Materials and Methods

Cell culture

As described in our previous research, MSCs were isolated from the umbilical cords of informed and healthy pregnant women (Yan et al., 2009). Isolated MSCs were maintained in α-MEM (Minimum Essential Medium-α) with 10% Fetal bovine serum（FBS). Human HSC cell line LX-2, human liver cancer cell line HepG2, and 293T cell lines were purchased from Fenghbio (Changsha, China) and cultured in H-DMEM (High glucose Dulbecco's Modified Eagle Medium) containing 10% FBS. Human liver cell line L02 was purchased from the Chinese Academy of Science and cultured in RPMI 1640 containing 10% FBS, maintained in a humidified incubator at 37 °C with 5% CO2. All cell lines were identified by STR and purified against mycoplasma contamination.

Cell transfection

Human miR4465 mimics, mimic negative control (NC), were purchased from GenePharma (Shanghai, China). MiR4465 mimics (5 nM and 10 nM) and NC were transiently transfected into LX-2 using Lipofectamine 2000 in Opti-MEM^{TM} medium (Invitrogen, USA) at 80%-90% confluency in 6-well culture plates in accordance with the manufacturer's instructions. At 4-6 h post-transfection, the culture medium was replaced with H-DMEM with 10% FBS for another 48 h. The transfected cells were collected for further research.

Dual-luciferase reporter assay

The wild-type or mutated LOXL2 possessing miR4465-binding sites were obtained from Fenghbio, which was later sub-cloned into the pmirGLO Vector. Then, miR4465 mimics or mimic negative control (NC), LOXL2-WT vector, or LOXL2-MUT vector were co-transfected into LX-2 by Lipofectamine 2000 (Invitrogen). Luciferase activity was measured 48h post-transfection using the Dual-Luciferase Reporter Gene Assay Kit (Vazyme, Nanjing, China) on the Dual-Glo® Luciferase Assay System (Promega, USA) as manufacturer's directions.

Western blot analysis

LX-2 and MSC-sEV were collected and lysed in RIPA buffer (Pierce, Rockford, USA). A BCA assay kit (Vazyme) was used to detect the concentration of the MSC-sEV protein sample. Protein samples were added into 10% (v/v) SDS-PAGE denaturing gel prepared. After obtaining the molecular weight of the target protein, it was transferred from the gel onto an activated polyvinylidene fluoride membrane with a constant current of 350 mA for 2 h. Later, when nonspecific binding was blocked owing to 5% (w/v) milk for 1 h, the membranes were incubated with primary antibodies against β-actin (Abclonal, AC026, Wuhan, China), LOXL2 (Abcam,

ab179810, UK), TSG101 (Bioworld, BS91381, CA, USA), Calnexin (Bioworld, BS1438), CD9 (Bioworld, BS3022), CD63 (Abcam, ab271286), α-SMA (Abcam, ab7817), Col3a1 (Boster, M00788, USA), Col4a2 (Boster, BA3626), TGFβR2 (proteintech, 66636-1-Ig, USA), p-Smad2/3 (SAB, 12241, USA), Smad2/3 (Bioworld, BS1838), N-cadherin (Abclonal, A0433), E-cadherin (Abclonal, A3044), Vimentin (Santa Cruz Biotechnology, SC-373717, USA) at 4°C overnight. The next day, the membrane was washed by TBS/T to remove the excessive primary antibody, followed by being incubated with horseradish peroxidase (HRP) labeled secondary antibodies (Abclonal) for 1 h at 37°C. Finally, the excessive secondary antibody was eliminated by TBS/T; immunoreactive proteins were visualized using the enhanced chemiluminescence reagents (Millipore, USA). Immunoreactive labeling was analyzed with Image J software and normalized to β-actin protein levels.

Quantitative reverse transcription PCR (qRT-PCR)

Total RNA from LX-2 transfected with miR4465 mimic was isolated using Trizol reagent (Gibco, Beijing, China) following the manufacturer's instructions. cDNA was synthesized using a reverse transcription process applied oligo(dT) primers and the SuperScript RT Kit (Vazyme). The QuantiTect SYBR Green PCR Kit (CWBIO) performed qRT-PCR in a Step One Plus Real-Time PCR System (Applied Biosystems; Life Technologies, Carlsbad, CA, USA). Then a $2^{-\Delta\Delta Ct}$ method was used to achieve relative gene expression normalized to β-actin. All samples were performed three times on different experimental replicates. Bio-Engineering Company (Shanghai, China) synthesized the primers involved in the experiment. The primer of LOXL2: 5'-TGT ACC GCC ATG ACA TCG AC-3' (forward); 5'-TAG CGG CTC CTG CAT TTC AT-3' (reverse). The primer of Col1a1: 5'-CCTGCGTGTACCCCACTCA-3' (forward); 5'-CGCCATACTCGAACTGGAA TC-3' (reverse); The primer of α-SMA: 5'-CGGACAGCGCCAAGTGAAG-3' (forward); 5'-TTGTGTCTAGTTTCTGGGCGG-3' (reverse); The primer of Col3a1: 5'-TGG ATG GTG GTT TTC AGT TTA GCT A-3' (forward); 5'-TTT ACA TTT CCA CTG GCC TGA TC-3' (reverse). The primer of Col4a2: 5'-ATA GGA GGG CCC AAG GGA TT-3' (forward); 5'-CAG GGT CCC CTC TAT CAC CA-3' (reverse). The primer of β-actin: 5'-GAC CTG TAC GCC AAC ACA GT-3' (forward); 5'-GAT AAG CCG TGG TTC TGG TC-3' (reverse).

Immunofluorescence staining

LX-2 transfected with miR4465 mimics or NC were placed on coverslips in a 24-well plate. When the cell density reached 50-60%, LX-2 was fixed with 4% (w/v) paraformaldehyde (Sigma-Aldrich) and infiltrated with 0.1% (v/v) Triton x-100. After blocking with 5% (w/v) bovine serum albumin (BSA), the first antibody against LOXL2, α-SMA (Abcam), and Col3a1 (Boster) were

incubated at 4 °C overnight. The next day, LX-2 was washed with PBS three times and incubated with fluorescence-labeled secondary antibodies (Abclonal) to make it visualized. Finally, the nucleus was dyed with Hoechst 3342 in the dark at room temperature. A confocal microscope can observe fluorescence intensity and distribution (Nikon, Tokyo, Japan).

Isolation and identification of MSC-sEV

When the degree of MSC fusion reached 60%, the supernatant was replaced with α-MEM containing 10% (v/v) exosome-free FBS for 48 h. Then MSC-sEV was extracted by MSC culture supernatant collected according to the ultracentrifugation method. Briefly, the supernatant collected was centrifuged at 2 000×g for 30 min followed by 10 000×g 30 min to remove cell debris. Subsequently, after concentration at 1 500×g with 100 kDa MWCO (Millipore), the concentrated solution was subjected to ultracentrifugation at 100 000×g for 75 min to achieve the pellet. Later, PBS was utilized to resuspend the pellet at the bottom and ultracentrifuged again at 100 000×g for 75 min. The pellet obtained with the final centrifugation was considered MSC-sEV. Then MSC-sEV was filtered with a 0.22 μm filter, and the filtrate was transferred to a new tube stored at −80 °C before use. The detection of the morphology of MSC-sEV was performed by transmission electron microscopy (TEM). Nanoparticle tracking analysis (NTA) (NanoSight, Amesbury, UK) was utilized to determine the size distribution and relative intensity of MSC-sEV. Western blot was used to detect the expression of the exosomal markers CD9, CD63, and TSG101.

MSC-sEV tracking in LX-2 and mice

To detect MSC-sEV in LX-2, MSC-sEV was labeled with lipophilic membrane dye CM-Dil (Invitrogen). The purified MSC-sEV was incubated with CM-Dil at 37°C for 30 min, and free dyestuff was removed by centrifugation at 1 500×g for 30 min with a 100 kDa MWCO. After incubation with LX-2, a confocal microscope could detect the CM-Dil labeled MSC-sEV in LX-2. For tracking MSC-sEV in mice, MSC-sEV was labeled with lipophilic membrane dye CM-Dil (Invitrogen), similar to CM-Dil labeled MSC-sEV. After injection via the tail vein, liver fibrosis mice were detected by a vivo imaging system (CRi, USA) to observe MSC-sEV in vivo. In vivo spectral imaging from 690–850 nm was performed using an exposure time of 150 ms per image frame.

MiR4465 Loading of MSC-sEV

MiR4465 mimics were passively loaded into MSC-sEV by sonication. MSC-sEV was diluted in PBS to 200 ng/μL, and miR4465 mimics (10 nM) were added to 100 μL of MSC-sEV and co-incubated for 30 min. Then the MSC-sEV mixture with miR4465 mimics (10 nM) was sonicated using a Qsonica Sonicator Q700 (Branson, USA) with the following settings: 10% amplitude, 4 s

pulse/2 s pause, cooled down on the ice for 2 min, and then sonicated again for six cycles. After sonication, the MSC-sEV mixture was incubated at 37°C for 60 min to recover the exosomal membrane. Next, miR4465 modified MSC-sEV was diluted 10 times with PBS and centrifuged at 1, 500×g for 30 min with a 100 kDa MWCO to remove free miR4465.

Mice model of liver fibrosis and MSC-sEV injection

Mice (female, ICR, 6-8 weeks) were purchased from the Laboratory Animal Center of Jiangsu University. All mice were allowed free access to food and water under SPF sterile conditions. In addition, all experimental procedures involved were approved by the ethics committee of Jiangsu University. The liver fibrosis model in mice was established by intraperitoneal injection of CCl4 at a dose of 0.1 ml/100 g body weight dissolved in mineral oil MSC-sEV for three days for two mouths. To analyze the effect of MSC-sEV-miR4465 and MSC-sEV on LOXL2, α-SMA expression, and collagen deposition, liver fibrosis mice were injected with PBS (n=5), MSC-sEV^{miR4465} (n=5), or MSC-sEV (n=5) twice a week by tail vein at a dose of 1×10^{12} particles/Kg body weight. Then the mice were sacrificed for liver samples three days after the last treatment. All experiments involving animals were conducted according to the ethical policies and procedures approved by the Jiangsu University ethics committee (Approval no. UJS-IACUC-AP-2020033127).

Sirius red staining and Masson staining

Sirius red staining assay kit (Chondrex, USA) and Masson staining assay kit (Solarbio, Beijing) was used to assess collagen deposition in liver slices according to the manufacturer's instructions. Fibrotic septa were randomly chosen from five different mice/groups to examine the distribution of hepatic collagen. Images were acquired using a digital slide scanner (3DHISTECH, Hungary). The percentage of each section's staining blue surface was the basis for calculating the collagen content.

Immunohistochemistry

After dewaxed in Xylene and ethanol, liver tissue sections were exposed to 3% (v/v) H_2O_2 to inactivate endogenous peroxidase. Then, liver tissues were boiled in citrate buffer (pH 6.0) for 30 min to retrieve antigen. Next, 5% (w/v) BSA was utilized to block the slides for 1 h at room temperature to avoid non-specific adsorption. Later, primary antibodies against LOXL2，α-SMA (Abcam) were incubated with liver tissue at 4 °C overnight. The next day, the excessive primary antibody over the slides was removed by PBS. Then the liver tissues were incubated with a biotin-conjugated anti-mouse/rabbit IgG antibody and streptavidin–biotin for 30 min at 37 °C. The chemical signals were detected with DAB Horseradish Peroxidase Color Development Kit (Boster, Wuhan, China) and counterstained with hematoxylin. Besides, the slides were dehydrated following

the reverse process of dewaxing above being sealed with neutral gum. Finally, a digital slice scanner was used to capture images.

Cell counting Kit-8 assay

To evaluate the cell viability, 5×10^3 cells were seeded in 96-well plates with 100 μL medium and cultured overnight. Then, various concentrations of MSC-sEV were added for five replications each group. 100 μL of Cell Counting Kit-8 (CCK8, Kum-amoto, Japan) solution was added to each well and incubated in the dark for 2 h after 24 h, 48 h, 72 h. The absorbance of each well was measured at a wavelength of 450 nm using a microplate reader (800TS, Biotek, USA). The average value SD value of each replicate hole was calculated by subtracting the OD value of each hole from the OD value of the blank control group.

Migration and invasion assays

For wound healing assays, cells were planted in 6-well plates to 100% confluency, and wounds were scraped along the bottom of the dish with a 100 μL plastic pipette. The wound healing process was recorded after 24 h. 6×10^4 cells were seeded into the top Transwell filter chambers (Corning, MA, USA) in a serum-free medium for cell migration and invasion assays. Besides, for invasion assay, the diluted Matrigel (BD Biosciences, NY, USA) was added to filter chambers for 30 min at 37°C before planting cells. Then complete medium containing 10% FBS was added to the lower chamber. After 24 h of the migration assay and 48 h of the invasion assay, the cells at the bottom of the membrane were stained with crystal violet (Yeasen, Shanghai, China) for 15 min. After being photographed, three regions each were randomly counted under a microscope.

Statistics analysis

All experiments were repeated at least three times. Statistical analysis was performed by GraphPad Prism 8.0 software. Data are presented as means ± standard deviation (SD). The Student's *t*-test is used to analyze the statistical significance between the two groups. The comparison between multiple groups was analyzed for significance by One-way analysis of variance (ANOVA). The difference was statistically significant when $P<0.05$.