Supplement Material

Methods and Materials

Materials

FVB/NJ mice and male FVB/NJ EGFP$^+$ transgenic mice were obtained from Jackson Laboratories (Bal Harbor, ME). AMD3100 Octahydrochloride and Monoclonocal Antibody to SDF-1 were obtained from Sigma- Aldrich (St. Louis, MO). Primary antibodies utilized were: GFP (1:1000, Invitrogen, CA), c-kit (1:1000, R&D Systems, MN), Sca-1 (1:200, Santa Cruz, CA and 1:50, CedarLane Labs, Ontario), Isl-1(1:100, Santa Cruz), SDF-1(1:200, R&D Systems), α-Smooth Muscle Actin (1:500, Sigma-Aldrich), and vWF (1:200, DAKO, CA). Secondary Antibodies utilized were: Biotinylated anti-mouse IgG (1:200, Vector, CA), HRP Conjugated Donkey Anti-Goat IgG (1:2000, Jackson Immunoresearch, PA), Goat Anti-Mouse IgG Alkaline Phosphatase (1:100, Sigma-Aldrich), and Goat Anti-Rabbit IgG-Peroxidase (1:100, Sigma-Aldrich).

Bone Marrow (BM) Transplantation

The BM of male FVB/NJ EGFP$^+$ transgenic mice was obtained by removing the femur and tibia and flushing with RPMI 1640 containing gentamicin 10 U/ml. Recipient neonatal (2 wk old) wild type FVB/NJ mice were lethally irradiated to 9.0 Gy whole body dose and under anesthesia, were given a retro-orbital injection of 1-2x 10^6 whole BM-derived cells obtained from the transgenic EGFP$^+$ mice. Success of BM transplantation was documented 4 wk post transplant by flow cytometry of peripheral blood; assay of > 50% GFP+ circulating nucleated blood cells.

After 4 wk of recovery, recipient mice were exposed to normobaric hypoxia (10% O_2) or normoxia (20.9% O_2) for 8 wk. This duration was chosen as the mice were no longer neonatal at the beginning of the exposure and as such their pulmonary arteries were less reactive.

Pulmonary Vascular Morphometry

A 23-gauge silastic catheter was introduced through the right ventricular wall and advanced into the pulmonary artery and fixed in this position by suturing to the ventricular wall. The catheter was connected to a reservoir containing phosphate buffered saline and 4% polyvinyl pyrrolidone (PVP) adjusted to a pH of 7.4. This solution was delivered at air driven pressure of 40 cmH_2O for 5 min and the atrium was punctured after distension. After completion of the vascular perfusion, a vascular fixative containing 2% glutaraldehyde, 1% paraformaldehyde and 4% PVP in 0.1 cacodylate buffer, pH 7.4 was delivered at the same pressure and duration. The airways were perfused through the trachea at a transpulmonary pressure of 13 cmH_2O for 5 min with the same fixative solution as vascular, but without the PVP.

After vascular perfusion of the pulmonary arteries and formalin inflation of the airways, the lungs and heart were removed en bloc. After the vascular and airway perfusion was completed, the heart was dissected and separated from the lungs. The right ventricle was dissected from the septum (S) and left ventricle (LV). The weight of the dissected tissues was used for the calculation of the RV to LV+ S ratio. This ratio was used to reflect the degree of right ventricular hypertrophy (RVH).

The lungs and hearts were fixed overnight in 4% paraformaldehyde and subsequently embedded in paraffin. In order to determine the degree of muscularization of the pulmonary artery, five micrometer paraffin embedded sections were stained with an antibody against α-smooth muscle actin (clone 1A4, Sigma-Aldrich). Alpha smooth muscle actin-positive vessels with external diameter 20-50 μm, were assessed for their degrees of circumferential staining of α-smooth muscle actin staining. The mean linear intercept (MLI) was used to evaluate alveolarization as previously described[1, 2]. An increased MLI signifies an increase in the distance between alveolar walls and therefore

decreased alveolarization. Five randomly selected fields in each section were utilized to calculate the MLI.

The number of proliferating pulmonary vascular cells was determined by proliferating cell nuclear antigen (PCNA) staining, whilst the number of apoptotic cells was determined by the terminal deoxynucleotidyl transferase (TdT)–mediated dUTP nick end-labeling (TUNEL) method as previously described[3]. PCNA or TUNEL -positive cells were quantified as the mean number of positively immuno-stained cells per pulmonary vessel. A total of 25-30 randomly chosen pulmonary vessels per mouse were assessed.

Western Blot

Protein homogenates were separated by SDS –PAGE and transferred to nitrocellulose membranes. The membranes were incubated in blocking solution (5% non–fat dried milk in 0.1% Tween 20) for one hour and then treated with the appropriate primary antibodies overnight at 4^0C. The membranes were then washed and incubated with appropriate secondary antibody-alkaline phosphatase enzyme conjugate. Band intensity was quantified with Quantity One software (Bio-Rad, CA).

Immunohistochemistry and Immunofluorescence

Paraffin-embedded lung sections were deparaffinized and rehydrated. Following antigen retrieval, endogenous peroxidase in the lung sections was blocked by incubating for 45 minutes with 0.3% H_2O_2 in methanol. Samples were washed and incubated for 1hour at room temperature with 10% donkey serum to saturate any non-specific binding sites of the antibodies, followed by overnight incubation at 4^0C with appropriate primary antibodies. After washing, the sections were incubated with HRP-conjugated $F(ab')_2$ secondary antibodies (1:2000, Jackson Immunoresearch) at room temperature for 1hour as previously described[4]. The signal was enhanced by using a tyramide signal

amplification kit (Perkin Elmer, Waltham, MA) according to manufacturers' instructions and sections were evaluated under a Zeiss Confocal Microscope (model LSM-510; Carl Zeiss Microimaging, Inc., Thornwood, NY).

ELISA

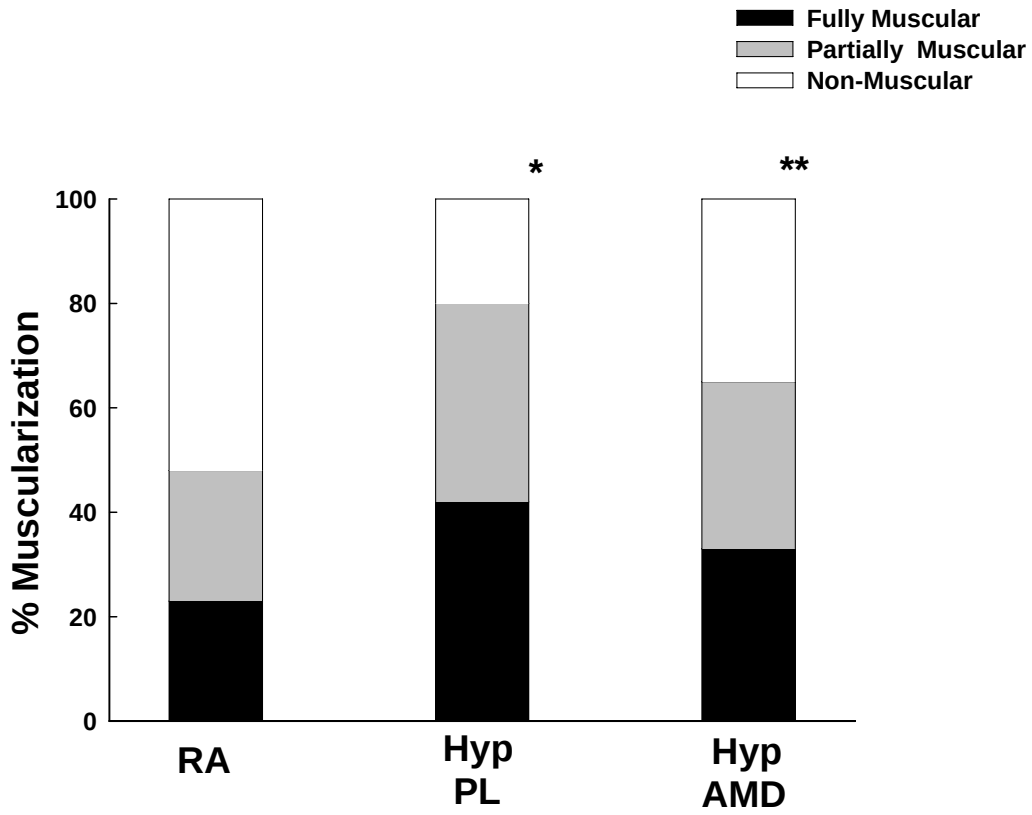
Lung and Right ventricular SDF-1α concentrations were determined by Quantikine ELISA kit (R&D Systems) as per the manufacturer specifications.

Statistical Analysis

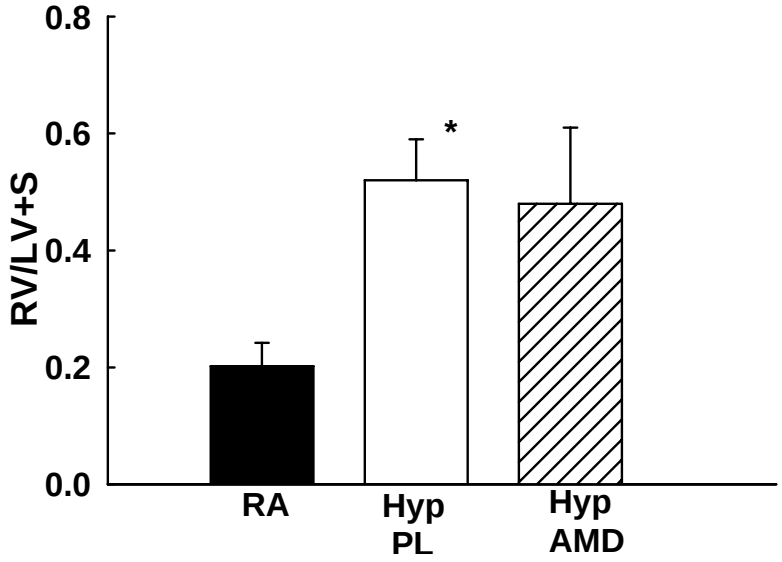
Data are expressed as mean ± SD. Data were analyzed by Student's t test and analysis of variance (ANOVA). A p value < 0.05 was considered significant.

Supplemental Figures

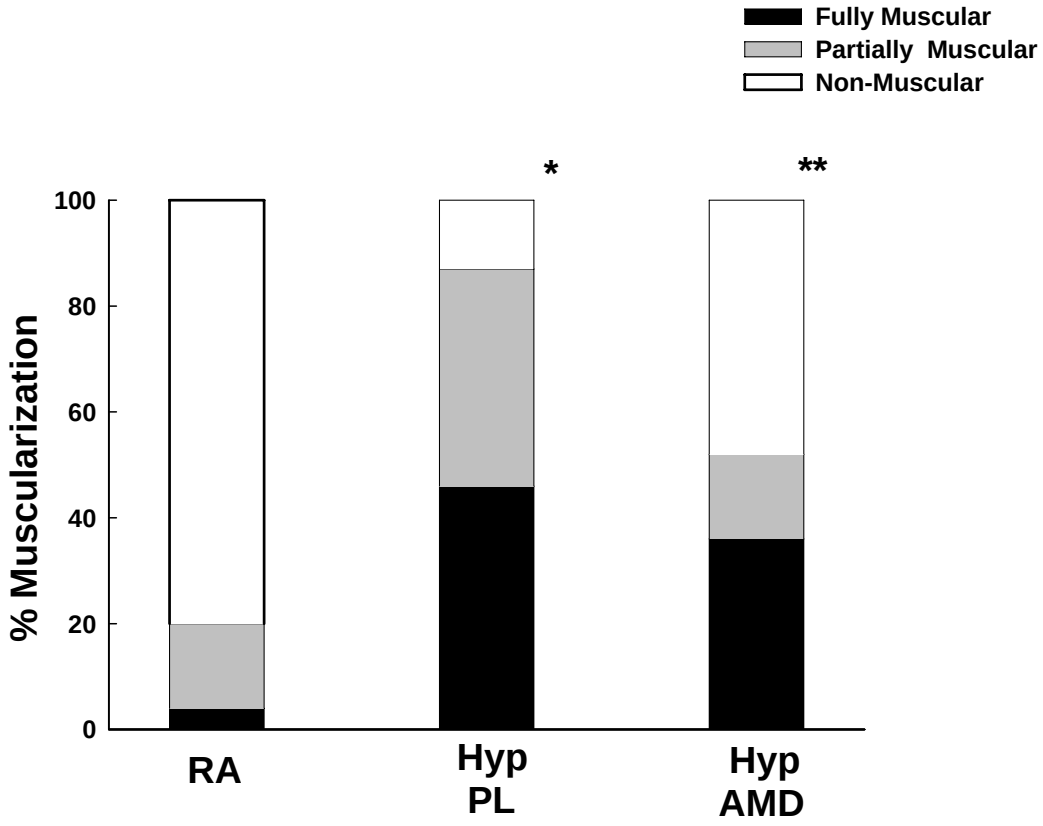
Online Figure I



Online Figure II



Online Figure III



Online Figure Legends

Online Figure I: The percentage of non-muscularized pulmonary arterioles was significantly increased in the hypoxia treated mice as compared to placebo (* $p < 0.001$ RA vs Hyp PL; ** $p < 0.04$ Hyp PL vs Hyp AMD; n=5/group). Non-muscularized, partially-muscularized and fully muscularized vessels were defined as α-smooth muscle actin staining 0-25%, 25-75% and > 75% of vessel circumference respectively.

Online Figure II: Exposure of neonatal mice to hypoxia (n=6) for 2 wk resulted in a marked RV Hypertrophy as compared to normoxia (n=8), (* $p < 0.0001$ RA vs Hyp PL), but this was not reversed following administration of AMD3100.

Online Figure III: Morphometric Analysis demonstrated that the percentage of non-muscular pulmonary arterioles was significantly increased in the hypoxia treated mice as compared to placebo (* $p < 0.03$ RA vs Hyp PL; ** $p < 0.05$ Hyp PL vs Hyp AMD; n=4/group).

References

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3. Tang J-R, Markham NE, Lin Y-J, McMurtry IF, Maxey A, Kinsella JP, Abman SH. Inhaled nitric oxide attenuates pulmonary hypertension and improves lung growth in infant rats after neonatal treatment with a VEGF receptor inhibitor. *Am J Physiol Lung Cell Mol Physiol.* 2004; 287: L344-351.

4. Davie NJ, Crossno JT, Jr., Frid MG, Hofmeister SE, Reeves JT, Hyde DM, Carpenter TC, Brunetti JA, McNiece IK, Stenmark KR. Hypoxia-induced pulmonary artery adventitial remodeling and neovascularization: contribution of progenitor cells. *Am J Physiol Lung Cell Mol Physiol.* 2004; 286: L668-678.

5. Ambalavanan N, Nicola T, Li P, Bulger A, Murphy-Ullrich J, Oparil S, Chen YF. Role of matrix metalloproteinase-2 in newborn mouse lungs under hypoxic conditions. *Pediatr Res.* 2008; 63: 26-32.