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# Serum-Free Medium Enhances the Immunosuppressive and Antifibrotic Abilities of Mesenchymal Stem Cells Utilized in Experimental Renal Fibrosis

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## ABSTRACT

Serum used in culture medium brings risks of immune reactions or infections and thus may hinder using ex vivo expanded mesenchymal stem cells (MSCs) for medical treatment. Here, we cultured MSCs in a serum-free medium (SF-MSCs) and in a medium containing 10% fetal bovine serum (10%MSCs) and investigated their effects on inflammation and fibrosis. MSC-conditioned medium suppressed transforming growth factor-β1–induced phosphorylation of Smad2 in HK-2 cells, with no significant difference between the two MSCs. This finding suggests that the direct antifibrotic effect of SF-MSCs is similar to that of 10%MSCs. However, immunohistochemistry revealed that renal fibrosis induced by unilateral ureteral obstruction in rats was more significantly ameliorated by the administration of SF-MSCs than by that of 10%MSCs. Coculture of MSCs and monocytic THP-1 cell-derived macrophages using a Transwell system showed that SF-MSCs significantly induced polarization from the proinflammatory M1 to the immunosuppressive M2 phenotype macrophages, suggesting that SF-MSCs strongly suppress the persistence of inflammation. Furthermore, the gene expression of tumor necrosis factor-α–induced protein 6 (TSG-6), which inhibits the recruitment of inflammatory cells, was higher in SF-MSCs than in 10%MSCs, and TSG-6 knockdown in SF-MSCs attenuated the anti-inflammatory responses in unilateral ureteral obstruction rats. These findings imply that SF culture conditions can enhance the immunosuppressive and antifibrotic abilities of MSCs and the administration of ex vivo expanded SF-MSCs has the potential to be a useful therapy for preventing the progression of renal fibrosis. STEM CELLS TRANSLATIONAL MEDICINE *2018;7:893–905*

## SIGNIFICANCE STATEMENT

This study showed that the serum-free medium for mesenchymal stem cells (MSCs) enhances the ability of MSCs to induce a change in the phenotype of macrophages to immunosuppressive M2. Serum-free conditions also enhance the gene expression of TSG-6, which inhibits the recruitment of inflammatory cells, in MSCs. Thus, MSCs cultured in serum-free conditions significantly ameliorate renal fibrosis compared with MSCs cultured in serum-containing conditions. These findings indicate that the serum-free culture medium can potentiate immunosuppressive and antifibrotic abilities of MSCs and is useful for culturing clinically used MSCs.

## INTRODUCTION

Mesenchymal stem cells (MSCs) are undifferentiated and multipotent adult stem cells that are mainly collected from the bone marrow, adipose tissue, and umbilical cord blood [1–3]. MSCs have been used in various experimental models of diseases [4–6]. Initially, the reparative and regenerative effects of MSCs were determined by their ability to be engrafted into various injured tissues [7, 8]. However, recent studies have demonstrated that the protective effects of MSCs result from their immunomodulatory properties, mediated by the release of various soluble factors such as prostaglandin E2 (PGE2), interleukin-10, tumor necrosis factor-α–induced protein 6 (TSG-6), and hepatocyte growth factor (HGF) [4, 5, 9–11].

 

The prevalence of chronic kidney disease (CKD) is estimated to be 8%-16% worldwide and it imposes a substantial socioeconomic burden [12]. To alleviate CKD progression, a renin–angiotensin–aldosterone system blockade has been developed [13, 14]. However, the beneficial effects are not radical and many CKD patients eventually develop renal failure, requiring renal replacement therapy (RRT). The number of patients receiving RRT is expected to more than double from 2.6 million people worldwide in 2010 to 5.4 million in 2030 [15]. Renal fibrosis is a histological manifestation that occurs in almost every type of CKD during progression to the end stage of the disease; chronic fibrosis is exacerbated by the persistence of inflammation, and the progression of fibrosis leads to more inflammatory cell infiltration [16, 17]. In addition, MSCs directly suppress the progression of fibrosis by inhibiting TGF-β1 signaling [9]. As MSCs exhibit immunosuppressive abilities mediated by the release of soluble factors [18], the administration of ex vivo expanded MSCs has the potential to be a useful therapy for preventing the progression of renal fibrosis.

Unilateral ureteral obstruction (UUO) is a procedure applied to rodents that induces tubulointerstitial fibrosis [19, 20]. Notably, several studies have shown that the administration of MSCs is effective in suppressing the tubulointerstitial fibrosis induced by UUO [18, 21, 22], but all of these studies used MSCs cultured in a serum-containing medium. Although adipose tissue-derived stromal cells cultured in a low-serum medium exerted renoprotective effects on experimental glomerular nephritis [23], no study has examined the effect of MSCs cultured in serum-free (SF) conditions on renal disorders.

Most previous studies involving MSCs cultured them in a medium supplemented with fetal bovine serum (FBS), fetal calf serum, or human serum [24, 25]. However, the serum in the culture medium may prevent the use of ex vivo expanded MSCs for medical treatment. Supplementation with serum is associated with several risks, not only in transmitting unknown viral diseases but also in provoking immune reactions [24, 26, 27]. Furthermore, the use of allogeneic human serum arrested human MSC proliferation [28]. In addition, acquiring a large amount of autologous serum would be difficult from patients with reduced renal function, and culturing MSCs in media with serum-containing uremic toxin may induce abnormal differentiation of MSCs [29]. Conversely, the use of serum-free media reduces the risk of infection and is useful for obtaining a sufficient number of MSCs for medical treatment in a short period. Chemically defined serum-free media for MSCs are currently commercially available and are experimentally used in cell culture [30–32]. STK1 (used only for primary culture) and STK2 are serum-free media (developed by DS Pharma Biomedical, Osaka, Japan) for MSC culture that contains growth factors, including FGF2, insulin, PDGF, and EGF, and lipids, including fatty acids and phospholipids, in addition to nutrients and minerals (see Patent No. U.S. 9,074,176 B2) and can promote the proliferation of MSCs without serum. However, no study has investigated whether MSCs cultured in serum-free media are superior in therapeutic efficacy to those cultured in a serum-containing medium when administered in animal experiments. In this study, we cultured MSCs in a serum-free medium and in a medium containing 10% FBS and investigated their effects on inflammation and fibrosis in an experimental renal fibrosis model.

## Materials and Methods

### Animals

We used male Sprague–Dawley (SD) rats (Charles River Laboratories Japan, Yokohama, Japan) and male CAG-enhanced green fluorescent protein (EGFP)-transgenic SD rats (Japan SLC, Shizuoka, Japan) at 6 weeks of age for collecting bone marrow. We used SD rats (8 weeks old) for inducing UUO. The animal studies were all conducted according to the "Guide for the Care and Use of Laboratory Animals, 8th ed., 2010" (National Institutes of Health, Bethesda, MD) and were approved by the Institutional Animal Care and Use Committee of Hiroshima University (Hiroshima, Japan; Permit Number: A13-34).

### MSC Preparation

We collected bone marrow from the SD and CAG-EGFP-transgenic SD rats as described previously [22, 33]. The cells were harvested in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich, St. Louis, MO) containing 10% FBS (Sigma–Aldrich) or STK1 (KBDSTC101; DS Pharma Biomedical) for the primary culture and were passaged after reaching 80%-90% confluence. The culture medium in the STK1 group was then substituted with STK2 (KBDSTC102; DS Pharma Biomedical). The cells established under STK2 and medium containing 10% FBS were designated as "SF-MSCs" and "10%MSCs," respectively. Human MSCs from bone marrow (hMSCs; Riken BRC, Ibaraki, Japan) and human MSCs derived from adipose tissue (AT-hMSCs; DS Pharma Biomedical) were similarly cultured in STK2 or cultured in DMEM containing 10% FBS. In human platelet lysate (HPL) analyses, rat MSCs and hMSCs were cultured in DMEM containing 10% heat-inactivated HPL (SCM141; Merck Millipore, Billerica, MA) with 2 U/l heparin (#07980; STEMCELL Technologies, Vancouver, Canada). To inactivate HPL, HPL was incubated at 56°C for 30 minutes prior to use. Cells within passage 5 were used in all experiments and counted by using a TC-20 (Bio-Rad, Hercules, CA).

### Experimental Animal Model

To establish the animal model, SD rats were randomly divided in four groups ($n$ = 6 in each group): the SF-MSCs, 10%MSCs, PBS and Sham groups. Rats were anesthetized with an intraperitoneal pentobarbital injection (about 40 mg/kg) and isoflurane inhalation. The left ureter was exposed by midline incision and ligated, inducing UUO. The sham-treated animals underwent the same surgical procedure without the ureteral ligation. At 4 days post-UUO, SF-MSCs and 10%MSCs ($5 \times 10^6$ cells/rat) in 0.5 ml PBS were injected through the tail vein. In the PBS and Sham groups, only 0.5 ml PBS was administered. At 5, 7, 10, or 21 days post-UUO, the rats were sacrificed and their kidneys were analyzed.

A similar procedure was performed for the TSG-6 knockdown analyses. SD rats were randomly divided in four groups ($n$ = 6 in each group). At 4 days post-UUO, TSG-6 siRNA/SF-MSCs and negative control (NC) siRNA/SF-MSCs ($5 \times 10^6$ cells/rat) in 0.5 ml PBS were injected through the tail vein. In the PBS and Sham groups, only 0.5 ml PBS was administered. At 10 days post-UUO, the rats were sacrificed and the kidneys were analyzed.

### Quantitative Real-Time Reverse Transcription PCR

Total RNA extraction, the synthesis of cDNA, and real-time reverse transcription polymerase chain reaction (PCR) were performed as previously described [34]. PCR experiments were analyzed by TaqMan Gene Expression Assays and 7500 Fast (Applied Biosystems, Foster City, CA). The mRNA levels of the samples were normalized to the level of 18 s rRNA.

### Western Blotting and ELISA

The sample collection and Western blotting was performed as previously described [35]. The intensity of each band was analyzed with ImageJ software (version 1.47v; NIH) and normalized to the level of either glyceraldehyde 3-phosphate dehydrogenase or α-tubulin. The enzyme-linked immunosorbent assay (ELISA) analyses were performed following the manufacturer's protocols and the concentrations were normalized to the total protein content.

### Immunofluorescence Assay

Immunofluorescence staining was performed on frozen tissue. Briefly, the frozen kidneys were cut into 5-μm sections on a cryostat and dried in air. The sections were then fixed in 4% paraformaldehyde, blocked with Block Ace (DS Pharma Biomedical) and incubated overnight at 4°C with the rabbit polyclonal anti-TGF-β1 antibody (1:200; SAB4502954; Sigma–Aldrich). Sections were then washed in PBS, incubated with Alexa Fluor 594 Donkey antirabbit IgG (1:500; R37119; Thermo Fisher Scientific, Waltham, MA) for 3 hours and mounted with VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA). The sections were visualized with a fluorescence microscope (KEYENCE, Osaka, Japan; ×200).

### Immunohistochemistry Analysis

Immunohistochemical staining was performed on paraffin-embedded tissue, as described previously [36]. Immunostaining for α-SMA and CD3 was accomplished with the EnVision System (Dako, Glostrup, Denmark). Other markers were identified with the ABC system (Vector Laboratories). The sections were all visualized using 3,3′-diaminobenzidine (Sigma–Aldrich). CD3-, CD68- and EGFP-positive cells and the positive areas for α-SMA, collagen type I, and collagen type III staining were assessed as the average of five randomly selected fields (×200) for each rat with ImageJ software (NIH).

### Histological Analysis

Sections of formalin-fixed, paraffin-embedded tissues (2 μm thick) were stained with Masson trichrome. Masson trichrome staining was performed as described previously [9]. The extent of interstitial fibrosis was assessed as the average of five randomly selected fields (×200), and the stained area was analyzed in selected fields using a Lumina Vision (Mitani, Osaka, Japan).

### Flow Cytometry Analysis

Flow cytometry analysis was performed as previously described [37]. The stained cells were analyzed using BD FACSVerse (Becton, Dickinson and Company, Franklin Lakes, NJ) and evaluated with FlowJo software (FlowJo, LLC; Ashland, OR).

### Preparation of Conditioned Medium

To prepare the conditioned medium from SF-hMSCs (SF-hMSC-CM) and 10%hMSCs (10%hMSC-CM), cells were seeded into 10 cm dishes (at $3 \times 10^5$ cells/dish). Once the cells reached 60%-80% confluence, they were washed and then cultured in DMEM containing no FBS for 48 hours. The supernatants were collected, centrifuged at 2,000 rpm for 10 minutes at 4°C, and sterilized by filtration through a 0.2-μm filter (Merck Millipore).

### Cell Culture and Treatments

HK-2 cells (American Type Culture Collection, Manassas, VA) and THP-1 cells (Riken BRC) were maintained as described previously [38, 39]. After the starvation of HK-2 cells with SF-hMSC-CM (with or without TSG-6 siRNA transfection), 10%hMSC-CM, or serum-free DMEM for 24 hours, the cells were exposed to 1 μM human angiotensin-II (Ang-II; Sigma–Aldrich) for 12 hours, 5 ng/ml recombinant human TGF-β1 (R&D Systems, Minneapolis, MN) for 12 hours, or 10 ng/ml TGF-β1 for 1 hours or 48 hours. Whole cell lysates were prepared in RIPA buffer (Sigma–Aldrich) and analyzed. Total RNA was extracted using TRIzol Reagent (Thermo Fisher Scientific) and subjected to PCR.

THP-1 cells were differentiated into M1 macrophages by treatment with phorbol 12-myristate 13-acetate (160 nM; Sigma–Aldrich) for 48 hours and then stimulated for a further 24 hours with recombinant human interferon-γ (20 ng/ml; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). A Transwell cell coculture system (Corning, Corning, NY) was used to examine the phenotypic change of macrophages by MSCs without direct cell contact. Upper inserts (pore size, 1.0 μm; Corning) with cultured SF-hMSCs, 10%hMSCs, or SF-hMSCs transfected with TSG-6 siRNA ($5 \times 10^4$ cells/insert) were dipped into the basal plate of cultured THP-1 macrophages. After 48 hours, the surface antigens of THP-1 macrophages were determined by flow cytometry.

### Transfection with TSG-6 siRNA

SF-hMSCs were transfected with 20 nM siRNA against human TSG-6 (sc-39819; Santa Cruz Biotechnology, Santa Cruz, CA) or NC siRNA (sc-37007; Santa Cruz Biotechnology) using Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific). The cells were then passaged and seeded into upper inserts for the coculture experiments or into 10 cm dishes for the preparation of CM. For the knockdown of rat TSG-6, SF-MSCs were transfected with 20 nM siRNA against rat TSG-6 (sc-270514; Santa Cruz Biotechnology) or NC siRNA. At 24 hours after transfection, the cells were collected and injected into the tail veins of UUO rats.

### Statistical Analysis

All data are expressed as the mean ± standard deviation. Statistical analyses were performed using one-way ANOVA followed by Bonferroni post hoc testing. Differences between two groups were analyzed by Student's *t* test. $p < .05$ was defined as statistically significant.

## RESULTS

### MSCs Cultured in Serum-Free Conditions Suppressed the Tubulointerstitial Fibrosis Induced by UUO

Even when MSCs are cultured over many passages in STK medium, a serum-free medium for MSCs, their spindle-shaped appearance is maintained over a long period compared with

cells cultured in a serum-containing medium (Supporting Information Fig. S1). Therefore, we investigated whether MSCs cultured in a serum-free medium were more effective in an experimental renal fibrosis model. At 4 days after the UUO procedure ("post-UUO"), we injected rats through the tail vein with $5 \times 10^6$ cells of rat MSCs cultured in STK2 serum-free medium (SF-MSCs), rat MSCs cultured in DMEM containing 10% FBS (10%MSCs), or with only PBS. At 3 or 6 days after administration (i.e., at 7 or 10 days post-UUO), the mRNA and protein levels of the profibrotic markers TGF-β1 and α-SMA were increased in the PBS group compared with the sham group; the administration of 10%MSCs suppressed these increases and the administration of SF-MSCs suppressed them even more significantly (Fig. 1A, 1C). Immunostaining of kidney sections showed that the number of TGF-β1-positive cells and α-SMA-positive area had increased in the PBS group at 10 days post-UUO and these expressions were more significantly suppressed in the SF-MSC group than in both the PBS group and the 10%MSC group (Fig. 1D, 1E).

### MSCs Cultured in Serum-Free Conditions Strongly Suppressed the Accumulation of Extracellular Matrix Proteins Induced by UUO

We next examined the expressions of collagen types I and III, which are markers of extracellular matrix (ECM) proteins. At 7 or 10 days post-UUO, collagen type I and III mRNA levels were increased in the PBS group, and these levels were reduced by MSC treatment, particularly in the SF-MSC group (Fig. 2A). Likewise, immunostaining and histology revealed that treatment with SF-MSCs more significantly suppressed the extent of the collagen type I- and III-positive areas and interstitial fibrosis area than 10%MSCs (Fig. 2B). At 21 days post-UUO, progression of tissue necrosis and severe fibrosis were found in UUO kidney, and treatment with SF-MSCs tended to suppress the expression of α-SMA and collagen type III, but there was no significance (Supporting Information Fig. S2). However, these findings indicate that SF-MSCs can significantly ameliorate the tubulointerstitial fibrosis induced by the UUO operation in rats.

### MSCs Cultured in Serum-Free Conditions Inhibited the Infiltration of Inflammatory Cells in UUO Rats

Damaged cells release inflammatory mediators, and the persistence of inflammation leads to the excessive accumulation of ECM and fibrotic changes [16, 17, 40]. Therefore, we investigated the expression of CD3 (a T lymphocyte marker), CD68 (a macrophage marker), and proinflammatory cytokines to evaluate the anti-inflammatory effects of MSCs in UUO rats. At 7 days post-UUO, the expression levels of CD3 and CD68 mRNA were elevated in the PBS group; administration of 10%MSCs suppressed the expression of these mRNAs, and administration of SF-MSCs did so to a greater extent (Fig. 3A). At 10 days post-UUO, the expression of CD68 mRNA was much lower in the SF-MSC group than in the 10%MSC group, but the CD3 mRNA levels were almost same in the three groups. Immunostaining revealed that the number of CD3- and CD68-positive cells had increased in the PBS group at 10 days post-UUO and that administration of SF-MSCs more significantly suppressed the infiltration of these cells than administration of 10%MSCs. In addition, the number of CD163 (immunosuppressive macrophage marker)-positive cells had increased in the 10%MSC group at 10 days post-UUO, and administration of SF-MSCs induced significantly more cell infiltration compared with 10%MSCs (Fig. 3B). The expression levels of monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-α (TNF-α) mRNA were also elevated in the PBS group; administration of MSCs reduced these increases, particularly in the SF-MSC group (Fig. 3C). These findings indicate that culturing MSCs in a serum-free medium enhances their immunosuppressive effects compared with MSCs cultured with serum.

### Serum-Free Culture Conditions Did Not Affect the Migration and Engraftment of MSCs

MSCs are known to migrate to sites of inflammation following tissue injury and to repair tissue injury [8, 41, 42]. Therefore, we investigated whether MSCs were present in the kidneys of UUO rats by using MSCs from EGFP-positive rats. No EGFP-positive SF-MSCs or 10%MSCs were observed in the kidneys of the rats in the sham operation group. EGFP-positive SF-MSCs and 10%MSCs were both observed in the UUO kidneys at 3 days but not at 6 days after their administration (Supporting Information Fig. S3). These results suggest that serum-free culture conditions do not increase MSC capacity for migration and engraftment.

### Conditioned Medium from MSCs Prevented Fibrotic Change Through Inhibiting TGF-β1–Induced Phosphorylation of Smad2

Having established that the serum-free culture conditions did not promote the capacity of MSCs for engraftment, we investigated whether serum-free culture conditions enhanced the antifibrotic effects in a paracrine manner. We prepared conditioned medium from human MSCs (hMSCs) cultured in STK2 serum-free medium (SF-hMSC-CM) or in 10% FBS-containing medium (10%hMSC-CM). We then stimulated HK-2 cells, a human proximal tubular cell line, with Ang-II with and without each hMSC-CM for 12 hours. Ang-II–induced TGF-β1 mRNA expression was suppressed in both hMSC-CM treatment groups, with no significant difference between them (Fig. 4A). Therefore, we stimulated HK-2 cells with TGF-β1 with and without each hMSC-CM. TGF-β1–induced phosphorylated Smad2 and α-SMA protein expressions were suppressed in both hMSC-CM treatment groups, with no significant difference between them (Fig. 4B, 4C). The mRNA levels of TNF-α and MCP-1 also increased with TGF-β1 stimulation and were significantly suppressed by both hMSC-CM treatments (Fig. 4D, 4E). Although SF-hMSC-CM suppressed TGF-β1–induced TNF-α expression more significantly than did 10%hMSC-CM, there was no significant difference between the two hMSC-CM treatments in the expression of MCP-1. These results indicate that SF-hMSC-CM and 10%hMSC-CM suppress fibrosis to a similar extent.

### MSCs Cultured in Serum-Free Conditions Enhanced a Change in the Phenotype of Macrophages from Proinflammatory M1 to Immunosuppressive M2

To investigate whether SF-MSCs mediated the polarization of immunosuppressive M2 macrophages, we cultured THP-1 cells, a human monocytic cell line, with either SF-hMSCs or 10% hMSCs using a Transwell system. As shown in Figure 5A, the expression of CD163 and CD206, two markers of M2 macrophages, were not detected to a great extent in the macrophages alone. Coculturing macrophages with 10%hMSCs significantly increased the number of CD163-positive cells and

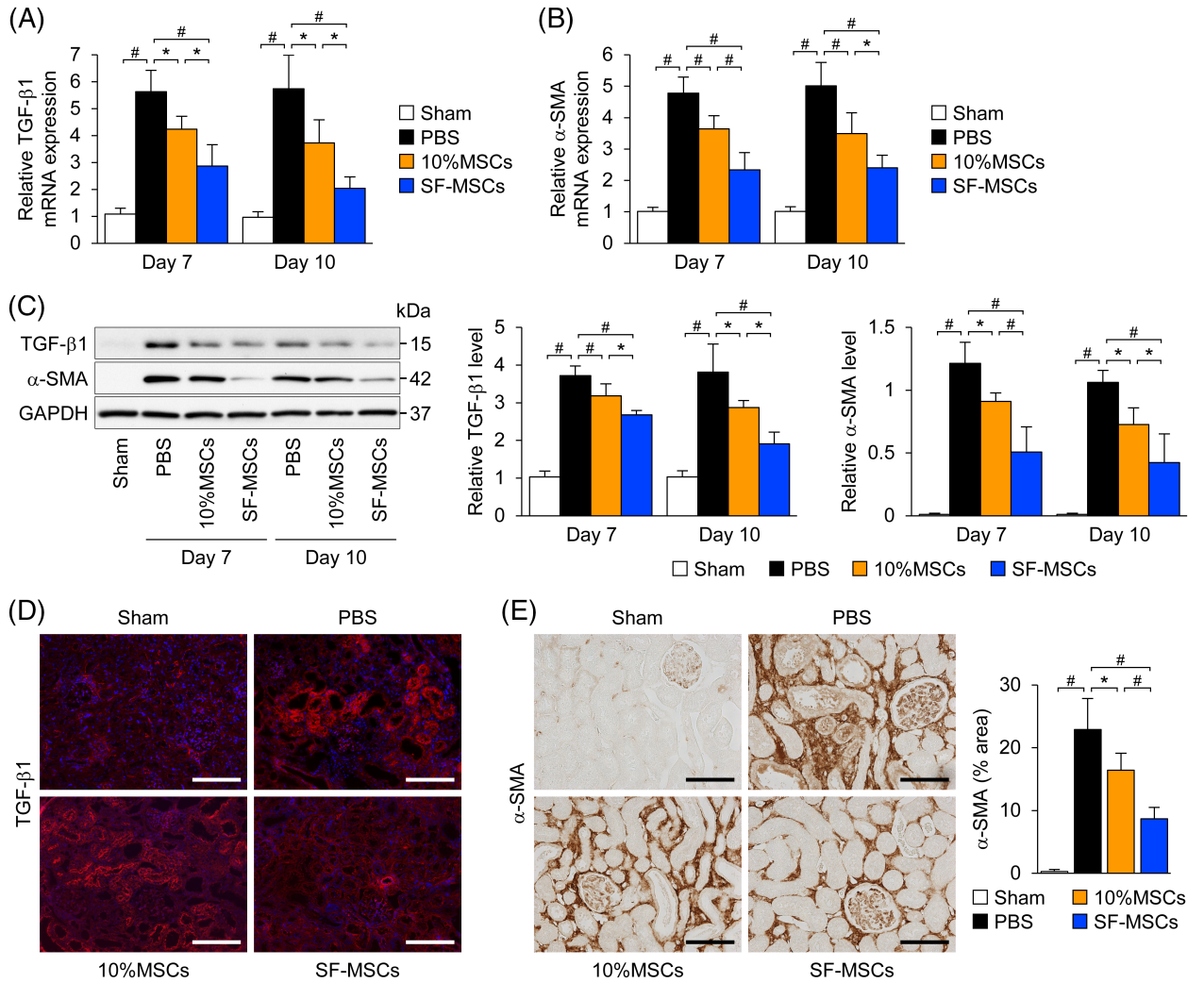


**Figure 1.** SF-MSCs inhibited transforming growth factor-β1 (TGF-β1) and α-smooth muscle actin (α-SMA) more strongly in rat kidneys with UUO than did 10%MSCs. Kidney injury was induced in rats using the UUO procedure. SF-MSCs, 10%MSCs, or PBS were injected through the tail vein at 4 days post-UUO. **(A, B):** Graphs showing the renal cortical expression of TGF-β1 and α-SMA mRNA in UUO rats injected with SF-MSCs, 10%MSCs, or PBS, at 7 or 10 days post-UUO ($n$ = 6 in each group). **(C):** Representative Western blot gel images of TGF-β1 and α-SMA in the kidney cortex of UUO rats at 7 or 10 days post-UUO. The graphs show densitometric analyses of TGF-β1 and α-SMA expression levels normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) level ($n$ = 6 in each group). **(D):** Representative immunofluorescence staining images of TGF-β1-positive areas in kidney sections at 10 days post-UUO (scale bar, 100 μm). The experiments were repeated at least 3 times, and similar results were obtained each time. **(E):** Representative immunohistochemical staining images of α-SMA-positive areas in kidney sections at 10 days post-UUO (scale bar, 100 μm). The graph shows the percentages of α-SMA-positive areas in each group ($n$ = 6 in each group). Data are presented as means ± SD. $^{\#}p < .01$, $^{*}p < .05$, analyzed by one-way ANOVA followed by Bonferroni post hoc testing. Abbreviations: 10%MSCs, MSCs cultured in 10% FBS containing DMEM; MSCs, rat mesenchymal stem cells; SF-MSCs, MSCs cultured in a serum-free medium; UUO, unilateral ureteral obstruction.

slightly increased the CD206-positive cells; both CD163- and CD206-positive cells were increased to higher levels by coculture with SF-hMSCs (Fig. 5B).

Several studies have reported that M2 macrophage polarization is prompted by PGE2 secreted by MSCs [5, 23]. Therefore, we used ELISA to measure the concentration of PGE2 in CM and found that the concentration was significantly lower in SF-hMSC-CM than in 10%hMSC-CM (Fig. 6A). Another study reported that interleukin-6 (IL-6) secreted by MSCs may shift the macrophage phenotype from M1 to M2 [43]. Indeed, when IL-6 was added to macrophages cocultured with SF-hMSCs, we observed a significant increase of CD163-positive cells compared with coculture without IL-6 (Supporting Information Fig. S4). However, the expression level of IL-6 mRNA was significantly lower in SF-hMSCs than in 10%hMSC (Fig. 6B). Although we previously reported that MSCs ameliorated peritoneal fibrosis by suppressing inflammation through secreting HGF [9], we found that HGF was poorly secreted by SF-hMSCs in comparison with 10%hMSCs (Fig. 6C).

## TSG-6 Enhanced the Immunosuppressive Effects of MSCs Cultured in Serum-Free Conditions

Several studies have reported that MSCs secrete TSG-6, an anti-inflammatory cytokine [4, 44]. TSG-6 inhibits the recruitment of

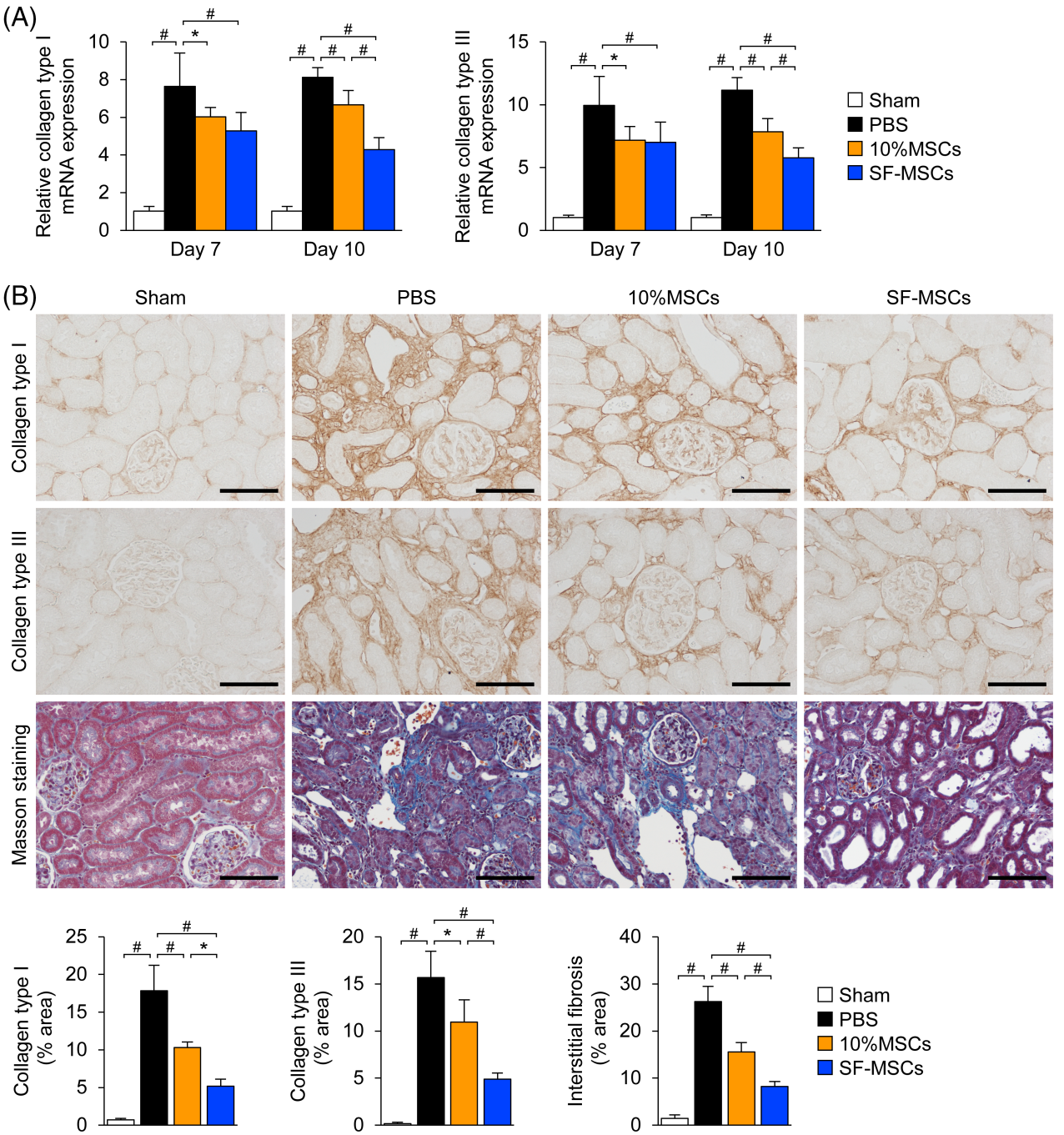


**Figure 2.** SF-MSCs suppressed the accumulation of extracellular matrix protein more strongly than 10%MSCs in rat kidneys with UUO. **(A):** Graph showing renal cortical collagen type I and III mRNA expression levels at 7 and 10 days post-UUO (on 3 or 6 days after the administration of SF-MSCs, 10%MSCs, or PBS; $n = 6$ in each group). **(B):** Representative immunohistochemical staining images of collagen type I- and III-positive areas and Masson trichrome staining images in kidney sections at 10 days post-UUO (scale bar, 100 μm). The graph shows the percentages of collagen type I- and III-positive areas and interstitial fibrosis area ($n = 6$ in each group). Data are presented as means ± SD. $^{\#}p < .01$, $^{*}p < .05$, analyzed by one-way ANOVA followed by Bonferroni post hoc testing. Abbreviations: 10%MSCs, MSCs cultured in 10% FBS containing DMEM; MSCs, rat mesenchymal stem cells; SF-MSCs, MSCs cultured in a serum-free medium; UUO, unilateral ureteral obstruction.

inflammatory cells and attenuates inflammatory cascades in damaged cells [45, 46]. We found that the TSG-6 mRNA level in hMSCs increased as the concentration of serum in the culture medium decreased, and the levels were highest in SF-hMSCs (Fig. 6D and Supporting Information Fig. S5). We next examined whether TSG-6 was involved in the anti-inflammatory effect of SF-hMSCs by knocking down TSG-6 with TSG-6 siRNA. The TSG-6 mRNA levels were decreased by approximately 80% in TSG-6 siRNA-transfected cells (TSG-6 siRNA/SF-hMSCs) compared with NC siRNA-transfected cells (NC siRNA/SF-hMSCs), confirming the successful knockdown efficiency of TSG-6 siRNA (Fig. 6E). We then cocultured differentiated THP-1 cells with TSG-6 siRNA/SF-

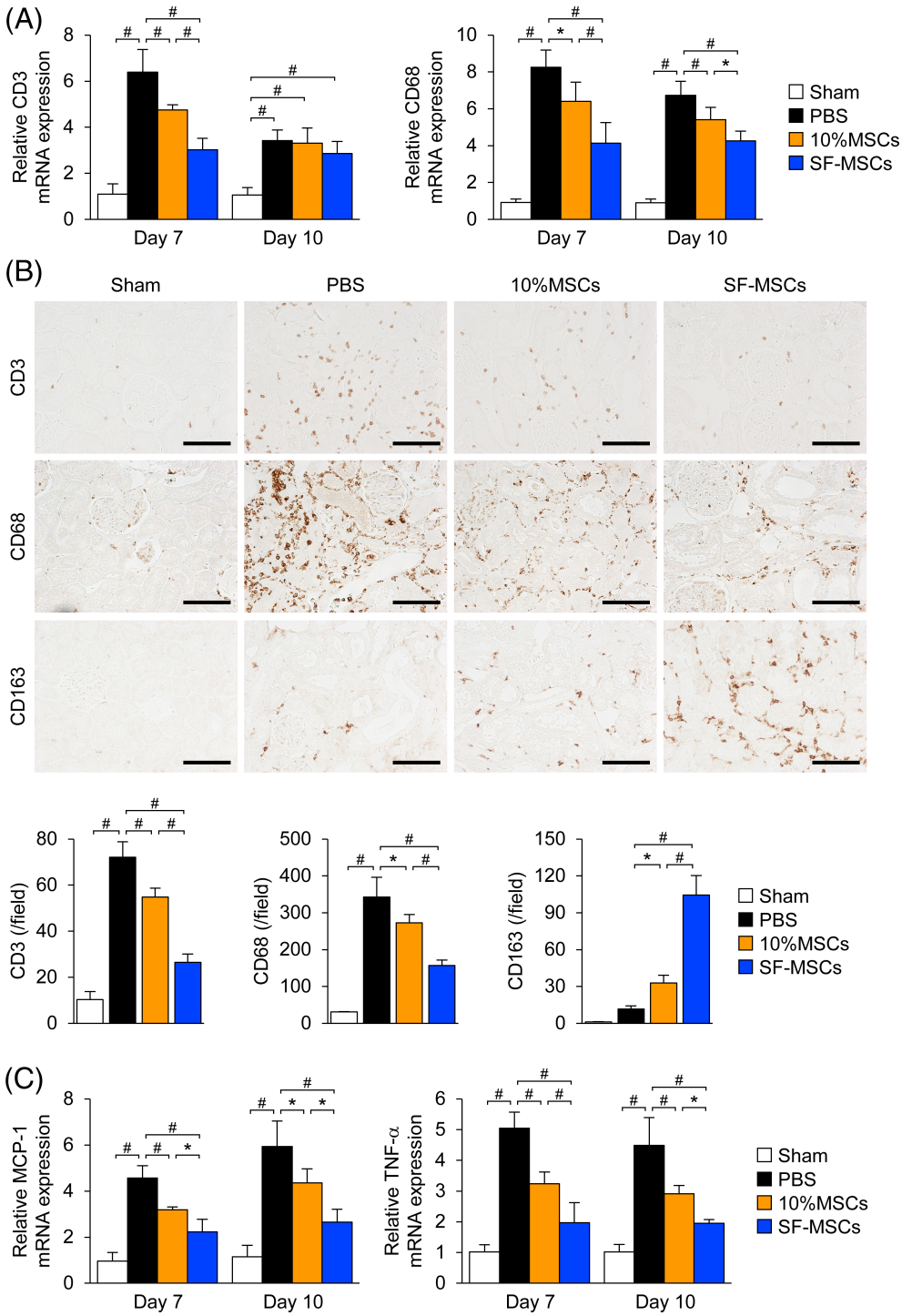


**Figure 3.** SF-MSCs showed the strongest inhibition of the infiltration of inflammatory cells and suppressed the gene expression of various proinflammatory cytokines in rat kidneys with UUO. Rat kidneys were analyzed at 7 or 10 days post-UUO (on 3 or 6 days after the administration of SF-MSCs, 10%MSCs, or PBS). **(A):** Graphs showing the renal cortical mRNA expression of CD3 and CD68 at 7 and 10 days post-UUO ($n$ = 6 in each group). **(B):** Representative immunohistochemical staining images of the infiltration of CD3, CD68, and CD163 at 10 days post-UUO (scale bar, 100 μm). The graphs show the number of CD3, CD68, and CD163-positive cells per field in each group ($n$ = 6 in each group). **(C):** PCR analyses of the expression of proinflammatory cytokines in kidneys of UUO rats after administration. The graphs show renal cortical mRNA expression levels of monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-α (TNF-α) at 7 and 10 days post-UUO ($n$ = 6 in each group). Data are presented as means ± SD. $^{\#}p < .01$, $^{*}p < .05$, analyzed by one-way ANOVA followed by Bonferroni post hoc testing. Abbreviations: 10%MSCs, MSCs cultured in 10% FBS containing DMEM; MSCs, rat mesenchymal stem cells; SF-MSCs, MSCs cultured in a serum-free medium; UUO, unilateral ureteral obstruction.

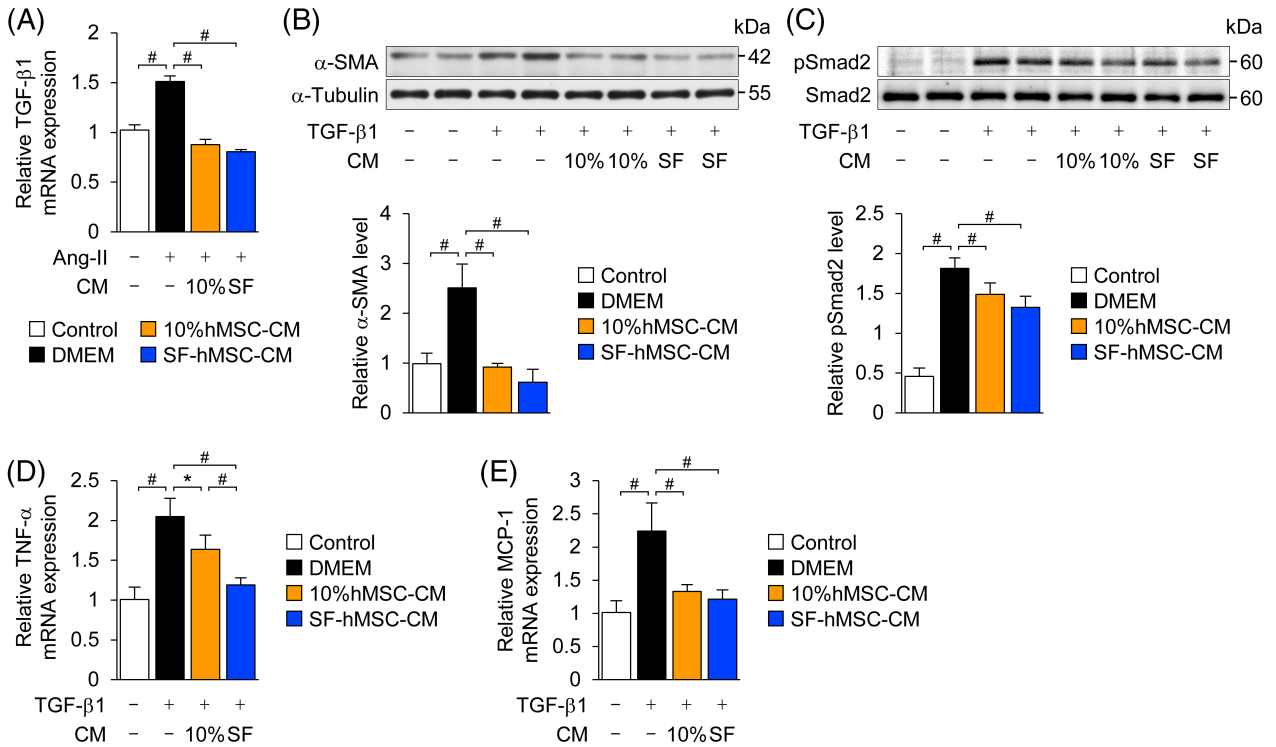


**Figure 4.** Human MSCs suppressed angiotensin-II (Ang-II)–induced TGF-β1 and TGF-β1–induced fibrotic changes in HK-2 cells. HK-2 cells were stimulated with Ang-II or TGF-β1 with or without the SF-hMSC-CM and 10%hMSC-CM, prepared by culturing in serum-free DMEM for 48 hours. **(A):** Graph showing the expression of TGF-β1 mRNA in HK-2 cells stimulated with Ang-II for 12 hours with or without hMSC-CM, as indicated ($n$ = 6 in each group). **(B):** Representative Western blot gel images of α-SMA protein in HK-2 cells stimulated with TGF-β1 for 48 hours with or without hMSC-CM. The graph shows the densitometric analysis of α-SMA expression normalized to GAPDH expression ($n$ = 6 in each group). **(C):** Representative Western blot gel images of phosphorylated Smad2 (pSmad2) in HK-2 cells stimulated with TGF-β1 for 1 hours with or without hMSC-CM. The graph shows the densitometric analysis of pSmad2 expression normalized to Smad2 expression ($n$ = 6 in each group). **(D, E):** Graphs showing the expression levels of TNF-α and MCP-1 mRNA in HK-2 cells stimulated with TGF-β1 for 12 hours with or without hMSC-CM ($n$ = 6 in each group). All experiments were repeated at least 3 times, and similar results were obtained each time. Data are presented as means ± SD. $^{\#}p < .01$, $^{*}p < .05$, analyzed by one-way ANOVA followed by Bonferroni post hoc testing. Abbreviations: 10%hMSCs, hMSCs cultured in 10% FBS containing DMEM; CM, conditioned medium; hMSCs, human mesenchymal stem cells; SF-hMSCs, hMSCs cultured in a serum-free medium.

hMSCs or NC siRNA/SF-hMSCs and analyzed the expression of CD163-positive cells. There was no significant difference in the number of CD163-positive macrophages between the two groups (Fig. 6F). Therefore, we prepared conditioned medium from TSG-6 siRNA/SF-hMSCs and stimulated HK-2 cells with TGF-β1 with or without the conditioned medium for 12 hours to evaluate the paracrine effect of SF-hMSCs through TSG-6. As shown in Figure 6G, the expression levels of TGF-β1–induced MCP-1 and TNF-α mRNA were significantly suppressed by conditioned medium from NC siRNA/SF-hMSCs. However, this suppression was abrogated by transfection with TSG-6 siRNA. These findings indicate that culturing MSCs in serum-free conditions enhances their TSG-6 expression and that this enhancement of TSG-6 may be responsible for the immunosuppressive effects of SF-MSCs.

### Knockdown of TSG-6 in SF-MSCs Weakened the Immunosuppressive and Antifibrotic Effects of SF-MSCs in UUO Rats

TSG-6 mRNA levels were also higher in SF-MSCs than in 10% MSCs in rats (Fig. 7A). We investigated whether TSG-6 secreted by SF-MSCs was related to the SF-MSC-mediated suppression of inflammation and fibrosis induced by UUO. At 4 days post-UUO, we injected rats through the tail vein with $5 \times 10^6$ SF-MSCs transfected with TSG-6 siRNA (TSG-6 siRNA/SF-MSCs) or NC siRNA (NC siRNA/SF-MSCs), or with only PBS. Figure 7A confirmed successful knockdown of TSG-6 in siRNA-transfected SF-MSCs. At 10 days post-UUO, the expression levels of TNF-α and IL-1β mRNA had increased in the PBS group and were significantly suppressed by the administration of NC siRNA/SF-MSCs (Fig. 7B). However, these reductions were abrogated by the administration of TSG-6 siRNA/SF-MSCs. Similarly, the protein expression levels of α-SMA and TGF-β1 were also increased in the PBS group and significantly suppressed by the administration of NC siRNA/SF-MSCs, but these reductions were weakened by administration of TSG-6 siRNA/SF-MSCs (Fig. 7C). In addition, the α-SMA- and collagen type I-positive areas and the number of CD68-positive cells were increased in the PBS group and the administration of NC siRNA/SF-MSCs reduced expression levels, whereas TSG-6 siRNA/SF-MSCs significantly attenuated these reductions (Fig. 7D, 7E). These results suggest that knockdown of TSG-6 in SF-MSCs weakens their ability to suppress inflammation and fibrosis.

## Discussion

This study has provided the first evidence that the administration of SF-MSCs ameliorated inflammation and the tubulointerstitial fibrosis induced by UUO in rats to a greater extent than

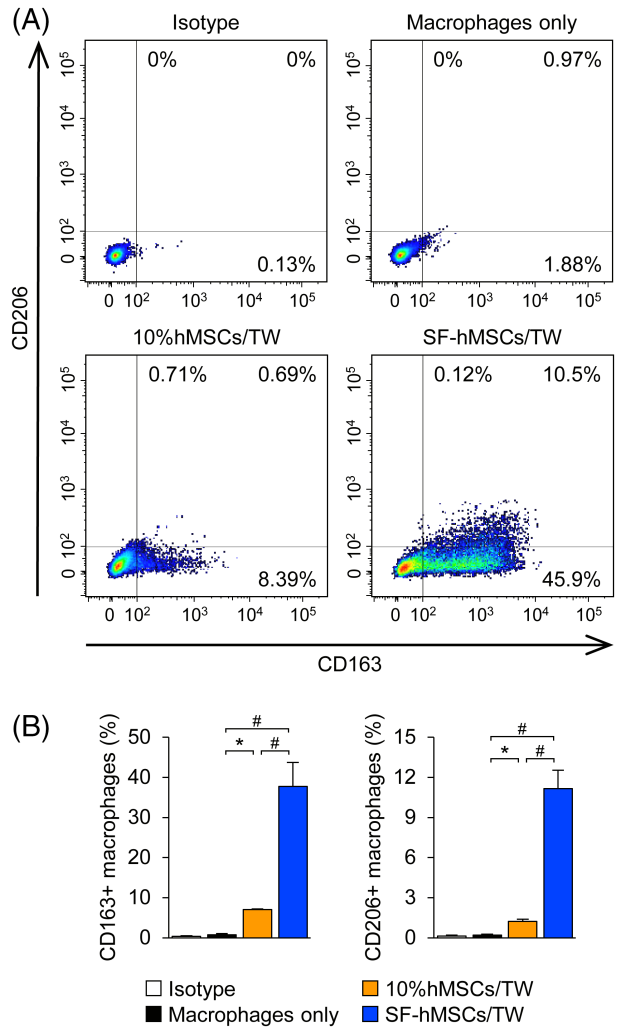


**Figure 5.** SF-hMSCs enhanced the polarization of the M1 macrophage phenotype toward the M2 phenotype. THP-1 cells were differentiated into macrophages with phorbol 12-myristate 13-acetate and interferon-γ, and then THP-1 macrophages were cocultured with hMSCs using a Transwell system for 48 hours. **(A):** The expression of CD163 and CD206 in THP-1 macrophages cocultured with SF-hMSCs (SF-hMSCs/TW) or 10%hMSCs (10%hMSCs/TW) was assessed by flow cytometry analysis. **(B):** Graph showing the percentages of CD163-positive and CD206-positive cells in THP-1 macrophages + SF-hMSCs/TW, THP-1 macrophages +10% hMSCs/TW, and THP-1 macrophages only ($n$ = 6 in each group). All experiments were repeated at least 3 times, and similar results were obtained each time. Data are presented as means ± SD. $^{\#}p < .01$, $^{*}p < .05$, analyzed by one-way ANOVA followed by Bonferroni post hoc testing. Abbreviations: 10%hMSCs, hMSCs cultured in 10% FBS containing DMEM; hMSCs, human mesenchymal stem cells; hMSCs/TW, cocultured with hMSCs using Transwell; SF-hMSCs, hMSCs cultured in a serum-free medium.

the administration of 10%MSCs. In addition, this study elucidated the paracrine effects of SF-MSCs: the conditioned medium from SF-MSCs suppressed TGF-β1 signaling, although there was no significant difference with the suppression by 10%MSCs. The coculture of monocytic THP-1 cell-derived macrophages with MSCs using a Transwell system showed that the SF-MSCs enhanced polarization to the immunosuppressive M2 phenotype macrophage, suggesting that they strongly suppress the persistence of inflammation. Finally, knockdown of TSG-6 in SF-MSCs weakened SF-MSC-mediated suppression of the infiltration of inflammatory cells. Although direct antifibrotic effects of SF-MSCs were similar to those of 10%MSCs, serum-free culture conditions enhanced the immunosuppressive abilities of MSCs, and administration of SF-MSCs ameliorated renal fibrosis to a greater extent than 10%MSCs.

EGFP-positive SF-MSCs and 10%MSCs were observed in the UUO kidneys at 3 days but not at 6 days after their administration, and serum-free culture conditions did not promote migration and engraftment by the MSCs. However, the administration of SF-MSCs ameliorated renal fibrosis more significantly than the administration of 10%MSCs. We consider that these effects are independent of the transdifferentiation of SF-MSCs into functional cells and are more likely because of the paracrine activities of SF-MSCs. Our previous study showed that conditioned medium from MSCs suppressed TGF-β1 signaling [9]. Therefore, we evaluated whether conditioned medium from SF-MSCs and 10%MSCs inhibited the Ang-II-induced TGF-β1 expression and TGF-β1–induced phosphorylated Smad2 and α-SMA expressions in HK-2 cells. However, we found no significant difference between the conditioned media from SF-MSCs and 10%MSCs. Thus, we concluded that the direct antifibrotic effect of SF-MSCs is virtually the same as that of 10%MSCs. On the basis of these findings, we focused our subsequent experiments on the immunosuppressive effect of SF-MSCs.

Several studies have reported that MSCs can induce a change in the phenotype of macrophages from proinflammatory M1 to immunosuppressive M2 [5, 23, 47, 48]. M2 macrophages play an important role in the resolution of inflammation by secreting anti-inflammatory mediators, such as interleukin-1 receptor antagonist and interleukin-10, which regulate Th1, Th2, and the regulatory T cell response and inhibit the persistence of inflammation [49]. In the present study, the coculture of macrophages with 10%MSCs significantly increased the number of CD163-positive cells and slightly increased the CD206-positive cells, with both cell types increased to higher levels in coculture with SF-MSCs. In addition, our results in macrophages cocultured with MSCs derived from adipose tissue indicated that serum-free culture conditions also potently enhanced macrophage polarization from M1 to M2 compared with culturing in a medium containing serum (Supporting Information Fig. S6). Hence, our results suggest that MSCs cultured in serum-free conditions enhance macrophage polarization toward the M2 phenotype and strongly inhibit the persistence of inflammation.

PGE2 secreted from MSCs plays an important role in polarizing macrophages toward the M2 phenotype [50], but we found that 10%MSCs secreted a greater amount of PGE2 and IL-6 compared with SF-MSCs. MSCs cultured in a medium with low levels of serum also secreted lower amounts of PGE2 compared with MSCs cultured in a medium containing 10% FBS [51]. Although other studies have shown that the proresolving lipid mediators lipoxin A4 and resolvin D1 enhance M2 polarization of macrophages and ameliorate lung and liver injury [52, 53], we only observed undetectable concentrations of lipoxin A4 and resolvin D1 in conditioned medium from SF-MSCs (data not shown). However, only the gene expression of TSG-6 was higher in SF-MSCs than in 10%MSCs. Moreover, the gene expression of TSG-6 in MSCs increased as the concentration of serum in the culture medium decreased. These results suggest that several factors in serum weaken the ability

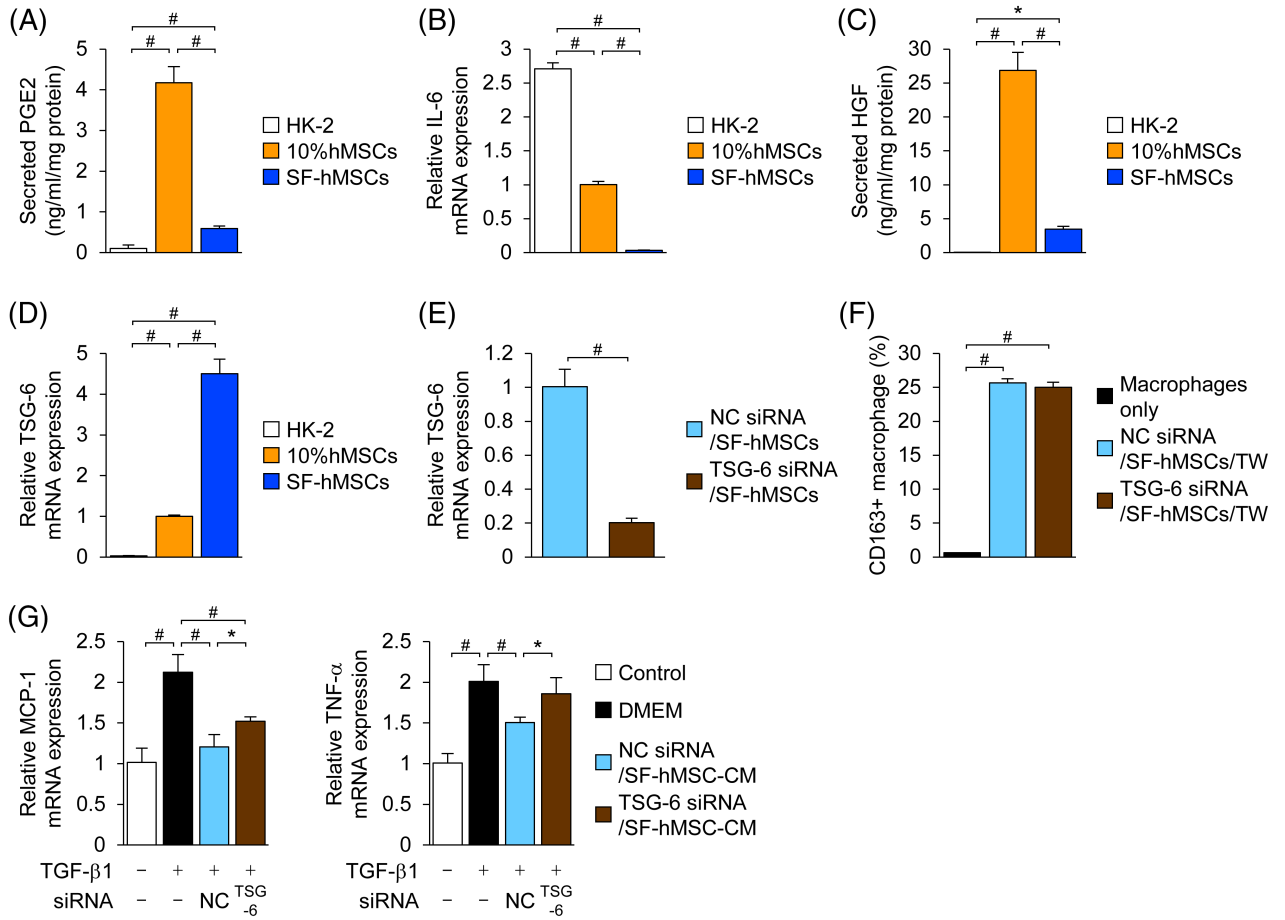


**Figure 6.** SF-hMSCs upregulated the production of tumor necrosis factor-α–induced protein 6 (TSG-6) and inhibited inflammation through TSG-6. SF-hMSCs and 10%hMSCs were cultured in serum-free DMEM for 48 hours and the supernatants were collected as CM. The concentrations of **(A)** prostaglandin E2 (PGE2) and **(C)** hepatocyte growth factor (HGF) in CM from SF-hMSCs, 10%hMSCs, and HK-2 cells were measured in triplicate with ELISA. SF-hMSCs, 10%hMSCs, and HK-2 cells were harvested for PCR analysis of **(B)** interleukin-6 (IL-6) and **(D)** TSG-6 mRNA ($n$ = 6 in each group). **(E):** The knockdown efficiency of TSG-6 siRNA in SF-hMSCs was analyzed in triplicate by PCR. **(F):** THP-1 cells were cocultured with SF-hMSCs transfected with TSG-6 siRNA (TSG-6 siRNA/SF-hMSCs/TW) or negative control siRNA (NC siRNA/SF-hMSCs/TW) for 48 hours and the expression of CD163 in THP-1 cells was analyzed with flow cytometry ($n$ = 6 in each group). **(G):** The CM from SF-hMSCs transfected with TSG-6 siRNA (TSG-6 siRNA/SF-hMSC-CM) or negative control (NC siRNA/SF-hMSC-CM) were collected by incubation for 48 hours with serum-free DMEM. HK-2 cells starved with TSG-6 siRNA/SF-hMSC-CM or NC siRNA/SF-hMSC-CM were stimulated with TGF-β1 for 12 hours and the expression levels of MCP-1 and TNF-α mRNA were analyzed by PCR ($n$ = 6 in each group). Data are presented as means ± SD. $^{\#}p < .01$, $^{*}p < .05$, analyzed by one-way ANOVA followed by Bonferroni post hoc testing in all figures except for panel E, in which the data were analyzed by Student's $t$ test. Abbreviations: 10%hMSCs, hMSCs cultured in 10% FBS containing DMEM; CM, conditioned medium; hMSCs, human mesenchymal stem cells; hMSCs/TW, cocultured with hMSCs using Transwell; SF-hMSCs, hMSCs cultured in a serum-free medium.

of MSCs to express TSG-6. Several studies have shown that TSG-6 inhibits the migration of inflammatory cells, such as neutrophils and macrophages, by affecting chemokine receptors and that it attenuates inflammatory cascades in the early inflammatory phase in damaged sites [45, 54]. Furthermore, TSG-6 secreted by MSCs inhibits the recruitment of inflammatory cells [4, 44, 46]. In our study, knockdown of TSG-6 in SF-MSCs did not affect the polarization of macrophages toward the M2 phenotype by SF-MSCs. However, TSG-6 siRNA treatment weakened the suppression by SF-MSCs on the expression of TGF-β1–induced MCP-1 and TNF-α. In addition, knockdown of TSG-6 in SF-MSCs weakened the immunosuppressive and antifibrotic effects of SF-MSCs in UUO rats. Indeed, intravenously injected MSCs with TSG-6 knockdown did not suppress the infiltration of inflammatory cells in experimental myocardial infarction [4]. In addition, implanting differentiated fat cells with TSG-6 siRNA treatment did not suppress the infiltration of inflammatory cells in experimental glomerulonephritis [10]. These studies support the idea that TSG-6 plays an important role in suppressing, at an early phase, the infiltration of inflammatory cells induced by tissue injury.

In analyses of MSC-conditioned media, 10%MSCs secreted a greater amount of PGE2, which induced the phenotypic change to M2 macrophages, compared with SF-MSCs. However, coculture of THP-1 macrophages with 10%MSCs slightly induced macrophage polarization from the M1 to M2 phenotype compared with SF-MSCs. Moreover, administration of SF-MSCs suppressed the infiltration of inflammatory cells and induced M2 macrophage polarization in UUO rats to a greater extent than 10%MSCs. However, 10%MSCs secreted a higher amount of HGF, which suppressed fibrosis, compared with SF-MSCs. Furthermore, administration of SF-MSCs ameliorated

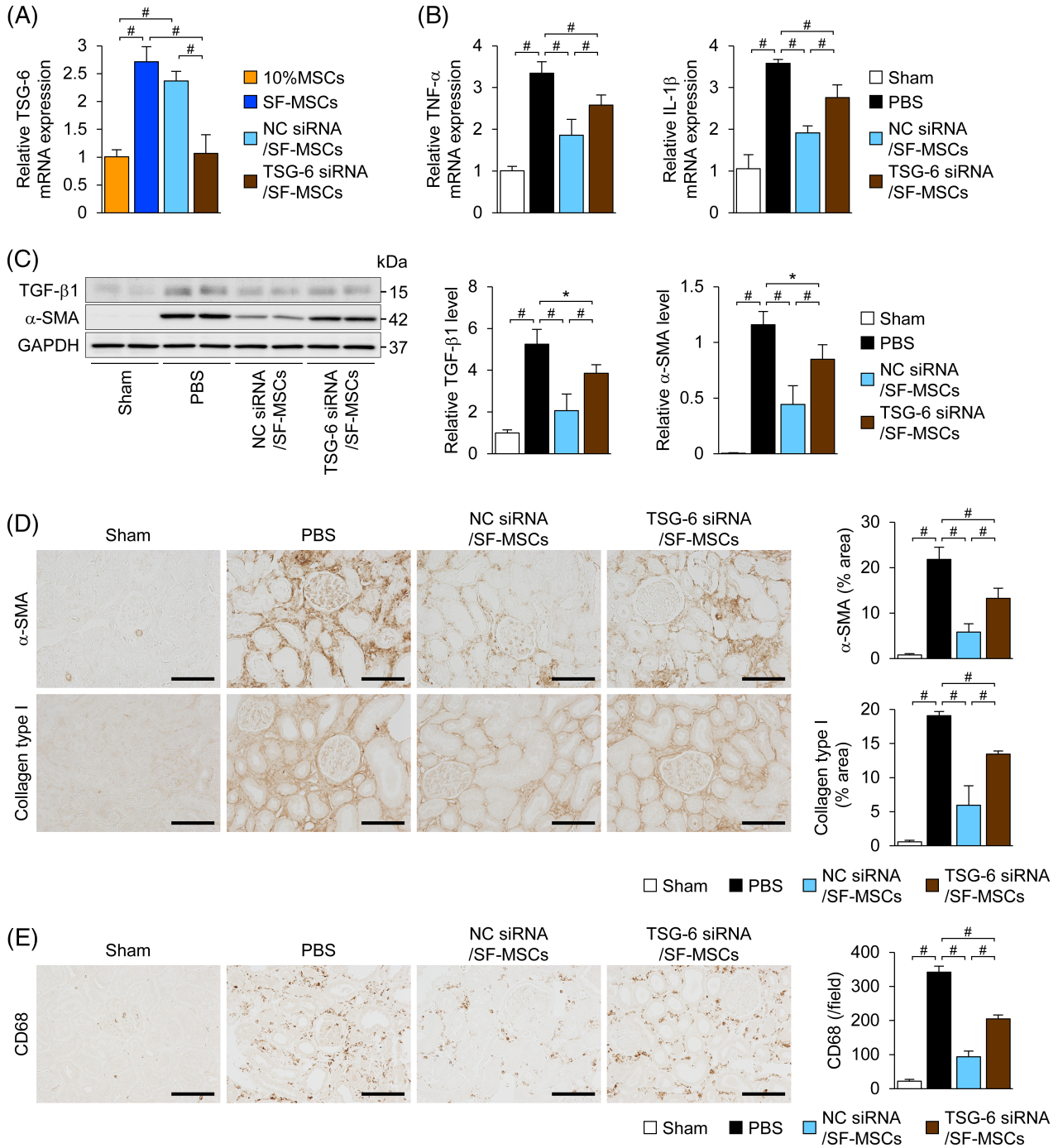


**Figure 7.** TSG-6 siRNA transfection of SF-MSCs attenuated the anti-inflammatory and antifibrotic effects of SF-MSCs in kidneys from UUO rats.Kidneys of UUO rats were analyzed 6 days after the administration of SF-MSCs transfected with TSG-6 siRNA (TSG-6 siRNA/SF-MSCs), SF-MSCs transfected with negative control siRNA (NC siRNA/SF-MSCs), or PBS (at 10 days post-UUO). **(A):** Graph showing the knockdown efficiency of TSG-6 siRNA in SF-MSCs ($n$ = 6 in each group). **(B):** Renal cortical mRNA expression levels of TNF-α and IL-1β analyzed by PCR ($n$ = 6 in each group). **(C):** Representative Western blot gel images of TGF-β1 and α-SMA in the kidney cortex of UUO rats at 10 days post-UUO. The graphs show the densitometric analysis of TGF-β1 and α-SMA expression levels normalized to the expression of GAPDH ($n$ = 6 in each group). **(D):** Representative immunohistochemical staining images of α-SMA-positive and collagen type I-positive areas in kidney sections at 10 days post-UUO (scale bar, 100 μm). The graphs show the percentages of α-SMA-positive and collagen type I-positive areas ($n$ = 6 in each group). **(E):** Representative immunohistochemical staining images of CD68-positive regions at 10 days post-UUO (scale bar, 100 μm). The graph shows the number of CD68-positive cells per field ($n$ = 6 in each group). Data are presented as means ± SD. $^{\#}p < .01$, $^{*}p < .05$, analyzed by one-way ANOVA followed by Bonferroni post hoc testing. Abbreviations: MSCs, rat mesenchymal stem cells; SF-MSCs, MSCs cultured in a serum-free medium; UUO, unilateral ureteral obstruction.

the tubulointerstitial fibrosis induced by UUO in rats to a greater extent than 10%MSCs. Although we cannot fully explain these mechanisms, the higher expression of TSG-6 in SF-MSCs may contribute to immunosuppressive and antifibrotic effects of SF-MSCs.

A recent representative medium supplement as an alternative to FBS is HPL. HPL contains various growth factors and can be used to expand human MSCs [27, 55]. Although we revealed that MSCs cultured in HPL (PL-MSCs) secreted more HGF and less TSG-6 than SF-MSCs, immunosuppressive and antifibrotic effects of PL-MSCs were inferior to those of SF-MSCs (Supporting Information Fig. S7). In rat UUO experiments, PL-MSCs and 10%MSCs suppressed inflammation and fibrosis to a similar extent, but SF-MSCs inhibited them more strongly compared with PL-MSCs. Additionally, previous reports showed that several serum-free media for MSCs such as StemPro MSC SFM XenoFree (Thermo Fisher Scientific), Mesencult-XF Medium (STEMCELL Technologies, Vancouver, BC, Canada), and BD Mosaic Mesenchymal Stem Cell Serum-Free Medium (Becton, Dickinson and Company) also caused a spindle-shaped morphology of MSCs and supported a significantly faster growth rate of MSCs compared with FBS-containing culture conditions [31, 32]. However, we have not compared the difference in the immunosuppressive and antifibrotic effects of MSCs cultured in between these serum-free media and the STK media in this study.

## Conclusion

In summary, SF-MSCs significantly suppress the persistence of inflammation through the enhanced induction of a change in the phenotype of macrophages from proinflammatory M1 to immunosuppressive M2. They also suppress the infiltration of inflammatory cells through the enhanced expression of TSG-6. In addition to these notable effects, serum-free media for MSCs, such as STK1 and STK2, are useful for culturing MSCs with no risk of infections or immune reactions compared with media that contain serum. Our results suggest that the administration of ex vivo expanded SF-MSCs has the potential to be a useful therapy for preventing the progression of renal fibrosis.

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## Author Contributions

K.Y., A.N., and T.M.: conception and design, data analysis and interpretation; K.Y., A.N., T.U., T.O., and K.K.: collection and/or assembly of data; S.D., M.K., Y.K., and Y.H.: data analysis and interpretation; K.Y. and A.N.: article writing; A.N. and T.M.: final approval of article.

## Disclosure of Potential Conflicts of Interest

Y.K. declares patent ownership for serum-free medium for MSCs and employment/leadership position with TWOCELLS Co. Ltd. K.Y., S.D., and T.M. declared patent ownership for serum-free medium for MSCs. A.N. declares patent ownership for serum-free medium for MSCs and research funding from TWOCELLS Co. Ltd. The other authors indicated no potential conflicts of interest.

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