Fenfluramine-induced gene dysregulation in human pulmonary artery smooth muscle and endothelial cells

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ABSTRACT

Fenfluramine is prescribed either alone or in combination with phentermine as part of Fen-Phen, an anti-obesity medication. Fenfluramine was withdrawn from the US market in 1997 due to reports of heart valvular disease, pulmonary arterial hypertension, and cardiac fibrosis. Particularly, idiopathic pulmonary arterial hypertension (IPAH), previously referred to as primary pulmonary hypertension (PPH), was found to be associated with the use of Fen-Phen, fenfluramine, and fenfluramine derivatives. The underlying mechanism of fenfluramine-associated pulmonary hypertension is still largely unknown. We reasoned that investigating drug-induced gene dysregulation would enhance our understanding of the fenfluramine-associated pathogenic mechanism of IPAH. Whole-genome gene expression profiles in fenfluramine-treated human pulmonary artery smooth muscle (PASMC) and endothelial (PAEC) cells (isolated from normal subjects) were compared with baseline expression in untreated cells. Fenfluramine treatment caused dysregulation in a substantial number of genes involved in a variety of pathways and biological processes. In addition to several common pathways and biological processes such as "MAPK signaling pathway," "inflammation response," and "calcium signaling pathway" shared between both cell types, pathways and biological processes such as "blood circulation," "muscle system process," and "immune response" were enriched among the dysregulated genes in PASMC. Pathways and biological processes such as those related to cell cycle, however, were enriched among the dysregulated genes in PAEC, indicating that fenfluramine could affect unique pathways (or differentially) in different types of pulmonary artery cells. While awaiting validation in a larger cohort, these results strongly suggested that fenfluramine could induce significant dysregulation of genes in multiple biological processes and pathways critical for normal pulmonary vascular functions and structure. The transcriptional and posttranscriptional changes in these genes may, therefore, contribute to the pathogenesis of fenfluramine-associated IPAH.

Key Words: anorexigen, gene expression profile, lysosome, mitochondria, pulmonary hypertension

INTRODUCTION

Fenfluramine (Fen, 3-trifluoromethyl-*N*-ethylamphetamine), a drug in the class of anorectics (appetite suppressants), has been prescribed either alone or in combination with phentermine (Phen) as part of Fen-Phen, an anti-obesity medication. The drug was withdrawn from the U.S. market in 1997 due to reports of valvular heart disease and pulmonary hypertension, including a condition known as cardiac fibrosis. Though the magnitude and prevalence of their deleterious cardiopulmonary effects remain undetermined,

the links between these anorectics and valvular heart disease and pulmonary hypertension are clearly established.[1] For example, idiopathic pulmonary arterial hypertension (IPAH), previously referred to as primary pulmonary hypertension (PPH), was found to be associated with the use of Fen-Phen, fenfluramine, and fenfluramine derivatives.[2-6] Fatal cases of pulmonary hypertension and valvular heart disease

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have been reported to be associated with even short-term fenfluramine usage.[2,7] Furthermore, based on a study of more than 5,700 former fenfluramine users, damage to the heart valve (e.g., regurgitant valvulopathy) continued long after discontinuing use of the medication.[8] Previous studies suggested that nitric oxide deficiency could predispose affected individuals to develop anorexigen-associated pulmonary hypertension.[9] In addition, Fen and its active metabolite norfenfluramine were found to, indirectly, activate mitogenic serotonin 2B (5-HT$_{2B}$) receptors,[10,11] thus potentially leading to the valvular abnormalities (e.g., abnormal valve cell division) found in patients taking fenfluramine.[12] Particularly, in cells expressing recombinant 5-HT$_{2B}$ receptors, norfenfluramine potently stimulates the hydrolysis of inositol phosphates, increases intracellular Ca^{2+}, and activates the mitogen-activated protein kinase (MAPK) cascade, the latter of which has been linked to mitogenic actions of the 5-HT$_{2B}$ receptors.[13]

The underlying cellular and molecular mechanism of fenfluramine-associated pulmonary hypertension is still largely unknown. Quantitative gene expression is an important intermediate phenotype that situates in the middle of DNA sequence variation, environmental influences (e.g., exposure to drugs) and other cellular/whole-body phenotypes/traits[14-17] including the susceptibility to complex diseases (e.g., IPAH). For example, a previous genomic study of the expression profiles in peripheral blood mononuclear cells from patients with pulmonary arterial hypertension and normal individuals demonstrated a significant number of dysregulated genes between the patient cohort and normal individuals, as well as between patients with IPAH and secondary pulmonary hypertension.[18] Distinct gene signatures derived from lung tissues in IPAH and secondary pulmonary hypertension patients were also identified based on genome-wide expression profiling.[19] Therefore, we reasoned that a comprehensive examination of fenfluramine-induced gene dysregulation (upregulation or downregulation of mRNAs after treatment with fenfluramine) in relevant normal lung tissues, i.e., pulmonary artery smooth muscle cells (PASMC) and pulmonary artery endothelial cells (PAEC), could potentially help shed light on the molecular pathogenesis of fenfluramine-associated pulmonary hypertension.

In order to examine the fenfluramine-associated gene dysregulation, we profiled transcriptional (mRNA) expression using a whole-genome cDNA array (covering 41,000 unique human transcripts with public domain annotations) in human PASMC and PAEC samples derived from normal individuals. We compared gene expression profiles in the PASMC and PAEC samples after treatment with fenfluramine and their baseline expression profiles. Known pathways from the Kyoto Encyclopedia of Genes and Genomes (KEGG),[20,21] Gene Ontology (GO)[22] categories (i.e., biological processes, molecular function, cellular components), and gene networks were further evaluated among the dysregulated genes between cells treated and untreated with fenfluramine. Our findings suggest that fenfluramine may contribute to the pathogenesis of IPAH through causing differential expression of genes in certain pathways and biological processes that are critical to the normal functions of pulmonary arteries.

MATERIALS AND METHODS

Cell culture, drug treatment and cell morphological experiments

Normal human PASMC and PAEC (3 samples for each cell type) were purchased from Lonza (Walkersville, Md.) and maintained in cell growth medium supplemented with 10% fetal bovine serum (FBS) and growth factors. Two days before the treatment, 5×10^5 cells were seeded in a 10-cm plate. The cell growth medium was replaced by the medium with 200 µM fenfluramine based on a previous publication,[23] and culturing was continued for 72 hrs. For cell morphological experiments, the cells were stained with the membrane-permeable nucleic acid stain, 4', 6'-diamidino-2-phenylindole (DAPI, 5 µM). The blue fluorescence emitted at 461 nm was used to visualize the cell nuclei. The smooth muscle α-actin antibody was used to evaluate expression of α-actin in DAPI-stained cells.

Mitochondrial and lysosome imaging

Normal human PASMC and PAEC samples were cultured in medium supplemented with 10% FBS and growth factors. The cover slips with cells were mounted on glass slides and phase contrast images of cells were taken using an Olympus microscopy system. For mitochondrial staining, the media in 6-well plate were removed and pre-warmed medium containing MitoTracker Green FM (100 nM, Invitrogen, Carlsbad, Calif.) was added. The cells were incubated in the incubator for 15 min. The staining solution was removed and cells were rinsed with pre-warmed medium, and cells were visualized with a 100× objective on a fluorescent microscope (Nikon, Japan) coupled to the Solamere Imaging System. For lysosome staining, the media in 6-well plate were removed and a staining solution containing LysoTracker Red (50 nM, Invitrogen, Carlsbad, Calif.) was used. The cells were incubated in the incubator for 30 min. before the staining solution was removed. The cells were then rinsed with pre-warmed medium and observed with a 20× objective on an Olympus microscope. Fluorescent images and phase contrast images, which were taken in the same field, were overlaid using the software included in the Olympus fluorescent imaging system.

RNA isolation and microarray hybridization

Total RNAs from treated and untreated PASMC and PAEC samples were isolated using standard molecular biology protocols. High-quality RNA samples with no signs of DNA contamination and RNA degradation were hybridized on the Agilent Whole Human Genome 4×44K Gene Expression Two-color arrays (Agilent Technologies, Santa Clara, Calif.), which contain 41,000 unique human transcripts (targeting 19,596 Entrez Gene RNAs) supported by public sources including RefSeq,[24] Golden Path Ensembl UniGene Human Genome (Build 33) and GenBank (http://www.ncbi.nlm.nih.gov/genbank/) databases, according to the manufacturer's recommended protocol at the UCSD Microarray Core Facility. Two PAEC samples did not pass the quality control of array hybridization. Therefore, in total, 4 samples (3 PASMC and 1 PAEC samples) were included in further analyses.

Microarray data preprocessing

The raw expression data were normalized and summarized with the robust multi-array average (RMA)[25] algorithm using GeneSpring GX v10 (Agilent Technologies, Santa Clara, Calif.). Since the ratio of the 2-color channels is most informative when the intensities are well over background for both the cy3 and cy5 channels, we removed those probesets if both the cy3 and cy5 channels had intensities in the lower quartile across all of the 4 samples. However, a gene could be interesting from a biological point of view, even if it has a meaningless ratio (e.g., a gene expressed in only 1 channel). Therefore, we included those genes with intensities above the cutoff (i.e., lower quartile) in either cy3 or cy5 channel. Only transcripts with unique, unambiguous gene annotations according to the manufacturer's information (retrieved from the Agilent eArray website at http://earray.chem.agilent.com/earray/) were analyzed. In total, ~18,000 gene-level transcripts were included in the final analysis set.

Identification of dysregulated genes

Differentially expressed genes between fenfluramine-treated samples and untreated controls were identified based on a series of fold-changes (e.g., 1.2, 1.5 and 2.0) in the 3 PASMC samples. Due to the exploratory nature of this study and the small sample size, our choices of statistical approaches were limited, so any genes meeting a cutoff (e.g., fold-change>1.5) in at least 2 PASMC samples were considered differentially expressed. For the 1 PAEC sample, a single relatively stringent cutoff (i.e., fold-change>2) was used to control false positives. The expression patterns (i.e., upregulation or downregulation of genes) were also compared between the two cell types.

Western blot validation of microarray data

Three dysregulated genes that met fold-change>1.5: *MMP1* (encoding metallopepetidase 1) for PAEC, *CYCS* (encoding cytochrome C, somatic) and *VIM* (encoding vimentin) for PASMC were selected for Western blot validation. Cells were washed with ice-cold PBS, suspended in lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 100 μg/ml phenylmethylsulfonyl fluoride, phosphatase inhibitors, and protease inhibitors), and incubated for 30 min. on ice. The cell lysates were then sonicated and centrifuged at 12,000 rpm for 10 min., and the supernatant was collected. Protein concentrations were determined by DC™ Protein Assay (Bio-Rad Laboratories, Hercules, Calif.) using BSA as a standard. Samples were applied on SDS-PAGE (4–20%), and proteins were transferred onto nitrocellulose membranes by electroblot. Membranes were blocked in 5% nonfat milk and incubated overnight at 4°C with primary antibodies and then with secondary antibodies. Blots were developed with the SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, Ill.).

Gene ontology and pathway analyses

We further searched the KEGG[20,21] and GO[22] databases for any enriched physiological pathways or biological processes among the dysregulated genes relative to the final analysis set using the Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/).[26,27] For pathway and GO analyses, genes dysregulated in at least one PASMC or PAEC were included. Significantly enriched pathways or biological processes were determined based on an adjusted P-value after the Benjamini-Horchberg (BH) procedure[28] (e.g., adjusted P-value<0.05) and the size of the gene sets (e.g., a minimum size of 5 or 10 hits). To obtain robust evaluation of enrichment patterns, we focused on those enriched pathways or biological processes across different cutoffs of differential expression.

Gene network analysis

We used the the Cytoscape (http://www.cytoscape.org/)[29] plug-in from the Reactome (http://www.reactome.org/)[30,31] to find gene networks among the fenfluramine-induced dysregulated genes. This plug-in accesses the Reactome Functional Interaction (FI) network, a highly reliable, manually curated pathway-based protein functional interaction (e.g., activation, inhibition) network covering close to 50% of human proteins.[30,31] We also evaluated the relative importance of the interacting genes based on betweenness centrality, a measure of a node's (i.e., a gene's) centrality in a network.

RESULTS

Cell and mitochondrial morphologies after the treatment of fenfluramine

Cell morphologies of untreated controls and fenfluramine-treated PASMC and PAEC were evaluated. In untreated PASMC and PAEC, the phase contrast images showed that the cells appeared to be flat and have a smooth surface in the cytosplasm, and no intracellular organelle structure could be seen in untreated cells (Fig. 1 left panels). Treatment of the cells with 200 μM of fenfluramine for 72 hrs. seemed to cause significant morphological changes. In fenfluramine-treated cells, the surface membrane of the cytoplasm became rough, while perinuclear organelles became strikingly swollen (Fig. 1, right panels). In addition, we observed significant changes in the mitochondrial morphology in PASMC treated with fenfluramine (Fig. 2). In untreated cells, photomicrographs showed typical images of mitochondria; the mitochondrial structure looked intact and distributed throughout the entire cytoplasm (Fig. 2 left panels). After treatment with fenfluramine, however, the mitochondrial structure was severely damaged; the volume and the tubular frequency were significantly dec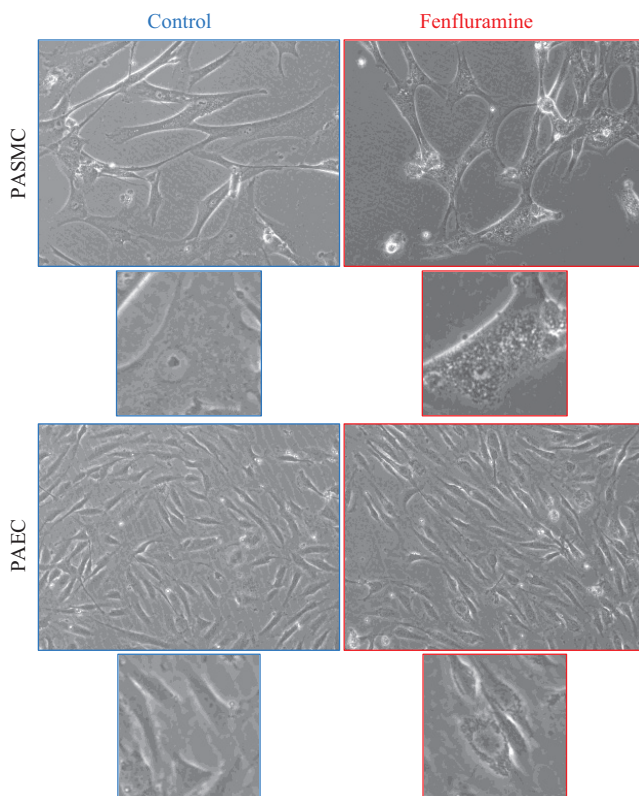reased (Fig. 2 right panels). These data imply that fenfluramine treatment may induce mitochondrial fragmentation in PASMC.

Furthermore, we observed the changes in lysosomes in PAEC treated with fenfluramine. We stained the untreated and fenfluramine-treated PAEC with LysoTracker Red to label the lysosomes. In untreated cells, the fluorescence intensity from lysosomes was relatively low; however, in fenfluramine-treated cells, the fluorescence intensity of lysosomes was significantly increased (Fig. 3). By overlaying the phase contrast images with the LysoTracker Red-stained images, it seemed that the swollen perinuclear organelles in fenfluramine-treated PAEC were lysosomes.

Genes dysregulated in fenfluramine-treated pulmonary artery cells

In total, 17,877 gene-level transcripts met our criteria

Figure 1: Morphological changes in human PASMC and PAEC treated with fenfluramine. Phase contrast images of untreated PASMC (upper left panels) and PAEC (lower left panels), and fenfluramine-treated PASMC (upper right panels) and PAEC (lower right panels). The small images depict an enlarged area of a single cell from each of the multi-cell images for PASMC and PAEC.
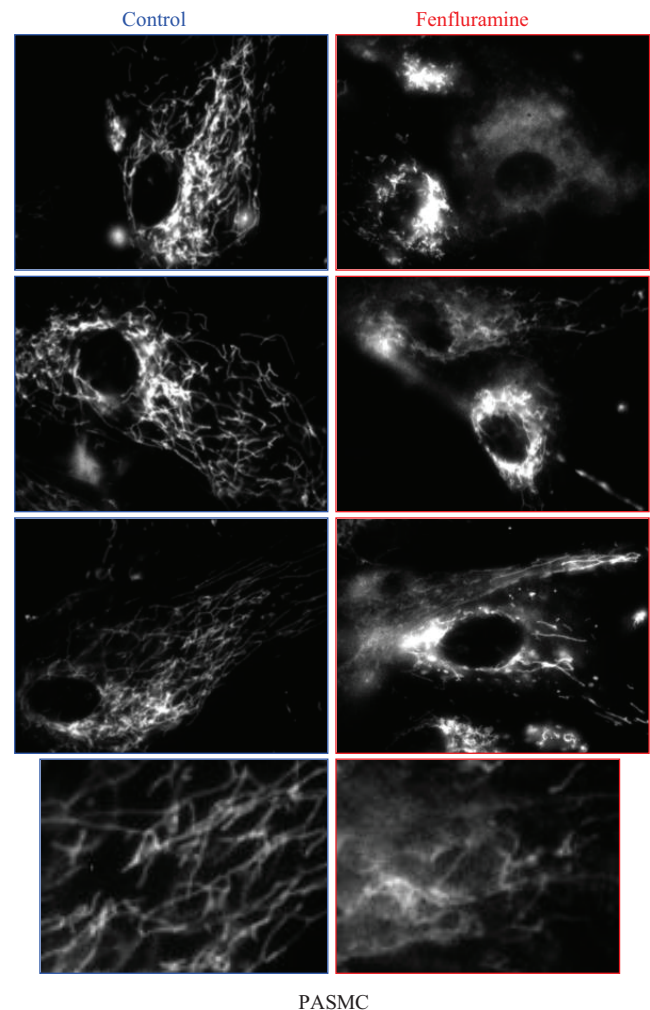

Figure 2: Mitochondrial morphological changes in human PASMC treated with fenfluramine. Photomicrographs show images of mitochondria in untreated PASMC (left panels) and PASMC treated with 200 μM of fenfluramine for 72 hrs. The enlarged images at the bottom show the perinuclear area of an untreated PASMC (left) and a fenfluramine-treated PASMC.

to be included in the analysis set. These transcripts had expression intensities above the lower quartile in at least 1 channel, as well as unique, unambiguous gene annotations (excluding putative genes encoding hypothetical proteins) according to the manufacturer's information. Figure 4 shows a general characterization of the dysregulated genes in fenfluramine-treated PASMC and PAEC samples using different fold-change cutoffs. At fold-change>1.5, 881 genes were found to be dysregulated (497 upregulated and 384 downregulated) in the fenfluramine-treated PASMC samples; and 2,534 genes were found to be differentially expressed in the fenfluramine-treated PAEC sample (1,226 upregulated and 1,308 downregulated) (Fig. 4a). In contrast, at
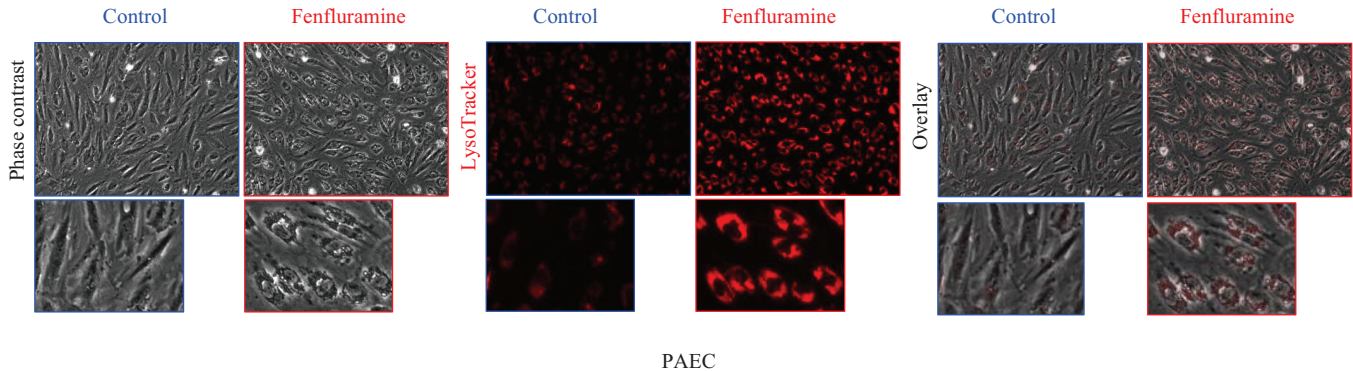

PAEC

Figure 3: The effect of fenfluramine on the lysosomes in human PAEC. Cells were treated with 200 μM fenfluramine for 72 hrs. and the lysosomes in the cells were stained with LysoTracker Red. The phase contrast images (upper panels) of the cells were overlaid with the fluorescent images of the lysosomes (in red, middle panels) to show the location of lysosomes in untreated and fenfluramine-treated PAEC (bottom panels). The LysoTracker Red-staining is mainly localized in the perinuclear area in fenfluramine-treated cells.
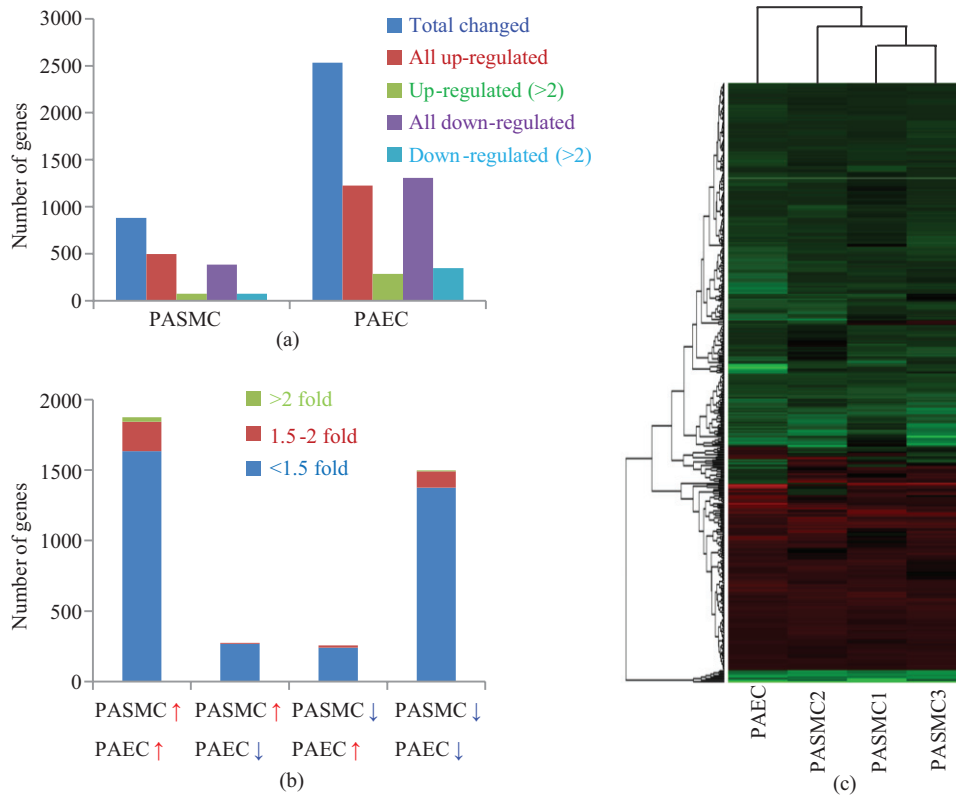

Figure 4: A general characterization of the genes dysregulated in PASMC and PAEC after fenfluramine treatment. (a) Numbers of genes that are affected by fenfluramine in PASMC and PAEC. The dysregulated genes are classified into five categories: expression level changed (fold-change>1.5) - "total changed," upregulated - "all upregulated," upregulated greater than 2-fold - "upregulated (>2)," downregulated - "all downregulated," and downregulated greater than 2-fold - "downregulated (>2)" in PASMC (left) and PAEC (right). (b) Numbers of genes that are upregulated in both PASMC and PAEC, upregulated in PASMC but downregulated in PAEC, downregulated in PASMC but upregulated in PAEC, or downregulated in both PASMC and PAEC at different fold-changes (<1.5-fold, 1.5- to 2-fold, or >2-fold). (c) A heatmap of the 384 dysregulated genes (fold-change>2.0) in PASMC or PAEC.

fold-change>2.0, there were 122 (74 upregulated and 48 downregulated) and 633 (285 upregulated and 348 downregulated) genes found to be dysregulated in the fenfluramine-treated PASMC and PAEC, respectively (Fig. 4a). Notably, there were more dyregulated genes with the same direction (e.g., upregulated in both cell types) in both PASMC and PAEC than those with different direction (e.g., upregulated in PASMC, but downregulated in PAEC) (Fig. 4b). At fold-change>2.0, there were 39 dysregulated genes (32 upregulated and 7 downregulated) in both

PASMC and PAEC samples (Table 1). Figure 4c shows a heatmap of the 384 differential genes (i.e., dysregulated in at least 1 cell type) in both PASMC and PAEC. Particularly, the PASMC samples were clustered together, showing a similar dysregulation pattern relative to PAEC.

Western blot validation of microarray data

Three dysregulated genes (fold-change>1.5), *MMP1*, *CYCS* and *VIM*, after fenfluramine treatment were selected for Western blot validation in the same PASMC

Table 1: Genes dysregulated in both PASMC and PAEC samples (fold-change > 2.0)				
Dysregulation pattern[a]	Gene symbol	Gene title	Fold-change in PASMC[b]	Fold-change in PAEC
PASMC↑ and PAEC↑	C12orf42	Chromosome 12 open reading frame 42	8.4	11.7
	CECR1	Cat eye syndrome chromosome region, candidate 1	3.1	2.7
	SC4MOL	Sterol-C4-methyl oxidase-like	2.1	2.7
	DKKL1	Dickkopf-like 1 (soggy)	2.0	2.0
	PPP1R12B	Protein phosphatase 1, regulatory (inhibitor) subunit 12B	4.5	2.6
	SMR3B	Submaxillary gland androgen regulated protein 3 homolog B (mouse)	4.3	3.0
	DOCK8	Dedicator of cytokinesis 8	2.9	2.1
	ITGA2	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	2.1	2.2
	DPCR1	Diffuse panbronchiolitis critical region 1	2.7	2.7
	CLCA3	Chloride channel, calcium activated, family member 3	2.1	2.2
	ZFYVE16	Zinc finger, FYVE domain containing 16	2.3	2.2
	LIG4	Ligase IV, DNA, ATP-dependent	2.0	2.0
	ZSCAN4	Zinc finger and SCAN domain containing 4	2.9	5.4
	HTR2B	5-Hydroxytryptamine (serotonin) receptor 2B	2.8	2.7
	GALC	Galactosylceramidase	2.6	2.4
	ANKRD19	Ankyrin repeat domain 19	2.4	2.3
	C5orf29	Chromosome 5 open reading frame 29	2.3	3.7
	PIK3AP1	Phosphoinositide-3-kinase adaptor protein 1	3.8	4.6
	ZDHHC15	Zinc finger, DHHC-type containing 15	2.5	5.8
	LOC541473	FKBP6-like	3.4	2.8
	KLF17	Kruppel-like factor 17	2.3	3.4
	CCDC62	Coiled-coil domain containing 62	2.6	2.0
	TPD52L3	Tumor protein D52-like 3	2.2	2.6
	OR2W3	Olfactory receptor, family 2, subfamily W, member 3	2.0	2.2
	COBL	Cordon-bleu homolog (mouse)	2.2	2.3
	NAT8L	N-Acetyltransferase 8-like	2.0	2.8
	FSTL5	Follistatin-like 5	3.2	2.8
	C9orf18	Chromosome 9 open reading frame 18	4.4	3.4
	ASAH1	N-Acylsphingosine amidohydrolase (acid ceramidase) 1	2.1	2.1
	IRF8	Interferon regulatory factor 8	4.2	5.1
	SLC6A15	Solute carrier family 6, member 15	3.0	3.1
	DYNC2H1	Dynein, cytoplasmic 2, heavy chain 1	4.0	2.1
PASMC↓ and PAEC↓	AGMAT	Agmatine ureohydrolase (agmatinase)	−2.9	−2.5
	WNT11	Wingless-type MMTV integration site family, member 11	−2.4	−2.1
	OXCT2	3-Oxoacid CoA transferase 2	−2.5	−2.1
	ANGPTL4	Angiopoietin-like 4	−2.7	−7.6
	C13orf21	Chromosome 13 open reading frame 21	−2.1	−3.0
	SLC16A14	Solute carrier family 16, member 14 (monocarboxylic acid transporter 14)	−2.1	−4.6
	MYOZ3	Myozenin 3	−2.1	−2.0
PASMC↑ and PAEC↓	–	–	–	–
PASMC↓ and PAEC↑	–	–	–	–

PASMC: Pulmonary artery smooth muscle cells; **PAEC:** Pulmonary artery endothelial cells. **a:** ↑ – Upregulation, ↓ – Downregulation; **b:** Median fold-change for the PASMC samples

and PAEC samples. The gene dysregulation patterns for these 3 genes were recaptured by the Western blot experiments (Fig. 5a and b). Particularly, the mRNA expression of *MMP1* in PAEC after fenfluramine treatment (our microarray data) was upregulated by approximately 200% (Fig. 5c), while the protein expression level was upregulated by 50% (Fig. 5b). The fenfluramine-mediated mRNA expression upregulation of *CYCS* and *VIM*, determined by the microarray data (Fig. 5c), was also consistent with the fenfluramine-mediated protein expression upregulation determined by Western blot analysis (Fig. 5b).

Enriched pathways and GO biological processes among the fenfluramine-induced dysregulated genes

We evaluated the enrichment of KEGG[20,21] pathways and GO[22] biological processes among the genes dysregulated in PASMC and PAEC samples after fenfluramine treatment. Figure 6 compares the top 15 enriched pathways and biological processes (enrichment significant at adjusted P-value<0.001 after the BH procedure;[28] a minimum gene set size of 5 hits) between different cutoffs for differential expression (i.e., fold-change>1.5 and 2.0), as well as between both cell types. A significant number of enriched KEGG[20,21] pathways (Table 2) and GO[22] biological processes (Table 3) were identified using both fold-changes of 1.5 and 2.0 for differential expression. Notably, 14 KEGG[20,21] pathways (e.g., "MAPK signaling pathway," "calcium signaling pathway," "cell adhesion molecules") (Table 2) and 7 GO[22] biological processes (e.g., "inflammatory response," "response to organic substance," "regulation of cell proliferation") (Table 3) were enriched among the dysregulated genes in both PASMC and PAEC. Furthermore, some pathways and biological processes were also found to be enriched specifically among the dysregulated genes in either PASMC or PAEC. For example, the KEGG[20,21] pathways: "dilated cardiomyopathy" and "complement and coagulation cascades" (Table 2), as well as the GO[22] biological processes: "regulation of blood pressure" and " muscle system process" (Table 3), were enriched among the dysregulated genes in PASMC. In contrast, the KEGG[20,21] pathways: "steroid biosynthesis" and "cell cycle" (Table 2), as well as the GO[22] biological process "cell proliferation" and "cell division" (Table 3), were enriched among the dysregulated genes in PAEC.

Gene network analysis

Some of the dysregulated genes in PASMC or PAEC after fenfluramine treatment were found to be connected with each other through certain FIs (e.g., activation, inhibition). Specifically, the Reactome[30,31] FI database was queried to identify FI relationships among the dysregulated genes after fenfluramine treatment in either PASMC (Fig. 7a) or
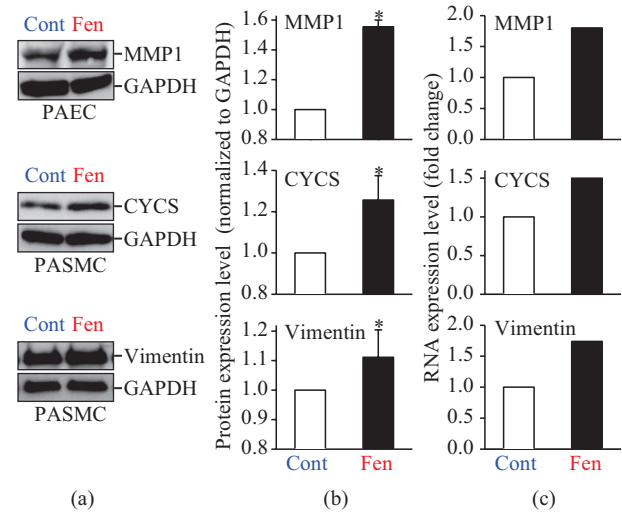

Figure 5: Western blot validation of microarray data. Validation of microarray expression data was performed using Western blot in PASMC and PEAC. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as control. (a and b) Western blot analysis on matrix metallopeptidase 1 (MMP1) in untreated and fenfluramine-treated PAEC, and on somatic cytochrome c (CYCS) and vimentin in untreated and fenfluramine-treated PASMC. The data shown in B are mean±SE; *P<0.05 vs. control (Cont). (c) The fold-changes of mRNA expression levels of *MMP1*, *CYCS* and vimentin in untreated and fenfluramine-treated cells as indicated by the microarray data.

PAEC (Fig. 8a). We also evaluated the relative importance of the genes in the identified FI networks based on betweenness centrality, a measure in graph theory for a node's (i.e., a gene's) centrality in a particular network (Fig. 7b for PASMC and Fig. 8b for PAEC). Particularly, encoding bone morphogenetic protein receptor, type IB (*BMPR1B*) was located in the hub based on betweenness centrality in the networks comprised of the dysregulated genes in PASMC, while encoding repulsive guidance molecule A (*RGMA*) was found to be the most important gene based on betweenness centrality in the networks comprised of the dysregulated genes in PAEC (Figs. 7 and 8).

DISCUSSION

Fenfluramine-associated IPAH is likely a complex phenotype caused by multiple genetic and non-genetic factors, as well as by molecular changes after exposure to the drug. Elucidating the effects of this drug on relevant lung tissues (i.e., PASMC and PAEC) can potentially provide much-needed information on the underlying mechanism of fenfluramine-associated IPAH. In terms of cell and mitochondrial morphologies, there were obvious differences observed in PASMC and PAEC before and after treatment with fenfluramine (Figs. 1-3). These morphological data suggest that fenfluramine treatment not only results in mitochondrial damage (or fragmentation) in PASMC but also causes lysosome swelling in PAEC. However, it is unclear how these
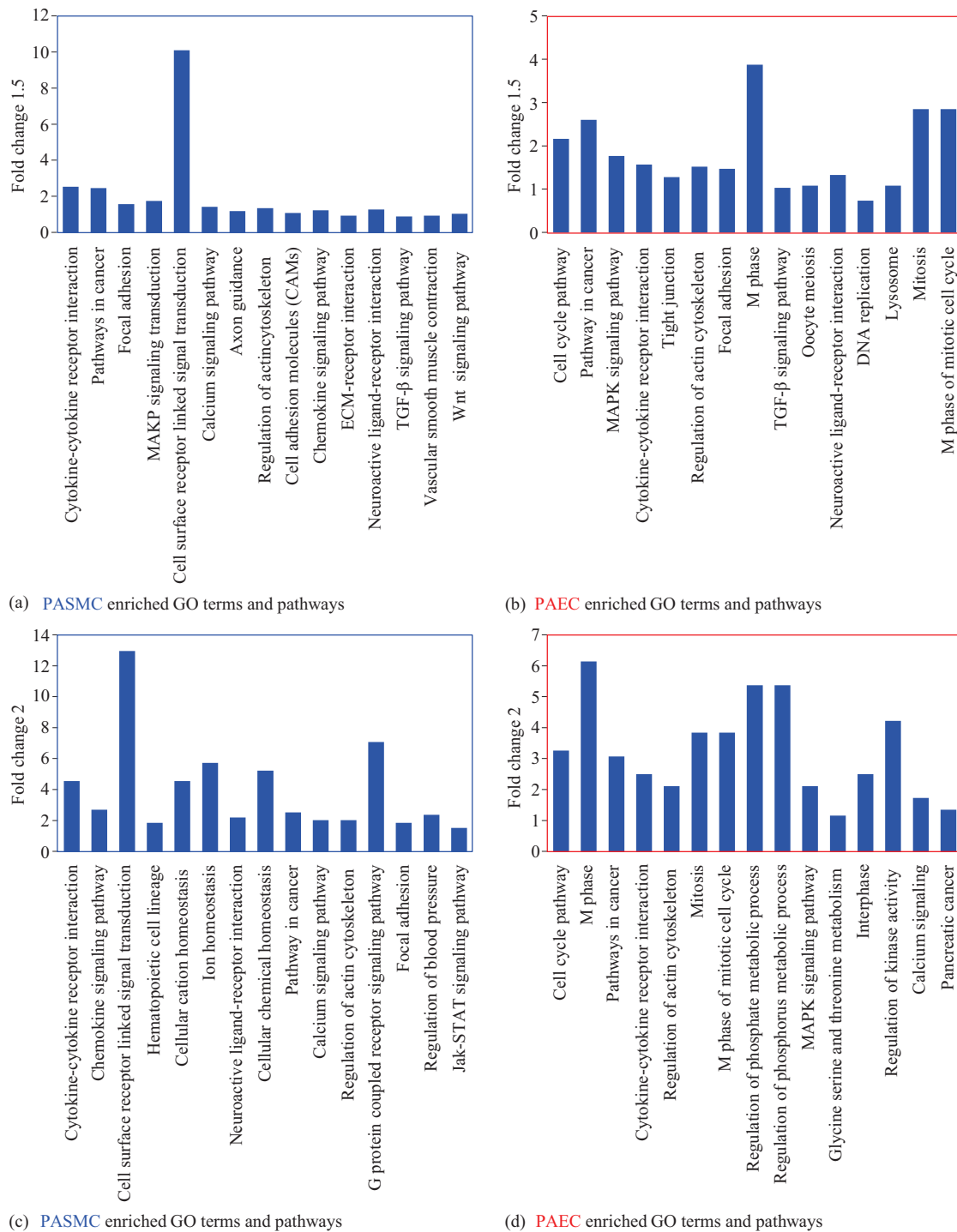
(a) PASMC enriched GO terms and pathways

(b) PAEC enriched GO terms and pathways

(c) PASMC enriched GO terms and pathways

(d) PAEC enriched GO terms and pathways

Figure 6: Enriched pathways and biological processes among the dysregulated genes. Top 15 enriched KEGG pathways and GO biological processes for the genes that are changed by fenfluramine in PASMC (a and c) and PAEC (b and d) at fold-change greater than 1.5 (a and b) or 2.0 (c and d).

effects are related to the development of pulmonary hypertension.

We hypothesized that gene expression dysregulation after fenfluramine treatment in normal pulmonary artery tissues might contribute to the pathogenesis of

fenfluramine-associated IPAH. Using a whole-genome cDNA microrray, a comprehensive evaluation of gene expression profiles in PASMC and PAEC samples showed a substantial number of dysregulated genes after fenfluramine treatment (Fig. 4), suggesting a wide range of molecular changes in lung tissues after fenfluramine

Table 2: Enriched KEGG pathways (FDR < 0.001) among the dysregulated genes after fenfluramine treatment

Cell type	Pathway	Fold enrichment of Pathway Genes[a] (2.0)	Fold enrichment of Pathway Genes[b] (1.5)
PASMC	hsa00230:purine metabolism	12.3	10.4
	hsa04115:p53 signaling pathway	25.4	16.5
	hsa04210:apoptosis	23.6	14.8
	hsa04512:ECM-receptor interaction	18.9	18.8
	hsa04610:complement and coagulation cascades	26.3	16.7
	hsa04620:Toll-like receptor signaling pathway	16.9	10.7
	hsa04621:NOD-like receptor signaling pathway	27.8	15.5
	hsa04630:Jak-STAT signaling pathway	18.2	12.5
	hsa04650:natural killer cell mediated cytotoxicity	18.2	13.6
	hsa04722:neurotrophin signaling pathway	12.4	8.9
	hsa04916:melanogenesis	15.4	12.3
	hsa04960:aldosterone-regulated sodium reabsorption	34.7	13.3
	hsa05014:amyotrophic lateral sclerosis	25.1	13.2
	hsa05414:dilated cardiomyopathy	17.3	15.8
	hsa05416:viral myocarditis	20.4	16.5
PAEC	hsa00100:steroid biosynthesis	71.9	32.4
	hsa00260:glycine, serine and threonine metabolism	50.6	23.8
	hsa04110:cell cycle	35.2	25.7
	hsa04114:oocyte meiosis	15.0	15.5
	hsa04142:lysosome	15.7	13.9
	hsa04350:TGF-beta signaling pathway	18.8	18.5
	hsa04360:axon guidance	14.8	12.5
	hsa04914:progesterone-mediated oocyte maturation	18.3	12.1
	hsa05212:pancreatic cancer	25.2	14.2
	hsa05220:chronic myeloid leukemia	21.0	9.8
Both[c]	hsa03320:PPAR signaling pathway	21.5/26.7	15.4/17.5
	hsa04010:MAPK signaling pathway	7.7/11.0	10.9/10.2
	hsa04020:calcium signaling pathway	18.1/14.0	13.6/10.5
	hsa04060:cytokine-cytokine receptor interaction	32.9/16.4	19.8/11.4
	hsa04062:chemokine signaling pathway	23.6/10.7	11.6/9.0
	hsa04080:neuroactive ligand-receptor interaction	17.0/10.8	10.6/10.3
	hsa04510:focal adhesion	14.4/10.9	13.2/11.5
	hsa04514:cell adhesion molecules	17.5/15.8	15.1/10.2
	hsa04640:hematopoietic cell lineage	37.7/21.3	17.2/14.0
	hsa04670:leukocyte transendothelial migration	16.7/14.8	11.4/13.2
	hsa04810:regulation of actin cytoskeleton	15.2/14.4	10.9/11.4
	hsa05200:pathways in cancer	12.0/13.3	12.6/12.4
	hsa05218:melanoma	22.9/19.7	15.5/14.4
	hsa05222:small cell lung cancer	23.9/21.7	13.6/14.0

PASMC: pulmonary artery smooth muscle cells; **PAEC:** pulmonary artery endothelial cells; **KEGG:** kyoto Encyclopedia of Genes and Genomes. **a:** among genes dysregulated at least 2-fold; **b:** among genes dysregulated at least 1.5-fold; **c:** enrichment fold shown in this category is for PASMC and PAEC, respectively

exposure. For example, 122 and 633 genes were identified to be dysregulated for at least 2-fold in PASMC and PAEC, respectively (Fig. 4).

In addition to a number of genes that were dysregulated in both PASMC and PAEC (i.e., upregulated or downregulated in both cell types) (Table 1), the majority of dysregulated genes after fenfluramine treatment showed changes in either PASMC or PAEC only (Fig. 4b), thus potentially indicating a differential effect of fenfluramine on different cell types. Therefore, the microarray results suggested that with the exception of some commonly dysregulated genes in PASMC and PAEC, fenfluramine exposure could cause unique or differential changes in different cell types. Notably, some commonly and cell type-specifically dysregulated genes have been implicated in either pulmonary hypertension or related biological processes, demonstrating the relevance of these genes with fenfluramine-associated IPAH. For example, *CECR1* (encoding cat eye syndrome chromosome region, candidate 1), which was upregulated (fold-change>2.0) in both PASMC and PAEC (Table 1), has been implicated in cat eye syndrome, a rare disease that features abnormal pulmonary venous return, potentially leading to pulmonary hypertension.[32] *PPP1R12* (encoding protein phosphatase 1, regulatory [inhibitor] subunit 12B), an upregulated (fold-change>2.0) gene in both PASMC and PAEC after fenfluramine treatment (Table 1), is a myosin

Table 3: Enriched GO biological processes (FDR<0.001) among the dysregulated genes after fenfluramine treatment

Cell type	Biological process	Fold enrichment of GO Genes (2.0)[a]	Fold enrichment of GO Genes (1.5)[b]
PASMC	GO:0001558-regulation of cell growth	4.6	2.9
	GO:0003012-muscle system process	5.6	4.0
	GO:0003013-circulatory system process	6.3	3.9
	GO:0006873-cellular ion homeostasis	4.5	2.5
	GO:0006874-cellular calcium ion homeostasis	6.3	3.4
	GO:0006875-cellular metal ion homeostasis	6.8	3.3
	GO:0006928-cell motion	3.0	2.7
	GO:0006935-chemotaxis	6.0	3.1
	GO:0006952-defense response	4.5	3.1
	GO:0006955-immune response	4.3	2.7
	GO:0007155-cell adhesion	3.5	2.6
	GO:0007166-cell surface receptor linked signal transduction	2.7	2.1
	GO:0007186-G-protein coupled receptor protein signaling pathway	3.0	1.8
	GO:0007204-elevation of cytosolic calcium ion concentration	7.6	4.8
	GO:0007242-intracellular signaling cascade	2.0	1.8
	GO:0007610-behavior	3.5	2.5
	GO:0007626-locomotory behavior	4.1	2.7
	GO:0008015-blood circulation	6.3	3.9
	GO:0008217-regulation of blood pressure	7.9	3.8
	GO:0008284-positive regulation of cell proliferation	3.3	2.7
	GO:0010941-regulation of cell death	2.5	2.1
	GO:0019725-cellular homeostasis	3.3	2.1
	GO:0019932-second-messenger-mediated signaling	4.7	2.9
	GO:0022610-biological adhesion	3.5	2.6
	GO:0030003-cellular cation homeostasis	5.9	2.9
	GO:0030005-cellular di-, tri-valent inorganic cation homeostasis	5.5	2.8
	GO:0030334-regulation of cell migration	4.8	3.5
	GO:0030335-positive regulation of cell migration	6.7	4.3
	GO:0040012-regulation of locomotion	4.5	3.2
	GO:0040017-positive regulation of locomotion	6.6	4.1
	GO:0042330-taxis	5.9	3.1
	GO:0042493-response to drug	4.2	2.4
	GO:0042592-homeostatic process	3.0	2.0
	GO:0042981-regulation of apoptosis	2.5	2.1
	GO:0043066-negative regulation of apoptosis	3.8	2.5
	GO:0043067-regulation of programmed cell death	2.5	2.1
	GO:0043069-negative regulation of programmed cell death	3.7	2.5
	GO:0043085-positive regulation of catalytic activity	3.4	2.3
	GO:0043434-response to peptide hormone stimulus	6.2	2.5
	GO:0044057-regulation of system process	3.9	3.3
	GO:0044093-positive regulation of molecular function	3.0	2.3
	GO:0048878-chemical homeostasis	4.3	2.5
	GO:0050801-ion homeostasis	4.3	2.5
	GO:0051247-positive regulation of protein metabolic process	4.1	2.2
	GO:0051270-regulation of cell motion	4.2	3.2
	GO:0051336-regulation of hydrolase activity	3.7	2.4
	GO:0051345-positive regulation of hydrolase activity	5.7	2.8
	GO:0051480-cytosolic calcium ion homeostasis	7.4	4.4
	GO:0055065-metal ion homeostasis	7.1	3.3
	GO:0055066-di-, tri-valent inorganic cation homeostasis	5.2	2.7
	GO:0055074-calcium ion homeostasis	6.4	3.3
	GO:0055080-cation homeostasis	5.3	2.8
	GO:0055082-cellular chemical homeostasis	4.4	2.5
	GO:0060191-regulation of lipase activity	9.1	3.8
	GO:0060548-negative regulation of cell death	3.7	2.5
PAEC	GO:0000087-M phase of mitotic cell cycle	5.2	3.9
	GO:0000278-mitotic cell cycle	4.8	3.5
	GO:0000279-M phase	5.4	3.5
	GO:0000280-nuclear division	5.3	4.0

(Continued)

Table 3: Continued

Cell type	Biological process	Fold enrichment (2.0)[a]	Fold enrichment (1.5)[b]
	GO:0007049-cell cycle	4.3	3.0
	GO:0007067-mitosis	5.3	4.0
	GO:0008283-cell proliferation	4.6	3.8
	GO:0019220-regulation of phosphate metabolic process	3.7	2.7
	GO:0022402-cell cycle process	4.8	3.2
	GO:0022403-cell cycle phase	5.8	3.6
	GO:0042325-regulation of phosphorylation	3.6	2.6
	GO:0043549-regulation of kinase activity	4.2	2.9
	GO:0045859-regulation of protein kinase activity	4.1	2.8
	GO:0048285-organelle fission	5.1	3.8
	GO:0051174-regulation of phosphorus metabolic process	3.7	2.7
	GO:0051301-cell division	4.0	2.9
	GO:0051325-interphase	8.0	5.1
	GO:0051329-interphase of mitotic cell cycle	7.6	4.9
	GO:0051338-regulation of transferase activity	4.0	3.2
	GO:0051726-regulation of cell cycle	4.3	3.1
Both[c]	GO:0006954-inflammatory response	5.5/4.0	3.4/2.6
	GO:0007267-cell-cell signaling	4.1/3.2	3.1/2.4
	GO:0008285-negative regulation of cell proliferation	3.5/3.8	3.0/3.2
	GO:0009611-response to wounding	4.3/3.1	3.0/2.7
	GO:0009719-response to endogenous stimulus	4.5/3.3	2.8/3.1
	GO:0010033-response to organic substance	3.1/2.6	2.3/2.4
	GO:0042127-regulation of cell proliferation	3.2/3.3	2.7/2.7

PASMC: pulmonary artery smooth muscle cells; **PAEC:** pulmonary artery endothelial cells; **GO:** gene ontology. **a:** among genes dysregulated at least 2-fold; **b:** among genes dysregulated at least 1.5-fold; **c:** enrichment fold shown in this category is for PASMC and PAEC, respectively
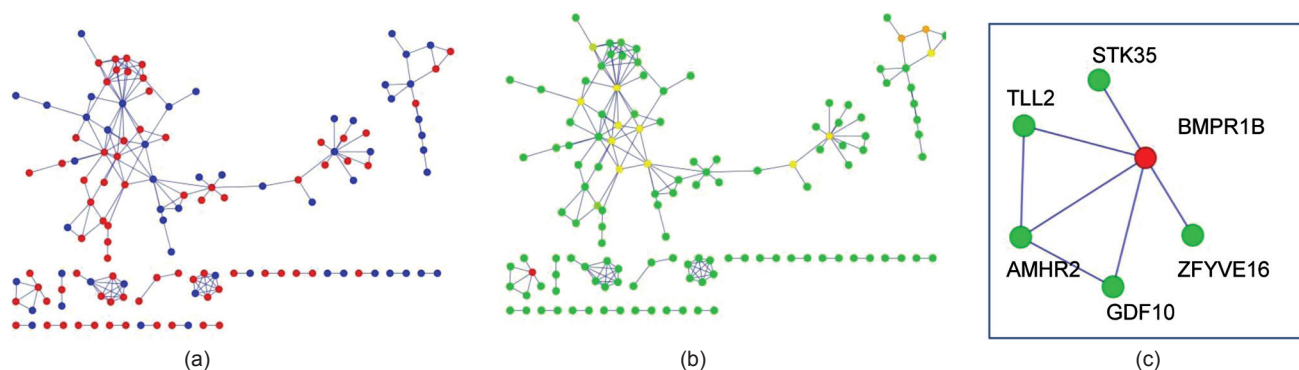

Figure 7: Gene networks comprised of the dysregulated genes in PASMC after fenfluramine treatment. A substantial proportion of the dysregulated genes after fenfluramine treatment (fold-change>2.0) are connected with each other through functional interactions. (a) A gene network comprised of the dysregulated genes in PASMC. Red: upregulated genes; Blue: downregulated genes. (b) A gene network comprised of the dysregulated genes in PASMC showing betweenness centrality, a measure for the relative importance of genes in a network. Green: low value; Red: high value; Orange/ Yellow: intermediate value. (c) Bone morphogenetic protein receptor, type IB (BMPR1B) is the hub based on betweenness centrality. AMHR2: Anti-Mullerian hormone receptor, type II; GDF10: Growth differentiation factor 10; STK35: Serine/threonine kinase 35; TLL2: Tolloid-like 2.

phosphatase (also known as MYPT2) that regulates muscle contraction, thus potentially affecting blood pressure.[33] In addition, mutations in BMP family including *BMPR1B*, an upregulated (fold-change>2.0) gene after fenfluramine treatment in PASMC, have been associated with IPAH.[34,35] In contrast, *MYLK* (encoding myosin, light chain kinase, transcript variant 1), an upregulated gene (fold-change>2.0) in PAEC, has been implicated in endothelial cell contraction and barrier dysfunction.[36-38]

We further examined whether these dysregulated genes were enriched in any biological processes or known physiological pathways, which could help elucidate the underlying mechanism of fenfluramine-associated pathogenesis of IPAH. Similar to the comparison of gene dysregulation, a number of common KEGG[20,21] pathways and GO[22] biological processes were identified among the genes dysregulated in PASMC and PAEC samples, respectively, in addition to other cell type-specific
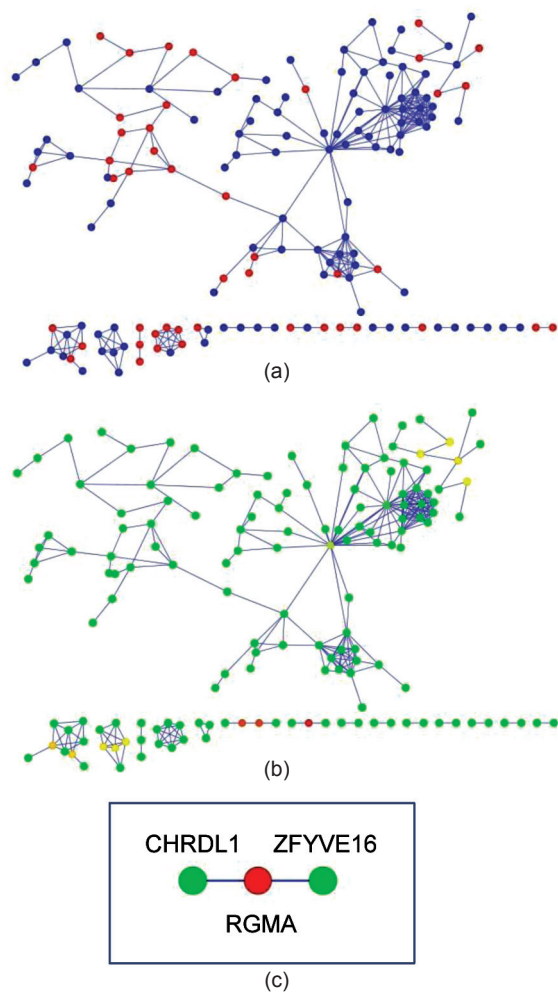
(a)

(b)

CHRDL1   ZFYVE16

RGMA

(c)

Figure 8: Gene networks comprised of the dysregulated genes in PAEC after fenfluramine treatment. A substantial proportion of the dysregulated genes after fenfluramine treatment (fold-change>2.0) can be connected with each other through functional interactions. (a) A gene network comprised of the dysregulated genes in PAEC. Red: upregulated genes; Blue: downregulated genes. (b) A gene network comprised of the dysregulated genes in PAEC showing betweenness centrality, a measure for the relative importance of genes in a network. Green: low value; Red: high value, Orange/Yellow: intermediate value. (c) Repulsive guidance molecule A (RGMA) is the hub based on betweenness centrality. CHRDL1: Chordin-like 1; ZFYVE16: Zinc finger, FYVE domain containing 16.

pathways and biological processes (Fig. 4, Tables 2 and 3). Notably, the enriched pathways (Table 2) and biological processes (Table 3) were robust across different cutoffs for differential expression (i.e., fold-change>1.5 and 2.0). Particularly, the dysregulated genes in PASMC samples were found to be enriched in a number of GO[22] biological processes including "immune response," "defense response," "blood circulation," and "circulatory system process," as well as processes related to cell adhesion and cell migration (Table 3). Similarly, a number of KEGG[20,21] pathways were enriched specifically among the dysregulated genes in PASMC, including "dilated

cardiomyopathy," and a number of signaling pathways such as "Toll-like receptor signaling pathway," and "JAK-STAT signaling pathway" (Table 2). Among the dysregulated genes in PAEC, however, there were enriched pathways and biological processes including "mitotic cell cycle," "regulation of phosphorylation," and "TGF-β signaling pathway" (Tables 2 and 3). In addition, there were several pathways and biological processes, including "inflammatory response," "PPAR signaling pathway," "MAPK signaling pathway," and "calcium signaling pathway," that were enriched among the dysregulated genes in both PASMC and PAEC (Tables 2 and 3). Clearly, some of these enriched pathways and biological processes have been implicated in pulmonary hypertension or related physiological processes. For example, the MAPK signaling pathway has been demonstrated to be activated by norfenfluramine, a derivative of fenfluramine, together with the increase of intracellular Ca^{2+} and the activation of the hydrolysis of inositol phosphates.[13] Previous studies have also implicated the PAAR signaling pathway in PAH.[39,40] Our pathway analysis results also indicated that genes involved in cell permeability, inflammation and immune response, such as "calcium signaling pathway," and "cell adhesion," could play critical roles in fenfluramine-associated pathogenesis.

Furthermore, our gene network analysis demonstrated that a substantial proportion of the dysregulated genes in PASMC and PAEC after fenfluramine treatment could be linked with each other through certain functional interactions (e.g., activation, inhibition) (Figs. 7 and 8), suggesting that fenfluramine exposure may cause changes in a series of interacting proteins. Particularly, BMPR1B (Fig. 7c) and RGMA (Fig. 8c) were among the most important genes in the networks comprised of the dysregulated genes in PASMC and PAEC, respectively, based on betweenness centrality, indicating their potentially critical roles in determining the fenfluramine-associated pathogenic process in these cells. Interestingly, repulsive guidance molecule, a BMP co-receptor has been found to alter utilization of bone morphogenetic protein (BMP) type II receptors,[41,42] which have been implicated in pulmonary hypertension.[43]

CONCLUSIONS

In summary, our findings strongly suggest that fenfluramine exposure could cause a wide range of gene dysregulation. Significantly, our results confirmed that fenfluramine-associated IPAH is likely due to a complex pathogenic process that could be caused by genes involved in a variety of pathways and biological processes, including those related to normal functions of blood vessels (e.g., cell permeability, cell adhesion), inflammation response,

immune response, and cell cycle. On the other hand, significant genetic and expression variations exist both within human populations and between normal subjects and patients with cardiopulmonary disease[44-47] that may in turn affect the cellular response to fenfluramine. Future investigations with more samples and populations are necessary to validate our findings. Finally, expanding molecular profiling to include other transcriptional targets such as microRNAs and integrating gene expression with genetic and epigenetic variations in the future could provide a more comprehensive picture of the complex cellular response to fenfluramine exposure as well as the pathogenesis of fenfluramine-associated IPAH or pulmonary arterial hypertension in general.

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