Supporting information

DNA-coated gold nanoparticles for tracking of hepatocyte growth factor secreted by transplanted mesenchymal stem cells in pulmonary fibrosis therapy†

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1. Experimental Section

Materials. Gold (III) chloride ($HAuCl_4 \cdot 3H_2O$) was purchased from Sigma-Aldrich. Trisodium citrate and triethylamine were bought from China National Medicine Corporation. All of the Deoxyribonucleic Acid (DNA) samples were ordered from Shanghai Sangon Biotech, Co., Ltd. The sequences and modifications of the DNAs were shown in Table S1. Cell counting kit-8 (CCK-8) was obtained from Shanghai Beyotime Biotechnology, Co., Ltd. DMEM/F12 basic medium, phosphate-buffered saline (PBS), TrypLE express, penicillin-streptomycin and fetal bovine serum (FBS) were acquired from Gibco. Human umbilical cord mesenchymal stem cell osteogenic differentiation medium, adipogenic differentiation medium, Alizarin Red S and Oil Red O were got from Cyagen Biosciences Inc. Rest of the reagents were analytically pure and purchased from Sinopharmaceutical Chemical Reagents Co., LtD. Rabbit anti-human HGF antibody and secondary antibody were purchased from Abcam Co., LtD. All chemicals and reagents were used as received without any further purification.

Preparation of Nanoflare Tracer. AuNPs were first prepared according to Frens's approach.[1] In a typical process, $HAuCl_4$ aqueous solution (100 mL, 2.43×10^{-4} M) was heated to boiling with vigorous stirring, to which trisodium citrate solution (2 mL, 1%) was added. The mixture was then kept boiling for 15 min. Afterwards, the solution was cooled to room temperature with continuous stirring, obtaining 14 nm AuNPs. Next, in a typical experiment, DNA1 (100 μM, 3 μL) and DNA2-FAM (100 μM, 3 μL) were mixed with the AuNPs (10 nM, 100 μL). The mixture was then placed in a laboratory freezer (set at -20 ℃) for 2 h, followed by thawing at room temperature, acquiring nanoflare tracer.

Fluorescence Experiments. The nanoflare tracer were diluted to a concentration of 1 nM in PBS and then treated with target DNA (1 μM). The fluorescence spectra were recorded on a Hitachi F4600 exciting at 750 nm and the emission was measured from 770 to 800 nm in 1 nm increments.

Cell Culture. MSCs originated from human umbilical cord (hMSCs) were generously provided by Nanjing Drum Tower Hospital (Nanjing, China). The hMSCs (passage 4-10) were cultured in DMEM/F12 basic medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin

under 37 °C and 5% CO_2 in a humidified atmosphere incubator (Thermo 3111).

Cell Viability Assay. hMSCs were seeded into 96-well plates at a density of 5×10^3 cells per well. After being incubated for 24 h, the medium containing nanoflare tracer at various Au concentrations (100, 200, 300, 350 and 400 pM) was added to co-incubate with hMSCs for different periods of time (24 and 48 h). The hMSCs incubated without the nanoflare tracer were used as control. Each sample was examined in triplicate. CCK-8 was used to evaluate the cell viability according to the instructions of manufacturer and then the optical density was measured at 450 nm by a microplate reader.

Cell Model. Recombinant human TGF-β1 was obtained from Peprotech, Inc. (Rocky Hill, NJ, USA) and dissolved in phosphate-buffered saline (PBS) solution. Mouse lung fibroblasts (MLg) generously provided by Nanjing Drum Tower Hospital (Nanjing, China) were firstly seeded into 6-well plates at a density of 2×10^5 cells per well, and then divided into two groups: control group and TGF-β1 induction group. When the MLg growth density reached 75-80%, the MLg in TGF-β1 induction group was treated with TGF-β1 (5 ng mL^{-1}) for 12 h. The control group was cultured without TGF-β1 for 12 h. Subsequently, the cells of each group were collected for RNA extraction. The relative mRNA expressions of fibronectin (FN), nuclear factor-κB p65 (P65), and α-smooth muscle actin (α-SMA) were determined by polymerase chain reaction (PCR). All primers used in this experiment were gained from Sangon Biotech (Shanghai) Co., Ltd., and the sequences were provided in Table S2.

Endogenous Secretion of HGF by hMSCs *In Vitro*. hMSCs were seeded into 24-well plates with 1×10^4 cells per well, and then cultured for 24 h. After that, the medium was replaced with the conditional medium from TGF-β1 induction group (TGF-β1-CM) to induce endogenous secretion of HGF in hMSCs for different periods of time (4, 8, 12 and 24 h). After induction, the cells were fixed with 4% paraformaldehyde, blocked for 1 h with 3% goat serum and 0.1% Triton-X-100 dissolved in PBS, incubated with primary antibody overnight at 4 °C, and then labeled with red fluorescence-conjugated secondary antibody for 1 h at room temperature, followed by staining with 4', 6-diamidino-2-phenylindole (DAPI) for 5 min. The immunostained cells were visualized by laser confocal microscope.

Lentiviral Transfection. For RfLuc labeling, hMSCs (1×10^6 cells) were incubated with lentivirus at 1×10^3 viral particles per cell (vp per cell) in serum-free $_L$-DMEM medium, obtaining $hMSCs^{RfLuc}$ lineage.[2]

***In Vitro* Fluorescence Imaging.** hMSCs were seeded into 24-well plates at a density of 1×10^4 cells per well and then divided into two groups: control group and TGF-β1-CM group. After 24 h culturing, the hMSCs in the two group were both incubated with the nanoflare tracer (300 pM Au) for another 24 h. Subsequently, the hMSCs in TGF-β1-CM group were cultured with TGF-β1-CM for 12 h. The hMSCs in control group were cultured with common culture medium for 12 h. Finally, hMSCs were fixed with 4% paraformaldehyde, and then stained with DAPI for 5 min. Laser confocal microscope was used to assess the release of dye-labeled recognition sequences.

***In Vitro* BL Imaging.** For *in vitro* BLI, $hMSCs^{RfLuc}$ (5×10^4 cells) in PBS (50 μL) were dispersed into black 24-well plates. The BL imaging was collected with the IVIS Lumima II system (Caliper Life Science) using an open filter immediately after addition of $_D$-luciferin (3 μL, 15 mg mL^{-1}). All BL images were analyzed by Living Image software, version 4.0 (Caliper Life Science).

Proliferation Assay. $hMSCs^{RfLuc}$ were incubated with the nanoflare tracer (300 pM Au) for 24 h. The hMSCs without any labeling were taken as control. The dual-labeled and unlabeled hMSCs were digested and counted, then seeded into 24-well plates at a density of 1×10^4 cells per well. At 1, 3, 5 and 7 d after incubation, the relative number of the cells was detected with CCK-8, and the absorbance was measured at 450 nm by a microplate analyzer.

Osteogenic Differentiation Assay. Osteogenic induction differentiation complete medium (Cyagen Biosciences Inc) was prepared according to the instructions of manufacturer. The dual-labeled hMSCs were seeded into 24-well plates with 1×10^4 cells per well, and cultured for 24 h. After that, the medium was replaced with osteogenic differentiation medium to induce differentiation. When the dual-labeled hMSCs were further cultured for at least 21 d, the cells were fixed with 4% paraformaldehyde, stained by Alizarin Red S for 3-5 min, and then washed with PBS. The calcium nodules were observed under a

microscope. For quantitative detection, dimethyl sulfoxide (DMSO) was used to dissolve the dye in cells, and the absorbance at 550 nm wavelength was determined by a microplate analyzer. The hMSCs without any labeling were employed as control.

Adipogenic Differentiation Assay. Adipogenic differentiation medium A (Induction medium) and medium B (Maintenance medium) (Cyagen Biosciences Inc) were prepared according to the instructions of manufacturer. The dual-labeled hMSCs were seeded into 24-well plates with 1×10^4 cells per well. After 24 h, the medium was first replaced with adipogenic differentiation medium A for 3 d, and then with adipogenic differentiation medium B for 1 d. After medium A and B inducted alternately for 3-5 times (12-20 d), the cells were further cultured with medium B for another 4-7 d. The cells were then fixed with 4% paraformaldehyde and stained with Oil Red O for 30 min, washing with PBS for three times. The formation of lipid droplets was observed under the microscope. For quantitative detection, DMSO was used to dissolve the dye in cells, and the absorbance at 490 nm wavelength was determined by a microplate analyzer. The hMSCs without any labeling were acted as control.

Animal Model. All animal experiments were carried out following the Guide for the Care and Use of Laboratory Animals, Suzhou Institute of Nano-Tech and Nano-bionics, Chinese Academy of Sciences (Approval number: SINANO/EC/2020-030). C57BL/6 mice (6 weeks old, about 25 g) were anesthetized and then exposed to bleomycin (BLM) as described.[3] Briefly, the trachea was exposed using a sterile technique, and then BLM (3 mg kg^{-1} each mouse) in 100 μL 0.9% NaCl was slowly instilled into the tracheal lumen. Control mice received the same volume of sterile saline. After exposure, the skin incision was closed and the animals were allowed to recover on a warming plate. The mice were sacrificed to take out the lung tissues at 7, 14 and 21 d post-treatment of BLM. Lung tissues were fixed with 4% paraformaldehyde and embedded in paraffin sections, and the sections were then observed by hematoxylin and eosin (H&E) and Masson's trichrome staining, according to standard protocols.[4]

***In Vivo* Imaging**. For the convenience of the *in vivo* observation, long wavelength Cyanine7 (Cy7) was used to prepare the nanoflare tracer instead of FAM. After 3 d post-treatment of BLM or sterile saline,

the dual-labeled hMSCs (4×10^6 cells each mouse) in 50 μL 0.9% NaCl were administered *via* intratracheal injection. Subsequently, *in vivo* NIRF and BL imaging were performed at 1, 2, 3, and 4 d after injection. The chloral hydrate anesthetized mice were received luciferin substrate (3 mg each mouse) by intraperitoneal injection and immediately imaged using an IVIS Lumina II system (Caliper Life Science). All images were analyzed by Living Image software, version 4.0 (Caliper Life Science).

Statistical Analysis. All statistical data were expressed as mean ± SD in triplicate each time. Differences between two groups were analyzed using Student's t-test. $P < 0.05$ was considered statistically significant.

2. Supporting Tables and Figures

Table S1. The sequences and modifications of the DNA samples used in this work.

DNA names	Sequences and modifications (from 5' to 3')
DNA1	SH-AAAAAAAAAGGCCTCGGCTG
DNA2-Cy7	CCCGATGGCCAGCCGAGGCC-Cy7
DNA2-FAM	CCCGATGGCCAGCCGAGGCC-FAM
Target DNA	GGCCTCGGCTGGCCATCGGG

Table S2. qRT-PCR primers.

Primer names	Sequences (from 5' to 3')
mouse-FN-forward	CGGCTACATCATCCGCCATC
mouse-FN-reverse	CTGACAACGTACTCGGTGCC
mouse-α-SMA-forward	GAGCATCCGACACTGCTGAC
mouse-α-SMA-reverse	GCATAGAGGGACAGCACAGC
mouse-p65-forward	TACCACCAAGACACACCCCA
mouse-p65-reverse	TAGTAGCCATCCCGGCAGTC
mouse-β-actin-forward	GAACCCTAAGGCCAACCGTG
mouse-β-actin-reverse	TGTGGTACGACCAGAGGCAT

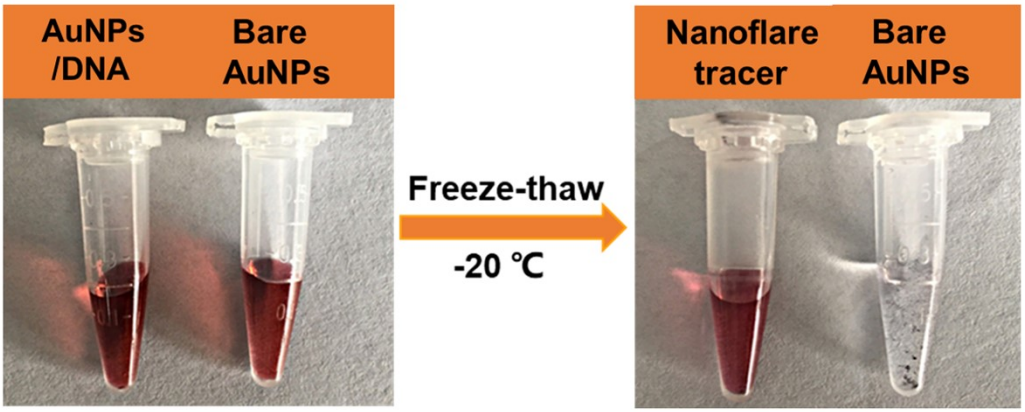

Fig. S1 Photographs of AuNPs before and after a freeze-thaw cycle. While bare AuNPs were aggregated, adding the oligonucleotide complementary sequences (DNA1, 3 μL, 100 μM) and the dye-labeled recognition sequences (DNA2-FAM, 3 μL, 100 μM) protected the AuNPs from aggregation

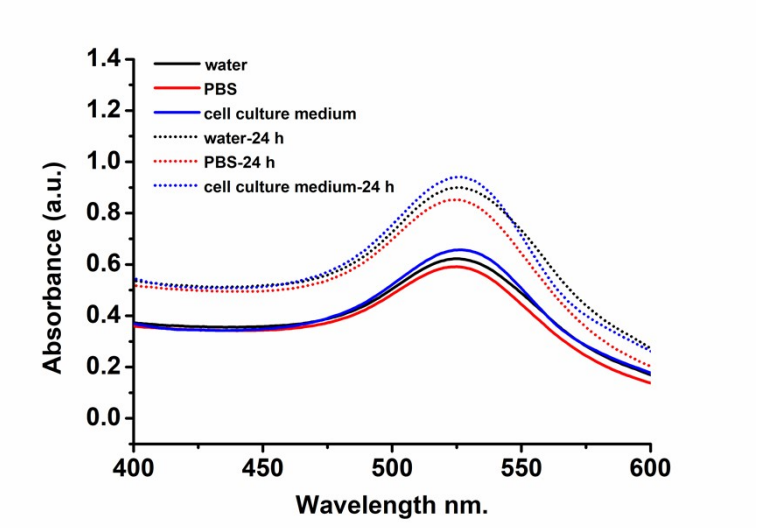

Fig. S2 Normalized UV-vis absorption spectra of the nanoflare tracer in water, PBS and cell culture medium, respectively.

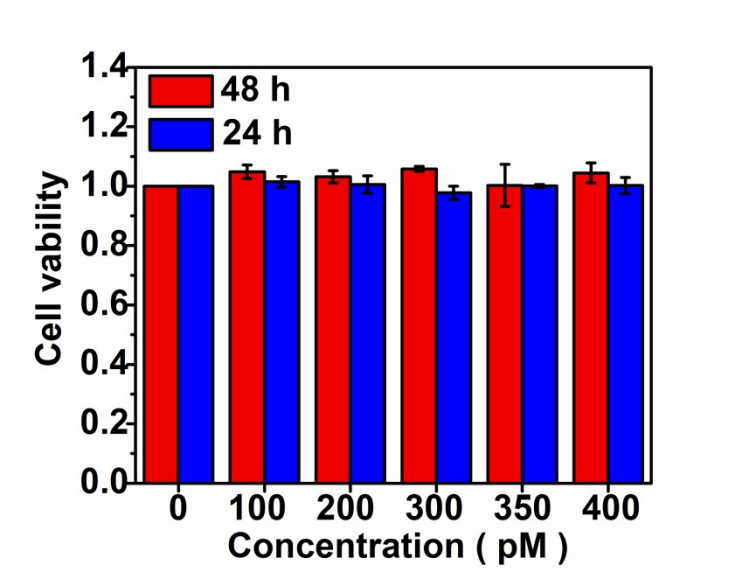

Fig. S3 Relative viability of hMSCs incubated with nanoflare tracer at various Au concentrations for different time points.

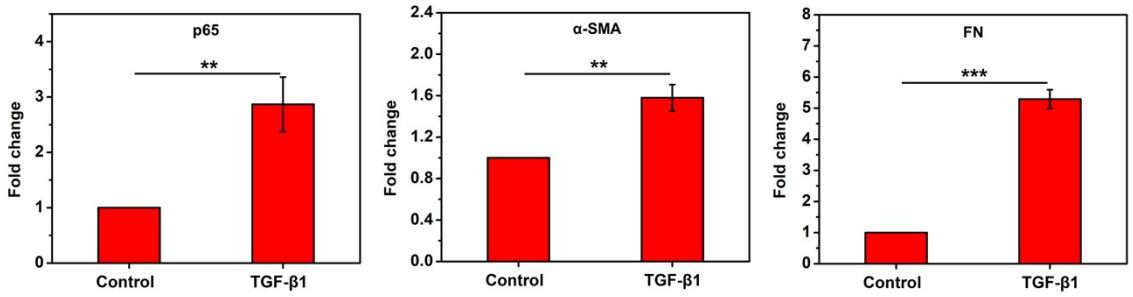

Fig. S4 Expressions of p65, α-SMA and FN in MLg with or without TGF-β1 induction, respectively.

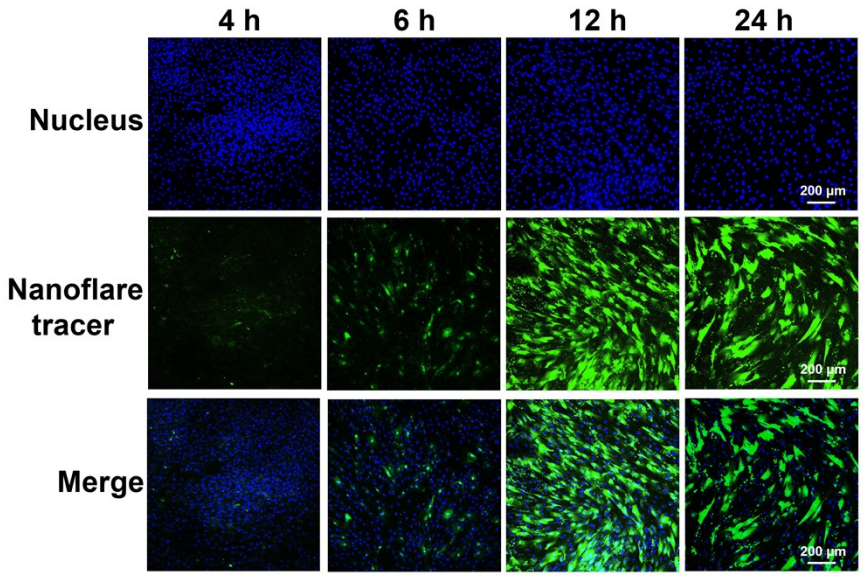

Fig. S5 Laser confocal fluorescence microscopy of hMSCs incubated with nanoflare tracer, followed by inducing with TGF-β1-CM at different time points.

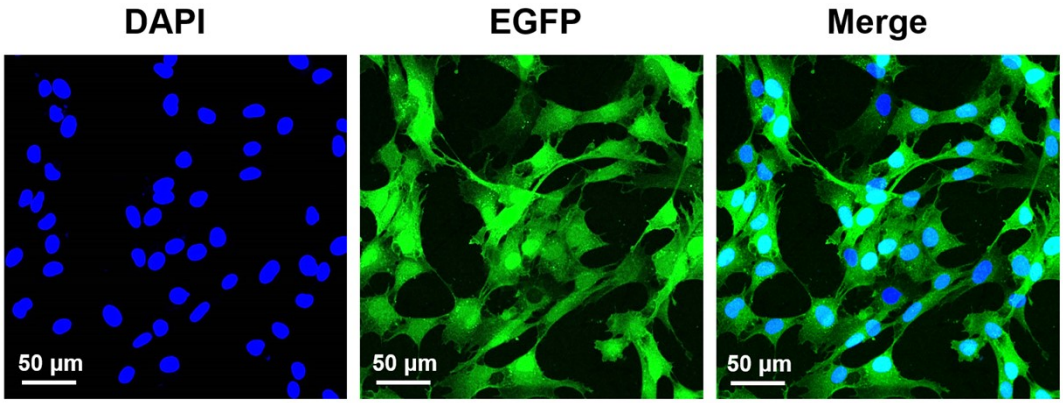

Fig. S6 Fluorescence images of RfLuc labeled hMSCs; green image and blue image indicate the EGFP-expressing hMSCs and the nucleus stained with DAPI, respectively.

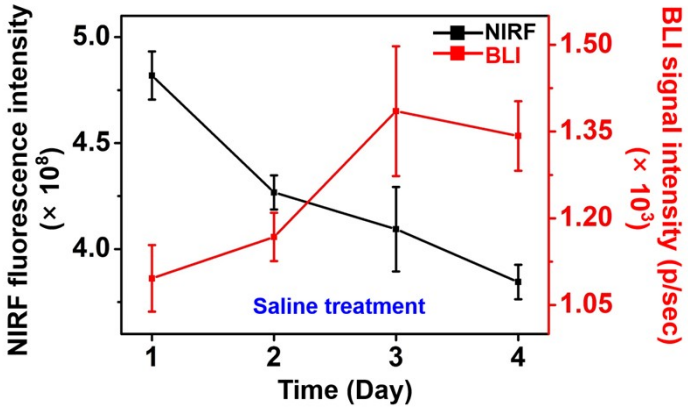

Fig. S7 NIRF and BL signal intensities of the dual-labeled hMSCs after transplantation into the saline treated mouse at 1, 2, 3 and 4 days.

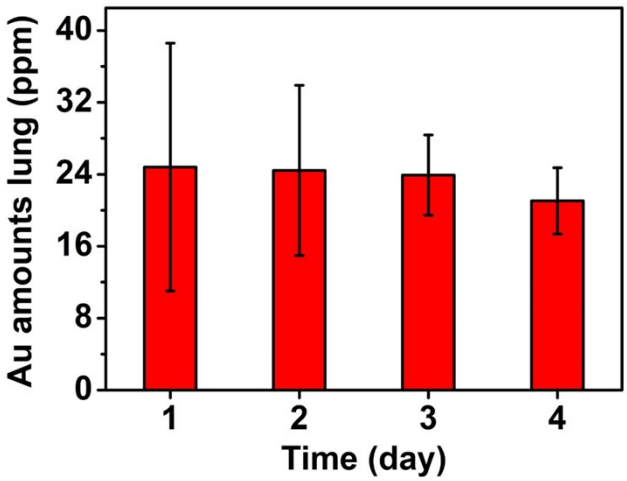

Fig. S8 Time-dependent concentration of Au in the lung of BLM treated mice after transplantation of the dual-labeled hMSCs.

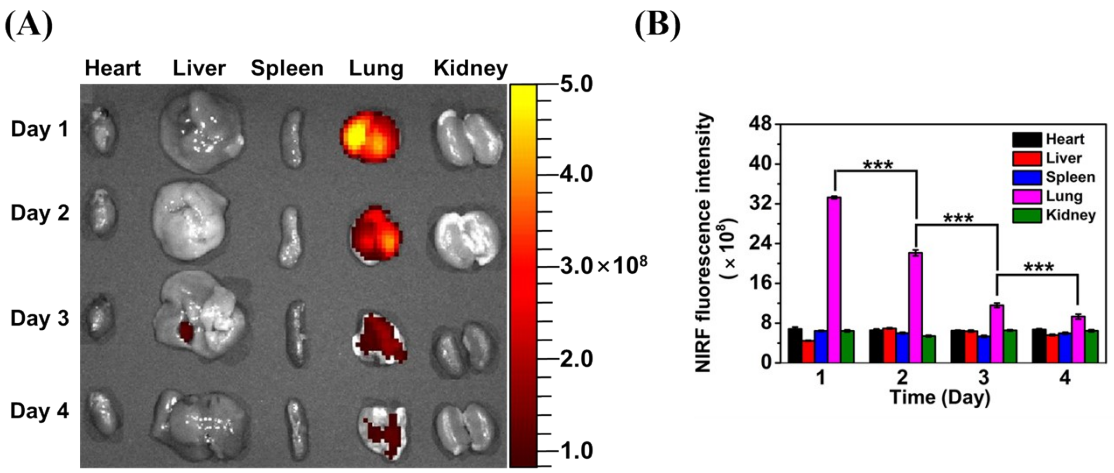

Fig. S9 (A) NIRF images and (B) average NIRF intensity of heart, liver, spleen, lung, kidney (from left to right) after tracheal injection of the nanoflare tracer labeled hMSCs at 1, 2, 3, and 4 days.

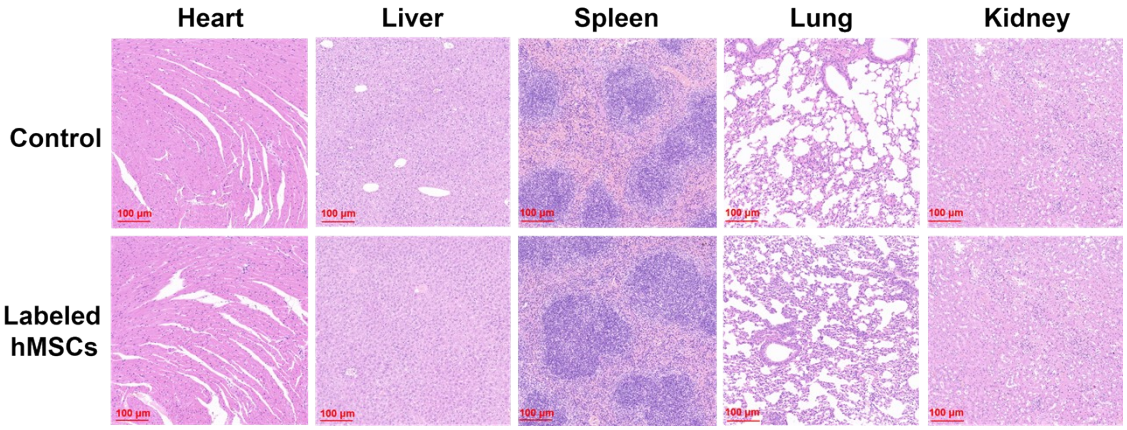

Fig. S10 HE staining for major organs (heart, liver, spleen, lung, and kidney). The mice treated with the nanoflare tracer labeled hMSCs were sacrificed on the 4th day. Age-matched healthy mice without treatment were used as a control group.

Notes and references

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