Endothelial Indoleamine 2,3-Dioxygenase Protects against Development of Pulmonary Hypertension

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Rationale: A proliferative and apoptosis-resistant phenotype in pulmonary arterial smooth muscle cells (PASMCs) is key to pathologic vascular remodeling in pulmonary hypertension (PH). Expression of indoleamine-2,3-dioxygenase (IDO) by vascular endothelium is a newly identified vasomotor-regulatory mechanism also involved in molecular signaling cascades governing vascular smooth muscle cell (vSMC) plasticity.

Objectives: To investigate the therapeutic potential of enhanced endothelial IDO in development of PH and its associated vascular remodeling.

Methods: We used loss and gain of function *in vivo* studies to establish the role and determine the therapeutic effect of endothelial IDO in hypoxia-induced PH in mice and monocrotaline-induced PH in rats. We also studied PASMC phenotype in an IDO-high *in vivo* and *in vitro* tissue microenvironment.

Measurements and Main Results: The endothelium was the primary site for endogenous IDO production within mouse lung, and the mice lacking this gene had exaggerated hypoxia-induced PH. Conversely, augmented pulmonary endothelial IDO expression, through a human IDO-encoding *Sleeping Beauty* (SB)-based nonviral gene-integrating approach, halted and attenuated the development of PH, right ventricular hypertrophy, and vascular remodeling in both preclinical models of PH. IDO derived from endothelial cells promoted apoptosis in PH-PASMCs through depolarization of mitochondrial transmembrane potential and down-regulated PH-PASMC proliferative/synthetic capacity through enhanced binding of myocardin to CArG box DNA sequences present within the promoters of vSMC differentiation-specific genes.

Conclusions: Enhanced endothelial IDO ameliorates PH and its associated vascular structural remodeling through paracrine phenotypic modulation of PH-PASMCs toward a proapoptotic and less proliferative/synthetic state.

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Expression of indoleamine-2,3-dioxygenase (IDO) by vascular endothelium is a newly identified vasomotor-regulatory mechanism also involved in molecular signaling cascades governing vascular smooth muscle cell (vSMC) plasticity.

What This Study Adds to the Field

This study shows that overexpression of IDO in pulmonary endothelium halts and attenuates vascular structural remodeling in experimental pulmonary hypertension (PH). The beneficial effect is related to reversal of the pathologic proliferative/synthetic and apoptosis-resistant vSMC phenotype in the PH vasculature. These studies identify endothelial IDO as a novel antivascular remodeling signal and thus may open a new avenue for the treatment of PH.

Keywords: smooth muscle cells; vascular remodeling; gene therapy

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Pulmonary hypertension (PH) is a fatal disease characterized by increased pulmonary vascular resistance that leads to progressive right ventricular hypertrophy (RVH) and eventual right heart failure. Remodeling of small pulmonary arteries, which is attributed primarily to excessive proliferation and impaired apoptosis of pulmonary arterial smooth muscle cells (PASMCs), is a key pathological change responsible for increased pulmonary vascular resistance in all categories of PH (1). Remarkably, PASMCs derived from patients with PH and animals with experimental PH (PH-PASMCs) retain their pathologic phenotype *in vivo* and *in vitro* (2–6), but, like other adult vascular smooth muscle cells (vSMCs), they remain highly plastic and capable of reversible change in their phenotype in response to extracellular cues (6–8). This unique feature raises an exciting possibility that phenotypic switch from normal vSMCs to the pathologic proliferative/synthetic and apoptosis-resistant state of PH-PASMCs could be directly targeted through therapeutic interventions. Such innovative approaches, however, have not yet been fully explored.

Indoleamine-2,3-dioxygenase (IDO) is a cytosolic enzyme that catalyzes the oxidative cleavage of the indole ring of tryptophan to *N*-formyl-kynurenine, which decomposes spontaneously to formate and L-kynurenine. This enzyme has antiinflammatory and immunosuppressive activities contributing to asthma (9) and cystic fibrosis inhibition (10), tumoral escape, and alloimmune tolerance (11–13). We recently reported that endothelial IDO shares functional similarities with NO synthase (NOS) and induces relaxation of arterial vessels through activation of cGMP-dependent

Figure 1. Endogenous indoleamine-2,3-dioxygenase (IDO) produced by pulmonary endothelium serves as a protective signal against hypoxia-induced pulmonary hypertension (PH). Mean pulmonary arterial pressure (mPAP) (A), right ventricular (RV) hypertrophy (B), and percentage of medial thickness of pulmonary resistance arteries (C) in wild-type (WT) and IDO^{-/-} mice studied at serial time points under hypoxic conditions of 10% oxygen. (C, right panel) Photomicrographs of hematoxylin and eosin-stained sections of pulmonary resistance arteries are representative of six mice in each group after 5 weeks of hypoxia (scale bars, 30 μ m). (D) Single lung cell suspensions were stained with fluorescence-labeled anti-CD31 antibody, and fluorescence-activated cell sorting was subsequently performed to isolate pulmonary ECs. (E) Endogenous IDO activity in pulmonary endothelial cells (ECs) and the rest of lung cells (non-ECs) measured at the indicated time points during hypoxia. Data are mean $\pm$ SEM; n = 5–6 animals or independent experiments for each group. LV = left ventricle; S = inter-ventricular septum. * P < 0.05, ** P < 0.01 versus WT B6 mice (A–C) or non-EC lung cells (E) at 2 weeks; † P < 0.05, †† P < 0.01 versus WT B6 mice (A–C) or non-EC lung cells (E) at 5 weeks.

protein kinase G (PKG) in vSMCs (14). A downstream target of PKG is the transcription factor myocardin, a crucial regulator of vSMC phenotype (15, 16). By physically binding with serum response factor at DNA sequences known as CArG boxes (CC[A/T]_nGG), myocardin activates most vSMC-specific genes, such as smooth muscle 22 α actin (SM22 α), SM-myosin heavy chain (SM-MHC), and calponin, to promote the differentiated/contractile phenotype and suppress the dedifferentiated/proliferative/synthetic vSMC phenotype (15–17). In addition, we have shown that mitochondrial transmembrane potential ($\Delta\Psi_m$) is decreased when cells are exposed to an IDO-high lung tissue microenvironment (18), whereas hyperpolarization of $\Delta\Psi_m$ is a critical determinant for maintaining PH-PASMCs in an apoptosis-resistant state (6, 19). These findings indicate that IDO has potential to direct phenotypic modulation of PH-PASMCs toward a less-proliferative/synthetic and proapoptotic state via a paracrine-way.

The objective of this study was to investigate whether sustained up-regulation of endothelial IDO could prevent and/or ameliorate pulmonary vascular remodeling and PH. To this end, we developed a *Sleeping Beauty* (SB)-based nonviral gene-integrating strategy

capable of long-lasting human IDO (hIDO) transgene expression within pulmonary endothelium (20).

METHODS

Animal Studies

All animal protocols were approved by the Animal Care and Use Committee of Boston Children's Hospital. For inducing PH in mice, 8- to 10-week-old male C57BL/6J (B6) and IDO^{-/-} mice on the B6 background (21, 22) were exposed to normobaric hypoxia (10% O₂) continuously for up to 5 weeks (23, 24). For induction of PH in rats, monocrotaline (MCT from Sigma-Aldrich, St. Louis, MO) was given as a single 60 mg/kg subcutaneous injection to male Sprague-Dawley rats weighing between 220 and 250 g (25, 26).

Plasmids Constructs and Establishment of hIDO-Overexpressing Cell Line

We modified our previous hIDO-encoding SB transposon with a CpG-depleted cytomegalovirus promoter by replacing the cytomegalovirus promoter with endothelin-1 promoter, and this new construct is referred to as pMSZ.ET1-hIDO (27, 28). A plasmid containing a hyperactive

Figure 2. Sleeping Beauty (SB)-mediated pulmonary endothelium-specific human indoleamine-2,3-dioxygenase (hIDO) transgene integration. (A) Plasmid vectors. (B) Expression of hIDO (bright red) in endothelium of small pulmonary artery was assessed by immunofluorescence staining (original magnification, $\times 800$). The lung sections were obtained from mice 6 weeks after intravenous injection of the SB-hIDO complex. The separated pulmonary endothelial cells (ECs) and the rest of lung cells (non-ECs) were analyzed for hIDO expression by real-time polymerase chain reaction (C) and Western blotting (D). (E) Integration sites of hIDO transgene in DNA from SB-hIDO-treated pulmonary ECs. In C and D, data are mean $\pm$ SEM; $n = 4$ –6 independent experiments for each group. CMV = cytomegalovirus; ET-1 = endothelin-1; IR/DR = inverted repeat/direct repeat elements; pA = poly(A) sequence. ** $P < 0.01$ versus other groups.

SB transposase (pTRUF-hSB17) was constructed as we previously described (12, 26). Constructs were confirmed by restriction and sequence analysis (12, 26). A stable hIDO-overexpressing pulmonary endothelial cell line was established by integrating hIDO gene into the genomic DNA of human pulmonary artery endothelial cells (PAECs from ATCC, Manassas, VA) using the SB-based nonviral gene transfer system (12, 28).

Quantitative Real-Time Polymerase Chain Reaction, Splinkerette Polymerase Chain Reaction, and Chromatin Immunoprecipitation–Polymerase Chain Reaction

We used real-time polymerase chain reaction (PCR) for quantitative analysis of hIDO mRNA level and used Splinkerette PCR to identify integration sites of hIDO transgene in genomic DNA extracted from the SB-hIDO-treated endothelial cells (ECs) (12, 26, 28). Chromatin immunoprecipitation assay was used for evaluation of myocardin binding to vSMC-specific CARG elements.

Additional details on all methods are provided in the online data supplement.

Statistical Analysis

Data are expressed as mean $\pm$ SEM, and statistical analyses were performed with the Prism statistical program (GraphPad, La Jolla, CA). Unpaired Student t test was used for comparisons between two means. One-way analysis of variance with the Newman-Keuls test was

used to evaluate differences between more than two groups. Linear correlation was evaluated with the Pearson r coefficient. In all cases, P value less than 0.05 was considered significant.

RESULTS

Expression of Endogenous Endothelial IDO Serves as a Protective Mechanism against PH

To examine the role of endogenous IDO in PH pathogenesis, we studied susceptibility of IDO $^{-/-}$ mice to hypoxia-induced PH. We found that both wild-type (WT) and IDO $^{-/-}$ mice manifested progressively elevated mean pulmonary artery pressure (mPAP) over a 5-week hypoxic exposure. However, at both 2 and 5 weeks of hypoxic exposure, IDO $^{-/-}$ mice demonstrated a significantly higher increase in mPAP compared with WT mice (Figure 1A). Consistent with this observation, the IDO $^{-/-}$ mice exhibited significantly increased RVH (Figure 1B) and vascular medial thickening (Figure 1C) compared with WT mice.

Next, we investigated the cellular source of endogenous IDO in the hypoxic lung. We separated ECs from the rest of lung cells based on CD31-positive selection (Figure 1D) and compared IDO expression between these two cell subpopulations (28). We found that IDO enzymatic activity (Figure 1E) and protein expression (see Figure E1 in the online supplement) were

Figure 3. Enhanced pulmonary endothelial indoleamine-2,3-dioxygenase (IDO) protects against monocrotaline (MCT)-induced pulmonary hypertension (PH). Mean pulmonary arterial pressure (mPAP) (A) and systemic blood pressure (SBP) (B) in rats measured at various time points after administration of MCT. The *Sleeping Beauty* human IDO (SB-hIDO) complex was given to animals on Day 0. (C) Level of hIDO mRNA in the isolated pulmonary endothelial cells (ECs) from vehicle- and SB-hIDO-treated PH lungs. (D) IDO activity in pulmonary ECs and the level of mPAP measured on Day 42 after MCT are inversely related. In separate studies, the SB-hIDO complex was given to animals on Day 21 after MCT and the level of mPAP (E), right ventricular (RV) hypertrophy (F), and percentage of medial thickness of resistance arteries (G) were measured at the indicated time point. (G, right panel) Representative hematoxylin and eosin-stained sections of resistance arteries from vehicle- and SB-hIDO-treated rats on Day 42 after MCT (scale bars, 30 μ m). Data are mean $\pm$ SEM; $n = 5$ –6 animals or independent experiments for each group. LV = left ventricle; S = interventricular septum. In A and E, * $P < 0.05$ versus vehicle on Day 21; † $P < 0.05$, †† $P < 0.01$ versus vehicle on Day 42 after MCT. In F and G, ** $P < 0.01$ versus control; † $P < 0.05$, †† $P < 0.01$ versus vehicle.

detectable in lung ECs of WT mice under normoxia and significantly increased in animals exposed to hypoxia. In contrast, IDO was undetectable or minimally detectable in non-EC lung cells from the same animals (Figure 1E, Figure E1).

Combined, these two sets of experiments provide initial evidence that endogenous IDO expression produced by pulmonary endothelium serves as a protective mechanism against PH development.

Sustained and Pulmonary Endothelium-Selective hIDO Transgene Expression through an SB-based Nonviral Approach

To achieve sustained overexpression of human IDO in pulmonary endothelium *in vivo*, we generated a hIDO-encoding SB transposon driven by the EC-selective promoter endothelin-1 (pMSZ.ET1-hIDO) along with a plasmid containing hyperactive transposase (pTRUF-hSB17) (Figure 2A). Furthermore, we used polymer polyethylenimine (PEI) as transgene carrier because this pharmacologic-grade reagent has been proven to

transfect lung tissue *in vivo* with high efficiency and specificity (12, 26–28). We then used the commercially available monoclonal anti-human IDO antibody that does not cross-react with rat or mouse IDO (US Biological, Swampscott, MA), to discern hIDO from endogenous IDO protein expression in mouse lungs. Our immunofluorescent studies (Figure 2B) showed that hIDO protein was predominantly expressed in the endothelium of small blood vessels in lung (red staining) at 6 weeks after a single intravenous administration of pET1-hIDO/pTRUF-hSB17/PEI complexes (SB-hIDO). To further confirm the pulmonary endothelium as the primary site for hIDO transgene expression, we compared hIDO mRNA and protein levels between pulmonary ECs and the rest of lung cells (non-ECs). As shown in Figures 2C and 2D, pulmonary ECs expressed approximately six times more hIDO mRNA and significantly higher levels of hIDO protein compared with non-ECs. Using splinkerette PCR technique to recover sequences flanking transposon insertion sites we identified three hIDO integration sites and confirmed no insertional disruption of genes in genomic DNA isolated from the SB-hIDO-treated

pulmonary ECs (Figure 2E). Importantly, the hIDO protein was highly functional, as shown by significantly higher total IDO activity in the SB-hIDO-treated pulmonary ECs compared with the vehicle-treated pulmonary ECs 6 weeks after the gene delivery (Figure E2). As seen previously with PEI-mediated gene delivery (12, 26–28), absent to barely detectable hIDO mRNA levels were found in other vascular tissues or organs including aorta, heart, liver, and kidney (Figure E3). Taken together, these results demonstrate that a single intravenous delivery of SB-hIDO complex preferentially (but not exclusively) integrates hIDO transgene into the host genome of pulmonary ECs and consequently endows these cells with the ability to persistently produce active hIDO protein *in vivo*.

Enhanced Endothelial IDO Ameliorates PH

We next examined the therapeutic potential of sustained overexpression of IDO within pulmonary endothelium in preventing and/or attenuating PH. Our first experiment tested a single administration of the SB-hIDO complex in rats on Day 0 after MCT injection (prevention protocol). We found significant PH by Day 21 in MCT-treated rats that received vehicle but not in rats that received SB-hIDO. Although mPAP increased further between Days 21 and 42 in all MCT-treated rats, the level of mPAP (Figure 3A) and RVH (Figure E4) on Day 42 in the SB-hIDO-treated rats was significantly lower than in rats receiving vehicle. In contrast, there was no change in systemic blood pressure in all animals (Figure 3B). In line with our previous studies showing that the duration and level of SB-mediated transgene was not altered by MCT treatment (26), ECs isolated from SB-hIDO, but not from vehicle-treated lung, contained a drastically increased level of hIDO mRNA (Figure 3C) and total IDO activity (Figure E5). Furthermore, pulmonary endothelial IDO activity was inversely correlated with the level of mPAP (i.e., higher endothelial IDO activity led to lower mPAP) (Figure 3D). In addition, we found that MCT-induced pulmonary inflammatory response, as indicated by the elevated levels of myeloperoxidase activity and protein carbonyl in the PH lung, was remarkably attenuated by treatment with the SB-hIDO complex (Figure E6).

We further evaluated the therapeutic potential of enhanced endothelial IDO in a PH reversal model, which is more clinically relevant. A single administration of SB-hIDO complex on Day 21 after MCT did not completely reverse but halted progression of PH, as assessed by mPAP (Figure 3E), RVH (Figure 3F), and pulmonary arterial wall thickness (Figure 3G) on Day 42 after MCT. In a parallel reversal study, we gave the SB-hIDO complex to WT mice after 2 weeks of hypoxic exposure, at which time point PH was developing. We then evaluated the mice after 3 additional weeks of hypoxia and found that hIDO transgene exhibited a strong therapeutic effect. Hypoxic mice that received SB-hIDO showed a restoration of mPAP, right ventricle, and vascular wall thickness to those found in the normoxic WT mice despite continuous exposure to hypoxia for 5 weeks (Figure E7).

Endothelial IDO Enhances Susceptibility of PH-PASMCs to Apoptosis

We found that conversion of tryptophan to kynurenine by hIDO transgene *in vivo* did not affect pulmonary vasoreactivity in MCT- (Figure E8) and hypoxia-PH animals (Figure E9). Our further studies therefore focused on the effect of endothelial IDO on structural vascular remodeling in PH.

One of the underlying mechanisms of structural vascular remodeling in PH is apoptosis resistance of PH-PASMCs; we thus investigated whether exposure to an IDO-high tissue microenvironment created by augmented endothelial IDO would alter PH-PASMC susceptibility to apoptosis. We used three complementary approaches. First, flow cytometry analysis revealed that

PH-PASMCs isolated from vehicle-treated PH rats have a lower percentage of apoptotic cells as compared with PASMCs isolated from normal animals, whereas SB-hIDO eliminated this difference (Figure 4A). Second, we challenged the subcultured PH-PASMCs with the apoptosis inducer, H_2O_2 (90 μ M), in the presence of WT- or hIDO-overexpressing PAECs. We found that PH-PASMCs were more resistant to H_2O_2 -induced apoptosis in comparison to normal PASMCs. In the presence of hIDO-overexpressing PAECs, PH-PASMCs were significantly more susceptible to apoptosis. The reduced resistance to apoptosis of PH-PASMCs cocultured with hIDO-overexpressing PAECs, notably, was almost completely recovered by addition

Figure 4. Enhanced pulmonary endothelial indoleamine-2,3-dioxygenase (IDO) *in vivo* and *in vitro* promotes apoptosis of pulmonary hypertension (PH)-pulmonary arterial smooth muscle cells (PASMCs). (A) Quantitative analysis by flow cytometry of percentage of apoptotic cells in total PASMCs and PH-PASMCs from vehicle- or *Sleeping Beauty* (SB)-hIDO-treated animals. Note that the SB-hIDO complex or vehicle was given to rats on Day 21 and the cells were collected on Day 42 after monocrotaline treatment. (B) Percentage of oxidant-induced apoptotic cells in PASMCs or PH-PASMCs cocultured with wild-type (WT)- or hIDO-overexpressing pulmonary artery endothelial cells (PAECs). Note that hIDO-overexpressing PAECs demonstrated a strong apoptosis-promoting effect on PH-PASMCs, which was abolished by IDO inhibitor 1-mT (1 mM). Data are mean $\pm$ SEM; n = 4–6 animals or independent experiments for each group. In A, **P < 0.01 versus other groups. In B, *P < 0.05, **P < 0.01 hIDO-PAEC-treated PH-PASMCs versus other PH-PASMC groups at indicated time point.

of the IDO inhibitor 1-methyl-DL-tryptophan (1-mT, 1 mM) (14, 18) (Figure 4B). We obtained similar results when we used 0.1% fetal bovine serum as the proapoptotic stimulus (data not shown). Last, the studies with subcultured PH-PASMCs were performed with exogenously added kynurenine (0–500 μ M). We found that addition of kynurenine, but not tryptophan, exhibited a similar apoptosis-promoting effect as hIDO-overexpressing PAECs on PH-PASMCs (Figure E10).

Apoptosis resistance in PH-PASMCs is linked to hyperpolarization of mitochondrial $\Delta\Psi_m$, which prevents translocation of cytochrome c from mitochondria to cytosol (6, 19). We confirmed this by showing that $\Delta\Psi_m$ was higher in PH-PASMCs relative to baseline $\Delta\Psi_m$ in normal PASMCs (Figure 5A). Notably, endothelial-derived IDO significantly decreased $\Delta\Psi_m$ of PH-PASMCs *in vitro* (Figure 5A) and *in vivo* (Figure E11). In line with these findings, we found that, in the presence of hIDO-overexpressing PAECs, H_2O_2 -induced release of cytochrome c and its downstream effectors caspase-9 and 3 cleavage in PH-PASMCs was elevated to levels seen in the H_2O_2 -treated normal PASMCs (Figure 5B). Using the IDO inhibitor 1-mT we confirmed that this apoptosis-promoting effect is mediated by IDO.

Together, these studies confirm that PH-PASMCs exhibit an apoptosis-resistant phenotype and demonstrate that endothelial IDO is able to reverse this phenotype by restoring mitochondrial $\Delta\Psi_m$.

Endothelial IDO Inhibits PH-PASMC Proliferation

Excessive proliferation of PASMCs is another key contributing factor to structural vascular remodeling in PH. We therefore examined whether enhanced endothelial IDO down-regulates PH-PASMC proliferation via a paracrine way. Flow cytometry analysis revealed

that expression of proliferating cell nuclear antigen (PCNA) was significantly higher in freshly isolated PH-PASMCs as compared with normal PASMCs, and SB-hIDO drastically reduced PCNA-positive PH-PASMCs to a basal level (Figures 6A and 6B). In line with this finding, subcultured PH-PASMCs from SB-hIDO-treated lung had roughly half the proliferation rate as PH-PASMCs from vehicle-treated lung (Figure 6C). Direct evidence for the inhibitory activity of endothelial IDO on PH-PASMC proliferation was obtained from a cell coculture study using the IDO-overexpressing PAECs and the IDO inhibitor 1-mT (Figure E12).

We next compared phenotypic patterns of PH-PASMCs from vehicle- and SB-hIDO-treated PH animals. In PH-PASMCs from vehicle-treated animals, expression of proteins associated with vSMC differentiated/contractile phenotype including SM22 α and SM-MHC were decreased in comparison to normal PASMCs. In contrast, the synthetic markers syndecan-1 (Figure 6D) and fibronectin (Figure E13) were more abundant in PH-PASMCs from vehicle-treated animals relative to normal PASMCs. These changes in differentiation-specific marker proteins in the PH-PASMCs from vehicle-treated animals were almost completely reversed by SB-hIDO treatment (Figure 6D and Figure E13). These results suggest that endothelial IDO is able to restore the differentiated state in PH-PASMCs and that the dedifferentiation of PH-PASMCs underlies, to a large extent, the proproliferative/synthetic phenotype of these cells.

Endothelial IDO Enhanced Myocardin-mediated Differentiation in PH-PASMCs

Our next experiments aimed at defining the molecular pathways involved in the IDO-induced restoration of PH-PASMC differentiation. We first studied myocardin expression by Western blotting and demonstrated that IDO produced from pulmonary

Figure 5. Depolarization of mitochondrial membrane potential contributes to endothelial indoleamine-2,3-dioxygenase (IDO)-induced apoptosis in pulmonary hypertension (PH)-pulmonary arterial smooth muscle cells (PASMCs). (A) Analysis of mitochondrial membrane potential ($\Delta\Psi_m$) in control PASMCs and PH-PASMCs, and the PH-PASMCs that have been cocultured with wild-type (WT)- and human IDO (hIDO)-overexpressing pulmonary artery endothelial cells (PAECs) for 48 hours. The PH-PASMCs were collected from PH rats on Day 42 after monocrotaline treatment. (B) Protein levels of cytochrome c in cytosolic fractions and cleaved caspase-9, -3 in whole cell lysate were analyzed by Western blotting. Data are mean $\pm$ SEM; $n = 4$ –5 independent experiments for each group. In A, * $P < 0.05$ versus other PH-PASMC groups; in B, ** $P < 0.01$ versus other PH-PASMC groups for the indicated protein. 1-mT = IDO inhibitor 1-methyl-DL-tryptophan (1mM).

Figure 6. Enhanced pulmonary endothelial indoleamine-2,3-dioxygenase (IDO) decreases proliferation of pulmonary hypertension (PH)-pulmonary arterial smooth muscle cells (PASCs). (A) Representative flow cytometry histograms for PCNA-positive cells. (B) Percentage of proliferating cell nuclear antigen (PCNA)-positive cells in control PASCs and PH-PASCs from vehicle- or *Sleeping Beauty* (SB)-human IDO (hIDO)-treated animals. Note that the SB-hIDO complex or vehicle was given to rats on Day 21, and the cells were collected on Day 42 after monocrotaline treatment. These cells were also used for determination of growth curve (C), protein levels of smooth muscle 22 α actin (SM22 α), SM-myosin heavy chain (SM-MHC), and Syndecan-1 in whole cell lysates by Western blotting (D). Data are mean $\pm$ SEM; $n = 4$ –6 independent experiments for each group. In B, * $P < 0.05$ versus other groups. In C and D, * $P < 0.05$, ** $P < 0.01$ versus other groups at indicated time point (C) or protein marker (D).

ECs up-regulated myocardin in PH-PASCs, and that this change was abolished by the presence of the PKG inhibitor DT-3 (50 μ M) (Figure 7A). This result expands on our previous observation that IDO activates cGMP-dependent PKG (14) and supports the notion that myocardin is a downstream effector of PKG (15). Because phosphorylated Elk-1 (p-Elk-1) is known to compete with myocardin for a common dock site on serum response factor, and formation of CArG-Elk-1-SFR represents an opposing transcriptional complex relative to CArG-myocardin-SFR complex (15–17), we next examined the effect of endothelial IDO on Elk-1 phosphorylation. We found increased abundance of p-Elk-1 in PH-PASCs, whereas coculturing with hIDO-overexpressing PAECs reduced Elk-1 phosphorylation in the PH-PASCs to a baseline level comparable to normal PASCs (Figure 7A). Notably, the IDO-induced dephosphorylation of Elk-1 was not affected by DT-3, suggesting the involvement of a pathway that is distinct from the cGMP-PKG pathway.

Finally, we investigated whether physical binding of myocardin or Elk-1 to the CArG-containing regions of vSMC-specific promoters was indeed affected by endothelial IDO. Using quantitative chromatin immunoprecipitation assays, we found a lower level of myocardin binding to the CArG-containing regions

of SM22 α (Figure 7B) and SM-MHC (Figure 7C) promoters in PH-PASCs as compared with normal PASCs. Coculturing with hIDO-overexpressing PAECs resulted in a significant increase in myocardin binding at each of the vSMC-specific promoters in the PH-PASCs. Furthermore, the IDO-dependent recruitment of myocardin to these CArG boxes was accompanied by the reduction of p-Elk-1 from the same sites, whereas the IDO inhibitor 1-mT significantly prevented this effect (Figures 7B and 7C). Taken together, we provide a comprehensive model explaining IDO-dependent phenotypic modulation of PH-PASCs toward a less proliferative/synthetic state, including transcriptional activation of vSMC-specific genes due to up-regulation of myocardin and dephosphorylation of Elk-1 through distinct pathways.

DISCUSSION

Current therapies for PH may improve hemodynamics but often fail to yield long-term benefits and improve mortality (29, 30). The limited efficacy of these therapies is somewhat expected, because their mechanism of action is mainly pulmonary vasodilation. The present study identifies endothelial IDO as a novel protective signal that targets structural vascular remodeling in PH lung. This

Figure 7. Enhanced pulmonary endothelial indoleamine-2,3-dioxygenase (IDO) activates vascular smooth muscle cell-specific gene transcription in pulmonary hypertension (PH)-pulmonary arterial smooth muscle cells (PSMCs). (A) Western blotting analysis of myocardin, phosphorylated and total Elk-1 in control PSMCs and PH-PSMCs, and the PH-PSMCs that have been cocultured with wild-type (WT)- and human IDO (hIDO)-overexpressing pulmonary artery endothelial cells (PAECs) for 48 hours. The PH-PSMCs were collected from PH rats on Day 42 after monocrotaline treatment. In separate studies, these cells were processed for chromatin immunoprecipitation assays and real-time polymerase chain reaction was performed on immunoprecipitants using primers for the CArG-containing regions of smooth muscle 22 α actin (SM22 α) (B) or SM-myosin heavy chain (SM-MHC)- (C) specific promoters. Data are mean $\pm$ SEM; $n = 4-5$ independent experiments for each group. In A, $^{**}P < 0.01$ versus myocardin level in other PH-PSMCs groups; $^{++}P < 0.01$ versus p-Elk1 level in control and WT-PAEC-treated PH-PSMCs. In B and C, $^{*}P < 0.05$, $^{**}P < 0.01$ versus other PH-PSMCs groups.

evidence is derived from our intriguing finding that, in an IDO-high tissue microenvironment created by endothelial IDO, the pathologic antiapoptotic and proproliferative/synthetic PH-PASMC phenotype is reversed toward the normal PASMC state.

The importance of PASMC phenotypic switch in experimental and human PH is supported by direct experimental evidence (7). Although direct targeting of PASMCs in experimental PH was accomplished with several interventions, including dichloroacetate (31) cyclosporin (32), and the platelet-derived growth factor receptor antagonist imatinib (33), the pulmonary vascular endothelium is a more accessible target for gene delivery than the vSMC layer. As shown in our study, sustained overexpression of endothelial IDO provided by hIDO transgene resulted in several favorable effects on PASMC homeostasis and was protective in experimental PH. In addition to reduced PH-PASMC proliferative/synthetic capacity and a shift to a more differentiated phenotype, enhanced endothelial IDO restored mitochondria-dependent apoptosis of PH-PASMCs to a level seen in normal PASMCs. We noted that, in the PH lungs that received SB-hIDO therapy, the hIDO-expressing PAECs themselves were also exposed to the IDO-high tissue environment. This appears to conflict with the observation that endothelial

IDO ameliorates and prevents PH development, as the IDO-high tissue environment may also induce apoptosis in these PAECs, thereby worsening PH (34). Our prior studies, however, demonstrated that IDO-overexpressing ECs are endowed with the ability to resist apoptosis, possibly through stabilization of mitochondrial redox status (28). This means that overexpression of IDO by PAECs can selectively induce apoptosis in the adjacent PH-PASMCs via a paracrine way, sparing PAECs themselves. Notably, the phenomenon that one active molecule exerts opposite effects under an identical tissue microenvironment is not unusual. For example, almost all tumor cells overexpress IDO (35), which facilitates their growth and survival while concurrently damaging tumor-infiltrating T cells (11, 35). Similarly, Zuckerbraun and colleagues showed paracrine effects of PAEC-derived NO that induces death of PH-PASMCs while preserving PAECs in the PH lung (36). It is important to note, however, that there is convincing evidence that PAECs from patients with PH actually demonstrate an apoptosis-resistant and proproliferative phenotype (37, 38). Thus, more studies are required to clarify the role of IDO in human PAEC homeostasis in the setting of PH.

Our findings indicate that enhanced endothelial IDO can inhibit inflammatory response in the MCT-treated lung. This anti-inflammatory effect may be significant, because a recent study by Vergadi and colleagues demonstrated that inflammatory cells directly promote PASMC proliferation in PH (23). Thus, the anti-vascular remodeling effects provided by endothelial IDO are likely mediated by multiple pathways, which regulate PH-PASMC apoptosis, differentiation/proliferation, and possibly lung inflammation. Some of these pathways do not overlap, and this feature is clinically important, because it may reduce the likelihood of emergence of therapy resistance as currently seen with anti-PH therapies. In support of this notion, studies from other groups have shown that therapeutic interventions targeting either PH-PASMCs apoptosis (39) or proliferation (33, 40) or lung inflammation (23) could ameliorate experimental PH.

The gene delivery system used in our study resulted in SB-mediated integration of hIDO gene into DNA of PAECs, which was confirmed by splinkerette PCR. This is essential for achieving IDO-dependent long-term therapeutic efficacy against PH and its associated vascular remodeling. However, this transposition event raises a theoretical concern for inducing tumorigenic mutations. Nonetheless, this has not been observed in our PH mice or rats or in our previous studies using animal models of lung transplantation (12), hemophilia A mice (41), and eNOS-treated PH rats (26). In all of these studies, we showed that SB-mediated integrations always occurred outside active genes or promoter regions. The safety profile of the SB-based gene-integrating approach has also been documented by other reports, some of which are already advancing into clinical trials (42).

In summary, our studies provide a novel, pulmonary circulation-specific therapeutic strategy for prevention and amelioration of multiple pathological processes associated with PH vascular remodeling, through up-regulation of pulmonary endothelial IDO activity using the clinically relevant SB-based non-viral gene-integrating approach. Remarkably, the regression of experimental PH is achieved by a single injection of the pharmacological-grade complex SB-hIDO. The safety profile and translational potential of this intriguing approach in treating human PH warrant further study.

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