Supplemantary Materials

Mesenchymal stem cells-derived exosomes alleviate liver fibrosis by targeting Hedgehog/SMO signaling

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Methods and Materials

Mouse liver fibrosis model and treatment. The C57BL/6 mice (6–7 weeks old) purchased from Hubei Provincial Center for Disease Control and Prevention (Wuhan, China) were bred at a temperature of 30 ± 2 °C with a 12-h light/dark cycle. Animal welfare and experimental procedures were performed according to the guide for the Care and Use of Laboratory animals.

Mice were randomly divided into seven groups: normal control (NC) group, saline + UC-MSCs-Exo group, saline + UC-MSCs group, liver fibrosis + PBS group, liver fibrosis + UC-MSCs-Exo group, and liver fibrosis + UC-MSCs group (n=5/group). The fibrosis group mice were fasted for 12 h before modeling and were intraperitoneally injected with 150 mg/kg of TAA for 3 times/week. Other mice were treated with the same amount of normal saline. Four weeks after liver fibrosis induction, the saline + UC-MSCs-Exo group and the liver fibrosis + UC-MSCs-Exo group mice were

intravenously injected with 75 μg/200 μL UC-MSCs-Exo. For the saline + UC-MSCs group and the liver fibrosis + UC-MSCs group, 1.0×10^6/200 μL UC-MSCs were intravenously injected into mice. The liver fibrosis + PBS group mice were intravenously injected with 200 μL PBS. The NC group was untreated to serve as blank control. Mice were sacrificed 6 weeks after TAA injection.

Preparation of UC-MSCs and UC-MSCs condition medium. The present study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The use of the human umbilical cord tissue from a healthy donor who gave birth and signed informed consent in Renmin Hospital was supported by the Institutional Ethics Review Board of Renmin Hospital of Wuhan University (Permit Number: WDRY2019-G001). UC-MSCs were isolated, cultured and identified as described in our previous study [27]. Briefly, the umbilical cord was cut into small pieces, washed with PBS, centrifuged and cultured in serum-free medium (Lonza, MD, Walkersville) at 37°C and 5% CO_2 concentration. The fifth generations of cells were applied for the following experiments. UC-MSCs were identified by induction differentiation experiments using Oil red O staining, Alizarin, Alcian blue staining and surface marker expression as described previously [27].

The supernatant of UC-MSCs was collected after 72 h of cell culture as UC-MSCs-conditioned medium (UC-MSCs-CM). The medium was centrifuged at 300 g for 10 min and 2000 g for 20 min to remove cell debris and passed through a 0.22-μm filter (Millipore).

The differentiation experiments of UC-MSCs were carried out using Oil red O staining to confirm adipogenesis, using Ali- zarin red staining to verify osteogenesis, and Alcian blue staining to examine chondrogenesis. Related cell surface markers, such as CD90, CD105, CD73, HLA-DR, CD34, CD19, and CD45 (Biolegend, USA), were detected by flow cytometry.

Isolation and characterization of UC-MSCs-Exo. The concentrated medium was ultracentrifuged at 300 g for 10 min, 2000g for 10 min and 10,000 g for 30 min at 4°C.

The supernatant was removed, and exosomes were resuspended in PBS. The solution was subsequently ultracentrifuged at 10,000 g for 70 min at 4°C. The exosomes were resuspended in PBS and stored at -80°C for further use.

The morphology of exosomes extracted from UC-MSCs (UC-MSCs-Exo) were observed by transmission electron microscope (TEM) (Tecnai G2 Spirit Bio TWIN, FEI, USA). Particle size and concentrations were measured by nanoparticle tracking analysis (NTA) (Particle Metrix, Germany). Positive biomarkers (Alix and CD9) and negative biomarker (Calnexin) were detected by western blot.

Cell Culture and transfection. LX-2 cells (human hepatic stellate cell line) were purchased from the Cell Bank of Wuhan University. They were cultured in DMEM (Gibco by Thermo Fisher Scientific, NY, USA) containing 10% fetal bovine serum (Gibco by Life Technologies, NY, USA) at 37°C, 5% CO2.

To obtain SMO over-expressed LX-2 cells, LX-2 cells were seeded at a density of 0.5×10^6 cells per well in a 6-well culture plate for 24 h. The Lipo8000 reagent (Beyotime, Shanghai, China) was used for siRNA and plasmid DNA transfection into LX-2 cells.

Histopathology and immunofluorescence staining. Fresh liver tissues were fixed in 4% paraformaldehyde and embedded in paraffin, and sectioned into 5 μm sections. To detect the degree of pathological damage and fibrosis of liver tissues, hematoxylin and eosin (H&E), Sirius Red and Masson's trichrome (Masson) staining were used to observe the liver tissue sections under a light microscope.

After deparaffinized and rehydrated, liver sections (5 μm) were processed for antigen retrieval in a pressure cooker with EDTA buffer (pH 8.0) for immunofluorescence staining. Then, sections were washed with PBS (pH 7.4). After slightly drying, fluorescent quencher was added to avoid spontaneous fluorescence and then rinsed off in running buffer. BSA was dropped onto the sections and incubated. Next, sections were incubated at 4°C overnight with 1:50 diluted rabbit anti-F4/80 antibody (sc-52664) and 1:200 diluted rabbit anti-Ly6G antibody (Bioss, China). After washing with PBS for 3 times, the sections were incubated at room temperature with the corresponding

secondary antibody for 50 min in dark. The nucleus was stained with DAPI. Negative controls without primary antibody were prepared with 2% BSA. Images were detected by a fluorescence microscope (CKX53, OLYMPUS, Japan).

Quantitative real-time PCR analysis. Total RNA was extracted from liver tissue or cell cultures using Trizol reagent (Invitrogen, CA) according to manufacturer's instruction. RNA (1 μg) was reverse transcribed into complementary first-strand cDNA using cDNA synthesis kit (Takara Bio, Inc., Otsu, Japan). The SYBR Green Mix (YEASEN) was used for quantitative real-time PCR (qRT-PCR). Target gene expressions were calculated by their ratios to the housekeeping gene *Gapdh*. Primer sequences are provided in suppl. table 1 for the amplification of *Gapdh*, *Acta2*, *Col1α1*, *Col3α1*, *Smo*, *Tnfα*, *Il-6*, *Mcp-1*, *Adgre1* and *Timp1*.

Western blot analysis. Snapped frozen liver tissues were treated with RIPA lysis buffer for protein extraction (Beyotime, Shanghai, China). The concentration of protein was examined by BCA assay kits (Beyotime, Shanghai, China). The 50 μg protein samples were loaded in 10% SDS-PAGE gels and transferred onto PVDF membranes (Millipore, Bedford, MA). After blocking with 5% skim milk for 1 h, the membranes were respectively incubated overnight at 4°C with primary antibodies and secondary antibodies. The nuclear and cytosolic fractions were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Themo Fisher Scientific, MA). The membrane was visualized using ECL Senicapture gel image acquisition software (Peiqing, Shanghai, China).

Statistical Analysis. All data were presented as mean ± SD by GraphPad Prism 5 software (GraphPad Software Inc., California, USA) and analyzed by SPSS 19 statistical software (SPSS Inc., Chicago, Illinois). Multiple group comparisons were performed using one-way analysis of variance (ANOVA). T test was used for two-group comparisons. P value < 0.05 was considered as statistically significant.