a marked decrease in mRNA expression of the renal tubular biomarker aquaporin 1 (Aqp1) and the increase of Twist1 and Sna1, two core pro-EMT transcription factors, as well as fibrogenic growth factors [including Tgfβ1 and connective tissue growth factor (Ctgf)] were detected after pIL-11 transfection (Figure 4, F–I and Supplemental Figure S4C). Moreover, the transcription of MTDH, a newly defined pro-EMT factor, was markedly up-regulated after IL-11 intervention (Figure 4J). These results suggest that tubular-derived IL-11 promotes renal fibrosis by inducing EMT in RTECs.

IL-11 Induces EMT in RTECs by Activating the STAT3 and ERK1/2/MTDH Signaling Pathways

The underlying mechanism by which IL-11 induced EMT was investigated further. The classical signaling pathways downstream of IL-11Rα1/gp130 include Janus kinase/STAT3, ERK1/2, and AKT signaling.21,28 As shown in Figure 5, A–D, phosphorylated STAT3 was rapidly up-regulated in mTECs treated with recombinant mouse IL-11 (50 ng/mL), and this up-regulation was most pronounced at 5 and 15 minutes. Similarly, under IL-11 stimulation (50 ng/mL), the ERK1/2 signaling pathway was activated but with a longer stimulating time; this effect was much more pronounced at 1 to 2 hours. Conversely, IL-11 stimulation failed to induce AKT phosphorylation within 24 hours. Subsequently, the effect of blocking these two pathways on IL-11–induced EMT in mTECs was investigated to verify whether IL-11 promoted EMT by activating the STAT3 and ERK1/2 pathways. As shown in Figure 5, E–J, pre-incubation with a specific inhibitor of the STAT3 (Stattic) or ERK1/2 (SCH772984) pathway suppressed α-SMA, fibronectin, and collagen type I expression induced by IL-11 overexpression, and halted E-cadherin loss. However, only the ERK1/2 pathway blockage inhibited the expression of MTDH.

To further clarify the role of MTDH in IL-11–mediated EMT, MTDH expression was knocked-down with specific MTDH-shRNA in addition to IL-11 overexpression and the expression of Twist1, Sna1, and EMT markers was detected. As shown in Figure 5K, the transcription of Twist1, up-regulated by IL-11 overexpression, was significantly suppressed after MTDH knockdown. Consistently, IL-11–induced Sna1, α-SMA, and collagen type I expressions were also inhibited, whereas E-cadherin expression was significantly restored after MTDH knockdown (Figure 5, L–Q and Supplemental Figure S4, F and G). These findings indicate that ERK1/2-dependent expression of MTDH is the key effector pathway in IL-11–mediated renal tubular EMT.

Characterization of the Interaction of MCL with IL-11

MCL, which is derived from parthenolide, antagonizes MTDH expression in TGF-β1–induced tubular EMT, but the mechanism is unknown.29 The chemical structures of parthenolide and MCL are depicted in Figure 6A. In a preliminary drug screening using virtual molecular docking, MCL was determined to likely act on IL-11. The preferred docking result with the lowest binding energy (binding energy −7.06, ranking first) showed that MCL formed hydrogen bonds with the amino acid residues ARG77, ASP134, and PRO132 on the active site of IL-11 through the hydrogen atom on the hydroxyl group and the oxygen atom on the carbonyl group (Figure 6B). The formation of three hydrogen bonds allows MCL to be stably embedded in the hydrophobic cavity of IL-11. The truncated conformation (Protein Data Bank identifier 6o4o) of IL-11 in the Protein Data Bank database was used for docking. The actual positions (UniProt p20809) of the whole amino acid sequence of IL-11 corresponding to the three sites were ARG98, ASP155, and PRO153. Hydrophobic interactions and hydrogen bonds make MCL strongly bind to IL-11. Whether the insertion of MCL affected the binding activity of IL-11 to its receptor, IL-11Rα1, was determined next. The interaction
Figure 2  Knockdown of IL-11 ameliorates renal fibrosis after unilateral ureteral obstruction (UUO). A: Animal experimental design. The red arrow indicates the injection of Ctrl-shR or IL-11-shR plasmids. Black arrows indicate the timing of UUO surgery. B–J: Western blot analyses show the effect of IL-11 knockdown on tubular injury and fibrogenic response in UUO mice. Representative Western blot (B) and graphical representations of IL-11 (C), IL-11 receptor subunit alpha-1 (IL-11Rα1) (D), E-cadherin (E), α-smooth muscle actin (α-SMA) (F), transforming growth factor-β1 (TGF-β1) (G), fibronectin (H), and collagen type I (I) in three groups, as indicated. J: Representative micrographs show kidney morphology in different groups, as indicated. Kidney sections were subjected to periodic acid-Schiff (PAS) or Masson trichrome staining. K: Graphical representation of kidney fibrotic lesion area in different groups after quantitative determination. L: Representative immunostaining micrographs show α-SMA, fibronectin, and TGF-β1 expression in different groups. M: Quantitative determination of α-SMA staining in different groups. Data are expressed as means ± SEM; statistical significance was determined by using one-way analysis of variance followed by Tukey-Kramer multiple comparisons test (C–I, K, and M). ***P < 0.001, ****P < 0.0001 compared with sham controls (n = 6); **P < 0.01, ***P < 0.001 compared with the UUO group alone (n = 6) (C–I). ****P < 0.0001 compared with sham controls (n = 6); ***P < 0.0001 compared with the UUO group alone (n = 6) (K). ****P < 0.0001 compared with sham controls (n = 6); ****P < 0.0001 compared with the UUO group alone (n = 6) (M). Scale bars = 50 μm (J and L).
between IL-11 (Protein Data Bank identifier 6o4o) and IL-11Rα (Protein Data Bank identifier 6o4p) was simulated using the macromolecular docking tool ZDOCK. As shown in Supplemental Figure S5, all three residues mediating the combination of MCL with IL-11 were in the main contacting surfaces of IL-11 and IL-11Rα1 proteins; therefore, inserting MCL in this region would probably severely interfere with the binding stability of IL-11 to IL-11Rα1 (Supplemental Table S1).

A pull-down experiment using biotin-labeled MCL (ie, the MCL-probe) and Ctrl-probe was performed to verify the results of virtual docking. As shown in Figure 6C, only the probe conjugated with MCL pulled down IL-11 effectively. Next, a competitive co-immunoprecipitation experiment was performed to further clarify whether MCL treatment influenced the combination of IL-11 with IL-11Rα1. As shown in Figure 6D, mTECs was transfected with an IL-11 expression plasmid carrying flag-tag (pflagIL-11), followed by stimulation with TGF-β1 for 24 hours. 

![Figure 3](https://example.com/f3.png)

**Figure 3** Knockdown of IL-11 ameliorates transforming growth factor-β1 (TGF-β1)—induced epithelial-to-mesenchymal transition in renal tubular epithelial cells. **A:** Co-localization of IL-11 and TGF-β1 in tubules of unilateral ureteral obstruction (UUO) mice. Paraffin-embedded kidney sections were immunofluorescence stained for IL-11 and TGF-β1. **B:** Co-localization of IL-11 receptor subunit alpha-1 (IL-11Rα1) and TGF-β1 in the tubules of UUO mice. Paraffin-embedded kidney sections were immunofluorescence stained for IL-11Rα1 and TGF-β1. **C:** Representative Western blot shows that the expression of IL-11 and IL-11Rα1 were up-regulated in TGF-β1—treated (5 ng/mL) mouse tubular epithelial cells (mTECs). **D:** Graphical representations of IL-11 and IL-11Rα1 expression levels in different groups. **E-J:** Western blot analyses show that IL-11 knockdown with shRNA significantly inhibited the expression of α-smooth muscle actin (α-SMA), fibronectin, and collagen type I, as well as the loss of E-cadherin induced by TGF-β1 in mTECs. mTECs were transfected with Ctrl-shR or IL-11-shR, followed by stimulation with TGF-β1 for 24 hours. Representative Western blot (**E**) and quantitative data on the relative abundance of IL-11 (**F**), E-cadherin (**G**), α-SMA (**H**), fibronectin (**I**), and collagen type I (**J**) in different groups are presented. Data are expressed as means ± SEM; statistical significance was determined by using the U-test (**D**) and one-way analysis of variance followed by Tukey-Kramer multiple comparisons test (**F-J**). **E-J**.

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**A:** DAPI TGF-β1 IL-11 Merged

**B:** DAPI TGF-β1 IL-11Rα1 Merged

**C:** Ctrl TGF-β1 - IL-11 - IL-11Rα1 - α-Tubulin

**D:** Protein expression (relative to α-Tubulin)

**E:** IL-11 E-cadherin α-SMA Fibronectin Collagen I

**F:** IL-11 expression

**G:** E-cadherin expression

**H:** α-SMA expression

**I:** Fibronectin expression

**J:** Collagen I expression

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**Figure 4** Knockdown of IL-11 ameliorates transforming growth factor-β1 (TGF-β1)—induced epithelial-to-mesenchymal transition in renal tubular epithelial cells. **A:** Co-localization of IL-11 and TGF-β1 in tubules of unilateral ureteral obstruction (UUO) mice. Paraffin-embedded kidney sections were immunofluorescence stained for IL-11 and TGF-β1. **B:** Co-localization of IL-11 receptor subunit alpha-1 (IL-11Rα1) and TGF-β1 in the tubules of UUO mice. Paraffin-embedded kidney sections were immunofluorescence stained for IL-11Rα1 and TGF-β1. **C:** Representative Western blot shows that the expression of IL-11 and IL-11Rα1 were up-regulated in TGF-β1—treated (5 ng/mL) mouse tubular epithelial cells (mTECs). **D:** Graphical representations of IL-11 and IL-11Rα1 expression levels in different groups. **E-J:** Western blot analyses show that IL-11 knockdown with shRNA significantly inhibited the expression of α-smooth muscle actin (α-SMA), fibronectin, and collagen type I, as well as the loss of E-cadherin induced by TGF-β1 in mTECs. mTECs were transfected with Ctrl-shR or IL-11-shR, followed by stimulation with TGF-β1 for 24 hours. Representative Western blot (**E**) and quantitative data on the relative abundance of IL-11 (**F**), E-cadherin (**G**), α-SMA (**H**), fibronectin (**I**), and collagen type I (**J**) in different groups are presented. Data are expressed as means ± SEM; statistical significance was determined by using the U-test (**D**) and one-way analysis of variance followed by Tukey-Kramer multiple comparisons test (**F-J**). **E-J**.

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**Figure 5** Knockdown of IL-11 ameliorates transforming growth factor-β1 (TGF-β1)—induced epithelial-to-mesenchymal transition in renal tubular epithelial cells. **A:** Co-localization of IL-11 and TGF-β1 in tubules of unilateral ureteral obstruction (UUO) mice. Paraffin-embedded kidney sections were immunofluorescence stained for IL-11 and TGF-β1. **B:** Co-localization of IL-11 receptor subunit alpha-1 (IL-11Rα1) and TGF-β1 in the tubules of UUO mice. Paraffin-embedded kidney sections were immunofluorescence stained for IL-11Rα1 and TGF-β1. **C:** Representative Western blot shows that the expression of IL-11 and IL-11Rα1 were up-regulated in TGF-β1—treated (5 ng/mL) mouse tubular epithelial cells (mTECs). **D:** Graphical representations of IL-11 and IL-11Rα1 expression levels in different groups. **E-J:** Western blot analyses show that IL-11 knockdown with shRNA significantly inhibited the expression of α-smooth muscle actin (α-SMA), fibronectin, and collagen type I, as well as the loss of E-cadherin induced by TGF-β1 in mTECs. mTECs were transfected with Ctrl-shR or IL-11-shR, followed by stimulation with TGF-β1 for 24 hours. Representative Western blot (**E**) and quantitative data on the relative abundance of IL-11 (**F**), E-cadherin (**G**), α-SMA (**H**), fibronectin (**I**), and collagen type I (**J**) in different groups are presented. Data are expressed as means ± SEM; statistical significance was determined by using the U-test (**D**) and one-way analysis of variance followed by Tukey-Kramer multiple comparisons test (**F-J**). **E-J**.

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**Figure 6** Pull-down experiment using biotin-labeled MCL (ie, the MCL-probe) and Ctrl-probe was performed to verify the results of virtual docking. As shown in Figure 6C, only the probe conjugated with MCL pulled down IL-11 effectively. Next, a competitive co-immunoprecipitation experiment was performed to further clarify whether MCL treatment influenced the combination of IL-11 with IL-11Rα1. As shown in Figure 6D, mTECs was transfected with an IL-11 expression plasmid carrying flag-tag (pflagIL-11), followed by stimulation with TGF-β1 for 24 hours. Representative Western blot (**E**) and quantitative data on the relative abundance of IL-11 (**F**), E-cadherin (**G**), α-SMA (**H**), fibronectin (**I**), and collagen type I (**J**) in different groups are presented. Data are expressed as means ± SEM; statistical significance was determined by using the U-test (**D**) and one-way analysis of variance followed by Tukey-Kramer multiple comparisons test (**F-J**). **E-J**.

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by incubation with or without MCL. The protein abundance of IL-11Rα1 being pulled down by the FLAG antibody was determined by Western blot analysis. The results indicated that the combination of IL-11Rα1 and exogenous IL-11 was markedly abolished by MCL treatment (Figure 6, D and E). These results show that MCL competitively inhibits the interaction of IL-11 and IL-11Rα1 by recognizing and binding to the active region of IL-11.

**Figure 4** IL-11 induces epithelial-to-mesenchymal transition and G2/M arrest *in vitro*. A and B: Western blot analysis shows that IL-11 stimulation significantly increased the expression of α-smooth muscle actin (α-SMA), fibronectin, and collagen type I and decreased the expression of E-cadherin in mouse tubular epithelial cells (mTECs). mTECs were treated with recombinant mouse IL-11 at 50 ng/mL for 24 hours. Representative Western blot (A) and quantitative data showing the means ± SEM of at least three independent experiments (B) are presented. C: Representative immunofluorescence micrographs of E-cadherin (green) and α-SMA (red) merged with DAPI (blue) in mTECs treated with or without the recombinant mouse IL-11 for 24 hours. D and E: Cell cycle analysis by propidium iodide staining and flow cytometry in mTECs at baseline (left) and after treatment with recombinant mouse IL-11 at 50 ng/mL for 24 hours (right). Representative cell cycle distribution shown by DNA contents (D) and quantitative data from three independent experiments are presented (E). F–J: Graphical representations of the mRNA level of AQP-1 (F), Twist1 (G), CTGF (H), TGF-β1 (I), and MTDH (J) relative to ACTB in mTECs transfected with control vector or IL-11 gene expression plasmid pEX-5-IL-11 (pIL-11) for 24 hours. Data are expressed as means ± SEM; statistical significance was determined by using the U-test (B, F, G, H, I, and J) and parametric t-test (E). *P < 0.05 compared with the control group (n = 3 or 4) (B). **P < 0.01 versus Ctrl group in G2/M phase (n = 3) (E). *P < 0.05, **P < 0.01 compared with the pEX-5 (vector) group (n = 3) (F–J). Scale bars = 200 μm (C). MTDH, metadherin; TGF-β1, transforming growth factor-β1.
Figure 5  IL-11 induces epithelial-to-mesenchymal transition (EMT) of renal tubular epithelial cells, mainly through the activation of STAT3 and extracellular signal–regulated kinase (ERK1/2)/metadherin (MTDH) signaling pathways. A–D: Western blot analyses of phosphorylated (activated) and total STAT3, ERK1/2, and AKT in IL-11–induced mouse tubular epithelial cells (mTECs) for different durations. Representative Western blots (A) and quantitative data on the relative abundance of phosphorylated (p)-STAT3/STAT3 (B), p-ERK1/2/ERK1/2 (C), and p-AKT/AKT (D) in five groups, as indicated. E: Representative Western blots show that the specific small-molecule inhibitor of ERK1/2 (SCH772984) or STAT3 (Stattic) blocks IL-11–induced EMT. mTECs were transfected with vector or pIL-11, then treated with pathway inhibitor SCH772984 (1 μmol/L) or Stattic (0.5 μmol/L) for 24 hours. F–J: Graphical representations of MTDH (F), E-cadherin (G), α-smooth muscle actin (α-SMA) (H), fibronectin (I), and collagen type I (J) protein expression levels in different groups, as indicated. K: Knockdown of MTDH reduced IL-11–induced Twist1 mRNA transcription in mTECs. mTECs were transfected with negative control plasmid (sh-NC) (vector) or MTDH shRNA expression plasmid (sh-MTDH) for 6 hours, then further transfected with pEX-5 or pIL-11. Cells were cultured for 24 hours. L: Representative Western blots show that knockdown of MTDH ameliorates IL-11–induced EMT response in mTECs. M–Q: Graphical representations of IL-11 (M), MTDH (N), E-cadherin (O), α-SMA (P), and collagen type I (Q) expression levels in different groups, as indicated. Data are expressed as means ± SEM; statistical significance was determined using one-way analysis of variance followed by Tukey-Kramer multiple comparisons test (B–D, F–K, and M–Q). *P < 0.05, ****P < 0.0001 compared with the group treated without IL-11 (n = 3) (B–D). *P < 0.05, ****P < 0.0001 compared with the pEX-5 group alone; †P < 0.05, ††P < 0.01, †††P < 0.001, ††††P < 0.0001 compared with the pEX-5 group alone; ‡P < 0.05, §P < 0.01, ¶P < 0.001, §§§P < 0.0001 compared with the pIL-11 group alone (n = 3) (F–J). **P < 0.01 compared with pEX-5 + sh-NC; †P < 0.05 compared with pIL-11 + sh-NC (n = 3) (K). *P < 0.05, **P < 0.01 compared with pEX-5 + sh-NC; †P < 0.05, ††P < 0.001 compared with pIL-11 + sh-NC (n = 3) (M–Q). ACTB, actin beta.
MCL Inhibits IL-11–Induced EMT through Double Blockage of STAT3 and ERK1/2/MTDH Signaling Pathways

The role of MCL in IL-11–induced EMT was evaluated further. Based on the findings that IL-11 induces EMT in mTECs primarily through the activation of STAT3 and ERK1/2 signaling pathways, the effect of MCL intervention on STAT3 and ERK1/2 signaling were examined first. As shown in Figure 7, A–D, 2.5, 5, and 10 μmol/L MCL drastically down-regulated IL-11–induced STAT3 phosphorylation in mTECs, although in a nondiscernible concentration-dependent manner. MCL at 5 and 10 μM concentrations significantly decreased IL-11–induced ERK1/2 phosphorylation. Based on these findings, 5 μmol/L was selected as the optimal MCL dose and its effect on IL-11–mediated EMT and extracellular matrix protein production in mTECs was tested. As shown in Figure 7, E–J, MCL intervention inhibited the upregulation of MTDH, α-SMA, fibronectin, and collagen type I, and restored E-cadherin expression in mTECs overexpressing IL-11. These results suggest that MCL is a potent inhibitor of IL-11–induced EMT, which may be achieved through the double blockage of the STAT3 and ERK1/2/MTDH–signaling pathways in tubular epithelial cells.
DMAMCL Ameliorates Renal Fibrosis Exacerbated by IL-11 Overexpression in UUO Mice

UUO mice were treated with DMAMCL, the pro-drug of MCL, immediately after transferring the IL-11 encoding vector (pIL-11) or control empty vector (pEX-5) to UUO kidneys. As shown in Figure 8A, DMAMCL is a dimethylamino addition derivative of MCL with better water solubility and druggability and can be administered via gavage. The experimental design is shown in Figure 8B, where the pIL-11 plasmid or empty vector (pEX-5) was transferred into the kidney through hydrodynamic-based gene delivery 3 days after UUO surgery. DMAMCL (25 mg/kg per day) or an equal volume of normal saline was administered every day for 1 week after plasmid injection. The pathologic morphology was detected through periodic acid-Schiff and Masson's trichrome staining. Ectopic IL-11 expression significantly aggravated the tubular injury and interstitial matrix deposition in the UUO model, and DMAMCL ameliorated UUO-induced fibrotic pathologic changes despite the overexpression of IL-11 (Figure 8, C and D). Western blot analyses revealed that IL-11 and IL-11Rα expression was up-regulated in UUO kidneys, and IL-11 expression was further enhanced through a single infusion of the pIL-11 plasmid. DMAMCL treatment markedly inhibited IL-11 and IL-11Rα expression, probably due to the improvement of overall kidney damage (Figure 8, E–G).

Immunostaining of kidney sections with antibodies against α-SMA, fibronectin, and collagen type I revealed...
Dimethylaminomicheliolide (DMAMCL) ameliorates renal fibrosis exacerbated by IL-11 overexpression in unilateral ureteral obstruction (UUO) mice. A: Chemical structures of micheliolide (MCL) and DMAMCL. B: Animal experimental design. The red arrow indicates the injection of pEX-5 or pIL-11 plasmids. Green arrows indicate the injection of DMAMCL. Black arrows indicate the timing of UUO surgery. C: Representative micrographs of periodic acid-Schiff (PAS) and Masson’s trichrome staining showed that the ectopic expression of IL-11 significantly aggravated the tubular injury and interstitial matrix deposition in the UUO model, whereas DMAMCL ameliorated UUO-induced pathologic changes despite the overexpression of IL-11. D: Fibrosis was quantitatively assessed by analysis of Masson trichrome staining in the five groups. E–G: Western blot analyses show renal expression of IL-11 and IL-11 receptor subunit alpha-1 (IL-11Rα1) proteins in five groups, as indicated. Representative Western blot of IL-11 and IL-11Rα1 (E) and quantification results (F and G) are presented. H: Representative micrographs of immunohistochemistry staining and for fibronectin, collagen type I, and α-smooth muscle actin (α-SMA) in five groups, as indicated. I–K: Quantitative determination of fibronectin (I), collagen type I (J), and α-SMA (K) staining in different groups. L: Western blot analyses show that the renal expression of α-SMA, fibronectin, and collagen type I was up-regulated by IL-11 overexpression and down-regulated by DMAMCL treatment in the UUO model. M–O: Graphical representations of renal protein abundance of α-SMA (M), fibronectin (N), and collagen type I (O) in five groups. Data are expressed as means ± SEM; statistical significance was determined using one-way analysis of variance followed by Tukey-Kramer multiple comparisons test (D, F, G, I–K, and M–O). ***P < 0.0001 compared with UUO group alone (n = 6). **P < 0.0001 compared with UUO group alone (n = 6). ***P < 0.0001 compared with the UUO with pIL-11 group (n = 6) (D). *P < 0.05, ***P < 0.001, ****P < 0.0001 compared with UUO group alone (n = 6); **P < 0.01 compared with UUO group alone (n = 6); ***P < 0.001 compared with UUO group alone (n = 6). #P < 0.05, ##P < 0.01 compared with the UUO with pIL-11 group (n = 6) (F and G). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 compared with UUO group alone (n = 6); **P < 0.01 compared with UUO group alone (n = 6); ***P < 0.001 compared with UUO group alone (n = 6); ****P < 0.0001 compared with UUO group alone (n = 6). #P < 0.05, ##P < 0.01, ###P < 0.001 compared with UUO group alone (n = 6). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 compared with the UUO with pIL-11 group (n = 6) (M–O). Scale bars = 50 μm (C and H).
that the in vivo expression of exogenous IL-11 exacerbated the deposition of major interstitial matrix proteins and the transdifferentiation of fibroblasts to myofibroblasts induced by UUO. DMAMCL treatment effectively alleviated these fibrogenic phenotypes in the UUO model, even with exogenous IL-11 expression (Figure 8, H–K). Similar results were observed with Western blot analyses of kidney lysates (Figure 8, L–O). The enhanced expression of phosphorylated STAT3 and phosphorylated ERK1/2 in UUO kidneys was also observed; overexpression of IL-11 in vivo exacerbated this phenomenon. Consistent with the in vitro results, DMAMCL not only inhibited the STAT3 and ERK1/2 signaling activated by UUO alone but also inhibited their activation intensified by IL-11 overexpression (Supplemental Figure S3, D–F). These findings show that the protective effect of DMAMCL on renal fibrosis is closely related to its inhibition effect on IL-11 and its downstream signaling.

Discussion

Renal fibrosis has been the subject of extensive research in the past few decades. However, the molecular mechanisms underlying its progression are still poorly understood, and no effective clinical approaches are available for its treatment. Based on previous research, the current study further showed that IL-11 contributed to renal fibrosis development, at least partly, through ERK1/2/MTDH induction and its resultant EMT response in RTECs. Moreover, MCL halted IL-11–induced EMT and renal fibrosis by blocking the STAT3 and ERK1/2/MTDH signaling pathways. These findings shed new light on the molecular mechanism of renal fibrosis and provide a potential avenue for its treatment.

In the last few years, the profibrotic role of IL-11 in different organs has been frequently reported, indicating that IL-11 might be a potential therapeutic target for inhibiting fibrosis progression.16–18 Recently, Widjaja et al29,30 showed that neutralization of IL-11 reduced albuminuria, improved renal function, and increased lifespan in multiple acute and chronic models of kidney disease, accompanied by reduced fibrosis and inflammation, attenuation of EMT, and increased expression of regenerative markers. Notably, an important study showing the profibrotic effect of IL-11 in the heart and kidney published earlier by this team showed that IL-11 and its receptor (IL-11Rα1) are expressed specifically in fibroblasts.16 The discrepancy in the main expression sites of IL-11 and its receptor in different organs aroused our interest because it reflects a huge difference in the profibrotic mechanism. In this study, a classical model of renal fibrosis (UUO) was used to detect the expression sites of IL-11 and IL-11Rα1 in kidneys. As verified by in situ hybridization and immunofluorescence, the positive staining of IL-11 and IL-11Rα1 mainly appeared in the renal tubules, at the mRNA transcription and protein expression levels. Only a negligible amount of IL-11 could be detected in the renal interstitium, which meant that the fibrotic response driven by IL-11 in UUO kidneys probably originated from RTECs. Interestingly, IL-11Rα1 is mainly expressed on the luminal surface of renal tubules, which led to the hypothesis that IL-11 might be secreted into the urine and could be measured as a noninvasive predictor of renal fibrosis; this hypothesis will be verified in our subsequent studies.

TGF-β1 is widely considered as a principal profibrotic factor in renal fibrosis, but its inhibition is associated with many side effects. Up-regulation of IL-11 is the dominant transcriptional response to TGF-β1 exposure and is required for its profibrotic effect.16 Co-localization of TGF-β1 with IL-11 and IL-11Rα1 was observed in renal tubules of UUO kidneys. Knockdown of IL-11 through a hydrodynamic-based gene delivery approach significantly ameliorated UUO-induced tubulointerstitial fibrosis. In mTECs, IL-11 knockdown markedly inhibited the TGF-β1–mediated EMT response. Based on these results, whether IL-11 intervention could directly induce EMT in RTECs was investigated further. Stimulation with recombinant IL-11 successfully induced the transdifferentiation of RTECs from the epithelial to the mesenchymal phenotype, arrested the cell cycle in the G2/M phase, and increased the synthesis of profibrotic mediators. Furthermore, the overexpression of exogenous IL-11 in UUO kidneys significantly exacerbated the progression of UUO-mediated renal fibrosis. IL-11 stimulation also up-regulated Tgfβ1 mRNA transcription in RTECs, indicating that the relationship between TGF-β1 and IL-11 is not simply upstream and downstream but more likely involves positive feedback, creating a vicious cycle in the EMT response.

The underlying mechanism by which IL-11 induced EMT was explored further by detecting the activation of three classical pathways downstream of IL-11Rα1/gp130, including STAT3, ERK1/2, and AKT. Specific inhibitors and regulation of IL-11 expression in cultured RTECs and UUO kidneys were used to show that IL-11–mediated tubular EMT and renal fibrosis were closely associated with activating the STAT3 and ERK1/2 pathways. However, the activation timing of these two signaling pathways was inconsistent. The STAT3 pathway was significantly activated after 5 minutes of IL-11 treatment, and its activity gradually decreased as the time was extended to 24 hours. In contrast, ERK1/2 pathway activation was gradually enhanced by IL-11 from 1 to 2 hours, and it remained highly active until the end of the intervention. Altogether, these results suggest that the STAT3 and ERK1/2 pathways may be sequentially involved in IL-11–mediated EMT in RTECs, in which activation of the STAT3 pathway may initiate the EMT response, and ERK1/2 is more likely to play an important role in maintaining the phenotype transition. This hypothesis needs to be confirmed in future experiments.

Importantly, the current results revealed that IL-11 mediated the EMT response, at least partly, through
MTDH induction. MTDH, also known as lysine-rich CEACAM1 co-isolated (LYRIC)/3D3 and astrocyte elevated gene-1, was initially discovered as an oncogene up-regulated in various cancers. It promotes tumorigenesis and metastasis by inducing EMT. MTDH up-regulation in RTECs is an important driving force of EMT in ischemia-reperfusion injury—induced and UUO-induced renal fibrosis; however, the mechanism mediating MTDH over-expression is unclear. The current study showed that IL-11 is an important inducer mediating MTDH expression, and MTDH knockdown significantly inhibited IL-11-mediated EMT responses in mTECs. Furthermore, selective blockade of the ERK1/2 pathway markedly inhibited IL-11-mediated MTDH expression. These findings indicate that IL-11 causes EMT, at least partly, through MTDH induction by activating the ERK1/2 pathway in RTECs. Interestingly, MTDH overexpression also induced ERK1/2 pathway activation, suggesting that there might be positive feedback between MTDH and the ERK1/2 pathway in RTECs.

DMAMCL, which gradually releases MCL into the plasma, is currently emerging as a promising anticancer therapeutic and has been authorized for clinical trials in Australia and China for treating glioblastoma multiforme. Accumulating evidence shows that MCL has anticancer activities in many oncologic diseases by promoting tumour-specific metabolic pathways, promoting tumour cell apoptosis, and inhibiting inflammatory and EMT responses. In addition to its therapeutic effect on oncologic diseases, DMAMCL ameliorates inflammatory and fibrotic lesions in the kidney, liver, and peritoneum in preliminary studies. DMAMCL/MCL blocks the MTDH/bone morphogenetic protein/mitogen-activated protein kinase pathway by inhibiting MTDH expression, which inhibits EMT in RTECs and delays UUO-mediated and ischemia-reperfusion injury—mediated renal fibrosis. However, the specific mechanism by which MCL inhibits MTDH expression is still unclear.

In the current study, molecular screening via virtual docking revealed multiple potential binding sites for MCL and IL-11. These sites were located in the main contacting surface of IL-11 and IL-11Rz1, suggesting that MCL treatment may interfere with the interaction of IL-11 with IL-11Rz1. Molecular probe—mediated protein pull-down and competitive co-immunoprecipitation were conducted to verify the results of virtual docking. Consistently, these experiments showed that MCL could bind IL-11 and competitively inhibit the combination of IL-11 and IL-11Rz1. The inhibitory effect of MCL on the downstream signaling of IL-11 was subsequently verified. Finally, the study confirmed that MCL competitively binds IL-11 and inhibits the interaction of IL-11 with IL-11Rz1, which in turn blocks the downstream signaling pathways STAT3 and ERK1/2, ultimately inhibiting the expression of MTDH and the subsequent EMT response in RTECs. In vivo, DMAMCL intervention had a significant inhibitory effect on IL-11—induced renal fibrosis, further validating the mechanism by which MCL exerts its antifibrotic effects by targeting IL-11 and its downstream effector pathways.

In summary, the results of this study show that aberrant up-regulation of IL-11 in RTECs is important in the progression of renal fibrosis and that MCL could treat renal fibrosis by targeting IL-11 and inhibiting its downstream effects. The study provides new insights into the pathogenesis and treatment of renal fibrosis.

**Author Contributions**

Z.X. and H.L. conceived the study; C.L. conceived and designed the experiments; Y.L. conducted the experiments; Y.Z., Z.Z., and D.T. analyzed the data; Q.L., Y.H., and S.W. provided the reagents and materials; and C.L. and Y.L. wrote the manuscript. All authors reviewed the manuscript and read, verified, and approved the final version.

**Disclosure Statement**

None declared.

**Supplemental Data**

Supplemental material for this article can be found at http://doi.org/10.1016/j.ajpath.2023.07.005.

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