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ORIGINAL ARTICLE

Mesenchymal Stem Cells Mediated Suppression of GREM2 Inhibits Renal Epithelial-Mesenchymal Transition and Attenuates the Progression of Diabetic Kidney Disease

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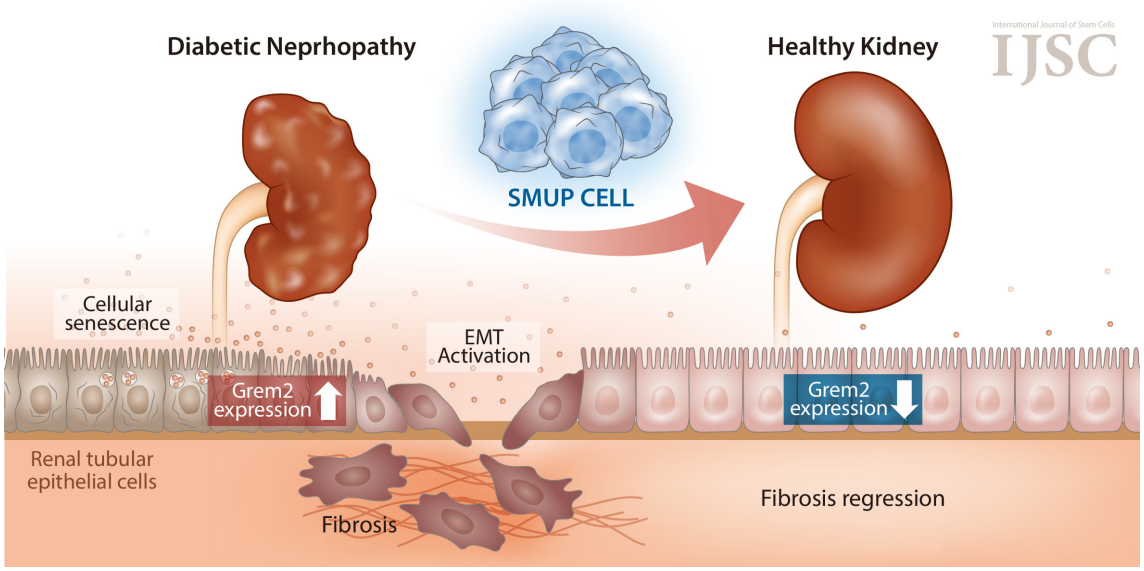

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Mesenchymal Stem Cells Mediated Suppression of GREM2 Inhibits Renal Epithelial-Mesenchymal Transition and Attenuates the Progression of Diabetic Kidney Disease

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Diabetic kidney disease (DKD) is the leading cause of end-stage renal disease worldwide. Despite advancements in various treatments, the prevalence of DKD continues to rise, leading to a significant increase in the demand for dialysis and kidney transplantation. This study aimed to evaluate the effects of a Small cell+Ultra Potent+Scale UP cell (SMUP-Cell), a type of human umbilical cord blood-derived mesenchymal stem cell, on DKD in the *db/db* mouse model of type 2 diabetes mellitus. After administering SMUP-Cells via tail vein injection in *db/db* mice, the animals were monitored over a three-month period. The *db/db* mice exhibited an increased urine albumin-to-creatinine ratio (UACR). However, the administration of SMUP-Cells resulted in a reduction of the UACR. The expression levels of desmin, α-smooth muscle actin, and fibronectin—markers of epithelial-mesenchymal transition (EMT)—as well as kidney injury molecule 1, a sensitive marker of tubular injury, were significantly elevated in *db/db* mice. Treatment with SMUP-Cells ameliorated all of these changes. Notably, Gremlin isoform 2 (*Grem2*) exhibited the most significant difference in expression according to the transcriptome analysis. The elevated expression of *Grem2* in *db/db* mice was significantly reduced following SMUP-Cell treatment. In vitro, treatment with high glucose and cholesterol induced *Grem2* expression in renal tubular epithelial cells (RTECs), while *Grem2* knockdown effectively prevented fibrosis and senescence induced by high glucose and cholesterol in RTECs. These observations suggest that SMUP-Cells inhibit the progression of DKD by inhibiting EMT through the reduction of *Grem2* expression in RTECs.

Keywords: Mesenchymal stem cells, Epithelial-mesenchymal transition, Diabetic kidney disease, Fibrosis

Introduction

Diabetic kidney disease (DKD) is a significant microvascular complication of diabetes mellitus (DM) and one of the leading causes of chronic kidney disease (CKD) and end-stage renal disease (ESRD) worldwide (1). Early and appropriate therapeutic interventions are crucial to preventing progression to ESRD, highlighting the importance

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of prompt diagnosis and proactive treatment strategies. Mitigating the progression of DKD necessitates a multifaceted approach that includes dietary modifications, strict glycemic control, effective blood pressure management, and inhibition of the renin-angiotensin-aldosterone system (2). Recent therapeutic outcomes for DKD highlight the limitations of current treatment options. Despite various approaches, managing this condition remains challenging, underscoring the need for innovative treatment alternatives. This necessity is driven by the persistently high rates of progression to ESRD, which require dialysis and kidney transplantation, particularly as the prevalence of DM continues to rise globally (3).

Stem cells, which are characterized by their self-renewal capabilities and potential to differentiate into various functional cell types under specific conditions, offer promising new avenues for the treatment of numerous human diseases due to their plasticity, unlimited proliferation, and ease of genetic manipulation. In particular, mesenchymal stem cells (MSCs) have shown potential as a cell-based therapy for kidney diseases through various mechanisms. Beyond their anti-inflammatory properties, MSCs exhibit anti-apoptotic, pro-angiogenic, antioxidant, anti-fibrotic, autophagy-regulating, and anti-aging effects, highlighting their potential in the treatment of renal diseases (4, 5). Notably, several studies have reported that systemic administration of MSCs in DKD animal models effectively suppressed the progression of advanced DKD (5, 6). Recently, we demonstrated that repeated intravenous administration of Small cell+Ultra Potent+Scale UP cells (SMUP-Cells), which are MSCs derived from human umbilical cord blood (7), improved DKD in a type 1 DM animal model. Specifically, SMUP-Cells prevent the progression of DKD by modulating macrophage function and improving mitochondrial biogenesis and function in tubular epithelial cells (8).

Growing evidence suggests that renal fibrosis is a key driver in the progression from DKD to CKD and ultimately ESRD (9, 10). Renal fibrosis, characterized by excessive deposition of extracellular matrix (ECM) and tubular atrophy, plays a pivotal role in the decline of renal function. This pathological process is further exacerbated by sustained hyperglycemia, inflammation, and oxidative stress, which collectively promote fibrosis through mechanisms involving both glomerular and tubular damage (10). Recent studies have demonstrated that mitigating renal fibrosis can slow the progression of DKD and delay the need for dialysis, emphasizing the importance of targeting fibrosis in the prevention of advanced kidney failure in diabetic patients. Despite these findings, effective therapeutic strategies to halt or reverse fibrosis in DKD remain limited (1). Given the critical role of renal fibrosis in the progression of DKD to CKD, this study aims to investigate the dose-dependent effects of SMUP-Cell administration and assess the durability of its therapeutic benefits in a type 2 DKD model.

Materials and Methods

Animals

All animal-based experimental protocols were approved by the Institutional Animal Care and Use Committee of the Asan Institute for Life Sciences, Seoul, Korea (Approval No: 2021-12-279). Mice were housed at an ambient room temperature of 22℃±1℃ with a 12-hour light-dark cycle and had free access to water and food. Eight-week-old male *db/db* mice and C57BL/6J mice were obtained from Jackson Laboratory. At 10 weeks of age, 24-hour urine samples were collected from the *db/db* mice to measure proteinuria. Based on these measurements, the mice were divided into four groups. At 12 weeks of age, mice in the vehicle group and the treatment groups received intravenous injections via the tail vein. The treatment groups were administered SMUP-Cells at dosages of 2×10^6 (low), 5×10^6 (mid), and 1×10^7 (high) cells/kg, respectively. Human SMUP-Cells were prepared as described previously (7). At 16, 20, and 24 weeks of age, 24-hour urine samples were collected to measure urinary albumin excretion. Mice were then sacrificed, and their kidneys were harvested for morphometric and histochemical analyses.

Urinary albumin/creatinine ratio measurement

At 10, 12, 16, 20, and 24 weeks of age, mice were placed in individual metabolic cages for 24-hour urine collections to measure urinary albumin excretion rates. Urinary albumin levels were measured using an enzyme-linked immunosorbent assay (E-90AL; Immunology Consultants Laboratory). Urinary creatinine levels were measured using a colorimetric detection kit (ADI-907-030A; Enzo Life Science). The urine albumin-to-creatinine ratio (UACR) was calculated by dividing the amount of urinary albumin by the amount of urinary creatinine (mg/g Cr).

Determination of plasma glucose

Plasma glucose levels were determined using the Asan set glucose (AM201-K; Asan Pharm) according to the manufacturer's protocol.

Histological analysis

Renal cortex tissues were fixed in 10% formalin for dehydration, embedded in paraffin, and sectioned. Sections

were stained with H&E, periodic acid-Schiff (PAS), and Masson's trichrome (MT). PAS-stained slides were analyzed using ImageJ software (National Institutes of Health) to measure glomerular and mesangial areas.

Immunohistochemistry

Paraffin-embedded tissue blocks were sectioned at a thickness of 3 μm, mounted on slides, and subjected to deparaffinization and rehydration. Slides were washed with Tris buffer (pH 7.6). For antigen retrieval and to prevent cross-link reactions, sections were incubated with Tris/Borate/EDTA buffer (pH 8.4) at 100℃ for 1 hour, followed by washing with the reaction buffer. Endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 at 37℃ for 4 minutes. After washing, primary antibodies were applied and incubated at 37℃ for 36 minutes, followed by another wash. The primary antibodies used were anti-Desmin (1 : 200, ab227651; Abcam), anti-Fibronectin (1 : 2,000, ab268021; Abcam), anti-KIM-1 (1 : 300, NBP1-76701; Novus Biologicals), and anti-Grem2 (1 : 200, 13892-1-AP; Proteintech). The secondary antibody (Discovery UltraMap anti-Rabbit HRP) was then applied and incubated at 37℃ for 12 minutes, followed by a wash. Sections were treated with DAB (UltraView DAB and DAB H2O2) at 37℃ for 8 minutes to develop the chromogenic reaction. After washing, sections were incubated with UltraView Copper (760-500; Ventana) at 37℃ for 2 minutes, washed again, and counterstained with hematoxylin at 37℃ for 4 minutes. A final nuclear staining was performed with Bluing reagent at 37℃ for 4 minutes. Sections were washed, dehydrated, and mounted with a coverslip. Images were quantified using ImageJ Fiji software.

Immunofluorescence staining

After sacrificing the animals, kidney tissues were harvested and prepared for frozen embedding. Tissues were fixed in 4% paraformaldehyde at 4℃, washed with distilled water, and dehydrated to enhance tissue penetration of the optical coherence tomography (OCT) compound. Prepared tissue blocks were mounted in a Thermo Cryocut microtome using OCT compound and frozen at −20℃. Sections were cut at a thickness of 7 μm. Slides were dried at room temperature for 30 minutes before being washed with phosphate-buffered saline (PBS) for 5 minutes, repeated three times. To enhance cell membrane permeability, 0.2% Triton X-100 containing 100 mM glycerol in PBS was applied, followed by incubation at room temperature for 20 minutes. The slides were washed three times with PBS. To block nonspecific antibody binding, sections were incubated with 2% bovine serum albumin in PBS at room temperature for 30 minutes, followed by three washes with PBS. Primary antibodies were applied and incubated overnight at 4℃. The primary antibodies used were anti-Synaptopodin antibody (1 : 50, 61494; Progen), anti-Nephrin (1 : 500, sc-19000; Santa Cruz Biotechnology) and anti-α-SMA (1 : 1,000, ab7817; Abcam). After washing, secondary antibodies (all at 1 : 200, Anti-Mouse IgG Alexa 488, A-11001; Anti-Mouse IgG Alexa 555, A-21424; Anti-Goat IgG Alexa 555, A-21432; Invitrogen) were applied and slides were incubated at room temperature for 1 hour in the dark. The slides were washed three times with PBS. For nuclear staining, DAPI (62248; Thermo Scientific) was applied for 15 minutes at room temperature, followed by two washes with PBS and a final quick rinse with distilled water. Immunofluorescence images were acquired using a Zeiss LSM 780 inverted confocal microscope (Carl Zeiss).

Detection of SMUP-Cells using immunostaining

SMUP-Cells were detected using immunofluorescence staining for human mitochondria (ab92824; Abcam) and visualized using an Alexa 488-labeled secondary antibody. Nuclei were counterstained with DAPI. Images were acquired using a Zeiss LSM 800 confocal microscope system (Carl Zeiss).

Cell culture and *GREM2* siRNA transfection

HK-2 cells, a human renal proximal tubular epithelial cell line, were cultured in RPMI-1640 medium (LM011-03; Welgene) supplemented with 10% fetal bovine serum (16000-044; Gibco) and 1% penicillin-streptomycin (15140-122; Gibco). HK-2 cells were seeded in 12-well plates or 60 mm dish and transfected with 50 μM of predesigned human *GREM2* siRNA (64338-1 and 64388-2; Bioneer) or 50 μM of negative control siRNA (SN-1003; Bioneer) using Lipofectamine 3000 reagent (L3000-015; Invitrogen) according to the manufacturer's protocol. Twenty-four hours after transfection, cells were treated with high glucose (25 mM) and cholesterol (50 μM) for 24 or 48 hours.

Western blot analysis

HK-2 cells were lysed in cell lysis buffer (9803; Cell Signaling Technology) supplemented with protease inhibitor cocktail (P8340; Sigma-Aldrich) and phenylmethanesulfonyl fluoride (P7626; Sigma-Aldrich). Lysates were incubated for 30 min at 4℃ and centrifuged at 14,000 *g* for 10 minutes at 4℃ and supernatants were quantified using the DC protein assay (500-0116; Bio-Rad Laboratories). Lysates (20 μg) were resolved by 4%～12% Bolt Bis-Tris plus mini protein gels (NW04125BOX; Invitrogen) and

transferred to nitrocellulose membranes (10600004; Cytiva). SMAD3 (9523) and phosphorylated SMAD3 (p-SMAD3, 9520) antibodies were obtained from Cell Signaling Technology and ACTB (A5441) antibody was purchased from Sigma-Aldrich.

Real-time polymerase chain reaction analysis

Total RNA was isolated using TRIzol (15596-018; Invitrogen) and reverse-transcribed into cDNA with the RevertAid First Strand cDNA Synthesis Kit (K1621; Thermo Fisher Scientific). Real-time polymerase chain reaction (PCR) was performed on a QuantStudio 5 using SYBR Green qPCR premix (RT501UM; Enzynomics). Gene expression levels were normalized to 18S rRNA, GAPDH, or TBP. Primer sequences are listed in Table 1.

Transcriptome analysis

Total RNA was isolated from kidney tissue using TRIzol and purified with the RNeasy Mini Kit (74104; Qiagen). RNA quality and integrity were assessed using the Agilent 2100 Bioanalyzer. High-quality RNA was used to construct sequencing libraries with the NEBNext Ultra II RNA Library Prep Kit (New England Biolabs), which were then sequenced on an Illumina NovaSeq 6000 platform. Sequencing reads were aligned to the reference genome using STAR, followed by differential gene expression analysis with DESeq2 (eBiogen).

Statistical analysis

Data are presented as the mean±SEM. Sample sizes were determined empirically based on preliminary experiments to ensure appropriate statistical power. Unpaired two-tailed Student's t-tests were used to compare the two groups. One-way ANOVA was used to compare variables among multiple groups. For comparisons involving multiple measurements at different time points, two-way ANOVA was used. Statistical analyses and graphing were performed using GraphPad Prism 10 (GraphPad Software).

Results

SMUP-Cell administration reduces albuminuria without dose dependency

The Lepr *db/db* (*db/db*) mouse model is frequently utilized in the study of type 2 DM. This model exhibits hyperphagia, obesity, and hyperinsulinemia due to a deficiency in leptin. Additionally, the progression of renal changes in *db/db* mice closely mirrors those observed in human DKD er time (11). Therefore, we used *db/db* mice as an animal model for type 2 DKD. To evaluate the effect of SMUP-Cells on the progression of DKD, we injected the mice with SMUP-Cells after the establishment of albuminuria at 12 weeks of age (12).

We examined the dose-dependent effects of SMUP-Cell administration over a three-month period. The mice were sacrificed three months after SMUP-Cell injection (Fig. 1A), and no residual SMUP-Cells were detectable within the kidney (Fig. 1B).

DKD is primarily characterized by persistent albuminuria and a progressive decline in renal function (13). At 12 weeks of age, *db/db* mice exhibited a significantly elevated UACR compared to control mice, with no noticeable effect from SMUP-Cell administration. However, starting from 16 weeks, SMUP-Cell treatment led to a reduction in UACR, which persisted up to 20 weeks (Supplementary Fig. S1). At 24 weeks of age, it significantly lowered UACR in a dose-dependent manner at low and mid doses (Fig. 1C). However, high-dose SMUP-Cell injections had no effect on UACR levels (Fig. 1C). However, SMUP-Cell administration did not reduce body weight, kidney weight, or plasma glucose levels (Fig. 1D-1F).

Table 1. Primer lists

Gene name	Forward primer	Reverse primer
Grem2	5′-GGTAGCTGAAACACGGAAGAA-3′	5′-TCTTGCACCAGTCACTCTTGA-3′
Tbp	5′-CCTTCACCAATGACTCCTATGAC-3′	5′-CAAGTTTACAGCCAAGATTCAC-3′
18S rRNA	5′-GTAACCCGTTGAACCCCATT-3′	5′-CCATCCAATCGGTAGTAGC-3′
ACTA2	5′-GTGTTGCCCCTGAAGAGCAT-3′	5′-GCTGGGACATTGAAAGTCTCA-3′
CDKN1A	5′-TGTCCGTCAGAACC ATGC-3′	5′-AAAGTCGAAGTTCCATCGCTC-3′
CDKN2A	5′-ATGGAGCCTTCGGCTGACT-3′	5′-GTAACTATTCGGTGCGTTGGG-3′
FN1	5′-CGGTGGCTGTCAGTCAAAG-3′	5′-AAACCTCGGCTTCCTCCATAA-3′
TGFB1	5′-CAATTCCTGGCGATACCTCAG-3′	5′-GCACAACTCCGGTGACATCAA-3′
TP53	5′-GAGGTTGGCTCTGACTGTACC-3′	5′-TCCGTCCCAGTAGATTACCAC-3′
TBP	5′-GAGCCAAGAGTGAAGAACAGTC-3′	5′-GCTCCCCACCATATTCTGAATCT-3′
GAPDH	5′-GGAGCGAGATCCCTCCAAAAT-3′	5′-GGCTGTTGTCATACTTCTCATGG-3′

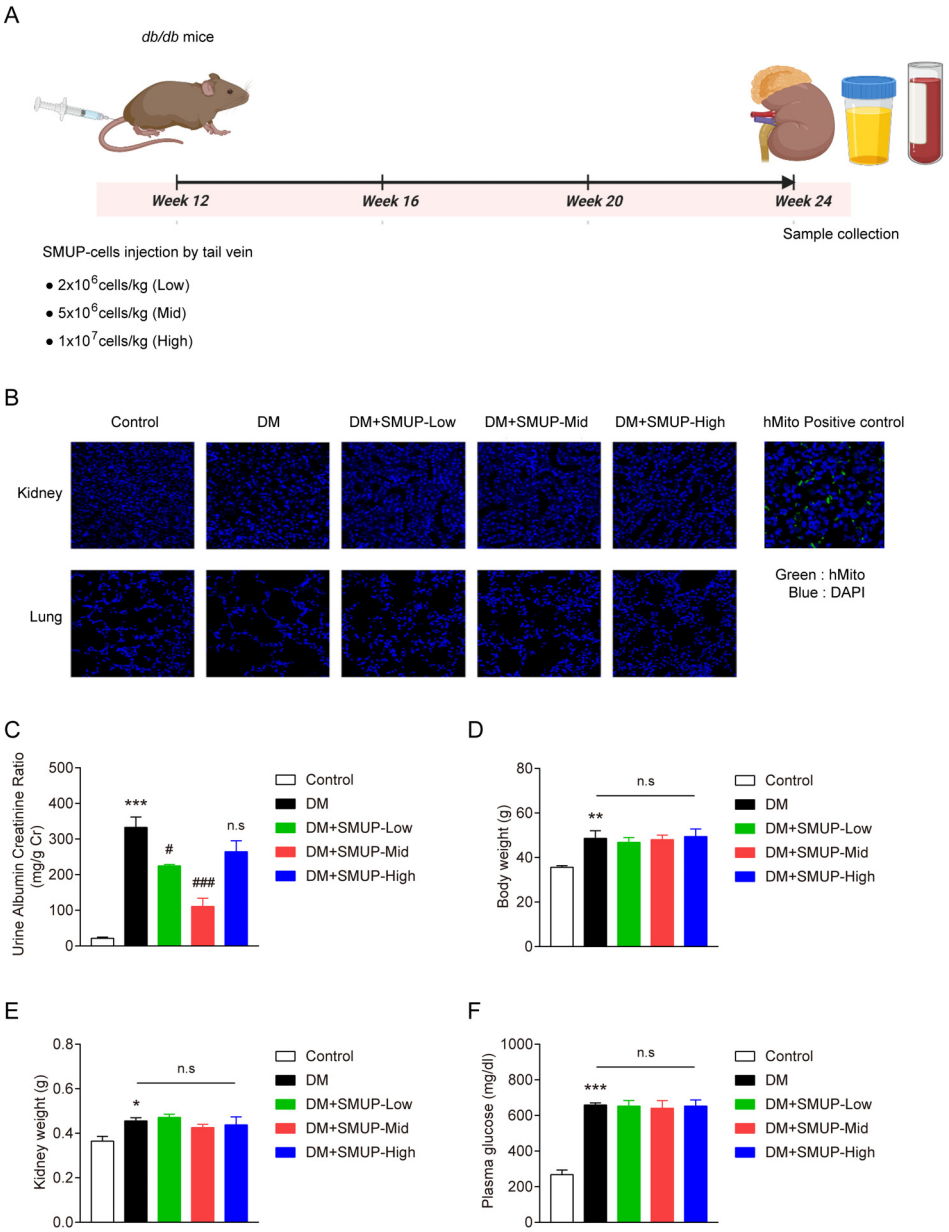

Fig. 1. Intravenous administration of Small cell+Ultra Potent+Scale UP cells (SMUP-Cells) reduces proteinuria in *db/db* mice over a 12-week period. At 12 weeks of age, *db/db* mice in both the vehicle and treatment groups received intravenous injections through the tail vein. The treatment groups were administered SMUP-cells at doses of 2×10^6 cells/kg (low), 5×10^6 cells/kg (mid), and 1×10^7 cells/kg (high), respectively. (A) Experimental protocol for SMUP-Cell therapy in diabetic mice. (B) Detection of SMUP-Cells in the kidney. Kidney tissue samples from mice were obtained and examined using confocal microscopy. SMUP-Cells were stained for human mitochondria (hMito). The staining for hMito (green) was merged with DAPI (blue). Original magnification, ×400. Each image is representative of three mice per group. (C) 24-hour urine albumin-to-creatinine ratio. (D) Body weight. (E) Kidney weight. (F) Plasma glucose under non-fasting conditions. Control: control nondiabetic C57BL/6J mice, DM: *db/db* mice, DM+SMUP: *db/db* mice with SMUP-Cells treatment. Data show the mean±SEM of 5~6 mice. *p<0.05, **p<0.01, and ***p<0.001 vs. Control; $^{\#}$p<0.05 and $^{\#\#\#}$p<0.001 vs. DM; n.s: non-significant.

SMUP-Cell administration reduces glomerular hypertrophy and fibrosis in *db/db* mice

Histological examination using H&E and PAS staining revealed that the control group exhibited normal-sized glomeruli and Bowman's capsule with appropriate spacing. The cellular density of mesangial cells within the glomeruli was maintained within normal limits, and there was no observed thickening of the basement membrane or ex-

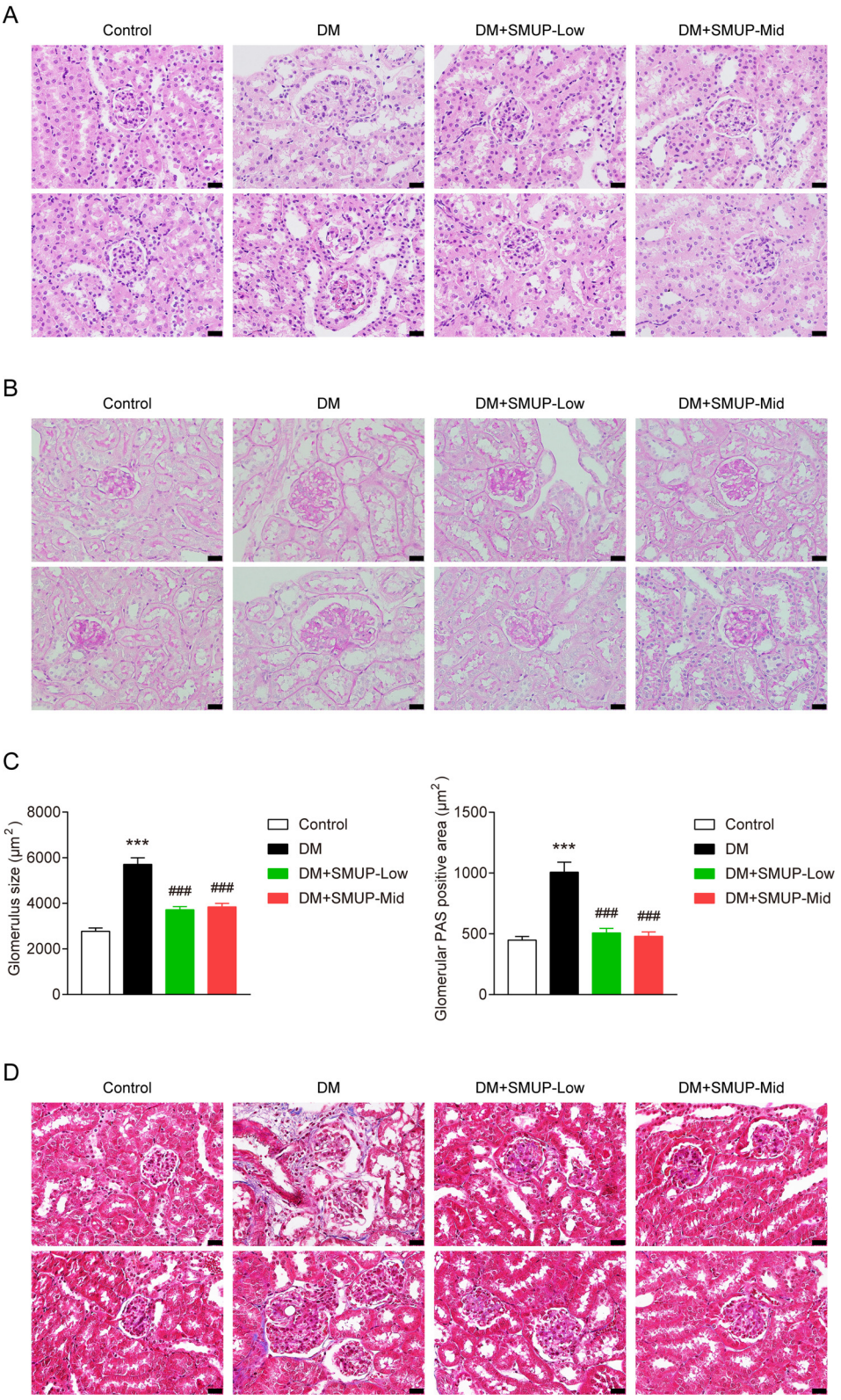

Fig. 2. Effects of Small cell+Ultra Potent+Scale UP cell (SMUP-Cell) treatment on renal fibrosis in the development of diabetic kidney disease. Representative staining of H&E (A) and periodic acid-Schiff (PAS) (B). (C) Quantitative analysis of glomerular area and PAS-positive areas in the kidneys. (D) Masson's trichrome staining of kidney sections. Scale bar=20 μm. Control: control non-diabetic C57BL/6J mice, DM: *db/db* mice, DM+SMUP: *db/db* mice with SMUP-Cells treatment. Data show the mean±SEM of 5 mice. ***p<0.001 vs. Control; $^{###}$p<0.001 vs. DM.

pansion/accumulation of the ECM, including the mesangial matrix. Additionally, there was no evidence of connective tissue proliferation or fibrosis (Fig. 2A, 2B).

In the diabetic control group (*db/db* mice), diffuse glomerular thickening was observed, leading to a reduction in the space of Bowman's capsule. Increased cellularity

within the glomeruli due to mesangial cell hyperplasia was noted, along with expansion of the ECM, including the mesangial matrix, corresponding with the characteristic pathological phenomena of DKD (14). In the SMUP-Cell-treated groups, both low and mid-doses showed an increase in glomerular and Bowman's capsule size compared to the control group. Cellular density increased due to mild mesangial cell proliferation, but the extent was significantly lower compared to the diabetic control group (Fig. 2A, 2B). Image analysis of the proportion of the mesangial matrix within the glomeruli indicated a significant reduction in the SMUP-Cell-treated groups compared to the diabetic control group (Fig. 2C). Additionally, MT staining, which highlights fibrotic areas in blue, focused on the fibrotic changes within the glomeruli. Compared to the control group, the diabetic control group showed prominent glomerular sclerosis, while SMUP-Cell treatment improved glomerular sclerosis relative to the diabetic control group (Fig. 2D).

SMUP-Cell administration attenuates podocyte injury and inhibits epithelial-mesenchymal transition

Podocytes are specialized epithelial cells that cover the outer surface of the glomerular basement membrane (12). The density of glomerular podocytes is a key predictor of albuminuria and the progression of DKD; consequently, damage to podocytes is a driving factor in the advancement of DKD (12). Synaptopodin, a component of the actin cytoskeleton, is crucial for maintaining podocyte function. Therefore, a decrease in synaptopodin expression reflects the reduction in the number of podocytes observed in DM (15). Compared to the control group, the diabetic control group showed a reduction in synaptopodin expression, while the group treated with SMUP-Cells demonstrated a recovery in synaptopodin expression (Fig. 3A).

Epithelial-mesenchymal transition (EMT) is a process in which epithelial cells transform into matrix-producing fibroblasts and myofibroblasts; this process plays a key role in tissue fibrosis following injury (16) and is a significant mechanism of podocyte injury in DKD (17). We evaluated the expression of nephrin, an epithelial cell marker, and desmin, a mesenchymal cell marker, as indicators of podocyte EMT (16). The diabetic control group showed a significant decrease in nephrin expression compared to the normal control group. However, in the SMUP-Cell-treated group, nephrin expression was notably restored (Fig. 3B). Additionally, immunostaining for desmin demonstrated that SMUP-Cell administration significantly reduced its expression compared to the diabetic control group (Fig. 3C). These data suggested that the administration of SMUP cells ameliorated podocyte injury and EMT, which are key pathological features of DKD.

EMT is inhibited upon SMUP-Cell administration

Another pathological feature of DKD is inflammation within the tubulointerstitium and subsequent accumulation of ECM, often involving EMT in renal tubular cells. The continuous progression of these changes ultimately leads to glomerulosclerosis and tubular atrophy (18). As expected (19), the expression of α-smooth muscle actin (α-SMA) and fibronectin was increased in the kidneys of diabetic mice. However, immunostaining for α-SMA and fibronectin revealed a significant reduction in their expression in the SMUP-Cell-treated group compared to the diabetic control group (Fig. 4A, 4B). Studies have reported that urinary tubular markers such as kidney injury molecule-1 (KIM-1) are independently associated with early albuminuria in nephropathy (20). In our study, we observed that the expression of KIM-1, which was prominently stained in the tubules of the diabetic control group, was reduced in the SMUP-Cell treatment group (Fig. 4C).

SMUP-Cells decrease *Grem2* expression in the kidney

To gain further insights into the protective mechanisms of SMUP-Cells on DKD, we assessed gene expression changes in the kidneys. Gene expression was measured using RNA sequencing, and pathways of interest were identified through differential gene expression analyses. Among them, we noted that *Grem2* was significantly upregulated in diabetic mice and downregulated following SMUP-Cell treatment (Fig. 5A-5C). *Grem2* belongs to the DAN family of bone morphogenetic protein antagonists (21). While *Grem2* expression is rarely detected in normal kidneys, it has been reported to be high in the kidneys of diabetic *ob/ob* mice (21). Additionally, overexpression of *Grem2* in podocytes has been shown to increase apoptosis by activating Smad2/3 and suppressing Smad1/5/8 (21). Real-time PCR analysis confirmed that *Grem2* levels in the kidneys were increased in diabetic mice and decreased by SMUP-Cell treatment (Fig. 5D). Consistently, immunostaining showed that GREM2 expression was increased in the kidneys of diabetic mice, with the highest expression observed in renal tubular epithelial cells (RTECs) rather than in the glomeruli. SMUP-Cell treatment decreased *Grem2* expression (Fig. 5E).

Grem2 induces EMT and cellular senescence in RTECs under high glucose and cholesterol conditions

The progression of DKD is significantly driven by EMT in renal tubular cells (22). In cultured RTECs, trans-

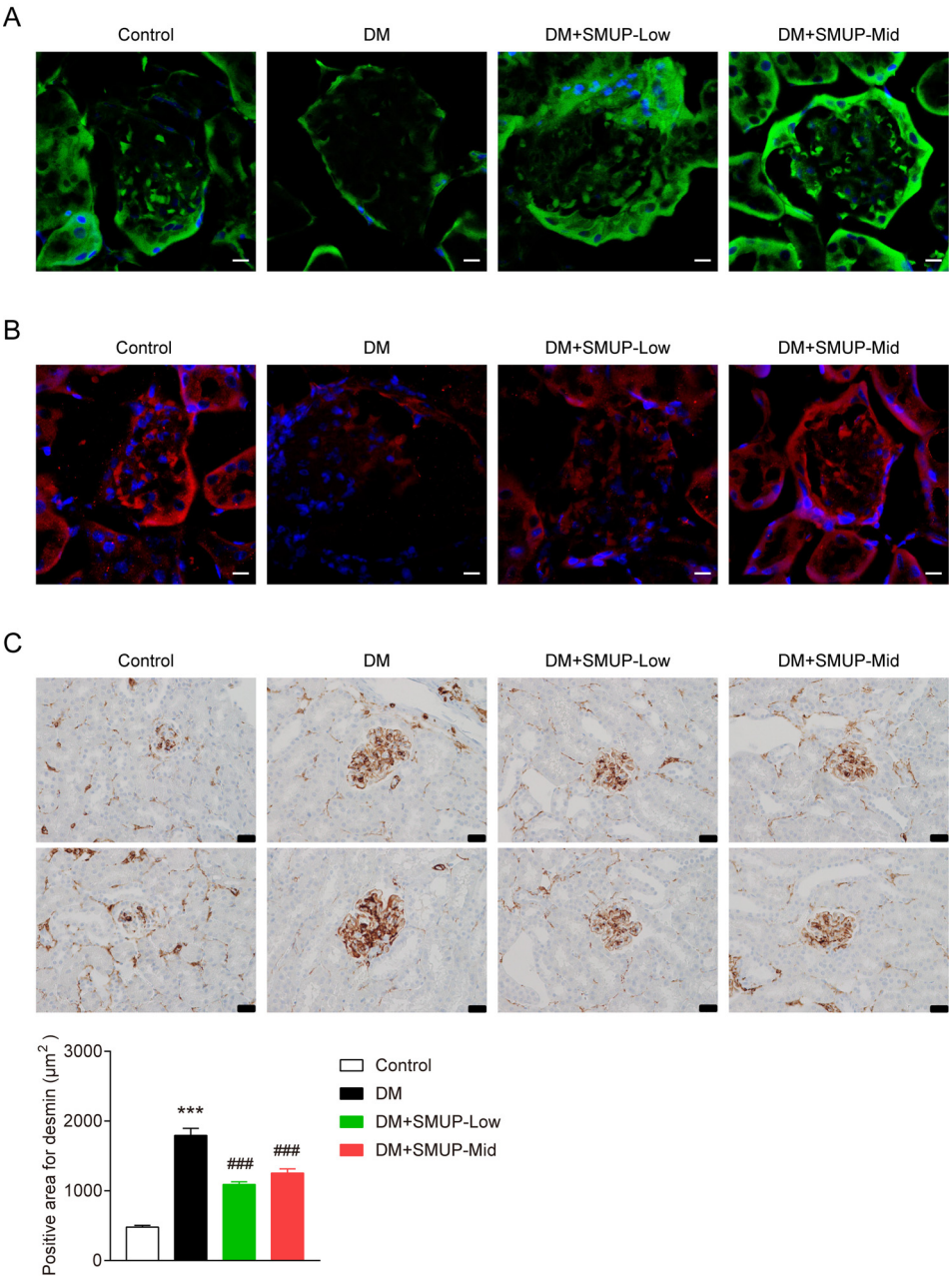

Fig. 3. Protective effects of Small cell+Ultra Potent+Scale UP cells (SMUP-Cells) on podocyte injury and epithelial-mesenchymal transition. Representative immunofluorescence image of synaptopodin (A) and nephrin (B). Scale bar=10 μm (n=4). (C) Immunohistochemical detection and semiquantitative analysis of desmin in the kidneys (n=5). Scale bar=20 μm. Control: control nondiabetic C57BL/6J mice, DM: *db/db* mice, DM+SMUP: *db/db* mice with SMUP-Cells treatment. Data are expressed as the mean±SEM. ***p< 0.001 vs. Control, $^{###}$p<0.001 vs. DM.

forming growth factor-β1 (TGF-β1) stimulates the production of Gremlin, which is linked to EMT phenotypic changes, suggesting that Gremlin may act as a downstream mediator of TGF-β1 (23). In RTECs, cellular senescence triggers the secretion of senescence-associated secretory phenotype, including pro-inflammatory and pro-fibrotic factors, which further promote EMT. This process leads to the proliferation and differentiation of renal interstitial fibroblasts into myofibroblasts, resulting in excessive ECM deposition, fibrosis, and ultimately contributing to the progression of renal failure (24).

Therefore, we investigated the relationship between *Grem2*, cellular senescence, and EMT in RTECs. We mimicked a diabetic milieu *in vitro* and examined the effects of potential pathogenic factors on cultured RTECs. Exposure to high levels of glucose and cholesterol consistently increased *GREM2* mRNA expression and triggered changes associated with EMT and cellular senescence (Fig. 6A, 6B). To evaluate the role of *Grem2* in EMT and senescence, we transfected the cells with *GREM2* siRNA and observed that the knockdown of *GREM2* reversed these changes (Fig. 6C-6E). Previous report showd that Gremlin

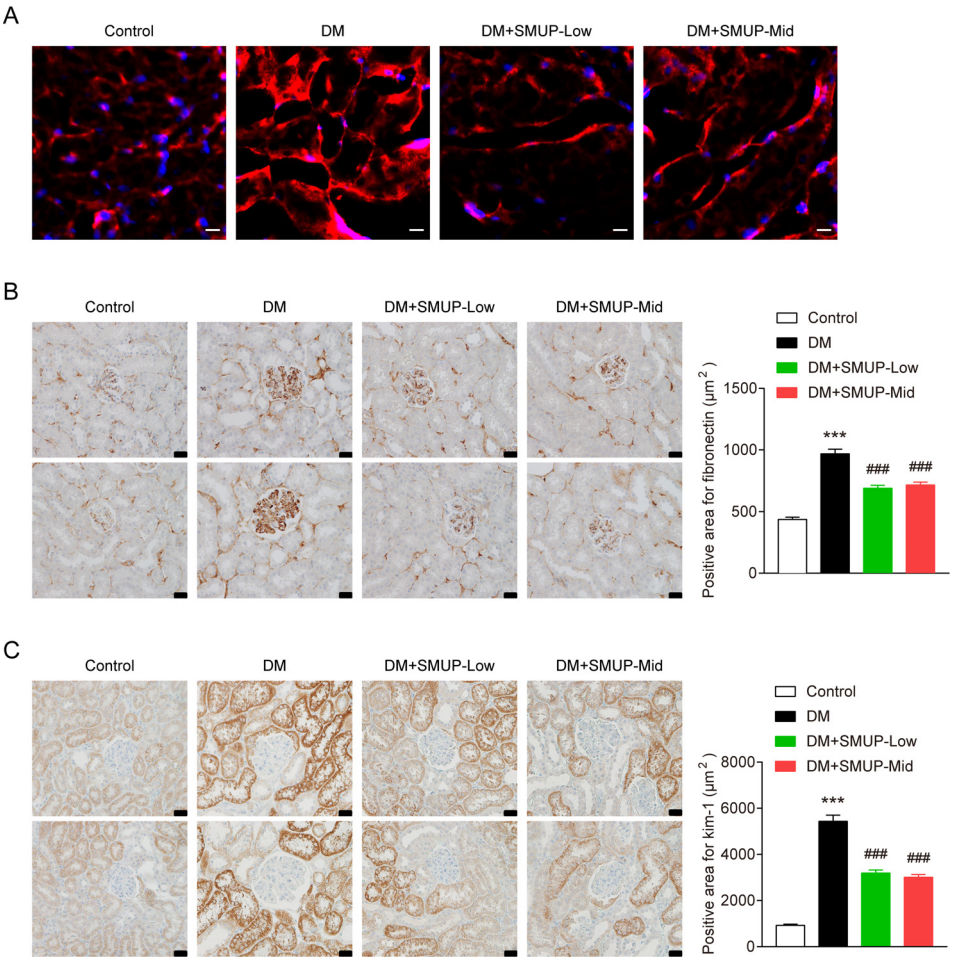

Fig. 4. Small cell+Ultra Potent+Scale UP cell (SMUP-Cell) treatment attenuates fibrosis and tubular injury in diabetic kidney tissue. (A) Representative immunofluorescence image of α-smooth muscle actin (n=4). Scale bar=10 μm. (B) Immunohistochemical detection and semiquantitative analysis of fibronectin in the kidneys (n=5). (C) Representative immunohistochemical staining and semiquantitative analysis of kidney injury molecule-1 in the kidneys (n=5). Scale bar=20 μm. Control: control nondiabetic C57BL/6J mice, DM: *db/db* mice, DM+SMUP: *db/db* mice with SMUP-Cells treatment. Data are expressed as the mean±SEM. ***p<0.001 vs. Control, ###p<0.001 vs. DM.

is implicated in renal fibrosis by inducing EMT in RTECs through direct activation of the Smad pathway (25). Based on this, we performed Western blot analyses to assess Smad3 expression in RTECs following treatment with cholesterol and glucose, with or without *Grem2* siRNA transfection. Our results demonstrated a significant increase in p-SMAD3 levels after cholesterol and glucose treatment, suggesting activation of the Smad pathway. In contrast, *Grem2* siRNA transfection led to a marked reduction in p-SMAD3 expression, indicating suppression of the pathway (Supplementary Fig. S2). These findings support the role of Gremlin in activating the Smad pathway and inducing EMT, contributing to renal fibrosis. Overall, these hese data suggest that *Grem2* is a crucial mediator of EMT and cellular senescence in RTECs.

Discussion

Stem cell therapy has been shown to prevent the onset of DKD in experimental animal models (5). Notably, in animal models of chronic DKD, systemic administration of MSCs has been reported to suppress the progression of DKD (6). In numerous preclinical studies, systemic infusion of MSCs has been demonstrated to reduce the expression of major renal inflammatory mediators such as TNF-α, IL-6, and IL-1β (26, 27). Furthermore, exosomes derived from MSCs offer a promising strategy to ameliorate DKD by reducing mesangial hyperplasia and renal fibrosis, as well as enhancing kidney function (28).

However, previous studies were mostly conducted using type 1 DM models, involving intravenous administration of single doses or repeated dosing at 1- or 2-week intervals, with observations of proteinuria and renal pathology from 2 to 12 weeks post-administration (29-31). Previously, we investigated the therapeutic effects of SMUP-Cells in a type 1 DM animal model induced by streptozotocin injection and unilateral nephrectomy. SMUP-Cells were administered intravenously three times at 4-week intervals, resulting in significant improvement of DKD. The underlying mechanism was identified as the reversal of mi-

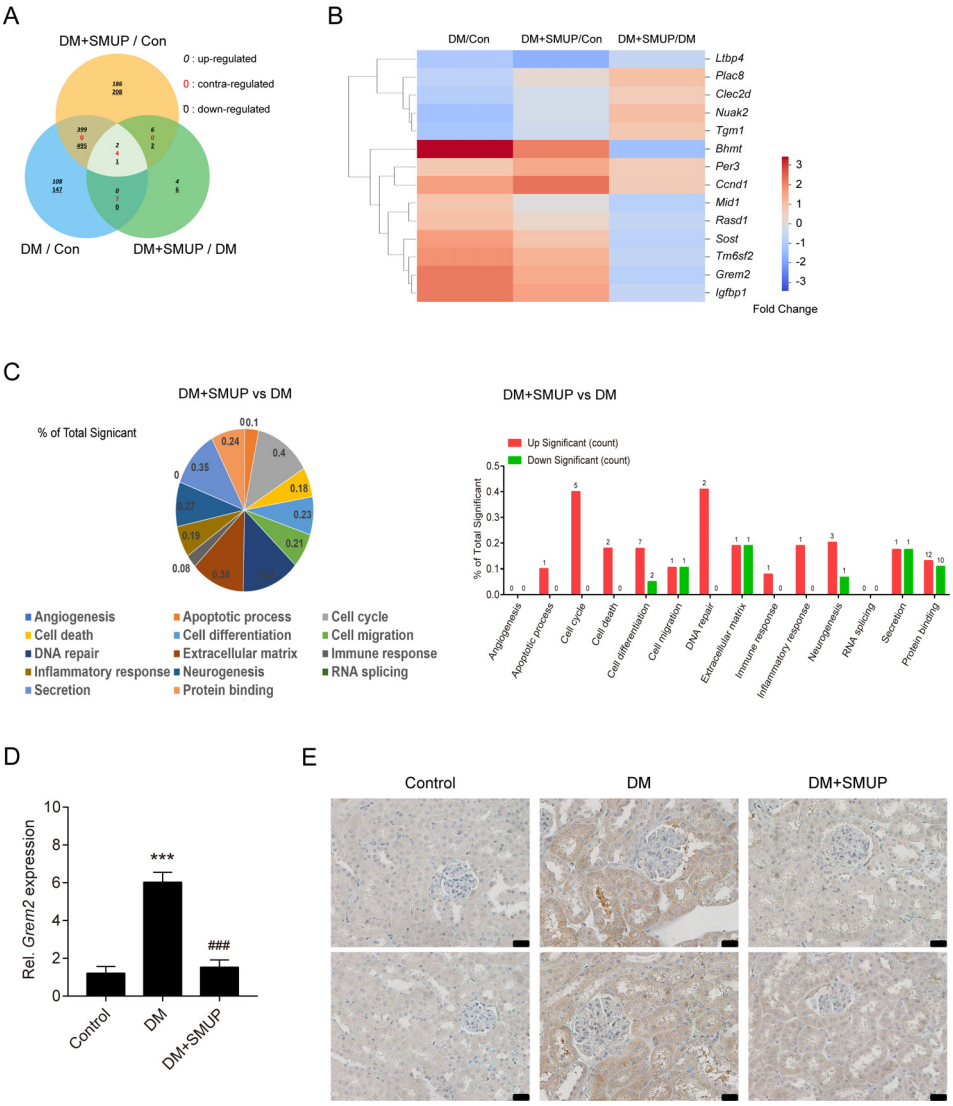

Fig. 5. *Grem2* is a key regulator in the effects of Small cell+Ultra Potent+ Scale UP cell (SMUP-Cell) treatment on diabetic kidney disease. (A) Venn diagram showing differentially expressed genes (DEGs) in the bulk RNA-sequencing (n=4). (B) Heatmap showing 14 intersected DEGs. (C) Distribution of the RNA-sequencing profiling annotated to the functional categories in the DM+SMUP (5×10^6 cells/kg) vs. DM. (D) *Grem2* mRNA expression in the kidney (n=5). (E) Representative immunohistochemical images of GREM2 in the kidneys (n=5). Scale bar=20 μm. Control: control nondiabetic C57BL/6J mice, DM: *db/db* mice, DM+SMUP: *db/db* mice with SMUP-Cells treatment. Data are expressed as the mean±SEM. ***p<0.001 vs. Control, ###p<0.001 vs. DM.

tochondrial dysfunction in RTECs, mediated through the induction of arginase 1 in macrophages (8). These observations suggest that SMUP-Cells exert their beneficial effects by targeting and correcting mitochondrial dysfunction in RTECs via macrophage modulation.

In contrast, research on type 2 DKD has been less extensive. For instance, stem cells from human exfoliated deciduous teeth inhibited advanced glycation end product-induced EMT in type 2 DM following a single intravenous dose, with observations made over an 8-week period (32). In the present study, we observed that a single administration of SMUP-Cells in a type 2 DKD animal model led to a reduction of EMT in podocytes and RTECs, as well as suppression of renal fibrosis.

In a type 2 DKD model using *db/db* mice, SMUP-Cells were administered via tail vein injection, and proteinuria was monitored for 3 months. Although SMUP-Cells did not persist in the renal tissue, administration at doses of 1×10^6 and 5×10^6 cells/kg significantly reduced proteinuria. However, high-dose SMUP-Cell administration (1×10^7 cells/kg) did not show any effect on proteinuria. Notably, the dose-dependent effects of stem cell administration do not always follow a linear or predictable pattern. Several studies have indicated that the therapeutic efficacy of stem cells does not necessarily increase with higher doses, challenging the assumption that greater cell numbers will yield better outcomes. MSC function and paracrine signaling may reach a plateau or diminish at higher concentrations (33). Stem cell efficacy can be limited by the saturation of cellular receptors or target sites, whereby additional cells fail to contribute further therapeutic benefit (33). Moreover, immune modulation

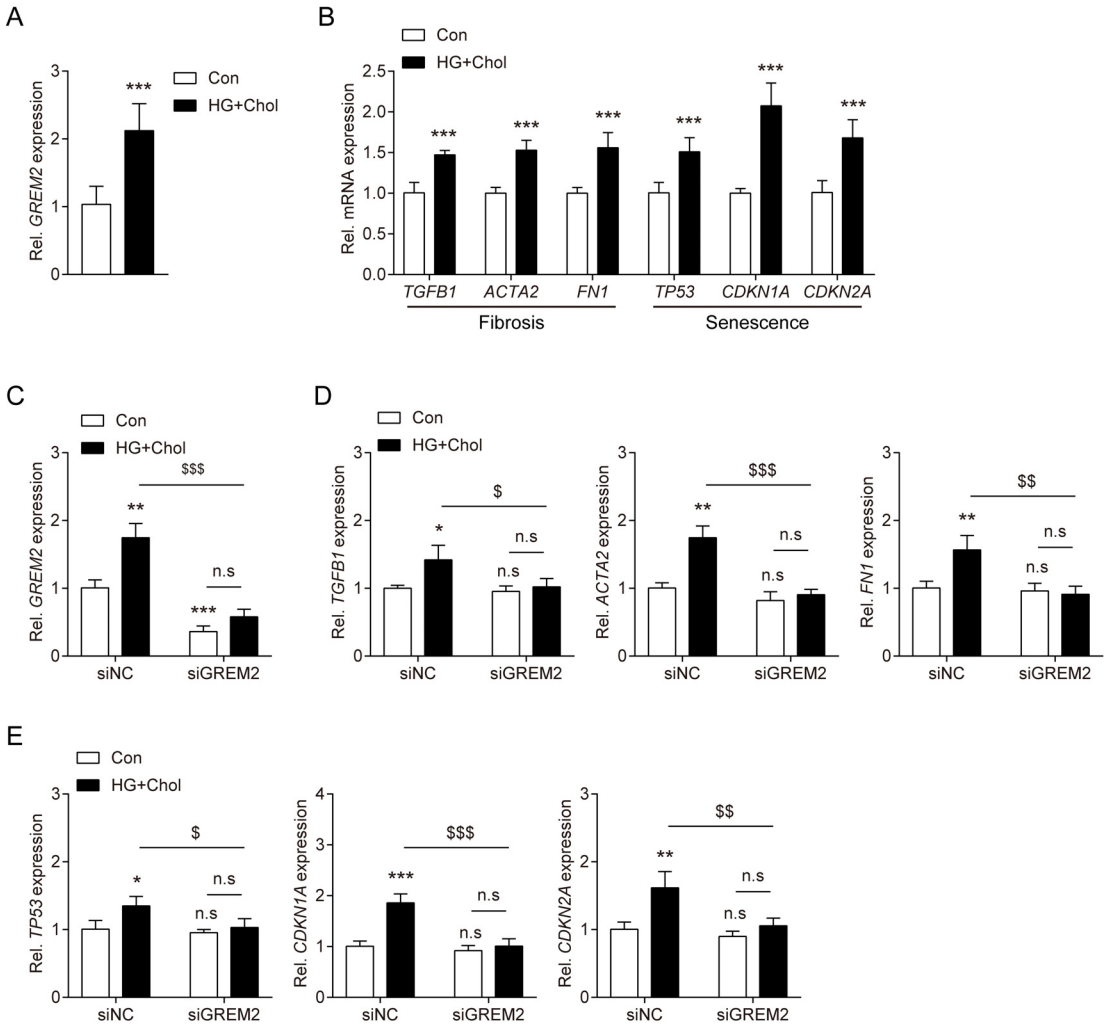

Fig. 6. *Grem2* is involved in the fibrosis and senescence in the renal tubular epithelial cells. (A) *GREM2* expression in HK2 cells exposed to high glucose (25 mM) and cholesterol (50 μM) treatment (HG+Chol) for 48 hours. (B) mRNA expressions of fibrosis and senescence markers under high glucose and cholesterol treatment (n=3). (C) *GREM2* mRNA expression following transfection with *GREM2* siRNA or control siRNA. (D, E) mRNA expression changes related to fibrosis and senescence under *GREM2* siRNA or control siRNA treatment. Data show the mean±SEM (n=3). *p<0.05, **p<0.01, and ***p<0.001 vs. Con; $^{\$}$p<0.05, $^{\$\$}$p<0.01, and $^{\$\$\$}$p<0.001 vs. HG+Chol; n.s: non-significant.

plays a significant role in the observed non-linear dose-response relationships. High doses of stem cells can elicit unintended immune responses or promote inflammation, which can counteract their therapeutic effects (34). Overall, the absence of a dose-dependent effect in stem cell therapy is not unexpected and reflects the intricate nature of cellular therapies.

In DKD, there is an increase in glomerular size and mesangial cell number, along with a reduction in podocyte numbers and foot process effacement. Another pathological feature of DKD is inflammation in the tubulointerstitial area and consequent ECM accumulation, driven by EMT in RTECs. The progression of these changes ultimately leads to glomerulosclerosis and tubular atrophy (35). Upon examining renal pathology and immunostaining, we observed that in DKD, the mesenchymal marker desmin in podocytes was increased, while the epithelial marker nephrin was decreased. However, SMUP-Cell treatment significantly mitigated the increase in desmin expression and restored nephrin levels. Proteinuria is closely associated with tubular injury, and the expression of KIM-1 was significantly elevated in the diabetic control group but notably reduced in the SMUP-Cell-treated group. As nephropathy progresses, interstitial myofibroblast activation occurs, with increased expression of α-SMA and fibronectin, which was reduced following SMUP-Cell admi-

nistration. These data suggest that the administration of MSCs effectively inhibits renal EMT, thereby curtailing the progression of renal fibrosis. This intervention plays a pivotal role in preserving kidney function by preventing the associated decline.

Gremlin 1 and Gremlin 2 belong to the family of bone morphogenetic protein antagonists (36). Recent research indicates that Gremlin plays a significant role in promoting fibrosis across various diseases, such as idiopathic pulmonary fibrosis and liver fibrosis (36, 37). Furthermore, experimental models in mice demonstrate that inhibiting Gremlin 1 reduces renal fibrosis, as evidenced in studies using streptozotocin-treated diabetic knockout mice with a heterozygous deletion of the *Grem1* gene (38). Our transcriptome analysis revealed that the effect of SMUP-Cell is mediated through the inhibition of *Grem2* expression. The *Grem2* expression has been reported to be high in the brain and low in the kidneys and lungs (39). Consistent with this, we found that *Grem2* expression was barely detected in the kidneys of control mice. However, we observed that *Grem2* expression is more pronounced in the renal tubules compared to the glomeruli. In environments mimicking diabetic conditions through glucose and cholesterol treatment, we noted an increase in *Grem2* expression. Suppression of *Grem2* led to a reduction in markers of fibrosis and senescence. This finding underscores the effectiveness of SMUP-Cells' inhibition of *Grem2*, which can mitigate EMT and cellular senescence by targeting its expression in RTECs.

Clinical studies have identified that RTECs play a pivotal role in senescence within the kidney, evident in early-stage CKD and in patients with proteinuria despite normal glomerular filtration. These cells accelerate CKD progression by secreting pro-inflammatory and pro-fibrotic factors, which promote EMT and fibroblast activity, leading to fibrosis and eventual renal failure (24). Furthermore, the senescence of these cells impairs the kidney's repair mechanisms, exacerbating the degeneration and dysfunction of renal structures.

Our data have revealed that *Grem2* plays a crucial role in cellular senescence in RTECs. Consequently, *Grem2* emerges as a vital therapeutic target in MSC treatments, not only for DM but also for other CKDs. The inhibition of *Grem2* expression upon MSC administration could potentially be explored as a therapeutic approach in these conditions.

One of the limitations of our study is that we did not elucidate the intermediary mediators that contribute specifically to the suppression of *Grem2* expression within the renal environment through SMUP-Cell treatment. Although *Grem2* expression is typically minimal in the kidneys, it has been reported to increase in human plasma under diabetic conditions and to rise further in cases of DKD (40). Future research should explore various secretory factors and exosomes to determine how MSC administration can sustainably inhibit *Grem2* expression in the kidneys.

In summary, intravenous administration of MSCs has been shown to suppress *Grem2* expression in the kidneys, thereby inhibiting EMT and senescence in RTECs. This suppression of renal EMT suggests potential therapeutic applications for MSCs in treating DKD. Given the recent surge in type 2 DM prevalence and the corresponding increase in DKD cases leading to dialysis and kidney transplantation, MSC therapy could represent a promising new treatment alternative.

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Potential Conflict of Interest

While the SMUP-Cells were provided by Medipost, this study was entirely funded through government research grants. Importantly, Medipost and the author affiliated with Medipost (HJJ) were not involved in the analysis or interpretation of the data. The author contribution paragraph states that HJJ was involved in funding acquisition and resources. Therefore, we believe that there are no conflicts of interest to declare regarding this study.

Authors' Contribution

Conceptualization: MSK, EHK. Data curation: MSK, JYY, SK, MOK, SG. Formal analysis: MSK, JYY, SK, MOK, SG. Funding acquisition: HJJ, EHK. Investigation: SG, EHK. Methodology: MSK, JYY. Project admin-

istration: EHK. Resources: HJJ, EHK. Software: MSK, JYY. Supervision: EHK. Validation: MSK, EHK. Visualization: MSK, JYY. Writing – original draft: MSK, JYY. Writing – review and editing: EHK.

Supplementary Materials

Supplementary data including two figures can be found with this article online at https://doi.org/10.15283/ijsc24113

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