ONLINE SUPPLEMENT

Left ventricular failure produces profound lung remodeling and pulmonary hypertension in mice: heart failure causes severe lung disease

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Expanded Methods

Induction of PAH with TAC in mice: Male mice at age 10-14 weeks were used for the minimally invasive TAC procedure as we have previously described. [1,2] Briefly, the mice are anesthetized with a mixture of 80 mg/kg ketamine and 30 mg/kg xylazine (i.p.). The neck and upper ventral chest are shaved, and the mice placed in the supine position. A horizontal incision 5 mm in length is made at the level of the suprasternal notch to allow direct access to the transverse aorta without entering the pleural space. Aortic constriction is performed by ligating the aorta between the right innominate artery and the left carotid artery over a 26-gauge needle using 5.0 silk suture with the aid of a dissecting microscope. The needle is then quickly removed, leaving the constriction in place. The skin is closed using wound clips. Final studies were performed at 8 weeks after TAC.

Induction of PAH with hypoxia in mice: Male C57B6J mice at age 10-14 weeks were exposed to hypobaric hypoxia as previously described. [3,4] Briefly, the pressure in the chamber was decreased progressively from 0.8 atm (16.9% O2) on Day 1 to 0.5 atm (10.5% O2) after Day 7, and was maintained at 10.5% O2. After exposure to 10.5% O2 for 2 more weeks, mice were removed from the hypoxia chamber for determination of right ventricular (RV) pressure and hypertrophy. The sham mice were kept in normobaric conditions.

Measurements of aortic pressure, LV and RV hemodynamics: At the end of the study protocol at four weeks, mice were anesthetized with 1.5% isoflurane. They were intubated with a 20-gauge Teflon tube attached to a MiniVent type 845 mouse ventilator (Hugo Sachs Elektronik). [10]

A 1.2-F pressure catheter (Scisense Inc. Ontario Canada) was introduced through the right common carotid artery into the ascending aorta for measurement of systolic and diastolic blood pressures, and LV hemodynamics as described previously. [1,2] For RV hemodynamics, open-chest RV catheterization was performed during anesthesia with 1.5% isoflurane. [3,5] Data were collected when steady state was reached.

Sample Preparation: After the final hemodynamic assessment, the mice were euthanized by exsanguination, and the heart, lung, and other major organs were harvested. The wet weight of RV and of left ventricle (LV) + septum (S) were weighed and the ratio of RV weight to LV + S was calculated as an index of RV hypertrophy. [3,6,7] Lung wet weight was determined and the left lung was snap-frozen in liquid nitrogen for biochemical analysis. The airways of the upper right lobe were subsequently perfused and fixed in 10% buffered formalin for histological analysis. Visual comparable inflation of the lung lobes were observed in all tissues.

Histological staining: The relative pulmonary vascular muscularization was determined under H&E staining. Briefly, in each mouse, 60 intra-acinar arteries were examined and categorized as nonmuscular (NM), partially muscular (PM) or fully muscular (FM). The relative percentage of NM, PM and FM arteries was calculated. Lung fibrosis was stained using Masson's Trichrome Stain Kit from Sigma-Aldrich. Heart sections were stained with Sirius Red (Sigma) for fibrosis, and FITC conjugated wheat germ agglutinin (AF488, Invitrogen, Carlsbad, CA) to evaluate myocyte size using methods as previously described. [1,2,3,8]

In addition, lung sections were stained with monoclonal antibodies to identify smooth muscle cells or myofibroblasts (using an antibody anti-mouse smooth muscle α-actin, Sigma-Aldrich), [2] macrophages (using an antibody against Mac-2, clone M3/38; Accurate Chemical, Westbury),[9] and neutrophils (using anti-mouse neutrophil antibody clone 7/4; Accurate Chemical).[9] Briefly, tissue sections (5μm) were deparaffinized, rehydrated and antigen recovered in Tris-EDTA buffer (pH=9.0) for 30 minutes at 95-100°C before being washed in PBS. The sections were incubated with 3% H_2O_2 in PBS for 20 minutes, followed by 3% BSA solution for 1 hour. Sections were then incubated with corresponding monoclonal primary antibodies (1:400) overnight at 4°C, and followed with avidin/biotin peroxidase-linked secondary antibody (1:1000) (Invitrogen).[9] Staining was visualized by using an avidin/biotin peroxidase-linked detection system (Vector Laboratories, Burlingame, CA).

Measure lung water content: Lung (lower right lobe) wet weight was first determined. The lung tissue was then dried at 58°C to a constant weight. The dry weight of lung tissue was then determined. The relative water content of lung tissue was calculated using the following equation: Lung water content = (lung wet weight – lung dry weight)/lung wet weight X 100%.

Quantitative RT-PCR: Isolation of total RNA from lung and determination of cDNA synthesis by reverse transcription and quantitative real-time PCR were performed as described previously.[3] Briefly, total RNA was isolated from lung tissue and reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR reaction was performed using the Fast SYBR® Green Master Mix (Applied Biosystems). Results were normalized to 18S rRNA levels. The sequences of the primers are provided in **Table S1**.

Statistical Analysis: Values of each group are expressed as mean ± standard error. Data of two groups was compared with unpaired t-test. One-way ANOVA was used to test for differences among groups. If analysis of variance demonstrated a significant effect, *post hoc* pair-wise comparisons were made using the Fisher least significant difference test. Statistical significance was defined as $p<0.05$.

References

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Table S1. Primers for real-time RT-PCR products.

Genes	Sense	Antisense
TGF-β	5’-CTGATACGCCTGAGTGGCTGTC-3’	5’-AGCGAAAGCCCTGTATTCCG-3’
Collagen I	5’-CATAAAGGGTCATCGTGGCT-3’	5’-TTGAGTCCGTCTTTGCCAG-3’
Collagen III	5’-GAAGTCTCTGAAGCTGATGGG-3’	5’-TTGCCTTGCGTGTTTGATATTC-3’
TNF-α	5’-TGGGCTCCCTCTCATCAGTTC-3’	5’-GGCTACGGGCTTGTCACTCG-3’
MCP-1	5’-CAAGATGATCCCAATGAGTCG-3’	5’-CTTCTTTGGGACACCTGCTGCT-3’
TLR4	5’-TTCAGAACTTCAGTGGCTGG-3’	5’-TGTTAGTCCAGAGAAACTTCCTG-3’
IL-1β	5’-CCTCTCAAGCAGAGCACAGACC-3’	5’-TGGTGAAGTCAACTATGTCCCG-3’
VCAM	5’-ACAAAGGCAGAGTACACAGAC-3’	5’-CACAGGATTTTGGGAGTTGG-3’
ICAM	5’-GAGAAGTTGGACAGAACCCTG-3’	5’-GTTACTTGGTCCCCTTCTGAG-3’
18S	5’-AGTCCCTGCCCTTTGTACACA-3’	5’-CGATCCGAGGGCCTCACTA-3’
GAPDH	5’-TCCTGCACCACCAACTGCTTAG-3’	5’-GATGACCTTGCCCACAGCCTTG-3’

Table S2. Anatomic data of control, M-HF, HF and hypoxia groups.

Parameters	Control	M-HF	HF	Hypoxia
Number of mice	10	11	10	11
Bodyweight (g)	28.0±0.88	29.3±0.67	27.1± 0.69	28.0 ±0.48
Tibia length (mm)	17.9±0.10	18.1±0.08	18.1±0.10	17.9±0.05
Left ventricular + septum weight (mg)	97.3±2.36	162±8.93*	187±4.87*†	99.6±4.01
Left atria weight (mg)	3.17±0.17	9.54±1.12*	16.3±2.53*†	3.21±0.13
Lung mass (mg)	139±2.88	185±16.1*	395±23.4*†	187±3.00*
Right ventricular weight (mg)	21.9±0.79	26.9±1.96	38.8±1.1.93*†	34.6±2.04*
Right atria weight (mg)	3.61±0.18	4.61±0.47	6.01±0.57*†	3.55±0.15
Ratio of RV weight to LV weight	0.23±0.01	0.165±0.01*	0.21±0.01†	0.35±0.01*
Ratio of RA weight to LA weight	1.19±0.01	0.52±0.05*	0.49±0.07*	1.13±0.07
Ratio of left ventricular weight to body weight (mg/g)	3.48±0.06	5.50±0.21*	6.87±0.13*†	3.55±0.10
Ratio of LA weight to body weight (mg/g)	0.11±0.01	0.32±0.03*	0.59±0.09*†	0.12±0.01
Ratio of lung weight to body weight (mg/g)	4.99±0.12	6.33±0.18*	14.7±0.89*†	6.68±0.09*
Ratio of right ventricular weight to body weight (mg/g)	0.79±0.04	0.91±0.06	1.43±0.08*†	1.23±0.06*
Ratio of RA weight to body weight (mg/g)	0.13±0.01	0.15±0.02	0.22±0.02*†	0.13±0.01
Ratio of left ventricular weight to tibia length (mg/mm)	5.44±0.11	8.96±0.48*	10.4±0.28*†	5.56±0.23
Ratio of LA weight to tibia length (mg/mm)	0.18±0.01	0.53±0.06*	0.90±0.14*†	0.18±0.01
Ratio of lung weight to tibia length (mg/mm)	7.78±0.14	10.2±0.87*	21.9±1.32*†	10.4±0.18*
Ratio of right ventricular weight to tibia length (mg/mm)	1.22±0.04	1.49±0.11	2.16±0.11*†	1.94±0.12*

*p<0.05 as compared with corresponding control conditions; † p<0.05 as compared with LVH.

Table S3. Hemodynamic of mice in control group, mild heart failure group (M-HF), severe heart failure group (HF), or hypoxia group.

parameters	Control	M-HF	HF	Hypoxia
Number of mice	6	10--12	5--8	7-10
Heart rate (beats per minute)	518±11.2	553±10.1	562±10.5	553±10.1
RV systolic pressure (mmHg)	21.3±1.02	38.7±3.54*	55.1±8.46*†	43.0±1.52*
RV end diastolic pressure (mmHg)	1.25±0.11	3.15±1.06	8.37±5.01*†	2.63±0.18*
LV systolic pressure (mmHg)	117±1.36	171±4.52*	149±9.75*†	117±5.3
LV end diastolic pressure (mmHg)	6.12±0.90	11.7±2.12*	24.5±1.59*†	8.17±4.0

*p<0.05 as compared with corresponding control conditions; † p<0.05 as compared with M-HF.

Expanded Results

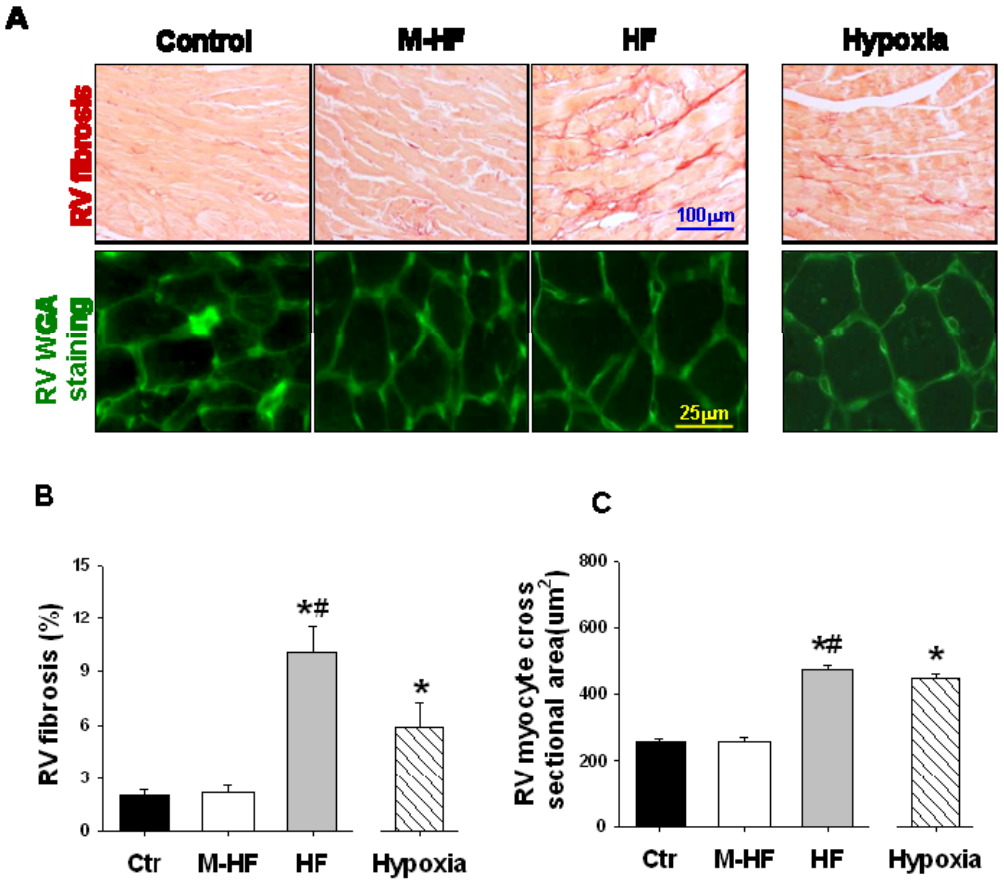

Figure S1. Relative RV fibrosis and RV cardiac myocyte hypertrophy in 4 experimental groups. Increased RV fibrosis is observed in hypoxia group and HF group (A,B). Increased RV cardiac myocyte size in hypoxia group and HF group (A, C). *p<0.05 vs control group; #p<0.05 vs. corresponding M-HF group.

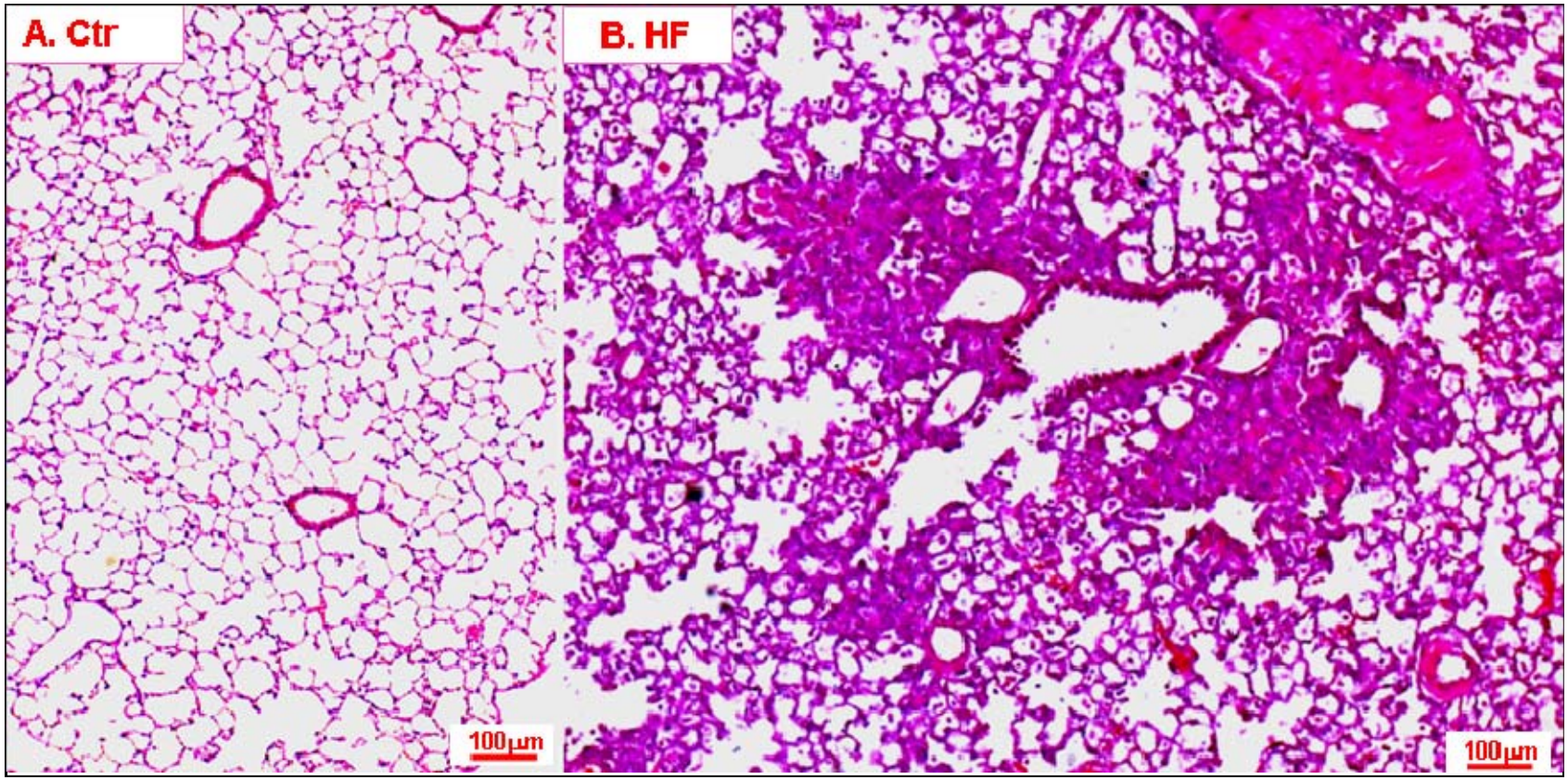

Figure S2. Representative H&E stained lung section shows increased pulmonary vascular wall thickness, and focal collapsed lung tissue in a HF mouse relative to control.

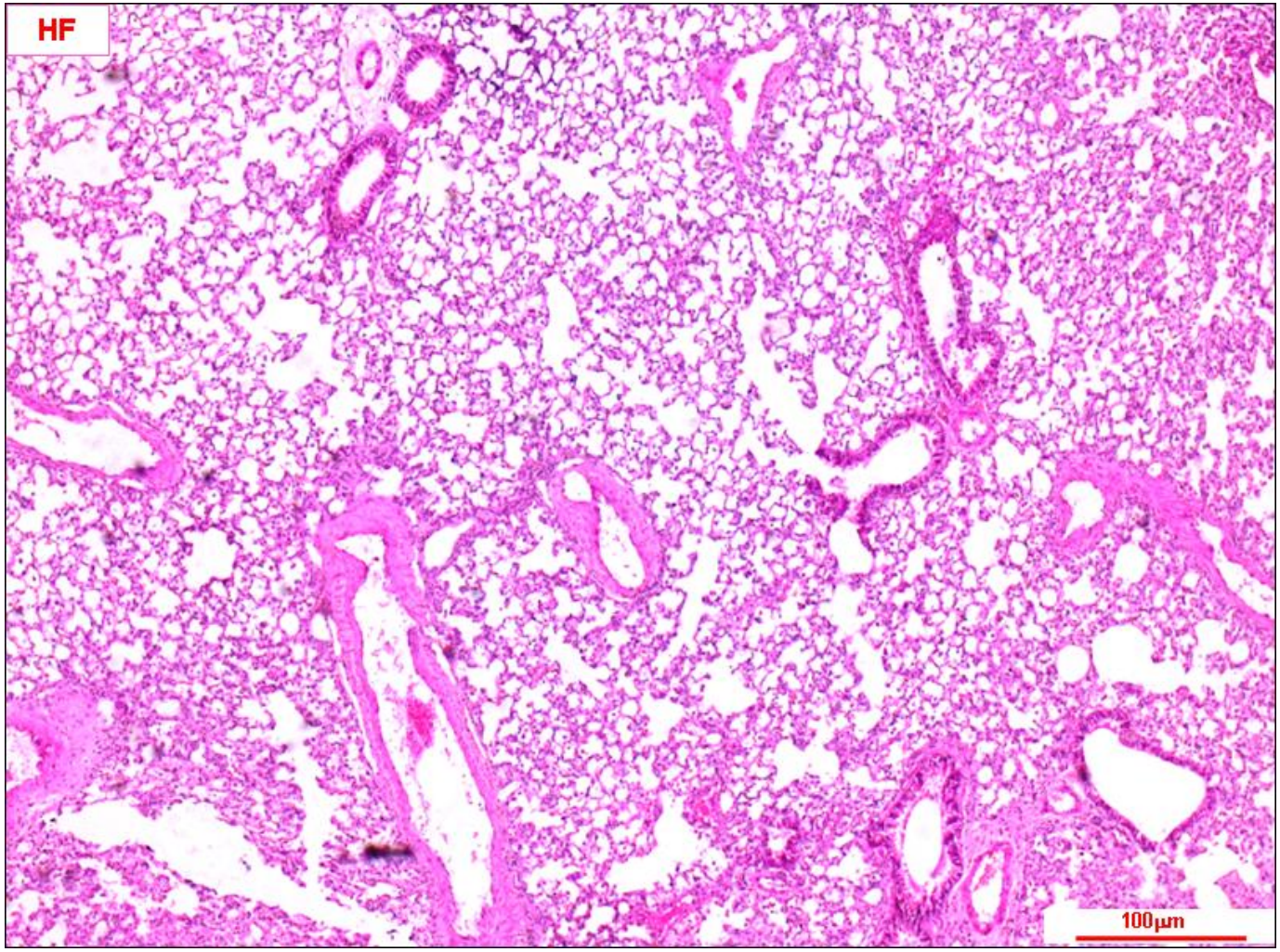

Figure S3. Representative H&E stained lung section shows marked pulmonary vascular remodeling in a HF mouse.

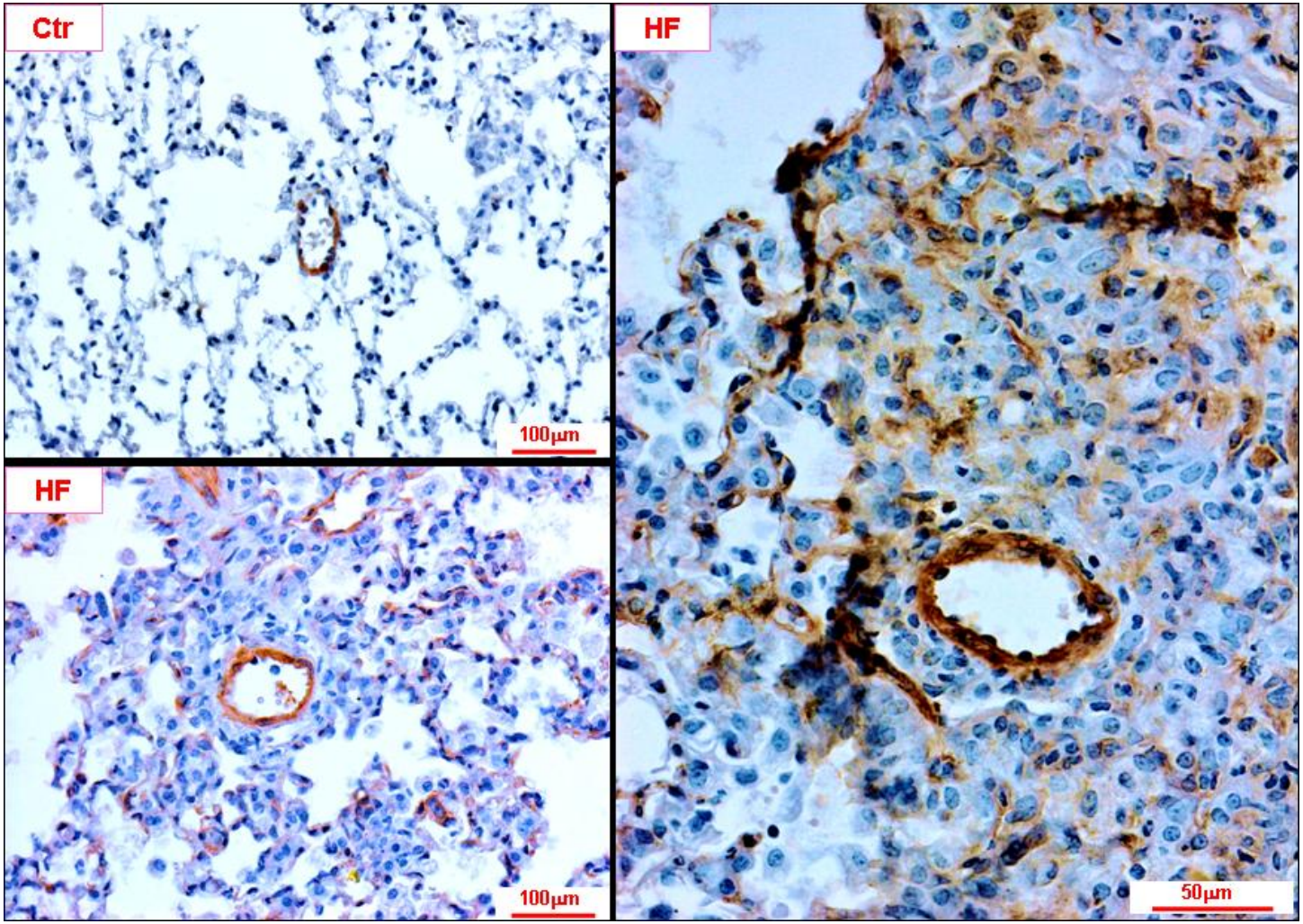

Figure S4. Representative lung sections show increased staining for smooth muscle α-actin in pulmonary vessels and collapsed lung tissue in HF mice, indicating proliferation of smooth muscle cells and myofibroblasts in lung tissues.

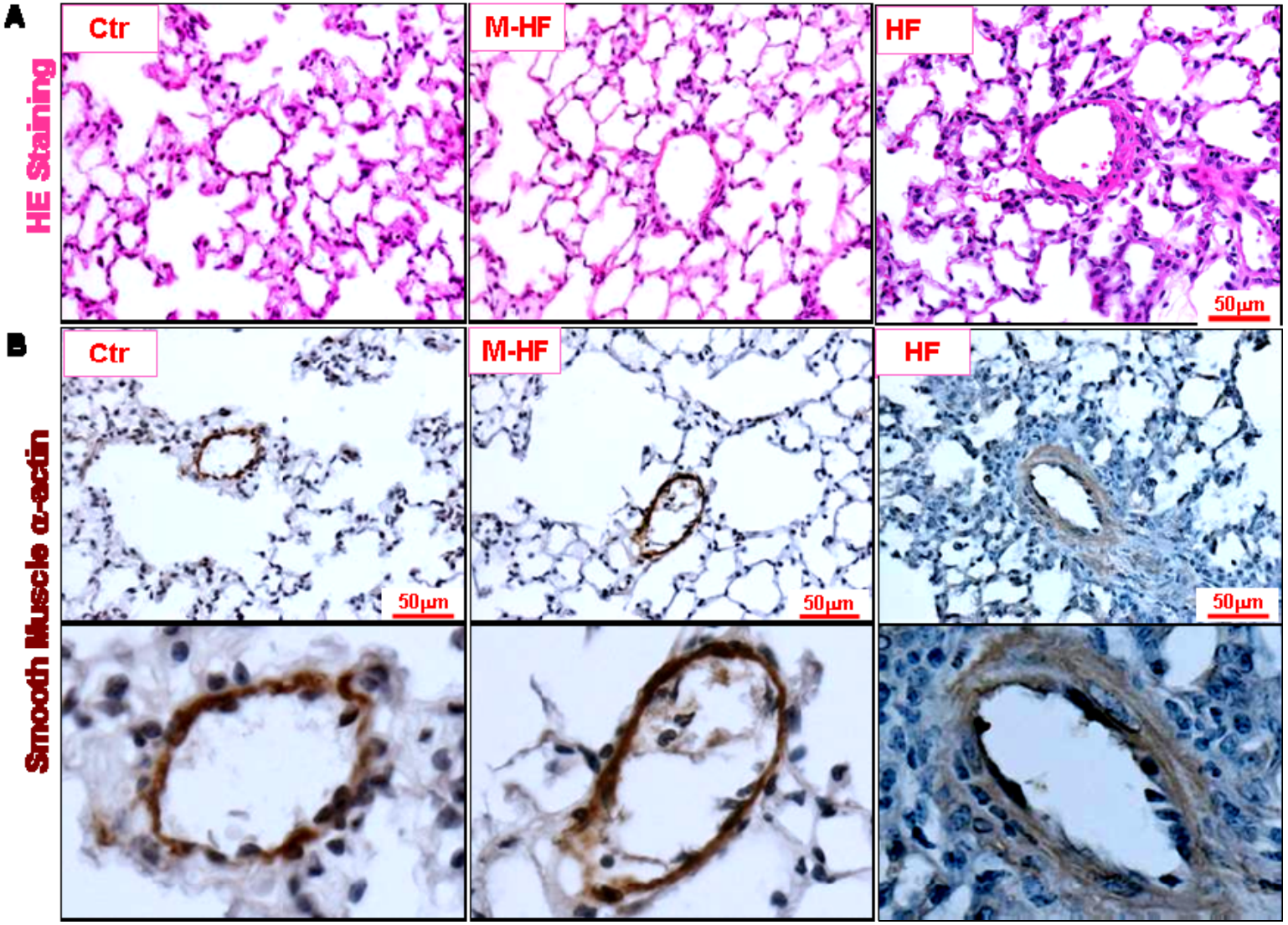

Figure S5. (A) Representative H&E stained sections show small arteries in control, M-HF and HF mice. (B) Representative staining of smooth α-actin shows increased proliferation of smooth muscle cells and/or myofibroblast in HF mice.

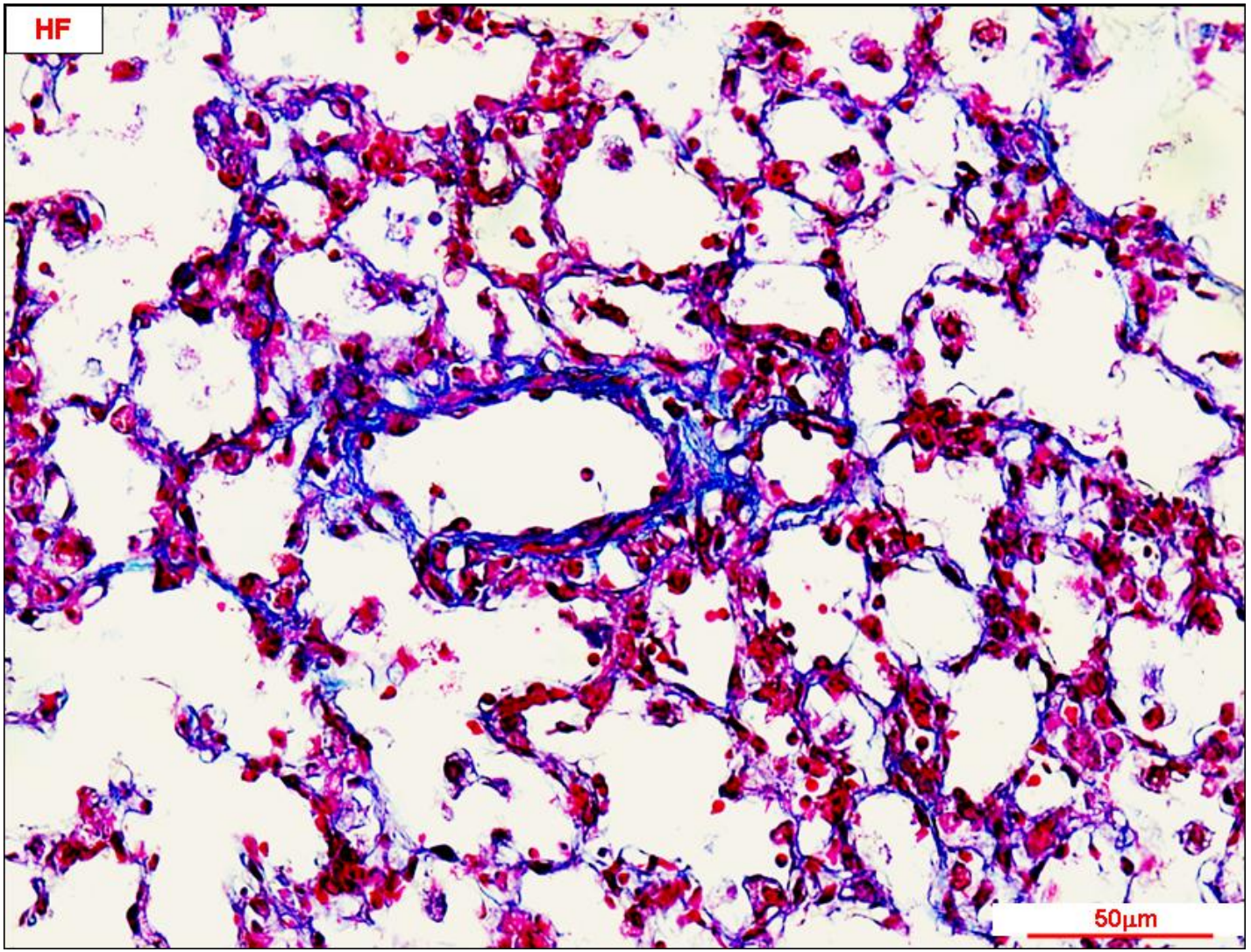

Figure S6. Representative Masson's Trichrome stained lung section shows widespread collagen deposition (blue staining) in the lung interstitial space and vessels in a HF mouse.

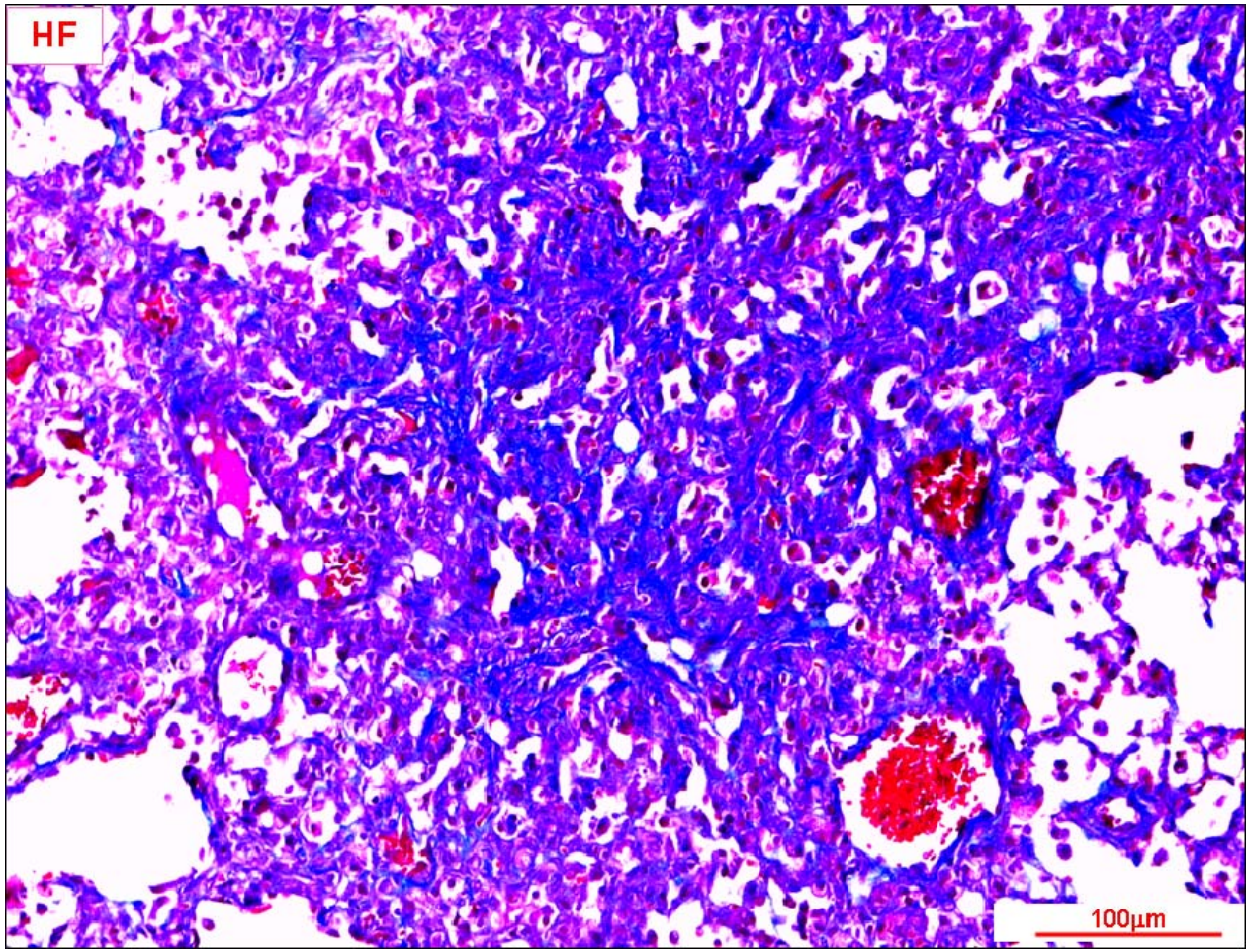

Figure S7. Masson's Trichrome stained lung section shows dense focal collagen deposition (blue staining) in the lung interstitial space and vessels in a HF mouse.

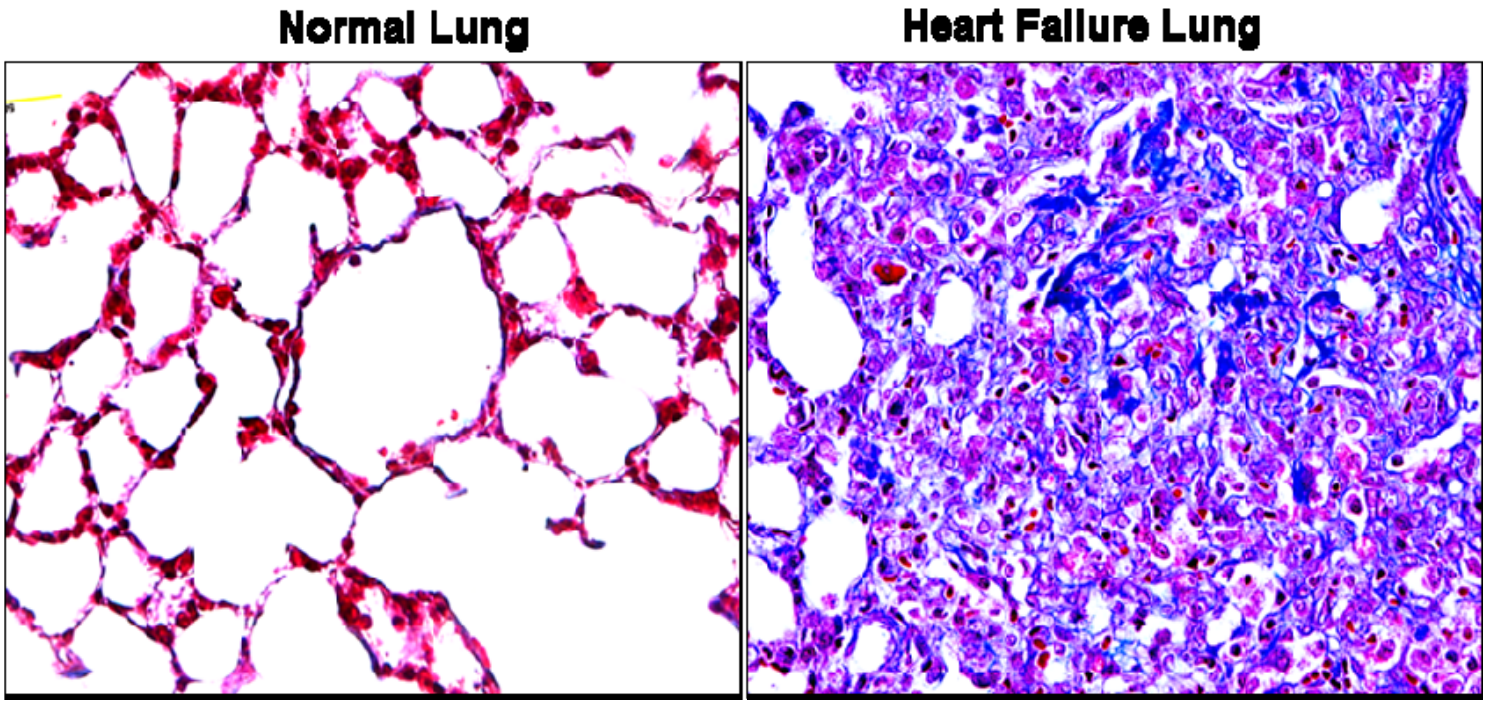

Figure S8. Masson's Trichrome stained lung sections show the destruction of normal lung structure and the dramatic focal collagen deposition (blue staining) in a mouse with severe heart failure.

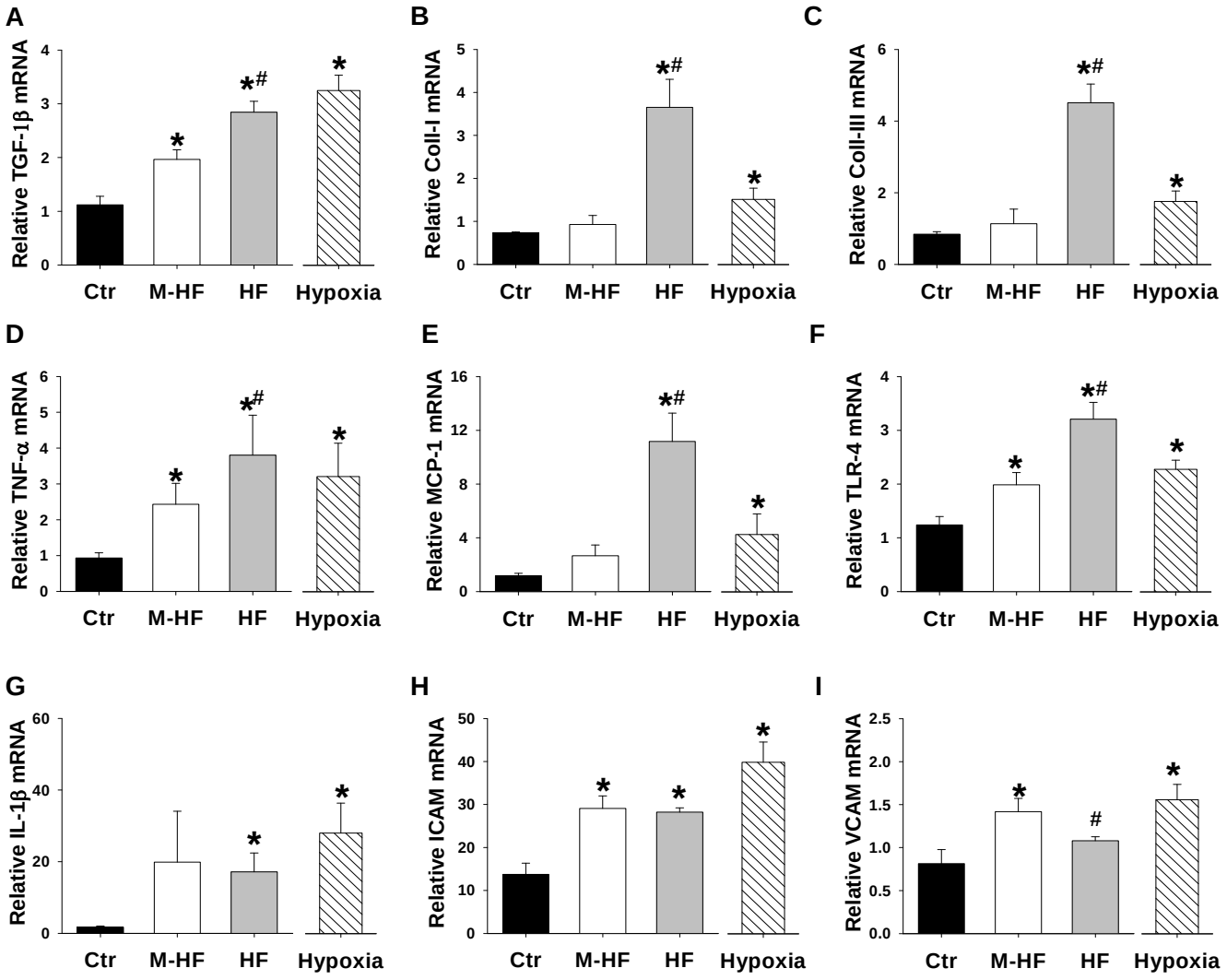

Figure S9. Chronic TAC and hypoxia increased expression of genes related to lung fibrosis and inflammation. (A) Expression of TGF-1β; (B) Collagen-I; (C) collagen-III; (D-F) pro-inflammatory cytokines; and (G-I) adhesion molecules ICAM and VECAM in lung tissues from control group, TAC-induced LVH or CHF groups, and hypoxia group. *p<0.05 vs control group; #p<0.05 vs. corresponding LVH group