Research Article

Idiopathic and heritable PAH perturb common molecular pathways, correlated with increased MSX1 expression

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ABSTRACT

The majority of pulmonary arterial hypertension (PAH) is not associated with BMPR2 mutation, and major risk factors for idiopathic PAH are not known. The objective of this study was to identify a gene expression signature for IPAH. To accomplish this, we used Affymetrix arrays to probe expression levels in 86 patient samples, including 22 healthy controls, 20 IPAH patients, 20 heritable PAH patients (HPAH), and 24 BMPR2 mutation carriers that were as yet unaffected (UMC). Culturing the patient cells removes the signatures of drug effects and inflammation which have made interpretation of results from freshly isolated lymphocytes problematic. We found that gene expression signatures from IPAH patients clustered either with HPAH patients or in a single distinct group. There were no groups of genes changed in IPAH that were not also changed in HPAH. HPAH, IPAH, and UMC had common changes in metabolism, actin dynamics, adhesion, cytokines, metabolism, channels, differentiation, and transcription factors. Common to IPAH and HPAH but not UMC were an upregulation of vesicle trafficking, oxidative/nitrosative stress, and cell cycle genes. The transcription factor MSX1, which is known to regulate BMP signaling, was the most upregulated gene (4×) in IPAH patients. These results suggest that IPAH cases have a shared molecular origin, which is closely related to, but distinct from, HPAH. HPAH and IPAH share the majority of altered signaling pathways, suggesting that treatments developed to target the molecular etiology of HPAH will also be effective against IPAH.

Key Words: BMPR2, heritable pulmonary arterial hypertension, idiopathic pulmonary arterial hypertension, PPH

INTRODUCTION

Pulmonary arterial hypertension (PAH) is a disease of progressively increasing pulmonary vascular resistance, which leads inexorably to failure of the right ventricle and death. Current therapies improve symptoms and function, but meta-analyses disagree over whether survival is improved. The problem with current therapies is a failure to address underlying molecular etiology.[1] The heritable form of disease (HPAH) is usually associated with mutation in BMPR2.[2] In 10 years of study, the molecular etiology of PAH secondary to BMPR2 mutation has become increasingly clear,[3-5] revealing a host of novel drug targets.[1]

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However, the majority of cases of PAH are not associated with BMPR2 mutation. While a few moderate risk factors for idiopathic PAH (IPAH) have been found through candidate gene approaches,[6,7] major genetic risk factors are still unknown. Because of relatively low patient numbers and a lack of a common genetic etiology, broad genetic approaches are unlikely to be successful, and have not been attempted. There have been several expression array experiments addressing molecular events in IPAH, recently reviewed,[8] using freshly isolated tissue from IPAH patients. These include 3 studies using lung tissue[9-11]

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and 3 using freshly isolated peripheral blood mononuclear cells (PBMCs).[12-14] A limitation of this approach is that, since they are taken directly from patients with end-stage disease, the data is contaminated by signatures of drug effects and inflammation or other host response, effectively masking or confounding signal derived from the primary pathogenesis and early molecular events which may still be present.

While the ideal would be to study pulmonary vascular tissues obtained prior to the development of disease, this is not feasible in humans for many reasons. We have previously demonstrated success in overcoming some of the confounding influences by studies of cultured lymphocytes. Using this methodology to compare BMPR2 mutation carriers who developed PAH to those who did not develop clinical disease (unaffected mutation carriers, UMC), we discovered that decreased expression of the estrogen metabolism gene CYP1B1 in women correlated to disease penetrance.[15] Follow-up studies found that estrogen metabolites measured in patient urine also correlated closely with disease penetrance in HPAH.[16] This methodology thus has proven valuable in producing robust, clinically relevant results. The theory behind this success is that genetically based alterations in gene expression will be present in any tissue, not just those affected by disease, and culturing the cells removes them both from drug effects and inflammatory effects or other host responses seen in end stage disease.

The goal of the present study is to extend this successful approach to determine genetic risk factors for idiopathic PAH. To accomplish this, we used Affymetrix arrays to probe expression levels in 22 healthy controls, 20 idiopathic PAH patients (BMPR2 mutation excluded), 20 heritable PAH patients, and 24 BMPR2 mutation carriers without clinical disease (Unaffected Mutation Carriers, UMC).

MATERIALS AND METHODS

Study population

The Vanderbilt Pulmonary Hypertension Research Cohort contains clinical and biologic specimens collected over 30 years, including detailed family pedigree and medical histories of patients with HPAH and IPAH. BMPR2 mutations have been detected in a large proportion of HPAH subjects tested to date, and several IPAH patients. The BMPR2 mutations vary in type, including nonsense mutations, insertion-deletion mutations that lead to splicing errors, frameshift mutations, and missense mutations.

The majority of patients were diagnosed and treated at Vanderbilt University Medical Center (VUMC). For those patients not diagnosed and treated at VUMC, specialist physicians in their geographic regions identified HPAH patients, and our investigators reviewed all medical records for accuracy of diagnosis. PAH was defined either by autopsy results showing plexogenic pulmonary arteriopathy in the absence of alternative causes such as congenital heart disease, or by clinical and cardiac catheterization criteria. These criteria included a mean pulmonary arterial pressure of more than 25 mmHg with a pulmonary capillary wedge or left atrial pressure of less than 15 mmHg, and exclusion of other causes of pulmonary hypertension in accordance with accepted international standards of diagnostic criteria.[17] Clinical information concerning survival in terms of death or lung transplantation was up to date as of March 2011, the closing date for this study.

Vanderbilt Pulmonary Hypertension Research Cohort study subjects were recruited via the Vanderbilt Pulmonary Hypertension Center, the Pulmonary Hypertension Association, and the NIH Clinical Trials website (http://clinicaltrials.gov). The VUMC Institutional Review Board approved all study protocols. All participants gave informed written consent to participate in genetic and clinical studies and underwent genetic counseling in accordance with published guidelines.[18] Samples were obtained following informed consent at the time of hospitalization, clinic visits, or by mail via a kit for collection of whole blood.

Ethylenediaminetetraacetic acid (EDTA) anticoagulated blood was collected from 86 individuals, including 20 idiopathic PAH patients, 22 healthy controls, 20 heritable PAH patients with BMPR2 mutation, and 24 BMPR2 mutation carriers who did not have evident PAH.

Genotyping and genetic analysis

We isolated genomic DNA using Puregene DNA Purification Kits (Gentra, Minneapolis, Minn.) according to the manufacturer's protocol. *BMPR2* gene mutation detection was performed by sequencing exons and exon intron boundaries of genomic DNA and by reverse transcriptase polymerase chain reaction (RT-PCR) analysis as described previously.[19,20] The *BMPR2* mutations in this study have been previously reported, and are included in a recent summary of detectable *BMPR2* mutations.[21]

Lymphocyte cultures

Lymphocyte cultures were performed as previously described.[15] Lymphocytes were isolated from anticoagulated whole blood within 48 hrs of collection and exposed to Epstein-Barr Virus (EBV) to induce cell immortalization. Two ml blood was diluted with 2 ml PBS, layered on top of 3 ml of Lympho Separation Medium (MP Biomedicals) and centrifuged for 10 minutes at 1,000×g at room temperature. Using a Pasteur pipet, the lymphocytes were removed from the serum/Lympho

Sep Media interface, washed in 10 ml PBS and then resuspended in 3 ml lymphoblast media (RPMI 1640 media containing L-glutamine, and 20% fetal bovine serum) containing 2 µg/ml cyclosporine. The lymphocytes were then infected with 3 ml Epstein-Barr virus (EBV) and transferred to a T-25 vent capped flask. The cells were incubated at 37°C/5% CO2 and fed weekly with lymphoblast media + cyclosporine until signs of growth occurred.

Affymetrix arrays

RNA was isolated from lymphocytes using a Qiagen RNeasy mini kit (Valencia, Calif.). First and second strand complimentary DNA was synthesized using standard techniques. Biotin-labeled antisense complimentary RNA was produced by an in vitro transcription reaction. Human Genome U133 Plus 2.0 microarrays (Affymetrix, Foster City, Calif.) were hybridized with 20 µg cRNA. Target hybridization, washing, staining, and scanning probe arrays were done following an Affymetrix GeneChip Expression Analysis Manual. All array results have been submitted to the NCBI gene expression and hybridization array data repository (GEO, www.ncbi.nlm.nih.gov/geo/), as series (pending).

Array analysis

The open source software, R2.13/Bioconductor2.8, was utilized for microarray analyses. Preprocessing of all cell files was carried out using the RMA algorithm, followed by duplicate probe removal to retain probes with higher IQR. The summarized data contained 19,701 features for each of the 59 arrays of HPAH, IPAH and control samples. Differential expression analysis was carried out using the standard moderated t-test procedure in package limma. The function decideTests with method="global" was used to make statistical tests comparable across probes and contrasts. Genes with an average expression above 7 in the group showing higher expression and having P value above 0.05 were considered significant and selected for further analysis.

Heirarchical clustering of both samples and genes was performed using algorithms within dChip,[22] according to established methods.[23] Rows were standardized by subtracting mean and dividing by standard deviation; correlation was used as the distance metric, using the centroid linkage method. Analysis of enriched gene function groups was performed using the 2010 release of Webgestalt,[24] using the hypergeometric test for enrichment of wither Gene Ontology consortium categories[25] or KEGG pathways.[26]

Western blot

Mouse lungs used were tissue archived in –80°C storage from prior experiments. Control mice had the Rosa26-rtTA2 transgene, which drives universal expression of the reverse tetracycline transactivator. Other mice included either the TetO7-Bmpr2^{R899X} or the TetO7-Bmpr2^{delx4+} transgenes,[5,27] which in combination with the Rosa26-rtTA2 allow doxycycline-inducible expression of two different Bmpr2 mutations. All mice were treated with six weeks of doxycycline starting at 8 weeks of age, and thus sacrificed at 14 weeks of age. The Institutional Animal Care and Uses Committee at Vanderbilt University approved the animal studies.

Mouse lungs were homogenized in RIPA buffer (PBS, 1% Ipegal, 0.5% sodium deoxycholate, 0.1% SDS) with proteinase and phosphatase inhibitor cocktails (Sigma-Aldrich St. Louis, Mo.) immediately upon sacrifice. Protein concentration was determined by Bradford assay. Primary antibodies used for Western blot included MSX1 (Abcam ab73883) and Beta-Actin (Abcam ab8227).

Statistics

Statistical methods for array analysis are described above. Correlation z-tests were performed using the JMP program (SAS, Cary, NC).

RESULTS

Experimental design

The goal of this experiment was to identify a gene expression signature for idiopathic pulmonary arterial hypertension (IPAH), using cultured, unaffected tissues, which are free from confounding effects of end-stage disease or drug effects. To accomplish this, we used Affymetrix arrays to probe expression levels in lymphocyte cell lines created from 22 healthy controls, 20 idiopathic PAH patients (BMPR2 mutation excluded), 20 heritable PAH patients, and 24 BMPR2 mutation carriers without clinical disease (Unaffected Mutation Carriers, UMC).

The overall experimental design is depicted in (Fig. 1), with patient population described in methods and in Table 1. Results of this analysis flow are presented below.

The same genes are altered in IPAH and HPAH, differing in magnitude

Comparing Affymetrix expression array data between 20 idiopathic PAH patients and 22 healthy controls, we found 168 genes upregulated and 118 genes downregulated, with $P<0.05$ (*see* Methods, above). Comparing Affymetrix expression array data between 20 heritable PAH patients and 22 healthy controls, we found 116 genes upregulated and 110 genes downregulated, with $P<0.05$ (*see* Methods, above).

The significant lists only overlap by 46 genes up and 27 down between IPAH and HPAH, but this paints an

Table 1: Patient characteristics

Group	Number	Female (%)	Age at diagnostic catheterization (Pts) or current age (Controls), yrs (S.D.)	Mean RAP, mmHg (S.D.)	Mean PAP, mmHg (S.D.)	Mean PCWP, mmHg (S.D.)	Cardiac output, L·min (S.D.)	Indexed PVR U·m² (S.D.)
Healthy controls	22	22 (100)	44.9 (23.6)	N/A	N/A	N/A	N/A	N/A
IPAH	20	18 (90)	40.1 (18.0)	10.8 (7.0)	56.3 (18.6)	10.1 (3.7)	3.4 (1.0)	15.7 (7.8)
HPAH	20	19 (95)	34.5 (16.7)	10.1 (6.1)	62.4 (12.8)	9.3 (4.0)	3.2 (0.9)	17.1 (4.5)
UMC	24	11 (46)	56.7 (17.0)	N/A	N/A	N/A	N/A	N/A

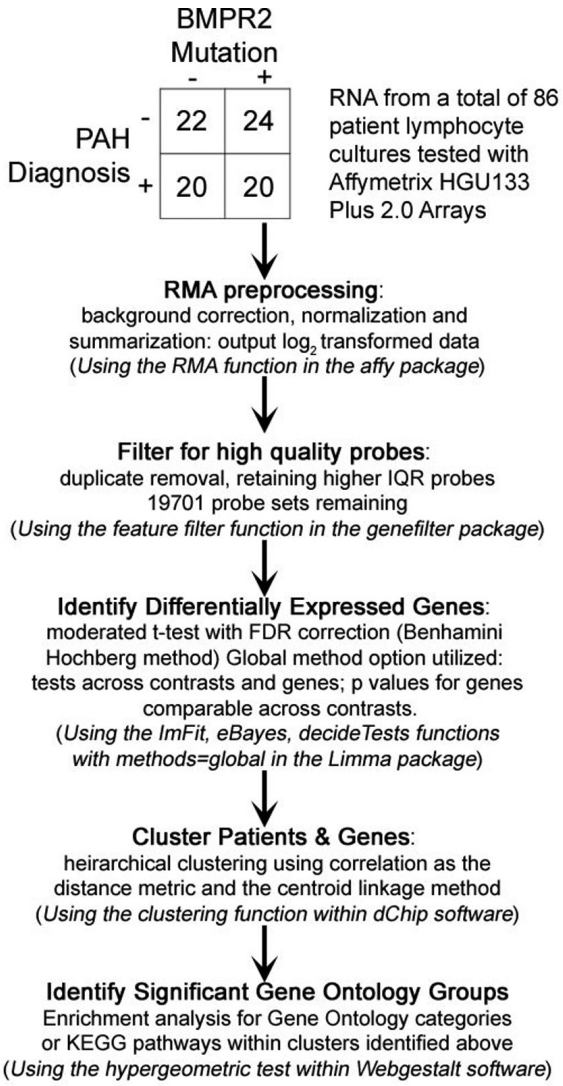

Figure 1: Experimental and analysis flow. The first three analysis steps, RMA preprocessing, Filter for high quality probes, and identification of differentially expressed genes, were performed within R version 2.13, Bioconductor version 2.8.

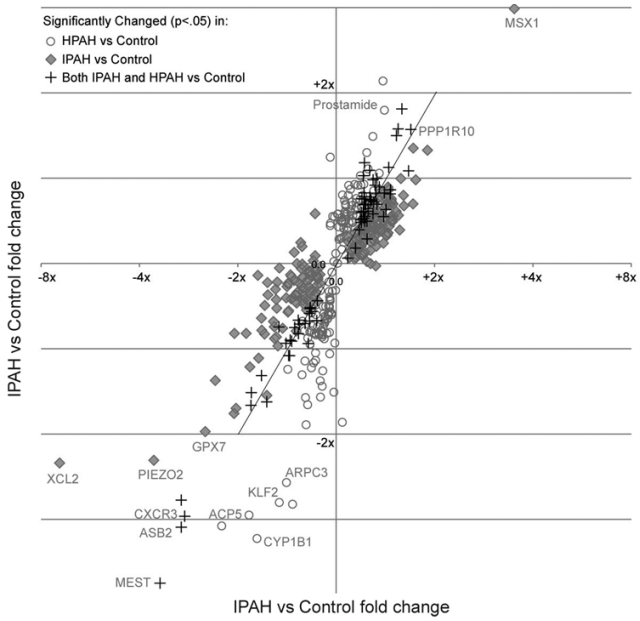

Figure 2: Gene expression changes in IPAH and HPAH are closely correlated. Grey diamonds represent genes that are only significantly changed in IPAH, but which are changed to nearly the same degree in HPAH. Open circles represent genes which are only significantly changed in HPAH, but which are changed to nearly the same degree in IPAH. Crosses indicate genes significantly changed in both.

incomplete picture. In (Fig. 2), filled grey diamonds depict fold change in genes significantly changed in IPAH vs. controls compared to fold change in those same genes in HPAH vs. controls. Correlation is excellent (0.922, P<0.0001 by correlation z-test): the difference is that while these genes are changed in both IPAH and HPAH, the magnitude of the average change in HPAH is half that in IPAH. The grey circles in (Fig. 2) are a plot of genes significantly changed in HPAH vs. controls compared to fold change in these same genes in IPAH vs. controls. Correlation is still very strong (0.88, P<0.0001 by correlation z-test). Once again, the difference is that the average magnitude of the change in IPAH is only a little over 40% of that in HPAH.

There is no feature of the study design or analysis that would be expected to predispose to correlation between significant genes in IPAH and HPAH cohorts, let alone a correlation of this strength. This pattern of similar changes, but with relative magnitudes inverted between IPAH and HPAH, implies that the same pathways are being altered, perhaps with a different initiating event.

There do not appear to be multiple molecular etiologies of IPAH

We powered this study to detect 20% average changes in individual genes, under the assumption that there might be multiple molecular etiologies of IPAH, and so average fold changes would be reduced by the fact that any individual gene would be changed in only a subset of the sample. To determine whether there were multiple molecular etiologies, we used unsupervised clustering of the samples, without assigning the samples to groups. The 86 patient samples naturally clustered into four groups (Fig. 3), which roughly corresponded to the 4 known a priori classifications (HPAH, UMC, IPAH, and healthy controls). Of the IPAH patient samples (purple color in top bar): 14/20 clustered in a single group; 1 clustered with the healthy controls; and 5/20 clustered with the HPAH, potentially representing IPAH with BMPR2 mutation not detected by sequencing or MLPA.

There are three primary conclusions that can be tentatively drawn from this pattern. First, while most genes altered in lymphocytes cultured from IPAH patients correlate nearly perfectly with those drawn from HPAH patients (Fig. 2), they are for most patients not identical in specific pattern. This once again implies that the molecular etiology for most IPAH patients ultimately perturbs the same pathways as in BMPR2 mutants, but with a different initiating molecular event. Second, some IPAH patients, even with BMPR2 mutation excluded by sequencing, probably still have a problem with signaling through BMPR2, either through unrecognized BMPR2 mutations or through pathway elements not previously screened (e.g., intracellular or secreted inhibitors). Third, the expression signature of those patients that do not cluster with the HPAH group are molecularly relatively homogenous. Thus, there do not appear to be multiple common molecular etiologies of IPAH.

Finally, it is informative that asymptomatic BMPR2 mutation carriers (UMC) primarily cluster with HPAH rather than with the healthy controls. This suggests, first, that the pattern of changes seen are not the consequence of end stage disease or drug effects, since they are also seen in UMC. Second, UMC are not very well separated from HPAH in this dendogram. It is our belief, based both on our BMPR2 mutant animal models[3,5] and on UMC exercise tests,[28] that many UMC may have silent pulmonary vascular disease as well, but under the threshold required to diagnose clinical disease (in fact, three samples collected as UMC developed clinical disease between collection and expression analyses, and so have been included as HPAH for these analyses using an *intention to treat* approach). Our previous

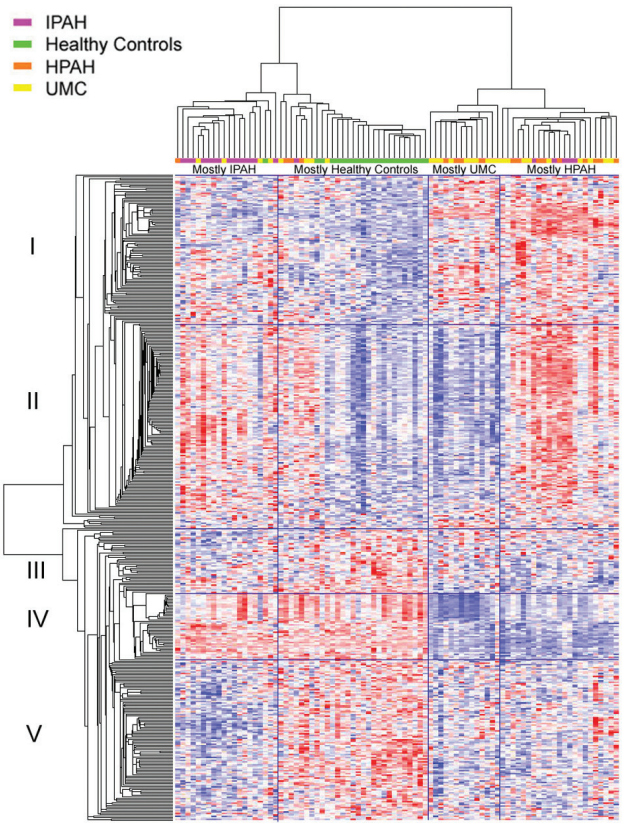

Figure 3: Clustered heat map of all 439 genes significantly changed in IPAH or HPAH as compared to controls. Clustering of patient expression profiles is depicted by the dendogram at top (color coding according to legend at top left). Clustering of expression patterns of dysregulated genes is depicted by the dendogram at left, which can be broken into roughly five groups of expression patterns, labeled I through V. Higher than average expression is depicted as increasing red: lower than average expression is depicted increasing blue.

study using cultured lymphocytes was designed through patient selection to determine gene expression patterns that distinguished UMC from HPAH;[15] the present study was not, and so UMC and HPAH are only poorly separated.

Clustering of gene function suggest vesicle trafficking, oxidative/nitrosative stress, and proliferation/apoptosis as critical risk factors

Unsupervised clustering was also performed on the 439 (402 named) genes significantly changed in either IPAH vs. control, HPAH vs. control, or both (Fig. 3). Genes that are clustered together have similar patterns of expression across the 86 patient samples. Genes clustered into five groups (labeled I through V).

Group I consists of genes that have increased expression in HPAH, UMC, and IPAH compared to controls. Gene ontology groups abundant in this group include regulation of the actin cytoskeleton (P=0.013 by KEGG), macromolecule metabolic processes (P=0.009 by GO, primarily g-protein

and lipid metabolism), and transcription regulation (P=0.010 by GO) (Fig. 4, and examples in top row of Fig. 5).

Group II consists of genes that have increased expression in HPAH and IPAH compared to healthy controls, but no change in UMC. These genes may correspond to critical risk factors for the development of PAH. They consist of 136 named genes involved in organelle and vesicle localization (P=0.004 by GO), oxidative and nitrosative stress (P=0.003 by GO), and cell cycle (P=0.0004 by GO). (Fig. 4, examples in Fig. 5 second row).

Groups III and V are similar; they consist of 145 named genes that show decreased expression in HPAH, UMC, and HPAH compared to controls. These include cell adhesion molecules (P=0.00004 by KEGG), cytokines (P=0.00008 by GO), potassium or calcium channels, and differentiation-related genes (Fig. 4, examples in Fig. 5 third row).

Group IV consists of 39 named genes downregulated in BMPR2 mutants, whether symptomatic or not, but unchanged in IPAH compared to healthy controls. It thus consists of genes altered by BMPR2 mutation, but probably not relevant to disease development (Fig. 4, examples in Fig. 5 bottom row). This group includes some endoplasmic-reticulum specific genes, which may reflect ER stress due to BMPR2 misfolding.[29] It also includes spliceosome-related genes (P=0.003, KEGG), many of which are markers of nonsense-mediated decay that may be affecting some BMPR2 mutations.[30]

One of the most important groups, though, is the one that isn't there: there does not exist a group of genes dysregulated in IPAH that is not also dysregulated in HPAH. On a molecular level, IPAH is a subset of HPAH.

It is important to note that the gene ontology groups which include the majority of the genes with altered regulation in IPAH are not new: they have all been extensively associated with BMPR2-related PAH in the past, and the connections between these groups are well established (Fig. 4). A central finding of this study is that most of the research that has been done on BMPR2-related heritable PAH is directly applicable to IPAH.

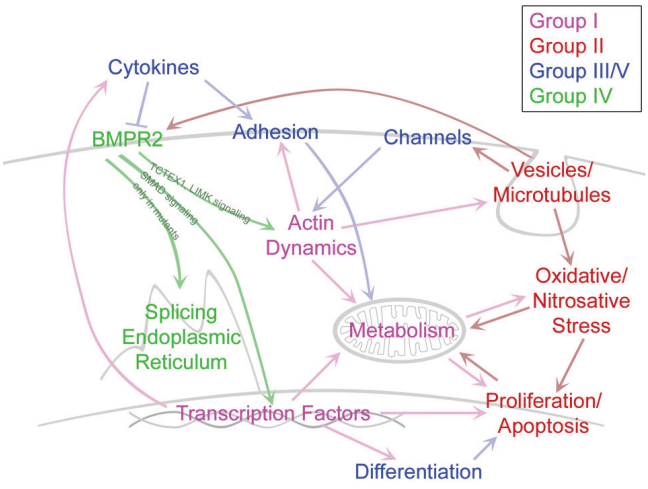

Figure 4: Relationship of gene ontology groups dysregulated in IPAH and HPAH, color coded according to the samples in which they are dysregulated. Groups I and III/V are changed in IPAH, HPAH, and UMC. Group IV is dysregulated only in HPAH and UMC. Group II is dysregulated in IPAH and HPAH, but not in UMC, and probably represents pathways critical for disease development. Arrows indicate interaction between groups indicated in the literature, but are not intended as exhaustive.

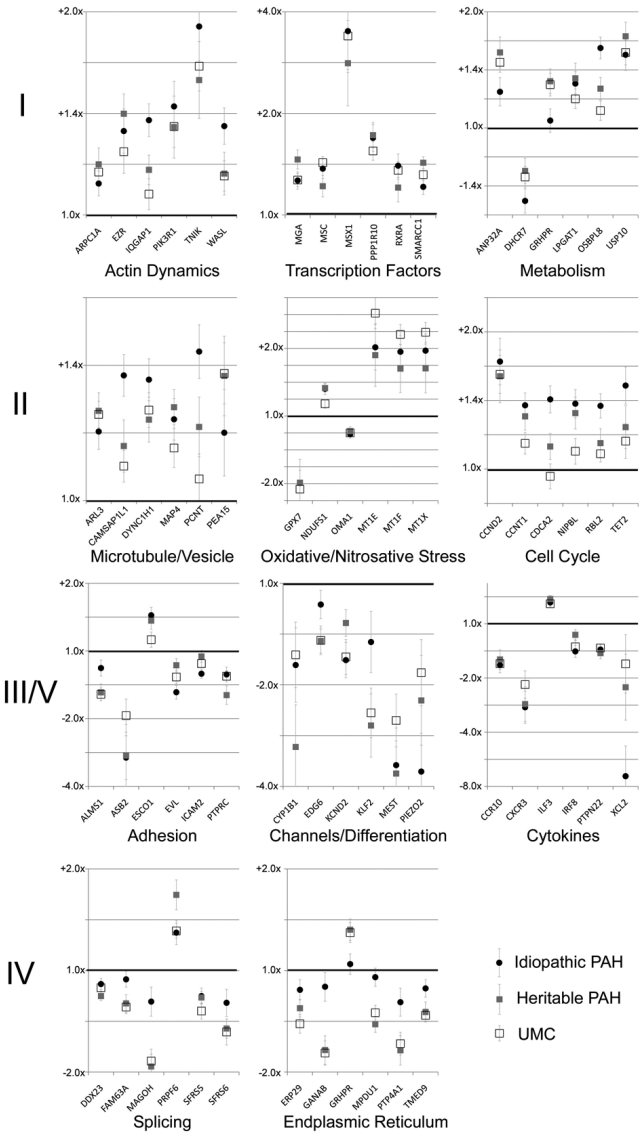

Figure 5: Example genes dysregulated in each ontology group. For each graph, fold change compared to healthy controls is plotted on the y axis. Symbols used for IPAH, HPAH, and UMC are listed in the lower right, with error bars giving SEM. The expression pattern most strongly corresponding to each group of genes is given in the left column (I-V, *see* Figs. 3 and 4).

MSX1 expression may drive other expression changes

Risk for IPAH is not caused by independently altered regulation of the 439 genes described above: rather, these alterations are all probably the consequence of alterations in a very small number of genes. MSX1 (muscle segment homeodomain-like homeobox 1) is a strong candidate as an upstream factor regulating much of the alteration seen in IPAH. It shows by far the strongest upregulation of any gene in IPAH, and as a transcription factor likely regulates the expression of many genes downstream. Roughly two thirds (276) of the 439 genes significantly ($P<0.05$ by correlation z-test) correlate with MSX1 expression, either positively or negatively. For instance, MSX1 has a correlation coefficient of 0.59 with the transcription factor Musculin (MSC) (Fig. 6a). This correlation is not a result of bias in selecting genes differentially regulated in disease: correlation between these two genes, considering only healthy controls, is a very high 0.57.

Moreover, it is possible that many of the 163 genes not directly correlated with MSX1 could still be regulated by it as a second order effect; for instance, as shown above, MSX1 expression correlates strongly with that of MSC, itself a transcription factor important in muscle development.[31] There are an additional 69 genes that significantly correlate with MSC expression that do not correlate with MSX1 expression. Thus, through a cascade of transcription factors, MSX1 could plausibly be responsible for the majority of the changes seen in IPAH. Note, however, that until it is defined by further experiments (e.g., a transgenic overexpression mouse), this remains only a correlation, not causation.

MSX1 targets may be altered in IPAH

Next, we considered whether the correlations between MSX1 and other genes altered in PAH were subgroup dependent. We found that the correlations between MSX1 and other genes were the same when those correlations considered only healthy controls as when those correlations considered only HPAH patients (Fig. 6b, correlation=0.83, $P<0.0001$ by correlation z-test). This

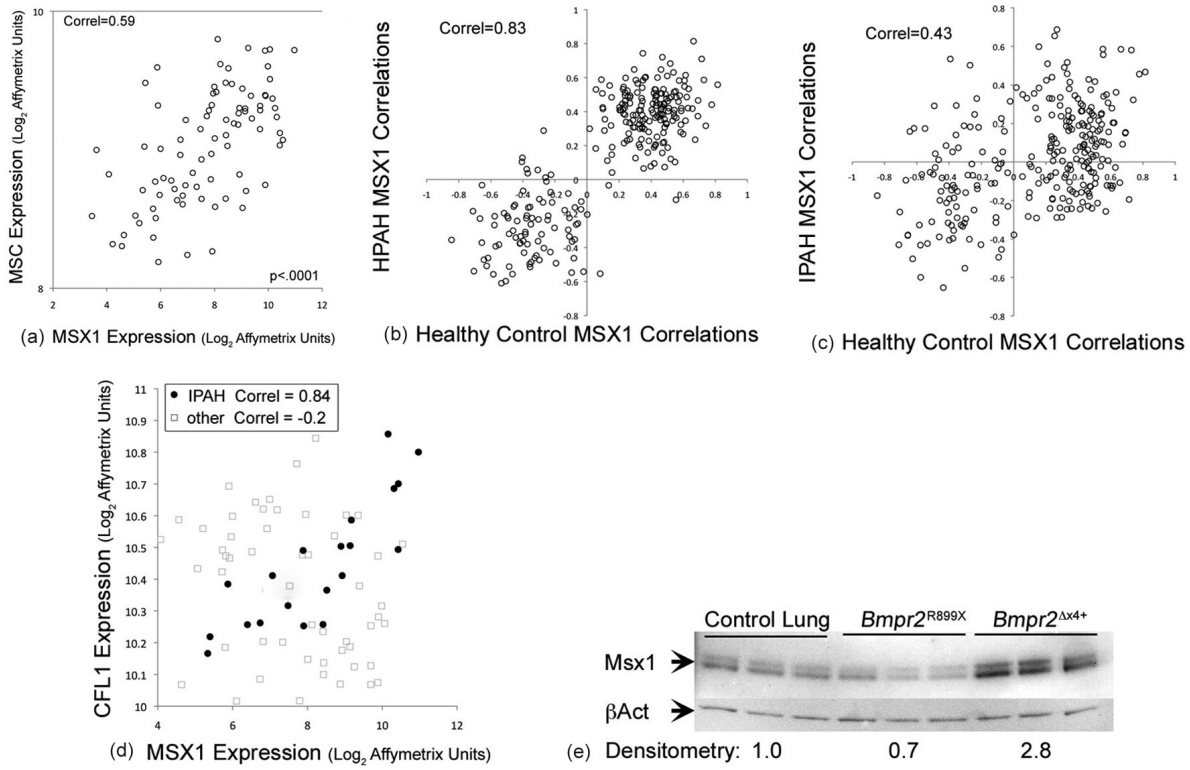

Figure 6: Transcription factor MSX1 alterations may be upstream of many other changes found. (a) MSX1 expression pattern correlates significantly with the pattern in most other genes; the transcription factor MSC is one example. Each point indicates the expression in a single patient of MSX1 (X axis) and MSC (y axis). (b) Strong MSX1 correlations with other altered gene expression patterns are not an artifact of how the genes were selected: correlations exist even within groups, not just between groups. Each point represents a gene, with correlation to MSX1 considering only healthy control data plotted on the x axis, and considering only HPAH patients plotted on the y axis. (c) MSX1 target genes may be changed in IPAH. Figure is similar to part B above, with the substitution of IPAH for HPAH on the y axis. Correlation between IPAH and healthy controls is much weaker (0.43) than correlation between HPAH and healthy controls (0.83). (d) F-actin regulator CFL1 expression (y axis) strongly correlates with MSX1 expression (x axis) in IPAH (filled circles) but not in HPAH (open circles). (e) MSX1 protein levels are increased roughly 3× in lungs from mice with a BMPR2 mutation inhibiting SMAD signaling ($BMPR2^{\Delta x4+}$), but not in lungs from mice with a BMPR2 mutation in which SMAD signaling is intact ($BMPR2^{R899X}$).

suggests that, while MSX1 is upregulated in HPAH as compared to controls, it is regulating transcription of the same genes in the same way as in healthy controls. However, when we make the same plot using IPAH rather than HPAH (Fig. 6c), the correlation becomes much weaker. This suggests that MSX1 targets may change, or change from repression to activation, in the context of IPAH.

One example of this is the central F-actin regulatory gene, cofilin (CFL1), which is functionally (not transcriptionally) regulated directly by BMPR2.[32] CFL1 expression correlates with MSX1 expression with a strength of 0.84 (Fig. 6d, $P<0.0001$), but only in IPAH: the overall correlation is −0.03. This suggests that while CFL1 has altered regulation through phosphorylation in HPAH, it may be transcriptionally regulated in IPAH.

MSX1 expression in lung is suppressed by SMAD signaling through BMPR2

Previous studies had suggested that MSX1 expression was regulated by BMP signaling, and in particular that its expression was upregulated by SMAD8 activity.[33] This finding contrasts with the current study, in which, at least in lymphocytes, loss of BMPR2 correlated with increased expression of MSX1.

To directly test the hypothesis that loss of BMP signaling in the lung led to increased MSX1 expression, we used archived frozen lung tissue from our existing BMPR2 mutant mouse models. Western blots were performed using protein from lung tissue from Rosa26-rtTA2 × $Bmpr2^{R899X}$, Rosa26-rtTA2 × $Bmpr2^{\Delta x4+}$, and control Rosa26-rtTA2 only mice. These mice have universal doxycycline inducible expression of a mutation that leaves SMAD signaling intact, R899X, or a mutation that destroys SMAD signaling, ΔX4+.[15,27,34] We found that Msx1 protein levels were nearly 3x increased in lungs from the Rosa26-rtTA2 x $Bmpr2^{\Delta x4}$ mice, but slightly downregulated in lungs from the Rosa26-rtTA2 × $Bmpr2^{R899X}$ mice (which have slightly elevated SMAD signaling, probably compensatory) (Fig. 6e). Pulmonary microvascular endothelial cells cultured from these mice showed a similar pattern of MSX1 protein expression (not shown).

These results show that increased MSX1 is a consequence of loss of SMAD signaling through BMPR2 in mouse lung. The discrepancy between this and earlier reports may relate to different tissue types assayed, or because the prior study's approach was indirect (they demonstrated SMAD binding to the MSX1 promoter, but never directly tested whether it was a repressor or an activator in a non-overexpressed setting).[33]

DISCUSSION

This study presents unique insights into the molecular pathogenesis of IPAH and how it relates to BMPR2 mutation, both with and without PAH. The central results are as follows. First, IPAH shares most altered molecular pathways with HPAH (Figs. 2-5). The exceptions to this are the pathways likely directly influenced by processing a mutant BMPR2, rather than the signaling consequences of mutation. Second, while much of the core molecular etiology is shared, global gene expression in most IPAH is molecularly distinct, both from HPAH and from healthy controls (Fig. 3). This indicates that risk for IPAH is at least partially genetic, but that it is usually not caused by cryptic problems with BMPR2. Third, at least in this sample, there seems to be only one IPAH molecular signature that is distinguishable from HPAH: suggesting there are not multiple common etiologies of adult onset IPAH (Fig. 3, dendogram at top). Finally, these points suggest that IPAH has a molecular origin very closely related to BMPR2 mutation, but is not BMPR2 mutation. One possibility is the transcription factor MSX1, which demonstrates the most prominent upregulation of any gene in IPAH patient samples.

The set of pathways dysregulated in IPAH, as presented in (Fig. 4), form a summary of much of the current state of PAH research. Examining these in detail is thus a topic more appropriate for a review article than for this discussion. However the mechanism by which BMPR2 mutation leads to PAH can be summarized as follows. BMPR2 mutation results in defects in SMAD signaling through a kinase domain,[35] and actin dynamics through direct BMPR2 interactors TCTEX1 and LIMK1.[32,36] Decreased SMAD signaling results in alteration in gene transcription, including dedifferentiation of smooth muscle,[4] increased adhesion of inflammatory cells and decreased cell-cell adhesion in endothelial cells,[3,37] and alterations in cell cycle, driving some cells to proliferate and others to apoptose (both mechanisms are increased, probably in different cell types). Altered signaling through TCTEX1 and LIMK1 lead to defects in actin dynamics and cytoskeletal trafficking mechanisms, causing metabolic problems[34] and vesicle trafficking defects,[38] and more cell-cell adhesion defects, among other problems.[5] The combination of these defects leads to increased oxidative and nitrosative stress,[34,39] which cause additional injury in a feedback mechanism that may drive PAH. All of these pathways are also deranged, to a very similar degree, in idiopathic PAH, suggesting a very closely related genetic origin.

One possibility as an initiating risk factor for IPAH is increased expression of the transcription factor MSX1. The MSX1 promoter is directly bound[33] and repressed

(Fig. 6) by SMAD transcription factors. MSX1 has roughly 20 known polymorphisms in the first kilobase of upstream promoter, but is also regulated by an antisense promoter, with the ratio of sense to antisense transcript determining protein level.[40] It thus has quite complex regulation. Functionally, BMPR2 mutations that decrease SMAD signaling result in increased MSX1 expression in lung (Fig. 6e). Moreover, MSX1 also regulates BMP expression; MSX1 knockouts have impaired BMP signal and blood vessel maturation.[41] Upregulated MSX1 has also been correlated with capillary regression.[42] Thus, MSX1 overexpression is a plausible candidate as a driver of IPAH based on the literature. To test it as a candidate, we plan studies both of MSX1 promoter polymorphisms in IPAH patients and effects of MSX1 overexpression in cell culture and mice.

There are several limitations to our study. First, interpretation of our study relies on the hypothesis that the gene expression differences we see are the functional outcome of genetic differences; that they are a cause of disease, not an effect of disease. This hypothesis is supported both by our previous success in using results of this methodology,[15,16] in the presence of many of the same changes in UMC, which do not have end-stage disease, and in the dissimilarity between these results and results from freshly isolated lymphocytes. Second, because lymphocytes are probably not a disease effector cell, there may be important pathways that can not be interrogated in this cell type. This does not invalidate the pathways discovered, but suggests that there may be additional pathways not able to be seen in this cell type. Third, our patient numbers, while substantially larger than in any previous array study, are sufficiently limited that we may not have seen etiologies associated with less common causes: genes with altered regulation in only one or two of our IPAH samples would not have been detected in our current study design. Further, good tests are not available to detect silent pulmonary vascular disease, so the patient categories can migrate: UMC develop clinical disease and become HPAH. Not all IPAH are clinically identical; for instance, one of the IPAH patients developed disease at age 3, but has survived for 30 years. One must imagine that this patient has protective factors, but once again, our study design cannot distinguish them from normal human variation. Finally, there is the question of scope. We have not yet tested functional consequences of any of these changes, although this is an important future direction.

CONCLUSIONS

In conclusion, we find that the molecular pathways altered in IPAH are very similar, but not identical, to HPAH. These molecular defects encompass much of the current state of the art in PAH research, indicating that while these pathways may seem separate, they must be part of an indispensable whole. As a practical consequence of these facts, treatments aimed at downstream molecular consequences of HPAH will also be effective against IPAH.

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