ONLINE SUPPLEMENTAL MATERIAL

Transplantation of mesenchymal stem cells attenuates pulmonary hypertension by normalizing the EndMT

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METHODS AND MATERIALS

Culture of mesenchymal stem cells (MSC) and isolation of MSC culture medium (MSC-CM)

Bone marrow derived GFP+ MSC of Sprague-Dawley (SD) rat (Cyagen Bioscience, China) were purchased at passage 2. As seem in Figure E1-A, the cells were positive for CD44 and CD29 and negative for CD34, CD45 and CD11b/c. Meanwhile, the cells could be induced to differentiate into osteogenic, chondrogenic and adipogenic cells under specific culture conditions according to previous studies (Figure E1-B). MSC were routinely cultured in Minimum Essential Medium α (MEMα; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin-streptomycin (Gibco, USA) in 5% CO_2-95% air incubator at 37°C. Cells at passage 3-6 were used for experiments. For the harvest of MSC-CM, when MSC grown to 70-80% confluence, replace the growth medium MEMα with 10% FBS with serum free basal MEMα, and subjected to hypoxia (3% O_2) for 48 hours. Then harvest the medium centrifuge at 1000g for 10 minutes, filtered with 0.22 μm filter and used as MSC-CM for the treatment of PMEC. Basically, a 2 ml MSC-CM was used for the treatment of a 35 mm culture dish with PMEC grown to 60% confluence. The PMEC were then exposed to normoxia or hypoxia (3% O2) for 7 days and subjected to the subsequent experiments.

Animal model

Male SD rats (203-233 g, 6-8 weeks) were purchased from Guangdong Provincial Medical Experimental Animal Centre and raised in the Specific Pathogen Free (SPF) grade animal room of the State Key laboratory of Guangzhou Medical University. Rats of CHPH model were housed in a hypoxic chamber (10% O_2) for 21 days as previous described. Rats of SuHx-PH model were subcutaneous injected with sugen (SU5416, 20mg/kg; MedChem Express, USA) at the first day during 3 weeks of exposure to hypoxia, followed by 2 weeks of exposure to normoxia. Four groups were divided in each experiment: (1) normoxia control group (NC group), (2) CHPH/SuHx-PH control group (CHPH/SuHx-PH group), (3) CH/SuHx + RF

group and (4) CH/SuHx + MSC group. Group (4) were intravenous injected with MSC (1×10^6 bone marrow derived MSC resuspended in 1 mL saline) via tail vein at Day 7 post hypoxic exposure, while group (3) were given the same amount of rat fibroblast and the other two groups were given the same volume of saline (Figure E2-A).

Hemodynamic parameters measurement and histological staining

Briefly, after the establishment and treatment of the animal models, rats were anesthetized with 3% pentobarbital sodium (30 mg/kg ip). An incision was made in the abdomen to expose the diaphragm. A 23-gauge hollow needle filled with 0.3% heparinized saline which was connected to a pressure transducer were inserted through the diaphragm into the right ventricle (RV). The pressure in RV was then recorded and measured with BIOPAC MP150 (BIOPAC systems, Inc., Santa Barbara, CA). Right ventricular hypertrophy was evaluated by the wet weight ratio of RV to left ventricle (LV) plus septum (S).

After the animals were sacrificed, the left lungs were isolated and fixed in 4% paraformaldehyde for 24 hours and then dehydrated and embedded in paraffin. The samples were cut into sections of 4 μm and stained with hematoxylin and eosin (H&E) for general histological examination and Masson's trichrome for fibrosis and collagen deposition. The vessel wall area, as well as the wall thickness of the 50 to 100μm (outer diameter) pulmonary arteries (PAs) were measured using Image-Pro Plus 6.0 software. Parafin-embedded, formalin-ixed tissues were immunostained for MMP2 (matrix metalloprotein-2) and MMP9 (matrix metalloprotein-9) protein using standard immunohistochemistry procedures according to the manufacturers' instruction.

Lung tissue immunofluorescence

Briefly, the pararffin embedded lung tissues were sectioned and deparaffinized in xylene followed by isopropanol dilutions. Antigen retrieval was performed in 10 mM Citrate Buffer. Tissue sections were blocked with antibody dilution buffer for 1 hour at room temperature, and then labeled with anti-vWF (von Willebrand factor, Santa Cruz) and anti-α-SMA (Sigma), anti-GFP (Sigma). The secondary antibodies included

Alexa Fluor 488-(Jackson, West Grove, PA, USA) and Cy3-(Jackson) conjugated secondary antibodies. Nucleus were stained with DAPI diluted in PBS for 10 min. The co-localization of endothelial and mesenchymal markers was detected by confocal microscopy (LSM 780, Carl Zeiss, Jena, Germany).

Flow cytometry analysis

Rats were anesthetized with 3% pentobarbital sodium (30 mg/kg i.p.). An incision was made in the abdomen to expose the diaphragm. Fill the syringe pump with ice cold sterile DPBS and attach an intravenous needle, then exhaust air. Insert the needle into the left ventricle of the heart and smoothly dispense 5 ml of DPBS into the heart. In the meantime, use tweezers to break the right atria to allow blood to drain from circulation. Remove the needle from the left ventricle and insert it into the right ventricle to perfuse the lung until it turns pale (5 ml).

Cut the heart away from the lung and then remove the lung from the thoracic cavity after cutting off the trachea and any remaining connective tissue. Place the lung into the well of a 12 well tissue culture plate filled with 2.5 ml of DPBS on ice. Rinse the lung with DPBS and transfer it into another well without DPBS. Mince the lung into very fine pieces using scissors. Pour off 5 ml of digestion buffer to a new well, resuspend the lung tissue with 2.5 ml digestion buffer with a 2.5 ml transfer pipette and transfer to a 15 ml conical tube. Rinse the well with the remaining 2.5 ml of digestion buffer, and pool the mixture in the 15 ml conical tube. Place tubes on a rocker with gentle rotation at 37°C for 30 min. Neutralize collagenase by adding 70μl of 0.5 M EDTA-Na2 (pH=8.0). Mix well by gentle invert and place the tubes on the ice for 5 min. Place a 70 μm cell strainer into a 60 mm tissue culture dish.

Pour the lung tissue in digestion buffer onto the cell strainer. Spin down lung cells for 5 min at 1,300 rpm at 4°C in a bucket table top centrifuge. Discard supernatant and resuspend the cells with 1 ml of ACK buffer, and then add another 2 ml. Gently rock the conical tube and incubated for less than 1 min at RT to lyse the remaining red blood cells. Neutralize the ACK buffer with 30 ml of ice cold DPBS (10 time of ACK volume in the tube). Spin down the cells for 5 min at 1,300 rpm at 4°C and resuspend the cells in 5 ml of ice cold complete RPMI 1640 medium. Count

the cells with trypan blue and a hemocytometer. Pass the cells through a 70 μm cell strainer into FACS tubes using a pipetman. Acquire FACS data using a flow cytometer and analyze data using Flowjo software.

Hydroxyproline assay

The protein levels of hydroxyproline in lung tissue homogenates were measured by hydroxyproline assay kits (Abcam). The determination of hydroxyproline is based on the oxidation to a pyrrole intermediate followed by reaction with Ehrlich's reagent dissolved in concentrated perchloric acid, where hydroxyproline gets oxidized to form a reaction intermediate, which further in reaction forms brightly-colored chromophore that can be detected at OD 560 nm.

The culture and treatment of rat pulmonary microvascular endothelial cells (PMEC)

After the animals were sacrificed, the heart and lungs of rat were removed from chests and transferred into a dish. Under a stereomicroscope, 2–5 g rat peripheral tissue was carefully dissected from the lungs within 30min. Adipose and connective tissue and any adventitia were carefully removed from the thoracic cavity. The tissue was digested at 37°C for 20min in M199 containing collagenase (type 1, 2mg/ml), neutral protease (0.6U ml-1). The cells were then centrifuged for 8min at 800 g. The re-suspended cells were incubated with Dynabeads (Invitrogen, CarIsbad, CA) bound with anti-PECAM-1 antibody for 1 hour. The cell-bead mixture was separated from the media by a magnetic particle concentrator (Invitrogen), and re-suspended and cultured in media (EGM-2 media that contained 2% fetal calf serum and growth factor bullet kit, Lonza). In order to validate the cultured PMECs, we have examined the cell purity with vWF staining (Figure E1-D). Cells with a positive staining rate of over 90% were used for further experiments. The cells at passage 4-6 were used for subsequent experiments.

Three groups were divided in the in vitro experiment: (1) normoxia control group (Nor+Veh group), (2) hypoxia control group (Hyp+Veh group) and (3) hypoxia + MSC-CM group (Hyp+MSC group). Group (3) were treated with MSC-CM (50%v/v) before hypoxia exposure, while the other two groups were

given the vehicle control (serum-free α-MEM). PMECs were collected 72 hours after hypoxia exposure. Please see Figure E2-B for the time course treatment detail.

RNA extraction, cDNA generation and quantitative PCR (qPCR)

Total RNA was isolated from frozen tissue and cells using TRIZOL reagent (Invitrogen, Carlsbad, CA) as previously described. The purity and quantity were assessed by a ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE).

For cDNA generation, 1 μg total RNA in a 20 μl volume was reverse transcribed using Takara RT kit (Takara, Dalian, China) incubated at 37°C for 15 min, 85°C for 5 seconds and 4°C forever. The synthesized cDNA was subjected to 1:10 dilution for qPCR.

qPCR was performed using Scofast TM EvaGreen SuperMix (Bio-Rad, Carlsbad, CA) in a CFX96 TM real-time system (Bio-Rad) according to the manufacturer's instruction. Negative controls contained water instead of cDNA. Quantitative normalization of cDNA in each tissue-derived sample was performed using expression of 18S rRNA as an internal control. The PCR protocol consisted of initial enzyme activation at 95°C for 3min, followed by 40 cycles at 95°C for 5s and at 60°C for 15s. The generated Ct value of each gene was normalized by its respective Ct value of 18S rRNA (ΔCt). The final fold expression changes were calculated using the equation $2^{-\Delta\Delta Ct}$.

Primer sequences were 5'-ATCCTGCCGATGTCGCTAT-3' (sense) and
5'-CCACAAGCGTGCTGTAGGT-3' (antisense) for collagen I;
5'-CTGGTCCTGTTGGTCCATCT-3' (sense) and
5'-ACCTTTGTCACCTCGTGGAC-3' (antisense) for collagen III; and
5'-GCAATTATTCCCCATGAACG-3' (sense) and
5'-GGCCTCACTAAACCATCCAA-3' (antisense) for 18s.

Western Blot

Both rat lung tissues and PMVECs were sonicated and lysed in RAPI lysis buffer (GBCBIO Technologies, China) containing 1% PMSF and protease inhibitor cocktail II and III. The total protein concentrations were measured by a BCA protein assay kit

(Pierce, Rockford, IL). After being denatured with the addition of a loading buffer and heating at 100°C for 10 min, protein expression was measured by immunoblotting assay as previously described. Briefly, Homogenate proteins were separated by electrophoresis using 10% SDS-PAGE calibrated with prestained protein molecular weight markers (Precision Plus, Bio-Rad, Carlsbad, CA). Separated proteins were transferred to polyvinylidene difluoride membranes (pore size 0.22 μM, Bio-Rad). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.2% Tween 20 for 1 h and then incubated overnight at 4℃ with primary antibodies. The membranes were then washed for 10 min 3 times and incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG for 1 h. The expression levels of the proteins were detected via an ECL reagent (Pierce, Rockford, IL) and analyzed via ImageJ Software. The specific primary antibodies we used were FN1 (Proteintech), PECAM-1 (Proteintech), vimentin (Proteintech), VE-cadherin (Invitrogen), MMP2 (Abcam), MMP9 (Abcam) and β-actin (Santa Cruz), Snail (Proteintech).

Cell migration assay

A count of 50,000 cells were seeded onto the top of a polycarbonate filter with 8-μm pores in 4 ml of basal medium. The cells were incubated for 24 h under normoxia or hypoxic conditions. Following exposure, the cells were fixed in 95% ethanol for 10 min and stained with brilliant blue (Pierce) for 5 min. Cells were visualized via microscope mounted camera and Q-capture software. For each filter, five randomly chosen fields were imaged to obtain a total cell count. Unmigrated cells were then scraped off the top of the filter and the migrated cells (bottom layer) were imaged. Migration was calculated as the percent of cells migrated to total cells number on the filter.

Reference:

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2. Lu W, Wang J, Shimoda LA, Sylvester JT. Differences in STIM1 and TRPC expression in proximal and distal pulmonary arterial smooth muscle are associated with differences in Ca2+ responses to hypoxia. *American journal of physiology Lung cellular and molecular physiology* 2008; 295: L104-113.
3. Wang J, Weigand L, Lu W, Sylvester JT, Semenza GL, Shimoda LA. Hypoxia inducible factor 1 mediates hypoxia-induced TRPC expression and elevated intracellular Ca2+ in pulmonary arterial smooth muscle cells. *Circ Res* 2006; 98: 1528-1537.
4. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic acids research* 2001; 29: e45.

Figure legends

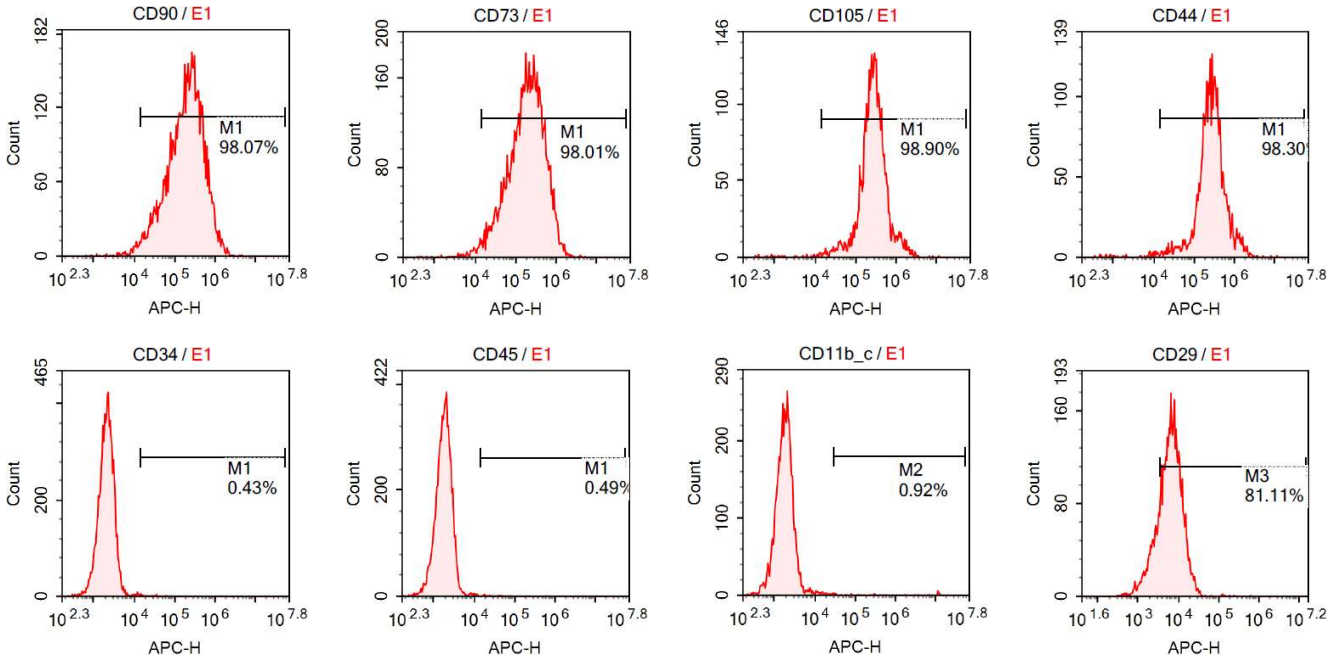
Figure E1. The validation of MSC and PMVEC.

Figure E2. The detailed exposure and treatment procedures of the MSC for both *in vivo* and *in vitro* design.

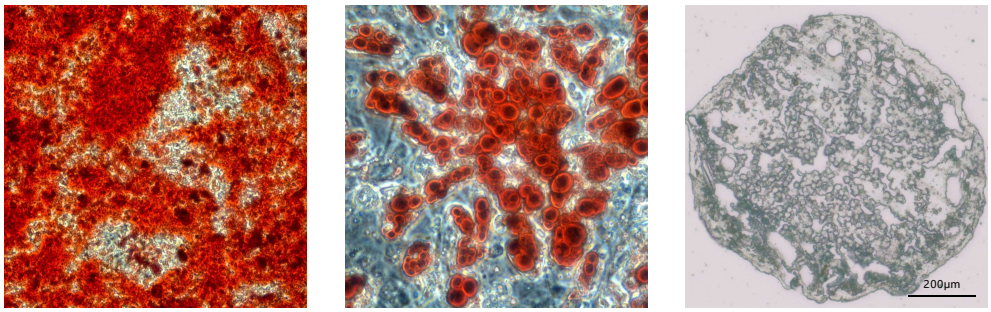
Figure E3. The intervention role of MSC transplantation on established SuHx-PH rat model.

Figure E4. The protective role of MSC transplantation on the protein expression of IL-RA, BMPR2 and TGF-β1 in the lung tissues of both CHPH and SuHx-PH rats.

A

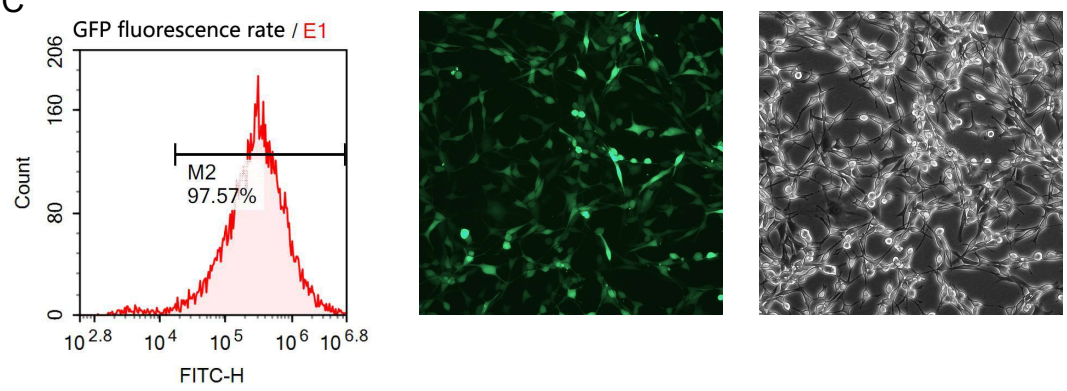


B

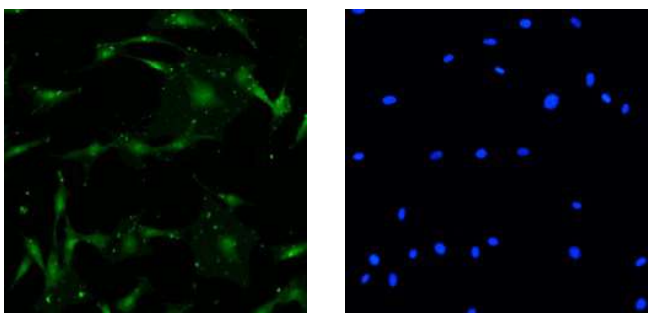


Osteogenesis Adipogenesis Chondrogenesis

C



D



vWF DAPI

Figure E1

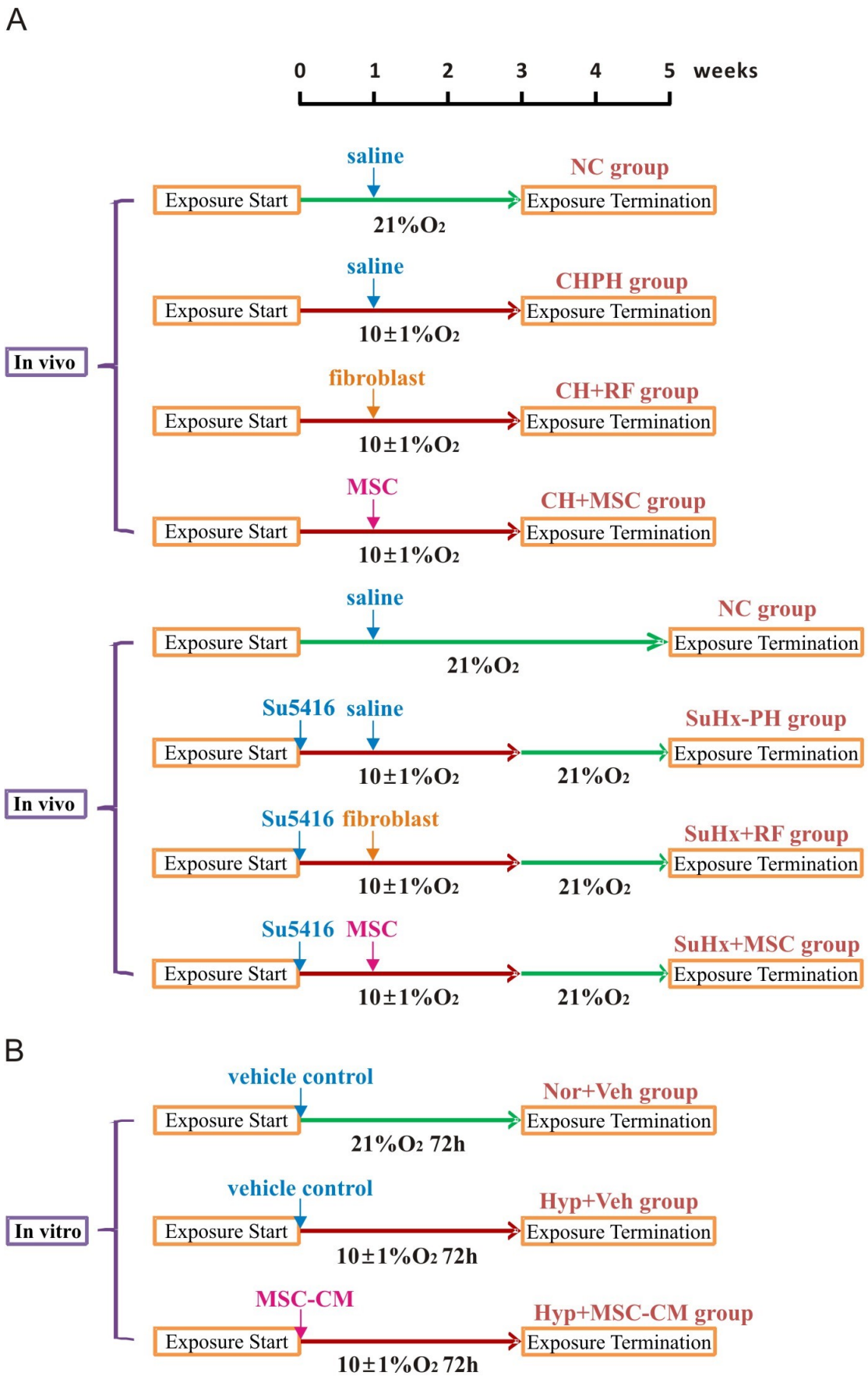
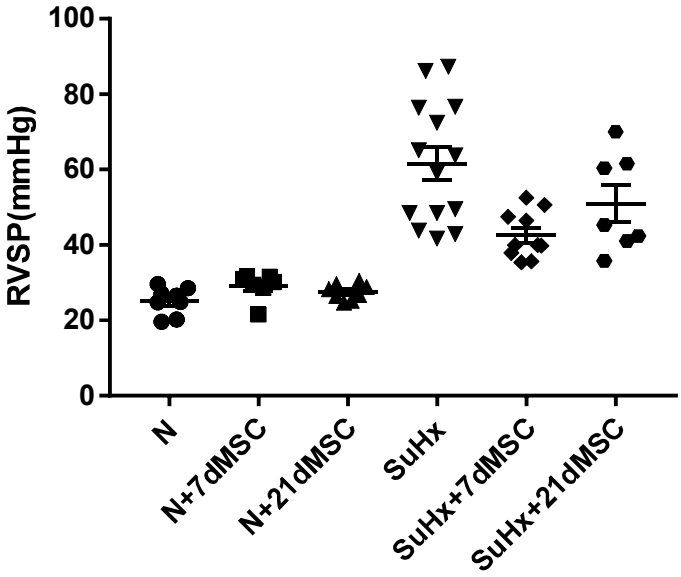
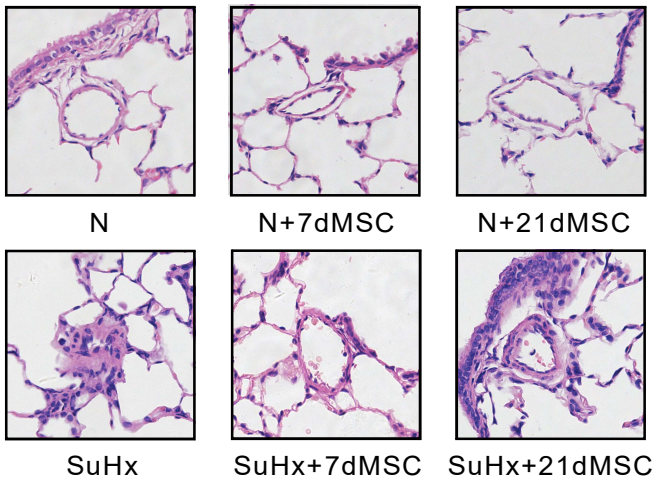
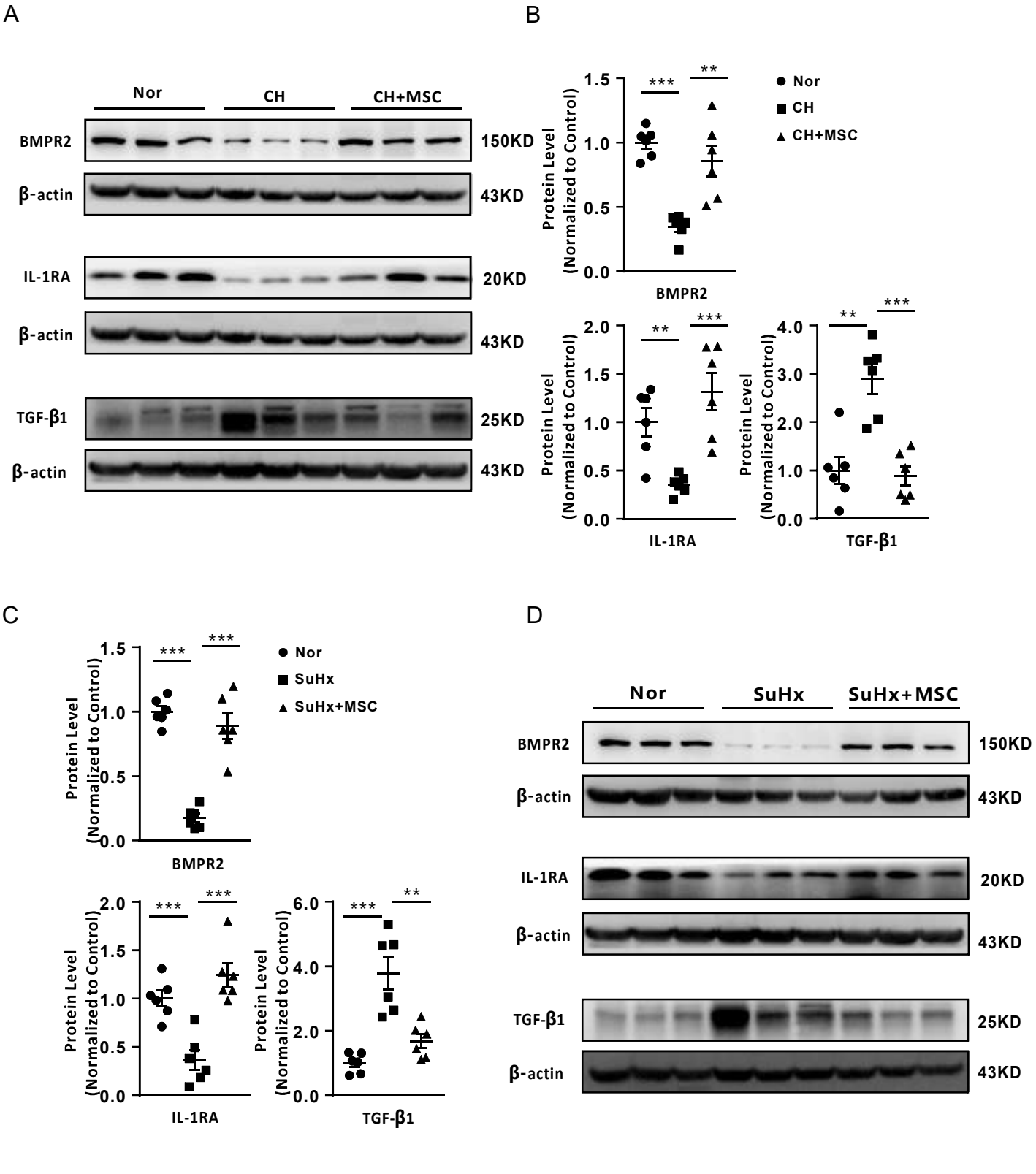

A
0 1 2 3 4 5 weeks
In vivo
saline
NC group
Exposure Start
Exposure Termination
21%O2
saline
CHPH group
Exposure Start
Exposure Termination
10±1%O2
fibroblast
CH+RF group
Exposure Start
Exposure Termination
10±1%O2
MSC
CH+MSC group
Exposure Start
Exposure Termination
10±1%O2
In vivo
saline
NC group
Exposure Start
Exposure Termination
21%O2
Su5416 saline
SuHx-PH group
Exposure Start
Exposure Termination
10±1%O2
21%O2
Su5416 fibroblast
SuHx+RF group
Exposure Start
Exposure Termination
10±1%O2
21%O2
Su5416 MSC
SuHx+MSC group
Exposure Start
Exposure Termination
10±1%O2
21%O2
B
In vitro
vehicle control
Nor+Veh group
Exposure Start
Exposure Termination
21%O2 72h
vehicle control
Hyp+Veh group
Exposure Start
Exposure Termination
10±1%O2 72h
MSC-CM
Hyp+MSC-CM group
Exposure Start
Exposure Termination
10±1%O2 72h

Figure E2


RVSP(mmHg)
100
80
60
40
20
0
N
N+7dMSC
N+21dMSC
SuHx
SuHx+7dMSC
SuHx+21dMSC


N
N+7dMSC
N+21dMSC
SuHx
SuHx+7dMSC
SuHx+21dMSC


A
Nor
CH
CH+MSC
BMPR2
150KD
β-actin
43KD
IL-1RA
20KD
β-actin
43KD
TGF-β1
25KD
β-actin
43KD
B
Nor
CH
CH+MSC
Protein Level (Normalized to Control)
BMPR2
IL-1RA
TGF-β1
C
Nor
SuHx
SuHx+MSC
Protein Level (Normalized to Control)
BMPR2
IL-1RA
TGF-β1
D
Nor
SuHx
SuHx+MSC
BMPR2
150KD
β-actin
43KD
IL-1RA
20KD
β-actin
43KD
TGF-β1
25KD
β-actin
43KD