PGC1 α -mediated Mitofusin-2 Deficiency in Female Rats and Humans with Pulmonary Arterial Hypertension

John J. Ryan¹, Glenn Marsboom^{1,2}, Yong-Hu Fang¹, Peter T. Toth¹, Erik Morrow¹, Nancy Luo¹, Lin Piao¹, Zhigang Hong¹, Kyle Ericson¹, Hannah J. Zhang¹, Mei Han³, Chad R. Haney⁴, Chin-Tu Chen⁴, Willard W. Sharp³, and Stephen L. Archer^{1,5}

¹Section of Cardiology, ³Section of Emergency Medicine, Department of Medicine, and ⁴Department of Radiology, University of Chicago, Chicago, Illinois; ²Immunology Department, University Hospital La Princesa, Autonomous University, Madrid, Spain; and ⁵Department of Medicine, Queen's University, Kingston, Ontario, Canada

Rationale: Pulmonary arterial hypertension (PAH) is a lethal, female-predominant, vascular disease. Pathologic changes in PA smooth muscle cells (PASMC) include excessive proliferation, apoptosis-resistance, and mitochondrial fragmentation. Activation of dynamin-related protein increases mitotic fission and promotes this proliferation–apoptosis imbalance. The contribution of decreased fusion and reduced mitofusin-2 (MFN2) expression to PAH is unknown.

Objectives: We hypothesize that decreased MFN2 expression promotes mitochondrial fragmentation, increases proliferation, and impairs apoptosis. The role of MFN2's transcriptional coactivator, peroxisome proliferator-activated receptor γ coactivator 1- α (PGC1 α), was assessed. MFN2 therapy was tested in PAH PASMC and in models of PAH.

Methods: Fusion and fission mediators were measured in lungs and PASMC from patients with PAH and female rats with monocrotaline or chronic hypoxia + Sugen-5416 (CH+SU) PAH. The effects of adenoviral mitofusin-2 (Ad-MFN2) overexpression were measured *in vitro* and *in vivo*.

Measurements and Main Results: In normal PASMC, siMFN2 reduced expression of MFN2 and PGC1 α ; conversely, siPGC1 α reduced PGC1 α and MFN2 expression. Both interventions caused mitochondrial fragmentation. siMFN2 increased proliferation. In rodent and human PAH PASMC, MFN2 and PGC1 α were decreased and mitochondria were fragmented. Ad-MFN2 increased fusion, reduced proliferation, and increased apoptosis in human PAH and CH+SU. In CH+SU, Ad-MFN2 improved walking distance (381 ± 35 vs. 245 ± 39 m; $P < 0.05$); decreased pulmonary vascular resistance (0.18 ± 0.02 vs. 0.38 ± 0.14 mm Hg/ml/min; $P < 0.05$); and decreased PA medial thickness (14.5 ± 0.8 vs. $19 \pm 1.7\%$; $P < 0.05$). Lung vascularity was increased by MFN2.

Conclusions: Decreased expression of MFN2 and PGC1 α contribute to mitochondrial fragmentation and a proliferation–apoptosis imbalance in human and experimental PAH. Augmenting MFN2 has therapeutic benefit in human and experimental PAH.

Keywords: mitochondrial fission; peroxisome proliferator-activated receptor gamma coactivator-1 α ; hypoxia-inducible factor-1 α ; optic atrophy 1; female sex

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Proliferation and apoptosis-resistance of pulmonary arterial smooth muscle cells (PASMC) contribute to vascular obstruction in pulmonary arterial hypertension (PAH). The mitochondrial network is fragmented in PAH PASMC and this disruption is mechanistically related to the PASMC proliferation–apoptosis imbalance.

What This Study Adds to the Field

Here we demonstrate a deficiency of peroxisome proliferator-activated receptor γ coactivator-1 α and mitofusin-2 (MFN2) in female patients with PAH and in two models of PAH created in female rats. Overexpression of MFN2 reverses fragmentation, reduces PASMC proliferation, and increases apoptosis. MFN2 augmentation also regresses experimental PAH *in vivo*.

Pulmonary arterial hypertension (PAH) is a syndrome characterized by obstructive vascular remodeling, inflammation, and vasoconstriction of small pulmonary arteries. PAH is predominantly a disease of females (1). Recent advances in understanding the mechanism of PAH include the identification of mutations in the bone morphogenetic protein receptor 2 in familial PAH (2–4) and the recognition that increases in proliferation and apoptosis-resistance of pulmonary arterial smooth muscle cells (PASMC), that have multifactorial etiology, contribute to vascular obstruction (reviewed in [5]). These discoveries have yet to be translated into approved therapies, and most PAH treatments are vasodilators. Perhaps because of this, the 1-year mortality rates remain high ($\sim 15\%$) (6). Although abnormalities of platelets, the endothelium, fibroblasts, and inflammatory cells contribute critically to the pathogenesis of PAH, the proliferation–apoptosis imbalance in PASMC contributes to the obstructive vasculopathy and thus is an attractive target for intervention.

We recently discovered that the mitochondrial network is fragmented in PAH PASMC (7–9) and noted that this disruption is mechanistically related to the PASMC proliferation–apoptosis imbalance (9). Fragmentation of the mitochondrial network reflects, in part, increased fission, caused by activation of dynamin-related protein 1 (DRP1) (9). Inhibiting DRP1 slows PASMC proliferation by preventing mitotic fission, a newly recognized mitotic checkpoint. When mitochondria cannot divide, mitosis does not proceed, and cells are arrested in G2-M phase of the cell cycle (9). *In vivo*, inhibition of DRP1 by the small molecule mitochondrial division inhibitor-1 (Mdivi1) partially regresses experimental PAH (9). In the same study, we noted that the fusion mediator

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Correspondence and requests for reprints should be addressed to Stephen L. Archer, M.D., Department of Medicine, Queen's University, Etherington Hall, Room 3041, 94 Stuart Street, Kingston, ON, K7L 3N6 Canada. E-mail: stephen.archer@queensu.ca

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mitofusin-2 (MFN2) was down-regulated in PAH (9, 10). In principal, impaired fusion could have the same effects on mitochondrial morphology and proliferation–apoptosis as increased fission. However, the role of impaired fusion in PAH has not been assessed.

Mitochondrial fusion is regulated by MFN1 and MFN2 (11). These GTPases reside in the outer mitochondrial membrane and tether adjacent mitochondria, permitting the docking required for fusion (12). Optic atrophy 1 (OPA1) also promotes fusion, through effects on the inner mitochondrial membrane (13). When cloned by Chen and coworkers (14), the MFN2 gene was initially named “hyperplasia suppressor gene” because of its antiproliferative effects on smooth muscle cells. MFN2 gene therapy reduces intimal-medial thickening in a carotid artery, balloon-injury model by reducing proliferation (14). These properties suggest that MFN2 deficiency could contribute to the mitochondrial fragmentation, proliferative diathesis, and apoptosis-resistance in PAH and suggests the therapeutic potential of augmenting MFN2.

We assessed the potential contribution of decreased expression of MFN2 and its transcriptional coactivator, peroxisome proliferator-activated receptor γ coactivator-1 α (PGC1 α), to PAH. PGC1 α can induce MFN2 transcription and PGC1 α positively regulates mitochondrial biogenesis (15). In part, the regulation of mitochondrial activity by PGC1 α depends on correct MFN2 expression (16). Although the role of PGC1 α in the pulmonary vasculature is largely unknown, its expression is decreased in several nonvascular tissues in experimental PAH (right ventricle myocytes, soleus muscle) (17, 18). PGC1 α mRNA levels in the peripheral blood of patients with PAH are inversely correlated with PAH severity (19).

Here we demonstrate a deficiency of PGC1 α and MFN2 in female patients with PAH and in two models of PAH created in female rats. Expression of PGC1 α and MFN2 is linked through positive feedback. Overexpression of MFN2 reverses fragmentation, reduces PASM C proliferation, and increases apoptosis. MFN2 augmentation also regresses experimental PAH *in vivo*.

METHODS

Human Lung Samples

Tissues for PASM C isolation and paraffin-embedded tissue sections were obtained from autopsied control patients (without PAH) or idiopathic patients with PAH under an Institutional Review Board approved protocol (see Table E1 in the online supplement for demographics). A total of 80% of the subjects were female.

Animal Studies

All protocols were approved by the University of Chicago Animal Care Committee. Female Sprague-Dawley rats were purchased from Charles Rivers Laboratories (Wilmington, MA). Two PAH models were used. For the chronic hypoxia plus Sugen-5416 (CH+SU) model, rats received a single subcutaneous injection of SU (20 mg/kg) and were exposed to normobaric hypoxia for 3 weeks (10% oxygen), as previously described (20). They were returned to normoxia for a period of 3–4 weeks before starting treatment. For the monocrotaline model, rats received a subcutaneous injection of monocrotaline (60 mg/kg; Sigma-Aldrich, St. Louis, MO), as previously described (21). Control rats for both models received a subcutaneous saline injection.

Small Interfering RNA Treatment of PASM C

Normal human PASM C were grown to approximately 80% confluence and then transfected using oligofectamine (Invitrogen, Carlsbad, CA) and 25-nM validated Silencer Select small interfering RNAs targeting MFN2 (Applied Biosystems, Carlsbad, CA) or PGC1 α (Sigma-Aldrich, St. Louis, MO) or a scrambled small interfering RNA control. Normal culture medium was added after 4 hours, and medium was changed after

48 hours. Gene knockdown was confirmed by quantitative reverse-transcriptase polymerase chain reaction and by immunoblot.

Live-Cell Imaging to Assess Mitochondrial Networks

Mitochondrial fusion. To quantify fusion in live cells, they were transiently transfected with mitochondrial matrix-targeted photoactivatable green fluorescent protein (mito-PA-GFP) and mitochondrial matrix-targeted DsRed and imaged with a Zeiss 510META confocal laser scanning microscope (Zeiss, Thornwood, NY) equipped with an environmental chamber to maintain humidity, temperature (37°C), and CO₂ (5%), as described (see METHODS in the online supplement) (9).

Mitochondrial fragmentation. Several techniques were used to image mitochondria morphology. In some experiments, mitochondria morphology was assessed using mito-GFP (22). This method requires transfection with a plasmid vector but creates high-resolution images of the mitochondrial network. To ensure effects on the network were not related to vector toxicity, we also imaged the mitochondria using the potentiometric dye tetramethylrhodamine (50 nM, 20 min in culture medium at 37°C; Molecular Probes, Eugene, OR), as described (23). Tetramethylrhodamine has the advantage of revealing the network in all cells without a vector.

Acquired images were background subtracted, filtered, thresholded, and binarized to identify mitochondrial segments using ImageJ (NIH, Bethesda, MD). Continuous mitochondrial structures were counted, and the number was normalized to the total mitochondrial area to obtain the mitochondrial fragmentation count (MFC) for each of 25 or more randomly selected cells, as described (23). Cells with greater fragmentation have a higher MFC.

MFN2 Immunohistochemistry

MFN2 immunohistochemistry was performed as previously described (see METHODS in the online supplement) (9).

Adenoviral Gene Transfer in PASM C

These experiments were performed in cultures of PASM C isolated from resistance PA of pulmonary hypertensive (monocrotaline and CH+SU) or control rats (passages 1–3) and in human PAH or control PASM C. Cells were grown to approximately 50% confluence and then were exposed to adenovirus carrying MFN2 (Ad-MFN2; multiplicity of infection range 50–500) or a control virus (either an empty adenovirus [Ad-empty] or LacZ adenovirus). PASM C were exposed to the virus under serum-free conditions for 12 hours, then returned to serum and imaged 48 hours after infection.

Treadmill, Echocardiography, Quantitative Reverse-Transcriptase Polymerase Chain Reaction, Immunoblot, Immunofluorescence, and Cell Proliferation Assays

Treadmill, echocardiography, quantitative reverse-transcriptase polymerase chain reaction, immunoblot, immunofluorescence, and cell proliferation assays were performed as described previously (24) (see METHODS in the online supplement for additional information).

In Vivo Therapies to Promote Mitochondrial Fusion

Table 1 details the design of the study and the number of rats in each protocol.

In vivo adenoviral therapy. Monocrotaline and CH+SU rats with echo-confirmed PAH (defined as a pulmonary artery acceleration time [PAAT] of <20 milliseconds on Doppler) were treated with Ad-MFN2 versus Ad-GFP (Table 1). Gene therapy was performed in anesthetized rats (ketamine, 80 mg/kg, plus xylazine, 8 mg/kg) by nebulization alone or by combined intravenous injection and nebulization (Table 1). Nebulization was performed by placing a micronebulizer (Penn Century, Wyndmoor, PA) in the trachea. Then, 0.1 ml of virus (in saline, 2×10^9 plaque-forming units) was administered, and the rat was allowed to recover. Intravenous administration was performed by injecting the same dose of virus by the internal jugular vein. Exercise capacity and hemodynamics were measured 2–3 weeks after therapy. Hemodynamics were

TABLE 1. ANIMALS STUDIED

Animal Model	Treatment	Number of Animals	Weight (g)	Heart Rate
Control	Nil	12	297 ± 8	391 ± 16
CH+SU	DMSO	10	287 ± 11	353 ± 12
CH+SU	Nebulized Ad-GFP	7	268 ± 7	351 ± 6
CH+SU	Nebulized Ad-MFN2	6	292 ± 7	321 ± 8
CH+SU	Nebulized Ad-GFP + intravenous Ad-GFP	8	281 ± 7	378 ± 18
CH+SU	Nebulized Ad-MFN2 + intravenous Ad-MFN2	10	293 ± 6	376 ± 13
CH+SU	Mdivi1	9	273 ± 7	388 ± 8
CH+SU	Intravenous Ad-MFN2 + Mdivi1	10	319 ± 7	349 ± 4
Monocrotaline	Nebulized Ad-GFP	8	308 ± 11	349 ± 20
Monocrotaline	Nebulized Ad-MFN2	8	306 ± 8	330 ± 8

Definition of abbreviations: Ad-GFP = adenovirus carrying green fluorescent protein; Ad-MFN2 = adenovirus carrying mitofusin-2; CH+SU = chronic hypoxia plus Sugen-5416; DMSO = dimethyl sulfate.

performed in anesthetized rats (ketamine, 80 mg/kg, plus xylazine, 8 mg/kg) by an open-chested technique, as previously described (25).

Ad-MFN2 and Mdivi1 therapy. Because of the previous demonstration of the therapeutic benefit of inhibiting mitochondrial fission in experimental PAH (9), we studied the effect of combining intravenous Ad-MFN2 and intraperitoneal DRP1 inhibitor, Mdivi1 (Enzo Life Sciences, Inc., Farmingdale, NY) in an additional cohort. After confirming the presence of elevated pulmonary artery pressure (PAP) on echocardiography, CH+SU rats received Ad-MFN2 or Ad-GFP through the internal jugular vein. A group of animals then received intraperitoneal injection of Mdivi1, 50 mg/kg, in dimethyl sulfate (DMSO) every 3 days for 18 days. The results in the combination therapy group were compared with groups receiving only Mdivi1, only Ad-MFN2, or only DMSO.

Statistics and Sample Size

Values were expressed as mean ± SEM. Sample sizes are shown in each figure and in Table 1. Prism 5 (GraphPad Software, La Jolla, CA) was used for data analysis. For comparisons between two groups, we used an unpaired Student *t* test (two-tailed as appropriate). For comparisons among multiple groups, an analysis of variance was used with *post hoc* testing using a Bonferroni correction for multiple comparisons. *P* < 0.05 was considered statistically significant.

RESULTS

Mitochondrial Fragmentation and Decreased MFN2 Levels in Human PAH

PASMC from patients with PAH have increased mitochondrial fragmentation (Figure 1A). There was a decrease in mRNA expression of fusogenic genes (MFN2) and an increase in fissionogenic gene expression (DRP1, fission 1 [FIS1]) (Figure 1B).

Immunoblots confirmed that MFN2 was significantly decreased in human PAH PASMC (Figure 1C). Despite a homogenous expression of translocase of the outer mitochondrial membrane, a measure of mitochondrial mass, there was considerable intersubject variation in MFN2 expression in PAH PASMC lines, with the protein being almost absent in some individuals. MFN1 levels were not significantly decreased in PAH PASMC (Figure 1C).

We qualitatively assessed expression of MFN2 in the small PAs within human lungs (*see* Table E1, *n* = 5 per group). Semi-quantitative assessment of MFN2 expression by immunohistochemistry revealed decreased MFN2 in the media of small PAs from patients with PAH versus control subjects (7.3 ± 0.7 vs. 2.1 ± 0.8 arbitrary units [AU]; *P* < 0.001) (Figure 1D).

CH+SU and Monocrotaline PASMC Mitochondria Are Fragmented

Like human PAH PASMC, CH+SU PASMC had more fragmented mitochondria versus control (Figure 2A). MFN2 protein and mRNA were likewise significantly decreased in CH+SU PASMC (Figures

2B and 2C). However, unlike human PAH there was no increase in the expression of mitochondrial fission mediators in CH+SU PASMC (Figure 2C). However, the activated form of DRP1 (which is phosphorylated at serine 616, DRP1 p-Ser616) was increased in CH+SU versus control PAs (Figure 2D).

Monocrotaline PASMC had increased PASMC mitochondrial fragmentation (Figure 2E). MFN2 protein and mRNA was also significantly decreased in monocrotaline rat lungs (Figure 2F; *see* Figure E1).

MFN2 Overexpression Increases Mitochondrial Fusion in Human PASMC

Mitochondrial fragmentation was increased in human PAH versus control PASMC (MFC 0.66 ± 0.03 vs. 0.54 ± 0.04 AU; *P* < 0.05) (Figures 3A and 3B). Overexpression of MFN2 reduced PAH PASMC proliferation rates to those of control PASMC (control 1.00 ± 0.03 vs. control+MFN2 0.50 ± 0.07 ; PAH 1.93 ± 0.10 vs. PAH+MFN2 0.85 ± 0.16 ; *P* < 0.05) (Figure 3C). Overexpression of MFN2 increased apoptosis in control and PAH PASMC (control 0.75 ± 0.23 vs. control+MFN2 1.74 ± 0.31 ; PAH 0.56 ± 0.39 vs. PAH+MFN2 1.96 ± 0.41 ; *P* < 0.05) (Figures 3D and 3E).

MFN2 Overexpression Increases Mitochondrial Fusion in Experimental PAH PASMC

Overexpression of MFN2 decreased mitochondrial fragmentation in CH+SU PASMC (MFC 3.89 ± 0.37 vs. 1.61 ± 0.11 AU; *P* < 0.05) (Figures 4A and 4B). Overexpression of MFN2 also decreased mitochondrial fragmentation in monocrotaline PASMC (MFC 2.79 ± 0.54 vs. 1.34 ± 0.21 AU) (Figures 4A and 4B). Ad-MFN2 decreased proliferation rates in monocrotaline PASMC compared with Ad-empty (Ad-empty 28.17 ± 0.47 vs. Ad-MFN2 24.92 ± 0.5 ; *P* < 0.01) (*see* Figure E2A) and increased percentage of apoptosis in PASMC (Ad-empty 1.5 ± 0.3 vs. Ad-MFN2 2.5 ± 0.15 ; *P* < 0.01) (*see* Figure E2B).

Hypoxia-inducible Factor-1 α -induced Mitochondrial Fission Is Decreased by MFN2 Overexpression

Normoxic activation of hypoxia-inducible factor-1 α (HIF1 α) is a feature of PAH that contributes to the mitochondrial fragmentation and hyperproliferative phenotype of PAH PASMC (8, 26). We previously showed that HIF1 α activation, with cobalt chloride (500 μ mol/L), fragments the mitochondrial network in normal rat PASMC within 2 hours (9). Here we show that Ad-MFN2 reverses the mitochondrial fragmentation induced by either hypoxia or cobalt chloride (Figures 4C and 4D). The increase in fusion achieved by MFN2 augmentation resulted in a proportionally more rapid diffusion (dilution) of mito-PA-GFP (Figure 4D).

Figure 1. Mitofusin-2 (MFN2) levels are decreased in human pulmonary arterial hypertension (PAH). (A) Representative image of increased mitochondrial fragmentation observed in human pulmonary arterial smooth muscle cells (PASMC) from patients with PAH compared with PASMC from control patients. Photoactivated mitochondria (pseudocolored to white/red) highlights the mitochondrial network (blue) connectivity in control and PAH hPASMCs. (B) Quantitative reverse-transcriptase polymerase chain reaction revealed decreased expression of the profusion protein MFN2 in human PAH PASMCs and increased expression of the profission proteins dynamin-related protein 1 (DRP1) and FIS1 ($n = 5$ independent control cell lines and 5 independent PAH cell lines). (C) Immunoblotting confirmed down-regulation of MFN2 ($n = 4$ independent control cell lines and 4 independent PAH cell lines). No significant difference in MFN1 expression was observed. Expression was normalized to translocase of the outer mitochondrial membrane (TOM20). (D) Immunohistochemistry for MFN2 in human lungs showing decreased MFN2 in vascular media in PAH versus control subjects. Lower panel is a higher magnification subsegment of the upper panel highlighting the decreased intensity in the vascular media. Staining intensity was quantified throughout the vasculature in arbitrary units (AU). *** $P < 0.001$, * $P < 0.05$. FIS1 = fission 1; MIEF = mitochondrial elongation factor; OPA1 = optic atrophy 1.

Ad-MFN2 Results in Increased MFN2 and PGC1 α Expression in PASMC

Ad-MFN2 caused the expected increase in MFN2 in control rat PASMC; however, it also increased expression of other fusion genes including MFN1 and OPA1 (Figure 5A). Ad-MFN2 also dramatically increased PGC1 α mRNA and protein (Figures 5A and 5B). There was no significant effect of MFN2 gene transfer on the expression of fission genes.

Decreased PGC1 α Expression in Human and Experimental PASMC

Immunoblots showed that PGC1 α levels were significantly decreased in human PAH PASMC (Figure 5C), CH+SU rats (Figure 5D), and monocrotaline rats (Figure 5E). PGC1 α mRNA was decreased in human PAH PASMC (Figure 5F), CH+SU rats (Figure 5F), and monocrotaline rats (see Figure E3).

Bidirectional Relationship between MFN2 and PGC1 α

siPGC1 α decreased MFN2 levels (Figure 6A). Conversely, siMFN2 also decreased PGC1 α levels in normal rat PASMC (Figure 6A), identifying a bidirectional relationship between MFN2 and PGC1 α expression.

MFN2 Down-regulation Increases Proliferation in Normal PASMC

siMFN2 was confirmed to decrease both MFN2 mRNA (Figure 6A) and protein (Figure 6D). siMFN2 and siPGC1 α increased mitochondrial fragmentation in normal PASMC (Figures 6B and 6C). siMFN2 increased proliferation rate of PASMC (Figure 6F). PGC1 α has been shown to be decreased in skeletal muscle in the setting of hypoxia (27), thus PASMC were grown in hypoxia (5% O₂) for 24 hours, and this also decreased expression of PGC1 α and MFN2 mRNA (see Figure E4).

Figure 2. Mitofusin-2 (MFN2) is decreased in the chronic hypoxia plus Sugen-5416 (CH+SU) and monocrotaline rat. (A) Representative image of increased mitochondrial fragmentation observed in CH+SU pulmonary arterial smooth muscle cells (PASC) compared with PASC from control rats. Mitochondrial fragmentation count (MFC) quantified the significantly increased fragmentation in CH+SU PASC ($n = 5$ cell lines in each group). Mitochondria labeled in green with BacMam virus. (B) Immunoblotting confirmed MFN2 is decreased in CH+SU versus control cell lines ($n = 4$ each). Expression was normalized to smooth muscle actin for each sample. (C) Quantitative reverse-transcriptase polymerase chain reaction revealed decreased expression of MFN2 in CH+SU versus control PASCs ($n = 5$ cell lines each). (D) Representative images show increased expression of the activated form of DRP1 (characterized by phosphorylation at Ser616) in the vascular media in CH+SU versus control lungs. DRP1 p-Ser616 was indexed to medial area and converted to arbitrary units (AU). Total of 25 vessels studied in each group. Blue = DAPI, green = DRP1 p-Ser616. $**P < 0.01$, $*P < 0.05$. (E) Increased mitochondrial fragmentation count in monocrotaline rat PASC compared with control. Representative images with BacMam virus. (F) Immunoblotting confirmed MFN2 is decreased in monocrotaline versus control rats ($n = 4$ each). Expression was normalized to actin for each sample. DRP1 = dynamin-related protein 1; FIS1 = fission 1; MIEF = mitochondrial elongation factor; OPA1 = optic atrophy 1; SD = Sprague-Dawley rats; TOM20 = translocase of the outer mitochondrial membrane.

In Vivo Therapy

Nebulized plus intravenous Ad-MFN2 partially regressed PAH in CH+SU rats. Ad-MFN2 therapy improved functional capacity versus Ad-GFP, as determined by treadmill walking distance (control 405 ± 26 vs. Ad-GFP 246 ± 39 vs. Ad-MFN2 381 ± 36 m; $P < 0.05$) (Figure 7A). MFN2 therapy significantly increased PAAT (control 31.3 ± 0.4 vs. Ad-GFP 16.2 ± 0.5 vs. Ad-MFN2 20.9 ± 0.9 millisecond; $P < 0.05$) (Figure 7A) reflecting decreases in mean PAP (mPAP) (control 17.14 ± 0.71 vs. Ad-GFP 28.31 ± 3.66 vs. Ad-MFN2 24.96 ± 1.76 mm Hg; $P < 0.05$) (Figure 7B). Right ventricular function improved with Ad-MFN2, as reflected by increased tricuspid annulus parastolic excursion (control 2.11 ± 0.01 vs. Ad-GFP 1.17 ± 0.08 vs. Ad-MFN2 1.74 ± 0.14 mm; $P < 0.05$) (Figure 7A) and increased cardiac output (control 108.4 ± 5.9 vs. Ad-GFP $66.3 \pm$

9.8 vs. Ad-MFN2 110.3 ± 7.3 ml/min; $P < 0.05$) (Figure 7B). Pulmonary vascular resistance (PVR) was also markedly reduced by Ad-MFN2 (control 0.10 ± 0.01 vs. Ad-GFP 0.40 ± 0.11 vs. Ad-MFN2 0.18 ± 0.02 mm Hg/ml/min; $P < 0.05$) (Figure 7B). Ad-MFN2 significantly reduced medial thickness of small PAs (control 12.1 ± 0.6 vs. Ad-GFP 18.9 ± 1.2 vs. Ad-MFN2 $14.5 \pm 0.8\%$; $P < 0.05$) (Figure 7C). There was a decrease in proliferating cell nuclear antigen-positive cells in PASC from CH+SU rats treated with Ad-MFN2 (control 0.6 ± 0.6 vs. Ad-GFP 5.2 ± 1.3 vs. Ad-MFN2 $2.1 \pm 0.5\%$; $P < 0.05$) (Figure 7C). MFN2 therapy restored MFN2 and PGC1 α expression to normal levels (Figure 7D).

MFN2 therapy increased lung vascularity. We performed microcomputed tomography pulmonary angiography on barium-perfused lungs. This technique confirmed that CH+SU rats had

Figure 3. Mitofusin-2 (MFN2) overexpression reduces mitochondrial fragmentation and proliferation in pulmonary arterial smooth muscle cells (PASMC). (A) Increased mitochondrial fragmentation is observed in pulmonary arterial hypertension (PAH) PASMC compared with control in cells loaded with the mitochondrial targeted dye tetramethylrhodamine methyl ester. Mitochondria are labeled in red. (B) MFN2 overexpression reduces the mitochondrial fragmentation count. Total of 20–25 cells studied in each group. (C) MFN2 overexpression reduced proliferation in control PASMC and PAH PASMC (multiplicity of infection = 50). (D) TUNEL staining of PASMC after adenovirus carrying MFN2 (Ad-MFN2) showing increased apoptosis in control and PAH PASMC compared with Ad-empty. TUNEL-positive stains in bright green. Nuclei stained blue with DAPI. (E) Ad-MFN2 increased the amount of TUNEL-positive cells in control and PAH PASMC. *** $P < 0.001$, * $P < 0.05$.

a decreased percentage of small blood vessels in the lung compared with control rats. Ad-MFN2 therapy increased the percentage of small blood vessels in CH+SU rats (Figure 7E), consistent with restored perfusion of the lung's resistance pulmonary vasculature.

MFN2 therapy by nebulization only. To exclude potential systemic effects of the intravenous MFN2 therapy, groups of CH+SU and monocrotaline rats were treated with nebulization of Ad-MFN2 alone. Nebulization-only therapy did show some therapeutic improvement in monocrotaline PAH, significantly lowering mPAP and increasing treadmill walking distance (see Figure E5). Nebulization-only therapy was also somewhat beneficial in CH+SU rats (see Figure E6), significantly lowering mPAP and PVR while raising cardiac output. The combination therapy of nebulized plus IV Ad-MFN2 was more beneficial than nebulized Ad-MFN2 alone likely caused by the higher level of MFN2 overexpression achieved by a dual approach resulting in greater therapeutic benefit (see Figure E7). Also, the increased MFN2 overexpression was more persistent in the combination therapy than observed in nebulized treatment alone. MFN2 mRNA was increased 2 weeks post-nebulization

(see Figure E8) but normalized by 4 weeks (see Figure E5C). The apparent failure of MFN2 gene therapy to elevate MFN2 at 4 weeks was a function of time of the measurement. Adenoviral gene transfer is well known to cause only transient elevation of transgene levels (usually for 2–3 wk) (28). Levels then decline as the virus is cleared.

Combination of profusion and antifission therapy in CH+SU. Because we previously showed a therapeutic effect of antifission therapy with Mdivi1 in animal models of PAH, we wanted to test whether there are any additive or synergistic effects of combining profusion (MFN2) and antifission therapy (Mdivi1). Ad-MFN2 and Ad-MFN2+Mdivi1 significantly improved exercise performance (control 309 ± 32 vs. DMSO 219 ± 12 vs. Mdivi1 262 ± 70 vs. Ad-MFN2 381 ± 36 vs. Ad-MFN2+Mdivi1 310 ± 22 m; $P < 0.05$) (Figure 8A). Ad-MFN2+Mdivi1 resulted in the greatest improvement in PAAT (control 34.9 ± 0.8 vs. DMSO 17.5 ± 1 vs. Mdivi1 20.5 ± 0.8 vs. Ad-MFN2 20.9 ± 0.9 vs. Ad-MFN2+Mdivi1 23.8 ± 0.7 milliseconds; $P < 0.05$) (Figure 8B). Ad-MFN2+Mdivi1 almost normalized mPAP (control 14 ± 1 vs. DMSO 34 ± 3 vs. Mdivi1 33 ± 4 vs. Ad-MFN2 25 ± 2 vs.

Figure 4. Fragmentation of mitochondria by hypoxia-inducible factor-1 α is reduced by overexpression of mitofusin-2 (MFN2). (A) Increased mitochondrial fragmentation observed in pulmonary arterial smooth muscle cells (PASM) of chronic hypoxia plus Sugen-5416 (CH+SU) and monocrotaline rats is decreased with overexpression of MFN2. Mitochondria labeled with BacMam virus and colored in green. (B) Graphic quantification of mitochondrial fragmentation count in animal models of pulmonary hypertension treated with adenovirus carrying MFN2. (C) We cotransfected PASM with mitochondrial matrix-targeted DsRed and photo-activatable green fluorescent protein (GFP). PASM were then studied in normoxia, hypoxia (5% O₂), and after exposure to cobalt chloride (CoCl₂). Hypoxia and cobalt cause mitochondrial fragmentation. MFN2 at multiplicity of infection 50 restored the mitochondrial network. Mitochondria network labeled in red with photo-activated GFP shown in green. (D) The rate of decline in the mito-PA-GFP signal (measured in relative fluorescence intensity units) is directly proportional to the extent of mitochondrial fusion. PASM from control rats with MFN2 overexpression had the fastest decrease in relative intensity of mito-PA-GFP, followed by control rats PASM. Control rat PASM exposed to either CoCl₂ or hypoxia had the highest relative intensity of mito-PA-GFP consistent with the fragmented nature of these mitochondria. SDR = Sprague-Dawley rats. * $P < 0.05$. MCT = monocrotaline.

Ad-MFN2+Mdivi1 19 ± 1 mm Hg; $P < 0.05$) (Figure 8C). Cardiac output improved in all treatment arms (control 98 ± 5 vs. DMSO 56 ± 7 vs. Mdivi1 77 ± 5 vs. Ad-MFN2 110 ± 7 vs. Ad-MFN2+Mdivi1 79 ± 3 ml/min; $P < 0.05$) (Figure 8D). There is a trend to a reduction in PVR between untreated CH+SU rats compared with combination therapy in CH+SU rats (control 0.08 ± 0.01 vs. DMSO 0.39 ± 0.15 vs. Mdivi1 0.42 ± 0.08 vs. Ad-MFN2 0.18 ± 0.02 vs. Ad-MFN2+Mdivi1 0.25 ± 0.01 mm Hg/ml/min; $P = 0.08$) (Figure 8E). Right ventricular hypertrophy was best attenuated by combination therapy (control 0.7 ± 0.1 vs. DMSO 1.5 ± 0.1 vs. Mdivi1 1.6 ± 0.2 vs. Ad-MFN2 0.9 ± 0.1 vs. Ad-MFN2+Mdivi1 0.5 ± 0.1 mm; $P < 0.05$) (Figure 8F). Combination therapy also caused the greatest reduction in %PA medial thickness (control 13 ± 0.5 vs. DMSO 27 ± 2 vs. Mdivi1 21 ± 1 vs. Ad-MFN2 15 ± 1 vs. Ad-MFN2+Mdivi1 13 ± 0.6 ; $P < 0.05$) (Figures 8G and 8H). There was increased prevalence of

vessels with TUNEL-positive cells in the vascular wall in CH+SU rats treated with Mdivi, Ad-MFN2, or combination therapy compared with untreated CH+SU rats (see Figure E9).

DISCUSSION

PAH is increasingly recognized as a syndrome that has similarities to cancer (29, 30), being marked by a hyperproliferative diathesis and apoptosis-resistance in lung vascular cells (29–33). There are many factors that contribute to the proliferative, apoptosis-resistant phenotype of PAH. These include mutation of the bone morphogenetic protein receptor 2 (34); metabolic abnormalities, notably a shift to aerobic glycolysis (21, 35); mitochondrial abnormalities, such as epigenetic down-regulation of superoxide dismutase 2 (7); *de novo* expression of the antiapoptotic protein survivin (36); increased expression and activity of

Figure 5. Mitofusin-2 (MFN2) overexpression in pulmonary arterial smooth muscle cells (PASC) increases profusion proteins. (A) Adenovirus carrying MFN2 (Ad-MFN2) infection of rat PASC in culture increased MFN2 and other fusion genes including MFN1 and optic atrophy 1 (OPA1). Ad-MFN2 also increased and peroxisome proliferator-activated receptor γ coactivator-1 α (PGC1 α). Note logarithmic scale. (B) Representative immunoblot and graph confirming Ad-MFN2 increased MFN2 and PGC1 α in human control PASC (n = 4 cell lines each). (C) Immunoblot showing decreased PGC1 α in PASC from patients with pulmonary arterial hypertension (PAH) compared with control (n = 4 cell lines each). (D) Immunoblot showing decreased PGC1 α in PASC from chronic hypoxia plus Sugen-5416 (CH+SU) rats compared with control (n = 4 cell lines each). (E) Immunoblot showing decreased PGC1 α in PASC from monocrotaline rats compared with control (n = 4 each). (F) Decreased mRNA expression of PGC1 α in PASC from patients with PAH and CH+SU rats compared with their respective controls (n = 5 cell lines in each group). *** $P < 0.001$, * $P < 0.05$. DRP1 = dynamin-related protein 1; FIS1 = fission 1; TFAM = transcription factor A, mitochondrial.

the serotonin transporter SERT (37); the platelet-derived growth factor receptor (38); tyrosine kinase activation (39); and changes in expression of microRNAs (40, 41). In addition, activation of transcription factors, including nuclear factor acting T lymphocytes (42) and HIF1 α , contributes to the PAH proliferative phenotype (8, 43).

We recently reported that mitochondrial division (fission) is a mitotic checkpoint that impacts the ability of cells to proliferate rapidly. Inhibiting mitotic fission using Mdivi1 fuses mitochondria and traps cells in the G2-M phase of the cell cycle, slowing proliferation and promoting apoptosis (9). In the current study, we examine the complementary means of arriving at a state of mitochondrial fragmentation: decreased fusion. We identify MFN2 down-regulation as a proliferative pathway and target this abnormality therapeutically. MFN2 deficiency contributes to hyperproliferation of PAH PASC in human PAH and in two well-established rodent PAH models. The basis for the MFN2 down-regulation relates to down-regulation of PGC1 α ,

a transcriptional coactivator of MFN2. Finally, we show that augmenting MFN2 in PAH inhibits PASC proliferation, increases PASC apoptosis, restores mitochondrial fusion, and significantly regresses PAH in CH+SU rats. Combining MFN2 therapy with recently identified antifission therapy (Mdivi1 [9]) has some additive effects, indicating that both impaired fusion and enhanced fission contribute to the pathogenesis of PAH.

Decreasing MFN2 in normal PASC recapitulates the PAH phenotype (fragments the mitochondrial network and enhances PASC proliferation) (Figures 6C–6E). This finding suggests that MFN2 acts as a tonic brake on proliferation. It also suggests that other fusion proteins, such as MFN1 and OPA1, cannot compensate for the loss of MFN2 (at least in the short term). The demonstration that MFN2 gene therapy restores mitochondrial networking in human and experimental PAH (Figures 3 and 4) indicates that the doses of MFN2 used were sufficient to correct not only the proliferative diathesis but also the mitochondrial fragmentation.

Figure 6. Peroxisome proliferator-activated receptor γ coactivator-1 α (PGC1 α) plays a regulatory role in determining mitofusin-2 (MFN2) expression and mitochondrial fragmentation in human pulmonary arterial smooth muscle cells (PASMC). (A) siMFN2 decreased in MFN2 and PGC1 α expression in control human PASMC. Conversely, administration of siPGC1 α decreases PGC1 α and MFN2 expression. (B) siPGC1 α increased mitochondrial fragmentation in human control PASMC. (C) siMFN2 increased mitochondrial fragmentation in human control PASMC. (D) Immunoblot showing decreased MFN2 expression in setting of siMFN2. (E) Representative images of mitochondrial fragmentation in the setting of siPGC1 α and siMFN2. (F) siMFN2 caused a significant increase in proliferation in human control PASMC, indicating that MFN2 acts as a brake on PASMC proliferation. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. GAPDH = glyceraldehyde phosphate dehydrogenase.

There are heritable diseases caused by mutations on MFN2, such as the motor neuron disease Charcot Marie Tooth disease 2A (44). However, decreased MFN2 in PAH seems to be an acquired defect because it occurs in two discrete PAH models created in normal female rats (Figures 2B and 2F).

Investigation of the mechanism by which MFN2 is down-regulated led to study of PGC1 α , a transcriptional coactivator of MFN2 transcription (45). Consistent with its putative role in MFN2 deficiency, PGC1 α expression in the PASMC was down-regulated in human PAH and in both experimental PAH models (Figure 5). Moreover, knockout of PGC1 α in a normal PASMC causes mitochondrial fragmentation (Figures 6B–6D). This effect likely reflects the depression of MFN2 levels that follows knockdown of PGC1 α . Although PGC1 α is known to regulate MFN2 expression, the current study offers a new finding, namely the bidirectional, positive-feedback, nature of the relationship between MFN2 and PGC1 α . Consistent with this feedback mechanism, augmenting MFN2 increases PGC1 α (in

PAH PASMC and *in vivo*) (Figure 7D). Conversely, inhibiting MFN2 decreases PGC1 α (Figures 6A and 6B). In the future we plan to assess whether directly augmenting PGC1 α has benefit in PAH. This would provide further insight on the relationship between expression of MFN2 and PGC1 α . Augmenting PGC1 α directly would also elucidate the role played by upstream regulators of PGC1 α , such as cAMP and cGMP, and might clarify the role of the glitazone receptor, peroxisome proliferator-activated receptor γ , PGC1 α 's downstream target.

Although PAH is predominantly a disease of women (with a ratio of four to one in the REVEAL registry [1]) most investigators have performed studies in male models of PAH. Although our study was not aimed at comparing sex differences, it is noteworthy that we use human samples that have a female/male ratio of four to one and that we exclusively used female rodents. Female sex may be relevant to the proposed PGC1 α -MFN2 pathway, because PGC1 α activates the MFN2 promoter by coactivating the nuclear receptor estrogen-related receptor

A) Treadmill and Echocardiography

B) Cardiac catheterization

C) Histology

D) Lung tissue

E) CT Angiography

Figure 7. Therapeutic benefit of mitofusin-2 (MFN2) in the chronic hypoxia plus Sugen-5416 (CH+SU) model. (A) CH+SU led to a significant decrease in treadmill exercise time compared with control subjects. Exercise capacity was improved in rats treated with adenovirus carrying MFN2 (Ad-MFN2). Pulmonary artery acceleration time (PAAT) and tricuspid annular plane systolic excursion (TAPSE), which were decreased in CH+SU rats, were improved by Ad-MFN2, reflective of an improvement of pulmonary artery pressures and right ventricular function, respectively. (B) Ad-MFN2 significantly increased cardiac output (CO) and reduced pulmonary vascular resistance (PVR) in CH+SU rats (PVR = mean pulmonary artery pressure [mPAP] – left ventricular end diastolic pressure divided by the cardiac output). (C) Ad-MFN2 significantly decreased PA muscularization in CH+SU and had an antiproliferative effect on the PA vasculature, as evidenced by the decreased percentage of proliferating cell nuclear antigen (PCNA) positive smooth muscle cells (SMC) in Ad-MFN2-treated animals. (D) MFN2 and peroxisome proliferator-activated receptor γ coactivator-1 α (PGC1 α) mRNA expression were reduced in CH+SU lungs and their expression was normalized in the Ad-MFN2-treated group. (E) Representative computed tomography (CT) angiogram. Note that compared with control, CH+SU rats have decreased percentage of small vessels ($< 0.1 \mu\text{m}$) and treatment with Ad-MFN2 significantly increased the percentage of small vessels. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. GFP = green fluorescent protein.

(ERR) α (46, 47). The ERR α is closely related to the estrogen receptor and modulates estrogen receptor signaling pathways. Moreover, ERR α is implicated in various types of female hyperproliferative diseases, including cancers of the breast and cervix (48, 49). Indeed in the right ventricle of rats with experimental PAH, PGC1 α and ERR α are both down-regulated (17). Changes in the PGC1 α -ERR α pathway do not alter MFN1, perhaps explaining why MFN2 but not MFN1 levels are reduced in PAH (46).

There is debate as to whether the antiproliferative effects of MFN2 are mediated by restoration of fusion or through nonfusogenic properties of MFN2 (50). Chen and coworkers (14) found that the antiproliferative effects of MFN2 in systemic arteries persisted when mutated forms of MFN2 that did not localize to the mitochondria were used. They suggested that

MFN2 was acting by inhibiting ERK/MAPK signaling and induction of cell-cycle arrest. This elegant study, however, was limited in that they did not measure fusion in cells exposed to the mutant MFN2. We find that overexpression of MFN2 does up-regulate expression of other fusion mediators, notably MFN1 and OPA1 in cells (Figure 5) and *in vivo* (see Figure E10). We document that the MFN2 therapy enhances fusion in human and experimental PAH, as documented using mitochondrial targeted probes (Figures 3 and 4). We also show that MFN2 overexpression decreases human PASMC proliferation *in vitro* and inhibits proliferation in the vascular smooth muscle layer in CH+SU *in vivo* (Figures 3C and 7C). It is unlikely that restoration of fusion and inhibition of proliferation in response to MFN2 therapy are unrelated, because achieving network fusion by inhibition of DRP1 yields concordant effects (9, 23).

Figure 8. Combination of fusion (adenovirus carrying mitofusin-2 [Ad-MFN2]) and antifission (mitochondrial division inhibitor-1 [Mdivi1]) therapy has additive effect in regressing established pulmonary hypertension in chronic hypoxia plus Sugen-5416 (CH+SU) rats. (A) Mdivi1 alone failed to improve treadmill distance in CH+SU animals, which was most markedly improved in Ad-MFN2-treated group (note this group has the highest cardiac output [CO]). *Treadmill distance of CH+SU + dimethyl sulfate (DMSO) was significantly less than control rats ($P < 0.05$). †Treadmill distance of CH+SU+Mdivi1 and treadmill distance of CH+SU+ Mdivi1+Ad-MFN2 was significantly longer than CH+SU+DMSO ($P < 0.05$). (B) Prolongation of pulmonary artery acceleration time (PAAT) (indicating a decreased mean pulmonary artery pressure [mPAP]) was achieved with Mdivi1 monotherapy and with the combination therapy of Mdivi1 plus Ad-MFN2. ****PAAT was significantly longer in control rats compared with all other groups ($P < 0.0001$). †PAAT is significantly longer in CH+SU+Mdivi1+Ad-MFN2 compared with CH+SU+DMSO ($P < 0.05$). (C) mPAP was significantly decreased by Ad-MFN2+Mdivi1, indicating the benefits of inhibiting fission and promoting fusion. ****mPAP was significantly lower in control rats compared with all other groups ($P < 0.0001$). †mPAP was significantly lower in CH+SU+Ad-MFN2 compared with CH+SU+DMSO ($P < 0.05$). §mPAP was significantly lower in CH+SU+Mdivi1+Ad-MFN2 compared with CH+SU+DMSO ($P < 0.01$). (D) The highest CO was observed in Ad-MFN2 treated group. ****CO was significantly reduced in CH+SU + DMSO rats compared with control ($P < 0.0001$). *CO was significantly lower in CH+SU+DMSO compared with all other treatment groups ($P < 0.05$). †CO is higher in CH+SU+Ad-MFN2 compared with the other treatment groups ($P < 0.01$). (E) Pulmonary vascular resistance (PVR) was significantly decreased in Ad-MFN2-treated CH+SU rats compared with untreated CH+SU rats or CH+SU rats treated with Mdivi1 alone (* $P < 0.05$). There was a trend to significance in difference in PVR between untreated CH+SU rats and Mdivi1+Ad-MFN2 treated CH+SU rats ($P = 0.08$). (F) Right ventricular free wall (RVFW) thickness was improved most successfully with combined Ad-MFN2+Mdivi1 with no observed benefit observed with Mdivi1 alone. **RVFW was thinner in control rats compared with CH+SU+DMSO and CH+SU+Mdivi1 ($P < 0.01$). †RVFW was significantly reduced in CH+SU+Ad-MFN2 compared with CH+SU+Mdivi1 ($P < 0.01$). §RVFW in CH+SU+Mdivi1+Ad-MFN2 was significantly reduced compared with CH+SU+Ad-MFN2 ($P < 0.01$). (G) Reduction in % medial thickness of small PAs (Ad-MFN2+Mdivi1 > Ad-MFN2 > Mdivi1 > DMSO). ****% medial thickness was less in control rats compared with CH+SU+DMSO and CH+SU+Mdivi1 ($P < 0.001$). †% medial thickness was less in CH+SU+Ad-MFN2 and CH+SU+Mdivi1+Ad-MFN2 compared with CH+SU+Mdivi1 ($P < 0.01$). (H) Representative images of smooth muscle actin staining showed decreased medial thickness in CH+SU animals treated with Mdivi1, Ad-MFN2, or Mdivi1 plus Ad-MFN2. Blue = DAPI; red = smooth muscle actin; green = proliferating cell nuclear antigen. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

Thus, we interpret our findings as indicating that forced fusion, achieved by overexpression of MFN2, inhibits proliferation by preventing mitochondrial division.

Impaired fusion was also observed in normal PASMC exposed to hypoxia (Figure 4C). Hypoxia (5% O_2) reduced MFN2 and PGC1 α expression, similar to the findings in PAH PASMC (see

Figure E4). In PAH PASMC there is a paradoxical normoxic activation of HIF1 α (7, 8). HIF1 α activation has previously been shown to cause mitochondrial fragmentation (9). In a prior report we showed that the HIF1 α activator, cobalt, activates DRP1 causing fragmentation that could be reduced by Mdivi1. The current study extends this observation, showing that mitochondrial fragmentation

caused by HIF1 α can be reversed by Ad-MFN2 (Figure 4). Thus, the normoxic activation of HIF1 α , a recognized feature of PAH (8), is likely an upstream stimulus for impaired mitochondrial fusion (and enhanced fission) in PAH. Hypoxia has also been observed to decrease PGC1 α in skeletal muscle (27). We speculate that HIF1 α may contribute to PAH in part by decreasing PGC1 α , which in turn suppresses transcription of MFN2. A recent publication proposed that MFN2 has a diametrically opposed role, driving proliferation in hypoxic pulmonary hypertension (51). However, a proliferative effect of MFN2 would be out of line with an extensive literature (14, 50, 52), and contradicts the findings in the current study, where we did not observe a proliferative effect of MFN2 (even in response to hypoxia).

The therapeutic benefit of augmenting MFN2 is clear in PAH. In each of the PAH models augmenting MFN2, whether performed with intravenous plus nebulized therapy or solely by nebulization (providing selective up-regulation of the transgene in the lung [53]), ameliorated PAH. The benefits of augmenting MFN2 include a reduction in hemodynamic severity of PAH, improved exercise capacity, and prevention of vascular pruning in the lung (Figures 7 and 8). Consistent with our prior work, inhibiting fission was beneficial in PAH and combining Mdivi1 with Ad-MFN2 almost normalized the pressure and %PA medial thickness in CH+SU PAH (Figure 8). However, combination therapy was not superior to monotherapy in reducing PVR. We cannot exclude the possibility that systemic therapy with Mdivi1 has an effect on the right ventricle. Similarly, MFN2 may play a role in right ventricular dysfunction. The role of mitochondrial fission and fusion proteins in the right ventricle will be evaluated in further work. DRP1 antagonism alone did not have a therapeutic effect on CH+SU, possibly because this is a more aggressive model of PAH.

Limitations

We did not explore the mechanism by which PGC1 α is initially down-regulated, although it does decrease with hypoxia, suggesting a potential role for HIF1 α . PGC1 α expression is also regulated by a variety of stimuli that interact with binding sites in its promoter, including the transcription factors forkhead box class-O, activating transcription factor 2, myocyte enhancer factor 2, and cAMP response element-binding protein. Further work is required to investigate this upstream pathway.

In addition, it remains uncertain whether MFN2 deficiency has effects on lung metabolism. It would not be surprising that loss of MFN2 reduced oxidative metabolism, because loss of function of MFN2 in Charcot Marie Tooth disease 2A decreases the oxidation rates of glucose, palmitate, and pyruvate in myotubes (54), and acute decreases in MFN2 levels reduce oxygen consumption and glucose oxidation in skeletal muscle cells (55).

Further study is required to more precisely define the role played by ERK/MAPK signaling in mitochondrial fusion versus a fusion-independent antiproliferative effect of MFN2, as suggested by Chen and coworkers (14).

We did not study the effects of overexpressing MFN2 on proliferation of CH+SU PASM in cell culture but did show that MFN2 has antiproliferative and proapoptotic effects on vascular smooth muscle cells *in vivo* (Figure 7C; see Figure E9).

The findings observed regarding the role of MFN2 deficiency in monocrotaline rats were not as conclusive as those observed in CH+SU rats, which may reflect in part the differences between the models. However, like human PAH and CH+SU, monocrotaline PAH PASM have deficient MFN2, mitochondrial fragmentation, and derive benefit from MFN2 gene transfer. Also, more effective means of augmenting MFN2 expression

are required as the adenoviral vector results in time-limited transgene expression (see Figure E8).

Conclusions

Down-regulation of MFN2 in the PAH PASM contributes to the mitochondrial fragmentation and excessive PASM proliferation observed in PAH and in female rodent models of PAH. MFN2 therapy, alone or in combination with inhibition of mitochondrial fission, regresses PAH in rodent models of PAH.

Author disclosures are available with the text of this article at www.atsjournals.org.

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