Impact of S-Adenosylmethionine Decarboxylase 1 on Pulmonary Vascular Remodeling

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Background—Pulmonary hypertension (PH) is a life-threatening disease characterized by vascular remodeling and increased pulmonary vascular resistance. Chronic alveolar hypoxia in animals is often used to decipher pathways being regulated in PH. Here, we aimed to investigate whether chronic hypoxia–induced PH in mice can be reversed by reoxygenation and whether possible regression can be used to identify pathways activated during the reversal and development of PH by genome-wide screening.

Methods and Results—Mice exposed to chronic hypoxia (21 days, 10% O2) were reoxygenated for up to 42 days. Full reversal of PH during reoxygenation was evident by normalized right ventricular pressure, right heart hypertrophy, and muscularization of small pulmonary vessels. Microarray analysis from these mice revealed s-adenosylmethionine decarboxylase 1 (AMD-1) as one of the most downregulated genes. In situ hybridization localized AMD-1 in pulmonary vessels. AMD-1 silencing decreased the proliferation of pulmonary arterial smooth muscle cells and diminished phospholipase Cγ1 phosphorylation. Compared with the respective controls, AMD-1 depletion by heterozygous in vivo knockout or pharmacological inhibition attenuated PH during chronic hypoxia. A detailed molecular approach including promoter analysis showed that AMD-1 could be regulated by early growth response 1, transcription factor, as a consequence of epidermal growth factor stimulation. Key findings from the animal model were confirmed in human idiopathic pulmonary arterial hypertension.

Conclusions—Our study indicates that genome-wide screening in mice from a PH model in which full reversal of PH occurs can be useful to identify potential key candidates for the reversal and development of PH. Targeting AMD-1 may represent a promising strategy for PH therapy. (Circulation. 2014;129:1510-1523.)

Key Words: cell hypoxia ■ hypertension, pulmonary ■ muscle, smooth

Pulmonary hypertension (PH) is a progressive and life-threatening disease resulting in a progressive increase in pulmonary vascular resistance, cardiac failure, and death.1 PH is characterized by vascular cell proliferation and structural changes in the vessel wall (pulmonary vascular remodeling).1 The remodeling process includes muscularization of distal and normally nonmuscularized vessels, hypertrophy of the medial (smooth muscle cells) and adventitial layers, intimal proliferation, and deposition of extracellular matrix proteins.2,3 A variety of growth factors and their receptors have been implicated to be involved in the development of PH.4,5 In addition to these mediators, chronic hypoxia is a profound trigger of PH with a well-characterized vascular remodeling process.6 Until now, research in the field of PH concentrates mostly on the onset and development of PH. In contrast, we focused here on the mechanisms underlying the reversal of PH. Although it has previously been shown in rodents that hypoxia-induced

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vascular remodeling can be reversed by re-exposure to normoxia, the factors implicated in reverse remodeling remain elusive. To identify such factors, we screened for potential candidates leading to reverse remodeling by using DNA microarrays. To determine the onset of expression changes among different genes and the time course of regulation, mice exposed to 3 weeks of chronic hypoxia for the full development of PH were re-exposed to normoxic conditions for 1, 3, 7, 14, and 21 days. In this setup, reoxygenation resulted in a full reversal of PH. In addition to other known genes involved in vascular remodeling processes such as elastin, our whole-genome approach revealed s-adenosylmethionine decarboxylase 1 (AMD-1), one of the major rate-limiting enzyme in polyamine synthesis, as one of the most downregulated genes. Here, against the background that other groups reported increased lung polyamine contents in hypoxia and that pharmacological inhibition of ornithine decarboxylase 1 (ODC-1), the second rate-limiting enzyme of polyamine synthesis, attenuated at least partially the symptoms of hypoxic PH, we focused in detail on AMD-1 and its impact on pulmonary vascular remodeling.

Methods
A detailed description of routine methodologies is provided in the online-only Data Supplement.

Experimental Design
Hypoxia pulmonary vascular remodeling was induced by exposure of adult mice (20–22 g; C57BL/6J) obtained from Charles River Laboratories (Sulzfeld, Germany) to chronic hypoxia (10% O2) in a ventilated chamber, as described previously. Briefly, animals were age-matched and randomly distributed to groups exposed to normoxia, chronic hypoxia (21 days), or chronic hypoxia with subsequent re-exposure to normoxia for 1, 3, 7, 14, 21, or 42 days.

ODC-1+/− and AMD-1+/− mice were kept for 28 days in a hypoxic chamber. The EGR1+/− animals were a kind gift from Patrick Charnay.

For interventional investigations, C57BL/6J mice were exposed to either normoxia or chronic hypoxia (28 days) and treated daily with either placebo (5% glucose) or 1 mg/kg body weight SAM486a. SAM486a was synthesized as previously described with slight modifications. All protocols were approved by the governmental Animal Ethics Committee (Regierungspraesidium Giessen).

Patient Characteristics and Measurements
Human lung tissue was obtained from 6 donors (3 female, 3 male; median age, 58 years; interquartile age range, 42–69 years) and 6 patients with idiopathic pulmonary arterial hypertension (4 female, 2 male; median age, 29 years; interquartile age range, 26–45 years) undergoing lung transplantation. Lung tissue was snap-frozen directly after explantation for further analysis. The investigations on human tissue were approved by the ethics committee of the Medical Faculty, Justus-Liebig-Universitaet Giessen (Germany).

Hemodynamic Measurements, Echocardiography, and Tissue Preparation
Please refer to the online-only Data Supplement.

Paraffin Embedding, Immunohistochemical Staining, and Microscopy
Please refer to the online-only Data Supplement.

Laser Microdissection, RNA Isolation, cDNA Synthesis, and Quantitative Polymerase Chain Reaction
For laser-assisted microdissection, RNA isolation, cDNA synthesis, and quantitative polymerase chain reaction, please refer to the online-only Data Supplement.

Isolation of Aorta and Carotid Artery
Please refer to the online-only Data Supplement.

Microarrays
RNA from 80 animals (20 controls and 12 per time point) was analyzed in a total of 40 dual-color hybridizations in a balanced dye-swap design in which each time point was directly compared with the control and adjacent time points. Stored data were evaluated with R software and the limma package from BioConductor. Gene-set enrichment analyses were done with the “geneSetTest” function, together with the KEGG pathway information given in the Bioconductor database package mgu4122a.db version 2.2.5. Significantly enriched pathways were selected on the basis of a 5% false-discovery rate. For further details, please refer to the online-only Data Supplement.

Nonisotopic In Situ Hybridization Combined With Immunofluorescence on Mouse Lung Sections
Nonisotopic in situ hybridization was carried out as previously described with slight modifications. For further details, please refer to the online-only Data Supplement.

Cell Culture
Human (h) pulmonary arterial smooth muscle cells (PASMCs) were purchased from Lonza (Cologne, Germany) and grown to near confluence. Murine (m) PASMCs were isolated from precapillary vessels modified from a previously reported protocol.

Proliferation and Apoptosis Assay
Please refer to the online-only Data Supplement.

Binding Sites
The human AMD-1 gene was screened 5000 bp downstream and upstream from the coding sequence for the presence of early growth response 1 (Egr1) binding sites (cgcgccgcg) and hypoxia-inducible transcription factor (HIF) consensus sequences (murine promoter, caggtggt; human, tacgtggg). The AMD-1 sequences were obtained from http://www.ncbi.nlm.nih.gov/mapview/.

Chromatin Immunoprecipitation
Chromatin immunoprecipitation assay was performed 1.5 hours after epidermal growth factor (EGF) treatment or exposure to hypoxia (2 hours, 1% O2) using the Chromatin Immunoprecipitation Assay Kit (Millipore, Billerica, MA) according to the manufacturer’s instructions. Antibodies were used against Egr1 (Cell Signaling, Boston, MA), HIF-1α, and HIF-2α (both Novus Biologicals, Littleton, CO). IgG (Millipore, Schwabach, Germany) served as negative control.

Electrophoresis Mobility Shift Assay
Electrophoresis mobility shift assay was carried out with nuclear extracts derived by the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, San Diego, CA) from hPASMCs that were stimulated with EGF for 1.5 hours. From equal amounts of nuclear proteins, electrophoresis mobility shift assay was performed using the LightShift Chemiluminescent Electrophoresis Mobility Shift Assay Kit (Pierce, Rockford, IL) according to the manufacturer’s instructions.
Plasmid Construction
Full-length human AMD-1 cDNA (accession number, NM_001634) was cloned into the pXIN vector (Clontech, Mountain View, CA). For more details, please see the online-only Data Supplement.

Western Blot Analysis
Please refer to the online-only Data Supplement.

Statistical Analysis
Data from animal experiments and from human tissue were analyzed similarly. Effect sizes are given as mean±95% confidence interval. Data are presented in box plots unless indicated differently. Boxes represent the 25th and 75th percentiles; black bars indicate the median; and whiskers display the 0th and 100th percentiles. P values for pairwise comparisons of groups were calculated from the Student t distribution. They are intended to represent a descriptive summary for the statistical significance of the difference in group means. Accordingly, P values from multiple comparisons were calculated from pooled variance estimates. Comparisons with values of P<0.05 are indicated in the diagrams. When the group sizes were between 6 and 8, symbols are shown only when the P value of the Wilcoxon test was also <0.05. When appropriate, data were analyzed by 2-factor ANOVA. The main research questions—whether AMD-1 mRNA expression was changed during the reversal of PH on reoxygenation or during the time course of exposure to hypoxia and whether AMD-1−/− mice were protected from hypoxia-induced increase in right ventricular systolic pressure (RVSP)—were tested at a 5% level of significance after Bonferroni correction for multiple testing. The antagonistic effect of the AMD-1 gene knockout on the hypoxia-induced increase in RVSP was tested as an interaction in the 2-factor ANOVA. They are intended to represent a descriptive summary for pairwise comparisons of groups were calculated from the Student t distribution. They are intended to represent a descriptive summary for the statistical significance of the difference in group means.

Results
Hypoxia-Induced Remodeling Can Be Reversed by Re-Exposure to Normoxia
To investigate the effect of reoxygenation after chronic hypoxic exposure on parameters characterizing PH, mice were randomly distributed to groups exposed to normoxia, chronic hypoxia (21 days, 10% O2), or chronic hypoxia with subsequent reoxygenation for the indicated time spans. Three weeks of chronic hypoxic exposure resulted in the development of PH, proven by an increase in RVSP, right heart hypertrophy, and degree of muscleaturation of small pulmonary vessels (Figure 1A–1D). Re-exposure of chronic hypoxic mice resulted in a reversal of PH (Figure 1A–1D). Vascular remodeling was accompanied by increased proliferation, whereas re-exposure to normoxia resulted in decreased proliferation, indicated by the expression of the proliferation markers Ki67 and proliferating cell nuclear antigen (Figure 1E and 1F). Furthermore, immunostaining identified proliferating cell nuclear antigen–positive cells within the vascular wall. The number of proliferating cells increased during chronic hypoxia but decreased after 14 days of re-exposure to normoxia (Figure 1G).

AMD-1 Expression Is Decreased During the Process of Reverse Remodeling
To decipher changes in gene expression during reverse remodeling, whole-genome DNA microarray analysis was performed. The experimental design is depicted in Figure 2A. Our screening confirmed several pathways known to be involved in the pathogenesis of PH such as calcium and mitogen-activated protein kinase signaling, as well as cell cycle and apoptosis factors, which were also identified for the reversal of PH (Figure 2B). Additionally, many genes implicated in vascular remodeling, including elastin or collagens, were differentially expressed (Figure 2C). Interestingly, one of the most downregulated genes during normoxic re-exposure was AMD-1 (Figure 2C and 2D). AMD-1 was consistently downregulated in all investigated time points during reverse remodeling (Figure 2C and 2E). Furthermore, AMD-1 was upregulated during exposure of mice to 3 weeks of hypoxia (Figure 2F). In contrast to the oxygen-dependent regulation in the lung, AMD-1 levels were mostly unchanged in the systemic vasculature (Figure IA and IB in the online-only Data Supplement) on hypoxic exposure.

AMD-1 Expression Is Elevated in Intrapulmonary Vessels
To determine the localization of AMD-1 in the mouse lung, we performed nonisotopic in situ hybridization combined with immunofluorescence staining. Higher AMD-1 mRNA signal intensity was detected in mouse lungs exposed to chronic hypoxia compared with normoxic animals. Immunostaining against α-smooth muscle actin (α-sma) localized AMD-1 expression to smooth muscle layers of the bronchi and pulmonary vessels (Figure 3A). Laser microdissection of intrapulmonary vessels with quantitative polymerase chain reaction verified the expression and upregulation of AMD-1 in this compartment (Figure 3B). Similar to our findings in mice, we observed higher expression of AMD-1 in the vessels from patients with idiopathic pulmonary arterial hypertension compared with donors (Figure 3C and 3D). Controls are shown in Figure II A and II B in the online-only Data Supplement.

AMD-1−/−Mice Develop Attenuated Hypoxia-Induced PH
To investigate whether AMD-1 is involved in the development of hypoxia-induced PH, AMD-1−/− mice were exposed to either chronic hypoxia (28 days) or normoxia. No differences in hemodynamic measurements were observed under normoxic conditions between AMD-1−/− and wild-type (WT) animals (Figure 4A). In contrast, AMD-1−/− mice developed a significantly lower degree of PH determined by RVSP (Figure 4A). Furthermore, loss of 1 AMD-1 allele resulted in a decreased ratio of right ventricular to left ventricular plus septal mass and a higher degree of nonmuscularized pulmonary vessels on chronic hypoxia exposure (Figure 4B and 4C and Figure IIIA in the online-only Data Supplement). Because the vascular remodeling process on chronic hypoxia was accompanied by increased proliferation (Figure 1E–1G), we were interested in whether depletion of AMD-1 had an impact on cell proliferation. Western blot analysis of whole-lung homogenate from AMD-1−/− mice demonstrated diminished proliferating cell nuclear antigen expression compared with WT littermates (Figure 4D). Furthermore, silencing of AMD-1 by siRNA in hPASMCs (Figure IIB in the online-only Data Supplement) led to decreased Ki67 mRNA levels and proliferation as indicated by total cell count (Figure 4E and 4F).
Figure 1. Reoxygenation reverses hypoxia-induced pulmonary hypertension. Assessment of (A) right ventricular systolic pressure (RVSP), (B) right heart hypertrophy (LV indicates left ventricle; and RV, right ventricle), and (C) degree of muscularization of pulmonary arterial vessels (outer diameter, 20–70 μm) in mice exposed to either normoxia or chronic hypoxia or during the time course of re-exposure to normoxia (n=5–7 animals per group, mean±SEM). Values are given as percentage of total vessel count for fully muscularized vessels from paraffin-embedded lung sections costained against α-sma and von Willebrand factor (vWF). D, Representative images of lung sections costained against α-sma and vWF displaying vessels (V) from mice exposed to either normoxia or chronic hypoxia or during the time course of reoxygenation. IgG served as negative control. Scale bars display 20 μm. B indicates bronchus. E, Relative Ki67 mRNA expression quantified by quantitative polymerase chain reaction from laser-microdissected pulmonary vessels (n=4–8 animals). F, Western blot analysis of lung homogenate for proliferating cell nuclear antigen (PCNA). Values are derived from n=5 to 6 animals (2 representative animals are shown). β-Actin is shown as loading control. G, PCNA immunostaining on mouse lung sections. One representative animal is shown per group. Scale bars, 20 μm. Inset, IgG served as negative control. Arrows indicate PCNA-positive nuclei. *P<0.05 vs respective controls.
Decreased proliferation was consistent with augmented apoptosis (Figure 4G). Consistently, overexpression of AMD-1 led to enhanced Ki67 mRNA expression and proliferation (Figure 4H and 4I), whereas apoptosis was attenuated (Figure 4J).

In contrast to AMD-1+/− mice, heterozygous ODC-1 knockout mice did not show any phenotypic differences compared with their WT littermates under chronic hypoxic conditions (Figure IVA–IVD in the online-only Data Supplement). Additionally, no changes in ODC-1

Figure 2. Gene regulation during reversal of hypoxia-induced pulmonary hypertension (PH). A, Schematic of the study design. Chronic hypoxic mice (21 days, 10% O2) were re-exposed to normoxia for the indicated time periods. B, Perturbed pathways during reverse remodeling assessed by DNA microarray analysis. Gray scale represents P values from the gene-set test ranging from <0.01 (black) to 1.00 (white). Values were derived from animals exposed to hypoxia (21 days, n=20) and mice re-exposed to normoxia (n=12 per time point). CAM indicates cell adhesion molecule; MAPK, mitogen-activated protein kinase; and TGF, transforming growth factor. C, Selected and perturbed genes during reverse remodeling assessed by DNA microarray analysis. Displayed genes in at least 2 time points were differentially regulated by a factor of 1.5 compared with hypoxic controls. Colors represent regulation ranging from downregulated (bright red) to upregulated (bright green). Values were derived from animals exposed to hypoxia for 21 days (n=20) and re-exposure to normoxia (n=12 per time point). D, Volcano plot: statistical significance versus regulation. Genes with log odds values ≥5 were considered to be regulated; black spots show AMD-1 regulation. The 2 different values for AMD-1 expression result from 2 different probes against AMD-1 spotted on the microarray chips used. E, AMD-1 mRNA expression quantified by quantitative polymerase chain reaction (q-PCR) during the reversal of PH. Values were derived from n=5 to 7 animals and normalized to B2M. F, AMD-1 mRNA expression levels during the time course of exposure to hypoxia as analyzed by q-PCR and normalized to B2M (n=5–6 animals). *P<0.05 vs respective controls.
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Expression were noted in mouse lung homogenate from chronic hypoxic (21 days) versus normoxic animals or during reoxygenation (Figure IVE and IVF) in the online-only Data Supplement. Similar effects were observed in PASMCs exposed to 1% O₂, with unchanged ODC-1 expression (hPASMCs) or its downregulation (mPASMCs; Figure VA and VB in the online-only Data Supplement). Heterozygous knockout in ODC-1+/− and AMD-1+/− animals was confirmed by reduced mRNA expression of the respective gene compared with WT mice (Figure VC and VD in the online-only Data Supplement).

AMD-1 mRNA Expression Is Stimulated by EGF

To investigate whether hypoxia can upregulate AMD-1 expression by its direct effect on PASMCs, for example, via HIF, mPASMCs and hPASMCs were exposed to hypoxia (1% O₂) for 24 hours. Contrary to our expectation, hypoxia had no effect on AMD-1 mRNA levels (Figure 5A and 5B). Although

**Figure 3.** S-adenosylmethionine decarboxylase 1 (AMD-1) is localized predominantly in pulmonary smooth muscle cells. A, Fluorescence in situ hybridization (FISH) for AMD-1 (red) combined with immunofluorescence staining for α-smooth muscle actin (SMA; green) of mice exposed to hypoxia for 21 days compared with normoxic controls. Representative images are shown. Scale bars, 20 μm for the lower and 10 μm for the higher magnification. B indicates bronchus; and V, vessel. B, AMD-1 mRNA regulation in laser-microdissected pulmonary vessels from chronic hypoxic mice compared with normoxic controls (n=6 each) and normalized to B2M. C, FISH for AMD-1 (red) combined with immunofluorescence staining for sma (green) of idiopathic pulmonary arterial hypertension (IPAH) or control (donor) tissue. Representative images are given. Scale bars, 50 μm in the lower and 20 μm in the higher magnification. Higher-magnification images for A and B were taken by different microscopic settings. D, AMD-1 mRNA expression in laser-microdissected pulmonary vessels from donor compared with IPAH lungs (n=5). *P<0.05 vs respective controls and normalized to B2M.
we identified an HIF-responsive element in both the murine and human AMD-1 promoter (Figure VIA in the online-only Data Supplement), we were not able to prove its functionality by chromatin immunoprecipitation (Figure VIB in the online-only Data Supplement) or electrophoresis mobility shift assay (not shown). Additionally, there was no difference in AMD-1 expression in hPASMCs after knockdown of HIF-1α or HIF-2α (Figure VIC in the online-only Data Supplement), and exposure to different degrees of hypoxia (1%, 3%, or 5% O2) led to a substantial change in AMD-1 expression at only 1

Figure 4. AMD-1+/− mice are partially protected from hypoxia-induced pulmonary hypertension. Functional and structural parameters of wild-type (WT) and AMD-1+/− mice exposed to either normoxia or 28 days of chronic hypoxia at 10% O2. A, Right ventricular systolic pressure (RVSP) quantified by right heart catheterization (n=7–8 animals each). *P<0.05 vs the indicated group. Two-way ANOVA was used to test the interaction of genotype and condition (−7.8±3.5 mm Hg; P=0.0004). B, Right heart hypertrophy given as the ratio of right ventricular (RV) to left ventricular (LV) plus septal mass (n=8–10). The interaction was −0.01±0.08 g/g. *P<0.05 vs the indicated group. C, The degree of muscularization of small pulmonary vessels is shown in bar graphs representing the mean±SEM. Values are given for nonmuscularized, partially muscularized, or fully muscularized vessels (outer diameter, 20–70 μm) from paraffin-embedded lung sections costained against α-smooth muscle actin (α-sma) and von Willebrand factor (n=7–8 animals each). *P<0.05 vs normoxic WT; †P<0.05 vs normoxic AMD-1+/−; ‡P<0.05 vs hypoxic wild-type (WT) mice. D, Western blot analysis for proliferating cell nuclear antigen (PCNA) in lung homogenate from WT and AMD-1+/− mice exposed to chronic hypoxia (n=4–5 each). β-Actin is shown as loading control. *P<0.05 vs WT controls. E, Relative Ki67 mRNA expression quantified by quantitative polymerase chain reaction (q-PCR) in human pulmonary artery smooth muscle cells (hPASMCs) 72 hours after knockdown of s-adenosylmethionine decarboxylase 1 (siAMD-1). n=7 to 10 per group (w/o indicates untransfected). *P<0.05 vs nonspecific knockdown (siR). F, Assessment of proliferation of hPASMCs 72 hours after AMD-1 silencing (72 hours) as assessed by the CaspACE Assay System, colorimetric (n=9–15 individual experiments). Staurosporine (stauro) served as positive control. *P<0.05 vs siR. G, Relative Ki67 mRNA expression quantified by q-PCR in hPASMCs 72 hours after overexpression of AMD-1 (AMD-1-plxin), n=8 to 9 per group. *P<0.05 vs empty vector (plxin). H, Assessment of proliferation of hPASMCs 72 hours after overexpression of AMD-1 was performed by total cell count (n=8). *P<0.05 vs siR. I, Apoptosis of hPASMCs after AMD-1 silencing (72 hours) as assessed by the CaspACE Assay System, colorimetric (n=9–15 individual experiments). Staurosporine (stauro) served as positive control. *P<0.05 vs siR. J, Apoptosis of hPASMCs after AMD-1 overexpression (72 hours) as assessed by the CaspACE Assay System, colorimetric (n=14 individual experiments). *P<0.05 vs empty vector (plxin). Staurosporine served as positive control.
investigated time point (Figure VIF and VIH in the online-only Data Supplement). To investigate the mechanisms underlying AMD-1 regulation in PH, hPASMCs were stimulated with growth factors known to be upregulated under hypoxic conditions in, for example, endothelial cells.20 Whereas there was no effect in response to platelet-derived growth factor-BB (data not shown), transforming growth factor-β1-induced AMD-1 silencing. The strongest time-dependent regulation method was applied for screening of potential Egr1 consensus sequences within the AMD-1 promoter region.

Electrophoresis mobility shift assay revealed an interaction of Egr1 with this consensus sequence (Figure VII in the online-only Data Supplement). Chromatin immunoprecipitation was carried out from untreated hPASMCs or after stimulation with EGF (50 ng/mL) with Egr1 or nonimmunogenic antibody (anti-IgG). One representative of 4 individual experiments is shown. I. Relative AMD-1 mRNA expression after 6 hours of EGF stimulation (50 ng/mL) in the presence or absence of AG1478 (20 μmol/L) was assessed by q-PCR (n=4–9). *P<0.05 vs EGF-treated cells.

**EGF Regulates AMD-1 Expression via Egr1 Transcription Factor**

Because EGF can induce multiple transcription factors, including early growth response (Egr1),22 we next studied whether EGF can induce Egr1 expression in hPASMCs. Stimulation of hPASMCs with EGF induced Egr1 mRNA and protein levels with the highest median values after 1 hour (Figure 5E and 5F). An automated computational analysis using a gene prediction method was applied for screening of potential Egr1 consensus sequences within the AMD-1 promoter region. Similar to previous findings,23 1 Egr1 consensus sequence (cgctcgcg) in position −386 from the transcription start site was detected (Figure VII in the online-only Data Supplement). Electrophoresis mobility shift assay revealed an interaction of Egr1 with this consensus sequence (Figure 5G, lane 2). The binding was enhanced in nuclear extracts stimulated with EGF (Figure 5G, lane 4). The specificity of binding was proven by a competition assay. Addition of excess unlabeled probe...
eliminated the specific complex (Figure 5G, lanes 3 and 5). This finding was further confirmed by chromatin immunoprecipitation (Figure 5H). With the use of primers flanking the Egr1-binding site in the AMD-1 promoter, a PCR product was detected in the input material and immunoprecipitated chromatin after EGF stimulation (Figure 5H, lanes 2 and 4). The EGF-induced increase in AMD-1 expression was attenuated by the EGFR inhibitor AG1478 (Figure 5I).

**EGF Regulates Egr1 Expression via Erk1/2**

Because accumulating evidence suggests that EGF can increase Egr1 levels via Erk1/2 signaling, we investigated whether this pathway also controls the Egr1 increase in hPASMCs. Indeed, EGF application induced Erk1/2 phosphorylation (Figure 6A), which was attenuated by the Erk1/2 inhibitor U0126 (Figure 6B). Additionally, application of U0126 prevented the EGF-dependent increases in Egr1 expression (Figure 6C and 6D). To further demonstrate the importance of Egr1 in regulating AMD-1, we examined AMD-1 expression in PASMCs from Egr1−/− and WT mice in response to EGF stimulation. In contrast to cells from WT mice, AMD-1 mRNA expression was not affected in PASMCs from Egr1−/− mice (Figure 6E). Additionally, EGF stimulation did not enhance the proliferation of PASMCs isolated from Egr1−/− knockout compared with WT mice (Figure 6F).

**AMD-1 Depletion Attenuates Phospholipase Cγ1 Phosphorylation**

In the next step, we assessed whether AMD-1 depletion has effects on phospholipase Cγ1 (PLC-γ1) phosphorylation. As depicted in Figure 7A, depletion of AMD-1 resulted in decreased PLC-γ1 phosphorylation. Similar effects were observed in lungs from AMD-1−/− mice (Figure 7B). Furthermore, EGF induced PLC-γ1 phosphorylation in hPASMCs, which was diminished by U0126 and the PLC inhibitor U73122 (Figure 7C).

**AMD-1 Inhibition Impairs Hypoxia-Induced PH**

Finally, we were interested in whether pharmacological inhibition of AMD-1 in vivo was able to inhibit hypoxia-induced PH. Therefore, the selective AMD-1 inhibitor SAM486a was used in WT mice during hypoxic exposure. Although RVSP was not reduced compared with hypoxic placebo controls (Figure 8A), right heart hypertrophy was improved considerably by AMD-1 inhibition (Figure 8B). In addition, the ratio of pulmonary artery acceleration time to pulmonary artery ejection time determined by echocardiography was ameliorated compared with hypoxic placebo controls (Figure 8C). Furthermore, maintained cardiac function was confirmed by the prevention of the hypoxia-induced decline in cardiac index and the avoidance of hypoxia-induced enhancement in
right ventricular internal diameter, total pulmonary resistance, and myocardial performance index (Figure 8D–8G). In addition, AMD-1 inhibition prevented the increase in the degree of muscularization in small pulmonary arteries because the percentage of fully muscularized vessels was lower than in hypoxic placebo controls (Figure 8H and 8I).

Discussion

The major findings of our study are that (1) PH induced in mice by chronic hypoxic exposure is fully reversible on re-exposure to normoxia, including a reversal of right heart hypertrophy and vascular remodeling; (2) this model in combination with genome-wide screening can be used to identify factors involved in the reversal of the disease and its development; and (3) AMD-1 is a potential key player for both the progression and the reversal of PH. Furthermore, we deciphered AMD-1 signaling in the pulmonary vasculature by a variety of different approaches, including cellular studies, knockout mice, and in vivo inhibition of AMD-1.

On exposure to chronic hypoxia, the pulmonary vasculature undergoes remodeling, resulting in sustained elevation in pulmonary vascular resistance and right heart hypertrophy. It has previously been shown that re-exposure of rats to normoxia reduces vascular changes, which are accompanied by decreased RVSP and lower right heart hypertrophy.2,24 Going beyond these data, we have shown in a detailed time kinetic that PH, the accompanying right heart hypertrophy, and the structural vascular alterations underlying hypoxia-induced PH are fully reversible on reoxygenation in mice.

The use of a microarray analysis during the course of PH reversal identified genes and pathways that are regulated during the course of PH and could be key factors for both the reversal and, as shown by our analysis, the development of PH. Of all genes differentially expressed during reverse remodeling, we focused here on further delineating the function of AMD-1 in vascular remodeling for the following reasons: AMD1 was one of the most downregulated genes in our screen; AMD-1 was demonstrated to be involved in embryonic development; AMD-1 has been reported to be associated with cancer progression; and AMD-1 has been linked to tissue repair.

AMD-1 is one of the major rate-limiting enzymes in polyamine synthesis. The polyamine precursor putrescine is generated by ODC-1; in turn, AMD-1 is required for the conversion of putrescine to polyamines. A large body of evidence suggests that polyamine suppression decreases cell growth, angiogenesis, and the expression of genes affecting tumor invasion and apoptosis.27,28 The prominent role of polyamines in directing cell proliferation, an important characteristic of vascular remodeling, revealed a possible involvement of AMD-1 in regulating responses to hypoxia in the pulmonary vasculature. In accordance with this assumption, we could show that hypoxic exposure in vivo led to the upregulation of AMD-1 not only in mouse lung homogenate but also, most importantly, in pulmonary vessels, the sites of pulmonary vascular remodeling.25,26 This is in line with previous findings in which substantial increases in AMD-1 activity in chronic hypoxic rat lungs were noted.10 Additionally, augmented AMD-1 activity has been described in monocrotaline-treated rats.30 AMD-1 and lung polyamine contents were found to be elevated in hypoxia.9,10 However, no direct molecular in vivo proof for the relevance of its synthesizing enzyme AMD-1 for the development or regression of PH previously existed. To determine the expression and localization of AMD-1, we used quantitative polymerase chain reaction and nonisotopic in situ hybridization instead of conventional protein detection methods such as Western blot analysis and immunohistological stainings because commercially available antibodies demonstrated unspecific stainings. In contrast to the pulmonary vasculature, AMD-1 levels in systemic vessels were almost unaffected by hypoxia. This underscores AMD-1 as a potential target in the pharmacotherapy of PH, which promises fewer side effects than other established treatments. AMD-1 was also upregulated in vessels in patients with human idiopathic pulmonary
Figure 8. The s-adenosylmethionine decarboxylase 1 (AMD-1) inhibitor SAM486a preserved right heart function and pulmonary ventricular resistance and attenuated pulmonary vascular remodeling in chronically hypoxic mice. Wild-type (WT) mice were exposed to either normoxia or 28 days of chronic hypoxia at 10% O₂ and treated with placebo or the AMD-1 inhibitor SAM486a (1 mg/kg body weight [BW] per day IP). 

A, Right ventricular systolic pressure (RVSP) was assessed by right heart catheterization (n=9–10 each).

B, Right heart hypertrophy given as the ratio of right ventricular (RV) to left ventricular (LV) plus septal mass from heart tissue (n=10 each).

C, The ratio of pulmonary artery acceleration time/pulmonary artery ejection time (PAAT/PAET), (D) cardiac index (CI), (E) right ventricular internal diameter (RVID), (F) total pulmonary resistance (TPR), and (G) myocardial performance index (MPI) were assessed by echocardiography (n=9–10 animals each). *P<0.05 vs the indicated groups.

H, The degree of muscularization of small pulmonary vessels is shown in bar graphs representing mean±SEM. Values are given for nonmuscularized, partially muscularized, or fully muscularized vessels (outer diameter, 20–70 μm) from paraffin-embedded lung sections costained against α-smooth muscle actin (α-sma) and von Willebrand factor (vWF; n=5 animals each). *P<0.05 vs hypoxic placebo control.

I, Representative images of lung sections costained against α-sma (violet) and vWF (brown) displaying vessels (V) from indicated mice exposed to normoxia or chronic hypoxia. IgG served as negative control. Scale bars, 20 μm. B indicates bronchus.

J, Schematic description of AMD-1 involvement in pulmonary hypertension. Enhanced endothelial growth factor (EGF) levels activate the EGF receptor, which leads to a dimerization and internal phosphorylation. Thus, Erk1/2 phosphorylation and subsequently early growth response-1 (Egr1) expression are augmented, resulting in induced AMD-1 transcription. Consequent enrichment of polyamines induces phospholipase Cγ1 (PLCγ1) phosphorylation and thus hydrolysis of PIP, Phosphatidylinositol 4,5-bisphosphate (PIP₂) into inositol 1,4,5-trisphosphate (IP₃) and diacyl glycerol (DAG). IP₃ induces the release of calcium from the sarcoplasmic reticulum, which increases vascular tone and thus leads to vascular remodeling.
arterial hypertension, which emphasizes AMD-1 as a novel target in PH therapy.

Because both homozygous AMD-1−/− and ODC-1−/− mice exhibit embryonic lethality,25,31 we exposed AMD-1+/− mice to chronic hypoxia to investigate the role of AMD-1 in vivo. Compared with WT littermates, AMD-1+/− mice developed less severe hypoxia-induced PH. This can be explained in part by our findings that AMD-1 depletion resulted in decreased proliferation and enhanced apoptosis. The clinical relevance of AMD-1 was further demonstrated by an intervention approach in which application of the inhibitor SAM486a led to improved cardiac function and decreased muscularization of intrapulmonary arteries.

In contrast to AMD-1, ODC-1 did not show changes in mRNA expression in our investigations in mouse lung homogenate. This was mirrored by constant or even decreased expression of ODC-1 in isolated human and mouse PASMCs exposed to hypoxia. Similarly, reduced ODC-1 expression was observed under hypoxic conditions in bovine PASMCs.32 In our experimental setup, ODC-1−/− mice did not demonstrate any protective phenotype when exposed to chronic hypoxia. In contrast, in another study, inhibition of ODC-1 in hypoxic rats improved mean pulmonary arterial pressure and medial thickness but did not affect right heart hypertrophy.11 Although we can speculate that AMD-1 is the key enzyme in polyamine metabolism responsible for hypoxia-induced vascular remodeling and PH, this assumption has to be drawn cautiously because only 1 ODC-1 allele is deleted in ODC-1−/− mice.

Interestingly, in human PASMCs, we could detect increased AMD-1 expression only after 24 hours of exposure to 5% O2 but at no other investigated condition. Although we cannot exclude that this may have a biologically important effect, we assume that the direct effect of hypoxia on AMD-1 regulation might be rather small. This might be explained by the fact that hypoxia-induced remodeling of the pulmonary circulation is a highly complex process. Multiple interacting events cooperate between various cell types, their differentiation, and the release of mediators such as growth factors.26,33 Numerous growth factors being regulated by hypoxia,34,35 including platelet-derived growth factor-BB, transforming growth factor-β, and EGF, have been implicated in human PH and animal models of PH.46 All of them can act as a stimulator of proliferation on different cells on release from, for example, adventitial fibroblasts31 or endothelial cells.20 They were also found to modulate AMD-1 levels in various cell types.37,38 However, little is known about the regulation of AMD-1 expression in PASMCs. From all the above-mentioned growth factors, we could show that EGF caused the most dramatic increase in AMD-1 expression. This might be of importance because previous studies demonstrated that its concentration can be augmented by hypoxia.35 Additionally, EGF expression has been elevated in cardiac and pulmonary fibrosis,39,40 indicating the involvement in proliferative diseases. Moreover, blockade of the EGF receptor increased survival and reversed PH in the animal model of monocrotaline-induced PH in rats.41 Because EGF induces a variety of mediators, including the transcription factor Egr1,42 which has been reported to be important for cell survival,42 we have analyzed whether the EGF/Egr1 axis directs AMD-1 expression. In this regard, we proved a direct interaction between Egr1 and the AMD-1 promoter, which was further enhanced by EGF.

To decipher the molecular mechanisms of the EGF/Erk1/2/Egr1/AMD-1 signaling cascade, we focused on potential downstream targets of AMD-1. Because our microarray analysis revealed phosphatidylinositol signaling to be strongly regulated, we investigated the potential contribution of PLC-γ1 to AMD-1 signaling. PLC-γ1 is a key player of phosphatidylinositol signaling known to increase cytosolic calcium,43 a process thought to mediate pulmonary vascular remodeling and PH.44

Indeed, downregulation of AMD-1 in human PASMCs or in mouse lung homogenate resulted in lower PLC-γ1 phosphorylation. Taken together, these findings indicate that AMD-1 might be responsible for the pulmonary remodeling process in response to hypoxia, the subsequent establishment of hypoxia-induced PH, and the regression of PH. This was further emphasized by our pharmacological intervention approach using the AMD-1 inhibitor SAM486a, which resulted in attenuated hypoxia-induced PH. From previous studies, we can speculate that all of our approaches may affect polyamines as 1) polyamines are upregulated during exposure to hypoxia,9–11 2) pharmacological inhibition of AMD-1 in vivo by 1 mg/kg body weight of SAM486a reduced spermine contents,45 and 3) prolonged EGF administration in rats was associated with increases in all 3 polyamines, along with augmented mean pulmonary arterial pressure.46

Conclusions

In this study, we demonstrated altered AMD-1 expression in vascular remodeling and reverse remodeling in hypoxia-induced PH. Furthermore, localization of AMD-1 in the pulmonary vasculature and attenuation of PH in AMD-1−/− mice strengthened evidence of its key role in PH. In addition, we could identify that upstream signaling of AMD-1 occurs by EGF, Erk1/2, and Egr1, whereas PLC-γ1 phosphorylation might be the downstream driving force for vascular remodeling (Figure 8J). Thus, we provided evidence for the first time that pharmacological inhibition of AMD-1 may be a new strategy for the treatment of PH because it resulted in decreased hypoxia-induced PH.

Acknowledgments

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Sources of Funding

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Disclosures

None.
References


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CLINICAL PERSPECTIVE

Pulmonary hypertension (PH) is a progressive and life-threatening disease characterized by an increase in pulmonary vascular resistance, which can culminate in cardiac failure and death. Until now, research in the field of PH has concentrated mostly on the onset and development of PH. Therefore, we focused here on the mechanisms underlying the reversal of PH. Although it has previously been shown in rodents that hypoxia-induced vascular remodeling can be reversed by re-exposure to normoxia, the factors involved in this process remain elusive. Using DNA microarray analysis, we screened for such potential factors in reverse remodeling of the pulmonary vasculature on re-exposure of chronically hypoxic animals with PH to normoxia. In addition to other known genes, our whole-genome approach revealed s-adenosylmethionine decarboxylase 1 (AMD-1), one of the major rate-limiting enzymes in polyamine synthesis, as one of the most downregulated genes during this process. The regulation was confirmed by different techniques not only in reverse remodeling but also during the hypoxia-induced remodeling process. Furthermore, attenuation of hypoxia-induced PH in wild-type mice treated with the AMD-1 inhibitor SAM486a (1 mg/kg body weight IP) and AMD-1+/− mice strengthened evidence of its key role in PH. Thus, pharmacological inhibition of AMD-1 may be a new strategy for the treatment of PH if these results are transferable to humans.
Impact of S-Adenosylmethionine Decarboxylase 1 on Pulmonary Vascular Remodeling

Friederike Christine Weisel, Christina Kloepping, Alexandra Pichl, Akylbek Sydykov, Baktybek Kojonazarov, Jochen Wilhelm, Markus Roth, Karen Marie Ridge, Kazuei Igarashi, Kazuhiro Nishimura, Wolfgang Maisen, Claudia Wackendorff, Walter Klepetko, Peter Jaksch, Hossein Ardeschir Ghofrani, Friedrich Grimminger, Werner Seeger, Ralph Theo Schermuly, Norbert Weissmann and Grazyna Kwapiszewska

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SUPPLEMENTAL MATERIAL

Impact of s-adenosylmethionine decarboxylase 1 in pulmonary vascular remodeling

Weisel FC, PhD, Kloeping C, DVM, Pichl A, PhD, Sydykov A, MD PhD, Kojonazarov B, MD PhD, Wilhelm J, PhD, Roth M, PhD, Ridge KM, PhD, Igarashi K, PhD, Nishimura K, PhD, Maison W, PhD, Wackendorff C, Klepetko W, MD, Jaksch P, MD, Ghofrani HA, MD, Grimminger F, MD PhD, Seeger W, MD, Schermuly RT, PhD, Weissmann N, PhD*, and Kwapiszewska G, PhD*

Supplemental Methods

Hemodynamic measurements, echocardiography, and tissue preparation.

For monitoring hemodynamics in the knockout mice and the wild-type mice during reverse remodeling, the animals were anesthetized interperitoneally with ketamine (40µg/g body weight) and Rompun 2% (0.6µg/g body weight). Measurement of right ventricular systemic pressure (RVSP) and systemic arterial pressure (SAP) was performed as described previously1. Briefly, the left carotid artery was cannulated, and a right heart catheter was inserted through the right jugular vein for measurement of RVSP with fluid filled pressure transducers. After exsanguination, the left lung was fixed for histology in 10% neutral buffered formalin, and the right lung was snap-frozen in liquid nitrogen. For right heart hypertrophy, the right ventricle (RV) was separated from the left ventricle plus septum (LV+S) and the RV/(LV+S) ratio was determined from the tissue. Hemodynamics in mice treated with SAM486a or placebo were measured as described previously2 with slight modifications. Briefly, anesthesia was induced with isoflurane gas (3%) after pretreatment with buprenorphine (0.05mg/kg, sc) and maintained with 1.5% isoflurane in room air
supplemented with 100% O₂. After intubation the mouse was placed supine on a homeothermic plate (AD Instruments, Spechbach, Germany) and connected to a small animal ventilator MiniVent type 845 (Hugo Sachs Elektronik, March-Hugstetten, Germany). The body temperature was controlled by the rectal probe connected to the control unit (AD Instruments, Spechbach, Germany) and was kept at 37°C during the catheterization. The right external jugular vein was catheterized with a high fidelity 1.4F micromanometer catheter (Millar Instruments, Houston, USA) and advanced into the right ventricle to assess RVSP. Subsequently, the high fidelity 1.4F micromanometer catheter was inserted into the aorta and the left ventricle through the right carotid artery for measurement of SAP. Data was collected and analyzed using the PowerLab data acquisition system (MPVS-Ultra Single Segment Foundation System, AD Instruments, Spechbach, Germany) and LabChart 7 for Windows software.

Echocardiography was assessed as previously described³,⁴. Briefly, anesthesia was induced with isoflurane gas (3%) and maintained with 1.5% isoflurane in room air supplemented with 100% O₂. Mice were laid in supine position on a heating platform with all legs taped to ECG electrodes for heart rate (HR) monitoring. Body temperature was monitored via a rectal thermometer (Indus Instruments, Houston, TX). The chest of the mice was shaved. To provide a coupling medium for the transducer, a pre-warmed ultrasound gel was spread over the chest wall. Transthoracic echocardiography was performed with Vevo770 high-resolution imaging system equipped by 30-MHz transducer (VisualSonics, Toronto, Canada). Cardiac output (CO) was calculated as the product of the velocity-time integral of the pulsed-Doppler tracing in the LV outflow tract, the cross-sectional area of the LV outflow tract, and the HR. CI was derived by dividing CO by body weight. The right ventricular internal diameter (RVID), pulmonary artery acceleration time (PAAT) and myocardial performance index (MPI) were measured. Additionally, total pulmonary resistance (TPR) was calculated⁵.
**Paraffin embedding and microscopy**

For vascular morphometry, lungs were fixed by vascular perfusion with Zamboni’s fixative through the pulmonary artery after flushing the lungs with saline at a vascular pressure of 22 cm H$_2$O and a tracheal pressure of 12 cm H$_2$O. Investigations were performed from 3 µm sections of paraffin embedded lungs. The degree of muscularization was determined from lung sections stained using antibodies against α-smooth muscle actin (α-sma) and von Willebrand factor (vWF) as described previously$^{1, 6}$. An antibody directed against IgG (Millipore, Schwalbach, Germany) served as negative control.

Lungs were fixed by immersion in a 3.5% paraformaldehyde solution. For paraffin embedding, the lungs were dissected in tissue blocks. The degree of muscularization of pulmonary arterial vessels was determined as described$^{7,8}$ from lung sections stained by antibodies against α-smooth muscle actin (mouse monoclonal, Sigma-Aldrich, Munich, Germany) and von Willebrand Factor to allow identification of vessels (Dako, Hamburg, Germany). Morphometric quantification was carried out microscopically using the Qwin software (Leica, Wetzlar, Germany). Vessels were categorized as fully- (>70% vessel circumference α-smooth muscle actin positive), partially- (5%-70% vessel circumference α-smooth muscle actin positive) or non-muscularized (<5% vessel circumference α-smooth muscle actin positive). 85 vessels of an outer diameter of 20-70µm were analyzed from each lung lobe in a blinded fashion. The degree of muscularization is given as percentage of total vessel count.

**PCNA staining**

For immunohistochemical localization of PCNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA), the AP-fast red kit (Zytochem, Berlin, Germany) was used on paraffin-embedded lung sections according to the manufacturer’s instructions. All stained sections were analyzed using digital slide scanning employing a mirax scanner and the mirax viewer software (Carl
Zeiss GmbH, Jena, Germany). An antibody directed against IgG (Millipore, Schwalbach, Germany) served as negative control.

**Laser-microdissection, RNA isolation, cDNA synthesis and q-PCR**

Laser-assisted microdissection was performed to isolate pulmonary arterial vessels from cryostat lung sections as previously described\(^9\). Total messenger RNA was extracted from frozen mouse or human lung tissue and microdissected vessels by using the RNeasy Mini or Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The isolated RNA from microdissected vessels was subsequently amplified and transcripted into cDNA with the WT-Ovation Pico RNA Amplification System (Nugen Technologies, San Carlos, CA, USA) according to manufacturer’s instructions and subsequently purified using Qiaquick PCR Purification Kit (Qiagen, Hilden, Germany). Q-PCR was performed after reverse transcription with equal amounts of RNA, using the iScript cDNA Synthesis Kit (BIO-RAD, Munich, Germany) according to manufacturer’s instructions. Q-PCR was performed with the iQ SYBR Green Supermix according to the manufacturer’s instructions (BIO-RAD, Munich, Germany). Q-PCR was carried out in a Mx3000P (Stratagene, Heidelberg, Germany) under the following conditions: 1 cycle at 95°C for 10 min, then 40 cycles at 95°C for 10s, 59°C for 10 s, 72°C for 10s, followed by a dissociation curve. The intron-spanning primers were designed by using sequence information from the NCBI database and purchased from Metabion (Martinsried, Germany). The Ct values were normalized to the endogenous control, β\(_2\) microglobulin (B2M), using the equation \(\Delta Ct = Ct_{\text{reference}} - Ct_{\text{target}}\). Thus, higher \(dCt\)-values indicate a higher relative expression.

**Microarray**

RNA from 80 animals (20 controls and 12 per time point) were analyzed in a total of 40 dual-color hybridizations in a balanced dye-swap design where each time point was directly compared to the control and to the adjacent time points. Table 1 shows which combinations
were hybridized in detail.

RNA was purified using the RNeasy Mini Kit (QIAGen, Hilden, Germany) following the kit’s instructions. RNA quality was assessed by capillary electrophoresis using the Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA).

Purified total RNA was amplified and Cy-labeled using the dual-color QuickAmp kit (Agilent Technologies, Palo Alto, CA) following the manufacturer’s instructions. Per reaction, 1µg of total RNA was used. The samples were labeled with either Cy3 or Cy5 to match a balanced dye-swap design. Cy3- and Cy5-labeled aRNA were hybridized overnight to 4x44K 60mer oligonucleotide spotted microarray slides (Mouse Whole Genome 4x44K; Agilent Technologies). Hybridization and subsequent washing and drying of the slides was performed following the Agilent hybridization protocol. The dried slides were scanned using a GenePix 4100A scanner (Axon Instruments, Union City, USA) at a resolution of 5 µm per pixel. Gain settings for the photomultiplier tubes were adjusted to use the entire dynamic range of the instrument and to get comparable fluorescence yields in both channels. Images of Cy3 and Cy5 signals were recorded as 2-layer TIFF-files and analyzed using GenePix Pro 5.0. Spots that could not be interpreted due to artifacts introduced by slide or hybridization processing were flagged bad and thus removed from further analysis. Calculated foreground and background intensities for all spots were saved as GenePix results files.

**Microarray data analysis**

Stored data were evaluated using the R software\textsuperscript{10} and the limma package\textsuperscript{11} from BioConductor\textsuperscript{12}. Data from technical replicates (hypoxia dye-swap data) were averaged. Data from replicate spots were averaged prior to further analysis. The M/A data were LOESS normalized\textsuperscript{13} before averaging using the "lmFit" function. Genes were ranked for differential expression using a moderated t-statistic\textsuperscript{14}. Candidate lists were created by adjusting the false-discovery rate to 5%. Geneset enrichment analyses were done using the “geneSetTest”
function together with the KEGG pathway information given in the Bioconductor database package mgug4122a.db version 2.2.5. Significantly enriched pathways were selected on the basis of a 5% false-discovery rate.

Isolation of aorta and carotid artery
After exposure to either normoxia or hypoxia for 1, 7 or 21 days, mice were anesthetized interperitoneally with ketamine and xylazine. Subsequently, the A. carotis interna and externa including the bifurcation were isolated. The aorta was separated from the heart close to the aortic arch and disconnected from the adventitia. Afterwards, the carotid arteries and the aortas were washed in sterile, ice-cold PBS and snap-frozen in liquid nitrogen.

Non-isotopic in situ hybridization (NISH) combined with immunofluorescence on mouse lung sections
Non-isotopic in situ hybridization on mouse lungs was carried out as previously described with slight modifications. The fluorescent substrate Alexa fluor 555 tyramide (Invitrogen, Karlsruhe, Germany) was used for visualization of an AMD-1 antisense probe. The mouse monoclonal FITC-labeled α-sma antibody (Abcam, Cambridge, MA, USA) was employed for immunostaining. As negative controls, a denatured sense AMD-1 probe as well as a 10-fold excess of an AMD-1 antisense unlabeled probe, which was applied two hours prior to the labeled probe, were used.

NISH on human lung tissue was carried out on 3µm paraffin sections. For deparaffinization, sections were treated twice with Xylol (10 min each) and rehydrated (100% Ethanol 5min, 80% Ethanol 5min, 70% Ethanol 5min, DEPC-water 5min). Following proteinase K digestion (Peqlab, Erlangen, Germany; 7.5µg/ml in DEPC-PBST) and washing in DEPC-PBST, sections were fixed in 4% PFA containing 0.2% glutaraldehyde for 25min. After an additional washing step, samples were incubated in 0.1 M acetylated triethanolamine (0.5ml acetic anhydride per 200ml) on a shaking platform for 10min. Further treatment of the samples was
according to the protocol on mouse tissue. Additionally, a poly-T probe (40nM, Exiqon, Vedbaek Denmark) was used as positive control.

**Cell culture**

hPASMCs were purchased from Lonza (Cologne, Germany) and grown to near confluence. mPASMCs were isolated from pre-capillary vessels modified from a previously reported protocol\(^\text{15, 16}\). SMCs were identified by immunohistochemical staining with α-sma and myosin antibodies (both from Abcam, Cambridge, MA, USA) as well as their morphology. The absence of endothelial cells was confirmed by staining with an antibody directed against vWF (Dako Cytomation, Hamburg, Germany).

For RNA interference, 100nM of AMD-1 siRNA (5’-gaa uuu cau gaa gcc uuc u-3’) (BioSpring, Frankfurt am Main, Germany) was transfected using 1µl X-tremeGENE siRNA Transfection Reagent (Roche, Mannheim, Germany) per cm\(^2\) of the well. For controls, a scrambled siRNA (Invitrogen, Karlsruhe, Germany) was employed.

For inhibitor experiments cells were serum-starved over-night. The compounds U0126 (Erk1/2 inhibitor, 10µM, Sigma-Aldrich, St Louis, MO, USA), U73122 (PLC inhibitor, 3µM, Merck, Darmstadt, Germany) and AG1478 (EGFR inhibitor, 20µM, Cayman Chemical Comp., Ann Arbor, MI, USA) were applied for 1h. Afterwards, starving medium was reintroduced and cells were treated as indicated. For stimulation experiments, the cells were serum-starved overnight and stimulated with mEGF (50ng/ml R&D Systems, Minneapolis, MN, USA), hEGF (50ng/ml, Peprotech, Hamburg, Germany) or hTGF-β1 (2ng/ml, Peprotech, Hamburg, Germany) for indicated time-points. Cells were then washed twice with DPBS and RNA or proteins were isolated.

**Proliferation assay**

HPASMCs were seeded on 6-well plates and transfected with control siRNA (siR) or siRNA against AMD-1 when grown to near confluence. 72h after transfection, cells were trypsinized
and counted in a Neubauer counting chamber (LO – Laboroistik GmbH, Bad Homburg, Germany). Values were normalized to siR.

**Apoptosis Assay**

hPASMCs were grown to near confluence and transfected against siR or siAMD-1, respectively. Apoptosis rate was analysed 72h after transfection using the CaspACE™ Assay System, Colorimetric Kit (Promega, Mannheim, Germany) according to manufacturer’s instructions. Staurosporine (1µm, Sigma-Aldrich, Munich, Germany) was incubated for four hours and served as positive control.

**Plasmid Construction**

Full-length human AMD-1 cDNA (accession number NM_001634) was amplified from a human cDNA clone (OriGene Technologies, Rockville, MD, USA) by PCR using primers containing restriction sites for XhoI or BamHI (shown in bold). Following restriction digestion with XhoI and BamHI, the insert was ligated into the pLXIN vector (Clonetech, Mountain View, CA, USA). For creation of the virus, the cloned plasmid or the empty pLXIN vector was incubated with the plasmids pCMV-VSV-G and pUMVC (both from Addgene, Cambridge MA, USA) and transfected into HEK293T cells using lipofectamine 2000 according to manufacturer’s instructions. The virus containing media was harvested during the following two days and after addition of 8µg/ml polyprene was filtered through a 0.45µm filter and snap-frozen. For infection of hPASMCs, 250µl of virus containing media was added three times a day per well of a six well plate. Experiments to assess the impact of AMD-1 overexpression were carried out after 72h post infection.

**Western Blot analysis**

Cells were scraped in 150 µl RIPA buffer, containing 1mM sodium vanadate, 0.1mM phenylmethylsulphonyl fluoride (PMSF), 40µl/ml protease-inhibitor mix complete (Roche, Mannheim, Germany) and 2.6µl/ml β-mercaptoethanol. Mouse lung homogenate was grinded
in liquid nitrogen and scraped in RIPA buffer. Subsequently the samples were centrifuged for 10 min at 8000g. The supernatant (containing 4x LDS loading buffer) was heated at 99°C for 10min and equal amounts of protein were loaded on a SDS polyacrylamide gel. The proteins were transferred to a polyvinylidene fluoride membrane (Pall Corporation, Dreieich, Germany) by the semidy-blotting method. The membrane was washed for 5min with wash buffer (20mM Tris, pH 7.6, 150mM NaCl, 0.1% (v/v) Tween 20) and subsequently blocked in 5% (w/v) non-fat dry milk powder dissolved in wash buffer at room temperature. For the quantification of Egr1, phospho-ERK1/2, ERK1/2, phospho-PLC-γ1, PLC-γ1 and PCNA in mouse lung homogenate and human PASMCs, the primary antibodies Egr1, phospho-ERK1/2, ERK1/2, phospho-PLC-γ1 Tyr(783), PLC-γ1 (all Cell Signaling, Danvers, MA, USA) and PCNA (Santa Cruz, Santa Cruz, CA, USA) raised in rabbits were used. For visualization of β-actin, the monoclonal β-actin antibody produced in mouse (Sigma-Aldrich, Munich, Germany) was employed. Before quantification, the band intensities were checked for saturation.

**Supplemental Tables**

**Table 1)**

Hybridizations in the reverse remodeling experiment. The numbers denote time points (in days), zero indicates the controls. Each combination was hybridized twice, using RNA from different animals.

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**Table 2)**

Primers employed in this study
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**NISH human**

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**EMSA human**

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**ChIP**

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AMD-1	AGCACTCTGGGAGGTTGAGG	GGTCAGTGCTCCATCTTTA
HRE
Cloning
AMD-1	NM_001634	ACCTTCTCGAGTTCGCTAGTCTCACGG
	TGATG	ATTCCGGATCTCAACTCTGCTTTG
	TTGCCGTTC
Supplemental figure 1

A

B

Rel. AMD-1 expression [ΔCt]

Normoxia 21 - - - - Hypoxia - 1 7 21 (days)

Normoxia 21 - - - - Hypoxia - 1 7 21 (days)
Supplemental figure 2

A

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<tr>
<th>AMD-1</th>
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<th>Cold competitor</th>
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B

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<th>Cold competitor</th>
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</table>
Supplemental figure 3

A

Supplemental figure 3

B

C

D

Rel. AMD-1 expression [ΔCt]

Rel. Ki67 expression [ΔCt]

Rel. AMD-1 expression [ΔCt]

Rel. Ki67 expression [ΔCt]
Supplemental figure 3

**E**

Relative proliferation

**F**

Apoptosis [405nm]

**G**

Rel. AMD-1 expression [ΔCt]

- Normoxia
- Hypoxia

siAMD-1
Supplemental figure 4

A

RVSP (mmHg)

WT ODC-1+/−
Normoxia Hypoxia

WT ODC-1+/−
Normoxia Hypoxia

Ratio RV/(LV+septum)

B

WT ODC-1+/−
Normoxia Hypoxia

WT ODC-1+/−
Normoxia Hypoxia

C

Degree of muscularization (% of total vessel count)

WT ODC-1+/−
Normoxia Hypoxia

WT ODC-1+/−
Normoxia Hypoxia

D

Degree of muscularization (% of total vessel count)

WT ODC-1+/−
Normoxia Hypoxia

Normoxia

Hypoxia (28 days)

E

Rel. ODC-1 expression

Normoxia Hypoxia

F

Rel. ODC-1 expression

Normoxia Hypoxia

after hypoxia (21 days)
Supplemental figure 5
Supplemental figure 6

A

-4630

HRE

AMD-1

-4273

HRE

AMD-1

B

Input

Hypoxia

IP: anti-Hif-1α

IP: anti-Hif-2α

IP: anti-IgG

C

D

E

F

G

Rel. AMD-1 expression

normalized ∆Ct [%]

Rel. AMD-1 expression

[∆Ct]

TGF-β1 stimulation (h)

Hypoxia (24h)

0 1 6 24 42

0 1 6 24 42

0 1 2 4 6 24

0 1 2 4 6 24

Input

Hypoxia

IP: anti-Hif-1α

IP: anti-Hif-2α

IP: anti-IgG

0 1 2 4 6 24

0 1 2 4 6 24

0 1 2 4 6 24

0 1 2 4 6 24

0 1 2 4 6 24

0 1 2 4 6 24

0 1 2 4 6 24

0 1 2 4 6 24
Supplemental figure 6

H

Rel. PGK expression
[ΔCt]

1% O₂ (h)

0 1 2 3 6 24 48
Supplemental figure 7

A

-386
Egr1

AMD-1
Figure Legends

Supplemental figure 1)

**AMD-1 mRNA levels are unchanged in systemic vessels upon hypoxia**

AMD-1 mRNA expression levels in mouse A) aorta and B) carotid artery during chronic hypoxic exposure derived from q-PCR. Values are assessed from n=5-6 animals per group and normalized to B2M. *p<0.05 vs respective controls.

Supplemental figure 2)

**Control stainings for NISH on mouse and human tissue**

A) Fluorescent *in situ* hybridization (FISH) for AMD-1 (red) combined with immunofluorescence staining for α-smooth muscle actin (sma, green) of mice exposed to hypoxia for 21 days. A sense probe as well as a 10-fold excess of unlabeled antisense probe (cold competitor) served as negative controls for NISH staining. Due to difficulties during fixation of the cryo sections, the images were not derived from serial sections. Bar represents 20µm. B) FISH for AMD-1 (red) combined with immunofluorescence staining for sma (green) on human tissue. A sense and a 10-fold-excess of cold competitor were employed as negative controls on the IPAH samples. A poly-T probe served as positive control on donor tissue. Bar represents 50µm.

Supplemental figure 3)

**Muscularization of pulmonary vessels after exposure to chronic hypoxia is less severe in AMD-1+/− mice compared to wild-type controls**

A) Immunohistochemical staining for α-sma (violet) and vWF (brown) on lung sections from wild-type (WT) and AMD-1+/− mice either exposed to normoxia or chronic hypoxia (28 days, 10% O₂), representative images. IgG served as negative control. Bar displays 20µm. B)
Relative AMD-1 mRNA expression in hPASMCs after knockdown of AMD-1 (72h). Values were derived from n=6-7 individual experiments and normalized to B2M. *Significant difference (p<0.05) compared to unspecific knockdown (siR). w/o = untransfected cells. Relative C) AMD-1 and D) Ki67 mRNA expression in hPASMCs after knockdown of AMD-1 (72h). Cells were either exposed to normoxia or hypoxia for the last 24 hours. Values were derived from 5-6 individual experiments and normalized to B2M. E) Comparison of proliferation of hPASMCs 72h after knockdown of AMD-1 and normoxic or hypoxic exposure (24h, 1% O₂) was performed by total cell count (n=8). F) Comparison of apoptosis of hPASMCs 72h after knockdown of AMD-1 and normoxic or hypoxic exposure (24h, 1% O₂) was performed by CaspACE Assay System, Colorimetric (n=11). G) Relative AMD-1 mRNA expression in hPASMCs after overexpression of AMD-1 (72h). Values were derived from 4 individual experiments and normalized to B2M. *p<0.05 compared to empty vector (plxin).

Supplemental figure 4)

Pulmonary hypertension in ODC-1+/- mice after exposure to chronic hypoxia

Hemodynamic measurements of wild-type (WT) and ODC-1+/- mice exposed to either normoxia or chronic hypoxia (28 days, 10% O₂). A) RVSP quantified by right heart catheterization from n=6-8 animals per group. *p<0.05 compared to indicated groups. B) Right heart hypertrophy given as the ratio of RV and LV+S mass from heart tissue. Values were derived from n=6-8 animals per group. C) The degree of muscularization of small pulmonary vessels (outer diameter 20-70 µm) is shown in bar graphs representing means ± SEM. Values are given as percentage of total vessel count for non-, partially- and fully-muscularized vessels from paraffin embedded lung sections co-stained against α-sma and
vWF (n=6-8). *p<0.05 compared to normoxic WT animals and †p<0.05 compared to normoxic ODC-1+/− mice. D) Representative images of lung sections co-stained against α-sma (violett) and vWF (brown) displaying vessels (V) from either WT or ODC-1+/− mice exposed to normoxia or chronic hypoxia. IgG served as negative control. Scale bars display 20µm. B = bronchus. percentage of total vessel count for non-, partially- and fully-muscularized vessels from paraffin embedded lung sections co-stained against α-sma and vWF (n=6-8). *p<0.05 compared to normoxic wild-type animals and †p<0.05 compared to normoxic ODC-1+/− mice. E) ODC-1 mRNA expression levels in mice exposed to chronic hypoxia (28 days, 10% O₂) analyzed by q-PCR. Values are derived from five animals per group and normalized to B2M. *p<0.05 compared to normoxic controls. F) ODC-1 mRNA expression levels carried out by q-PCR during the process of reverse remodeling compared to hypoxia. Values were derived from six animals in each group. All values were normalized to B2M. *p<0.05 compared to hypoxic controls.

Supplemental figure 5)

ODC-1 mRNA levels are unchanged in hPASMCs and downregulated in mPASMCs upon hypoxic exposure and ODC-1 and AMD-1 mRNA expression in heterozygous knockout mice

q-PCR analysis for ODC-1 mRNA expression in A) hPASMCs and B) mPASMCs exposed to hypoxia (24h, 1%O₂). Values were derived from n=4 animals each and normalized to B2M. C) Relative ODC-1 and D) AMD-1 mRNA expression in WT and AMD-1+/− mice after 28 days of hypoxic exposure (10% O₂) assessed by q-PCR. Values were derived from n=8-9 animals each and normalized to B2M. *p<0.05 compared to respective controls.
Supplemental figure 6)

Hypoxia does not affect mRNA expression in hPASMCs

Bioinformatic analysis revealed one potential HIF binding site in the murine (upper panel) and the human (lower panel) AMD-1 promoter. B) ChIP was carried out from normoxic or hypoxic (1% O\textsubscript{2}, 2h) hPASMCs using antibodies directed against HIF-1α and HIF-2α or non-immunogenic antibody (anti-IgG). One representative out of four individual experiments is shown. C) Relative AMD-1 mRNA expression in hPASMCs after knockdown of Hif-1α or Hif-2α compared to non-specific knockdown (siR). Values were derived from 3 individual experiments and normalized to B2M. D), E) Relative AMD-1 mRNA expression in hPASMCs after normoxic or hypoxic exposure under indicated conditions. Values were derived from n=4-15 individual experiments, compared to normoxic controls and normalized to B2M. Relative AMD-1 mRNA expression in hPASMCs during 24h of hypoxic exposure including stimulation with F) TGF-β1 (2ng/ml) and G) EGF (50ng/ml) quantified by q-PCR compared to non-stimulated hypoxic controls (n=5-7 individual experiments) and normalized to B2M. H) Relative PGK mRNA expression in hPASMCs after normoxic or hypoxic (1% O\textsubscript{2}) exposure for indicated time points. Values were derived from n=6-7 individual experiments, compared to normoxic controls and normalized to B2M. *p<0.05 compared to respective controls.

Supplemental figure 7)

The AMD-1 promoter possesses one potential Egr1 binding site

A) Bioinformatic analysis of the human AMD-1 promoter revealed one potential binding site for Egr1.
Supplemental References


bioinformatics. *Genome Biol.* 2004;5:R80


