

SUPPLEMENTAL DATA

Prevention of Pulmonary Hypertension by Angiotensin Converting Enzyme 2 Gene Transfer

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Methods:

Production of Lentiviral-Mediated Overexpression of ACE2 Viral Particles

Lentiviral particles containing reporter genes, human placental alkaline phosphatase (EF1 α -IRES-PLAP, lenti-PLAP) or enhanced green fluorescent protein (GFP; EF1 α -IRES-EGFP, lenti-GFP) and murine ACE2 (EF1 α -ACE2-IRES-EGFP, lenti-ACE2) were prepared by methods described previously.¹ Viral medium containing lenti-GFP or lenti-ACE2 was collected, concentrated, and titered. Concentration of viral particles was determined with the use of HIV-1 p24 antigen ELISA assay (Beckman Coulter) following the manufacturer's instructions. Efficacy of lenti-ACE2 in producing active ACE2 enzyme has been established previously.²

Determination of transduction efficiency of lung by lentivirus

Mice were anesthetized with isoflurane and the trachea was exposed through a midline incision. Lenti- PLAP particles (3×10^6 transducing units (TU) in 30 μ l of PBS) were injected into the trachea followed by air injection. Seven days following gene transfer, animals were sacrificed; lungs were perfused first with PBS, pH 7.4, followed by 4% paraformaldehyde (PFA) in PBS and postfixed with 4% PFA for 1 h by immersion.³ Tissues were incubated at 72 °C for 3 h, cooled, and subjected to PLAP staining as described previously.¹

Hypertrophy and histological analysis

The right ventricle (RV) was separated from the left ventricle (LV) plus ventricular septum (S) and the wet weights were determined. RV hypertrophy was expressed as the ratio of RV to LV plus ventricular septum (RV/LV+S) (n=6-8 in each group). Left lungs were perfused and fixed as described above. After fixation and paraffin embedding, 5 μ m-thick lung sections were cut and stained with anti- α smooth muscle actin (SMA) (1:600, clone 1A4, Sigma, St Louis, Mo), as described previously.³ Sixty to eighty intra-acinar vessels with diameter between 20 to 80 μ m, accompanying either alveolar ducts or alveoli were analyzed in each mouse. Each vessel was categorized as nonmuscularized (i.e. no apparent muscle), partially muscularized (i.e. with only a crescent of muscle), or fully muscularized (i.e. with a complete medial coat of muscle).⁴ A population of vessels was expressed as a percentage of the total vessel numbers counted from the section. The external diameter and medial wall thickness were measured in 30 muscular arteries per lung section for analysis of the medial wall thickness of the pulmonary arterioles. The medial thickness was calculated as follows: percent wall thickness = [(medial thickness x 2)/external diameter] x100 (n=4-5 mice per group).⁵

Immunohistochemical analysis

Paraffin embedded lung sections were first incubated with 0.3% H₂O₂ in PBS for 15 min followed by incubation with 1.5% goat serum in PBS containing 0.3% Triton X100 for 1 h. Sections were incubated overnight at 4°C with one of the following antibodies diluted in PBS containing 0.3% Triton X100 and 0.3% BSA: rabbit polyclonal anti-ACE2 (1:500, GTX15348, GeneTex), rabbit polyclonal anti-Ang-(1-7) (1:600) or rabbit polyclonal anti-Ang II (1:100, Abcam, Cambridge, MA), rabbit polyclonal anti-MCP-1 (1:100, Santa Cruz, CA) or rabbit polyclonal anti-TNF-alpha (1:100, Santa Cruz, CA) .^{6,7} After 4-5 rinses in PBS, biotinylated goat anti-rabbit IgG secondary antibody was added for 1 h followed by incubation with avidin-biotin-peroxidase complex reagents for 1 h, stained with diaminobenzidine solution for 4 min (Vector Laboratories), and analyzed using an Olympus BX 41 microscope. Each step was followed by washing the sections with PBS containing 0.3% Triton X100. Sections incubated without primary antibodies were used as negative controls.

RNA Isolation and Real-time PCR

Total RNA was extracted from frozen lung tissues and real-time RT-PCR (qRT-PCR) was performed as described previously using Bio-Rad PCR Master Mix to determine the expression levels of AT₁ receptor, AT₂ receptor, ACE, ACE2, renin, MCP-1, TNF- α , and IL-6 by using ABI Prism 7900 sequence detection system.⁸ mRNA levels were normalized to 18s RNA from the same samples (n=3-10 in each group).

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Disclosures:

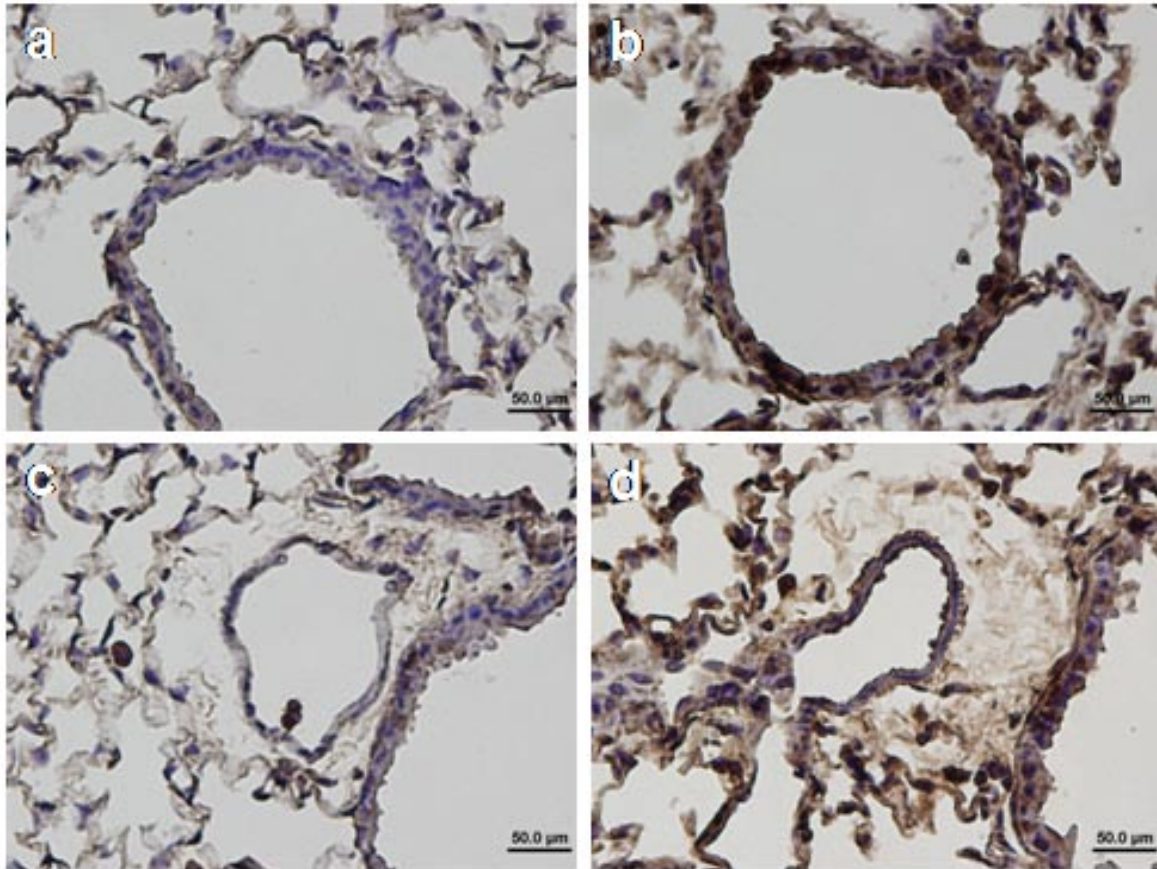
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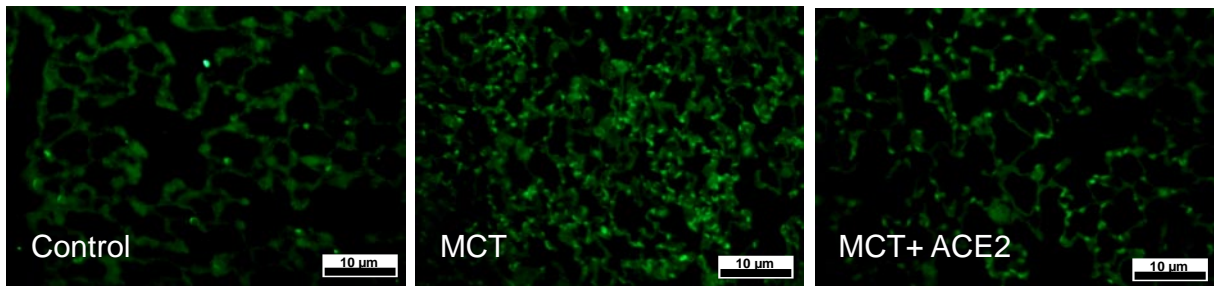
Supplemental Figure 1



Representative photomicrographs of Ang-(1-7) immunoreactivity in lungs of lenti-ACE2 treated mice:

Mice were injected with 3×10^6 TU of lenti-GFP or lenti-ACE2. Eight weeks following transduction, lungs were inflated, fixed and subjected to Ang-(1-7) immunohistochemistry as described in the Methods section. Ang-(1-7) immunoreactivity was significantly higher in (b and d) lenti-ACE2-treated lungs compared to (a and c) control lenti-GFP-treated lungs.

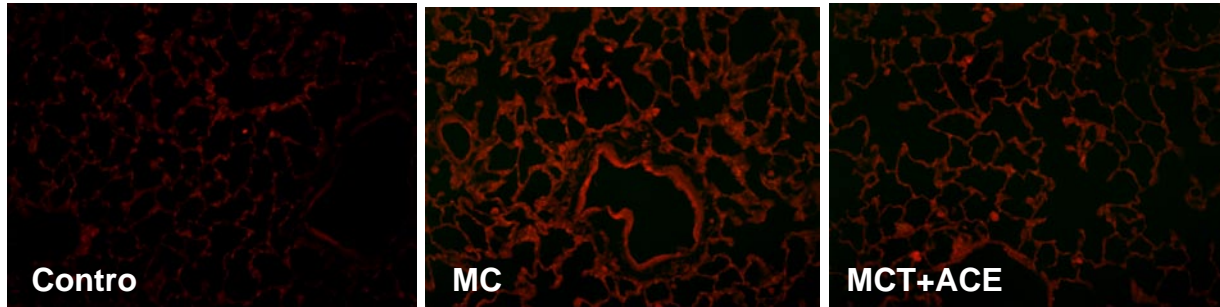
Supplemental Figure 2



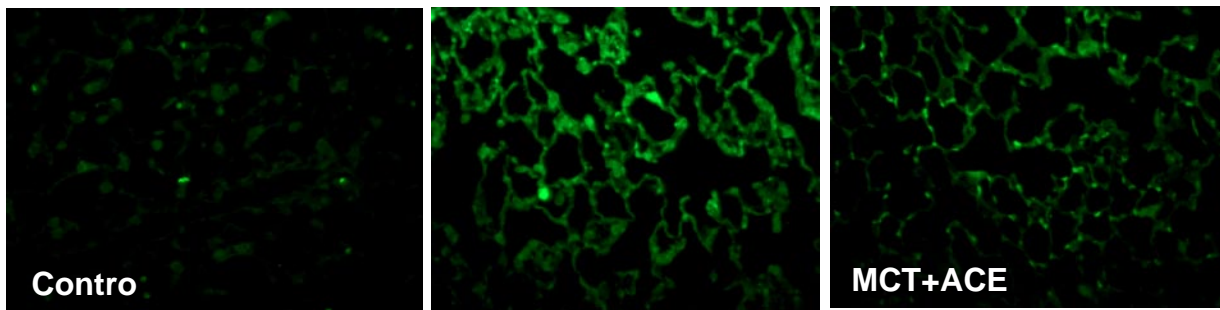
Effects of lenti-ACE2 on Ang II immunoreactivity in the lungs of MCT-treated mice:

Sections of lungs from control, MCT and MCT+ lenti-ACE2 mice from the reversal protocol were fixed and incubated with anti-Ang II antibody. This was followed by incubation with FITC-labeled secondary antibody as described in the Methods section.

Supplemental Figure 3



Supplemental Figure 4



Representative photomicrographs of MCP-1 and TNF- α immunostaining in lungs of lenti-ACE2-treated mice:

Sections of lungs from control, MCT-treated and MCT+lenti-ACE2-treated mice from the prevention study were prepared as described in the Methods section. They were subjected to immunohistochemistry with the use of antibodies specific for MCP-1 (Figure 3) and TNF- α (Figure 4) and rhodamine and FITC labeled secondary antibody, respectively. ACE2 overexpression attenuates levels of MCP-1 and TNF- α immunostaining induced by MCT treatment.