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Histone deacetylation contributes to low extracellular superoxide dismutase expression in human idiopathic pulmonary arterial hypertension

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Abstract

Epigenetic mechanisms, including DNA methylation and histone acetylation, regulate gene expression in idiopathic pulmonary arterial hypertension (IPAH). These mechanisms can modulate expression of extracellular superoxide dismutase (SOD3 or EC-SOD), a key vascular antioxidant enzyme, and loss of vascular SOD3 worsens outcomes in animal models of PAH. We hypothesized that SOD3 gene expression is decreased in patients with IPAH due to aberrant DNA methylation and/or histone deacetylation. We used lung tissue and pulmonary artery smooth muscle cells (PASMC) from subjects with IPAH at transplantation and failed donors (FD). Lung SOD3 mRNA expression and activity was decreased in IPAH vs. FD. In contrast, mitochondrial SOD (Mn-SOD or SOD2) protein expression was unchanged and intracellular SOD activity was unchanged. Using bisulfite sequencing in genomic lung or PASMC DNA, we found the methylation status of the SOD3 promoter was similar between FD and IPAH. Furthermore, treatment with 5-aza-dC did not increase PASMC SOD3 mRNA, suggesting DNA methylation was not responsible for PASMC SOD3 expression. Though total HDAC activity, HAT activity, acetylated histones and acetylated SP1 were similar between IPAH and FD, treatment with two selective class I HDAC inhibitors increased SOD3 only in IPAH PASMC. Class I HDAC3 siRNA also increased SOD3 expression. TSA, a pan-HDAC inhibitor decreased proliferation in IPAH, but not in FD PASMC. These data indicate that histone deacetylation, specifically via class I HDAC3, decreases SOD3 expression in PASMC and HDAC inhibitors may protect IPAH in part by increasing PASMC SOD3 expression.

Key words: extracellular superoxide dismutase, idiopathic pulmonary arterial hypertension, DNA methylation, histone deacetylation

Introduction:

Extracellular superoxide dismutase (EC-SOD or SOD3) has a prominent role in the protection of the pulmonary circulation against oxidative stress. SOD3 is the extracellular isoform of the superoxide dismutases, an important family of antioxidant enzymes that catalyze the rapid dismutation of superoxide ($O_2^{\bullet -}$) to hydrogen peroxide (H_2O_2). SOD3 is the most abundant isoform in the vasculature, accounting for 60-70% of total SOD activity (13, 32, 35) and the loss of SOD3 in multiple animal models of lung or vascular injury, including PH, increases disease severity (6, 11, 12, 19, 28, 31, 36, 47). One clinical study has reported diminished SOD3 protein in the bronchus of patients with idiopathic pulmonary arterial hypertension (IPAH) (28).

The mechanisms responsible for SOD3 expression and activity include post-translational modifications, genetic polymorphisms and epigenetic regulation. Post-translational events include proteolytic cleavage of the C-terminal heparin-binding domain with loss of binding to the extracellular matrix, altered protein folding and disulfide bond formation (37, 39). Genetic studies show that polymorphisms in the promoter region or heparin binding domain of SOD3 impact disease outcome for diabetic vasculopathy, ischemic heart disease, and COPD (6, 11, 18, 41, 43). There is now strong evidence that epigenetic mechanisms, in particular DNA methylation of the SOD3 promoter and histone deacetylation with change in histone occupancy of the promoter, can also regulate SOD3 expression (20, 25, 44, 46, 55, 57). These data provide a strong rationale to further evaluate the changes in SOD3 expression, and study the epigenetic regulation of this important antioxidant enzyme in patients with IPAH.

Epigenetic mechanisms are inheritable factors that regulate genetic expression without changing the DNA sequence, and include DNA methylation, histone modification, and small regulatory RNAs. DNA methylation involves modification of cytosine nucleotides within the promoter region, specifically cytosines adjacent to guanosine (CpG islands). Regulation of histone acetylation/deacetylation influences chromatin structure and the access to transcriptional machinery. The histone acetyltransferases and histone deacetylases work in concert to tightly regulate this process. Their activity, critical for normal cellular homeostasis, can be disrupted in disease states, leading to pathologic gene expression. Epigenetic mechanisms contribute to the pathogenesis of diseases such as cancer and atherosclerosis, which have features in common with IPAH including proliferation, inflammation and vascular remodeling (40, 42). Furthermore, epigenetic mechanisms have been implicated in the regulation of the mitochondrial isoform of SOD, SOD2 (2). It is therefore important to identify the epigenetic pattern in these cells, which in turn can be used as an epigenetic biomarker for new and improved therapeutic development. To date, there is limited information on the epigenetic regulation of SOD3. We tested the hypothesis that SOD3 gene expression is silenced in IPAH by epigenetic mechanisms, and thus enhances pulmonary artery smooth muscle cell (PASMC) proliferation. We utilized lung tissue and PASMC provided by the Pulmonary Hypertension Breakthrough Initiative (PHBI) obtained from subjects with IPAH at the time of lung transplantation and failed donors to measure SOD3 gene and protein expression, and to test if decreased DNA methylation and/or increased histone acetylation regulate SOD3 expression**.**

Methods:

Human lung tissue and PASMC

All explanted lungs were collected by the Pulmonary Hypertension Breakthrough Initiative Research Network. RNA and DNA isolated from lung tissue, flash frozen lung tissue and pulmonary artery smooth muscle cells (PASMC) were provided as deidentified samples from lung explants not suitable for lung transplantation (failed donor, FD) and IPAH patients at the time of lung transplantation. Human PASMC were isolated from distal muscularized small PA. The study was deemed IRB exempt by all institutions involved in the study. The age, gender and race for the individuals are shown in Table 1 for lung RNA, lung tissue, and PASMC. Control human PASMC for selected experiments were purchased from Lonza.

Cell culture growth conditions and treatments:

Human PASMC were maintained in Sm-GM2 (Lonza, Basel, Switzerland) at 37°C, 5% CO_2 in a humidified incubator. PASMC were used between passage 5-8. To inhibit DNA methyltransferase activity, cells were seeded into 6-well plates at 60,000 cells/well and grown to 80% confluence. Cells were then treated on 4 consecutive days with 5-Aza-2'-deoxycytidine (5-aza-dC)(1 μM)(Sigma-Aldrich, St. Louis, MO) or fresh media, and harvested on day 5. To inhibit HDAC activity, cells were treated with the general HDAC inhibitor, trichostatin A (TSA)(200 nM) (Sigma-Aldrich), selective class I HDAC 1, 2 and 3 inhibitor mocetinostat (MGCD0103)(1 μM) (Selleck Chemicals), class I HDAC 1 and 3 inhibitor entinostat (MS275)(1 μM) (Selleck Chemicals), class I HDAC 1 and 2 inhibitor biaryl-60 (BA-60)(1 μM),or class IIb inhibitor tubastatin A (TubA)(1 μM). Each HDAC inhibitor was dissolved in dimethyl sulfoxide (DMSO) (Fisher Scientific, Waltham, MA) and diluted 1:1000 in fresh media. The cells were treated with media containing HDAC inhibitors or DMSO alone and harvested 24 hours post treatment. Lonza PASMC were transfected with Silencer Select siRNAs (Life Technologies, Carlsbad, CA) targeting class I HDAC1 (s73), HDAC2 (s6495), HDAC3 (s16877), combinations of the

three HDAC siRNA, or a Silencer Select Negative Control #1 siRNA using Lipofectamine RNAiMAX transfection reagent (Life Technologies) according to manufacturer's instructions and harvested at 48, 72, or 96 hours. A second siRNA molecule against class I HDAC3 (s16876) was also tested to confirm results.

Cell proliferation:

Cell growth in PASMC from FD and IPAH subjects was determined after treatment with 200 nM TSA by two methods, manual cell counts and doubling time. For cell counts, 25,000 cells were seeded in 6 well plates and allowed to adhere for 24 hours. Cells were treated with either TSA or DMSO on day 0. The media was changed to fresh media without inhibitors on day 1. Cell counts were performed in triplicate on days 2 and 4 with a hemocytometer. Doubling time was measured using the xCELLigence Real-Time Cell Analyzer (ACEA Biosciences, San Diego, CA). Cells were treated as described above with either DMSO or TSA. Cells were plated at 1,000 cells per well in fresh media into an E-plate 16 (ACEA Biosciences) 24 hours after treatment. Growth was measured in real time over a 48 hour period and cell doubling time was determined and plotted (48).

Quantitative Real-Time PCR:

RNA was isolated from lung and PASMC using RNeasy Kits (Qiagen, Venlo, Limburg), and cDNA was synthesized using Maxima First Strand cDNA Synthesis Kits (Thermo Scientific, Waltham, MA) or iScript (Bio-Rad, Hercules, CA) cDNA Synthesis Kits. RT-qPCR was performed on an Applied Biosystems 7300 Real-Time PCR, StepOnePlus Real-Time PCR or a QuantStudio 6 Real-Time PCR machine (Applied Biosystems, Carlsbad, CA) using TaqMan Universal PCR Master Mix or Taqman Fast Advanced Master Mix (Life Technologies) and TaqMan Gene Expression Assays (Life

Technologies) designed for human SOD3 (Hs00984230_m1), HDAC1 (Hs02621185_s1), HDAC2 (Hs00231032_m1), HDAC3 (Hs00187320_m1), SP1 (Hs00916521) and housekeeping gene β2 microglobin (β2M) (Hs00162090_m1); and rat Sod3 (Rn00563570_m1) and housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (Gapdh) (Rn00563570_m1).

Protein preparation:

Cells and tissue were homogenized in 300 mM NaCl, 0.5% Triton X-100 in phosphate buffered saline with the addition of Halt Protease & Phosphatase Inhibitor Cocktail (Thermo Scientific). Histone extractions were performed following Abcam's Histone Extraction Protocol. Nuclear proteins were extracted with the EpiQuick Nuclear Extraction Kit (Epigentek, Farmingdale, NY) with the addition of 10 mM sodium butyrate (Sigma-Aldrich) to the lysis buffer. Immunoprecipitation was performed with 125 µg of nuclear extracts and 0.5 µg rabbit polyclonal SP1 (EMD Millipore, Billerica, MA) using the Universal Magnetic Co-IP Kit (Active Motif, Carlsbad, CA). Protein concentration was assayed using Pierce 660 nm Protein Assay Reagent (Thermo Scientific).

Western Blot:

15-20 µg of total protein, 5 µg of histone extracts, or the immunopreciptated protein were separated by gel electrophoresis using Criterion XT 4-12% Bis-Tris gels (Bio-Rad) with MES SDS running buffer (Life Technologies). Proteins were transferred to polyvinylidene fluoride membranes,(Bio-Rad) with NuPAGE transfer buffer using a Novex Semi-Dry Blotter (Life Technologies). Membranes were activated in methanol and blocked in 5% nonfat dry milk in Tris buffered saline containing 0.05% Tween20 (TBST) for 1 hour. Membranes were incubated in the following primary antibodies prepared at 1:1,000 in 5% milk in TBST at 4°C, overnight unless otherwise noted: rabbit

polyclonal SOD3, rabbit polyclonal SOD2 (EMD Millipore, Billerica, MA), rabbit polyclonal Calnexin (H-70) (Santa Cruz Biotechnology, Dallas, TX), mouse monoclonal β-actin clone AC-74 (1:10,000 at room temperature for 1 hour) (Sigma-Aldrich), rabbit polyclonal histone H3ac (1:1,500)(Active Motif, Carlsbad, CA) at 1:1,500, rabbit polyclonal histone H4ac (Active Motif) at 1:1,000, rabbit polyclonal histone H3 (1:10,000)(Active Motif) at 1:10,000 , rabbit polyclonal acetyl-Lysine (Cell Signaling), and rabbit polyclonal SP1 (EMD Millipore) in TBST. The appropriate horseradish peroxidase conjugated anti-rabbit or mouse secondary antibody (EMD Millipore) was applied at 1:10,000 in TBST for 1 hour at room temperature. Detection was accomplished using SuperSignal West Pico or Femto Chemiluminescent substrates (Thermo Scientific). Bands were quantified by densitometry using Image Lab Software (Bio-Rad) or FluorChem HD 9900 Software (ProteinSimple, San Jose, CA).

SOD Activity:

Lung tissue was homogenized in SOD assay buffer, containing 50 mM potassium phosphate pH 7.4, 0.3 M potassium bromide, 0.5 mM phenylmethylsulfonylfluoride (PMSF), and 3 mM diethylenetriaminepentaceic acid (DTPA). Concanavalin A-Sepharose 4B or Sepharose 4B were equilibrated in 250 mM NaCl in 50 mM HEPES, pH 7.0. Beads were spun and supernatant removed. Beads were washed in SOD assay buffer, spun and supernatant removed. Homogenized tissue was applied to the beads and incubated at 4°C for 30 minutes. Samples were spun and supernatant was assayed for SOD activity with a SOD assay kit-WST according to instructions (Dojindo, Rockville, MD). The supernatant in the Concanavalin A-Sepharose 4B beads reflected intracellular SODs, as SOD3 remained bound to the beads. The supernatant in the Sepharose 4B beads contained total SOD activity. Adequate separation with this protocol was confirmed by Western blot analysis for SOD3 and SOD2 (data not shown).

To calculate the SOD activity, several dilutions of the samples were performed to find 2 dilutions that contained between 0.5 – 2 U/ml SOD activity, which fell within the linear portion of a standard curve. The concentrations were determined by linear regression for the two dilutions and averaged to obtain the final SOD activity level. The activity was expressed as units/mg protein. Intracellular SOD activity was subtracted from total SOD activity to determine SOD3 activity within each sample.

Bisulfite Conversion and Sequencing:

Genomic DNA was isolated using DNeasy Blood and Tissue Kits (Qiagen). Bisulfite conversion of genomic DNA was performed using EpiTect Bisulfite Kits (Qiagen). The region of interest in the SOD3 promoter, containing the 18 CpG sites, was amplified using the following primers:

Hs SOD3 NI BS F1: CCATAAACAACCTCACACCCCCATTTTAC

Hs SOD3 NI BS R2: CCGTATTAATTTTTTAGAGTAGTTAGGGAAAGT

PCR was performed using EpiMark Hot Start *Taq* DNA Polymerase (New England Biolabs, Ipswich, MA). Recommended reaction conditions were followed and PCR products were purified using a QiaQuick PCR Purification Kit (Qiagen). The PCR products were inserted into pCR2.1 TOPO vector using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, MA) and transformed using TOP10 cells. 10-20 colonies per patient were picked for Miniprep cultures and plasmid DNA was subsequently extracted. DERC Molecular Biology Core (Aurora, CO) sequenced plasmids containing the PCR product using M13F-20 and M13R primers. Sequences were analyzed using CLC Main Workbench software.

HDAC Activity: Class I, IIa and IIb HDAC activity was measured in lung tissue and PASMC using a previously published protocol(27). Briefly, the HDAC activity was

determined by incubating lung or cell extracts with specific synthetic HDAC substrates: class I HDAC substrate (custom synthesis by Genscript, Piscataway, NJ), class IIa HDAC substrate (I-1985)(Bachem, Torrance, CA), class I/IIb substrate (I-1875)(Bachem). The class specific HDAC substrates are based on ε-N-acylated lysine, derivatized on the carboxyl group with 7-Amino-4-methylcoumarin (AMC)(Alfa Aesar). Subsequent to deacylation by HDAC activity, samples were treated with trypsin to release AMC, and the signal was detected as an increase in fluorescence using a BioTek Synergy 2 plate reader, with excitation and emission 360 nm and 460 nm, respectively, along with a 400 nm dichroic top mirror. Background signals were subtracted from buffer blanks and data normalized to FD.

HAT activity: HAT activity was measured in PASMC nuclear extractions using an EpiQuick HAT Activity/Inhibition Assay Kit (Epigentek). Data were expressed as ng/min.

Chronically hypoxic rat model: RNA was isolated from lung tissue harvested from rats exposed to 3 weeks of hypobaric hypoxia from a published study demonstrating protection against chronic hypoxic pulmonary hypertension by daily intraperitoneal injections of the HDAC inhibitor MGCD0103 (10 mg/kg in 50:50 DMSO:PEG-300) (4). The animal experiments were conducted in accordance with the National Institute of Health's "Guide for the Care and Use of Laboratory Animals" and were approved by the University of Colorado Denver Institutional Animal Care and Use Committee.

Statistical Analysis: Data were analyzed by unpaired t-test, one-way or two-way ANOVA followed by multiple comparisons test using Prism software (GraphPad Software Inc, La Jolla, CA). Data are expressed as mean +/- standard error.

Significance was defined as $p<0.05$.

Results:

Decreased lung and PASMC SOD3 expression and activity in IPAH. Lung SOD3 mRNA expression was significantly decreased in IPAH compared to FD (relative SOD3 expression 1.0 ± 0.09 FD vs. 0.47 ± 0.07 IPAH, $p<0.001$, n=13-14)(Figure 1A). Lung SOD3 protein expression was variable, tending to decrease in IPAH (1.0 ± 0.40 FD vs. 0.58 ± 0.11 IPAH, n=6)(Figure 1B). In these samples, lung SOD3 activity was significantly less in IPAH (SOD3 U/mg protein: 201.7 ± 61.7 FD vs. 42.8 ± 26.2 IPAH, $p<0.05$, n=6)(Figure 1C). PASMC were also evaluated for SOD3 content at baseline. Both SOD3 mRNA and protein tended to decrease in the untreated IPAH PASMC compared to FD, though the FD values were highly variable and the differences did not reach statistical significance ($p>0.05$, n=6)(Figure 2A,B).

No evidence for decreased lung SOD2 expression or activity in IPAH Lung SOD2 expression was evaluated in the same samples used for SOD3 analysis. No change in SOD2 expression was observed ($p>0.05$)(Figure 3A). Furthermore, intracellular SOD activity in the lung, which included SOD1 and SOD2, was similar in IPAH and FD ($p>0.05$)(Figure 3B).

No evidence that the low SOD3 gene expression in IPAH is regulated by DNA methylation of the SOD3 promoter. DNA methylation of the SOD3 promoter contributes to low SOD3 mRNA expression in several types of tumors, therefore we examined the lung and PASMC from individuals with IPAH to see if we could observe a similar increase in DNA methylation of the same 18 CpG sites in the promoter of SOD3.

To increase the probability of observing a difference between FD and IPAH, we selected 4 FD lungs with high SOD3 mRNA expression and 4 IPAH with low SOD3 mRNA expression. Following bisulfite conversion and DNA sequencing, we found that the overall % methylation of the SOD3 promoter was not different in FD vs. IPAH, with 36.8% methylation in FD and 31.0% in IPAH ($p>0.05$)(Figure 4A). Furthermore, there was no difference at any of the 18 CpG sites between FD and IPAH ($p>0.05$), though there was a difference in % methylation at particular CpG sites, ranging from 11% to 65% ($p<0.001$)(Figure 4B). The % methylation of the SOD3 promoter in PASMC was significantly lower than the % methylation observed in the lung samples. Furthermore, in contrast to tumors, there was not an increase in DNA methylation in the IPAH PASMC and, in fact, it was lower than the FD PASMC (7.9% ± 2.0 vs. 2.8% ± 0.6, $p<0.05$)(Figure 4C). The findings were similar when analyzed for each of the CpG sites, with very low to absent methylation, particularly in IPAH PASMC DNA ($p<0.001$)(Figure 4D). To further evaluate the contribution of methylation on SOD3 expression, cells were treated with 5-aza-dC and data expressed as change in SOD3 from baseline for each individual. There was no significant increase in SOD3 mRNA expression following treatment with 5-aza-dC and the change from baseline was not different between FD vs. IPAH (Figure 4E).

Blocking class I HDAC activity increased SOD3 mRNA expression and reduced proliferation in IPAH PASMC. We next evaluated whether increased histone deacetylation could contribute to low SOD3 expression in IPAH. We first measured Class I, Class IIa and Class IIb HDAC activity in lung and PASMC (Figure 5A-F). We did not observe a difference in HDAC activity between FD and IPAH lung or PASMC. In PASMC, HAT activity and histone acetylation also did not differ between FD and IPAH (Figure 5G-J).

We then tested whether histone deacetylase activity contributed to low EC-SOD mRNA expression in IPAH using a series of specific HDAC inhibitors. We evaluated the SOD3 mRNA expression in PASMC from 5 different IPAH and FD subjects. One outlier from each group with very high SOD3 content at baseline was excluded from analysis (data not shown). There was an overall significant difference in SOD3 gene expression in IPAH PASMC compared to FD and in response to HDAC inhibitors ($p<0.001$)(Figure 6A). Specifically, IPAH PASMC SOD3 mRNA expression increased following treatment with the selective class I HDAC 1, 2 and 3 inhibitor MGCD0103; or class I HDAC 1 and 3 inhibitor MS275, with no significant response to the class I HDAC 1 and 2 inhibitor BA-60 or class IIb inhibitor TubA. There was also a trend towards an increase in SOD3 expression with the pan-HDAC inhibitor TSA in the IPAH cells (Figure 6A). Cell proliferation was evaluated by two methods, cell counts and doubling time, following treatment with the pan-HDAC inhibitor, TSA. Overall the IPAH cells showed enhanced growth at baseline and had a more robust response to TSA than FD. By cell counts, we measured a higher number of IPAH cells at 4 days compared to FD. TSA decreased proliferation in IPAH PASMC, with lower cell counts at both 2 days and 4 days compared to untreated cells. In FD PASMC, TSA did not significantly decrease proliferation at 2 days, but did decrease FD cell counts by 4 days (Figure 6B). IPAH PASMC also had a shorter doubling time at baseline compared to the FD PASMC, reflecting more rapid growth (26.99 ± 3.29 hours in FD vs. 20.82 ± 1.10 hours in IPAH). When cells were treated with TSA, only the IPAH PASMC significantly increased the doubling time (43.2 ± 5.4 hours) compared to the FD cells (36.9 ± 4.0 hours)($p<0.05$))(Figure 6C).

Silencing class I HDAC3 increased SOD3 expression in PASMC. To confirm the effect of class I HDAC inhibitors on SOD3 expression, and further define the

contribution of specific class I HDACs, we measured SOD3 expression after siRNA knock down of class I HDAC1, 2 or 3 in human PASMC. In pilot experiments, we observed that class I HDAC1 siRNA decreased HDAC1 protein at 48 and 72 hours, but increased HDAC2 protein expression, with no change in SOD3 protein (data not shown). Therefore, we tested each siRNA molecule 72 hours after transfection individually and in combination to account for potential compensatory responses in HDAC expression. Figure 7A-C shows the greater than 90% knock-down of class I HDAC1, HDAC2 or HDAC3 mRNA at 72 hours with the respective siRNA molecule. SOD3 mRNA expression after class I HDAC knock-down was highly variable and showed a different response and time-course each time the experiment was repeated. Overall, we could not demonstrate a consistent or significant change in SOD3 gene expression after siRNA treatments at 48 or 72 hours (data not shown). SOD3 protein expression, however, significantly increased 72 hours after knock-down of class I HDAC3 siRNA, but not class I HDAC1 or HDAC2 siRNA (Figure 7D). We pooled the protein expression data into three groups for analysis: 1) siHDAC1 alone; 2) siHDAC2 and siHDAC1&2; and 3) siHDAC3, siHDAC1&3 and siHDAC1,2&3. This approach best demonstrates the increase in SOD3 expression whenever HDAC3 is included in the pool of siRNA (Figure 7E).

SP1 acetylation: SOD3 expression is regulated by the transcription factor SP1, and acetylation of SP1 by HDAC1 or HDAC2 can decrease DNA binding in promoter regions (49, 56). Therefore, we evaluate the nuclear expression of SP1 in PASMC nuclear extracts and immunoprecipitated SP1 to evaluate for lysine acetylation. We observed no difference in total nuclear SP1 expression between FD and IPAH (Figure 8A). We did detect acetylation in immunoprecipitated SP1 from PASMC, though there was no difference between FD and IPAH (Figure 8B).

Treatment of chronically hypoxic rats with the class I HDAC inhibitor MGCD0103 increased lung Sod3 mRNA expression. To further evaluate the role of histone deacetylation on Sod3 mRNA expression, we examined the lungs of chronically hypoxic rats treated with the HDAC inhibitor MGCD0103. We previously reported that treatment with MGCD0103 protected against chronic hypoxic PH, including a 30% decrease in pulmonary artery systolic pressures measured by right heart catheterization as well as normalization of the PA acceleration time by echocardiography (4)**.** We analyzed lung tissue from these rats for SOD3 mRNA expression. We observed an increase in Sod3 mRNA expression in rats exposed to 3 weeks of hypoxia during HDAC inhibitor treatment compared to normoxic or hypoxic rats ($p<0.001$)(Figure 9).

Discussion:

We utilized human lung tissue and PASMC obtained through the PHBI to test whether SOD3 is decreased in IPAH lung due to DNA methylation or histone deacetylation. We report a significant decrease in lung SOD3 gene expression and enzyme activity in lung tissue from individuals with late stage IPAH at time of lung transplantation compared to lungs from failed donors. Furthermore, SOD3 expression in IPAH PASMC was regulated by class I HDAC3 activity. This conclusion was based on our data showing selective class I HDAC inhibitors with activity against HDAC3 increased SOD3 gene expression and reduced the rate of cell proliferation in IPAH PASMC. Furthermore, siRNA knock-down of class I HDAC3 but not HDAC1 or HDAC2, increased SOD3 gene expression. Finally, SOD3 mRNA expression was increased in the lungs of chronically hypoxic rats treated with MGCD0103, the selective class I HDAC inhibitor with activity against HDAC3. These studies provide new insight in the

385 regulation of SOD3 in the pulmonary circulation and add to the accumulating literature
386 providing a rationale to test the therapeutic role of selective HDAC inhibitors for the
387 treatment of IPAH.

388 We focused our studies on SOD3 in IPAH due to its function as a major vascular
389 antioxidant enzyme, and the published evidence from our group and others implicating
390 a critical role for low SOD3 on disease pathogenesis in animal models of pulmonary
391 hypertension (1, 12, 19, 31, 33, 47, 51). Furthermore, we performed *in vitro* with
392 PASMC because this is the primary cellular source of SOD3 in the vessel wall (33, 57).
393 The observation that SOD3 gene expression is decreased in the lungs of individuals
394 with IPAH is consistent with an earlier report that SOD3 mRNA is decreased in the
395 bronchial tissue of individuals with IPAH (28). We found that the changes in SOD
396 expression and activity were selective for SOD3 isoform. This is in contrast to a
397 previous study that reported that SOD2 gene expression is decreased in the lungs of 3
398 individuals who died of IPAH or non-lung related conditions, and was consistent with the
399 loss in SOD2 in the fawn hooded rat model of PH (2). One major challenge in the study
400 of PAH in humans is the limited availability of human lung and cell culture samples. As
401 IPAH specimens are obtained either at lung transplantation, as in our study, or at the
402 time of autopsy, the data reflect the state in end-stage disease. Furthermore, the control
403 population is not uniform; in our study, the lungs provided by the PHBI tissue bank were
404 not accepted for lung transplantation therefore the reproducibility of the data from
405 control samples may have been confounded by the presence of underlying lung
406 diseases or acute lung injury that could impact SOD expression or activity. We
407 speculate that these factors contributed to the high variability in the outcome measures
408 including SOD3 expression, particularly in FD PASMC. In the face of these limitations,
409 the decrease in lung SOD3 gene expression in IPAH was notable, so we proceeded to
410 evaluate the role of DNA methylation and histone acetylation on the regulation of SOD3.

We first evaluated DNA hypermethylation of the SOD3 promoter because this epigenetic mechanism is responsible for low SOD3 expression in lung, breast and pancreatic cancers and contributes to enhanced tumor growth, survival, and invasion (30,34, 44, 45, 55). In addition, DNA methylation of the SOD3 promoter contributes to lower baseline SOD3 expression in normal human PAEC compared to PASMC (57)**.** In the study by Zelko et al, they report low levels (<10%) of DNA methylation of the SOD3 promoter in PASMC, similar to what we measured in the FD and IPAH PASMC. The observation that the lung had a higher overall level of DNA methylation of the SOD3 promoter sites compared to PASMC suggests that DNA hypermethylation could regulate low SOD3 in other important cell types in IPAH. The clinical significance of a further decrease in DNA methylation in IPAH from a low baseline level in FD PASMC is unclear. DNA hypermethylation can also lower SOD3 expression in leukocytes in the setting of coronary artery disease, and regulates the differential expression of SOD3 in different monocyte lines (20, 25). Others have evaluated SOD2 and found that methylation of the SOD2 promoter inhibits SOD2 transcription and contributes to cell proliferation in several cancer lines and in the fawn hooded rat model of IPAH described above (2, 14, 15, 17). Overall, our results indicate that, in contrast to cancer, low SOD3 expression in IPAH is not regulated by increased DNA methylation. These findings prompted us to consider the contribution of histone deacetylation to low SOD3 expression.

Our data collectively indicate that class I HDAC3 activity regulates SOD3 expression in IPAH PASMC. There is emerging interest in the role of histone deacetylation in the pathogenesis of cardiovascular diseases including human IPAH, and advances in the development of new selective HDAC inhibitors (9,10,21,26,29,42, 58) In animal studies, it is clear that HDAC inhibitors may not benefit every model of

PH, and a pan-HDAC inhibitor may even be harmful, both important points necessitating a better understanding of this process in human disease (3,7). To date, there are few human studies examining histone deacetylation in IPAH, and they are limited to testing HDAC subtype expression, rather than activity (21,23,58). Zhao et al, in collaboration with our group, reported an increase in class I HDAC1, and class IIa HDAC4 and HDAC5 protein expression in lung tissue from 12 individuals with IPAH compared to control lobectomy tissue (58). Interestingly, when Korfei and colleagues examined HDAC expression in lung tissue from individuals with idiopathic pulmonary fibrosis, a lethal lung disease that can be complicated by pulmonary hypertension, they reported an increase in class I HDAC subtypes that included HDAC3, the subtype implicated in this study in SOD3 expression (23). It is thus possible that different HDAC isoforms are altered in different forms of pulmonary hypertension.

Our observations implicating class I HDAC3 in SOD3 regulation build upon a limited but important series of published studies evaluating SOD3 regulation by histone deacetylation. Zelko and Folz were the first investigators to demonstrate that histone deacetylation could regulate SOD3 gene expression in cell lines derived from mouse liver, kidney and lung fibroblasts, and histone deacetylation contributed to the low expression of SOD3 in PAEC compare to PASMC (54, 57). They recently also showed histone H3 and H4 acetylation in the SOD3 promoter region in PAEC, further implicating histone acetylation in the normal low PAEC SOD3 expression. They also did not see an increase in SOD3 in commercially available PASMC with two class I and II HDAC inhibitors, scriptaid or HDAC-42, similar to our observation that control FD PASMC did not significantly increase SOD3 expression in response to class I HDAC inhibitors, (53). In a published study using neonatal ovine PASMC, class I HDAC inhibition with apicidin increased SOD3 expression, providing further evidence that class I HDACs regulate SOD3, and suggesting that there may be age or species-dependent variability (52).

Though we focused on class I HDACs, because of their recognized role in cardiovascular diseases, there are also a number of recent though discrepant studies examining sirtuins, in particular SIRT1 and SIRT3, in PH; the role of sirtuins warrant future interrogation (5, 38, 50). To the best of our knowledge, this is the first study to show that class I HDAC3 activity regulates SOD3 expression.

Class I HDACs, including HDAC3, can associate with SP1 and SP3, ubiquitous hypoxia-responsive transcription factors implicated in both repression and activation of genes. (16, 20, 22, 24). This has potential relevance in the regulation of SOD3 in IPAH, particularly given the known role of SP1/ SP3 in the regulation of SOD3 gene expression, and the impact of HDAC inhibitors on SOD3 expression. In addition to modulating HDAC activity, SP1 is also a target of acetylation; acetylation of SP1 decreases its promoter binding affinity and can be reversed by HDAC inhibitors (16, 20, 22, 24, 49). Our data confirms SP1 acetylation in PASMC, indicating that it may be a mechanism involved in SOD3 expression. However we did not observe SP1 hyperacetylation in IPAH, suggesting that it did not appear to be responsible for the low SOD3 expression in IPAH. The specific mechanism(s) by which HDACs regulate SOD3 in different cell types in IPAH or other forms of pulmonary hypertension will be an important future direction. We propose that selective class I HDAC inhibitors may protect not only by restoring normal PASMC SOD3 expression, but potentially by increasing SOD3 in other cells including PAEC in which expression is repressed by histone acetylation.

Though total HDAC activity, HAT activity and histone acetylation were similar between FD and IPAH, there are a number of points to consider when interpreting these data. A decrease in specific HDAC isoforms may not be reflected by the measurement of total HDAC activity. Though the substrates are useful to differentiate different

489 classes, they are not specific for the different HDAC subtypes within a class. A similar
490 problem could contribute to the measures of histone acetylation, as site-specific
491 changes in histone acetylation may not be reflected by the total histone measurements.
492 This critical area of inquiry continues to require further investigation.

493 In conclusion, we provide new evidence that the lung expression and activity of the
494 key vascular antioxidant enzyme, SOD3, is selectively decreased in IPAH. In contrast to
495 a series of cancer studies, we did not find evidence that DNA hypermethylation was
496 responsible for the decrease in SOD3 expression. We provide strong evidence that
497 class I HDAC3 activity contributes to the impaired SOD3 expression and enhanced cell
498 proliferation in IPAH PASMC. Further studies are necessary to establish the
499 mechanisms responsible for cell-specific regulation of SOD3 in different forms of
500 pulmonary hypertension and determine if selective HDAC inhibitors can improve SOD3
501 activity, and contribute to their therapeutic efficacy to improve outcomes in this
502 devastating disease.

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Figure Legends:

Figure 1: Decreased lung SOD3 mRNA expression and protein activity in IPAH. **A**. Lung SOD3/β2M mRNA expressed relative to FD. (n=13-16) **B**. Western blot analysis for lung SOD3 and calnexin, with corresponding densitometry data showing SOD3/calnexin relative to FD. (n=6) **C.** SOD3 in lung homogenates was separated from the intracellular SODs (IC-SOD) using Concanavalin A-Sepharose 4B beads to pull down SOD3. SOD3 activity was determined as the difference in activity in supernatant after incubation with Concanavalin A-Sepharose 4B (intracellular SOD) or plain Sepharose 4B beads (total SOD) SOD activity was assayed with the SOD assay kit-WST (Dojindo) and expressed as units SOD activity per mg protein (U/mg protein). (n=6) FD: failed donor, IPAH: idiopathic pulmonary arterial hypertension, *$p<0.05$ vs. FD by unpaired t-test.

Figure 2: Variable PASMC SOD3 mRNA and protein expression tends to decrease in IPAH. **A**. PASMC SOD3/β2M mRNA by qPCR expressed relative to FD **B.** Western blot for PASMC SOD3 and β-actin, with corresponding densitometry data for SOD3/β-actin relative to FD. (n=6) $p>0.05$ vs. FD by unpaired t-test.

Figure 3: No change in lung SOD2 expression or intracellular SOD activity in IPAH. A. Western blot for lung SOD2 and calnexin, with corresponding densitometry data for SOD2/calnexin relative to FD. (n=6) $p>0.05$ vs. FD by unpaired t-test. SOD2 expression was tested in the same membrane shown in Figure 1B. **B.** Intracellular SOD

activity in lung expressed as units SOD activity per mg protein. (U/mg protein) (n=6) p>0.05 vs. FD by unpaired t-test.

Figure 4. **No evidence that the low SOD3 gene expression in IPAH is regulated by DNA methylation of the SOD3 promoter**. Lung genomic DNA was subject to bisulfite conversion and sequencing for the 18 CpG sites in the SOD3 promoter. **A**. Percent methylation in the SOD3 promoter region in FD and IPAH lung. (n=4) p>0.05 vs. FD by unpaired t-test. **B.** DNA methylation at each of the 18 CpG sites in the SOD3 promoter in FD and IPAH lung**.** p>0.05 between FD and IPAH for each CpG site by two way ANOVA. **C.** Percent methylation in the SOD3 promoter region in FD and IPAH PASMC. (n=6). **D.** DNA methylation at each of the 18 CpG sites in the SOD3 promoter for FD and IPAH PASMC. n=6, *p<0.05 vs. FD by unpaired t-test. **E.** PASMC SOD3/β2M mRNA expression following treatment with DNA methyltransferase inhibitor, 1 μM 5-aza-dC, on days 1-4 with harvesting on day 5 in FD vs. IPAH. Data are expressed as change in SOD3/β2M from baseline for each individual. (n=6) p>0.05 vs. FD by unpaired t-test.

Figure 5. No change in HDAC activity, HAT activity or total histone H3 and H4 acetylation between FD and IPAH.

Class specific HDAC activity was determined by incubating tissue or cell extracts with specific synthetic HDAC substrates against class 1, class IIa or class IIb HDACs. Activity levels were measured in FD and IPAH lung **(A,C,E)** or PASMC **(B,D,F**). Data are expressed as the fluorescent signal relative to the FD. (n=14-16 for lung; n=6 for PASMC). p>0.05 vs. FD by unpaired t-test. **G.** HAT activity was measured in nuclear extracts isolated from PASMC and expressed as ng/min. (n=6) p>0.05 vs. FD by unpaired t-test. **H.** Western blot analysis for acetylated histone H3 (H3ac), aceylated

histone H4 (H4ac) and total histone H3 (H3) in histone extracts from FD and IPAH with corresponding densitometry for H3ac **(I)** or H4ac **(J)** expressed relative to total H3. (n=5-6) p>0.05 vs. FD by unpaired t-test.

Figure 6. Treatment with HDAC inhibitors increase SOD3 mRNA expression in PASMC and enhanced cell proliferation in IPAH A. SOD3 mRNA expression following treatment with the following HDAC inhibitors, expressed as SOD3/β2M. Cells were treated for 24 hours with the general HDAC inhibitor, trichostatin A (TSA)(200 nM), selective class I HDAC 1, 2 and 3 inhibitor MGCD0103 (MGCD)(1 μM), class I HDAC 1, 2 and 3 inhibitor entinostat (MS275)(1 μM), class I HDAC 1 and 2 inhibitor biaryl-60 (BA-60)(1 μM), class IIB HDAC6 inhibitor tubastatin A (TubA)(1 μM) or dimethyl sulfoxide (DMSO) (1:1000). (n=5) **B.** Cell counts at 2 days and 4 days after a 24 hour treatment with TSA (200 nM) in FD and IPAH PASMC (n=4-5). **C.** Doubling time was measured using the xCELLigence Real-Time Cell Analyzer (ACEA Biosciences) to provide a real time measurement of cell proliferation. Cells, treated with either TSA (200 nM) or DMSO (1:1000), were plated 24 hours post treatment in fresh media on an E-plate 16 (1000 cells/well) and monitored continuously over a 48 hour period (n=4-5). *p<0.05 vs. FD DMSO, #p<0.05 vs. IPAH DMSO by two way ANOVA.

Figure 7. siRNA knock-down of Class I HDAC3 in PASMC increased SOD3 protein expression. PASMC (Lonza) were transfected with siRNA (Life Technologies) against HDAC1, HDAC2 and/or HDAC3 and combinations of the three siRNA molecules. HDAC1-3/β2M mRNA by qPCR expressed relative to siNC **(A-C)**. The data were pooled into three groups: 1) HDAC1 alone, 2) HDAC2 and HDAC1&2 and 3) HDAC3, HDAC1&3, and HDAC1,2,&3. **D.** Representative Western blot of SOD3 and β-actin for each experimental condition. **E.** Densitometry data for SOD3 expression. Data are

expressed as SOD3/β-actin relative to HDAC1. Experiments were repeated at least 3 times. n=3-4 *p<0.05 vs. HDAC1 and #p<0.05 vs. HDAC2 and HDAC1&2 group by one way ANOVA. siNC: negative control siRNA.

Figure 8. Acetylation of SP1 did not differ between FD and IPAH PASMC. A. Representative Western blot data of SP1 and total Histone 3 expression in nuclear extracts of FD and IPAH PASMC along with densitometry data**. B.** SP1 was immunoprecipitated from PASMC nuclear extracts and evaluated for protein acetylation. The Western blot and corresponding densitometry is shown for acetylated lysine (Ac-lysine) and SP1. (n=3) p>0.05 by unpaired t-test.

Figure 9. Treatment of chronically hypoxic rats with the HDAC inhibitor MGCD0103 increased lung Sod3 mRNA expression. Sod3 mRNA expression in the lungs of 3-week chronically hypoxic rats treated daily with the HDAC inhibitor MGCD0103 (10 mg/kg) intraperitoneal injections (HX + HDACi) compared to sham treated normoxic (NX) and hypoxic (HX) rats.(2) (n=6) *p<0.001 vs. NX, #p<0.001 vs. HX by one way ANOVA.

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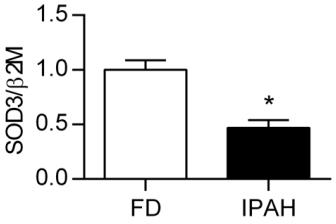
A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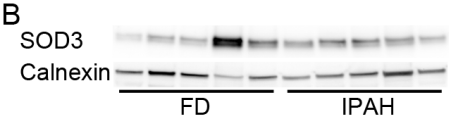
SOD3/β2M
*
FD
IPAH

B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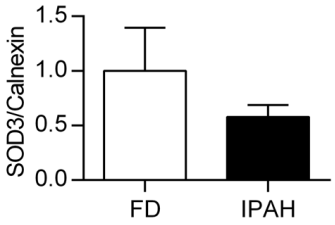
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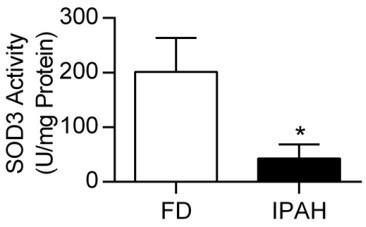
FD
IPAH

C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SOD3 Activity
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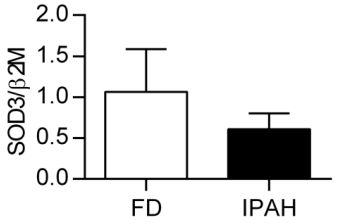
IPAH

A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SOD3/β2M
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IPAH

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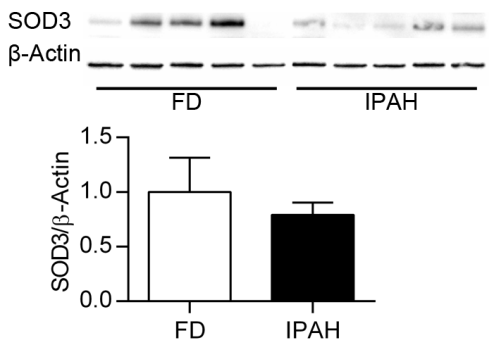
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SOD3/β-Actin
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IPAH

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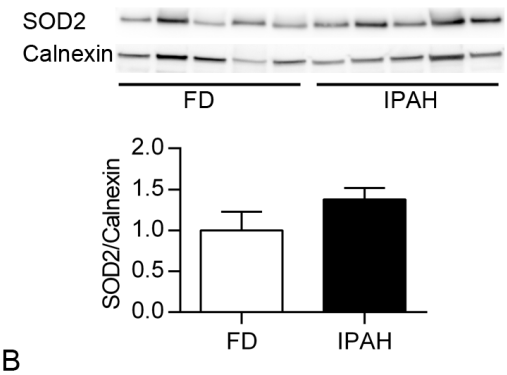
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IPAH

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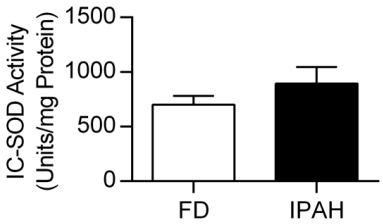
B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1000
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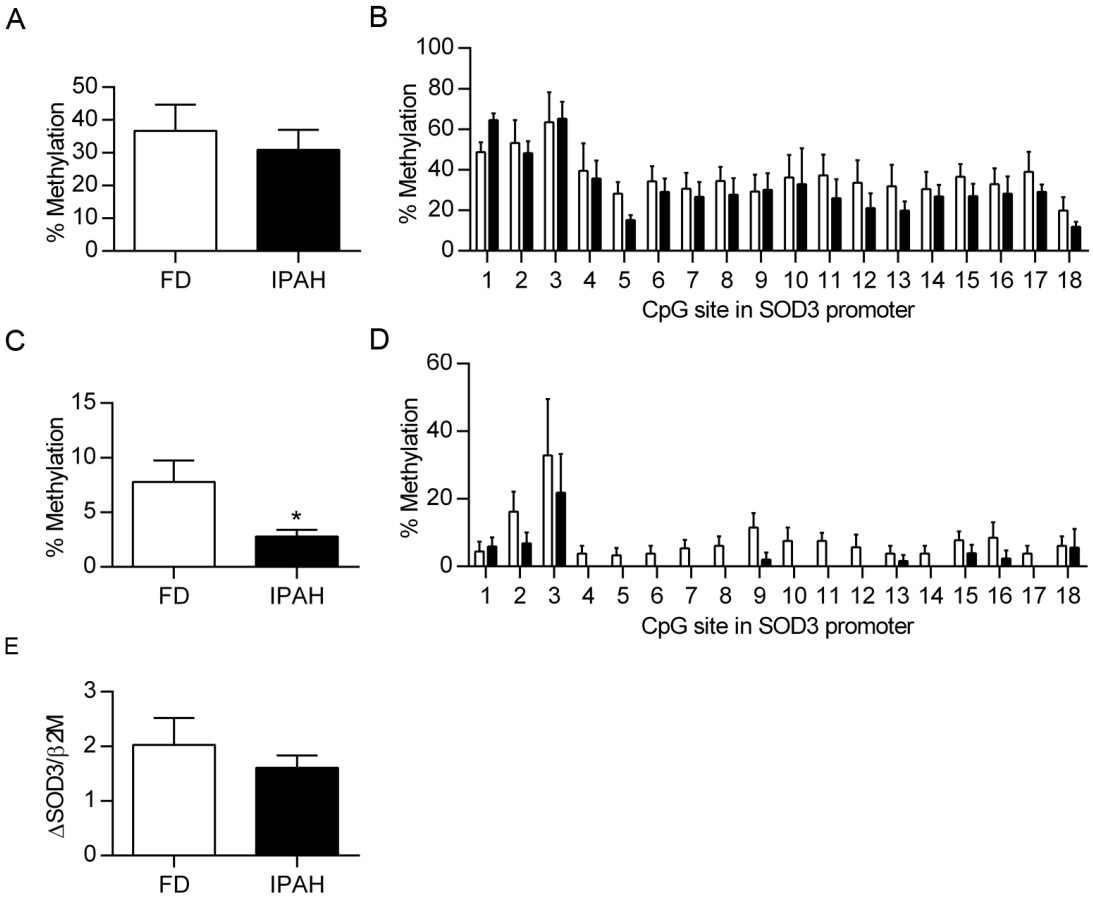
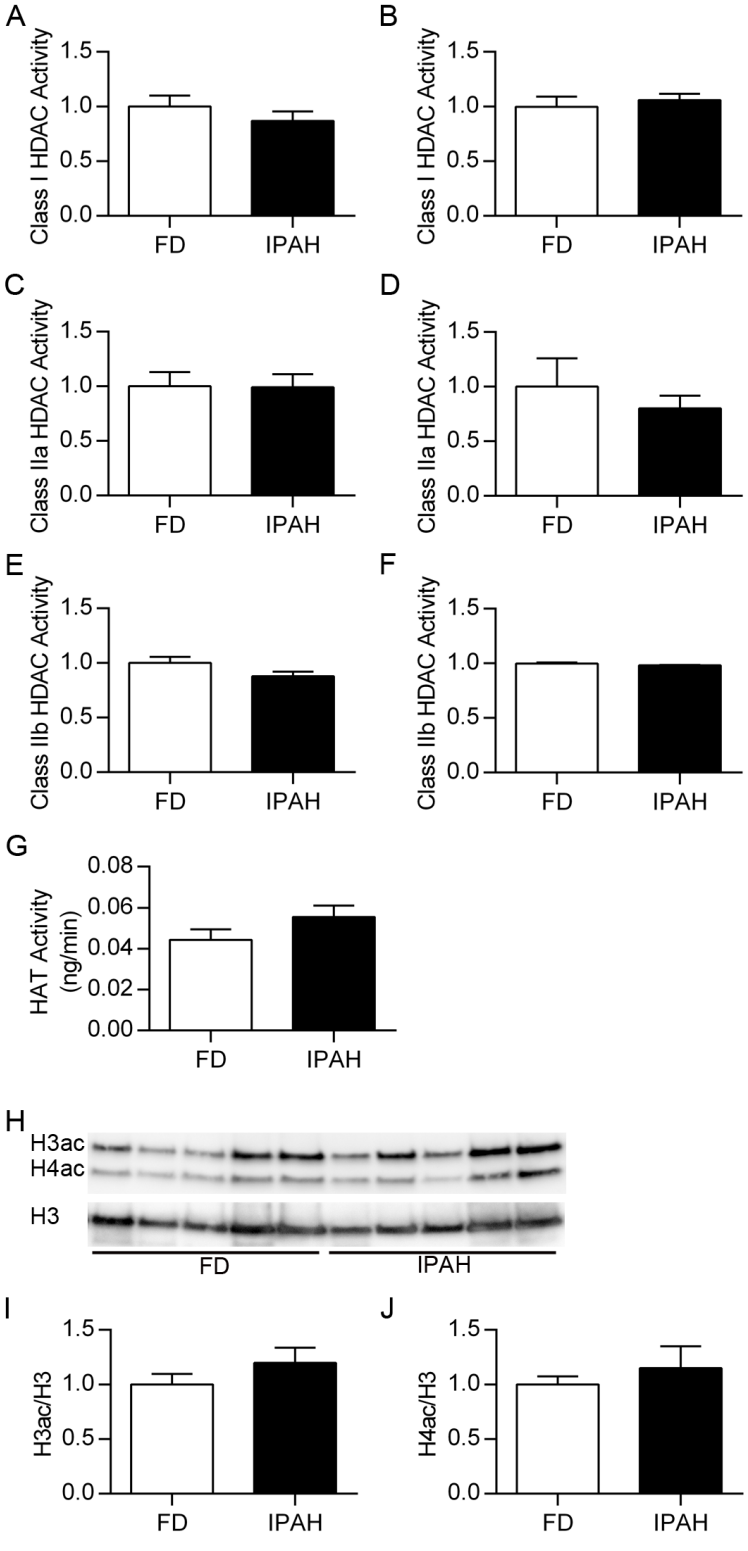
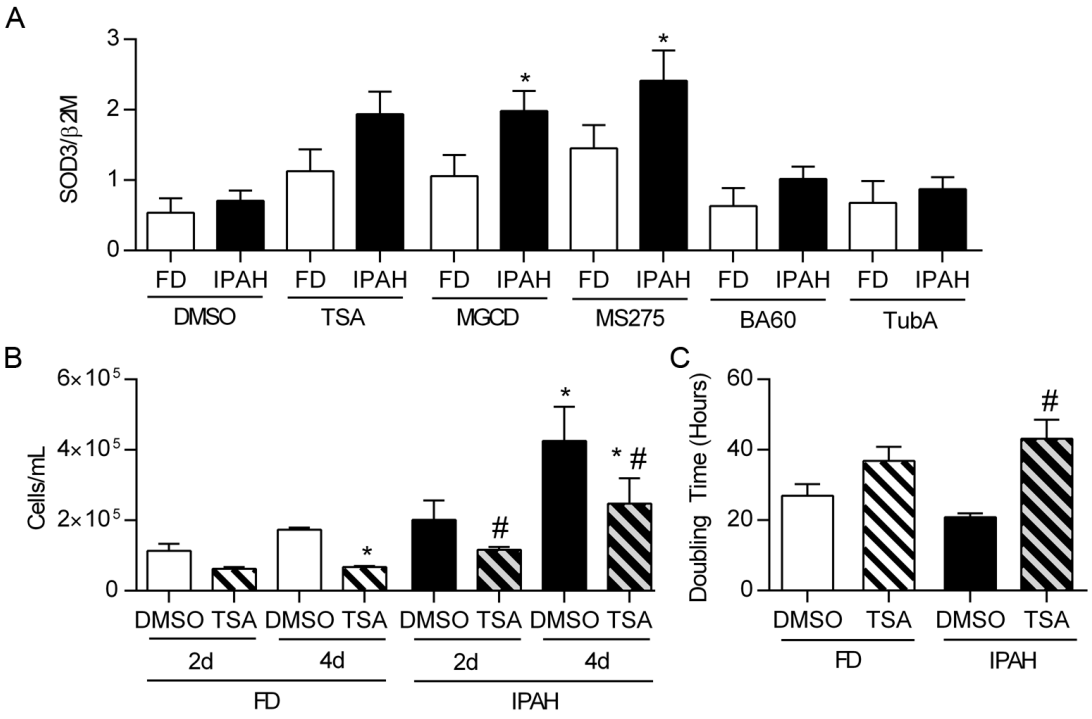
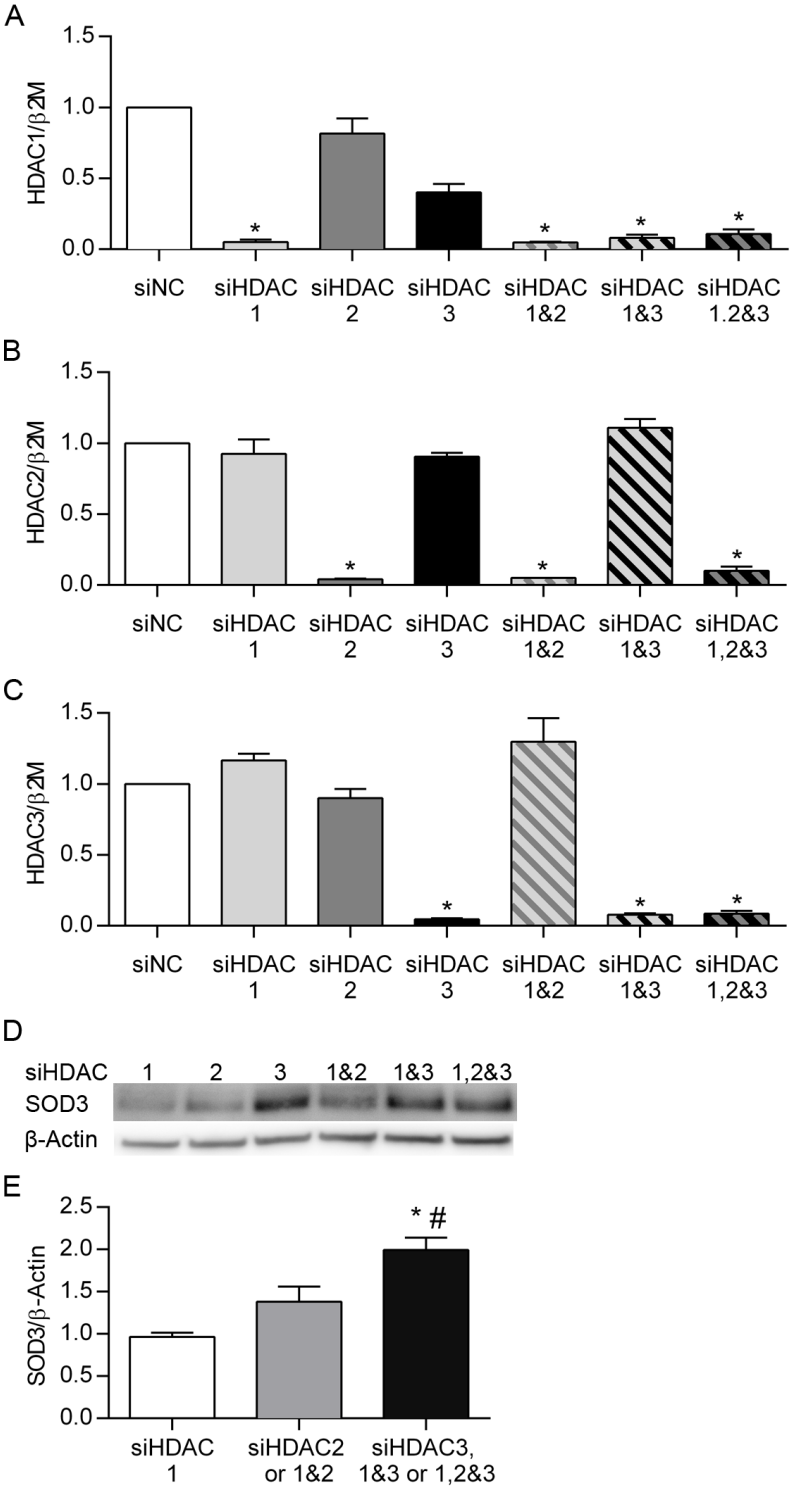
0
FD
IPAH

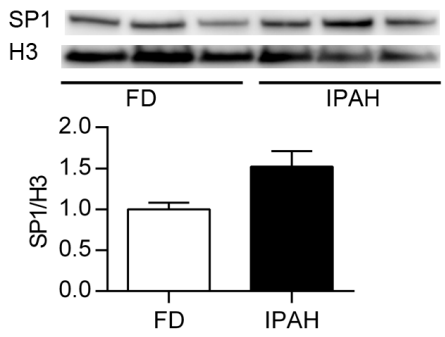

A
% Methylation
50
40
30
20
10
0
FD
IPAH
B
% Methylation
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
CpG site in SOD3 promoter
C
% Methylation
15
10
5
0
*
FD
IPAH
D
% Methylation
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
CpG site in SOD3 promoter
E
ΔSOD3/β2M
3
2
1
0
FD
IPAH


A
Class I HDAC Activity
1.5
1.0
0.5
0.0
FD
IPAH
B
Class I HDAC Activity
1.5
1.0
0.5
0.0
FD
IPAH
C
Class IIa HDAC Activity
1.5
1.0
0.5
0.0
FD
IPAH
D
Class IIa HDAC Activity
1.5
1.0
0.5
0.0
FD
IPAH
E
Class IIb HDAC Activity
1.5
1.0
0.5
0.0
FD
IPAH
F
Class IIb HDAC Activity
1.5
1.0
0.5
0.0
FD
IPAH
G
HAT Activity (ng/min)
0.08
0.06
0.04
0.02
0.00
FD
IPAH
H
H3ac
H4ac
H3
FD
IPAH
I
H3ac/H3
1.5
1.0
0.5
0.0
FD
IPAH
J
H4ac/H3
1.5
1.0
0.5
0.0
FD
IPAH


A
SOD3/β2M
3
2
1
0
FD IPAH FD IPAH FD IPAH FD IPAH FD IPAH FD IPAH
DMSO
TSA
MGCD
MS275
BA60
TubA
B
Cells/mL
6×10^5
4×10^5
2×10^5
0
DMSO TSA DMSO TSA DMSO TSA DMSO TSA
2d
4d
2d
4d
FD
IPAH
C
Doubling Time (Hours)
60
40
20
0
DMSO TSA DMSO TSA
FD
IPAH

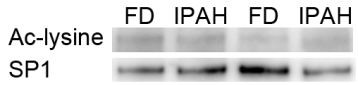

A
HDAC1/β2M
1.5
1.0
0.5
0.0
siNC
siHDAC 1
siHDAC 2
siHDAC 3
siHDAC 1&2
siHDAC 1&3
siHDAC 1.2&3
B
HDAC2/β2M
siNC
siHDAC 1
siHDAC 2
siHDAC 3
siHDAC 1&2
siHDAC 1&3
siHDAC 1,2&3
C
HDAC3/β2M
siNC
siHDAC 1
siHDAC 2
siHDAC 3
siHDAC 1&2
siHDAC 1&3
siHDAC 1,2&3
D
siHDAC 1 2 3 1&2 1&3 1,2&3
SOD3
β-Actin
E
SOD3/β-Actin
2.5
2.0
1.5
1.0
0.5
0.0
* #
siHDAC 1
siHDAC2 or 1&2
siHDAC3, 1&3 or 1,2&3

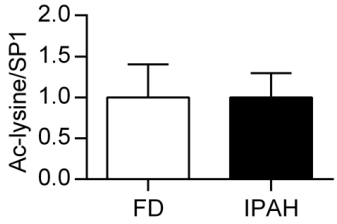
A


SP1
H3
FD
IPAH

SP1/H3
2.0
1.5
1.0
0.5
0.0
FD
IPAH

B


FD
IPAH
FD
IPAH
Ac-lysine
SP1


Ac-lysine/SP1
2.0
1.5
1.0
0.5
0.0
FD
IPAH

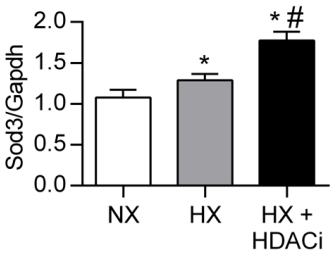

2.0
1.5
1.0
0.5
0.0
Sod3/Gapdh
*
*#
NX
HX
HX +
HDACi

Table 1. Age, gender and race of subjects

	Lung RNA		Lung Tissue		PASMC	
	FD	**IPAH**	**FD**	**IPAH**	**FD**	**IPAH**
Total Numbers	14	16	6	6	6	6
Mean Age (years ± SD)	39.00 +/- 16.55	37.75 +/- 16.20	42.00 +/- 18.87	38.33 +/- 15.40	45.67 +/- 11.22	39.17 +/- 10.55
	n (%)	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**n (%)**
Female	5 (38.5)	12 (75.0)	1 (16.67)	2 (33.33)	6 (100)	6 (100)
White	13 (100.0)	10 (62.5)	6 (100.00)	3 (50.00)	5 (83.3)	3 (50)
Black	0 (0)	2 (12.5)	0 (0)	1 (16.67)	0 (0)	2 (33.3)
Hispanic	0 (0)	2 (12.5)	0 (0)	1 (16.67)	1 (16.7)	1 (16.7)
Asian American	0 (0)	2 (12.5)	0 (0)	1 (16.67)	0 (0)	0 (0)

FD: failed donor, IPAH: idiopathic pulmonary arterial hypertension, PASMC: pulmonary artery smooth muscle cells