Detailed methods

Animals

A total of 75 SD rats (male, 6–8 weeks old, weighing 200–250 g) were obtained from the Beijing Vital River Laboratory Animal Technology Co., Ltd. We selected only male rats to limit the potential gender differences. Sixty rats were randomly assigned to three groups: the M2b macrophage transplantation group (MT, n=24), the control group (CK, n=24) and the sham operation group (SO, n=12). We included 12 rats in the SO group due to the homogeneity of this group and to meet the requirements of "the rules of 3R": replacement, reduction and refinement. The other 15 rats were sacrificed to harvest the macrophages or CFs. All animals were allowed free access to food and water and were housed with a constant temperature (22±2 ℃), humidity (45%±5%), and a 12-h day and 12-h night cycle. Experiments were performed under a project license (No. gdpulacSPF2017026) granted by the Animal Ethical Committee of Guangdong Pharmaceutical University, in compliance with the relevant guidelines for the care and use of laboratory animals.

Isolation and in vitro polarization of macrophages

Rats that were used to harvest the macrophages were sacrificed by the dislocation of the cervical vertebrae. Bone marrow was collected from the tibias and femurs, washed with complete Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Grand Island, NY, USA) and centrifuged at 500 g for 5 min. Cells were then cultured in flasks (Corning, NY, USA) at 37 ℃ in a 5% CO_2 incubator in RPMI 1640 for the first 3 days and then in Dulbecco's modified Eagle's medium (DMEM; Gibco) for the next 3 days to generate mature BMDMs. Both the RPMI 1640 and DMEM were supplemented with 10% fetal bovine serum (FBS; Gibco) and 10 ng/mL macrophage colony stimulating factor (PeproTech, Rocky Hill, NJ, USA). On day 6, BMDMs were replated in 24-well plates (Corning) to differentiate into M2b macrophages with 50 μg/mL IgG (Sigma Aldrich, St. Louis, MO, USA) and 100 ng/mL LPS (Sigma Aldrich). After incubation for 24 h, the cells were harvested for macrophage marker analysis or the cells were replated and cultured with fresh DMEM without stimulation for another 24 h. Then, the cell-free supernatants were collected at 24 h for the stimulation of primary CFs *in vitro*.

Identification of M2b macrophages by flow cytometry

Cells were conjugated with LIGHT antibody (Abcam, Cambridge, MA, USA) or isotype control (Abcam) and then with Alexa Fluor 488-conjugated antibody (Invitrogen, Carlsbad, CA, US). After that, the cells were combined with APC A750-rat CD45 (eBioscience, San Diego, CA, USA) or isotype control (eBioscience). Finally, flow cytometry was performed using a CytoFLEX flow cytometer (Beckman Coulter, Miami, FL, USA). The results were analyzed with FlowJo software.

CFs isolation and culture

CFs were enzymatically isolated from the hearts of adult male SD rats. Briefly, the rats were sacrificed by the dislocation of the cervical vertebrae. The hearts were removed and perfused with 0.08% collagenase I (Gibco). After mincing, the hearts were placed in a solution of 0.08% collagenase I and 0.05% trypsin (Gibco) and underwent sequential 10 min periods of digestion with constant stirring at 37 ℃. After enzymatic digestion, CFs were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin solution at 37 ℃ in humid air with 5% CO_2. After 2 h, the culture medium was changed to remove any myocytes and endothelial cells still weakly attached to the dish. All studies used CFs (passages 1 through 3) that were grown to subconfluence in serum-containing media and that were growth-arrested for 24 h in serum-free medium before treatment.

Animal model and M2b macrophage transplantation

The polarized M2b macrophages were washed in 1× PBS and resuspended in normal saline before the operation. After anesthesia with 2% Nembutal Sodium (50 mg/kg), the rats received endotracheal intubation to provide artificial ventilation (4–5 mL tidal volume, 90 breaths/min) with a rodent ventilator. Then, the chest was opened with a left thoracotomy through the fourth intercostal space, and the left anterior descending (LAD) coronary artery was occluded by an 8-0 prolene suture for 30 min, followed by reperfusion after the removal of the suture. One minute after artery occlusion, the ischemic area was identified, either 1×10^6 M2b macrophages (MT group) in 50 μL or the same volume of normal saline (CK group) was injected into the border zone at five scattered points. Rats in the SO group underwent the same operation but without

ligation of the coronary artery. Rats that experienced severe arrhythmia, cardiac arrest, or respiratory failure in the procedure were excluded. There were four, four and two rats that showed these effects in the CK, MT and SO groups, respectively. Two weeks after reperfusion, echocardiography was performed, and the rats were sacrificed by exsanguination. Blood was collected, and the hearts were harvested. Ten hearts from the CK and MT groups and five from the SO group were processed, were 4% paraformaldehyde-fixed and were used for Sirius red staining and immunohistochemical staining. RNA and protein were extracted from the rest of the hearts using an AllPrep DNA/RNA/Protein Mini Kit (QIAGEN, Duesseldorf, Germany) according to the manufacturer's instructions.

Echocardiography

Rats were anaesthetized with 1.5% isoflurane/oxygen, and cardiac function was assessed using transthoracic echocardiography (VisualSonics system, Toronto, Ontario, Canada) performed on day 14. M-mode and two-dimensional echocardiography were performed to assess the cardiac parameters and ventricular function.

Histological analysis

Excised hearts were fixed in 4% paraformaldehyde, paraffin embedded, and sectioned at a 5 μm thickness. Deparaffinized sections were stained with Sirius red. Image-Pro Plus software (Media Cybernetics, Rockville, MD, USA) was used to measure the fibrosis in the whole heart sections.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from the hearts using an AllPrep DNA/RNA/Protein Mini Kit (QIAGEN) according to the manufacturer's instructions. RNA was transcribed to cDNA with a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). Then, qRT-PCR was performed on a Light Cycler 480 system (Roche Applied Science, Mannheim, Germany) using a Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific) according to the manufacturer's instructions. For data analysis, the comparative threshold cycle (C_T) value for GAPDH was used to normalize the loading variations in the PCRs. The primer sequences for the target genes are as follows (5'-3'): GAPDH Fw: GGTCATCCATGACAACTT, Rev: GGGGCCATCCACAGTCTT; Collagen I Fw: AACTCCCTCCACCCCAATCT, Rev: CCATGGAGATGCCAGATGGTT; collagen III Fw: ACGTAAGCACTGGTGGACAG, Rev: GGAGGGCCATAGCTGAACTG; TGF-β Fw: ATCGACATGGAGCTGGTGA, Rev: TTGGCATGGTAGCCCTTGG; CCN2 Fw: TGTCTTCGGTGGGTCGGTGT, Rev: CAGGCAGTTGGCTCGCATCATAG; HGF Fw: TCAGCACCATCAAGGCAAGG, Rev: GCACATCCACGACCAGGAACAAT.

Western blot analysis

Total protein from the hearts was extracted using an AllPrep DNA/RNA/Protein Mini Kit (QIAGEN) according to the manufacturer's instructions, and proteins from cells were lysed by RIPA buffer (Beyotime, China). Protein concentrations were measured with a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, CA, USA). Proteins were fractionated with 8% to 15% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked and then incubated with GAPDH (60004-1-Ig at 1/10,000 dilution, Proteintech, Rosemont, IL, USA), α-SMA (ab32575 at 1/1,000 dilution, Abcam), p-PDGFRα (Tyr720) (ab134068 at 1/1,000 dilution, Abcam), PDGFRα (ab203491 at 1/1,000 dilution, Abcam), CCN2 (sc-365970 at 1/500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA), p-PDGFRβ (Tyr740) (#3168 at 1/1,000 dilution, Cell Signaling Technology, Danvers, MA, USA) and PDGFRβ antibodies (#3169 at 1/1,000 dilution, Cell Signaling Technology). Subsequently, the membranes were incubated with an HRP-conjugated secondary antibody (31430 and 31460 at 1/100,000 dilution, Thermo Fisher Scientific, Waltham, MA, USA) at room temperature for 1 h and were visualized using enhanced chemiluminescence reagents (Sigma-Aldrich) according to the manufacturer's instructions.

Immunocytochemistry

CF cells were seeded on chamber slides and cultured for 24 h with different treatments. Cells were then fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton

X-100 at RT. After blocking with donkey serum in PBS containing 0.2% Trion X-100 at RT for 0.5 h, CF cells were incubated with primary antibodies against α-SMA (ab32575 at 1/100 dilution, Abcam) at RT for 1 h. After washing three times with PBS, CF cells were incubated at RT for 1 h with a secondary antibody [donkey anti-rabbit IgG (H&L) PE; ab7007 at 1/100 dilution, Abcam]. Then the slides were washed three times with PBS, mounted with fluoroshield mounting medium containing DAPI (Sigma-Aldrich), and sealed with nail polish. Slides were observed with confocal microscopy (Carl Zeiss, Jena, Germany).

Statistics

Data are presented as the mean ± SD. Statistical analysis was performed using GraphPad Prism software and SPSS. The differences in the results between two groups were analyzed with a Student's *t*-test. Comparisons among groups were assessed with one-way ANOVA followed by Bonferroni's post hoc test. For all tests, $P<0.05$ was considered statistically significant.