Using Mouse Genomics to Understand Idiopathic Interstitial Fibrosis

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Idiopathic interstitial pneumonia represents a broad category of lung disorders characterized by scarring or fibrosis of the lung accompanied by varying degrees of inflammation. A number of important hypotheses based on clinical observations have substantially contributed to our understanding of the pathogenesis of the most insidious and devastating of the idiopathic interstitial pneumonias, idiopathic interstitial fibrosis (IIF). Patients with IIF usually present late in the course of their illness; thus, animal models of the early, preclinical stage of these diseases are needed. Although no model faithfully recapitulates the clinical course of disease or the histopathology observed in humans, all result in scarring of the lung and may therefore be used to understand the biological processes that contribute to this scarring. The purpose of this article is to summarize the application of mouse genetic and genomic tools to these models to advance our understanding of IIF and to describe emerging agnostic approaches to identifying genes important to the fibroproliferative component of IIF.

Keywords: fibrosis; QTL; genomics; microarray

Idiopathic interstitial fibrosis (IIF) is an insidious and devastating lung disorder characterized by scarring or fibrosis of the lung accompanied by varying degrees of inflammation (1, 2). In a recent historical perspective on the clinical aspects of human pulmonary fibrosis, Paul Noble describes how thinking about IIF has evolved from the "inflammation hypothesis," which focused on the observed inflammation as being critical to the end result of fibrosis, to the current view that there is an interaction between epithelial and mesenchymal cells that takes place that predisposes toward pulmonary fibrosis (3, 4). Growth factors were and still are considered to be pathogenically important soluble factors released by macrophages that play key roles in the evolution of pulmonary fibrosis (3, 4). These hypotheses (inflammation and growth factors) and many other more focused areas of investigation (e.g., injury/repair of the alveolar epithelia, alterations in fibrinolysis, and matrix homeostasis) have substantially contributed to our understanding of the pathogenesis of IIF. However, patients with IIF usually present late in the course of their illness, raising the possibility that the biological processes that are thought to be involved in disease pathogenesis may represent responses to the fibroproliferative process rather than primary or pathogenic events that cause IIF. To understand the primary events in IIF, animal models and strategies to identify patients at early, preclinical stages of their disease are needed.

Although there are a number of animal models of IIF, all are limited in being at least one step removed from the human

condition. Moreover, no model faithfully recapitulates the clinical course of the disease or the histopathology observed in humans. In specific diseases (e.g., sarcoidosis) (5), there are no animal models, and even in forms of interstitial lung disease where the cause of the disease is known (e.g., hypersensitivity pneumonitis, silicosis, and asbestosis), the animal models of these disorders are suboptimal (6). These limitations include the required large concentrations of organic or inorganic material, the method of administration (intratracheal bolus or extensive inhalation of high concentrations of dust), and the relatively rapid onset and progression of disease (7). However, despite having little in common with the clinical course of IIF, these different exposures (silica, asbestos, thoracic radiation, and bleomycin) produce scar tissue in the lung. Thus, even in the absence of a perfect animal model, these approaches represent important tools that may be used to map and clone genes and to understand the biology that contributes to lung scarring and fibroproliferation.

Many methods can be used to map and clone genes. The most powerful of these rely heavily on identifying phenotypic differences between inbred mouse strains. These comparisons between inbred strains combined with subsequent mapping of regions of DNA that are closely linked with a phenotype by using recombinant inbred mouse strains can identify a quantitative trait locus (QTL), or a region of the genome that is associated with a particular phenotype (8). Theoretically, each QTL should contain at least one gene or regulatory element that is pathogenically associated with the disease being studied (9). However, the record for proceeding from identification of a QTL to the identification of single genes that are unambiguously important in contributing to a particular disease phenotype is staggeringly poor (9), and such analyses have only identified some dozen or so genes (9). This highlights the need to improve our genetic tools and to use other strategies to identify disease genes. As a strategy to improve our genetic tools, efforts are underway to fully genotype 16 strains of inbred mice (10) and selectively target or knock out every gene in the mouse genome and to make this resource publicly available (11).

In silico mapping is a genomic approach that uses single nucleotide polymorphisms (SNPs) between inbred strains of mice to identify novel loci associated with a particular phenotype (12, 13). *In silico* mapping uses patterns of SNPs that define regions of the genome (haplotypes) that are inherited as a block of DNA and serve to define the relationship between different strains of mice. Thus, *in silico* mapping uses a computer algorithm to search for association between these SNP haplotypes and a particular phenotype among multiple strains of mice. Given the large amount of genetic information contained in the mouse genome, one needs to phenotype between 10 and 20 strains of mice or more to identify a specific locus with a reasonable degree of certainty.

Global transcriptional (microarray) analysis is an increasingly common methodology that is facilitated by hybridizing total RNA extracted from a biological sample onto a collection of microscopic DNA spots attached to a solid surface, such as glass, plastic, or silicon, forming an array for the purpose of examining simultaneous expression of tens of thousands of genes. Bioinformatic tools

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are then used to mine the extensive data and relate the gene expression changes to various pathophysiologic responses. Studies involving global analysis of gene expression are most often focused on genes that are significantly elevated in response to the disease-causing agent. However, we and others have identified long lists of genes that have significantly reduced gene expression compared with control subjects, suggesting that there are negative regulators that remain to be studied. All the tools being brought to bear on studies of genes that are elevated can just as easily be used to analyze genes that show decreased expression. Thus, characterizing global transcriptional responses to fibrogenic agents by microarray in mouse models of IIP have identified and will continue to identify broad categories of genes that are or may be involved in the development and progression of lung fibrosis in samples obtained from mice or humans.

In combination, these agnostic approaches (QTL mapping, *in silico* mapping, and global transcriptional analysis) have the potential to identify regions of DNA that contain genes that are differentially expressed and may provide clues to the pathogenesis of IIP. The purpose of this article is to discuss genetic and genomic approaches using mouse models and to improve our understanding of the mechanisms associated with the initiation and progression of idiopathic interstitial fibrosis and to highlight some emerging and novel approaches to genomic analyses that will meet the current challenge in the use of this technology.

GENETIC APPROACHES TO UNDERSTANDING LUNG FIBROSIS

Most of the advances in our understanding of genetic diseases over the last century have come from the identification of structural variation in single, so-called major genes. However, many diseases, such as pulmonary fibrosis, are complex and are not attributable to a single gene defect but are likely due to many gene–gene and gene–environment interactions. Heroic work has been done in the identification of QTL that contribute to the development and progression of pulmonary fibrosis using the radiation and bleomycin mouse models (14–19), and a solid QTL has been mapped on mouse chromosome 17 closely linked to the major histocompatibility complex (14), a region of the genome intimately involved in the immune response to insult and injury. In two subsequent studies using recombinant congenic mice (20), these same authors confirmed the QTL on chromosome 17 and further identified a QTL on chromosome 1 (20) specifically linked to radiation-induced fibrosis. In a further study (21), a QTL on chromosome 11 was shown to contain bleomycin hydrolase, demonstrating the sensitivity of this type of analysis. Because it is unlikely that bleomycin hydrolase evolved in response to evolutionary pressure resulting from bleomycin exposure, the authors speculate that this protein may function as a major histocompatibility complex, epitope-processing protease (21). The QTL on chromosome 17 has been confirmed in an independent study (14), and this locus has appeared in other QTL analyses for other lung injury phenotypes, including ozone-induced inflammation (22), particle exposure (23), and asthma (24, 25), suggesting that this might be a genomic region that contains a common lung injury-response gene. Therefore, the investigation of other models of fibrosis and the comparison of these results to current findings should provide a useful tool in attempting to identify other candidate genes that might be essential mediators of the fibroproliferative process.

MICROARRAY STRATEGIES USED TO CHARACTERIZE MOUSE MODELS OF IIP

The initial application of microarray technology to characterize a mouse model of interstitial lung disease (26) compared the

response in over 6,000 genes and expressed sequence tags from two inbred mouse strains that are susceptible to bleomycin-induced pulmonary fibrosis and compared these results to a dataset obtained from one of the strains (129) harboring a single gene deletion ($\beta 6$ integrin) that they had previously shown protected these mice from the fibroproliferative response but not the inflammatory response to bleomycin (26). These investigators used a straightforward cluster analysis (27) to identify subsets of genes involved in the inflammatory and fibrotic components of the response to bleomycin in the wild-type mice (26). To evaluate the genes that distinguished responsive from unresponsive mice, these investigators compared wild-type mice with the $\beta 6$ -integrin-deficient mice as a means of further delineating the differences between the inflammatory and the fibrotic gene expression profiles. In this analysis, the gene historically most strongly associated with the development of pulmonary fibrosis, transforming growth factor (TGF)- β , was not significantly differentially expressed (26). However, the analysis was able to identify a pattern of gene expression attributable to TGF- β activation, confirming the importance of $\beta 6$ -integrin activation of TGF- β (28). The hope expressed by these authors was that comprehensive data on the development and progression of fibrosis in mice would lead to more effective strategies for intervention in human patients (26).

Another early study that used an array of just over 4,000 genes (29) confirmed the findings of Kaminski and colleagues (26) that there are distinct patterns of gene expression in the bleomycin model that are associated with an inflammatory phase or a fibrotic phase (29). This analysis compared exposed mice with control mice (29), whereas Kaminski compared susceptible mice with mice with a known single gene defect that confers protection from fibrosis even in the presence of an inflammatory response typically associated with bleomycin treatment (26). Nonetheless, this study confirmed the fundamental finding of Kaminski and colleagues, and these studies taken together validate the use of microarray as a means to understand the etiology of the fibroproliferative response. In addition, the findings of Kaminski directly addressed the inflammation hypothesis as put forward by Crystal and colleagues (30) and provided support to the growth factor hypothesis suggested by Bitterman and colleagues (31) inasmuch as the $\beta 6$ integrin proved to be essential to TGF- β activation in this model (26).

The finding that TGF- β was not differentially expressed in the Kaminski study (26) highlights an important feature of microarray analyses, namely that if a gene is not regulated at the transcriptional level, the contribution of that gene can be overlooked unless deeper analyses of the data are undertaken. Kaminski and colleagues (26) knew what they were looking for and had reagents ($\beta 6$ -integrin-deficient mice) with which to address the expression profile of TGF- β -inducible genes.

There are some emerging methodologies for examining the effects of genes that are not regulated at the transcriptional level, such as genes regulated at the protein level (e.g., TGF- β and other proteins, such as transcription factors, whose activity is regulated by phosphorylation and dephosphorylation). To study a gene or genes whose general function is known, such as TGF- β , one can use a method such as that described by Sadlier and colleagues (32) in an analysis of tubulointerstitial fibrosis (TIF) in kidney. The approach as used by Sadlier and colleagues (32) identified novel genes involved in TIF by examining genes with expression profiles similar to those known to be involved in the disease process. These researchers used hierarchical clustering (27) of their dataset focused on mRNAs encoding matrix proteins followed by a secondary “baited”-global cluster analysis of gene expression (32). This two-step cluster analysis used the first-step cluster to identify patterns of extracellular matrix (ECM)

gene expression (genes known to be involved) over time during the experiment, and the second round of clustering identified genes that exhibited the same pattern of expression during the experiment (novel genes). In this way, these investigators identified molecules and pathways already implicated in the pathogenesis of TIF (e.g., TGF- β 1–connective tissue growth factor [CTGF]–fibronectin-1 pathway) and novel TIF-associated genes (32). This methodology has not been applied to pulmonary fibrosis, but, given the similarities between the disease models, this would be a viable approach to studying the late fibroproliferative phase and the early inflammatory phase in the bleomycin model or in another mouse model of pulmonary fibrosis.

Another perspective can be gained by recognizing that genes with similar expression profiles may be regulated by the same transcription factors (33). PRIMA (Promoter Integration of Microarray Analysis) and other algorithms of this type examine transcription factor binding site motifs in promoter regions and identify motifs that appear more frequently within a given group of genes (e.g., a cluster) than would be expected by chance. Burch and colleagues (34) recently used this approach to identify the ISRE (Interferon Stimulated Response Element) as an important transcriptional element in the response to inhaled LPS showing for the first time that IFN- γ plays a previously undefined role in neutrophil recruitment in this model system. Figure 1 illustrates our analysis of a publicly available dataset, obtained from the GEO (Gene Expression Omnibus) website (series accession number, GSE485), in which we demonstrate that there are subsets of genes associated with an inflammatory and a fibrotic phase. Using PRIMA analysis on the cluster of genes associated with the inflammatory response to bleomycin (Figure 1), we find the AP-1 and c-Rel transcription factor binding site motifs overrepresented ($p < 0.05$). It has long been known that AP-1 is a downstream target of mitogen-activated protein (MAP) kinase signaling (35); thus, it is not surprising that AP-1 appears in this analysis. Likewise, c-Rel, as a member of the nuclear factor- κ B family of transcription factors, is likely important in the inflammatory phase of the response to bleomycin. There were no transcription factor binding motifs overrepresented in an analysis of the genes associated with the late fibroproliferative phase from this dataset. This suggests that, although the initial inflammatory phase might be more broadly regulated, the fibroproliferative phase might be regulated by a few key genes with few regulatory components in common.

Although the focus of this article is on insights into lung fibrosis from mouse genetics and genomics, the larger goal is to understand human disease. Human lung fibrosis is a complex disease that has heretofore been characterized histologically. One recent study has made some sense of the complex transcriptional profiles of idiopathic pulmonary fibrosis (IPF), hypersensitivity pneumonitis, and nonspecific interstitial pneumonia (NSIP) (36) by identifying transcriptional profiles that are specific to IPF and hypersensitivity pneumonitis and comparing these with NSIP. That this study was not able to clearly identify a specific profile of genes associated with NSIP (36) underscores the complexity of this human disease and the limitations of histologic classification. To help address this complexity, we must return to mouse genomic studies and consider their relevance to human disease. Previous studies have identified genes from microarray data from human subjects that have been confirmed in gene-targeted mice deficient in matrix metalloproteinase 7 and osteopontin (as examples) (37–39), demonstrating that there are lessons to be learned from human studies that can be modeled in mice. For example, our laboratory has identified genes that are differentially expressed in 19 patients with IPF compared with six normal control subjects at a 5% false discovery rate and with greater than twofold over- or underexpression (Figure 2;

Table 1; and Ivana Yang, unpublished observations). Analysis of Gene Ontology categories and Kyoto Ensemble of Genes and Genomes pathways has revealed a significant enrichment of ECM genes, genes involved in skeletal development, and complement genes (Table 1). In a further analysis, we searched for mouse homologs of the genes on this list that show responsiveness to bleomycin exposure (Table 2). For this purpose, we used the gene expression data from a study of mice sensitive (C57BL/6) and resistant (Balb/C) to the fibroproliferative effects of bleomycin; the data were obtained from the GEO website (series accession number, GSE485). Among the mouse homologs to the genes identified from the human fibrosis dataset, we find that 20 homologs seem to be responsive to bleomycin and that 13 are most differentially expressed (vs. saline) at the 2 wk post-bleomycin time point, which is associated with the fibroproliferative phase of the bleomycin response (Table 2 and Figure 2). In Figure 2, the mouse data have been clustered (number of clusters k chosen as 2), showing that most of bleomycin-responsive mouse homologs are differentially expressed at the later fibroproliferative time points. However, this represents a single time point in the context of a complex and dynamic biological response; in this example, we see that most of the differential expression observed in this human dataset can be identified with the late (2 wk) fibroproliferative phase of the bleomycin response rather than the inflammatory component. Of the genes on this list, a majority of them have been previously identified as playing a role in fibrosis in mouse models, in human disease, or in both. These include the procollagens III α I and V α 2, IGF1 (40), tenascin C in human (41) and rat (42), Spp1 (osteopontin) (43), integrin α V (associated with β 6) (28), TIMP-1 in human (44) and mouse (45), tissue plasminogen activator (46), and surfactant protein C in human (47) and mouse (48). Genes on the short list in Table 2 that have recently been characterized with respect to their role in a fibroproliferative phenotype include CXCL12 (49) and thrombospondin 2 (50). CXCL12 has recently been shown to play a role in trafficking circulating fibrocytes to the lung in response to bleomycin (49), and the role of CXCR4, the receptor for CXCL12, has recently been demonstrated to be important in the development of fibroproliferative lesions in response to intratracheal bleomycin (I. V. Yang, unpublished data). This short list of genes derived from mouse homologs to genes identified by microarray of human IPF samples demonstrates that there remains much to be learned about the fibroproliferative process and has identified some genes that are worthy of further investigation in mouse model systems.

LESSONS FROM OTHER MODEL SYSTEMS

Although there is a substantial amount of information to be derived from directly modeling human IIF in mice, a hypothesis in our laboratory is that there are a few critical mediators of a fibroproliferative response regardless of the location in the lung and independent of the etiologic agent. Thus, an approach to understanding this generalized fibroproliferative response is to consider genes that are involved in other model systems. For example, there is increasing evidence that there is a fibroproliferative component to airway remodeling seen in reactive airway diseases such as asthma and chronic obstructive pulmonary disease (51, 52). Our laboratory has focused attention on environmental exposures known to produce asthma-like symptoms in agricultural workers. In mice, we have shown that repeated long-term exposure to inhaled LPS causes all of the classical features of asthma, including reversible airway obstruction, repeated episodes of inflammation, and airway remodeling, with a strong fibroproliferative component that persists over time and that is similar in many respects to human asthma (53–56). In C57BL/6

Figure 2. Cluster analysis (*left*) of genes that are differentially expressed in 19 patients with idiopathic pulmonary fibrosis compared with six normal control subjects at a 5% false discovery rate and with greater than twofold over- or underexpression (Table 1). (*Right*) Expression changes observed in the mouse homologs of these genes over the course of a bleomycin exposure experiment in mice (data obtained from the Gene Expression Omnibus [series accession number, GSE485]); the genes have been clustered (grouped by similarity in expression) into two clusters to highlight the large fraction of genes observed to be up-regulated toward the late stage of the bleomycin exposure (contrast with the early/sustained inflammatory response shown in Figure 1). The graph (*bottom right*) shows this component of expression from a different perspective to illustrate the stronger response seen in sensitive versus resistant mice. Red bars, Balb/C saline; orange bars, Balb/C bleomycin; green bars, C57BL/6 saline; black bars, C57BL/6 bleomycin.

Figure 3. Venn diagram of the intersection between LPS- and bleomycin-responsive gene list.

mice, we identified by microarray analysis over 600 genes that are significantly differentially expressed when compared with age-matched unexposed control mice. Because we have demonstrated that chronic LPS-induced airway remodeling is a fibroproliferative disorder (55, 56, 57), we have interrogated our gene list with a publicly available dataset in which there are 186 significantly differentially expressed genes at 14 d after bleomycin instillation in C57BL/6 mice. By interrogating our LPS-induced gene list with the bleomycin list, we have identified 49 genes that are significantly differentially expressed in common between these disparate model systems (Figure 3 and Table 3). Because there is a profound inflammatory component associated with LPS-induced airway remodeling and bleomycin-induced fibrosis, it is not surprising to see such genes as *Saa3* and many chemokines at the top of the list. Many of these genes have previously been reported to be involved in more traditional

TABLE 1. GENES DIFFERENTIALLY EXPRESSED IN LUNG TISSUE FROM SUBJECTS WITH PULMONARY FIBROSIS AS COMPARED WITH CONTROL SUBJECTS

Symbol	Fibrosis/Control	Name
COL3A1	11.5	Collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)
COL1A1	10.4	Collagen, type I, alpha 1
COL1A2	8.0	Collagen, type I, alpha 2
POSTN	7.3	Periostin, osteoblast specific factor
CXCL14	5.5	Chemokine (C-X-C motif) ligand 14
MMP7	5.2	Matrix metalloproteinase 7 (matrilysin, uterine)
IGFBP5	4.8	Insulin-like growth factor binding protein 5
COL6A3	4.6	Collagen, type VI, alpha 3
IGF1	4.5	Insulin-like growth factor 1 (somatomedin C)
COL15A1	4.5	Collagen, type XV, alpha 1
SFTPC	0.2	Surfactant, pulmonary-associated protein C /// surfactant, pulmonary-associated protein C
C1S	4.2	Complement component 1, s subcomponent
SRP68	0.2	Signal recognition particle 68kD
ASPN	3.9	Asporin (LRR class 1)
MMP1	3.9	Matrix metalloproteinase 1 (interstitial collagenase)
SPP1	3.8	Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)
HNRPDL	3.7	Heterogeneous nuclear ribonucleoprotein D-like
CFH /// CFHL1	3.7	Complement factor H /// complement factor H-related 1
RARRES1	3.7	Retinoic acid receptor responder (tazarotene induced) 1
CYP1B1	3.6	Cytochrome P450, family 1, subfamily B, polypeptide 1
S100A2	3.6	S100 calcium binding protein A2
FOSB	0.3	FBJ murine osteosarcoma viral oncogene homolog B
CXCL12	3.4	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)
COL14A1	3.3	Collagen, type XIV, alpha 1 (undulin)
COL5A2	3.3	Collagen, type V, alpha 2
SRPX	3.2	Sushi-repeat-containing protein, X-linked
CSPG2	3.2	Chondroitin sulfate proteoglycan 2 (versican)
TIMP1	3.1	Tissue inhibitor of metalloproteinase 1 (erythroid potentiating activity, collagenase inhibitor)
THBS2	3.1	Thrombospondin 2
FBN1	3.1	Fibrillin 1 (Marfan syndrome)
CA4	0.4	Carbonic anhydrase IV
EGR1	0.4	Early growth response 1
COL5A1	2.8	Collagen, type V, alpha 1
KRT15	2.8	Keratin 15
ITGAV	2.8	Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)
C1orf63	2.8	Chromosome 1 open reading frame 63
D15F37 /// LOC440248	2.8	D15F37 gene /// hypothetical LOC440248
PLA2G2A	2.7	Phospholipase A2, group IIA (platelets, synovial fluid)
SERPINF1	2.7	Serine (or cysteine) proteinase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium-derived factor), member 1
TNC	2.7	Tenascin C (hexabrachion)
IGFBP3	2.7	Insulin-like growth factor binding protein 3
LXN	2.7	Latexin
SPON1	2.7	Spondin 1, extracellular matrix protein
OGT	2.6	O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine: polypeptide-N-acetylglucosaminyl transferase)
CLK1	2.6	CDC-like kinase 1
STEAP1	2.6	Six transmembrane epithelial antigen of the prostate 1
CCNG1	2.5	Cyclin G1
FKBP11	2.5	FK506 binding protein 11, 19 kD
SEC22L1	2.5	SEC22 vesicle trafficking protein-like 1 (<i>Saccharomyces cerevisiae</i>)
MMP2	2.4	Matrix metalloproteinase 2 (gelatinase A, 72 kD gelatinase, 72 kD type IV collagenase)
PLSCR4	2.4	Phospholipid scramblase 4
C7	2.4	Complement component 7
TMEM45A	2.4	Transmembrane protein 45A
TIA1	2.4	TIA1 cytotoxic granule-associated RNA binding protein
GDF15	2.3	Growth differentiation factor 15
SMPDL3A	2.3	Sphingomyelin phosphodiesterase, acid-like 3A
SEC23A	2.3	Sec23 homolog A (<i>S. cerevisiae</i>)
IF	2.3	I factor (complement)
USP34	2.3	Ubiquitin-specific protease 34
TU3A	0.4	TU3A protein
ROBO1	2.3	Roundabout, axon guidance receptor, homolog 1 (<i>Drosophila</i>)
PPIC	2.3	Peptidylprolyl isomerase C (cyclophilin C)
NPR3	0.4	Natriuretic peptide receptor C/guanylate cyclase C (atrionatriuretic peptide receptor C)
METTL3	2.3	Methyltransferase like 3
TRIM2	2.3	Tripartite motif-containing 2
COL16A1	2.2	Collagen, type XVI, alpha 1
LOC161527	2.2	Hypothetical protein LOC161527
MAF	2.2	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)
IARS	2.2	Isoleucine-tRNA synthetase

(Continued)

TABLE 1. (Continued)

Symbol	Fibrosis/Control	Name
<i>PLOD2</i>	2.2	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2
<i>ChGn</i>	2.2	Chondroitin beta 1,4 N-acetylgalactosaminyltransferase
<i>MXRAS</i>	2.2	Matrix-remodeling associated 5
<i>COMP</i>	2.1	Cartilage oligomeric matrix protein
<i>PRPF4B</i>	2.1	PRP4 pre-mRNA processing factor 4 homolog B (yeast)
<i>CSRP2</i>	2.1	Cysteine and glycine-rich protein 2
<i>PPAP2A</i>	2.1	Phosphatidic acid phosphatase type 2A
<i>FHL2</i>	2.1	Four and a half LIM domains 2
<i>ANKRD10</i>	2.1	Ankyrin repeat domain 10
<i>CFH</i>	2.1	Complement factor H
<i>PPP1R3C</i>	2.1	Protein phosphatase 1, regulatory (inhibitor) subunit 3C
<i>FLRT2</i>	2.1	Fibronectin leucine-rich transmembrane protein 2
<i>FUBP1</i>	2.1	Far upstream element binding protein 1
<i>CTSK</i>	2.1	Cathepsin K (pseudosyndactyl)
<i>KIAA0907</i>	2.1	KIAA0907
<i>SULF1</i>	2.1	Sulfatase 1
<i>ATR</i>	2.1	Ataxia telangiectasia and Rad3 related
<i>GPI7</i>	2.1	GPI7 protein
<i>ELTD1</i>	2.0	EGF, latrophilin, and seven transmembrane domain containing 1
<i>SNAI2</i>	2.0	Snail homolog 2 (<i>Drosophila</i>)
<i>PLAT</i>	2.0	Plasminogen activator, tissue
<i>BTA1</i>	2.0	BTA1 RNA polymerase II, B-TFIID transcription factor-associated, 170 kD (Mot1 homolog, <i>S. cerevisiae</i>)
<i>ZNF83</i>	2.0	Zinc finger protein 83 (HPF1)
<i>APOD</i>	2.0	Apolipoprotein D
<i>FLJ43663</i>	0.5	Hypothetical protein FLJ43663

False discovery rate < 5% by significance analysis of microarray data (SAM) and fold difference > 2 profiled on the Affymetrix U133A array. All but eight show increased expression with fibrosis. Analysis of Gene Ontology terms and Kyoto Ensemble of Genes and Genomes pathways finds a significant enrichment of extracellular matrix genes (*POSTN*, *MMP1*, *SPP1*, *MMP7*, *COL6A3*, *COL15A1*, *TIMP1*, *CSPG2*, *TNC*, *SPON1*, *FBN1*, *THBS2*, *COMP*, *COL16A1*, *MMP2*, and *FLRT2*), genes involved in skeletal development (*COL1A1*, *POSTN*, *COL1A2*, *IGF1*, *FBN1*, *NPR3*, and *COMP*), and complement genes (*C1S*, *IF*, *C7*, *CFH*, and *PLAT*).

fibrosis models, and two, *Tnc* and *Col3a1*, appear on the list of mouse homologs of genes identified in human IPF discussed previously. Many of these genes have not been investigated in this context, however, providing us with a new starting point for further investigation of the relationship between inflammation and fibrosis that has yet to be resolved.

Another model system that will yield new insights into the fibroproliferative process is the vanadium pentoxide (V_2O_5) model of airway fibrosis (58). Vanadium is a transition metal found in ambient particulate matter that has been shown to induce profound airway fibrosis in rats (58) and mice (59). Walters and colleagues (60) have identified the *C57BL/6* and

TABLE 2. MOUSE HOMOLOGS OF THE GENES IN TABLE 1 (DIFFERENTIALLY EXPRESSED IN PULMONARY FIBROSIS) AND SEEN RESPONSIVE TO BLEOMYCIN EXPOSURE IN C57 MICE

Time of Maximum Deviation from Saline	Symbol	Time Compared with Saline Exposure			Name
		1 d	3 d	2 wk	
1 d	<i>Gdf15</i>	35.6	12.8	2.2	Growth differentiation factor 15
	<i>Ccng1</i>	4.1	3.8	1.2	Cyclin G1
	<i>Robo1</i>	0.2	1.0	1.1	Roundabout homolog 1 (<i>Drosophila</i>)
	<i>Thbs2</i>	0.2	0.3	1.9	Thrombospondin 2
3 d	<i>Apod</i>	2.2	4.8	3.2	Apolipoprotein D
	<i>Plat</i>	1.0	2.3	1.6	Plasminogen activator, tissue
	<i>Sftpc</i>	1.2	0.3	2.1	Surfactant-associated protein C
2 wk	<i>Tnc</i>	1.8	4.0	9.2	Tenascin C
	<i>Spp1</i>	1.0	4.2	6.7	Secreted phosphoprotein 1
	<i>Col3a1</i>	1.2	2.5	5.0	Procollagen, type III, alpha 1
	<i>Igf1</i>	1.3	1.3	5.0	Insulin-like growth factor 1
	<i>Col5a2</i>	0.9	2.4	4.3	Procollagen, type V, alpha 2
	<i>Cxcl12</i>	2.0	2.3	2.9	Chemokine (C-X-C motif) ligand 12
	<i>Cyp1b1</i>	0.5	2.1	2.7	Cytochrome P450, family 1, subfamily b, polypeptide 1
	<i>Col6a3</i>	0.7	1.2	2.6	Procollagen, type VI, alpha 3
	<i>Srpx</i>	1.0	1.1	2.6	Sushi-repeat-containing protein
	<i>Cxcl14</i>	2.0	1.2	2.3	Chemokine (C-X-C motif) ligand 14
	<i>Itgav</i>	1.3	0.8	0.5	Integrin alpha V
	<i>Timp1</i>	1.7	2.1	0.4	Tissue inhibitor of metalloproteinase 1
	<i>Prpf4b</i>	0.9	1.0	0.4	PRP4 pre-mRNA processing factor 4 homolog B (yeast)

The genes listed show at least twofold deviation from the average in saline-exposed (C57) mice at any of the time points (1 d, 3 d, or 2 wk). Most genes are seen most strongly up-regulated at the latest (2 wk) time point. The most highly up-regulated gene, *Gdf15*, is involved in the injury response in liver and lung.

TABLE 3. GENES RESPONSIVE TO LIPOPOLYSACCHARIDE AND BLEOMYCIN*

Symbol	Bleomycin/ Saline (C57)	Lipopolysaccharide/ Unexposed	Name
<i>Saa3</i>	9.84	82.66	Serum amyloid A 3
<i>Ccl8</i>	8.99	3.14	Chemokine (C-C motif) ligand 8
<i>Cxcl9</i>	6.30	9.29	Chemokine (C-X-C motif) ligand 9
<i>Ccl17</i>	5.79	1.84	Chemokine (C-C motif) ligand 17
<i>Cxcl10</i>	5.77	38.16	Chemokine (C-X-C motif) ligand 10
<i>Adfp</i>	0.21	2.17	Adipose differentiation related protein
<i>Socs3</i>	4.24	3.89	Suppressor of cytokine signaling 3
<i>Tnc[†]</i>	4.03	5.76	Tenascin C
<i>Ccl9</i>	3.95	7.85	Chemokine (C-C motif) ligand 9
<i>Ccl12</i>	3.83	2.93	Chemokine (C-C motif) ligand 12
<i>Hc</i>	0.27	1.96	Hemolytic complement
<i>Mt1</i>	3.63	3.25	Metallothionein 1
<i>C1qb</i>	3.60	1.54	Complement component 1, q subcomponent, beta polypeptide
<i>C330016K18Rik</i>	0.28	0.61	RIKEN cDNA C330016K18 gene
<i>Rhou</i>	3.55	2.42	ras homolog gene family, member U
<i>Ch25h</i>	3.53	3.20	Cholesterol 25-hydroxylase
<i>Msr1</i>	3.46	3.11	Macrophage scavenger receptor 1
<i>Gadd45g</i>	3.43	1.63	Growth arrest and DNA-damage-inducible 45 gamma
<i>Fcer2a</i>	0.30	1.89	Fc receptor, IgE, low affinity II, alpha polypeptide
<i>Cspg2</i>	3.32	2.27	Chondroitin sulfate proteoglycan 2
<i>Pon1</i>	0.30	0.29	Paraoxonase 1
<i>2410015N17Rik</i>	3.23	0.65	RIKEN cDNA 2410015N17 gene
<i>Cdkn1a</i>	3.08	1.88	Cyclin-dependent kinase inhibitor 1A (P21)
<i>Slc23a1</i>	0.35	0.67	Solute carrier family 23 (nucleobase transporters), member 1
<i>Mt2</i>	2.86	5.90	Metallothionein 2
<i>Ifit1</i>	2.74	3.03	Interferon-induced protein with tetratricopeptide repeats 1
<i>Cyp7b1</i>	2.72	1.96	Cytochrome P450, family 7, subfamily b, polypeptide 1
<i>Fcgr2b</i>	2.67	4.14	Fc receptor, IgG, low affinity IIb
<i>Gp49a /// Liltrb4</i>	2.58	3.29	Glycoprotein 49 A /// leukocyte immunoglobulin-like receptor, subfamily B, member 4
<i>Ccr1</i>	2.50	6.47	Chemokine (C-C motif) receptor 1
<i>G1p2</i>	2.47	1.36	Interferon, alpha-inducible protein
<i>Col3a1[†]</i>	2.46	3.05	Procollagen, type III, alpha 1
<i>Irf7</i>	2.38	1.90	Interferon regulatory factor 7
<i>Junb</i>	2.18	1.59	Jun-B oncogene
<i>Ptger4</i>	2.15	1.85	Prostaglandin E receptor 4 (subtype EP4)
<i>Gclc</i>	2.10	2.55	Glutamate-cysteine ligase, catalytic subunit
<i>Akr1b8</i>	2.03	1.86	Aldo-keto reductase family 1, member B8
<i>S100a8</i>	2.00	9.14	S100 calcium binding protein A8 (calgranulin A)
<i>Anxa1</i>	1.99	3.18	Annexin A1
<i>Osmr</i>	1.95	2.75	Oncostatin M receptor
<i>Cyp2a4 /// Cyp2a5</i>	0.53	0.27	Cytochrome P450, family 2, subfamily a, polypeptide 4 /// cytochrome P450, family 2, subfamily a, polypeptide 5
<i>Il18bp</i>	1.86	2.70	IL-18 binding protein
<i>Gfra1</i>	0.55	0.39	Glial cell line-derived neurotrophic factor family receptor alpha 1
<i>Plaur</i>	1.79	1.67	Urokinase plasminogen activator receptor
<i>Cebpd</i>	1.73	2.81	CCAAT/enhancer binding protein, delta
<i>Il4ra</i>	1.71	2.31	IL-4 receptor, alpha
<i>Ldh2</i>	0.59	0.54	Lactate dehydrogenase 2, B chain
<i>Casp11</i>	1.71	1.83	Caspase 11, apoptosis-related cysteine protease
<i>Al607873</i>	1.64	3.17	Expressed sequence Al607873

The expression ratio for bleomycin is the average over the time course (1 d, 3 d, 2 wk) relative to saline; for lipopolysaccharide, it is the average over four replicates at 1 wk relative to unexposed.

* Shown in the intersection in Figure 3.

[†] Appears in Table 2.

the DBA/2 mice as being differentially sensitive to the effects of intratracheally instilled V₂O₅ and are conducting QTL analysis using C57BL/6×DBA/2J recombinant inbred mice to identify QTL associated with susceptibility to V₂O₅-induced airway fibrosis. They will then perform microarray analysis specifically targeted to the identified QTL to further narrow the list of candidate genes identified in their analysis. In addition, this group has phenotyped 35 inbred mouse strains that will be used for *in silico* mapping as a separate approach to identifying a short list of candidate genes.

COMBINING QTL AND GENE EXPRESSION

QTL-specific microarray, such as that planned by Walters and colleagues (60), is a powerful approach that has previously been

applied by this laboratory in an investigation of genes involved in the response to inhaled LPS (61). Combining these approaches serves as a powerful method to focus on genes within a QTL that might be pathogenically involved in the development of lung scarring. In a recent investigation of bleomycin-induced fibrosis in mice, QTL-specific microarray analysis (62) identified genes that are differentially expressed between susceptible and resistant mouse strains within previously identified QTL (21). This analysis identified a short list of genes that were under two identified QTLs and that showed unambiguous differential gene expression (62). Furthermore, using an NCBI database query for SNPs, the authors were able to identify a manageable number of nonsynonymous sequence variations that can be tested for their contribution to the development and progression of

pulmonary fibrosis in this model system (62). This approach identified groups of genes associated with DNA damage and repair, the oxidative stress response, apoptosis, immune and proinflammatory pathways, and extracellular matrix deposition (62, 63). These broad categories of genes have been implicated in the development and progression of bleomycin-induced fibrosis in mice, and these studies have generated short lists of novel candidate genes that can be investigated.

An approach that has not been applied to studies of fibroproliferation in the lung was recently described in three recent reports (64–66). This approach uses gene expression as determined by microarray analysis as the phenotype on which QTL analysis is performed (67). This approach, referred to as “genetical genomics” or “expression genetics,” allows the mapping of QTL that directly affect differential gene expression. Such an approach allows the identification of QTL that affect the abundance of transcripts from genes within the QTL (*cis*-acting QTL) and those that act at a distance (*trans*-acting QTL) (67). Applying such an approach to mouse models of fibroproliferative lung disorders would yield tremendous benefits in coming to understand the etiology of this disease.

CONCLUSIONS AND FUTURE DIRECTIONS

Although these genetic and genomic studies in mice are important, we constantly need to remind ourselves that gene lists are not an answer—rather, they pose a new set of questions that need to be asked—and that validation is needed in focused, hypothesis-driven studies. Moreover, we need to develop innovative approaches to understanding the importance of these genes in the development and progression of IIP in humans. In this way, we will identify novel pathways and critical regulatory genes that point toward novel therapeutic interventions for these diseases that are extraordinarily difficult to treat.

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