# Supplemental Information

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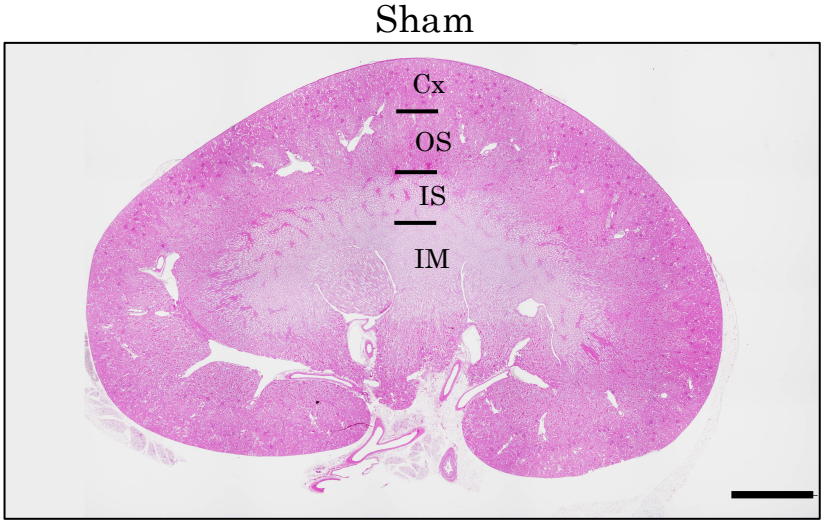
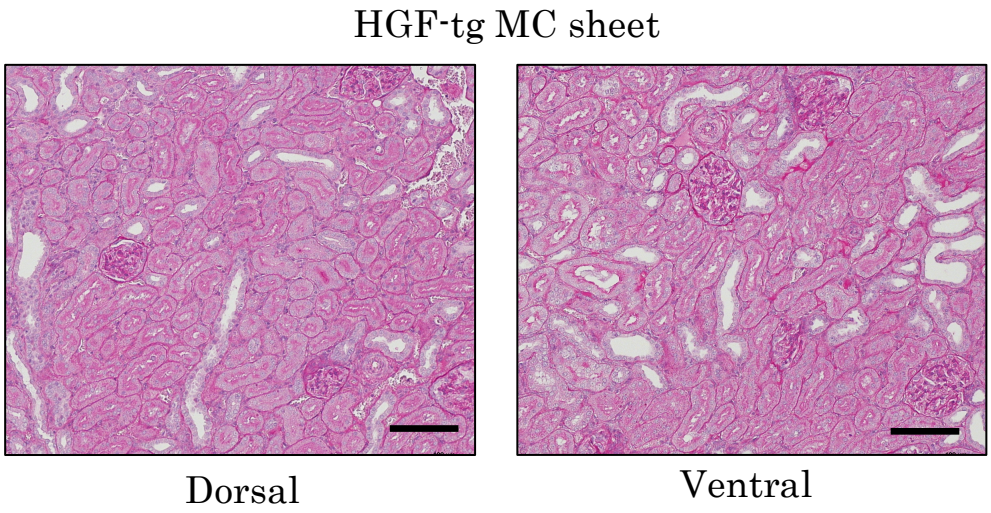
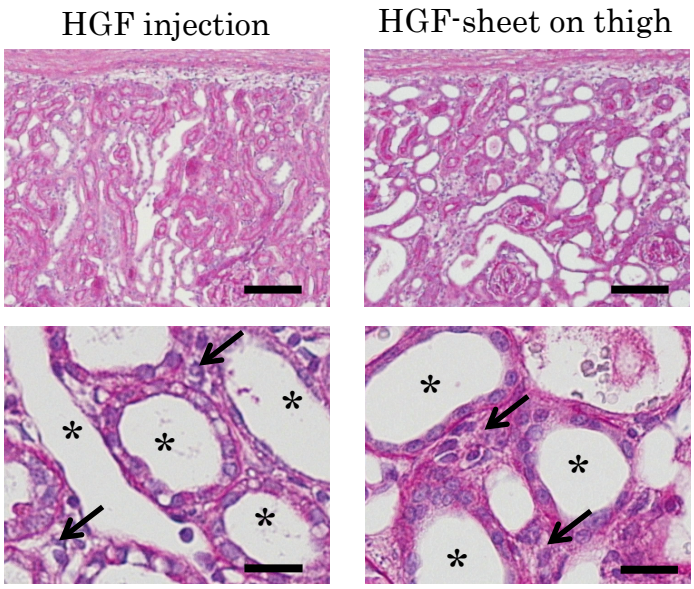
## SUPPLEMANTAL FIGURES



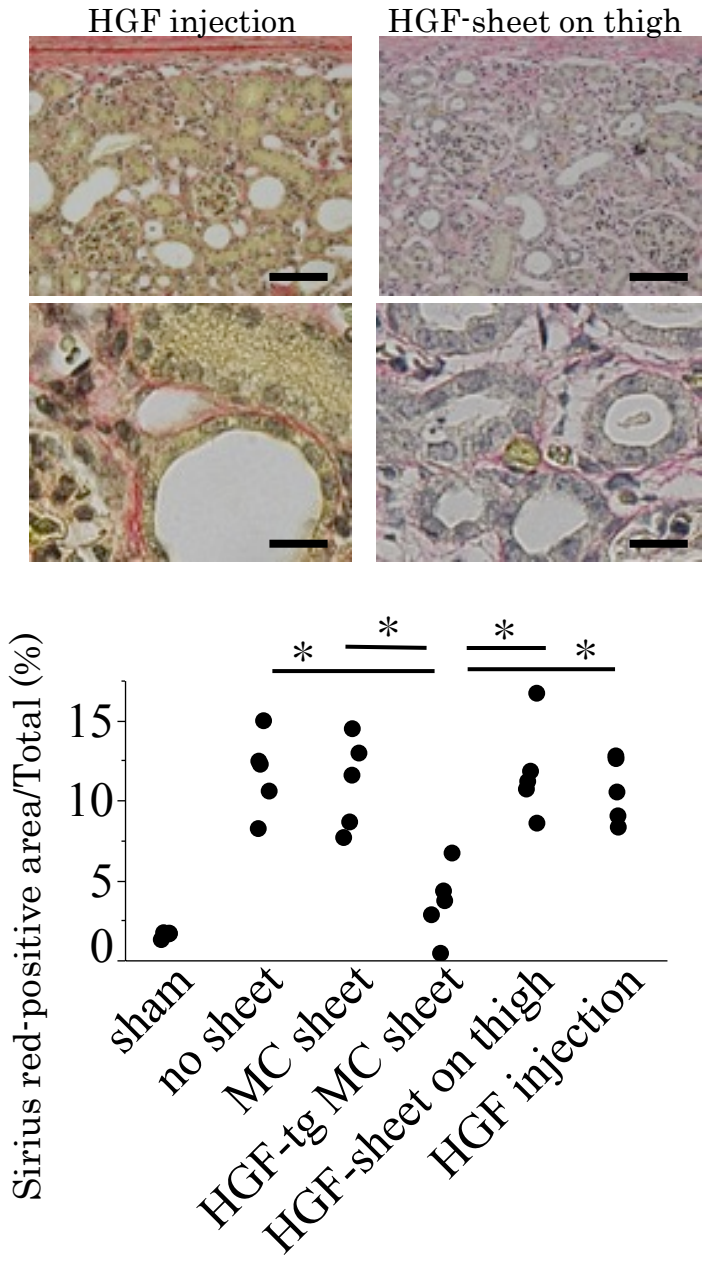

**Supplemental Fig. S1.** Periodic acid–Schiff-stained image of a whole-kidney cross-section on day 7 after the sham operation in the sham group. Scale bar: 500 μm. Cx cortex; IM inner medulla; IS inner stripe; OS outer stripe



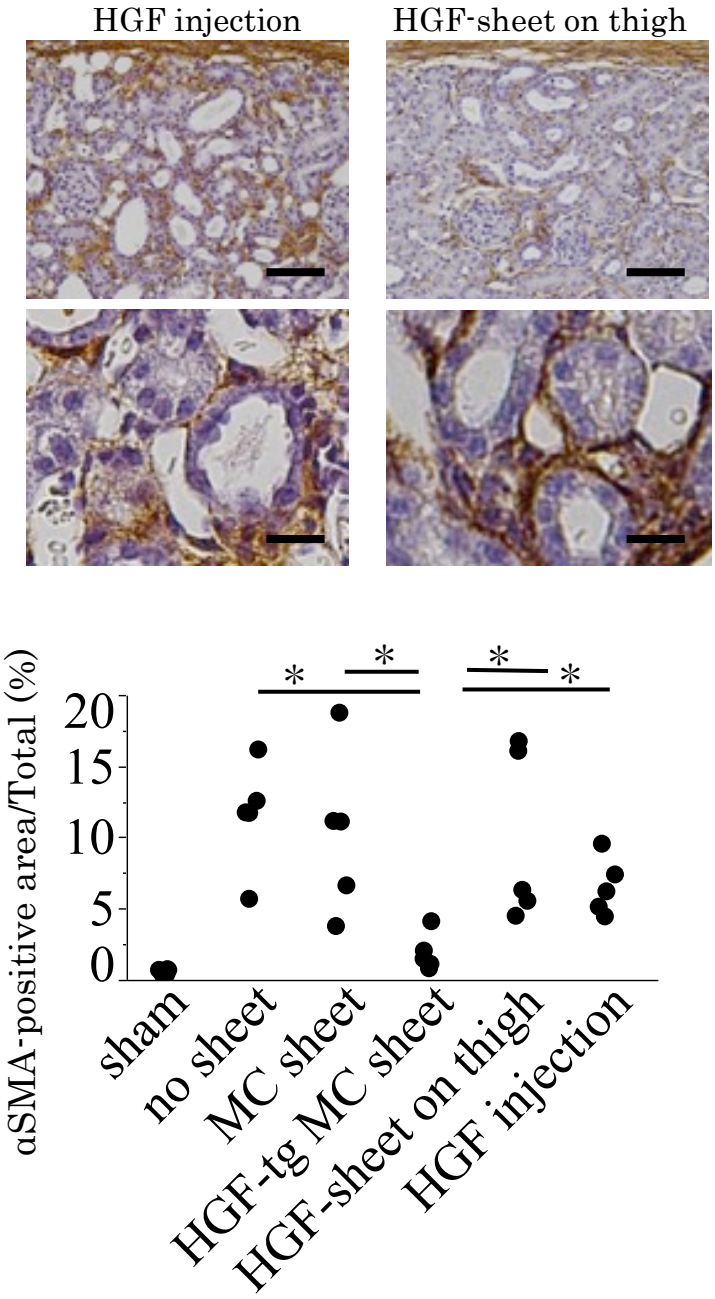

**Supplemental Fig. S2.** High-magnification, periodic acid–Schiff-stained images of an axial section of a UUO-treated kidney from the HGF-tg MC-sheet group on day 7. An HGF-tg MC sheet was transplanted to the dorsal site in the kidney (left), whereas the ventral (right) site was not subjected to transplantation. Scale bars: 100 μm




**Supplemental Fig. S3**. Each pair of representative periodic acid–Schiff-stained images show low (top) and high (bottom) magnifications of the renal cortical interstitial area on day 7 after UUO. Treatments included intravenous administration of HGF (HGF injection) and transplantation of an HGF-tg MC sheet onto the thigh vascular bed (HGF-sheet on thigh). Scale bars: 100 μm (top) and 20 μm (bottom). *indicates a dilated tubule. The black arrows indicate instances of increased cells in the expanded interstitium.




**Supplemental Fig. S4.** Each pair of representative Sirius red-stained images show low (top) and high (bottom) magnifications of the renal cortical interstitial area on day 7 after UUO. Scale bars: 100 μm (top) and 20 μm (bottom). Percentages of Sirius red-positive areas in all groups. Quantification was performed using ImageJ software. *P < 0.01 ($n$ = 5 for all groups)




**Supplemental Fig. S5.** Low (top) and high (bottom) magnifications of representative αSMA-immunostained images of the renal cortical interstitial area on day 7 after UUO. Scale bars: 100 μm (top) and 20 μm (bottom). Percentages of αSMA-positive areas in all groups. Quantification was performed using ImageJ software. *P < 0.01 (*n* = 5 for all groups)

## COMPLETE METHODS

### hHGF transfection

The HGF plasmid vector used for hHGF protein expression was kindly provided by Kunio Matsumoto (Cancer Research Institute, Kanazawa University, Japan). This pcDNA3.1(+) (Invitrogen)-based vector confers resistance to neomycin, but lacks the PMV promoter, and expression of the insert was driven by the SRα promoter. Met5A cells, obtained from the American Type Culture Collection, are nonmalignant human pleural MCs immortalized by simian virus 40 (SV40). To generate hHGF-secreting cells, plasmids were transfected into Met5A cells by the lipofection procedure. The HGF plasmid and Lipofectamine 2000 (Invitrogen) were mixed with Opti-MEM (Invitrogen); subsequently, this mixture was added to Met5A cells on the culture dishes. At 6 h post-transfection, the medium was replaced with complete medium (Medium 199, Thermo Fisher Scientific) containing 10% fetal bovine serum (Japan Bio Serum), 3.3 nM epidermal growth factor (Wako), 400 nM hydrocortisone (Sigma-Aldrich), 870 nM zinc-free bovine insulin (Wako), 1% penicillin/streptomycin (Sigma-Aldrich), and 500 μg/mL Geneticin G418 (Sigma–Aldrich). Continuous exposure to G418 resulted in the

selection of drug-resistant cells. Cells secreting HGF were selected and used for the preparation of cell sheets, and for the measurement the HGF concentration from the cell sheets.

**Generation of cell sheets**

HGF-tg MCs were cultured in complete medium with 500 ng/mL G418 for at least 4 passages to grow a sufficient number of cells required for this study. MCs and HGF-tg MCs were seeded onto 35-mm temperature-responsive culture dishes (Cell Seed), at a density of $1.2 \times 10^6$ cells/dish. Cells were cultured at 37°C in a 5% $CO_2$ incubator in 2 mL of complete medium, with or without G418, for 4 days. The cells were detached as sheets from temperature-responsive dishes without enzyme treatment by reducing the temperature through incubation at 20°C for 30 min. The cell sheets formed as circles on the bottom of the dishes. The prepared cell sheets were transplanted into rats in the animal study described below.

**Animal study**

All experimental protocols were approved by the Animal Welfare Committee of the Tokyo Women's Medical University School of Medicine (Tokyo, Japan; Permit number #14-87). F344/NJcl-rnu/rnu rats, 5–8 weeks of age (CLER Japan), were used. Rats were divided into 5 groups: the no-sheet (n = 10), MC-sheet (n = 10), HGF-tg MC-sheet (n = 10), HGF-injection (n = 5), and HGF-sheet on-thigh (n = 5) groups.

**UUO procedure**

Rats were anesthetized with 2–4% inhaled isoflurane and placed on a heated table to maintain their body temperature at 37–38°C. The rats were incised from the back to expose the left kidney and ureter. The kidney, which remained connected to blood vessels and the ureter, was carefully removed from the body and placed on wet gauze. The left ureter was separated from the surrounding tissues, and 3 ligatures were placed approximately 2–3 mm apart around the ureter near the renal pelvis with 4-0 nylon sutures.

After UUO processing, rats were subjected to various treatments. On day 7 after UUO and treatment, 5 rats were sacrificed from each group. On day 28, the

remaining rats were sacrificed in the no-sheet, MC-sheet, and HGF-tg MC-sheet groups (n = 5 in each case). On days 7 and 28 after UUO, the vasculature was perfused from the left ventricle with 50 mL of 4% paraformaldehyde (PFA) (Muto Pure Chemicals). After perfusion, the UUO-treated kidneys were harvested and bisected. The parenchymal thickness and longitudinal and horizontal length of the kidney pelvis were then measured.

**Transplantation of cell sheets onto the kidney**

When the UUO procedure was finished in the MC-sheet and HGF-tg MC-sheet groups, the left kidney was exposed from the back, and the renal capsule on the dorsal surface of kidney was peeled away with tweezers under a microscope. Two cell sheets were gently placed on the site where the capsule was removed, and the kidney surface was covered. After standing for 5 min, the cell sheets were attached to the kidney surface. In the no-sheet group, rats were not given any therapy after UUO. In the sham group, rats were not subjected to UUO or given any therapy. In all cases, the kidney was returned inside the body carefully so as not to touch surrounding tissues, and the incision was

sutured.

**Cell sheet transplantation on the thigh vascular bed**

Rats in the HGF-sheet on-thigh group had HGF-tg MC sheets transplanted onto the thigh vascular bed to examine the effect of these sheets at a distant site on the kidneys. To create a vascular bed in the thigh muscle, 2 ligatures were placed approximately 2 mm apart around the left femoral artery and vein with 5-0 nylon sutures 7 days before UUO. New blood vessels developed and formed an immature vessel network (vascular bed) after 7 days. After UUO, 2 HGF-tg MC sheets were placed gently on the thigh vascular bed for 5 min. The cell sheets were applied to this site, and the incision was sutured.

**HGF injection**

Rats in the HGF-injection group were administered hHGF protein intravenously to examine the effect of systemic administration on the kidneys. After UUO, rats in the HGF-injection group received 200 pg recombinant hHGF (R&D Systems) in 100 μL of

phosphate-buffered saline, via the tail vein, every 24 h for 7 days. This daily dose approximated that produced by 2 HGF-tg MC sheets in 24 h.

**Measuring hHGF secretion by ELISA**

To analyze hHGF secretion by the cell sheets, the hHGF concentrations in the supernatant were measured. Under the same conditions used to prepare the cell sheets for transplantation, HGF-tg MCs and non-tg MCs were seeded onto temperature-responsive culture dishes at a density of $1.2 \times 10^6$ cells/dish and cultured at 37°C for 4 days. After replacing the medium with fresh complete medium (with or without G-418) and culturing at 37°C for 24 h, the supernatants were collected and the HGF concentrations were detected using ELISA kits (R&D Systems) according to the manufacturer's recommendations.

**Micro-CT experiments**

Quantitative analysis of UUO-kidney volumes was performed using a micro-CT system (R_mCT2; Rigaku). During the micro-CT imaging process, rats were anesthetized with

isoflurane and injected with 100 μL/g of a non-ionic iodine-based contrast agent (Omnipark 350) as a bolus into the lateral tail vein. The UUO-treated kidney was scanned using contrast-enhanced micro-CT over a 17-s time frame on days 7, 14, 21, and 28 after UUO. Renal dimensions were measured from the CT images of UUO-treated kidneys. The renal length and width were measured from sagittal slices, and the thickness was measured from transversal slices. Kidney volumes were estimated from the linear dimensions using the ellipsoid formula (Eq. 1)[36]:

$$\text{kidney volume} = \text{length} \times \text{width} \times \text{thickness} \times \pi/6 \quad \text{(Eq. 1)}$$

The ratio of the kidney volume to the body weight was compared for 3 groups (no-sheet, MC-sheet, and HGF-tg MC-sheet) from day 7 to 28 after UUO.

Using the sagittal CT images including the kidney median, we measured the parenchyma thickness and pelvis transverse length on the center line and pelvis longitudinal length on the orthogonal center line on day 7 after UUO in three groups. The average values for each dimension in three groups were compared. With the axial and sagittal CT images including the kidney median, two section areas divided by the center line were measured using ImageJ day 7 after UUO in the HGF-tg MC sheet

group. The average cross-sectional area in each group was compared.

**Intravenous urography (IVU)**

Because the contrast agent accumulated in the kidney and was excreted through the urinary tract, the kidney pelvis and ureter were observed in the live X-ray moving-image mode of the micro-CT device. IVU was performed to evaluate urinary flow into the renal pelvis and urinary ureter, and to identify obstructed sites in the urinary tract for the no-sheet, MC-sheet, and HGF-tg MC-sheet groups from days 7 to 28.

**Histological and immunohistochemical analyses**

Harvested kidneys or cell sheets were fixed in 4% PFA, dehydrated, and embedded in paraffin. Sections were cut using a microtome (Leica Microsystems), deparaffinized, and stained with hematoxylin–eosin, periodic acid–Schiff, or Sirius red. To quantify the degree of renal fibrosis, 6 micrographs (magnification, × 200) of the renal cortex of each rat were acquired randomly. The area fraction of Sirius red staining was analyzed

using ImageJ software (National Institutes of Health).

For immunohistochemistry, paraffin sections were cut using a microtome, deparaffinized, and incubated in citrate buffer (pH 6.0) for 30 s at 125°C. Sections were immunolabeled using antibodies to αSMA (M0851, Dako), SV40 T-antigen (ab16879, Abcam), RECA-1 (ab9774, Abcam), or hHGF (kindly provided by Kunio Matsumoto) for 1 h at room temperature. Only when phosphor-cMet staining, the sections were stained by Pathology Institute Corp. (Toyama, Japan) using an antibody against phospho-cMet (ab5662, Abcam). Bound antibodies were detected using secondary antibodies and a peroxidase-conjugated polymer reagent (REAL EnVision, Dako). Sections were developed with 3,3′ -diaminobenzidine staining and counterstained with hematoxylin. For quantifying myofibroblasts, 6 micrographs (magnification, × 200) of the renal cortex of each rat were acquired randomly. The area fraction of α SMA staining was analyzed using ImageJ software. To quantify SV40-T-positive cells, 3 micrographs (magnification, × 100) of the renal capsule were acquired for each rat. Using ImageJ software, the numbers of total and SV40-T-positive cells in the renal capsule were counted to determine the fraction of SV40-T-positive cells.

**Capillary quantification**

RECA-1-stained sections were used to analyze capillary density and the filled-area fraction. The numbers of capillaries and tubules were counted per 5 fields (magnification, × 400) in the cortex region. The ratio of capillaries to tubules was used to assess the capillary density. Capillaries with different staining patterns were counted as described previously.[37]

The filled-area fraction of capillaries was analyzed using 5 micrographs (magnification, × 400) in the cortex region using computer-based measurements. The filled RECA-1 area fraction represented the vascular volume occupied by PTCs and was determined using a previously described custom macro implemented for ImageJ software.[37,39]

**Ultrasonography**

The left UUO-treated kidney was observed using an ultrasound imaging system (Vevo2100; Fujifilm VisualSonics) on day 7 after UUO in the no-sheet, MC-sheet, and

HGF-tg MC-sheet groups. Left renal artery (RA) blood flow was estimated from velocity time integral (VTI), left RA diameter (RAD) by PW-Doppler echo on day 7 after UUO using Eq. 2.[38]

RA blood flow (mL/min) = VTI (m/min) × CSA ($mm^2$) × $10^{-3}$ (Eq. 2)

* CSA: cross-section area = $\pi /4 \times (RAD)^2$

The RA blood flows in the no-sheet, MC-sheet, and HGF-tg MC-sheet groups were compared on day 7.

**Statistical analyses**

Differences between the means of 2 groups were analyzed by performing an unpaired Student's *t*-test. Multi-group differences were analyzed by ANOVA, followed by the Tukey–Kramer *post-hoc* comparison test using JMP Pro 13.0.0 software (SAS). For more than 2 groups, time-dependent data were analyzed by a one-way repeated-measures ANOVA. $P < 0.05$ was considered to represent statistical significance, and all tests were two-sided.