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Supplemental Information

Evidence of Accumulated Endothelial Progenitor Cells in the Lungs of Rats with Pulmonary Arterial Hypertension by ^{89}Zr-oxine PET Imaging

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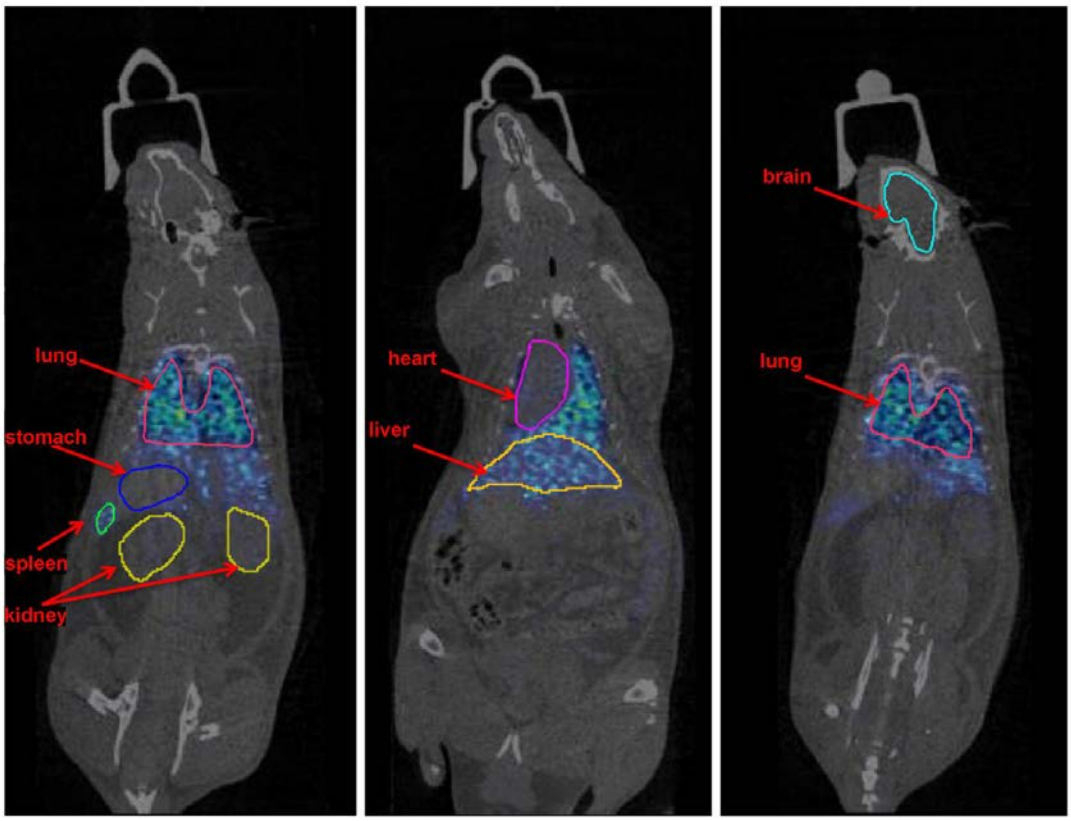
Supplemental Figure



S Figure 1 Representative microPET/CT graphs with the delineated ROIs of main organs of rats marked out.

Supplemental Table

S Table 1 *Ex vivo* biodistribution of ^{89}Zr-EPC in organs of rats (%ID/g, mean±SDM, n = 5 per time point)

Organs	Time point				
	1 h	24 h	72 h	168 h	254 h
Lung	5.99±1.74	0.72±0.36	0.63±0.35	0.49±0.20	0.53±0.34
Liver	2.59±0.66	5.07±0.62	6.13±1.20	5.94±0.28	5.43±1.29
Spleen	1.24±0.37	2.51±0.56	2.48±0.56	2.67±1.66	1.77±0.79
Heart	1.14±0.53	0.48±0.25	0.29±0.11	0.27±0.11	0.23±0.18
Kidney	0.38±0.20	0.59±0.19	0.81±0.27	1.15±0.62	0.90±0.29
Joints	0.14±0.05	1.17±0.13	1.61±0.35	1.76±0.22	2.10±1.02
Stomach	0.26±0.15	0.26±0.08	0.44±0.24	0.42±0.17	0.16±0.12
Bladder	0.24±0.20	0.07±0.02	0.07±0.05	0.05±0.09	0.04±0.08
Intestine	0.07±0.04	0.09±0.08	0.09±0.04	0.07±0.09	0.00±0.00
Bone	0.05±0.04	0.33±0.11	0.36±0.09	0.19±0.15	0.05±0.07
Brain	0.33±0.63	0.10±0.07	0.10±0.04	0.11±0.02	0.04±0.02
Muscle	0.11±0.19	0.05±0.02	0.03±0.02	0.01±0.01	0.00±0.00

Supplemental Videos

S Video 1 The reconstructed three-dimensional video of the microPET/CT imaging of rats, showing the radioactive distribution of ^{89}Zr-oxine-EPCs in main organs of rats in vivo.

S Video 2 Thevideo of the CellVizio confocal scope(additional explanation of Figure 4f), showing the DiO-labeled EPCs attached to the pulmonary microvasculature of rats 1h after injection in vivo. DiO-labeled cells are green, and blood with Evans blue is red. Scale bar = 20 μm. From the video, we could see the labeled EPCs stay in blood vessels and do not flow with the blood.

Supplemental Methods

1. EPCs culture and identification

A 50 mL sample of peripheral blood from a healthy volunteer was used for the isolation and generation of endothelial progenitor cells (EPCs). Peripheral-blood mononuclear cells were isolated by Ficoll (BD Biosciences) density-gradient centrifugation. The mononuclear cell suspension (5×10^5 cells/cm^2) was plated into a T-75 flask coated with type I collagen (BD Biosciences) and maintained in EGM-2MV medium (Lonza) supplemented with 10% FBS (HyClone) at 37 °C. The culture was maintained through days 7-14 till appearing EPC colonies. Colonies were sub-cultured with the trypsin digestion when they grow to 1000-2000 cells per clone, the initial seeding density was about 5000/cm^2. The cells of passages 4-6 were applied to all the assays. The cells were assessed by immunostaining and flow cytometry using CD31 (BD Biosciences, clone WM59, 1:100), CD144 (CST, 2500S, 1:100), and vWF (CST, 65707S, 1:500) CD146 (Abcam, ab10558, 1:100), KDR (CST, 2479S, 1:500), CD34 (Abcam, ab187284,1:100), CD45 (Abcam, ab10558, 1:100), CD14 (Abcam, ab28061, 1:100). For tube formation assay, a total of 2×10^4 cells were seeded into 48-well plate pre-coated with matrigel. The tube formation was detected at 4 h post-seeding.

2. Establishment of MCT-induced PAH rat model

Specific pathogen free (SPF) male Sprague-Dawley (SD) rats weighing 160-200 g (6-week-old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. Rats were housed in SPF animal house in controlled temperature of (25 ± 2) °C and a 12 h light/dark cycle and allowed free access to a standard rodent chow diet and

water. All experiments were conducted in compliance with the relevant laws and institutional guidelines of Beijing Union Hospital. Eight-week-old male SD rats were randomly separated into two groups. MCT-PAH group rats were subcutaneously injected with monocrotaline (Sigma-Aldrich) 55 mg/kg, Control group rats were subcutaneously injected with an equal volume of vehicle (saline).

The details of randomization and determination of sample size are as follows. First, all rats were named (e.g. a1, b3,), and the animal names were entered into an Excel spreadsheet. Then we used the RAND () function to assign a random number to each animal name, and we sorted the animal names according to the sizes of their random numbers. After sorting in ascending order, the first 15 were selected as control group, and the last 15 were used as model group. The sample size was determined by a formula: $N = 2[(a + b)^2 \sigma^2]/(\mu1 - \mu2)^2$. We chose a power of 0.80, an alpha of 0.05, then it could be determined that a = 1.96, b = 0.842 according to the multipliers for conventional values of alpha and beta. Based on the results of our previous MCT-induced PAH rats, we chose 5 mmHg of RVSP as the minimal relevant difference, which means $\mu1 - \mu2 = 5$. And according to our previous results of MCT-induced PAH model, the standard deviation of RVSP is about 4, which means $\sigma = 4$. Therefore, we could get $N = 2[(1.96 + 0.842)^2*4^2]/5^2 = 10.049536$. In addition, MCT-induced PAH rats would have 30%-40% mortality rate based on experience, so we determined 15 as the sample size of each group.

3. Hematoxylin-Eosin (HE) & Immunofluorescence (IF) staining

We opened the chest of the rats and perfused the heart with saline injection (about 20

ml per rat) immediately after the rats were killed by carbon dioxide. After that, the rat lung tissue was harvested. Then lung tissue was perfused with formaldehyde by pulmonary artery injection, after which the lung tissue was fixed in formaldehyde and embedded in paraffin. After dewaxing, the rat lung slices were treated with routine operation of HE staining and imaged on a microscopy from ZEISS (Axio Lab, A1). As for IF staining and observation, after intravenous injection of EPCs (2×10^6 cells per rat), rats were killed by carbon dioxide at 1 h later. The following animal operations and perfusion methods were the same as above to get formalin-fixed and paraffin-embedded lung tissue sections. Slices for IF staining were treated with heat mediated antigen retrieval by 0.1 mol/L sodium citrate. The anti-human CD31 (Dako, M0823, 1:200) and anti-rat SM22α (Abcam, ab14106, 1:200) antibodies were used to detect the location of the transplanted EPCs in rat lungs. Alexa Fluor594 labeled anti-rabbit or Alexa Fluor488 labeled anti-mouse secondary antibodies (Invitrogen, 1:100) were subsequently applied. Nuclei were counterstained with DAPI reagent, and the slices were observed and imaged on an Olympus FV1000 confocal laser scanning microscopy.

4. Assessment of cell viability and proliferation

The viability of the labeled cells was assessed by trypan blue exclusion assay test at 0 h, 24 h, 48 h, 72 h and 96 h post-labeling. The culture medium was replaced daily and maintained at 37 °C and 5% CO_2. Unlabeled cells served as control. ^{89}Zr-EPCs were preserved in EPCs complete medium. Radio-iTLC and cell viability were detected after being placed at 2-8 °C for 13 h. To assess the effect of radiolabeling on cellular proliferation, CCK-8 assay (Dojindo Laboratories, Japan) was performed according to

the manufacturer's instruction. The labeled cells as well as the unlabeled cells were plated in 48-well plate in triplicate. The cell numbers were counted at 0 h, 24 h, 48 h, 72 h and 96 h after seeding.

5. Transwell assay

Transwell chamber: 24-well, 8.0 μm pore membranes (Corning USA) was used for the transwell migration assay. 4×10^4 EPCs per well were seeded in the upper chamber in 200 μL of serum-free medium (0.1% BSA), and 500 μL of basic medium contain 10% plasma (plasma from healthy rats, MCT rats, healthy volunteers or iPAH patients) was added to the lower chamber. After incubated for 4 h at 37 °C, the cells remaining at the upper surface of the membrane were removed by cotton swab, and the cells on the lower surface of the membrane were fixed with methanol and stained with 0.1% crystal violet solution, the cells that passed through the filter (the migrated cells) were photographed by inverted microscope. The light microscopy images were used to calculated the average number of migrated EPCs (5 fields were counted for each condition). After the microscopic observation, the crystal violet in the migrated cells was eluted by 33% acetic acid solution, the OD value (at 570 nm) of that acid solution could be used to indirectly represent the number of the migrated cells, the specific steps are as follows. Add 33% acetic acid solution to a new 24-well plate (1 ml per well). Then put the chambers into each well. After ten minutes of immersion, the crystal violet in the cells was eluted, removed the chambers from the 24-well plate. Detect the OD value of the acid solution in each well at 570 nm by microplate spectrophotometer. The results of microscope photo and OD values were combined to reflect changes in the number of

migrating cells.

6. Attachment assay

2×10^5 HPAECs per well were seeded in the 12-well plate with complete medium. Then, HPAECs were treated with basic medium containing different plasma (from healthy volunteers or iPAH patients) with or without A-205804 (100 nM) (specific inhibitor of E-selectin and ICAM-1, from TargetMol). After incubation for 4 h, 2×10^4 EPCs per well labeled with DiO (Invitrogen, V-22886) were added to the medium of HPAECs and mixed well. After 45 min incubation in 37 °C, upper medium and suspended cells were removed, all wells were washed with PBS twice. The attachment of EPCs and HPAECs were observed and imaged on a fluorescence microscope. The fluorescent and light microscopy images were used to calculated the average number of EPCs attached (5 fields were counted for each condition).