Supplementary information

Human umbilical cord mesenchymal stem cells attenuate diabetic nephropathy through the IGF1R-CHK2-p53 signalling axis in male rats with type 2 diabetes mellitus

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Materials and methods

Cell culture, plasmids, and antibodies

HEK293T cells were maintained in DMEM (Gibco, cat. #11965118, USA). The cell line was obtained from ATCC (American Type Culture Collection, ATCC). The anti-IGF1R antibody was obtained from ABclonal (A0243). The anti-IGF1 antibody was obtained from Cell Signaling Technology (73034S). The anti-P53 antibody was obtained from ABclonal (A3185). The anti-phospho-p53 antibody for western blotting (WB) was obtained from Cell Signaling Technology (9287S). The anti-phospho-p53 antibody for IHC was obtained from ABclonal (AP0984). The anti-CHK2 antibody was obtained from Cell Signaling Technology (2662S). The anti-phospho-CHK2 antibody was obtained from ABclonal (AP0590).

HUcMSC preparation

Human umbilical cord mesenchymal stem cells (HUcMSCs) of clinical grade were provided by the Good Manufacturing Practice (GMP) laboratory of Shanghai East Hospital. The use of HUcMSCs was approved by the Hospital's Medical Ethics Council. The cell product was certified by the National Institutes for Food and Drug Control (Report number: SH201903852). HUcMSCs were isolated from fresh healthy umbilical cords and washed with normal saline. The blood vessels were removed when the tissue was peeled from the Fahrenheit glue, cut to 1–2 mm^3 in size, and then cultured in a serum-free stem cell medium (Life Technologies) at 37 °C in an incubator including 5% CO_2. The cells were digested with Trypl-Express when they grew to nearly 80% confluence. The cells were induced to differentiate into adipocytes, osteoblasts, and chondrocytes. The National Institutes for Food and Drug Control has certified this cell product. Fifth-passage cells were used in the subsequent experiments.

Animal experiments of male Sprague-Dawley rats

Male Sprague-Dawley rats (four weeks old and weighing approximately 100 g) were selected for the experiments and were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). The rats were placed in an animal facility with precisely controlled temperature ((23±1) °C), humidity (45%–65%), and a 12-h light/dark cycle with free access to chow and water. For the streptozotocin (STZ)-induced type 2 diabetes mellitus (T2DM) model, the rats were fed a high-fat diet (HFD; 40% fat, 40% carbohydrate, and 20% protein; Shanghai Pluteng Biological Technology Co., Ltd., Shanghai, China) for four weeks, followed by intraperitoneal injection of 30 mg/kg STZ (Sigma Aldrich, Louis, MO, USA) dissolved in sodium citrate buffer (pH 4.5) after overnight (12 h) fasting. After three days, the rats with a random glucose level

of >16.7 mmol/L for three continuous days were identified as diabetic rats (Xie et al., 2016; Reed et al., 2000;). Control rats were fed a regular chow diet.

The rats were randomly assigned to three groups: the control group, the saline-treated T2DM group (referred to as the DM group), and the HUcMSC-treated group (referred to as the MSC group), with eight rats in each group. T2DM rats were infused with 1×10^7 human HUcMSCs suspended in 0.5 mL saline (for the MSC group) or with 0.5 mL saline alone (for the DM group) through the tail vein once per week for four consecutive weeks. We did not observe severe infection after HUcMSC infusion. Body weight and random blood glucose in each group were measured at the beginning of the experiment, weekly during the experiment, and at sacrifice to document the progression of diabetes.

Glomerular basement membrane thickness observation

The rats were anaesthetized with 40 mg/kg pentobarbital, and the abdominal cavities were surgically opened after sectional disinfection. The rats were sacrificed by cervical dislocation. Kidney specimens were subsequently collected. First, the kidney was cut longitudinally along the long axis, fixed with 10% neutral formalin, embedded in paraffin, and cut into several 5-μm-thick slices continuously. Then, the 1-mm-long sections were fixed with 2.5% (volume fraction) glutaraldehyde for 6 h at 4 °C. The samples were rinsed with 0.1 mol/L phosphate buffer and treated with a 1% OsO_4 solution (TED PELLA, Inc.; Microscope Products for Science and Industry) for 3 h, dehydrated with a graded series of ethanol (30%, 50%, 80%, and 90%) and acetone, and embedded in epoxy resin (TED PELLA, Inc.; Microscope Products for Science and Industry). The specimens were then dropped into EPON-812 (TED PELLA, Inc.; Microscope Products for Science and Industry) and the capsules were heated and polymerized in an oven. Semithin sections (1 μm) were stained with 2% uranyl acetate (Electron Microscope Sciences, Inc.) for 30 min and lead citrate (TED PELLA, Inc.; Microscope Products for Science and Industry) for 15 min at room temperature. Sections were analyzed and images were captured under a transmission electron microscope (Electron Optics Laboratory Co., Ltd., JEM-1400 PLUS, Japan). Glomerular basement membrane thickness was determined using the orthogonal intercept method.

Blood chemistry

Blood samples (5–10 mL) were collected from the abdominal aorta, maintained at room temperature for 15 min prior to centrifugation in an Eppendorf tube containing ethylenediamine tetraacetic acid (EDTA; 1 mg/mL), and were subsequently centrifuged for 20 min at 2000*g* at 4 °C. The supernatant was kept in an Eppendorf tube and stored at −70 °C for the next step. Random blood glucose measurements can verify the onset of diabetes. One drop of tail blood was analyzed weekly with a standard glucometer (Sinocare Inc., Changsha, China), beginning at 8 weeks of age until 16 weeks of age, to document the development of diabetes. Serum interleukin (IL)-6 (BioLengend, cat#437107, USA), IL-10 (Abcam, cat#ab100764, UK), insulin (Crystal Chem, cat#90060, China), tumor necrosis factor-α (TNF-α) (Abcam, cat#ab236712, UK), monocyte chemoattractant protein-1 (MCP-1) (Simuwubio, cat#SDR0059 96T, China), 8-hydroxydeoxyguanosine (8-OHdG) (Elabscience, cat#E-EL-0028c, China), and insulin-like growth factor 1 (IGF1) (R&D Systems, cat#SMG100, USA) levels were determined with specific enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocol.

Renal function analysis

Serum samples were sent to the Shanghai Model Organisms Centre, Inc., for biochemical tests. Scr (serum creatinine reagent kit, Rayto, cat# S03076, China), BUN (blood urea nitrogen reagent kit, Rayto, cat# S03036, China), and uric acid (uric acid reagent kit, Rayto, cat# S03035, China) were analyzed using an automatic biochemistry analyzer (Shenzhen Redu Life Science Co., Ltd., Chemray 800, China). The concentrations of serum cystatin C were determined using specific ELISA kits (Abcam, cat#ab201281, UK) according to the manufacturer's protocol.

H&E and immunohistochemical staining

Kidney tissues were fixed with 4% paraformaldehyde, embedded in HistoGel and paraffin, and sectioned for haematoxylin and eosin (H&E) and immunohistochemistry (IHC) staining with antibodies. The paraffin-embedded tissue sections were deparaffinized, rehydrated, and then incubated in 0.3% (volume fraction) H_2O_2 for 30 min at room temperature. Antigen retrieval was performed using a 10 mmol/L sodium citrate buffer (pH 6.0) for 30 min. Anti-IGF1, anti-IGF1R, anti-p53, anti-phospho-p53, anti-CHK2, and anti-phospho-CHK2 antibodies were added and incubated at 4 °C for 12 h, and then a poly-HRP anti-rabbit IgG detection system was used. The streptavidin-biotin-peroxidase method was performed, and detection was completed using a 2,4-diaminobutyric acid (DAB) substrate kit. The tissue sections were then counterstained with haematoxylin and were washed with running water for an appropriate length of time. The paraffin-embedded tissue sections were H&E-stained. The slides were examined, and representative images were captured with a Leica DMI6000 microscope. The images were analyzed using ImageJ. Mean optical density (MOD) was calculated by the ratio of integrated optical density (IOD) to positive area. Significant differences were determined using two-way analysis of variance (ANOVA).

TUNEL assay

A terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay kit was purchased from Roche (11684795910, CH). Paraffin kidney sections were permeabilized (0.1% sodium citrate and 0.1% Triton X-100) on ice for 2 min. Then, the sections were incubated with the TUNEL reaction mixture at 37 °C for 60 min in the dark. Representative images were captured with a Leica DMI6000 microscope, and images were analyzed using ImageJ.

Western blot analysis

Cells were lysed in buffer (0.5% Nonidet P-40 (NP-40), 2% 1 mol/L Tris HCl pH 8.0, 2% 5 mol/L NaCl, 0.2% 0.5 mol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 10 mmol/L sodium fluoride (NaF)) for 30 min and centrifuged at 12 000 r/min for 10 min. Cell lysates were boiled for 10 min with 5× sodium dodecyl sulfate (SDS)-loading buffer and were subjected to immunoprecipitation with antibodies to determine protein expression.

Statistical analysis

Data were analyzed using SPSS 26.0 for Windows (SPSS Inc., Chicago, Illinois). Data were expressed as mean±standard deviation (SD). Significant differences were determined using one-way ANOVA, followed by Tukey's post-hoc test. The data were graphed using GraphPad Prism 6.0 software (GraphPad Software, Inc., San Diego, CA, USA). Differences between mean values were considered significant at two-tailed $P<0.05$.

References

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