SUPPLEMANTARY INFORMATION

Plasma Exosomes Confer Hypoxic Pulmonary Hypertension by Transferring LOX-1 Cargo

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SUPPLEMENTARY MATERIALS AND METHODS

1. Histological and immunofluorescence (IF) analysis

For histological analysis, the lung tissues were fixed in 4% paraformaldehyde, embedded in parafilm, and routinely cut into 3μm thickness. The slices were stained with hematoxylin-eosin and were examined with an Olympus microscope to visualize the morphology of pulmonary arteries. The percentage of medial wall thickness (WT%) and the percentage of medial wall area (WA%) were determined. WT% = (External diameter - Internal diameter)/External diameter ×100%; WA% = (Medial WA)/(Total vessel area) ×100%. For IF analysis, the slices were deparaffinized and incubated in citrate buffer (pH7.4) for 20 min in boiling water for antigen retrieval. Then, 0.1% triton was used to permeate cell membrane and nuclear membrane for 10 min. After 5% BSA block, the slices were incubated with specific primary antibodies anti-SM22α (1:200; Santa Cruz) and anti-PCNA (1:200; Proteintech) at 4 ℃ overnight. Incubation with Cy3-labeled goat anti-rabbit IgG (Beyotime, China) and FITC-labeled goat anti-mouse IgG (Beyotime, China) at room temperature for 1 h. Then, the sections were stained with DAPI for 5 min (Beyotime, China). Finally, the sections were imaged under fluorescence microscopy (Olympus IX71, Tokyo, Japan).

2. Cell viability assay

The cell viability assay was measured by using a colorimetric method to determine the cytotoxic and proliferative effect of SCH772984 at various concentrations. PASMCs were plated into 96-well flat bottom culture plates before SCH772984 treatment. After 24 h, assays were performed by adding 10 μl of CCK8 (Beyotime, China) to each well. The plate was incubated for additional 1 h at 37 ℃, and then the absorbance at 450 nm was measured with a 96-well microplate reader. The results were presented as the mean of

five independent experiments.

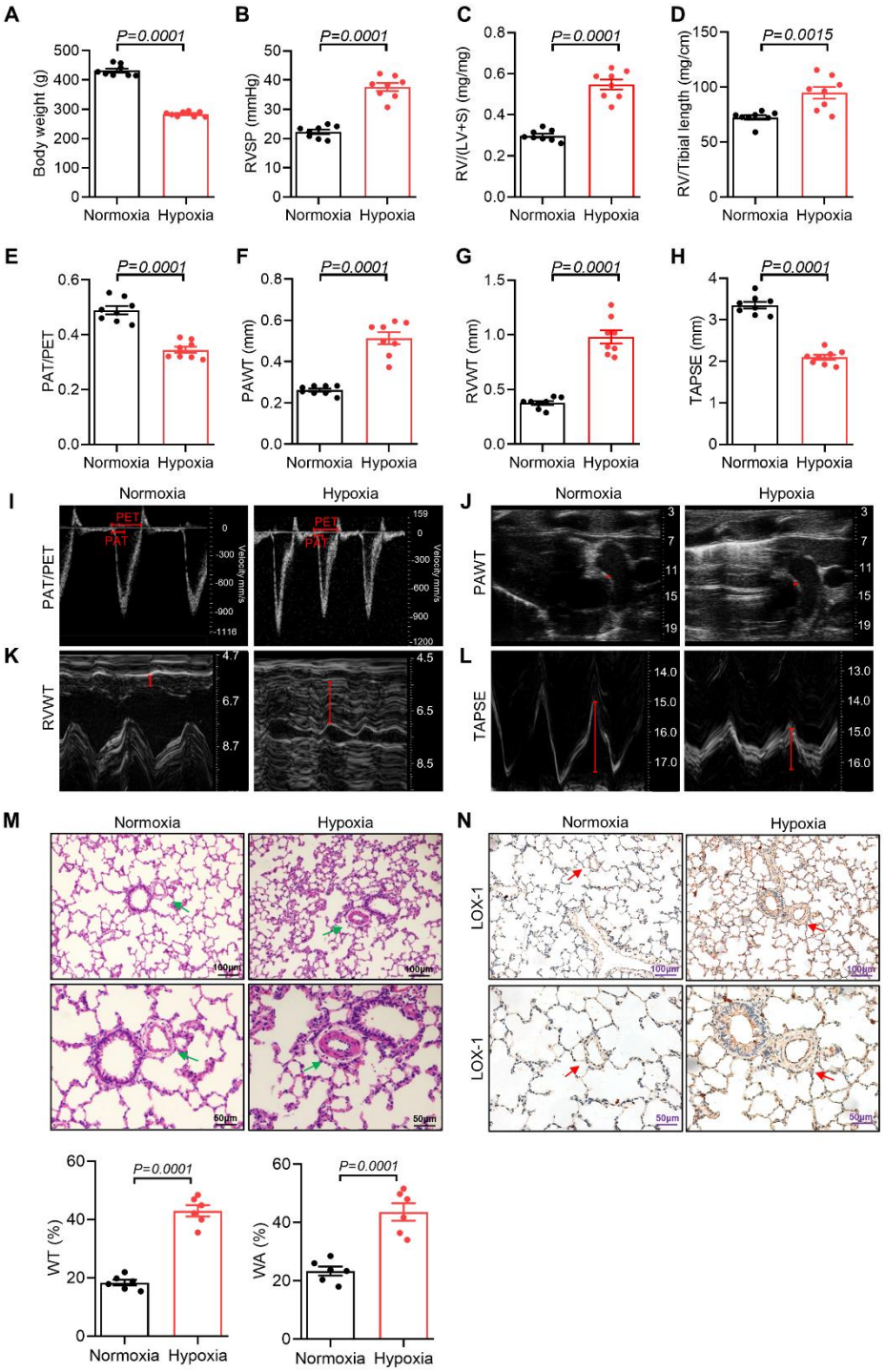
3. Immunofluorescence

PASMCs were plated into 96-well flat bottom culture plates and treated as designed for 24 h. Then, the PASMCs were incubated with primary antibody at 4℃ for 16 h, followed by a secondary antibody. The cells on plates were imaged by fluorescence microscopy. Primary antibodies used in this set of experiments are: anti-α-SMA (1:200; Santa Cruz), anti-PCNA (1:200; Proteintech), anti-KLF4 (1:100; Abcam).

4. Western blot analysis

Proteins were extracted from exosomes derived from 5 ml plasma, pulmonary arteries or PASMCs lysates with RIPA buffer (containing 1% PMSF) on ice for 30 min. The lysates were centrifuged at 12,000 g for 15 min at 4 ℃. BCA Protein Assay Kit (Beyotime, China) was used to determine the total protein concentrations. Then, equal amounts of protein from each sample were separated by 10% sodium dodecyl sulfate polyacrylamide gels and transferred to polyvinylidene fluoride membrane. The membranes were blocked with 5% non-fat milk for 1 h under room temperature and incubated with primary antibodies at 4℃ for 16 h. The membranes were washed with PBS thoroughly and then incubated with secondary antibodies for 1 h at room temperature. The target proteins were detected by Easy See Western Blot Kit (Beijing TransGen Biotech, Beijing, China) according to the manufacturer's recommendations. Image J 1.43 software (National Institutes of Health, New York City, NY, USA) were used for densitometric analysis. Primary antibodies used in these experiments are: anti-LOX-1 (1:1000; Abcam), anti-CD9 (1:2000; Abcam), anti-α-SMA (1:2000; CST), anti-SM22α (1:2000; CST), anti-PCNA (1:2000; Proteintech), anti-p-ERK1/2 (1:2000; ABclonal), anti-ERK1/2 (1:200; ABclonal), anti-KLF4 (1:1000; Abcam), anti-β-actin (1:5000; ABclonal), anti-Tubulin (1:5000; ABclonal).

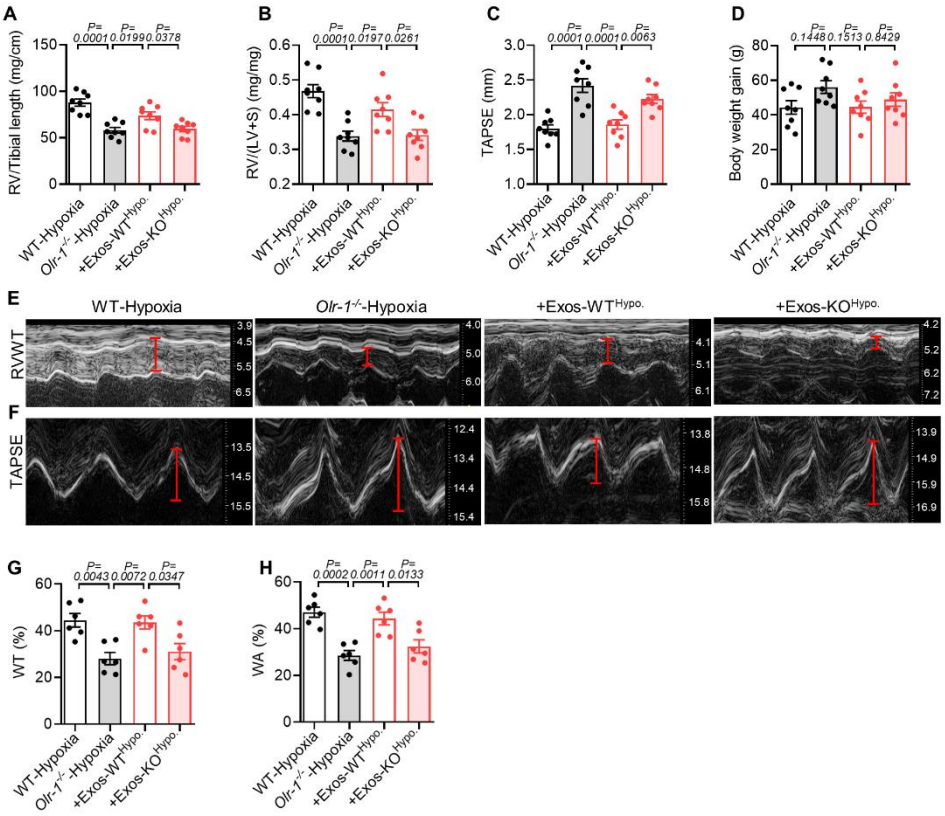
SUPPLEMENTARY FIGURES



Supplementary Fig. 1. Hypoxia resulted in PH in rats; related to Fig. 1

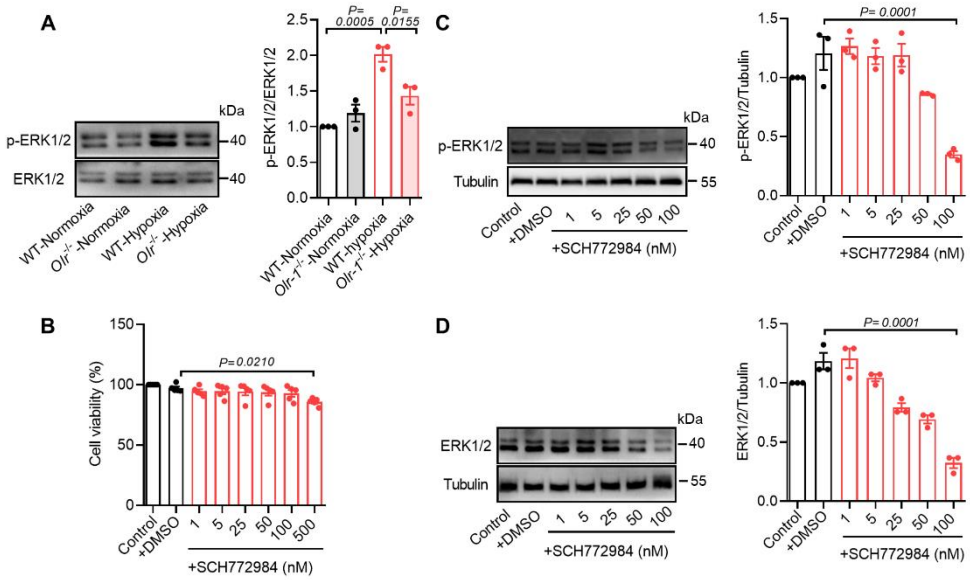
(A) The body weight of rats; **(B)** RVSP; **(C, D)** The ratio of RV weight to that of tibial length and LV plus IS; **(E)** PAT/PET; **(F)** PAET; **(G)** RVWT; **(H)** TAPSE; **(I)**The

representative echocardiographic images of PAT/PET; **(J)** The representative echocardiographic images of PAWT; **(K)** The representative echocardiographic images of RVWT; **(L)** The representative echocardiographic images of TAPSE; **(M)** Hematoxylin-eosin staining of lung tissues; **(N)** The expression of LOX-1 protein in pulmonary arteries was analyzed by immunohistochemistry. All values are expressed as mean ± S.E.M. n=6-8.



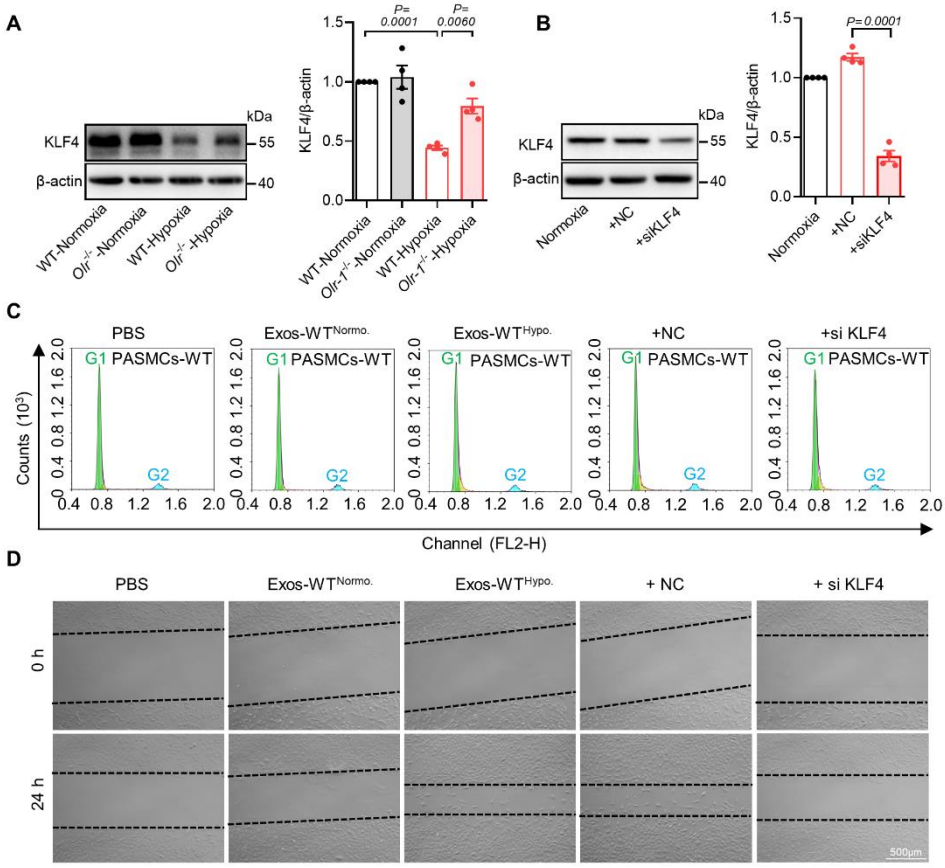
Supplementary Fig. 2. Effect of plasma exosomal LOX-1 on hypoxia-induced PH in *Olr*$^{-/-}$ rats; related to Fig. 3

(A, B) The ratio of RV weight to that of tibial length and LV plus IS; **(C)** TAPSE; **(D)** The body weight gain; **(E)** The representative echocardiography images of RVWT; **(F)** The representative echocardiographic images of TAPSE; **(G)** WT%; **(H)** WT%; All values are expressed as mean ± S.E.M. n=6-8.



Supplementary Fig. 3. p-ERK1/2 and ERK1/2 expression in the pulmonary arteries of hypoxic *Olr*$^{-/-}$ rats and the effect of SCH772984 on the cell viability and the expression of p-ERK1/2 and ERK1/2 in PASMCs; related to Fig. 5

(A) The expression of p-ERK1/2 and ERK1/2 in pulmonary arteries was analyzed by Western blotting (n=3); **(B)** Effect of SCH772984 on PASMCs viability measured by CCK8 (n=5); **(C)** The expression of p-ERK1/2 in PASMCs was analyzed by Western blotting (n=3); **(D)** The expression of ERK1/2 in PASMCs was analyzed by Western blotting (n=3); All values are expressed as mean ± S.E.M.



Supplementary Fig. 4. The expression of KLF4 in the pulmonary arteries of hypoxic *Olr*$^{-/-}$ rats and the effect of siKLF4 on the proliferation and migration of PASMCs; related to Fig. 6

(A) The expression of KLF4 in pulmonary arteries was analyzed by Western blotting (n=4); **(B)** The expression of KLF4 in PASMCs was analyzed by Western blotting (n=3); **(C)** Flow cytometry to evaluate PASMCs proliferation; **(D)** PASMCs migration was evaluated by scratch wound healing assay; All values are expressed as mean ± S.E.M.