Gene expression profiles reveal molecular mechanisms involved in the progression and resolution of bleomycin-induced lung fibrosis

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Running title: Gene expression in lung fibrosis

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Abstract

Lung fibrosis is the final result of a large number of disorders and is usually considered an irreversible process. However, some evidence suggests that fibrosis could eventually be reversible. In this study we aimed to document the time-related reversibility of bleomycin-induced lung fibrosis, and to examine the gene expression profile associated with its initial progression and subsequent resolution. C57BL/6 mice were instilled with a single dose of bleomycin and sacrificed at 1, 4, 8, 12, and 16 weeks. Control animals received an equal volume of saline. Lung fibrosis was examined by morphology and hydroxyproline content, and the transcriptional signature by gene microarray analysis. Our results showed that bleomycin-injured mice developed prominent inflammation at 1 week, followed by fibrosis that peaked at 2 months. Then fibrosis resolved until lungs displayed almost normal architecture at 4 months. Genome wide–transcriptional profiling revealed 533 significantly changed genes. Self organizing maps analysis of these genes identified 4 clusters based on the temporal pattern of gene expression. Clusters 1 and 2 contained genes upregulated during the inflammatory and fibrotic response and were enriched for extracellular matrix related genes including several collagens, matrix metalloproteinases and TIMP-1. Cluster 3 identified upregulated genes during the fibrotic response and cluster 4 contained genes decreased during inflammation and fibrosis that increased during resolution. Most enriched pathways included genes involved in cell-cycle and in regulation of transcription. Our findings corroborate the reversibility of bleomycin-induced lung fibrosis and reveal transcriptional signatures that characterize the progression and resolution.

Keywords: pulmonary fibrosis; fibrosis resolution, MMPs, NFkB2
Introduction

Lung fibrosis represents the final common pathway of a variety of lung injuries, and it is characterized, independently of the etiology, by the expansion of the fibroblast/myofibroblast population and by the abnormal accumulation of extracellular matrix (ECM) replacing normal functional parenchyma (18). In general, there is no effective therapy, and currently the only helpful available treatment for advanced lung fibrosis is transplantation.

However, a growing body of evidence suggests that tissue fibrosis may be a potentially reversible process providing new insights in the understanding of the mechanisms of remodeling as well as in the achievement of novel therapeutic approaches. In other tissues, evidence for regression has been observed usually after the causative agent is removed or if animals are treated effectively. For example, resolution of experimental kidney fibrosis has been obtained by blocking or antagonizing the action of the renin-angiotensin system, and fibrosis following steatohepatitis may regress when diet is controlled (2, 16). Furthermore, antiviral treatment for infection with either hepatitis B or C virus supports that viral eradication is associated with at least partial regression of liver fibrosis in humans (11). Spontaneous resolution of liver fibrosis has been also demonstrated in a model of CCl4-induced liver injury that has been associated with the presence of macrophage-derived MMP13 (4, 6).

The notion that lung fibrosis may resolve spontaneously in experimental models is suggested by some observations in bleomycin treated animals (13, 15). However, the molecular mechanisms likely implicated in lung fibrosis resolution in this model are unknown.

In the present study we aimed: 1) To document the time-related reversibility of bleomycin-induced lung fibrosis, and 2) To determine the gene expression patterns
associated with the initial progression and the subsequent regression of the fibrotic response by microarray in this bleomycin mouse model of lung fibrosis.

Methods

Bleomycin Treatment

Mice (C57BL/6) aging 8 to 10 weeks were instilled intratracheally with 0.10 U/10 g of bleomycin (Blenoxane, Bristol-Myers Squibb Co.) in 50 μl sterile saline. Control animals received an equal volume of sterile saline (1, 19). Mice were housed in specific pathogen-free conditions and provided with food and water ad libitum. All studies and procedures were approved by the Science and Ethic Committee at the National Institute of Respiratory Diseases and were performed according to the protocols and guidelines of the institutional animal care and use committee. Mice were sacrificed at 1, 4, 8, 12 and 16 weeks after bleomycin or saline treatment.

Morphology

Lungs were lavaged with saline solution through the main pulmonary artery, the right lung was removed and fixed by inflation with 4% paraformaldehyde in phosphate-buffered saline (PBS) at continuous pressure of 25 cmH₂O and were embedded in paraffin. Sections were stained with hematoxylin-eosin and Masson's trichrome stain. Lung fibrosis was quantified by using a semiquantitative score, as previously described (9).

Hydroxyproline assay

To quantify collagen concentration, left lungs were hydrolyzed in 6 N of HCl for 24 h at 110°C, and hydroxyproline was quantified as described elsewhere (26). Each sample was tested in triplicate. Data are expressed as micrograms of hydroxyproline per left lung.
**RNA extraction and preparation of labeled cRNA**

RNA was prepared from right lungs using TRIzol reagent (Life Technologies, Grand Island, New York, NY) following the manufacturers' instructions. An additional clean up of total RNA was carried out using the RNeasy Mini kit (Qiagen). Purity of the samples and efficiency of the extractions were verified by spectrophotometry (NanoDrop; Wilmington, DE) and bioanalysis (Agilent; Palo Alto, CA). Total RNA was used as template for double-stranded cDNA synthesis. cDNA was then purified using a QIAquick purification kit (Qiagen, Valencia, CA, USA) and used to obtain biotin-labeled cRNA by an in vitro transcription reaction. Biotin-labeled cRNA was recovered using the RNeasy kit.

**Microarray analysis**

Biotin-labeled cRNA from each mouse lung was fragmented and hybridized with an individual CodeLink Mouse Uniset 20K BioArray (Amersham Biosciences/GE Healthcare). After hybridization, arrays were stained with Cy5-streptavidin and washed before scanning. The arrays were scanned using a GenePix 4000B microarray scanner (Axon Instruments, Union City, CA). Image of each array were analyzed using Codelink Expression Analysis software (Amersham BioSciences). Raw Intensity values for each spot were corrected for background by subtracting Median Local Background Intensity determined using the default setting in CodeLink Expression Analysis software as suggested by the manufacturer (27).

Microarray analyses were performed using GeneSpring GX software (Agilent, Santa Clara, CA) or BRB array-tools (21, 22). Temporally differentially expressed genes were identified using one way ANOVA (P<0.05, FDR<5%). Hierarchical clustering and self organizing maps (SOM) were performed using GeneSpring. For
clustering and SOM, all sample values were compared to the geometric mean of the saline controls at the initial time point. This was performed by log based 2 transformation of the data and subtraction of the geometric mean of the saline control mice expression level. Microarray data were submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) accession number #GSE42301.

**Quantitative Real-Time RT-PCR**

Total lung RNA was extracted from mouse lungs using TRIzol reagent (Invitrogen Life Technologies, Grand Island, NY). 1 μg of RNA was treated with 1 unit of DNAase and reversed transcribed into cDNA (Advantage RT-for-PCR Kit; Clontech, Palo Alto, CA) according to the manufacturer's instructions. Quantitative real-time PCR amplification was performed using specific FAM dye-labeled TaqMan probes for MMP-8, TIMP-1, NFkB2 and 18S rRNA (PE Applied Biosystems, CA) and i-Cycler iQ Detection System (Bio-Rad, Hercules, CA). PCR was performed under the following conditions: 95°C for 10 min; 40 cycles at 95°C for 15 s, and 60°C for 1 min. Results were expressed as the ratio of the target gene normalized to 18S rRNA.

**Immunohistochemistry.**

Mouse lung sections were incubated with rabbit polyclonal anti NFkB2 (Abcam ab. 31409 Cambridge, UK) as previously reported. A secondary biotinylated anti-immunoglobulin followed by horseradish peroxidase-conjugated streptavidin (BioGenex, San Ramon, CA), was used according to the manufacturer. 3-Amino-9-ethylcarbazole (BioGenex) was used as substrate (1).

**Statistical analysis**

Statistical differences between groups were determined by one way ANOVA followed by Tukey’s test for qPCR and hydroxyproline measurement. Fibrosis score was evaluated by the nonparametric Kruskal-Wallis test followed by nonparametric
Results

Bleomycin injury induced a spontaneously reversible lung fibrotic reaction

To evaluate the putative long-term resolution of the fibrotic changes induced by bleomycin, lung collagen content and histopathology were assessed at 1, 4, 8, 12 and 16 weeks after 0.1U/10g bleomycin instillation. Intratracheal treatment with this dose of bleomycin resulted in 36% mortality during the first week, and 50% through the following 8 weeks without additional mortality after this time point. Representative photomicrographs of the lung histology are shown in Figure 1A-F. Lungs of saline treated animals were normal. After 1 week of bleomycin treatment, tissue sections showed a prominent inflammatory response, characterized by neutrophils, lymphocytes, and macrophages infiltrating the alveolar spaces and the interstitium. At 4 weeks, multifocal areas of interstitial fibrosis and epithelial bronchiolization near to fibrotic areas were observed. Fibrosis was more extensive after 8 weeks post-bleomycin and was characterized by intra-alveolar and septal fibrosis with dense collagen accumulation and loss of pulmonary architecture. At 12 weeks after bleomycin the extent of the lesions markedly decreased and at 16 weeks the lungs showed minimal lesions. Semi-quantitative analysis of the histopathological changes corroborated these observations (Figure 1G). In accordance with the results of the morphological scoring, collagen accumulation, examined by hydroxyproline content was markedly increased in the lungs of mice at 4 and 8 weeks after bleomycin treatment. As illustrated in Figure 1H, lung hydroxyproline increased at 4 weeks post-bleomycin (90.1 ± 5.5 versus 50.1 ± 4.3
μg/left lung in saline controls), reaching a peak at 8 weeks (187.2 ± 22.0, p < 0.001) which was followed by a significant reduction at 12 and 16 weeks (92.2 ± 23.0 and 70.9 ± 13.0 respectively; p < 0.05 compared with 8 weeks). Hydroxyproline levels at 16 weeks showed no statistical difference with saline control levels.

Microarrays analysis identified 533 differentially expressed genes in time course of bleomycin-induced lung injury.

To better understand the mechanisms involved in epithelial injury, inflammation and fibroproliferation during the time course of bleomycin-induced lung injury, and to identify candidate genes that might be involved in lung repair and reversibility after fibrosis, we performed microarray-based analysis of global transcription on whole lung mRNA from saline control and bleomycin-treated mice sacrificed at 1, 4, 8, 12 and 16 weeks post-instillation. Three representative animals of each group were selected for microarray analysis (Red points from Figure 1C). Using one way ANOVA, we identified 533 genes that were differentially expressed (P<0.05, FDR <5%; Figure 2A). Consistent with the histopathological fibrosis score and total lung collagen content, several genes which encode proteins that constitutes ECM, such as fibrillar collagens (Col1a1, Col1a2, Col5a1, Col5a2, Col5a3), and genes that play a role in ECM remodeling, such as matrix metalloproteinases (Mmp8, Mmp12, Mmp14, Mmp15, Mmp19) and specific inhibitors, such as Timp1, were significantly increased during the fibrotic response and decreased during fibrosis resolution (Figure 2B).

SOM clustering of these genes revealed four clusters with distinct gene expression patterns. To approach the mechanistic nature of the genes that differentiate the 4 clusters we examined the enrichment of functional annotations in each cluster using the National Institutes of Health DAVID (http://david.abcc.ncifcrf.gov/) (10). The
gene expression pattern and functional enrichment of every cluster are shown in figures 3 and 4.

Genes in cluster 1 increased (2 fold change) after 1 week in the inflammatory phase and stayed up during the fibrotic phases until 8 weeks post-bleomycin, then, they decreased at 12 weeks and returned to baseline (similar to saline control expression levels) at 16 weeks (Figure 3A). Cluster 1 contained 125 genes grouped in 20 functional categories, among them the most highly represented categories were extracellular region, extracellular matrix, cell adhesion, and kinase activity (Figure 3B, Table 1). Extracellular region and extracellular matrix functional annotations include Col1a1, Col1a2, Col5a1, Col12a1, Fmod, Mmp12, Mmp8, Tnc, Timp1 and Tgfbi genes. These genes are involved in the development and progression of lung fibrosis.

Genes in cluster 2 shared a similar pattern of gene expression with cluster 1 during inflammatory, fibrotic, and resolution phases (Figure 3C); however, these genes reach lower levels of expression compared with genes in cluster 1. Cluster 2 contained 82 genes grouped in 6 functional categories (Figure 3D, Table 2). The most representative categories were receptor activity, intracellular signaling cascade and protein kinase cascade suggesting that genes involved in signal transduction that influence extracellular matrix synthesis, chemotaxis, angiogenesis, proliferation and apoptosis are up-regulated during inflammation and fibrosis.

Cluster 3 revealed genes that were up-regulated at 4, 8, and 12 weeks and returned to baseline at 16 weeks post-bleomycin (Figure 4A). This cluster contained 185 genes grouped in 13 functional categories including transcription activator activity, intracellular signaling cascade, phosphoprotein, acetylation and cytoskeleton (Figure 4B, Table 3). Finally, in cluster 4 we identified genes that became down-regulated during inflammatory and fibrotic response and returned to normal values or increased
This cluster was composed by 141 genes grouped in 10 functional categories. Most enriched pathways for microarray transcripts that decreased during the inflammatory and fibrotic response and increased during resolution included nucleus, DNA-binding and transport (Figure 4D, Table 4). Nucleus and DNA-binding categories grouped genes involved in cell-cycle such as Cdc14b, Arid3 and G0/G1 switch gene 2, and in regulation of transcription, genes such as Fkhrl1, Mybl1, T2, Nr2e3, Pax3, Phtf1, Surf 6, Xpa and Ikbkb.

Transcript levels of 2 candidate genes that were upregulated in cluster 1 (MMP8 and TIMP1) and 1 upregulated in cluster 3 (NFκB2) were validated by quantitative real time RT-PCR. As illustrated in figure 5-A, -B and -C the results obtained correlated with the temporal patterns measured by microarray. TIMP-1 and MMP-8 displayed the higher levels at 1 week, during the inflammatory response, decreasing mildly after 8 weeks post-bleomycin instillation reaching almost normal values at 12-16 weeks. NFκB2 increased at first week, and showed a marked upregulation at 4 and 8 weeks with a significant decrease at 12 and 16 weeks.

We explored by immunohistochemistry the expression NFκB2 in the lungs of mice instilled with bleomycin. As shown in Figure 6 strong NFκB2 staining was observed in fibrotic areas mainly at 4 and 8 weeks post bleomycin (Figure 6 C and D) and localized in hyperreactive cuboidal epithelial cells and macrophages. Less immunoreactive NFκB2 was present at 1 and 12 weeks (Figure 6 B and E), mostly in macrophages whereas it was virtually negative at 16 weeks post bleomycin (Figure 6F).

Discussion

Lung fibrosis represents the outcome of a repair process characterized by the replacement of the alveolar-capillary structures by permanent scar tissue, and in general,
is considered an irreversible process. In other words, pulmonary fibrosis is regarded as the final result of virtually all chronic lung disorders where the tissue is unable of regeneration and, as consequence, without option to return to the pre-existing normal structure. However, a growing body of evidence indicates that “tissue fibrosis” is a highly dynamic pathological state and may eventually be reversible although key questions remain. Reversibility has been reported mainly in kidney and hepatic fibrosis, primarily in experimental models, although the feasibility to transfer this scientific promise to human fibrosis is presently unclear (2, 4-6, 11, 16, 23). Also, studies in animal models suggest that hypertrophic cardiomyopathy, characterized by cardiomyocyte hypertrophy, myofibrillar disarray, and fibrosis may be potentially reversible. However, current treatments have not been proved to reverse, or even ameliorate cardiac hypertrophy in patients (17). Usually, regression is associated to both the removal of the etiological agent or condition, and an effective therapy while research on spontaneous regression is scanty. Regarding lung fibrosis, some evidence suggests that, at least bleomycin-induced fibrosis may have a self-limiting response (13, 15).

In this study we corroborated that lung fibrosis induced by a single intratracheal instillation of bleomycin is a spontaneously reversible process. Following the inflammatory response, evaluated here at 1 week post-instillation, fibrosis developed reaching a peak at 8 weeks. Progressive lung collagen accumulation was confirmed by histology and fibrosis score as well as by the biochemical increase of hydroxyproline. Then, this fibrotic response was followed by a noteworthy reduction at 12 weeks with almost total regression at 16 weeks.

In order to better understand the mechanisms underlying progression and regression of the lung fibrotic response, we compared the transcriptional signatures of
lung tissues at different times after bleomycin-instillation to the initial saline time point. Although, some contribution of aging can not be ruled-out after 12-16 weeks of aging, we thought that the analysis of the global temporal changes in gene expression during development and resolution of pulmonary fibrosis, comparing to one time point was an acceptable approach.

Self Organizing Maps from 533 differentially expressed genes revealed 4 clusters that grouped the transcripts that increased during the inflammatory and fibrotic response (clusters 1 and 2), those that increased during the fibrotic response (cluster 3), and those that decreased during fibrosis and increased during regression (primarily cluster 4).

As expected, a number of genes implicated in the extracellular matrix remodeling were upregulated during the development of fibrosis, including collagens, fibromodulin and tenascin-C as well as several matrix metalloproteinases and TIMP-1. Actually, Gene Ontology and functional enrichment analysis revealed that the highly enriched and overrepresented genes belong to this category. We choose to validate two genes from the cluster 1 (MMP-8 and TIMP-1) by quantitative real-time PCR, because they have been implicated in lung fibrosis. Thus, several studies have demonstrated that increased expression of TIMP-1 plays a critical role in the development of experimental lung fibrosis (1). Moreover, TGF-β1 induces a strong over-expression of TIMP-1 in fibroblasts and lungs of fibrosis-prone mice in contrast to weak induction in fibrosis-resistant mice suggesting that differences in the fibrotic response might be in part due to the increase of this inhibitor (12). On the other hand, MMP-8, an enzyme produced mainly by neutrophils, have been recently implicated in the migration and homing of fibrocytes, one of the likely sources of myofibroblasts, to lung tissue (7). Moreover, MMP-8 deficient mice appear to be resistant to bleomycin-induced lung fibrosis (8).
Other extracellular matrix genes revealed in cluster 1 are involved in stabilization of fibrillar structures, such as lysyl oxidase-like-2 that catalyzes the formation of crosslinks in collagens and elastin proteins.

Lung fibrosis is associated with altered expression of TGFβ receptors, increased activity of TGFβ1 signaling pathways and upregulation of several TGFβ-regulated genes. In this context, cluster 1 also included several genes involved in TGF-β signaling pathway including the type II TGFβ receptor (TβRII). Ligand engagement by this receptor is a major step in TGFβ signaling, and mice with epithelium-specific deletion of TGFβRII are protected from bleomycin-induced pulmonary fibrosis, which is associated with reduced phosphorylation of Smad3 (14).

Several genes involved in the immune and inflammatory response, including two chemokines receptors Ccr1, and Cxcr7 were also upregulated. Ccr1, a receptor for CCL3/MIP-1α, CCL4/MIP-1β and RANTES, play a crucial role in the recruitment of immune cells to the site of inflammation, and interestingly, Ccr1-null mice are protected from experimentally-induced lung and liver fibrosis (20, 29). Cxcr7 is a chemokine receptor expressed by fibrocytes, but its role in fibrosis has not been explored. Collectively, our findings indicate that most of the Cluster 1 genes are implicated in extracellular matrix structure, fibroproliferative response, and recruitment and homing of inflammatory cells and fibrocytes, suggesting significant activity in tissue remodeling and reorganization in the lung.

On the other hand, the cluster 2, that followed the same time-course pattern than the cluster 1 included as major categories of significantly upregulated genes those implicated in diverse signaling cascades, including Wnt signaling pathway that may sustain fibrogenesis enhancing the secretion of oxidants, inflammatory cytokines and growth factors involved in lung fibroblast activation and proliferation. Importantly, the
pro-inflammatory and profibrotic genes from these two clusters significantly decrease after 8 weeks post-bleomycin instillation, returning to normal values at 16 weeks.

Cluster 3 identified genes that were upregulated during the fibrotic response and returned to control levels at 16 weeks post-bleomycin. This cluster included genes associated with cytoskeleton and cell adhesion such as Adducin 1, actin bundling protein 1 and Caldesmon 1, and genes involved in proteolysis and cell-cell and cell-matrix interactions like a disintegrin and metalloprotease 8 and 9 (Adam8 and Adam9).

There were also several genes related to signaling cascade and inflammatory response such as interferon (alpha and beta) receptor 2 (Ifnar2) and the chemokine Cxcl4.

NFκB2, a member of the NFκB family of transcription factors, was one of the upregulated transcripts in cluster 3, and was also selected for validation by qPCR. NFκB 2 plays an important role in inflammation and actually, recent genetic evidence indicates that negative feedback control mechanisms are necessary to limit the inflammatory effect of sustained activation of NF-κB2 signaling (28).

Interestingly, although NFκB2 fulfills a central role in inflammation we found it by real-time PCR and immunohistochemistry highly increased during the fibrotic response.

In parallel, a number of genes were downregulated during the fibrotic reaction, increasing during regression (cluster 4), suggesting that their decrease is involved in fibrogenesis. Functional categories in this cluster included nucleus and DNA-binding genes involved in cell-cycle and in transcription regulation. An interesting down-regulated gene in this category was G0/G1 switch gene 2 (G0s2), a novel target gene of Peroxisome Proliferator-Activated Receptors (PPARs). Interestingly, the reduction of fibroblast proliferation and its differentiation into myofibroblasts by PPARδ is associated with upregulation of this cell cycle inhibitory G0s2 gene (24). Likewise, a
zinc finger protein involved in DNA excision repair (Xpa) that is part of the nucleotide excision repair (NER) complex and it is involved in the DNA damage recognition was also decreased during the inflammatory/fibrotic reaction. Recently it was demonstrated that down-regulation of NER-associated genes (including \textit{Xpa}) are associated with LPS-induced lung inflammation (9).

In summary, in this study we identify transcriptional signatures that characterize the progression and regression of bleomycin-induced lung inflammation and fibrosis. Enrichment of functional annotations and network analyses identified several areas of biological importance, yielding new insight into mechanisms involved in fibrosis development and resolution. In general terms, the regression of the fibrotic response was caused by the turn off of the complex extracellular matrix remodeling program. However, some important questions remain. For example, why this model is spontaneously reversible? It is likely that, at least in part, the reversibility observed in this study was caused by the use of a single-dose injury. In this context, it has been recently found that the use of repetitive instillation of bleomycin by laryngeal intubation provokes a marked fibrotic response that persisted 10 weeks after the last dose (3). Perhaps as it has been suggested, the term ‘lung remodeling’ should be used to describe the dynamic repair multi-step process that occurs in the lung following inflammatory injury, which may be reversible with resolution and return to the lung to the pre-existing structure (i.e. after a single dose of bleomycin), or progress to the point of irreversible scarring, as was reported with repetitive bleomycin injuries (25).
References


Legend for figures

**Figure 1:** Histological assessment and collagen content in lung tissue during the time course of bleomycin-induced lung fibrosis. **Panels A-F:** Representative light micrographs in lung tissue sections of saline and bleomycin-instilled mice stained with hematoxylin-eosin. **A:** saline-instilled mouse. **B-F:** Bleomycin-instilled mice at 1, 4, 8, 12, and 16 weeks respectively. Scale bars indicate 50 μM. Histopathology shows dense areas of lung fibrosis at 4 weeks (C) with a more severe and diffused fibrotic reaction at 8 weeks (D). At 12 weeks (E) there was a focal and markedly less-pronounced fibrosis and at 16 weeks (F) most areas of the lung parenchyma were preserved. **G.** Fibrosis score. Data are expressed as mean ± SD (n = 6-9 mice in each time point). **H.** Total lung collagen levels were measured by hydroxyproline content (n = 6-9 mice in each time point). Red points represent mice selected for microarray analysis. *p<0.05; **p<0.001.

**Figure 2:** Gene expression infograms for time course of bleomycin-induced lung fibrosis. **A:** gene expression infogram for all 533 differentially expressed genes. **B.** gene expression infogram for most informative genes that code for ECM components or play a significant role in ECM metabolism. Every row represents a gene and every column a mouse lung sample. Increased genes are shown in progressively brighter shades of yellow, and decreased genes are shown in progressively darker shades of purple. Genes shown in gray are not different between the groups. The baseline per gene was defined as the geometric mean of the expression of the gene in the saline controls mice at the initial time point.

**Figure 3:** Gene expression patterns of Clusters 1 and 2 and enrichment of genes in each cluster with functional categories. SOM clustering of differentially expressed genes revealed four clusters with distinct gene expression patterns. Time-course profiles of cluster 1 (A) and cluster 2 (C) and enrichment of functional annotations in cluster 1.
(B) and cluster 2 (D) are shown. Genes contained within each cluster are listed in table 1 and 2, respectively.

Figure 4: Gene expression patterns of genes in Clusters 3 and 4 and enrichment of genes in each cluster with functional categories. Time-course profiles of cluster 3(A) and cluster 4 (C) and enrichment of functional annotations in cluster 3 (B) and cluster 4 (D) are shown. Genes contained within each cluster are listed in table 3 and 4, respectively.

Figure 5: Expression levels of representative genes upregulated in clusters 1 and 3. Lung mRNA was isolated and transcript expression levels of TIMP-1 (A), MMP-8 (B), and NFκB2 (C) were measured by real-time quantitative reverse transcription-polymerase chain reaction. Data are expressed as mean ± SD (n=6 mice in each time point). (*p <0.05; **p <0.001).

Figure 6: Immunolocalization of NFkB2 in lung tissue during the time course of bleomycin-induced lung fibrosis. Representative photomicrographs of immunohistochemical staining performed with specific antibody against NFkB2 at 1 (panel B), 4 (panel C), 8 (panel D), 12 (panel E), and 16 (panel F) weeks after bleomycin instillation. Saline-instilled mouse lung is shown in panel A, and negative control of 8 weeks post bleomycin in which the primary antibody was omitted is shown in panel G. Immunoreactive protein was observed in alveolar epithelial cells (C, D) and macrophages (B, inset, E and F). All sections were counterstained with hematoxylin. Scale bars indicate 50μM.
## Table 1. Genes in Cluster 1

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predicted gene ICRFP703B1614Q55 (ICRFP703B1614Q.55)
processing of precursor 7, ribonuclease P family, (S cerevisiae) (Pop7)
procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3 (Plod3)
Rap2 interacting protein (Rap2ip)
Ras-related associated with diabetes (Rrad)
Rho GDP dissociation inhibitor (GDI) alpha (Arhgdia)
rhomboid, veinlet-like 6 (Drosophila) (Rhbdl6)
RIKEN 0 day neonate head cDNA clone 4833434E01 3'
RIKEN 13 days embryo forelimb cDNA clone 5930414A15 3'
RIKEN 16 days neonate cerebellum cDNA clone 9630015L23 3'
RIKEN adult male liver tumor cDNA clone C730027P07 3'
RIKEN B16 F10Y cells cDNA clone G370076M11 3'
RIKEN cDNA 2310022B05 gene (2310022B05Rik)
RIKEN cDNA 4933440M02 gene (4933440M02Rik)
RIKEN cDNA 6330416G13 gene (6330416G13Rik)
RIKEN cDNA D030606M11 gene (D030606M11Rik)
RIKEN mammary gland RCB-0527 Jyg-MC(B) cDNA cDNA clone G930023C15 3'
RIKEN osteoclast-like cell cDNA clone I420003J16 3'
ring finger protein 36 (Rnf36)
serine/threonine kinase 22A (spermiogenesis associated) (Stk22a)
solute carrier family 10 (sodium/bile acid cotransporter family), member 3 (Slc10a3)
solute carrier family 12, member 4 (Slc12a4)
solute carrier family 7 (cationic amino acid transporter, y+ system), member 7 (Slc7a7)
sortilin-related VPS10 domain containing receptor 2 (Sorcs2)
sprouty protein with EVH-1 domain 2, related sequence (Spred2)
sterile alpha motif domain containing 8 (Samd8)
solubilized androgen regulated protein 2 (Smr2)
thymic stromal-derived lymphopoietin, receptor (Tslpr)
trans-acting transcription factor 5 (Sp5)
transient receptor potential cation channel, subfamily V, member 3 (Trpv3)
transmembrane 7 superfamily member 1 (Tm7sf1)
tweety homolog 2 (Drosophila) (Ttyh2)
ubiquitin associated domain containing 1 (Ubadc1)
ubiquitin-conjugating enzyme E2E 2 (UBC4/5 homolog, yeast) (Ube2e2)
unc-119 homolog (C elegans) (Unc119)
WD repeat domain 23 (Wdr23)

Table 3. Genes in Cluster 3

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<td>ADP-ribosylarginine hydrolase (Adprh)</td>
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Table 4. Genes in Cluster 4

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RIKEN adult male adrenal gland cDNA clone 7330424A17 3'
RIKEN adult male corpus striatum cDNA clone 7630402G19 3'
RIKEN adult male diencephalon cDNA clone 9330154D11 3'
RIKEN adult male diencephalon cDNA clone 9330197K07 3' similar to AL112552 Botrytis cinerea strain T4 cDNA library under conditions of nitrogen deprivation
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RIKEN adult male thymus cDNA clone 5830463H17 3'
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RIKEN cDNA B230342M21 gene (B230342M21Rik)
RNA binding motif and ELMO domain 1 (Rbed1)
Sestrin 1, mRNA (cDNA clone MGC:67135 IMAGE:6414521)
sodium channel 21 mRNA
sodium channel, voltage-gated, type XI, alpha polypeptide (Scn11a)
solute carrier family 19 (sodium/hydrogen exchanger), member 1 (Slc19a1)
solute carrier family 30 (zinc transporter), member 2, mRNA (cDNA clone IMAGE:4239648)
stereoidogenic acute regulatory protein (Star)
surfeit gene 6 (Surf6)
tafazzin (Taz)
ubiquitin specific protease 29 (Usp29)
ug27c05x1 Soares_NMpu cDNA clone IMAGE:1532840 3'.
UI-M-BH3-asm-e-08-0-UIs1 NIH_BMAP_M_S4 cDNA clone UI-M-BH3-asm-e-08-0-UI 3'.
UI-M-BH3-aty-a-09-0-UIs1 NIH_BMAP_M_S4 cDNA clone UI-M-BH3-aty-a-09-0-UI 3'.
UI-M-BH3-brr-e-07-0-UIr1 NIH_BMAP_M_S4 cDNA clone UI-M-BH3-brr-e-07-0-UI 3'.
UI-M-FY0-ccn-e-21-0-UIr1 NIH_BMAP_FY0 cDNA clone IMAGE: 6821854 5'.
un02d06x1 Sugano mouse kidney mkia cDNA clone IMAGE:2372651 3'.
uo73f07x1 NCI_CGAP_Mam1 cDNA clone IMAGE:2648197 3'.
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<tr>
<th>clone ID</th>
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<td>uv65e07x1</td>
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<td>vt55b07x1</td>
<td>Barstead mouse irradiated colon MPLRB7 cDNA clone IMAGE:1166965 3'</td>
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</tbody>
</table>

vomeronasal 1 receptor, C7 (V1rc7), mRNA
vq62d06y1 Barstead mouse irradiated colon MPLRB7 cDNA clone IMAGE:1106891 5'
vq64c03s1 Knowles Solter mouse 2 cell cDNA clone IMAGE:1107076 3'
vt55b07x1 Barstead mouse irradiated colon MPLRB7 cDNA clone IMAGE:1166965 3'
xeroderma pigmentosum, complementation group A (Xpa)
zinc finger protein 143 (Zfp143)
zinc finger protein 278 (Zfp278)
zinc finger protein 386 (Kruppel-like) (Zfp386)
G

Fibrosis Score (arbitrary units)

G

Weeks

**

H

Hydroxyproline (ug/lung)

H

Weeks

**

*
Figure 2
Figure 3

A

Weeks after bleomycin

B

Genes in a functional group (%)

- SMAD binding
- leukocyte mediated immunity
- basement membrane
- cytokine production
- chemotaxis
- phosphorylation
- collagen
- epithelium development
- ECM-receptor interaction
- response to wounding
- protease
- cell motility
- cell proliferation
- apoptosis
- lysosome
- blood vessel development
- kinase
- cell adhesion
- extracellular matrix
- extracellular region

C

Normalized Intensity Values

D

- cell junction organization
- cell-substrate junction assembly
- LDL-receptor activity
- protein kinase cascade
- intracellular signaling cascade
- receptor

Genes in a functional group (%)
Figure 4
**Figure 5**

(A) MMP8/18S rRNA E07

(B) TIMP1/18S rRNA E07

(C) NFKB2/18S rRNA E07