Inhibition of the SDF-1/CXCR4 Axis Attenuates Neonatal Hypoxia-Induced Pulmonary Hypertension

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Abstract—Exposure of the neonatal lung to chronic hypoxia produces significant pulmonary vascular remodeling, right ventricular hypertrophy, and decreased lung alveolarization. Given recent data suggesting that stem cells could contribute to pulmonary vascular remodeling and right ventricular hypertrophy, we tested the hypothesis that blockade of SDF-1 (stromal cell–derived factor 1), a key stem cell mobilizer or its receptor, CXCR4 (CXC chemokine receptor 4), would attenuate and reverse hypoxia-induced cardiopulmonary remodeling in newborn mice. Neonatal mice exposed to normoxia or hypoxia were randomly assigned to receive daily intraperitoneal injections of normal saline, AMD3100, or anti–SDF-1 antibody from postnatal day 1 to 7 (preventive strategy) or postnatal day 7 to 14 (therapeutic strategy). As compared to normal saline, inhibition of the SDF-1/CXCR4 axis significantly improved lung alveolarization and decreased pulmonary hypertension, right ventricular hypertrophy, vascular remodeling, vascular cell proliferation, and lung or right ventricular stem cell expressions to near baseline values. We therefore conclude that the SDF-1/CXCR4 axis both prevents and reverses hypoxia-induced cardiopulmonary remodeling in neonatal mice, by decreasing progenitor cell recruitment to the pulmonary vasculature, as well as by decreasing pulmonary vascular cell proliferation. These data offer novel insights into the role of the SDF-1/CXCR4 axis in the pathogenesis of neonatal hypoxia-induced cardiopulmonary remodeling and have important therapeutic implications. (Circ Res. 2009;104:1293-1301.)

Key Words: pulmonary hypertension ■ hypoxia ■ progenitor cells ■ SDF-1 ■ vascular remodeling

Despite marked improvements in medical care, approximately 2 in 1000 neonates are perinatally exposed to intermittent or chronic periods of hypoxia.1 Unlike that of the adult, the neonatal pulmonary vascular response to chronic hypoxic exposure is much more rapid and severe2 and results in failure of the fetal circulation to adapt to a response that supports postnatal life. This in turn contributes to the pathogenesis of persistent pulmonary hypertension (PH) of the newborn, chronic lung disease of prematurity, and congenital heart disease. It is typically characterized by profound proliferation of smooth muscle and adventitial cells in the pulmonary vasculature and abnormal extension of smooth muscle into peripheral arteries, along with impairment in alveolar development in preterm neonates.3,4

Although the mechanisms underlying neonatal hypoxia-induced cardiopulmonary remodeling remain unclear, recent studies have suggested that stem cells may contribute to systemic and pulmonary vascular remodeling. We therefore sought to examine the role of a key stem cell mobilizer, the chemokine SDF-1 (stromal cell–derived factor 1) and its receptor CXCR4 (CXC chemokine receptor 4) in neonatal chronic hypoxia–induced cardiopulmonary remodeling.

SDF-1 or CXCL12 is a chemokine that is secreted by several tissues following exposure to hypoxia,5,6 in turn leading to the release of progenitor cells along a chemical gradient to the zone of tissue injury.7–9 Its receptor CXCR4 is a G protein–coupled receptor that is widely expressed on several tissues, including endothelial cells, smooth muscle cells, monocytes, and hematopoietic and tissue-committed stem cells.10–14 Binding of SDF-1 to CXCR4 induces several signal transduction pathways that regulate cell survival and proliferation.15

We therefore first tested the hypothesis that increased release of SDF-1 in chronically hypoxic neonatal mice would facilitate an increased number of progenitor cells in the pulmonary vasculature and right ventricle. Secondly, to establish the functional relevance of SDF-1 in this process, we tested the hypothesis that inhibition of SDF-1 or its cognate receptor CXCR4 would attenuate cardiopulmonary remodeling by decreasing the recruitment of progenitor cells to the pulmonary vasculature and right ventricle, as well as by decreasing vascular cell proliferation and apoptosis.
Twenty-one FVB/NJ neonatal mice (1 to 2 days old) were exposed to normobaric hypoxia (10% O₂) or normoxia (20.9% O₂) for 1 week. After 1 week of this exposure, the mice were randomly assigned to receive daily intraperitoneal injections of normal saline (PL) (n=19), a CXCR4 antagonist, AMD3100 (7.5 mg/kg; n=10), or anti–SDF-1 antibody (25 μg/kg; n=19) for 7 days, from postnatal days 1 to 7.

**Therapeutic Strategy**

Twenty-one FVB/NJ neonatal mice (1 to 2 days old) were exposed to normobaric hypoxia (10% O₂) or normoxia (20.9% O₂) for 2 weeks. After 1 week of this exposure, the mice were randomly assigned to receive daily intraperitoneal injections of normal saline (PL) (n=6) or AMD3100 (7.5 mg/kg; n=7) for 7 days, from postnatal day 7 to 14.

The experimental protocol was performed according to guidelines set forth by the University of Miami Animal Care and Use Committee.

**Hemodynamic Measurements**

After a set duration of hypoxic or normoxic exposure, mice were weighed and anesthetized with Avertin (tribromoethanol) 0.375 mg/g body weight injected intraperitoneally. A tracheostomy was performed with a 22-gauge angiocatheter and secured in place with a 4.0 silk suture. Mice were ventilated with a Harvard Mini-Vent with a stroke volume of 325 μL and rate of 150 strokes per minute. Anesthesia was maintained throughout with 1% isoflurane mixed with room air (RA) or 10% O₂. After thoracotomy, a 25-gauge needle fitted to a pressure transducer was inserted into the right ventricle. Right ventricular systolic pressure (RVSP) was measured and continuously recorded on a Gould polygraph (model TA-400, Gould instruments, Cleveland, Ohio). Immediately after RVSP measurements were obtained, the mice were euthanized.

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org and provides details of all materials, bone marrow transplantation, pulmonary vascular morphometry, Western blot, immunohistochemistry, immunofluorescence, and statistical analyses.

**Results**

**SDF-1 Expression in Neonatal PH**

We first tested the prediction that neonatal hypoxia upregulates pulmonary SDF-1. Indeed, after hypoxia for 1 or 2 weeks, lung protein expression of SDF-1 increased 2-fold (Figure 1a). Similarly, within the right ventricle, SDF-1 expression was also markedly elevated (Figure 1b). In contrast, left ventricular SDF-1 was not elevated in response to hypoxia.

**Bone Marrow–Derived Cells Migrate to the Pulmonary Vasculature During Hypoxia**

We next examined whether bone marrow–derived cells were recruited to the pulmonary vasculature during chronic hypoxia. Six-week-old mice whose bone marrow had been reconstituted with enhanced green fluorescent protein–positive (EGFP⁺) cells were exposed to hypoxia for 8 weeks. Following this exposure, GFP⁺ cells were visualized in the smooth muscle and adventitial layers of the hypoxic pulmonary arteries (Figure 2a and 2b).

Given this, we sought to determine whether neonatal mice (1 to 2 days old) exposed to hypoxia had increased numbers of c-kit⁺ cells in their lungs and right ventricles. Indeed, we demonstrated a 2.5- and 4-fold increase in the number of c-kit⁺ cells in the lungs and right ventricles, respectively, of neonatal mice with PH and right ventricular hypertrophy (RVH), (Figure 3a and 3b). These c-kit⁺ cells were localized mainly to the...
adventitia of the hypoxic pulmonary arterioles. Additionally, within the hypertrophied right ventricles, double immunofluorescence study also demonstrated c-kit\(^{+/+}\), Sca-1\(^{+/+}\), and Isl-1\(^{+/+}\) cells colocalized with GATA-4 and Ki-67, suggesting that they were proliferating and committed to a cardiac fate (Figure 3c and 3d).

Inhibition of SDF-1/CXCR4 Axis Decreases Progenitor Cells in the Lungs and Right Ventricles of Mice With PH

Given that SDF-1 is an integral component of stem cell mobilization during hypoxia, and that c-kit\(^{+/+}\) and Sca-1\(^{+/+}\) cells were increased in the lungs and right ventricles of neonatal mice with hypoxia-induced PH, we next evaluated whether inhibition of the SDF-1/CXCR4 axis would decrease the expression of c-kit and Sca-1 in the lungs and right ventricles of mice with PH. Administration of AMD3100 or anti–SDF-1 antibody to mice exposed to 1 week of hypoxia significantly decreased the protein expression of these stem cell markers to baseline or near baseline values (Figure 4a through 4e).

Inhibition of the SDF-1/CXCR4 Axis Prevents and Reverses Pulmonary Vascular Remodeling Preventative Strategy

We next sought to elucidate whether inhibition of the SDF-1/CXCR4 axis would prevent or reverse pulmonary vascular remodeling. Exposure of neonatal mice to 1 week of hypoxia resulted in a significant increase in RVSP (11±2 versus 24±6 mm Hg; RA versus hypoxia [Hyp], P<0.001) and the ratio of the weight of the right ventricle to left ventricle and septum (0.2±0.1 versus 0.5±0.2; RA versus Hyp PL, P<0.01). To test the prediction that the increased release of SDF-1 in the lungs of hypoxic neonatal mice exacerbates PH, we administered anti–SDF-1 antibody or AMD3100 daily to neonatal mice exposed to 1 week of hypoxia. Importantly, both strategies to inhibit the SDF-1/CXCR4 axis restored RVSP and the ratio of the weight of the right ventricle to left ventricle and septum close to baseline values (Figure 5a and 5b). In addition, as compared to hypoxic placebo mice, hypoxic AMD3100-treated mice had a marked decrease in the medial wall thickness (Figure 5c and 5d) and a concomitant increased percentage of nonmuscular vessels (Online Figure I).

Moreover, because chronic hypoxia is also known to significantly alter lung alveolarization in the neonate, we measured the mean linear intercept to evaluate the effect of the SDF-1/CXCR4 axis on lung alveolarization. Whereas exposure to chronic hypoxia was associated with a significant increase in the mean linear intercept, blockade of the SDF-1/CXCR4 axis significantly decreased the mean linear intercept (Figure 5e).
Figure 4. Inhibition of the SDF-1/CXCR4 axis decreased stem cell marker expression in the lungs and right ventricles of neonatal mice with PH. Neonatal mice with PH had a marked increase in lung c-kit (2-fold, *P<0.0001) (a) and lung Sca-1 (1.9-fold, *P<0.02) (b), right ventricular c-kit (5-fold; *P<0.0001) (c), right ventricular Sca-1 (1.6-fold; *P<0.001) (d), and right ventricular Isl-1 (2-fold; *P<0.02) (e) expressions as compared to RA (n=5 to 7/group). As compared to hypoxia (Hyp) PL mice, administration of anti–SDF-1 significantly decreased lung c-kit (**P<0.0008) (a), lung Sca-1 (**P<0.02) (b), and right ventricular Sca-1 (**P<0.0005) (d) expressions to near baseline values. Similarly, administration of AMD3100 significantly decreased lung c-kit (**P<0.0001) (a), lung Sca-1 (**P<0.006) (b), right ventricular c-kit (**P<0.01) (c), right ventricular Sca-1 (**P<0.0001) (d), and right ventricular Isl-1 (**P<0.05) (e) expressions to near baseline values as compared to Hyp PL (n=5 to 7/group).
Therapeutic Strategy

Exposure of neonatal mice to 2 weeks hypoxia also resulted in a marked increase in RVSP (12±2 versus 29±4 mm Hg; RA versus Hyp PL, \( P<0.001 \)) and RVH (0.2±0.0 versus 0.5±0.1; RA versus Hyp PL, \( P<0.01 \)). In contrast, administration of AMD3100 in mice with established PH resulted in a significant decrease in RVSP (Figure 5f) but no significant difference in RVH (Online Figure II). There was, however, a significant increase in the percentage of nonmuscularized pulmonary arterioles in the hypoxia-treated mice as compared to placebo (Online Figure III).

Inhibition of the SDF-1/CXCR4 Axis Decreases Pulmonary Vascular Cell Proliferation and Apoptosis

To ascertain other mechanisms by which inhibition of the SDF-1/CXCR4 axis attenuates hypoxia-induced pulmonary vascular remodeling, proliferating-cell nuclear antigen (PCNA) immunostaining and TUNEL assay were used to determine cell proliferation and survival, respectively. Although exposure to hypoxia resulted in a marked increase in the number of PCNA\(^+\) and TUNEL\(^+\) cells in the pulmonary vasculature, this was significantly attenuated in the AMD3100 hypoxic mouse pups (Figure 6a through 6e). Moreover, on further questioning of the downstream signaling mechanisms that were responsible for this effect, we demonstrated a marked decrease in phosphorylated Akt expression in the treated mouse pups as compared to placebo (Figure 6c).

Discussion

In this study, we provide direct systematic evidence of the participation and the mechanism of action of SDF-1 and its receptor CXCR4 in the pathogenesis of neonatal chronic hypoxia–induced cardiopulmonary remodeling. We demonstrate that inhibition of the SDF-1/CXCR4 axis improves alveolarization, prevents the development of hypoxia-induced pulmonary vascular remodeling in neonatal mice, and significantly decreases pulmonary artery pressure in neonatal mice with established disease. These findings were associated with decreased expression of progenitor cells in the lungs and right ventricles of treated mice, as well as decreased pulmonary vascular cell proliferation and apoptosis. This study, therefore, offers important pathophysiologic insights into the role...
of the SDF-1/CXCR4 axis in neonatal chronic hypoxia-induced cardiopulmonary remodeling and has potential therapeutic implications for neonatal hypoxia-induced PH.

One of the major roles of SDF-1 is the mobilization of stem cells from the bone marrow to injured sites. In proof of this concept, we demonstrated that inhibition of the SDF-1/CXCR4 axis decreased the pulmonary expression of c-kit and Sca-1, known stem cell markers. The role of c-kit+ or Sca-1+ cells in neonatal hypoxia-induced cardiopulmonary remodeling, however, is unclear. Davie et al showed an increased number of c-kit cells in the pulmonary artery adventitia of neonatal animals with hypoxic PH, whereas bone marrow-derived cells were previously shown to express smooth muscle actin in hypoxia remodeled pulmonary arteries and selective depletion of circulating mesenchymal precursors prevented pulmonary adventitial remodeling, suggesting that these cells may have functional relevance. Our present study adds further credence to this theory as inhibition of the SDF-1/CXCR4 axis both inhibited and reversed pulmonary vascular remodeling, and this was associated with decreased stem cell expression.

This is plausible as SDF-1 has been suggested to play a role in adult systemic vascular remodeling. Although the remodeled systemic vasculature clearly does not mirror a

Figure 6. Inhibition of SDF-1/CXCR4 axis decreases pulmonary vascular proliferation and apoptosis. a, Representative immunofluorescence images of lungs obtained from RA, hypoxia (Hyp) PL, and Hyp AMD neonatal mice following staining with anti-PCNA antibody (green) and DAPI (blue). PCNA+ cells (green) were more abundantly present in Hyp PL mice as compared to Hyp AMD. Magnification, ×400. b, Bar graph demonstrating significantly decreased number of PCNA+ cells/pulmonary vessel in the Hyp AMD mice as compared to Hyp PL (*P<0.005 RA vs Hyp PL; **P<0.04 Hyp PL vs Hyp AMD; n=4/group). c, Phosphorylated Akt expression was markedly decreased in the lungs of neonatal mice with PH following inhibition of the SDF-1/CXCR4 axis (*P<0.0005 RA vs Hyp PL; **P<0.03 Hyp PL vs Hyp AMD; n=4/group). d, TUNEL assay revealed significantly less apoptotic (pink) pulmonary vascular cells following inhibition of the SDF-1/CXCR4 axis. Magnification, ×400. Scale bar=50 μm. e, Bar graph demonstrating significantly decreased number of TUNEL+ cells/pulmonary vessel in the Hyp AMD mice as compared to Hyp PL (*P<0.02 RA vs Hyp PL; **P<0.05 Hyp PL vs Hyp AMD; n=4/group).
remodeled neonatal pulmonary arteriole, this study does suggest that SDF-1 may be a crucial factor in both processes. In agreement with this, Satoh et al demonstrated increased expression of SDF-1 in the plasma of hypoxic adult rats and, following administration of a statin, demonstrated decreased pulmonary vascular remodeling associated with decrease SDF-1 and progenitor cell expression.26 Our study extends this finding and, more importantly, provides direct evidence that inhibition of the SDF-1/CXCR4 axis actually does decrease neonatal pulmonary vascular remodeling.

Another possible mechanism by which the SDF-1/CXCR4 axis could participate in hypoxia-induced pulmonary vascular remodeling is through its role in cell proliferation and apoptosis. Vascular cell proliferation is known to be an important component of pulmonary vascular remodeling, whereas early PH is known to be associated with increased endothelial cell apoptosis and loss of small capillaries. In this present study, we demonstrated that inhibition of the SDF-1/CXCR4 axis decreased pulmonary vascular cell proliferation, as well as apoptosis, and this was associated with decreased expression of phosphorylated Akt. Binding of SDF-1 to its receptor CXCR4 is known to induce several signal transduction pathways including activation of phosphatidylinositol 3-kinase (PI3K). The PI3K/Akt axis has been shown to affect the calcium currents that govern smooth muscle cell contraction through coupling membrane receptors to calcium channels,27 and, most recently, investigators demonstrated that SDF-1 may be a major regulator of smooth muscle cell proliferation through its involvement in the PI3K/PTEN signaling pathway.25 Our data support these findings and further suggest that blockade of the SDF-1/CXCR4 pathway is not only therapeutically beneficial in PH through its effects on cell migration but also via its role in cell proliferation and survival.

Another important aspect of this study is the finding that inhibition of the SDF-1/CXCR4 axis also decreased RVH, and this was associated with increased right ventricular expression of c-kit, Isl-1, and Sca-1. Moreover, cells that expressed these stem cell markers colocalized with GATA-4 and Ki67, suggesting that they were committed to a cardiac fate and also proliferating. Although we did not evaluate directly whether these stem cells were bone marrow–derived or resident stem cells, we speculate that a significant fraction may be indeed derived from the bone marrow, because following inhibition of the SDF-1/CXCR4 axis, we demonstrated a significant decrease in the expression of these stem cell markers in the right ventricle. Moreover, bone marrow–derived cells have been suggested by several investigators to be involved in the ventilricular hypertrophic response.28,29 Fukuda and colleagues found that bone marrow–derived cells were involved in the pathogenesis of cardiac hypertrophy through cell fusion, as well as transdifferentiation,28 whereas Spees et al demonstrated that some of the bone marrow–derived cells in the hypertrophied right ventricles of rats with monocrotaline-induced PH were vascular cells as well cardiomyocytes.30 Nonetheless, the possible role of resident cardiac stem cells cannot be understated because several investigators, including our group, have documented several resident stem niches within the myocardium that differentiate and proliferate in response to injury.31,32

It is unclear whether these stem cells in the hypertrophied right ventricle have adaptive or maladaptive roles. We speculate that these cells do contribute significantly to the compensatory increase in myocardial mass that is necessary to adapt to the increase overload, and our findings extend those of other investigators who have implicated stem cells in pressure-overload induced ventricular hypertrophy.28,30,33 Urbanek et al previously demonstrated that in a model of left ventricular overload induced by aortic stenosis, there was intense new formation of myocytes resulting from the differentiation of stem-like cells,34 whereas Lee and colleagues demonstrated that stem cells or precursor cells could regenerate cardiomyocytes in hearts subjected to pressure overload.35 It should be noted, however, that the roles of these stem cells may not be equivalent. Whereas c-kit+ cells have been shown by several investigators to have significant reparative potential, only one other published report has suggested that transplantation of Sca-1+ cells into the peri-infarct zone could attenuate left ventricular structural and functional remodeling after myocardial infarction, and no other studies have suggested that Isl-1–committed cardiomyoblasts could participate in repair.36–39 This is the first study to demonstrate that Isl-1+ cells could also contribute to RVH. This was a surprising finding because these cells were previously shown to be extremely sparse in the native heart after postnatal day 5.40,41 We postulate that this response could be developmentally determined because our pilot studies evaluating the role of stem cells in pressure overload–induced RVH showed a progressive decline in the role of these cells with increasing age. It should also be noted that although these cells were significantly increased in the hypertrophied right ventricle, they were not changed in the left ventricle, suggesting that the right ventricular stem cell response was not attributable to global hypoxia of the animal but, rather, was a direct reflection of the increase in the SDF-1 expression noted in the right but not the left ventricle.

This finding is clearly surprising because hypoxia is known to upregulate SDF-1 expression in several tissues in a manner proportional to the degree of hypoxia.6,42 Additionally, the promoter region of the SDF-1 gene contains at least 2 binding sites for the master regulator of hypoxic cell signaling, hypoxia-inducible factor-1α, and the latter is known to be a major participant in hypoxia-induced cardiopulmonary remodeling.6,43 Nonetheless, this present study does suggest that factors that are uniquely released in the pressure-overloaded right ventricle may also regulate SDF-1 production. Indeed, chronic hypobaric hypoxia has been shown to induce a differential transcriptional profile in the right and left ventricle,44 including genes involved in apoptosis, and SDF-1 secretion was triggered in medial smooth muscle cells following exposure to apoptotic bodies.23

In conclusion, this study clearly demonstrates that SDF-1 participates in the pathogenesis of neonatal hypoxia-induced cardiopulmonary remodeling through several mechanisms. We propose that during neonatal hypoxia, there is increased release of SDF-1, which results in the mobilization of progenitor cells to the pulmonary vasculature and right
ventricle, and that these mobilized cells participate directly in pulmonary vascular remodeling and RVH. Moreover, following binding of the locally released SDF-1 to its receptor CXCR4, downstream signaling pathways regulate pulmonary vascular cell proliferation and apoptosis, resulting in the findings evidenced in the lungs and hearts of hypoxic neonates. Finally, but most importantly, given the tremendous need for new therapies, the demonstration that inhibition of SDF-1/CXCR4 axis significantly improves alveolarization and attenuates neonatal PH, vascular remodeling, and RVH suggests a novel and potentially highly effective therapeutic strategy for this disease.

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Disclosures

None.

References


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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 10⁶ 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% O₂) or normoxia (20.9% O₂) 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\(_2\)O 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\(_2\)O 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 \(\alpha\)-smooth muscle actin (clone 1A4, Sigma-Aldrich). Alpha smooth muscle actin-positive vessels with external diameter 20-50 \(\mu\)m, were assessed for their degrees of circumferential staining of \(\alpha\)-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. 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°C. 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% H₂O₂ in methanol. Samples were washed and incubated for 1 hour at room temperature with 10% donkey serum to saturate any non-specific binding sites of the antibodies, followed by overnight incubation at 4°C 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 1 hour as previously described. 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

[Bar chart showing % Muscularization across different diagnoses: RA Hyp, Hyp PL, Hyp AMD. The chart indicates the percentage of muscularization into fully muscular, partially muscular, and non-muscular categories.]

Online Figure II

[Bar chart showing RV/LV+S across different diagnoses: RA, Hyp PL, Hyp AMD. The chart indicates statistical significance (*) and highly significant (**).]
Online Figure III

The figure illustrates the percentage of muscularization (% Muscularization) in different conditions: RA, Hyp PL, and Hyp AMD. The categories are: Fully Muscular, Partially Muscular, and Non-Muscular.

- RA: 100% Non-Muscular
- Hyp PL: 40% Fully Muscular, 50% Partially Muscular, 10% Non-Muscular
- Hyp AMD: 20% Fully Muscular, 60% Partially Muscular, 20% Non-Muscular

Significant differences are indicated by asterisks:*

**Hyp PL versus Hyp AMD (p < 0.05)**

**Hyp PL versus RA (p < 0.001)**
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


