Introduction

Pulmonary hypertension (PH) refers to a spectrum of diseases where the pulmonary artery pressure is elevated. A new classification of PH has recently been proposed [1]. No cause can be elucidated in primary (or sporadic, idiopathic) pulmonary hypertension (PPH). Secondary forms of PH can occur in association with congenital heart disease, thromboembolic disease, HIV, anorexigen usage, and a variety of connective tissue disorders. Familial primary pulmonary hypertension (FPPH) has been associated with heterozygous germline mutations in the bone morphogenetic protein type II receptor gene (BMPR2) [2,3]. While this recent discovery has generated extreme interest, the pathobiology of severe PH remains enigmatic. Recent genomic approaches to investigate PH are reviewed. Early studies investigated the alterations of vasoactive and growth factor related genes. Animal models, using either pharmaceutical approaches, transgenics, or targeted disruption of genes, have allowed for whole animal modeling of specific pathways in the development of PH. Progress in medical genetic investigations has lead to the discovery of a gene (BMPR2) associated with FPPH. Finally, microarray gene expression analysis has been utilized to investigate animal models, and has shown to be a useful tool providing novel information and better characterization of the molecular pathobiology of distinct clinical phenotypes of PH.

Genes involved in the pathobiology of PH

Most investigations of the role of specific genes in the pathobiology of PH have focused either on the balance of vasoconstriction and vasodilation or on specific growth
factors, inflammatory mediators, or ion channels. Another approach has been to compartmentalize the vasculature, and focus the investigations on the endothelium, smooth muscle cells, and the adventitia/extracellular matrix. Christman et al initially reported an imbalance of prostacyclin (PGI₂) and thromboxane metabolites in the urine of patients with both primary and secondary forms of PH, with more vasoconstrictor thromboxane metabolites in patients with PH [4]. Giaid et al similarly studied the expression of endothelin-1 in the lungs of patients with PH, and showed increased expression by both in situ hybridization and immunohistochemistry [5]. Overexpression of 5-lipoxygenase and 5-lipoxygenase activating protein was shown in endothelial cells of plexiform lesions and inflammatory cells in patients with PPH, suggesting that overexpression of enzymes involved in generation of inflammatory mediators may play a role in the pathogenesis of PPH [6]. As there is an imbalance of PGI₂ and thromboxane, we wondered whether PPH patients had diminished synthetic enzyme for PGI₂. We demonstrated, by in situ hybridization, western analysis and immunohistochemistry, that patients with PPH have decreased lung tissue prostacyclin synthase (PGIS) [7]. A comprehensive histochemical analysis of plexiform lesions was performed by Cool et al [8]. This analysis showed that the endothelial cells of plexiform lesions express, intensely and uniformly, the vascular endothelial growth factor receptor KDR. The analysis by Cool et al also showed that the cells segregate phenotypically into cyclin-kinase inhibitor p27/kip1-negative cells in the central core of the plexiform lesion and p27/kip1-positive cells in peripheral areas adjacent to incipient blood vessel formation. Using immunohistochemistry and three-dimensional reconstruction techniques, the plexiform lesions were shown to be dynamic vascular structures characterized by at least two endothelial cell phenotypes. Despite these powerful investigations, a unifying pathobiological scheme has remained elusive.

Animal models of PH
Commonly utilized models of PH in animals are the chronic hypoxic model and the monocrotaline model. Interestingly, monocrotaline causes PH in the rat, but not the mouse. Exactly how closely the animal models recapitulate human disease remains a source of debate. These two models have, however, been useful for hypothesis testing and determining the response of genetically altered animals. Several specific genes have been targeted for investigation in rodent models.

5-Lipoxygenase
Mice with targeted disruption of 5-lipoxygenase were subjected to chronic hypoxia [9]. These mice developed less right ventricular hypertrophy than matched controls, supporting the hypothesis that 5-lipoxygenase is involved in pulmonary vascular tone in rodent hypoxia models.

Nitric oxide synthase
Targeted disruption of the endothelial nitric oxide synthase (eNOS) gene results in mice with increased susceptibility to hypoxic-induced PH [10]. These studies conclude that eNOS-derived nitric oxide is an important modulator of the pulmonary vascular response to chronic hypoxia, and more than 50% of eNOS expression is required to maintain normal pulmonary vascular tone [10].

PGIS and prostacyclin receptor
We hypothesized that selective pulmonary overexpression of PGIS may prevent the development of PH. Transgenic mice were created with selective pulmonary PGIS overexpression using a construct of the 3.7 kb human surfactant protein-C promoter and the rat PGIS cDNA. Transgenic mice (Tg+) and nontransgenic littermates (Tg−) were subjected to a simulated altitude of 17,000 feet for 5 weeks. After exposure to chronic hypobaric hypoxia, Tg+ mice have lower right ventricular systolic pressure than do Tg− mice. Histologic examination of the lungs revealed nearly normal arteriolar vessels in the Tg+ mice in comparison with vessel wall hypertrophy in the Tg− mice. The Tg+ mice were thus protected from the development of PH after exposure to chronic hypobaric hypoxia. We conclude that PGIS plays a major role in modifying the pulmonary vascular response to chronic hypoxia. Additional data investigating the prostacyclin receptor knockout mice support the important modulating role of PGI₂ since chronic hypoxic PH is more severe in these prostacyclin receptor knockout mice when compared with the wild-type animals [11]. This has important implications for the pathogenesis and treatment of severe PH [12].

Matrix metalloproteinase and serine elastase
Important changes occur in PH in the vascular adventitia, with increased production of the extracellular matrix. Matrix metalloproteinases can stimulate the production of mitogenic co-factors, such as tenascin. Cowan et al recently showed that direct inhibition of serine elastases led to complete regression of pathological changes in experimental PH caused by monocrotaline [13].

Vascular endothelial growth factor
In contrast to the human disease, classical rodent models of hypoxia and monocrotaline lack the clustered proliferation of endothelial cells. Taraseviciene-Stewart et al recently showed that chronic administration of a vascular endothelial growth factor-2 inhibitor in chronically hypoxic rats lead, first, to endothelial cell death, then to obliteration of the vessel lumen by proliferating endothelial cells and, finally, to PH [14]. A broad spectrum caspase inhibitor blocked this proliferation. This model more accurately depicts the cellular events seen in the human condition.
Gene transfer
The promise of gene transfer therapy remains the ‘Holy Grail’ for many genetic diseases as well as diseases that exhibit a specific enzyme deficiency. PH is no exception. Adenoviral gene transfer has been used in rats to show diminished response to acute hypoxia. This has been accomplished by transfer of eNOS [15] and by gene therapy with PGIS [16]. Long-term benefit in chronic hypoxia has not been reported. Repeated adenoviral PGIS transfection has shown some effectiveness in decreasing PH in rats using the monocrotaline model [17].

Microarray expression analysis of animal models
We performed microarray analysis of our PGIS Tg+ animals to determine the global changes in gene expression caused by PGIS overexpression. Transgene negative littermates were examined as controls. The mRNA from five transgenic mouse lungs was pooled and compared with five nontransgenic, sex-matched littermates. Using strict criteria (a twofold change in expression), we determined that a definable number of genes was differentially expressed between the lungs of transgenic and nontransgenic animals. Of the 6500 genes surveyed, 32 genes showed an increase in expression and 26 showed a decrease in expression. Table 1 presents genes that demonstrate the most significant changes in expression (at least a 2.2-fold change) when comparing the lung mRNA from transgenic and nontransgenic mice.

Array analysis importantly demonstrated changes in both peroxisome proliferator-activated receptor (PPAR) λ and PPAR δ, and we have followed up these studies with work demonstrating that prostacyclin activates PPAR δ in colorectal cancer [18]. Histochimical analysis in human colorectal tumors demonstrated colocalization of PPAR δ and cyclooxygenase-2. An experimental condition was created in which PGIL2 production could be correlated with PPAR δ transcriptional activity. Transient transfection assays established that endogenously synthesized PGIL2 could serve as a ligand for PPAR δ. A stable PGIL2 analog also induces transactivation of PPAR δ in human colon cancer cells, demonstrating that endogenous PPAR δ is transcriptionally responsive to PGIL2 [18].

Human medical genetics
FPPH is an autosomal dominant disorder that is indistinguishable from sporadic PPH. The disease has reduced penetrance, and over 90% of patients have no known family history of the disease [19]. Linkage analysis in affected families enabled the locus to be defined within a 3 cM region of chromosome 2q33. Using a positional candidate-gene strategy, two groups were subsequently able to independently confirm that heterozygous germline mutations in BMPR2 cause FPPH [2,3]. Using a high-throughput denaturing high-performance liquid chromatography approach [20] has enabled the rapid identification of numerous mutations responsible for haploinsufficiency of BMPR2 [2]. Furthermore, germline mutations of BMPR2 have also been identified in ~26% of sporadic cases of PPH [21]. ‘Sporadic’ cases sometimes actually represented occult familial cases of PPH [21]. The molecular spectrum of BMPR2 mutations is more fully elucidated in an analysis of 47 European families [22]. The majority of mutations (58%) are predicted to lead to premature termination codons. However, mutations in BMPR2 have not been found in 45% of families with PPH [22]. A number of possible explanations for this fact are possible, including mutations in intronic and 3′-untranslated regions that are heretofore not examined, rearrangements in the transcribed gene that may occur, or genetic heterogeneity perhaps playing a role.

BMPR2 encodes a type II receptor member of the transforming growth factor-β (TGF-β) superfamily. Type II receptors, which have serine/threonine kinase activity, act as cell-signaling molecules. Following ligand binding, type II receptors form heteromeric complexes with membrane-bound type I receptors. This initiates phosphorylation of the type I receptor and downstream intracellular Smads [23]. This pathway is diverse and the specificity in cell growth and differentiation appears to be mediated through transcriptional control. The importance of the TGF-β pathway in vascular disorders is evidenced by the fact that two other components of this pathway, endoglin and the activin receptor-like kinase-1 gene, are mutated in hereditary hemorrhagic telangectasia [24,25].

Mutational analysis
Lee et al [26] recently demonstrated that the endothelial cells within plexiform lesions of patients with PPH expand in a monoclonal fashion, whereas secondary PH lesions develop via polyclonal expansion of endothelial cells.

| Genes demonstrating the most significant changes in expression | Genes with significantly increased expression | Genes with significantly decreased expression |
|-------------------------------------------------------------|---------------------------------------------|---------------------------------------------|
| PPAR γ                                                     | PPAR δ                                     |                                             |
| RAS GTPase                                                 | Cyclooxygenase-2                           |                                             |
| Focal adhesion kinase                                       | Multidrug resistance protein               |                                             |
| Keratinocyte growth factor receptor                        | α-Catenin                                  |                                             |
| Epidermal growth factor                                    | TGF-β and TGF-β receptor                   |                                             |
| IL-7 and IL-17 receptors                                    | Wilm’s tumor gene                          |                                             |
| Cathepsins C, D, and E                                      | BCR-abl                                    |                                             |

PPAR, Peroxisome proliferator-activated receptor; TGF, transforming growth factor.
lesions demonstrating microsatellite instability; second, within PPH lesions are genetically unstable, with 50% of [31]. The results showed that: first, the endothelial cells with sporadic PPH and those with secondary forms of PH formed microdissection of plexiform lesions from patients microsatellite instability signaling genes, Yeager exhibit microsatellite instability and mutations in TGF-

To investigate whether cells within plexiform lesions exhibit microsatellite instability and mutations in TGF-

mutations in regulatory genes such as TGF-

Expression analysis of human PPH

Gene microarray technology [32] now permits the analysis of the gene expression profile of lung tissue obtained from patients with primary PH to compare with that found in normal lung tissue. Because the vascular lesions are homogeneously distributed throughout the entire lung, a tissue fragment of the lung is probably representative of the whole lung. RNA extracted from such fragments is likely to provide meaningful information regarding the changes in gene expression pattern in PPH when compared with structurally normal lung tissue. We can model the range of normality by examining a sufficient number of lung tissue samples. Methods exist for determining coordination in expression data using cluster expression profiles. Cluster analysis can give clues to the pathogenesis by displaying genes whose expression is altered in a coordinate manner. Finally, an important goal is to discern sets of genes that differentiate between normal and disease states – or discrimination analysis. Building discrimination models has a long history in statistical pattern recognition and machine learning, and has been applied to cancer paradigms using gene expression data [33]. For our study, we used Affymetrix oligonucleotide microarrays (human FL) to characterize the expression pattern in the lung tissue obtained from six patients with PPH, including two patients with FPPH, and from six patients with histologically normal lungs [34].

Although the number of patient samples was small, gene dendogram, cluster analysis and concordant expression differences show that there are categorical and robust differences in the profile of expressed genes between structurally normal lungs, lungs from patients with sporadic PPH, and lungs from patients with FPPH. We began our study of differential gene expression in PPH with the assumption that sporadic PPH is a disease with typical and dramatic histological features, which are sufficiently distinct from the structurally normal lung but essentially indistinguishable from those features found in FPPH lungs. We found that only 307 genes were significantly different in their expression when PH tissues were com-
pared with structurally normal lung tissues. Genes encoding ribosomal, mitochondrial and cytoskeletal proteins and genes encoding ion channels and enzymes were differentially expressed between PH and normal lungs. Several transcription factor genes and genes related to cyclin-dependent kinases were different in their expression, indicating that the PH gene signature reflects a profound imbalance in the control of genes involved in cell proliferation and apoptosis. Furthermore, as shown in Figure 1, whole-tissue total RNA expression profiles demonstrate striking differences in the expression signatures between sporadic and familial PPH. Importantly, the differences in expression profiles are complemented by independent gene mutation analysis. Only the plexiform lesions in the lungs from patients with sporadic PPH [31], not those lesions in FPPH lungs, display mutations of the Bax and TGF-βRII genes. It is possible that these mutational differences may lead to gene expression changes. The RNA expression data and the DNA mutation data taken together [31] lead to the conclusion that sporadic and familial PH are mechanistically distinct. In summary, microarray gene expression analysis and profiling is a useful molecular tool that provides a better characterization and understanding of the pathobiology of distinct clinical phenotypes of PH.

Conclusions
Genomic approaches to the investigation of PH in animals or relevant tissues have vastly expanded our knowledge about the pathobiology of pulmonary hypertensive diseases. Human genetic analysis will undoubtedly expand and discover further gene mutations involved in the pathogenesis of PH. Gene expression profiling of different animal models of PH, and comparison of these profiles with human PH, will assist in determining the complex pathways that comprise the response that we term ‘pulmonary hypertensive tissue remodeling’.

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