cMet agonistic antibody prevents acute kidney injury to chronic kidney disease transition by suppressing Smurf1 and activating Smad7

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Running title: cMet antibody and prevention of AKI-to-CKD transition

Abbreviation: AKI = acute kidney injury; CKD = chronic kidney disease; cMet Ab = cMet agonistic antibody; CRRT = continuous renal replacement therapy; GFR = glomerular filtration rate; HGF = hepatocyte growth factor; hPTECs = human proximal tubular epithelial cells; UIRI = unilateral ischemia-reperfusion injury

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Clinical perspectives

- A higher increase in soluble plasma cMet level is a prognostic indicator for AKI-to-CKD transition, and the TGF-β1/Smad signaling pathway has been shown to be involved in hypoxia-induced AKI-to-CKD transition both in vivo in mice and in vitro in human primary tubular cell models.

- Treatment with a cMet agonistic antibody successfully modulated the TGF-β1/Smad signaling pathway (through Smad7 dependent- and independent-mechanisms) via the suppression of Smurf1 and activation of Smad7, thus reversing inflammatory responses and kidney fibrosis.

- The cMet agonistic antibody can prevent AKI-to-CKD transition by suppressing Smurf1 and activating Smad7.
ABSTRACT

We aimed to investigate the role of cMet agonistic antibody (cMet Ab) in preventing kidney fibrosis during acute kidney injury (AKI) to chronic kidney disease (CKD) transition. Additionally, we explored the effect of cMet Ab on TGF-β1/Smad pathway during the pathogenesis of kidney fibrosis. A unilateral ischemia-reperfusion injury (UIRI) mouse model was established to induce AKI-to-CKD transition. Furthermore, we incubated human proximal tubular epithelial cells under hypoxic conditions as in vitro model of kidney fibrosis. We analyzed the soluble plasma cMet level in patients with AKI requiring dialysis. Patients who did not recover kidney function and progressed to CKD presented a higher increase in the cMet level. The kidneys of mice treated with cMet Ab showed fewer contractions and weighed more than the controls. The mice in the cMet Ab-treated group showed reduced fibrosis and significantly decreased expression of fibronectin and α-smooth muscle actin. cMet Ab treatment decreased inflammatory marker (MCP-1, TNF-α, and IL-1β) expression, reduced Smurf1 and Smad2/3 level, and increased Smad7 expressions. cMet Ab treatment increased cMet expression and reduced the hypoxia-induced increase in collagen-1 and ICAM-1 expression, thereby reducing apoptosis in the in vitro cell model. After cMet Ab treatment, hypoxia-induced expression of Smurf1, Smad2/3, and TGF-β1 was reduced, and suppressed Smad7 was activated. Down-regulation of Smurf1 resulted in suppression of hypoxia-induced fibronectin expression, whereas treatment with cMet Ab showed synergistic effects. cMet Ab can successfully prevent fibrosis response in UIRI models of kidney fibrosis by decreasing inflammatory response and inhibiting the TGF-β1/Smad pathway.

Keywords: HGF, cMet, kidney fibrosis, AKI-to-CKD transition, Smad7, Smurf
Introduction

Acute kidney injury (AKI) is clinically important and may result in the progression of chronic kidney disease (CKD), thus, worsening the overall prognosis of the patients [1, 2]. Evidence indicates that AKI is not just a benign or reversible condition, but can also induce CKD development [3-5]. Thus, if the pathophysiological changes associated with AKI are well-managed, CKD incidences caused by AKI can be reduced [6]. Kidney fibrosis is an important pathological feature of CKD and is involved in the process of AKI-to-CKD transition [7]. Nevertheless, effective treatment options against the development of kidney fibrosis remain limited [8].

cMet is a transmembrane tyrosine kinase receptor for hepatocyte growth factor (HGF) and is involved in cell survival, growth, and regeneration [9]. The activation of HGF/cMet axis improves kidney diseases by inhibiting oxidative stress, apoptosis, fibrosis, and inflammation [10, 11]. In particular, cMet monoclonal antibody has been suggested as a potential therapeutic agent [12]. Recently, we confirmed the involvement of the HGF/cMet pathway in kidney fibrosis and showed that the cMet agonistic antibody (cMet Ab) reduces fibrosis and improves apoptosis in both glomerular endothelial cell model and unilateral ureteral obstruction model [13, 14]. However, the inhibitory activity of cMet Ab on kidney fibrosis during AKI-to-CKD transition remains to be fully elucidated.

The aim of this study was to investigate whether the cMet Ab can prevent CKD development by inhibiting kidney fibrosis using a unilateral ischemia-reperfusion injury (UIRI) induced in vivo AKI-to-CKD transition model. We also explored the regulatory mechanisms associated with the TGF-β1/Smad pathway in an in vitro hypoxia-induced kidney cell model and investigated whether cMet Ab treatment can modulate the TGF-β1/Smad pathway and prevent kidney fibrosis.
**Materials and methods**

**Measurement of plasma HGF and cMet levels in AKI patients.**

Clinical information of the 131 AKI patients who were admitted to intensive care unit and required continuous renal replacement therapy (CRRT) were collected, and their plasma samples were obtained to explore the relationship between plasma HGF or cMet concentrations and kidney-disease related prognosis. The study was approved by the Institutional Review Board of Seoul National University Boramae Medical Center (No. 2017-2/062). Informed consents were obtained from the study participants before enrollment. When a patient was started with CRRT, we prospectively collected the plasma samples on the day of starting dialysis and thereafter on days 2 and 7 [15]. Plasma HGF and cMet concentrations were measured using human HGF DuoSet ELISA kit (DY294, R&D systems), and soluble cMet Human ELISA kit (KHO2031, Invitrogen, ThermoFisher), respectively. The patient data corresponding to 3-month kidney-disease related outcomes including renal replacement therapy, serum creatinine, and estimated glomerular filtration rate (GFR) levels were collected. AKI to CKD progression was considered for patients who remained on dialysis and whose 3-month estimated GFR decreased ≥ 15% from the baseline estimated GFR levels.

**Animal models of UIRI and CKD transition.**

The animal experiments were conducted at the animal laboratory of Seoul National University Boramae Medical Center under specific pathogen-free conditions in accordance with the National Research Council’s ‘Guidelines for the Care and Use of Laboratory Animals’ after approval from the Seoul National University Boramae Medical Center Institutional Animal Care and Use Committee (No. 2019-0002). C57BL/6 male mice (20-22 g, aged 7 weeks) were purchased from (Koatech, Kyeonggi-do, Korea) and acclimatized to the laboratory conditions
for 1 week. The animals were randomly divided into 4 experimental groups: sham-operated normal control with vehicle treatment (saline, n = 5), cMet Ab treatment (20 mg/kg/day, n = 5), UIRI operated mice with vehicle treatment (saline, n = 6) and UIRI operated mice with cMet Ab treatment (20 mg/kg/day, n = 6).

Four-week AKI-to-CKD transition model was established by UIRI and sham surgery, as reported previously [16]. Briefly, the mice were anesthetized with intraperitoneal injection of Rompun™ (xylazine 10 mg/kg; Bayer korea Co., Ansan, Korea) mixed with Zoletil™ (zolazepam 30 mg/kg; Virbac Korea). During the procedure, the temperature of the animals was maintained at around 37 °C by placing them on heating pads. The left kidney of the mice was pulled out following a left flank incision, and the kidney pedicles were exposed to allow easy manipulation. Pedicles containing left renal artery were cross-clamped with proper devices (Roboz Surgical Instrument Co., Gaithersburg, MD) for 27 min. Left kidneys of sham operated groups were also exposed but renal artery clamping was not performed. cMet Ab (VM507), produced by the R&D Center for Innovative Medicines, Helixmith (Seoul, Korea) [14], was intravenously injected via tail vein 1 day before surgery and biweekly thereafter. The mice in the control group were administered with saline following the same schedule as that of treatment group. Mice were sacrificed at post-operative day 28 (4 weeks) followed by harvesting of left kidney. The harvested kidney was cut in transverse direction. The upper half of the kidney was dissected transversely into 3 tissue specimens from top to center and was used for mRNA extraction, frozen tissue specimen, and for estimating protein expression using western blot. Lower half was cut into 2 tissue specimens; upper section was used for preparing paraffin tissue block and lower section was used for estimating protein expression using western blot. Animal experiments were performed three times to evaluate statistical significance.
Histological examination for establishing fibrosis.

Paraffin embedded kidney tissue sections (4 μm thick) were stained with Masson’s trichrome and Sirius red (All from ScyTek, Logan, Utah, USA) to evaluate the extent of tissue fibrosis [16]. For each kidney section, at least 8 fields were randomly selected and photographed using light microscope (BX53F2; Olympus, Tokyo, Japan). The area of fibrosis and total tissue were measured at ×100 magnification using ImageJ 1.52d software (Wayne Rasband, National Institute of Health, USA).

Immunohistochemistry.

Paraffinized kidney tissue blocks were sliced to obtain 4 μm-thick sections, deparaffinized in xylene, and rehydrated in ethanol. Sliced specimens were heated in a microwave oven for 5 min repeatedly 3 times with 10% citrate buffer solution (pH 6.0) to retrieve the antigen. Endogenous streptavidin activity was blocked with 3% hydrogen peroxide in methanol for 10 min at room temperature. Kidney sections were probed with primary antibodies against TGF-β1, Smad2/3, and Smad7 and incubated overnight at 4 °C. Polink HRP DAB detection kit was used to detect rabbit primary antibodies (GBI Labs, Bothell, WA, USA). The sections were counterstained with Mayer’s hematoxylin (ScyTek Laboratories, Logan, UT, USA). The stained slides were evaluated at ×200 magnification and images were captured in at least 5 selected fields. The percentage of TGF-β1, Smad2/3, and Smad7 positive areas were measured by using ImageJ 1.52d software (Wayne Rasband, National Institute of Health, USA).

Quantitative reverse-transcription PCR (qRT-PCR).
The mRNA expression was measured using qRT-PCR. Total RNA from the kidney tissue and cell lysates was extracted using the TRIzol reagent (Bioline, Luckenwalde, Germany). One microgram of RNA from each tissue sample was used for cDNA synthesis using the Reverse Transcription System kit (Promega, Madison, WI, USA). Quantitative RT-PCR in a LightCycler®-480 instrument II (LifeScience, Roche Molecular Systems Inc., Pleasanton, CA, USA) was performed using assay-on-demand TaqMan® probes and primers for Bax, p53, IL-1β, TNF-α, MCP-1, Smurf1, and GAPDH genes, whereas, SYBR Green and specific primers were used for αSMA, COL1A1, fibronectin, TGF-β1, Smad2/3, Smad7, and Smurf1 (Supplementary Table 1). The mRNA levels of different genes were normalized to that of GAPDH mRNA levels and relative quantification was performed using the ΔΔCT method.

Western blot analysis.

Kidney tissues harvested 4 weeks after inducing UIRI and human proximal tubular epithelial cells (hPTECs) incubated for 72 hours were homogenized, followed by protein extraction using RIPA buffer containing complete protease inhibitors cocktail (Thermo fisher, Rockford, IL, USA). Protein concentrations were determined using the bicinchoninic acid (BCA) assay (Thermo scientific, Rockford, IL, USA) and equal amounts of protein extracts were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred and immobilized onto the membranes (Millipore Corporation, Bedford, MA, USA). After blocking the non-specific proteins, the membranes were incubated with specific primary antibodies overnight at 4 °C to probe target proteins (Supplementary Table 2). Anti-rabbit IgG or anti-mouse IgG antibodies (All from Cell Signaling Technology, Danvers, MA, USA) were used as secondary antibodies. Protein bands were visualized on an
enhanced chemiluminescence system (Advansta, CA, USA) and quantified using ImageJ 1.52d software (Wayne Rasband, National Institute of Health, USA).

**Establishment of the in vitro model of AKI-to-CKD transition.**

In this study, we established an *in vitro* model of hypoxia-induced AKI-to-CKD transition model using hPTECs [17, 18]. The protocol for obtaining and processing human kidney specimen was reviewed and approved by the institutional review board of Seoul National University Hospital (IRB no. 1002-045-309). Human donors of kidney specimens provided written informed consent before nephrectomy. Human proximal tubule segments were isolated from the kidneys surgically removed from patients diagnosed with renal cell carcinoma. After dissecting the cortex, the unaffected specimens were minced and digested with Hank’s balanced salt solution (HBSS) containing 3 mg/mL collagenase (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37 °C for 1 hour. The digested kidney cells were washed through a series of sieves (120, 70, and 40 μm in diameter) using phosphate buffer saline, followed by centrifugation at 500 g for 5 min. hPTECs were recovered from the pellet and incubated in DMEM/F12 for 4 hours. Tubules floating in the media were collected and cultured on collagen-coated petri dishes (BD Biosciences, Franklin Lakes, NJ, USA) until the establishment of epithelial cell colonies. Cells after 2–3 passages were used in the current study.

The hPTECs (2 × 10^5 cells/well) were seeded into six-well plates. Following serum starvation, hPTECs were incubated under normoxic (21% O_2, 5% CO_2, and 74% N_2) or hypoxic conditions (1% O_2, 5% CO_2, and 94% N_2). We observed the fibrosis through hypoxic injury for 3 days. One hour before the start of hypoxic treatment, the cells were treated with 0.25, 0.5, or 1 μg/mL cMet Ab or human IgG (R&D Systems, Wiesbaden, Germany). Furthermore, we established oxidative stress-induced cell fibrosis using H_2O_2. The hPTECs were treated with 0.5 mM H_2O_2,
cMet Ab (0.25, 0.5, or 1 μg/mL), or human IgG. After 2 days of treatment, the cell fibrosis was observed. *In vitro* experiments were performed at least three times to evaluate the statistical significance.

**Suppression of Smurf1 and Smad7 by siRNA-based transfection.**

To suppress the expression of Smurf1, we transfected hPTECs with Smurf1 and Smad7-siRNA (sc-41673 and sc-36508; Santa Cruz Biotechnology, Dallas, TX, USA; sequence listed in Supplementary Table 3) according to the manufacturer’s protocol. The hPTECs (2×10⁵ cells/well) were cultured in an antibiotic-free growth medium supplemented with fetal bovine serum (FBS) and incubated in a CO₂ incubator at 37 °C, until the cells attained a confluency of 60-80%, one day before transfection. Transfection Reagent mixture (siRNA duplex solution diluted with the Transfection Medium, both purchased from Santa Cruz Biotechnology) was used for cell transfection. After 19 h of Smurf1 and Smad7 siRNA transfection, the cultured hPTECs were treated with cMet Ab (1 μg/mL) and incubated under normoxic or hypoxic conditions.

**Immunofluorescence staining.**

Immunofluorescence staining was performed on hPTECs, which were seeded onto 4-chamber slides. After stimulation, the cells were fixed and permeabilized using 4% paraformaldehyde and 0.1% Triton X-100. The cells were then incubated with primary antibodies (Supplementary Table 2) overnight at 4 °C after blocking the slides with 2% bovine serum albumin (BSA). Following overnight incubation, the cells were incubated with anti-rabbit antibody and anti-mouse antibody conjugated with Alexa Fluor 488 or 594 (Invitrogen, Eugene, OR, USA) for 1 hour in dark. The cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-
Aldrich, St. Louis, MO, USA). In addition, we performed immunofluorescence staining to detect the level of apoptosis in 4-week-old mouse tissues. The primary antibodies were not considered for negative controls during the staining procedure. The stained slides were scanned by confocal microscopy using a Leica TCS SP8 STED CW instrument (Leica, Wetzlar Germany). The mean values of signal intensity were expressed with the total intensity per region of interest or per field calculated by the MetaMorph image analysis software.

**Flow cytometry analysis.**

A single-cell suspension was prepared by filtering the homogenate using 40 μm pore cell strainer (BD Pharmingen, San Diego, CA, USA). Cells were incubated with Fc Block anti-CD16/32 (IC1918F, BD Pharmingen) and stained with FITC-conjugated anti-fibronectin or isotype control (IC002F, R&D) for 1 h. Fibronectin positive cells were analyzed using a BD FACS Diva instrument version 8.0 (BD Biosciences, Franklin Lakes, NJ, USA). Cell apoptosis and necrosis was quantified by annexin V/PI assay (Supplementary Table 4) using flow cytometry. The hPTECs stained with PI and FITC-conjugated annexin V were incubated for 30 min in dark, followed by analysis with BD FACS Diva instrument.

**Statistical analysis.**

Data in the manuscript are expressed as means with standard deviations. Bar graph are presented with mean values with standard deviations. Statistical analyses were performed using IBM SPSS 20.0 and GraphPad Prism 8.0 (GraphPad Software, San Diego, CA). Differences between groups were analyzed using independent t-test for AKI patients. Pearson correlation method was used to show the relationship between soluble cMet levels and change percentage of eGFR. Two-way ANOVA was applied for animal experiments and post-hoc Tukey test in
comparison between groups including UIRI/vehicle vs. UIRI/cMet Ab groups. One-way ANOVA post-hoc Tukey tests were applied for cell culture related experiments. Detailed statistics are listed in the Supplementary Data. A value of $P < 0.05$ was considered as a threshold for statistical significance.
Results

Relationship between plasma HGF and cMet concentrations and AKI-to-CKD transition.

A total of 66 AKI patients (among the 131 patients enrolled) survived after three months following the initiation of the CRRT. Of these, for 60 patients, 3-month serum creatinine and estimated GFR related data was available and were considered for further analysis. Thirty patients did not recover to attain normal kidney functions (CKD progressors whose estimated GFR decreased ≥15% from the baseline levels) including the 14 patients who remained on dialysis. CKD progressors showed statistically decreased eGFR at three months compared with that with the CKD non-progressor (25.9 ± 33.3 vs. 80.4 ± 35.2 ml/min/1.73 m², respectively) despite the similar baseline clinical and demographical characteristics (Supplementary Table 5). CKD progressors had lower baseline log-transformed plasma cMet levels than non-progressors (Figure 1A, 6.40 ± 0.51 ln(pg/ml) vs. 6.69 ± 0.52 ln(pg/ml), \( P = 0.034 \)). In addition, change fraction in day 2 plasma log-transformed cMet levels from day 0 were higher among the CKD progressors (100.5% ± 3.9% vs. 98.3% ± 4.2%, \( P = 0.048 \)). The correlation between change of eGFR from baseline to 3 months and increase of soluble cMet from day 0 to day 2 was statistically significant (\( P = 0.034 \)). Log-transformed plasma HGF levels were not different between groups at day 0, day 2, and day 7 (data not shown).

cMet agonistic antibody treatment ameliorates kidney fibrosis following AKI.

Body weights of mice were comparable at the time of UIRI induction and sacrifice. Figure 1B shows the representative gross morphology of both kidneys in each group. Compared to the right kidneys, left kidneys had contracted and decreased in size in the UIRI and vehicle treated groups. However, the left kidneys of the UIRI models treated with cMet Ab were relatively
less contracted than those of vehicle-treated group. Furthermore, the weight of the left kidneys per body weight was significantly higher in the UIRI models treated with cMet Ab than in the vehicle-treated group ($P = 0.004$). The areas of interstitial fibrosis as observed by Masson’s trichrome or Sirius red staining were increased in the UIRI group compared to those in the sham-operated group (Figure 1C,S1A). The UIRI models treated with cMet Ab showed less degree of fibrosis, and areas of fibrosis were significantly lower in the cMet Ab-treated group (UIRI+cMet Ab vs. UIRI, 3.90% ± 1.74% vs. 20.24% ± 2.57%, $P < 0.001$ in Masson’s trichrome staining and 5.54% ± 1.35% vs. 19.97% ± 4.19%, $P = 0.005$ in Sirius red staining, Figure 1D). The mRNA expression of $\alpha$-smooth muscle actin ($\alpha$SMA) and fibronectin increased in the kidney tissue of UIRI and vehicle-treated groups and decreased following treatment with cMet Ab (Figure 1E). Furthermore, cMet Ab treatment downregulated the overexpression of COL1A1 and $\alpha$SMA in the UIRI groups and upregulated the decreased expression of E-cadherin protein (Figure 1F,G).

Changes in the expression of apoptosis and inflammation-related markers following treatment with cMet agonistic antibody.

We investigated the changes in apoptosis-related markers in the UIRI models treated with and without cMet Ab antibody. The tissue expression of phospho-p21 and cleaved caspase-3 increased in the UIRI group compared with that in the sham group (Figure 2A,S2). Treatment with cMet Ab decreased the expression of cleaved caspase-3 and phospho-p21 in mice kidney tissue. Furthermore, the administration of cMet Ab downregulated the overexpression of Bax and p53 mRNA in the ischemia-reperfusion model (Figure 2B). Moreover, the overexpression of cleaved caspase-3, Bax and p21 in the UIRI models was downregulated following cMet Ab treatment (Figure 2C,D). In addition, downregulated Bcl-2 expression was activated after cMet
Ab treatment. The overexpression of mRNAs coding inflammation-related markers including IL-1β, TNF-α, and MCP-1, and IL-1β in the UIRI and vehicle-treated groups was downregulated following cMet Ab treatment (Figure 2E,F).

cMet activation controls TGF-β1/Smad pathway in kidney fibrosis.

We investigated whether the increased activity of TGF-β1 in the UIRI models induced AKI-to-CKD transition and whether cMet Ab reduced the TGF-β1 activity in the kidney tissues. The tissue expression of TGF-β1 was higher in the UIRI group than the sham-operated group (Figure 3A,B, S1B). However, the UIRI models treated with cMet Ab showed downregulated expression of TGF-β1 compared with the vehicle-treated mice (UIRI+cMet Ab vs. UIRI, 3.01% ± 1.40% vs. 17.75% ± 4.70%, P < 0.001). Similarly, the overexpression of Smad2/3 observed in the UIRI models was downregulated following the cMet Ab antibody treatment (UIRI+cMet Ab vs. UIRI, 8.81% ± 2.30% vs. 24.93% ± 4.23%, P < 0.001). In contrast, cMet Ab treatment activated the expression of Smad7 in the UIRI models (UIRI+cMet Ab vs. UIRI, 35.51% ± 16.31% vs. 2.67% ± 1.60%, P < 0.001). Furthermore, the increased mRNA expression of TGF-β1 and Smurf1 in the UIRI models was downregulated following cMet Ab treatment (Figure 3C). Western blot analysis of TGF-β1 pathway markers showed increased expression of phospho-Smad2/3 and Smurf1 and TGF-β1, and decreased expression of phospho-cMet and Smad7 in the UIRI models. We identified that cMet Ab treatment activated successfully phospho-cMet tissue expression that was downregulated in the UIRI/vehicle group. Treatment of the UIRI models with cMet Ab reversed the trend of phospho-Smad2/3, Smurf1, TGF-β1, and Smad7 expression (Figure 3D,E).
cMet activation attenuates hypoxia induced cellular response to adhesion, apoptosis, and fibrosis in human proximal tubular epithelial cells.

hPTECs expressed cMet under normoxic condition. However, under hypoxia the cMet expression was decreased, whereas expression of ICAM-1 and COL1A1 was increased (Figure 4A,B,S3). Following hPTEC treatment with cMet Ab, an increase in cMet expression and decrease in ICAM-1 and COL1A1 expression were observed in a dose-dependent manner. As observed in the Annexin V/propidium iodide (PI) assay, hypoxia induced apoptotic response in hPTECs; however, the treatment with cMet Ab significantly reduced the percentage of apoptotic cells (Figure 4C).

Inhibition of Smurf1 and activation of Smad7 as a protective mechanism for kidney fibrosis following cMet agonistic antibody treatment.

Confocal microscopy showed changes in TGF-β1/Smad signaling in hPTECs subjected to hypoxia for 72 hours. Under hypoxic condition, cMet expression was downregulated whereas that of Smurf1 was upregulated when compared to normoxic conditions (Figure 5A,S4A). The treatment with cMet Ab led to significant overexpression of cMet, whereas that of Smurf1 decreased the expression. In addition, suppressed Smad7 expression in hypoxia was reversed in response to decreased Smurf1 expression following cMet Ab treatment (Figure 5B,S4B). Hypoxia-induced Smurf1 activation and Smad2/3 overexpression were inhibited following treatment with cMet Ab (Figure 5C,S4C). The treatment of hPTECs subjected to hypoxia and treated with cMet Ab led to decreased mRNA expression of TGF-β1, Smad2, Smad3, and Smurf1, and increased expression of Smad7 (Figure 5D). The expression pattern of these genes was further confirmed at the protein level by western blotting following cMet Ab treatment (Figure 5E,F).
We investigated the effect of Smurf1 down-regulation during hypoxia with si-RNA based knockdown of Smurf1. The flow cytometric analysis of hPTECs subjected to hypoxia for 72 h showed that the overexpression of fibronectin was reduced by Smurf1 knockdown irrespective of cMet Ab treatment (si-Smurfl vs. IgG control under hypoxia, 47.20% ± 1.77% vs. 60.90% ± 0.56%, \( P < 0.001 \)) (Figure 6A). The downregulation of Smurf1 combined with cMet Ab treatment synergistically inhibited fibronectin expression (si-Smurfl+cMet Ab vs. si-Smurfl under hypoxia, 28.00 ± 0.56% vs. 47.20 ± 1.77%, \( P < 0.001 \)). Furthermore, the effects of Smurf1 suppression on TGF-\( \beta \)/Smad signaling were confirmed using western blotting (Figure 6B,C). Smurf1 suppression increased Smad7 expression but decreased Smad2/3 expression. Although, cMet Ab treatment showed decreased Smurf1 and Smad2/3 and increased Smad7 expression with the subsequent breakdown of TGF-\( \beta \), Smurf1 downregulation alone did not have any additional effect on TGF-\( \beta \) expression.

In addition, we investigated whether the regulation of the TGF-\( \beta \)/Smad signaling pathway by cMet Ab was dependent on Smad7 by blocking Smad7. Smad7 suppression by si-Smad7 successfully downregulated the protein expression of Smad7 under normoxic condition. Under hypoxia condition, the protein expression of Smad7 decreased, and the administration of si-Smad7 did not show additional changes in Smurf1, Smad7, or phospho-Smad2/3. The treatment with cMet Ab under hypoxic condition inhibited Smurf1, activated Smad7, and suppressed the protein expression of phospho-Smad2/3 and fibronectin. Interestingly, cMet Ab treatment under Smad7 suppression by si-Smad7 successfully inhibited Smurf1 and suppressed the subsequent phospho-Smad2/3 and fibronectin expression (Figure 7A,B).
Discussion

We investigated the prognostic effect of soluble cMet level in patients with AKI requiring CRRT and the role of cMet Ab in the prevention of AKI-to-CKD transition using a UIRI mouse model of kidney fibrosis (in vivo model), and hypoxia induced human primary tubular epithelial-mesenchymal transition (in vitro model). A higher increase in the soluble cMet level during the initial phase of AKI and initiation of CRRT was significantly associated with poor kidney function outcomes and the subsequent development of chronic kidney disease. Our experimental results showed that cMet Ab treatment can successfully prevent kidney fibrosis in UIRI-induced AKI-to-CKD transition. Inflammatory markers including IL-1β, MCP-1, and TNF-α play an important role in the development of kidney fibrosis in UIRI models. The treatment with cMet Ab decreased the inflammatory responses. We also investigated the anti-fibrosis effects of cMet Ab in the 2-week UIRI mice model (Figure S5). Furthermore, the TGF-β1 and Smad signaling pathways were found to be involved in hypoxia-induced AKI-to-CKD transition in in vivo and in vitro models. cMet Ab simultaneously inhibits Smurf1 and activates Smady7, and these modulations in Smurf1 and Smad7 independently suppress the TGF-β1 and Smad signaling pathway, thereby leading to the reversal of kidney fibrosis. The anti-fibrosis effects of cMet Ab were also proved in the hydrogen peroxide-challenged in vitro human primary tubular epithelial cell models (Figure S6).

HGF is a pleiotropic protein that binds to its receptor cMet, activates downstream signaling pathways, and performs various biological functions including organ development, cellular proliferation, tissue regeneration, injury repair, and wound healing [19, 20]. In cancer cells or malignant tumors, HGF/cMet activation is closely related to cancer progression or metastasis through abnormal cellular proliferation and angiogenesis [21]. HGF/cMet tissue expression levels were elevated, and elevated serum or plasma HGF/cMet levels were associated with
cancer burden and poor survival prognosis in various malignancies including hepatocellular carcinoma, gastric and colorectal cancer, non-small cell lung cancer, renal cell cancer, head and neck cancer, acute leukemia, and multiple myeloma [22]. Soluble cMet is generated via cMet ectodomain shedding, and soluble cMet can inhibit the HGF/cMet signaling pathway via receptor binding and cMet homodimerization [23]. In general, an elevation in the soluble plasma or serum HGF/cMet levels is associated with a poor prognosis in other medical conditions such as diabetes mellitus and ischemic stroke [24, 25]. On the contrary, the prognostic value of soluble HGF/cMet levels in various kidney diseases has not been well investigated. Plasma HGF levels were elevated in an animal model of AKI and during the hemodialysis session of patients with end-stage kidney disease [26, 27]. Recently, Kim et al. reported that the elevated urinary cMet level was significantly associated with poor kidney function outcomes including the development of end-stage kidney disease among CKD patients with diabetes mellitus [28]. Urinary cMet levels were also associated with the higher degrees of proteinuria, tissue cMet expression levels, and poor kidney outcomes in IgA nephropathy patients’ group [29]. In this study, we showed that higher increase in the plasma cMet level just after the development of AKI and initiation of CRRT was associated with non-recovery from AKI and progression of AKI to CKD among critically ill patients. The severity of AKI and comorbidities, higher inflammatory responses, and higher CRRT dose might affect the increase in soluble cMet level in patients with AKI requiring CRRT [30]. In addition, increased soluble cMet levels can be a factor contributing to poor prognosis by inhibiting the HGF/cMet signaling pathway [31].

Activation of HGF and cMet is important for recovering from AKI via protection from apoptosis, injury repair, and cellular regeneration [32]. HGF and cMet signaling cascade inhibits kidney fibrosis via modulating TGF-β1 pathway [33, 34]. Antifibrotic effects of HGF and cMet have been explored in various in vitro and in vivo models of liver, lung, and kidney
fibrosis [35-39]. Therefore, HGF has attracted attention as a novel therapeutic modality for kidney diseases [40]. However, its short half-life (< 10 min) has reduced its efficacy as a therapeutic agent in AKI and kidney fibrosis. To overcome these limitations, our group investigated the role of cMet Ab in kidney disease using *in vitro* and *in vivo* models and demonstrated that cMet Ab treatment can inhibit kidney fibrosis in an unilateral ureteral obstruction mouse model, and TGF-β1 challenged glomerular endothelial cells and hPTECs [14, 28].

Ischemia-reperfusion injury (IRI) is a well-known cause of AKI [41, 42]. Mason et al. showed that HGF and cMet are upregulated after ischemic injury and that cMet activation is required in the recovery of AKI after ischemia using an *in vivo* selective knockdown mouse model of cMet in the proximal tubules [43]. Recently, Miyabe et al. reported that renal subcapsular implantation of HGF-producing mesothelial cell sheets after IRI attenuated AKI and the subsequent tubular necrosis and kidney fibrosis through the paracrine effects of HGF [44]. Researchers have shown that IRI can result in kidney fibrosis; animal models of chronic IRI can be used to study AKI-to-CKD transition and CKD development as reported by us previously [45, 46]. Different mechanisms have been suggested to be involved in the AKI-to-CKD transition including endothelial dysfunction, tubular epithelial injury, interstitial inflammation, and tissue fibrosis [47]. Kidney cell death including apoptosis and necrosis and inflammatory responses contribute to AKI following IRI [42]. Increased inflammatory responses associated with the presence of TGF-β1 in kidney tissue contribute to fibrosis and progression to CKD, even after functional recovery from AKI. In mouse models of cardiac arrest and cardiopulmonary resuscitation, tubular necrosis accompanied by cellular apoptosis and tissue inflammation occurs prior to recovery from AKI, and increased TGF-β1 and tissue fibrosis were observed in the CKD progression group [48].
TGF-β1 is an important profibrotic factor that plays a key role in the development of kidney fibrosis via activation of myofibroblast, promotion of inflammation, and accumulation of extracellular matrix proteins [49, 50]. TGF-β1 increases the production of extracellular matrix components including collagens, proteoglycans, and glycoproteins, and inhibits extracellular matrix degradation by inhibiting the synthesis of metalloproteinases [51, 52]. Among various downstream pathways, TGF-β1/Smad signaling is a major pathway associated with the progression to kidney fibrosis [53]. Smad signaling is up-regulated by phosphorylation of receptor-regulated R-Smads (eg. Smad1-Smad3, Smad3, Smad5, Smad8) and inhibited by activation of inhibitory Smads (I-Smad, such as Smad6 and Smad7). Smad7 is a representative I-Smad and negatively regulates Smad signaling by negative feed-back mechanism. Smad7 recruits and binds to E3 ligases, inhibits Smad3 phosphorylation, and down-regulates TGF-β1 receptor via ubiquitylation and degradation. Thus, increased Smad7 expression exerts protective effects on kidney fibrosis, whereas decreased Smad7 levels are observed in various models of kidney disease, including AKI, allograft rejection, and unilateral ureteral obstruction [54, 55].

Negative feedback mechanism of Smad signaling is regulated by E3 ubiquitin ligases including Smurfs. Smurfs bind to Smad7 and down-regulate TGF-β1 receptor via ubiquitin-mediated degradation pathway. In pathological conditions, Smurf/Smad7 complex formation is inhibited, resulting in activation of Smurfs, which down-regulates and degrades Smad7. During the progression of kidney fibrosis, the negative feedback mechanism is impaired. Further, activation of Smurf has been reported in various models of kidney fibrosis and diseases including in vitro model of TGF-β1 challenged kidney tubular cell model and in vivo models of unilateral ureteral obstruction, nephritis, diabetic nephropathy, and surgically partial nephrectomized kidney fibrosis [56-59]. Multiple studies have shown the importance of TGF-β1/Smad signaling pathway as a therapeutic target for kidney fibrosis by modulating...
Smad7/Smurfs. Ma et al. showed that in partial nephrectomized kidney fibrosis model, increased levels of Smurf2 and decreased expression of Smad7 were reversed after anti-fibrotic drug treatment [60]. Song et al. reported that Smurf1 silencing by siRNA increased the expression of Smad7 and in turn activated Smurf1 [61]. Further, the authors proved that Nrf2 activating drug Bardoxolone prevented TGF-β1 associated kidney fibrosis via increasing Smad7 in a 2-week aristolochic mouse kidney fibrosis model. In our study, we showed that treatment with cMet Ab attenuated and prevented kidney fibrosis by improving apoptosis and tissue inflammation in mouse-based in vivo and human primary tubular epithelial cell based in vitro models. We demonstrated that the cMet Ab treatment inhibited Smurf1 and Smurf inactivation by either cMet Ab or si-Smurf1, or both successfully activated Smad7. In addition, simultaneous treatment of cMet Ab and downregulation of Smurf1 showed synergistic activation of Smad7, suggesting the direct activation of Smad7 by cMet Ab. Moreover, the treatment effects of cMet Ab on the suppression of Smurf1 and Smad2/3 and inhibition of fibronectin formation were not reversed by Smad7 downregulation. This indicates Smad7-independent TGF-β1/Smad signaling pathway regulation by Smurf1 after cMet Ab treatment (Figure 7C).

In conclusion, a higher increase in the soluble plasma cMet level is a useful prognostic indicator of kidney function non-recovery and the subsequent CKD development. Treatment with cMet Ab can successfully prevent fibrosis in UIRI-induced kidney fibrosis model by decreasing apoptotic and inflammatory responses. Furthermore, cMet Ab inhibited hypoxia-induced fibrosis response by decreasing inflammatory and apoptotic responses. On the basis of our results, we propose that the regulation of the TGF-β1/Smad pathway might be a key mechanism underlying cMet antibody-induced inhibition of kidney fibrosis.
Data Availability

All data associated with this study are available in the main text or supplementary materials.

Competing Interests

The authors declare no conflict of interests.

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Author Contribution

L.L and J.L.(Jeonghwan) conducted experiments on patient-derived, mouse-derived, and cell line samples, analyzed the data, and wrote the manuscript. J.L.(Junghun) and S.-S.Y. provided the cMet agonistic antibody, interpreted the results, and provided valuable feedback. J.H.K., S.M.Z., A.C., and W.J. conducted mouse experiments and contributed to data acquisition. J.N.A. designed and conducted experiments on patient and mouse samples and analyzed the data. J.H.P, C.S.L., D.K.K., Y.S.K., S.H.Y. and J.P.L contributed to supervision, conception, and design of the study; S.H.Y. and J.P.L wrote and confirmed the manuscript.

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Figure Legends

Figure 1. Changes associated with kidney fibrosis in patients with acute kidney injury (AKI) and mouse model of unilateral ischemia-reperfusion injury.

(A) Baseline (day 0) log-transformed cMet concentrations [ln(pg/ml)] and the correlation between the increase of soluble cMet (from day 0 to day 2) and change percentage of 3 months eGFR from baseline during the progression of AKI to CKD in patients (n = 30 in both CKD progressor and non-progressor group). (B) Representative gross morphology of both the kidneys of mice in sham (n = 5 in each Sham/Vehicle and Sham/cMet Ab group) and unilateral ischemia-reperfusion group (n = 6 in each UIRI/Vehicle and UIRI/cMet Ab group), and comparison of left kidney weight per total body weight. (C) Interstitial fibrosis displayed by Masson's trichrome or Sirius red staining (×100 magnification). (D) Measurement of interstitial fibrosis area. (E) mRNA expression of fibronectin, COL1A1, and αSMA. (F) Expression of COL1A1, αSMA, and E-cadherin proteins. A representative band for each protein from triplicate experiments with same experimental design is shown. (G) Measurement of western blot results. Comparison of means between two groups were analyzed using independent t-test and Pearson correlation method was in panel (A). Two-way ANOVA test and post-hoc Tukey test was used in comparison between groups (panel B-G). cMet Ab, cMet agonistic antibody; UIRI, unilateral ischemia-reperfusion injury.
Figure 2. Changes in apoptosis and inflammation-related markers in unilateral ischemia-reperfusion injury animal models.

(A) Tissue expression levels of cleaved caspase-3 and phospho-p21 in unilateral ischemia-reperfusion injury mice models after treatment with cMet Ab. (B) Results of qRT-PCR performed to analyze apoptosis-related markers Bax and p53 (n = 5 in each Sham/Vehicle and Sham/cMet Ab group, n = 6 in each UIRI/Vehicle and UIRI/cMet Ab group). (C) Western-blot results for cleaved caspase-3, Bax, Bcl-2, and p21. (D) Western-blot measurement of apoptosis-related markers, cleaved caspase-3, Bax, Bcl-2 and p21. (E) Results for qRT-PCR analysis of the inflammation-related markers, IL-1β, TNF-α, and MCP-1. (F) Western-blot results for IL-1β. Two-way ANOVA with post-hoc Tukey test was used in comparison between groups (panel B-F). cMet Ab, cMet agonistic antibody; UIRI, unilateral ischemia-reperfusion injury.
Figure 3. Effect of cMet agonistic antibody treatment on TGF-β1/Smad pathway in mouse model of kidney fibrosis.

(A) Immunohistochemical staining for TGF-β1, Smad2/3, and Smad7 proteins (×200 magnification; n = 5 in each Sham/Vehicle and Sham/cMet Ab group, n = 6 in each UIRI/Vehicle and UIRI/cMet Ab group). (B) Tissue expression areas of TGF-β1, Smad2/3, and Smad7. (C) mRNA expression of TGF-β1 and Smurf1. (D) Protein expression of phospho-cMet, TGF-β1, Smad7, phospho-Smad2/3, and Smurf1. A representative band is shown for each protein from triplicate experiments performed under same experimental design. (E) Measurement of western blot results. Two-way ANOVA with post-hoc Tukey test was used in comparison between groups (panel B-E). cMet Ab, cMet agonistic antibody; p-cMet, phospho-cMet; p-Smad2/3, phospho-Smad2/3; UIRI, unilateral ischemia-reperfusion injury.
Figure 4. Effect of cMet agonistic antibody treatment on cellular adhesion, fibrosis-related markers, and apoptosis in human primary tubular epithelial cells under hypoxia.

(A) Changes in ICAM-1 expression under hypoxia and after cMet agonistic antibody treatment.

(B) Hypoxia-induced COL1A1 expression decreased by cMet agonistic antibody treatment in a dose-dependent manner. (C) Changes in the ratio of apoptotic cells. Reversal of hypoxia-induced apoptosis after cMet agonistic antibody treatment (n = 3 in each group). The results are from triplicate experiments. One-way ANOVA with post-hoc Tukey test was used in comparison between groups (panel C). cMet Ab, cMet agonistic antibody.
Figure 5. Effect of cMet agonistic antibody treatment on TGF-β1/Smad pathway in human primary tubular epithelial cells under hypoxic condition.

(A) Hypoxia-induced Smurf1 activation and its inhibition after cMet agonistic antibody treatment. (B) Interaction of Smurf1 and Smad7 under hypoxia and after cMet agonistic antibody treatment. (C) Inhibition of Smurf1 and Smad2/3 after cMet agonistic antibody treatment. (D) mRNA expression of TGF-β1, Smad2, Smad3, Smad7, and Smurf1 (n = 6 in each group). (E) Protein expression of TGF-β1, Smad2/3, Smad7, and Smurf1. (F) Western blot results (n = 6 in each group). One-way ANOVA with post-hoc Tukey test was used in comparison between groups (panel D-F). cMet Ab, cMet agonistic antibody.
Figure 6. Improvement in fibrosis in human primary tubular epithelial cells after downregulation of Smurf1 by siRNA or cMet agonistic antibody treatment.

(A) Fibrosis response under hypoxia, measured by increased fibronectin using FACS (n = 3 in each group). (B) Western blot results representing suppression of Smurf1 by si-RNA knockdown activates Smad7 and inhibits TGF-β1. (C) Quantification of western blot (n = 4 in each group). One-way ANOVA with post-hoc Tukey test was used in comparison between groups. ns$P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; cMet Ab, cMet agonistic antibody.
Figure 7. Improvement of fibrosis in human primary tubular epithelial cells after cMet agonistic antibody treatment under downregulation of Smad7 by siRNA.

(A) Western blotting results representing the anti-fibrotic effect of cMet Ab under hypoxic condition and the role of Smad7 in the TGF-β1/Smad signaling pathway regulation after cMet Ab treatment (n = 3 in each group). The cMet Ab treatment inhibited Smurf1, activated Smad7, and suppressed phospho-Smad2/3 and fibronectin expression. The suppression of Smad7 by si-RNA knockdown did not show additional changes in Smurf1, phospho-Smad2/3, and fibronectin expression. (B) Western blotting measurement results. (C) Summary diagram of the mechanism of acute kidney injury to chronic kidney disease transition and prevention after cMet Ab treatment. One-way ANOVA with post-hoc Tukey test was used in comparison between groups (panel A-B). cMet Ab, cMet agonistic antibody.
**A**

- CKD non-progressor
- CKD progressor

Log cMet [ng/mL]

Change percentage of eGFR%

Correlation coefficient: $R = -0.281$  $P = 0.034$

P = 0.004

Left Kidney / Body Weight (%)

**B**

- Sham
- UIRI

**C**

MT staining

Sirius Red staining

**D**

Aniline-blue positive area (%)

P = 0.001

P = 0.005

**E**

Fold increase

Fibronecint

COL1A1

αSMA

P = 0.010

P = 0.059

P = 0.005

**F**

- COL1A1
- αSMA
- E-cadherin
- GAPDH

**G**

COL1A1 / GAPDH

αSMA / GAPDH

E-cadherin / GAPDH

P = 0.029

P < 0.001

P = 0.002
(A) Normoxia

Control

DAPI  ICAM-1

Hypoxia

IgG control  cMet Ab (0.25 μg/ml)  cMet Ab (0.5 μg/ml)  cMet Ab (1 μg/ml)

(B) DAPI  COL1A1  cMet

(C) Normoxia

Control

IgG control  cMet Ab (0.25 μg/ml)  cMet Ab (0.5 μg/ml)  cMet Ab (1 μg/ml)

Annexin / PI

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(A) Control

(B) Hypoxia

(C) IgG control

(D) Hypoxia

(E) TGF-β1

(F) Smad7

[Graphs and images showing comparisons and fold increases under different conditions.]
**A**

![Graph A](image)

**B**

![Graph B](image)

**C**

![Graph C](image)
(A) Fibronectin
Smad7
Smurf1
p-Smad2/3
Smad2/3
β-actin

(B) 72 hours

(C) TGFβ
HGF

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