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Radiation Induced Pulmonary Fibrosis as a Model of Progressive Fibrosis: Contributions of DNA Damage, Inflammatory Response and Cellular Senescence Genes

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Abstract

Purpose of Study: Studies of pulmonary fibrosis (PF) have resulted in DNA damage, inflammatory response, and cellular senescence, being widely hypothesized to play a role in the progression of the disease. Utilizing these aforementioned terms, genomics databases were interrogated along with the term, “pulmonary fibrosis,” to identify genes common among all 4 search terms. Findings were compared to data derived from a model of radiation-induced progressive pulmonary fibrosis (RIPF) to verify that these genes are similarly expressed, supporting the use of radiation as a model for diseases involving PF, such as human idiopathic pulmonary fibrosis (IPF).

Materials and Methods: In an established model of RIPF, C57BL/6J mice were exposed to 12.5 Gy thorax irradiation and sacrificed at 24 hours, 1, 4, 12, and 32 weeks following exposure, and lung tissue was compared to age-matched controls by RNA sequencing.

Results: Of 176 PF associated gene transcripts identified by database interrogation, 146 (>82%) were present in our experimental model, throughout the progression of RIPF. Analysis revealed that nearly 85% of PF gene transcripts were associated with at least 1 other search term. Furthermore, of 22 genes common to all four terms, 16 were present experimentally in RIPF.

Conclusions: This illustrates the validity of RIPF as a model of progressive PF/IPF based on the numbers of transcripts reported in both literature and observed experimentally. Well characterized genes and proteins are implicated in this model, supporting the hypotheses that DNA damage, inflammatory response and cellular senescence are associated with the pathogenesis of PF.

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Introduction:

Pulmonary Fibrosis

The term “pulmonary fibrosis” (PF) defines a spectrum of pathological changes associated with a heterogeneous group of diseases in which excessive lung fibrogenesis disrupts the normal architecture and the gaseous exchange function of the lung [1]. The initiating events of PF are multifactorial; indeed, PF is associated with a number of diseases, including rheumatoid arthritis or sarcoidosis, exogenous exposure to agents such as asbestos, silica, sulphur mustard gases, medical radiation treatment or accidental exposure, or as part of the toxic response to some drugs, such as amioderone or bleomycin [2]. Familial and sporadic forms of PF have been associated with certain gene mutations (Tert, Muc5, Sftpc), however, ideopathic forms of PF with unknown origin (IPF) are also known to occur [2]. Unfortunately, IPF results in approximately 40,000 deaths annually in the United States, with a median survival of 3.5 years following diagnosis and a 20% survival rate at 5 years [3]. Interestingly, IPF appears to share symptoms and progression characteristics with a similarly progressive, (currently) irreversible disease, radiation-induced pulmonary fibrosis (RIPF); in both cases, unresolved injury and/or persistent cellular dysfunction lead to elevated levels of radical oxygen species (ROS), accelerated senescence and disrupted cytokine signaling. These events culminate in the hallmarks of pulmonary fibrosis: fibroblast recruitment, proliferation and activation leading to excess collagen deposition and restrictive lung disease [4]. Although affecting fewer people than IPF per se, similar pathological changes are seen in a minority of adult cancer survivors exposed to lung irradiation as part of their treatment, described as RIPF [5]; childhood lung cancer survivors also show a four-fold increased risk of developing pulmonary fibrosis after radiation treatment [6]. Currently, there are no approved treatment options for patients with RIPF, although regimens for IPF, such as Pirfenidone and Nimatinib, slow the progression of the disease [3, 7]. Efforts to halt or reverse the progression of pulmonary fibrotic disease have, to date, ended in failure. Therefore, with so few effective treatment options, strategies to predict or prevent the development and progression of these and similar diseases need to be developed.

Animal models of disease have proven useful to many researchers since they offer the ability to study the pathology of a disease and, importantly, elucidate the pathways which may drive underlying mechanisms, for example, and pertinent to this paper, those leading to the pathogenic remodeling of lung tissue. Indeed, numerous animal models of pulmonary fibrosis exist which are deemed to be reflective of the pathogenic mechanisms of PF; in 2013, Moore et.al. published a review of those most commonly used [8]. Despite the many diverse forms of PF, there nonetheless appear to be several characteristics common to all: an association with aging; persistent and/or progressive epithelial injury; alveolar septal thickening; cytokine and growth factor imbalance; fibroblast proliferation and activation; and collagen deposition culminating in restrictive lung disease. These commonalities are important since a critical component of developing treatment options for diseases such as IPF, is the use of an animal model which closely mimics the disease, i.e. one that presents with fibrosis of a similar chronic and progressive nature [8]. Importantly, some of the commonly-used lung fibrosis models, e.g. those that make use of injuries induced by bleomycin, asbestos, or silica, etc., have been shown to present with a number of the early

events which lead to PF. However, these injury models are often found to be dependent on the route of administration and, more significantly, exhibit a form of fibrosis that is not always progressive in nature [8]. In contrast, a major characteristic of models of RIPF is that effects do not vary by administration route, and repetitive dosing is not required to mimic the pathology of human fibrotic lung disease. It is, in fact, the dose-dependent and progressive nature of RIPF which we believe is essential in order to provide an acceptable model of human IPF. Therefore, for the purposes of this paper, we have considered a model of RIPF as a means of better understanding the alterations in the transcriptional profile of lung tissue which occur during the onset and progression of PF and, specifically, to evaluate its worth as a model of IPF.

DNA Damage, Senescence, and Inflammatory Response in Pulmonary Fibrosis

RIPF models have been well characterized over several decades [9–13], yet the underlying mechanisms culminating in the fibrotic disease state remain poorly understood, as evidenced by our current lack of effective treatment strategies. Nonetheless, from such models, it is known that doses of ionizing radiation sufficient to result in RIPF produce significant levels of DNA damage and the subsequent response includes, but is not limited to, promotion of cell death pathways, including apoptosis, as well as acute and chronic phases of inflammation and cellular senescence, all of which have been associated with IPF [14–17]. In a model proposed by Morgan et. al. [18], it was suggested that the effects of radiation injury lead to clinically described outcomes of radiation-induced lung damage through cyclically up-regulated inflammatory signaling; this is maintained through a feedback loop with senescence and DNA damage-associated cytokines (Figure 1), which are known to be immediately and persistently altered in response to radiation [19]. These biological processes are known to result in a variety of cellular effects, such as the activation of p53 and endoplasmic-reticulum stress pathways, altered NF- κ B signaling, and accumulating mitochondrial DNA damage. Indeed, the observed prolonged alteration in signaling levels of both pro-fibrotic and pro-inflammatory cytokines, implicated in the onset and progression of RIPF [13, 20], also may affect the phenotypes of infiltrating immune cells and/or resident stromal cells. Importantly, many of these same cytokines (IL-8, IL-13, CXCL5, IL-1 β , TGF β , and PGDF) have been implicated in the initiation of IPF, being thought to promote leukocyte recruitment and activation, myofibroblast proliferation, and extracellular matrix deposition [21].

A number of studies have linked both RIPF and IPF to senescence [7, 14, 22–25], DNA damage [17, 26–29], and the inflammatory response [11, 15, 30–33]. For example, studies have shown increases in the senescence markers SA- β gal, p21 or p16, within hours of, and up to 30 weeks following radiation treatment [23, 34–36], with the resultant cells displaying a defined senescence-associated secretory phenotype (SASP) [24, 36–38]. Senescent epithelial cells in culture have been shown to up-regulate a large number of pro-inflammatory gene transcripts, many of which have been recapitulated in whole lung models. These include, but are not limited to, KC (Gro α /Cxc11), MCP-1 (Ccl2), and IL-6 [36, 37], all of which have been implicated in the onset of RIPF [39]. In fact, within days to weeks following pulmonary irradiation, a number of pro-fibrotic and pro-inflammatory cytokines, such as TNF α , TGF β , IL-6, IL-1 α , fibrocyte growth factor 1 (FGF-1) and MCP-1,

show rapid elevations in mRNA abundance, which correlates highly with collagen deposition and the onset of RIPF [12, 19, 20, 40, 41]. While several of these same factors have been associated with the initiation of IPF, the timing between their production and onset of IPF has remained elusive, largely limited by the logistics of sample collection following a clinical diagnosis of IPF.

Overall, findings support the concept that radiation-induced DNA damage leads to dysregulated wound healing, creating a chronic inflammatory state, senescence and recurrent DNA damage, with a number of groups supporting the suggestion that multiple signaling pathways could overlap during the injury response [35, 42–45]. However, we are unaware of any studies that have sought to find commonalities between these 3 important biological processes and the onset and progression of IPF and RIPF. We therefore have hypothesized that the expression of a temporally-defined sequence of genes, specifically associated with chronic DNA damage, a persistent inflammatory response and senescence, is involved in the pathogenesis of idiopathic pulmonary fibrosis (IPF), and that common gene transcripts will be confirmed experimentally in a model of RIPF through RNA sequencing. This comparison will allow us to evaluate the relevance of our RIPF model as a surrogate for human IPF and, if validated, will offer the potential for adopting a multi-pathway approach to identifying molecular targets which play a critical role in the pathogenesis of PF. Indeed, to date, no single cell type or molecular entity has been shown to be responsible for the pathogenic outcome of IPF/RIPF. This is likely a consequence of the heterogeneous nature of the lung, comprised as it is of an estimated 40 cell types, and with a broad range of cellular processes and molecular pathways contributing to the fibrotic disease state. As a result, the commonly-used reductionist approach to determining the origins of PF has yet to yield a breakthrough in treatment strategy. Instead, we believe that the most effective treatment strategies for this multifactorial disease state will be derived by targeting a combination of genes or proteins from groups of common genes that are active in several critical pathways. Indeed, it may be argued that the modestly effective Pirfenidone is an example of one such drug – its success in slowing the progression of PF appears to be the result of inhibition of multiple pro-inflammatory and pro-growth signaling factors; Il1b, Il-6, MCP-1, Cxcl12, TNF α , Tfgb1, and Fgf2 have all been shown to be elevated in a bleomycin model of PF, and their combined fibrogenic effects have been shown to be attenuated by Pirfenidone treatment [46].

Application of new genomics tools, such as RNA sequencing, allows for a relatively unbiased comparison of transcriptional changes, as compared to the more traditional method of selecting individual gene transcripts for rt-PCR analysis. We believe that utilization of this exploratory approach to data collection and analysis in the context of an RIPF injury model will allow us to interrogate a rich source of genomic information, comparing our in vivo laboratory results to those reported in the literature. The resultant RIPF RNA seq data will expand our understanding of the interactions between genes involved in the development of fibrosis, and allow us to elucidate potential associations among senescent, inflammatory and pro-fibrotic pathways in whole lung tissue. Additionally, it may serve to identify potential biomarkers of radiation-induced lung injury and, more importantly, indicate multiple potential targets for therapeutic intervention in RIPF and, potentially, IPF.

Materials and Methods:

Gene identification by database interrogation:

In order to identify those genes that are associated with important cellular processes that we believe are critically associated with, and may be predictive of, the onset and progression of pulmonary fibrosis, we utilized three genomics databases: Gene Cards (www.genecards.org) [47], UniProtKB (<http://www.uniprot.org>) [48], and Ingenuity Knowledge (www.qiagen.com/ingenuity) [49]. Each database was interrogated using the search terms “cellular senescence”, “inflammatory response”, “DNA damage”, and “pulmonary fibrosis”; lists were compiled for each term and official gene IDs were cross-referenced across all three databases. Genes that were associated with each individual term in a minimum of two of three databases were compared to the mouse genome defined by the Genecode M6 gene annotation library [50] used in our RNA sequencing experiment. Non-matching gene IDs were examined for the orthologous mouse genes, as identified by the Gene Cards database and contained within the Genecode library. Genes which did not have a mouse ortholog, matching Gene ID in our library, or were unidentifiable in the Gene Cards database were eliminated as either human specific genes, repeated data, or simply unidentifiable; less than 1.5% of genes across all four search terms fell into this category. The remaining genes which met this thorough and in-depth examination provided a relatively comprehensive set of mouse genes associated with each term. The complete list of genes identified for each search term can be found in Supplementary Table 1.

In order to visualize the overlap between the four search terms/gene sets, we next constructed a Venn diagram (Figure 2) [51]. Increasing commonality between processes was associated with a decreasing total number of genes. Based on our database interrogation, this Venn diagram identified 22 genes that were not only indicated as participating in pulmonary fibrosis, but also in cellular senescence, inflammatory response, and DNA damage, hereafter referred to as “common genes” in PF. The official name and reported canonical function of these common genes was determined by examination of the NCBI Entrez Gene database (www.ncbi.nlm.nih.gov/gene) [52]. In order to elucidate the experimentally observed expression of these genes or their resultant protein as related to the occurrence of pulmonary fibrosis, the official gene name or ID was cross-referenced with the terms pulmonary fibrosis or fibrosis in both the GeneCards and PubMed databases. The results of this literature and database review can be seen in Table 2.

Experimental determination of genes involved in RIPF:

Animals—Female C57BL/6J mice, 6–8 weeks of age, were obtained from Jackson Laboratory (Bar Harbor, ME), housed five animals per cage under controlled conditions and acclimated for one week prior to experimentation. All animals received a standard laboratory diet and water *ad libitum*. All experiments were performed under protocols approved by the University of Rochester Committee on Animal Resources.

Irradiation—Animals were restrained in plastic jigs and irradiated with 12.5 Gy (thorax only) using a ^{137}Cs γ -ray source operating at a dose rate of approximately 1.5 Gy/min. Age-matched sham-irradiated (0 Gy) control mice received similar handling.

Sample Collection—Animals were euthanized at 24 hours, 1, 4, 12 and 32 weeks post-irradiation by intraperitoneal injection of sodium pentobarbital and their excised lungs used for low input RNA sequencing. Early time points (24 hours, 1 and 4 weeks) were presumed to reflect effects of the immediate wound healing (early inflammatory) phase of radiation injury, whereas the 12 week time point represented changes during the pneumonitic period of radiation injury prior to the onset of RIPF, and 32 weeks reflected the late effects occurring during the progression of pulmonary fibrosis[9]. Lung digests were obtained via instillation with 1.8 units/ml dispase (Gibco® Life Technologies, Grand Island, NY) in Dulbecco's modified Eagle medium (DMEM; Gibco Life Technologies) and incubated at room temperature for 45 min. Lungs were then disrupted by mincing and the cell solution filtered through 100 µm, 40 µm and 25 µm cell strainers (Fisher Scientific, Waltham, MA) using DMEM plus 0.01% DNase 1 (Sigma-Aldrich, St. Louis, MO) as a wash buffer, and then transferred to DMEM plus 10% fetal bovine serum (FBS; BD Biosciences, San Jose, CA) for counting. Approximately 5×10^6 cells were pelleted, washed and transferred into 600µl RLT Lysis buffer (Qiagen, Hilden, Germany) and stored at -80°C until processing for low input RNA sequencing. Additionally, lungs were inflated and fixed in 10% buffered zinc formalin (Anatech, Battle Creek, MI) and paraffin embedded. Tissue sections (6µm) were prepared and stained with Hematoxylin and Eosin or Gomori trichrome and examined by light microscopy at 16, 24, and 32 weeks post treatment. Images were acquired with an Olympus BX40 microscope (Olympus Imaging America Inc., Center Valley, PA).

RNA isolation and analysis by low input RNA sequencing—RNA quality and concentration per sample was determined through the use of the Agilent 2100 bioanalyzer (Agilent technologies, Santa Clara, CA). Samples meeting a minimum RNA integrity value (RIN) of 7 were considered suitable for analysis. RNA concentrations were normalized and 3–5 mice per time point and treatment group were pooled for low input RNA seq analysis utilizing the Illumina HiSeq 2500 Sequencer (Illumina, San Diego CA). Low input RNA sequencing required a minimum of 1ng mRNA. The differential expression of transcripts between irradiated and non-irradiated whole lung tissue was compared across all 5 time points. RNA sequencing data was trimmed and quality controlled through the use of trimmomatic 0.32 and fastqc software applications [53], mapped using star 2.4.2a mouse genome, and aligned to the Genecode M6 gene annotation library prior to comparison of differential expression of transcripts using cuffdiff2[54]. An acceptably small number of transcripts did not pass our quality control tests and were excluded from analysis largely due to technical limitations, such as transcripts not detected, detected at limits not reliably measured, or an overly abundant number of reads that caused numerical exception and prevented analysis. Changes in the relative abundance of 16 experimentally observed common genes over the course of 32 weeks were displayed in the form of a heatmap utilizing the Multi Experiment Viewer (MeV) software application version 4.9.0 [55]. The obtained from this RNA sequencing experiment can be found in GEO database accession number GSE94852.

Results:

From the mouse lung irradiation study, genes expressed and reliably quantified at each time point resulted in 14,401 genes available for experimental analysis. This list of genes was cross-referenced against the gene sets identified by the database searches found in Supplementary Table 1, creating a compilation of gene sets which were expressed and reliably measured across all time points, indicating their potential role in the pathogenesis of RIPF. The list of genes contained in the experimentally observed gene sets in our RIPF model can be found in Supplementary Table 2. A comparison of the genes identified by database search and the experimentally observed genes involved in DNA damage, inflammatory response, cellular senescence and PF can be seen in Table 1. In order to visualize the overlap between the experimentally observed four gene sets, we constructed a Venn diagram (Figure 3) similar to Figure 2. By this approach, 16 genes were observed to be associated with all 4 of the search terms, hereafter referred to as “experimentally observed common genes” in RIPF. Differential expression of these gene transcripts across all 5 time points is represented in supplemental figure 1. These changes coincide with the onset and progression of RIPF, which can be seen by blue collagen staining in fibrotic foci observed in irradiated mice at 32 weeks following radiation treatment (supplementary figure 2).

Database interrogation for the search terms, pulmonary fibrosis, cellular senescence, inflammatory response, and DNA damage, identified a number of genes involved in these processes (Table 1). Based on the 45,786 genes represented in the Genecode gene annotation library, these values represent a relatively small percentage of the mouse genome. Further comparison to our experimental gene set revealed that a majority of the genes identified by database interrogation were represented in our experimental data set (>74%) regardless of the stringent filters in place.

Examination of these results revealed a large number of genes associated solely with these cellular responses, as determined by our database interrogation (Figure 2) or experimental model (Figure 3). Importantly, the majority of pulmonary fibrosis-associated genes also were identified in one or more of the hypothesized critical processes. From our review of the literature, although 176 transcripts were associated with PF, only 27 were linked solely to pulmonary fibrosis, whereas nearly 85% of all transcripts were associated with at least one additional process. Furthermore, of the 176 transcripts identified by literature review to have been studied in the context of PF, nearly 83% (146) were identified in our RIPF RNA sequencing data. Indeed, of the 146 transcripts identified in our experimental RIPF model, only 24 were associated strictly with pulmonary fibrosis and nearly 84% of all gene transcripts were associated with at least one other process, a similar percentage to that found through our database interrogation. In both the literature search and our experimental model, greater than 50% of the genes identified in PF/RIPF were shown to be involved in combination with DNA damage, inflammatory response and/or cellular senescence. A complete list of the genes represented in each section of the Venn diagrams can be found in Supplementary Table 3 (determined by database interrogation) and Supplementary Table 4 (experimental observation).

Of the 22 “common genes” indicated as being involved in pulmonary fibrosis, cellular senescence, inflammatory response, and DNA damage by database interrogation (Figure 2), 6 were not identified in the “experimentally observed common genes” data set (Figure 3) at two or more time points. Gene transcripts that were not reliably detected at any time point are Il-10, Il-13, Tert, and Plg. Both Il-6 and Plau were detected at 32 weeks during the progression of RIPF, but were not reliably detected or measured at earlier time points, thus were excluded from Figure 3. Table 2 lists the 22 common genes, their reported expression patterns or changes in protein abundance in a pulmonary fibrosis model, and their canonical role in the genome.

Overall, as a result of the analysis from the literature, 22 “common genes” that play important roles in senescence, inflammatory response and DNA damage, are likely to contribute to the onset and progression of PF. This statement is supported by the fact that each of these transcripts or their protein has a reported pattern of expression or abundance across a variety of PF models and/or in IPF. Furthermore, of the 22 gene transcripts identified in the literature as having overlapping roles in pulmonary fibrosis, senescence, inflammatory response, and, 16 were reliably expressed during the onset and progression of our model of RIPF. While the determination of the abundance of proteins associated with these common genes is outside the scope of this paper, several studies utilizing similar pulmonary radiation injury models in C57BL/6J mice provide strong evidence of their presence. Specifically, Il-6, TNF α , SOD1, Akt1, Bax, p53, Ifng, Il-4, Il-13 have been shown to increase either in lung tissues or serum at similar (single) doses of thorax only radiation ranging between 12 and 16 Gy [72, 95–98].

Discussion:

In order to assess the effectiveness of RIPF as a model for human IPF, we made a simple comparison of the number of PF-associated gene transcripts identified in literature (176) with those generated experimentally (146) and collected across a number of time points that ranged across the development and progression of RIPF. This study served to support the contention that RIPF is a valid model of IPF, with almost 83% of all molecular targets examined being present in our experimental model at the time points selected. Given the number of animal models utilized within the literature search and the multifactorial nature of PF which may affect the expression of gene transcripts, the ability to measure 83% of them at any point examined during the progression of the disease is a strong indicator of the robustness of RIPF as a model of progressive PF. 6. We examined the Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo) in order to determine whether similar gene expression data exists in a comparable bleomycin model of pulmonary fibrosis. The “common genes” present in our model of RIPF appear in studies that fall under the broad classification of “pulmonary fibrosis” in our literature search, which would certainly capture studies of bleomycin induced PF. Therefore it is not surprising that these same common genes are present in both types of data sets. However, in these single dose models of mice receiving between 80–125 units/kg of bleomycin, it may be that the data presented in these studies represent an acute injury response occurring within a few weeks or months of administration, rather than a gradual progression of disease over the course of several months which occurs in our RIPF model. Many of the commonly-used animal models of PF,

and particularly those utilizing bleomycin, have been shown to induce only a transient or partly reversible PF and therefore do not mimic the progressive nature of human IPF [8, 99, 100].

The interrelationships between DNA damage, inflammatory response genes and cellular senescence have been the subject of a number of excellent reviews [35, 38, 101–103]. Understandably, several of the molecular pathways and select molecular targets associated with these processes have been examined in the context of IPF and animal models of PF due to their potential roles in organismal injury and/or interstitial lung disease. As stated earlier, 176 molecular targets associated with either cellular senescence, inflammatory response or DNA damage were identified as also being associated with PF, although only 27 (15%) of those were associated with PF alone. Interestingly, through database interrogation, 106 (60%) PF-associated gene transcripts were linked to the inflammatory response alone or to the inflammatory response in conjunction with cellular senescence. This overlap is understandable when considering the likelihood of a connection between PF and aging and/or the wholly or partially successful historical use of anti-inflammatory therapies in the treatment of PF [4, 7, 15, 42, 104]. Indeed, the inflammatory response has been shown to be an integral component of RIPF [12, 13, 19, 20, 40] and is best explained by examining what is known with respect to the overall normal tissue response to ionizing radiation. For example, it is known that the majority of normal tissues initiate an inflammatory response following radiation injury as part of canonical wound healing; although this response can appear to resolve normally, pro-inflammatory signaling may reappear in “waves” in the weeks and months following exposure. It has been hypothesized that these waves of molecular signaling are the result of failed attempts at tissue repair, and are considered to be evidence of a dysregulated immune response [105]. Interestingly, the up-regulated expression of pro-inflammatory Th1 type cytokines, such as Il-1 β , Ifn γ , Tnf α , and Il-6, is seen in association with the inflammatory and pneumonitic phases of radiation induced injury, whereas the expression of anti-inflammatory Th2 type cytokines, such as Il-13, Il-10, Il-4, and Tgf β , is not only thought to play a vital role in injury resolution, but also in the dysfunctional tissue remodeling seen in many types of organ/tissue fibroses [106]. This so-called perpetual cascade of cytokines results in the tissue’s failure to return to homeostatic conditions, thought to foster either a damaging, pro-inflammatory (Th1) or an overly aggressive fibrotic (Th2) pulmonary response associated with the imbalanced immune response [106]. Importantly, all of the aforementioned cytokines are present in the set of common genes (Figure 2), and the majority of these Th1/Th2 cytokines are also present in the experimentally observed common genes (Figure 3), implicating them as potential targets in the treatment of both RIPF and IPF. The question of whether restoration of the Th1/Th2 balance is a relevant treatment strategy in human IPF is complicated by the lack of available data, particularly during the onset of disease, as the majority of tissues from IPF patients are collected late in disease progression following a clinical diagnosis. While to date there is little evidence of prominent inflammation during early disease [107], increases in pro-fibrotic Th2 type cytokines, Il-4, Il-13, and Tgf β , have been shown in tissue samples collected from IPF patients [58, 67]. This has led to clinical trials of several anti-Il-13 antibodies [7], although the results of these studies have yet to be published at the current time.

Fewer transcripts have an association with both DNA damage and PF (39 of 176, 22%). Not surprisingly, the majority of studies linking DNA damage to PF have largely been performed in radiation or bleomycin models, both of which are known to exert their toxic injury through DNA damage, in contrast to human IPF studies where little data exist. A slightly greater number of transcripts have revealed the association between senescence and PF (80/176, 45%), with the majority of these genes also being associated with the inflammatory response (49/80, 61%). Numerous studies have reported an increasing incidence of IPF which correlates with aging[108], and given the obvious connection between senescence and aging, it is no surprise that numerous studies have undertaken elucidating the role of senescence and the pro-inflammatory senescence associated secretory phenotype in numerous PF models [14, 22–25, 35, 37, 38, 109].

The identification of genes that are common to PF, the three pathways that we have hypothesized as being etiologically important, and which also are transcriptionally regulated throughout RHPF pathogenesis, enables us to potentially pinpoint factors that may play critical roles in disease outcome. However, whether targeting a single one of these 22 common genes will be effective in the treatment of fibrosis remains to be seen. Certainly, in clinical trials to date, this does not appear to be the case. In 2007, the INSPIRE clinical trial of IPF patients administered 600 µg interferon-γ 1b (Ifng) weekly, was discontinued when found to have no survival benefit [110]. Similarly, a clinical trial using the Tnf inhibitor Etanercept was also shown to have no survival benefit [111]. Nonetheless, several treatments impacting other molecular targets identified in the common gene set, such as, Tgfb, Il-13, Il-10, SOD1, and Cxcl12, have shown beneficial antifibrotic effects in bleomycin models[99]; however, our data suggests that further experimentation utilizing other, more applicable, models of PF may be warranted. Furthermore, the multifactorial nature of IPF suggests that a more global approach to treating this complicated disease, possibly by inhibiting multiple genes/proteins indicated in the set of common genes may prove to be more effective. In support of this more systems-wide approach, is the previously mentioned evidence that the moderately effective Pirfenidone acts through several such pathways [46], and Nintedanib, a tyrosine kinase inhibitor, has also shown some ability to slow the progression of the disease. Although data on the effect of Nintedanib in animal models of PF are limited, it has been shown to target VEGF, FGF, and PGDF receptor signaling and decrease Tgfβ mRNA abundance along with Il-1b and Il-6 protein levels in lung tissue [112].

An interesting question raised by this study is whether or not these transcriptional responses are driven by a particular cell type that can then be deemed responsible for the manifestation of PF, or if they reflect the activity and interactions of multiple cellular (sub)types found throughout the lung tissue. It is unlikely that every pulmonary cell population contributes equally to the progression of this disease, instead a limited number of cell types may play critical roles at various stages in the biological process. The inter-relationship between cell types and the spectrum of functions performed within even single populations makes it difficult to identify critical roles through the examination of whole lung tissue. For example, interleukins are thought to be primarily secreted by leukocytes [113], and their presence in the list of common genes would imply that the adaptive immune response plays an important role in the fibrogenic response. And, indeed, the inflammatory response does appear to play

an important role in animal models of radiation-induced PF, although its role in human IPF is still somewhat controversial [107]. However, given the heterogeneous nature of lung tissue, particularly in the alveolar region where PF manifests, it seems unlikely that a single molecular target, pathway or cell type can be responsible for the development or progression of the complex fibrotic disease state. The resultant need to target multiple pathways offers an explanation for the, albeit limited, success seen with the current recommended therapies, Pirfenidone and Nintedanib [114], both of which affect multiple molecular targets and/or pathways that are implicated by our common gene set. Nonetheless, despite these limitations, we believe that, by examining the RNA transcriptional profile of individual cell types in the RIPF model, it may be possible to determine whether or not late acting, effector type cells, such as activated fibroblasts (myofibroblasts) represent suitable targets for treatment following disease onset or whether protecting epithelial cells as part of a more preventative approach may be more appropriate. While the inflammatory response appears to be particularly important based on the 60% overlap with PF-associated genes, this study was not able to determine whether the inflammatory-related molecular targets identified were primarily derived from immune type cells. Therefore, an important extension of this project is to isolate specific cell types from the pre- and fibrotic lung and identify their potential contributions to the manifestation of this disease.

In conclusion, through the utilization of RNA sequencing technology, we attempted to identify genome-wide changes that occur in response to radiation injury through a non-selective approach rather than limiting the study to specific genes/transcripts which have been studied previously. This unbiased approach identified a number of overlapping molecular targets involved in DNA damage, inflammatory response and cellular senescence that also appeared to be crucial to the onset and progression of RIPF. Furthermore, the development of a “common gene” set, by combining data from our experimental model with results gleaned from database interrogation, provided a degree of validation, since previous studies with alternative models also showed potential links between many of the identified gene transcripts and the onset and progression towards PF. Based on these data and the high degree of overlap seen between the various pathways with the fibrosis endpoint (Figure 3), it appears that the “magic bullet” approach to either preventing or treating IPF is unlikely to succeed. Rather, the ability to target or interfere with multiple pathways and molecular targets that play a role in PF provides an approach with a higher probability of success. While we acknowledge the weakness of the whole lung approach to RNA sequencing, much can be gained by applying this same strategy to specific cell types, an approach that can be utilized in future studies. Lastly, we suggest that these data provide compelling evidence that RIPF is an appropriate model of progressive interstitial pulmonary fibrosis, revealing important transcriptional changes occurring as late as 32 weeks following a single initiating event.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1:
Processes hypothesized to play a role in the development and progression of RIPE.

DNA damage

Inflammatory response

Cellular senescence

Pulmonary fibrosis

Gene Lists:

- DNA damage only:** Parp1 Irf5 Lep Egfr Nos1 Sod2 Chek2 Myc Bcl2l1 Ep300 Trp53bp2 Nox4 Calr Fos Ybx1 Vcp Nr2c2 Fgf2 Rela Ccnd1 Rb1 Xrcc6 Psen1 Gadd45g Pomc Pik3r1 Trp73 Il24 Sqstm1 Runx1 Mtor Esr1 Polg Twist1 Fn1 Gapdh Kras Mapk14 Ccr5 Ahr Hspb1 Cdkn2a Igf2 Junb Pik3cg Xdh Hmox1 Ddit3 Mdm2 Ppara Egl3n Lyn Nbn Cat Msh2 Ogg1 Birc5 Lmna Ctnnb1 Hdac2 Elavl1 Casp8 Cdkn1a Hspa1l Map3k5 Mcl1 Sirt1 Mapk3 Stub1 Bcl2 Ar Psmb5 Pten Fanccl2 Il2 Irf1 Prkcd App Hdac1 Hmgbl1 E2f1 Nup85 Hif1a Mapkapk2 Bcl6 Casp3 Rassf1 Hspa1a Tyr Apc Hras Gadd45a Trp63 Cdk2 Egf Prckb Olr1 Rara Atm Clu Sirt6 Foxo3 Idh1 Bmp7 Mif Eif2ak2 Bdkrb2 Il3 Tnfsf13 Wnt1 Gstp1 Hspa1b Jun Foxo1 Stat3 Stat5a Agt
- Inflammatory response only:** Foxm1 Yy1 Tinf2 Top1 Acd
- Cellular senescence only:** Il13 Il10 Trp53 Bax Il4 Tgfb1 Sod1 Il1b Ifng Flt3 Abl1 Akt1 Plau Rac1 Tnf Il6 Igf1 Cd44 Plg Tert Cxcl12 Tgfb2
- Pulmonary fibrosis only:** Cxcr3 Il1r1 Kit Cd40lg Fas Tgfa Ppard Plat Mapt Tslp Stat1 Nfkb1 Nr3c1 Tgfb2 Fgfr1 Csf2 Vtn Egr1 Col3a1 Il5 Ager Alox5 Pdgfrb Fgfr3 Fasl Krt8 Ptgs2 Pparg Sphk1 Mmp7 Src Serpine1 Ada Il1rn Cav1 Cd36 Lgals3 Sparc Terc Fgfr2 Icam1 Ncf1 Selp Smad3 Agrt1a Flt1 Il6ra Vhl Elane

Overlap Counts:

- DNA damage & Inflammatory response: 88
- Inflammatory response & Cellular senescence: 281
- Cellular senescence & Pulmonary fibrosis: 4
- DNA damage & Cellular senescence: 117
- Inflammatory response & Pulmonary fibrosis: 57
- DNA damage & Pulmonary fibrosis: 5
- Inflammatory response & Cellular senescence & Pulmonary fibrosis: 1
- DNA damage & Inflammatory response & Cellular senescence: 22
- Inflammatory response & Cellular senescence & Pulmonary fibrosis: 11
- DNA damage & Cellular senescence & Pulmonary fibrosis: 49
- All four conditions: 27

Figure 3: Intersection of genes identified for each search term observed to be differentially expressed in RIPE.
Venn diagram showing the intersection of genes associated with each search term identified by RNA sequencing in an animal model of RIPF as listed in Supplementary Table 2. Lists for genes for each section of the Venn diagram can be found in Supplementary Table 4.

Table 1:

Number of genes associated with each search term as determined by Gene Cards, UniprotKB, and/or Ingenuity Knowledge Databases in contrast to experimental observation in the RIPF model.

Search Term/Process Interrogated	Total Number of Genes Identified by Database Search	Estimated Percentage of Genome Represented	Number of Genes Represented in RNA seq data	Percent Experimentally Represented Genes Compared to Literature Search
Pulmonary Fibrosis	176	0.4	146	82.9
Cellular Senescence	1529	3.3	1293	84.6
Inflammatory Response	1701	3.7	1266	74.4
DNA damage	1007	2.2	896	89.0

Table 2:
Common Genes associated with DNA Damage, Cellular Senescence, Inflammatory Response, and Pulmonary Fibrosis

Gene ID	Gene Name	Characteristic Gene Expression, Role or Protein Abundance in Pulmonary Fibrosis	Canonical Role (NCBI Entrez Gene Database)
Il13	Interleukin 13	Increases at 4 weeks following proton and ⁵⁶ Fe radiation (56) Increased (up to 12 days) in response to intratracheal instillation of bleomycin (57) Elevated in IPF tissues (58)	Encodes an immunoregulatory cytokine primarily produced by activated Th2 cells. Associated with B-cell maturation and differentiation, up-regulation of CD23 and MHC class II expression, and promotes IgE isotype switching of B cells. Down-regulates macrophage activity, inhibiting production of pro-inflammatory cytokines and chemokines
Il10	Interleukin 10	Increased at 24 hours post intratracheal silica instillation (59) Increased up to 120 days following 22 Gy ⁶⁰ Co thoracic radiation (60)	Anti-inflammatory cytokine secreted by both innate and adaptive immune cells
Trp53	Transformation related protein 53, Tumor protein p53	Rapid and sustained increase in silica-induced fibrosis (61) Upregulated in response to bleomycin induced DNA damage (62)	Encodes tumor protein p53, which responds to stress by regulating target genes that induce cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism
Bax	Bcl-2 associated X protein	Upregulated in response to bleomycin intratracheal instillation (63–65),	Apoptosis regulator of the Bcl-2 family
Il4	Interleukin 4	Elevated in IPF tissues (58) Significantly elevated in patients with connective tissue/interstitial lung disease (66) Elevated in biopsy samples taken from IPF patients (67) Elevated in a transgenic mouse model of pulmonary fibrosis, post 13 weeks of age (68)	Pleiotropic cytokine which promotes the lineage commitment, activation and proliferation of T cells, and B cell activation
Tgfb1	Transforming growth factor beta	Increased up to 120 days following 22 Gy ⁶⁰ Co thoracic radiation (60) Elevated in biopsy samples taken from IPF patients (67) Immediate increase up to 12 hours following 12.5 Gy ¹³⁷ Cs thoracic radiation, followed by resolution and a second period of increased expression from 2–24 weeks post RT (69) Significantly increased at 7 and 14 days following intratracheal bleomycin instillation (70)	Encodes a multifunctional cytokine which undergoes proteolytic processing to generate a mature, functional protein with biological functions such as regulation of cell proliferation, differentiation and growth. The secreted protein can modulate expression and activation of other growth factors including interferon gamma and tumor necrosis factor alpha
Sod1	Superoxide dismutase 1	Persistently increased in response to repeated subcutaneous bleomycin injection (71) Significantly increased at 5 weeks following 12 Gy Thoracic radiation (72)	The protein encoded by this gene binds copper and zinc ions and is one of two isozymes responsible for destroying free superoxide radicals in the body. The encoded isozyme is a soluble cytoplasmic protein, acting as a homodimer to convert naturally-occurring, but harmful, superoxide radicals to molecular oxygen and hydrogen peroxide
Il1b	Interleukin 1 beta	Increased up to 120 days following 22 Gy Co thoracic radiation (60) Significantly decreased at 14 days post (12.5 Gy) thoracic radiation treatment in C57BL/6J mice (19) Significantly elevated protein levels in lung tissue at 10 and 28 days post bleomycin intravenous injection (46)	The protein encoded by this gene is a member of the interleukin 1 cytokine family, and plays a role in thymocyte proliferation and the inflammatory response
Ifng	Interferon gamma	Significantly decreased protein levels in lung tissue at 10 and 28 days post bleomycin intravenous injection (46) Significantly increased at 6 days following intratracheal instillation of bleomycin (73)	This gene encodes a soluble cytokine that is a member of the type II interferon class. The protein is secreted by cells of both the innate and adaptive immune systems which trigger a cellular response to viral and microbial infections

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		No significant difference in serum of patients with connective tissue/interstitial lung disease and healthy individuals (66)	
Flt3	FMS like tyrosine kinase 3	Stimulation of the Flt3 kinase receptor by CSF-1 promotes fibroblast proliferation (74)	This gene encodes a class III receptor tyrosine kinase that regulates hematopoiesis. The activated receptor kinase subsequently phosphorylates and activates multiple cytoplasmic effector molecules in pathways involved in apoptosis, proliferation, and differentiation of hematopoietic cells in bone marrow.
Abl1	c-abl oncogene 1, non-receptor tyrosine kinase	Activated in fibroblasts isolated from human IPF patients (75) c-Abl is required for fibroblast morphologic alteration and proliferation mediated by TGF- β (76)	This gene is a proto-oncogene that encodes a protein tyrosine kinase involved in a variety of cellular processes, including cell division, adhesion, differentiation, and response to stress
Akt1	Thymoma viral proto-oncogene 1	Alveolar macrophage Akt1 is required in the pathogenesis of pulmonary fibrosis (77) Found to be elevated in fibrotic foci of patients with IPF(78)	This gene encodes the founding member of the Akt serine-threonine protein kinase gene family that also includes Akt2 and Akt3. This kinase is a major downstream effector of the phosphatidylinositol 3-kinase (PI3K) pathway that mediates the effects of various growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin and insulin-like growth factor I (IGF-I). Plays a pivotal role in mediating a variety of cellular processes, such as glucose metabolism, glycogen biosynthesis, protein synthesis and tumor over, inflammatory response, cell survival (anti-apoptosis) and development
Plau	Plasminogen Activator, Urokinase (u-PA)	u-PA is significantly reduced in BAL fluid of IPF patients compared to health controls (79) u-PA is decreased in lung tissue of mice given intratracheal bleomycin (80)	Encodes a serine protease (u-PA) involved in degradation of the extracellular matrix and possibly tumor cell migration and proliferation
Rac1	RAS-related C3 botulinum substrate 1	Activity of mitochondrial Rac1 was found to be nearly 4-fold higher in alveolar macrophages obtained from patients with asbestosis compared with normal subjects (81) Rac1 activation in WT monocytes dramatically increased up to 1 hour after exposure to asbestos. (82)	The protein encoded by this gene is a GTPase which belongs to the RAS superfamily of small GTP-binding proteins. Members of this superfamily appear to regulate a diverse array of cellular events, including the control of cell growth, cytoskeletal reorganization, and the activation of protein kinases
Tnf	Tumor necrosis factor	Increased up to 120 days following 22 Gy Co thoracic radiation (60) Significantly elevated in BAL fluid of mice at 2, 8 and 16 days following intratracheal instillation of bleomycin (83) Significantly increased at 14 days post (12.5 Gy) thoracic radiation treatment in C57BL/6J mice (19)	Encodes a multifunctional pro-inflammatory cytokine that belongs to the tumor necrosis factor (TNF) superfamily. It plays an important role in the innate immune response, as well as regulating homeostasis, but is also implicated in diseases of chronic inflammation
Il6	Interleukin 6	Elevated in a transgenic mouse model of pulmonary fibrosis, post 13 weeks of age (68) Significantly elevated protein levels in lung tissue at 10 and 28 days post bleomycin intravenous injection (46)	Encodes a member of the interleukin family of cytokines that have important functions in immune response, hematopoiesis, inflammation and the acute phase response
Igf1	Insulin like growth factor 1	Igf1 protein is significantly elevated in BAL fluid obtained from IPF patients (84) Significantly elevated (mRNA) up to 29 days following repeated intraperitoneal bleomycin injection (85)	Encodes a member of the insulin-like growth factor (IGF) family of proteins that promotes growth and development during fetal and postnatal life
Cd44	Cd44 antigen	Western blot showed that CD44+ protein level was increased from 3 to 21 days weeks after intratracheal bleomycin treatment, and increased the invasive capacity of human lung fibroblasts(86)	The protein encoded by this gene is a cell-surface glycoprotein involved in cell-cell interactions, cell adhesion and migration. It is a receptor for hyaluronic acid (HA) and can also interact with other ligands, such as osteopontin, collagens, and matrix metalloproteinases (MMPs). This protein participates in a

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		Th9 activation increases expression of Ccd44 in normal human lung fibroblasts (87)	wide variety of cellular functions, including lymphocyte activation, recirculation and homing, hematopoiesis, and tumor metastasis
Plg	Plasminogen	Induces fibroblast apoptosis (88) Plasminogen deficient mice showed greater susceptibility to fibrosis compared to wild type controls as measured by fibrin and hydroxyproline content in lung following intratracheal bleomycin instillation (89)	The protein encoded by this gene is a secreted blood zymogen that is activated by proteolysis and converted to plasmin and angiotatin. Plasmin dissolves fibrin in blood clots and is an important protease in many other cellular processes, particularly extracellular matrix disassembly, while angiotatin inhibits angiogenesis
Tert	Telomerase reverse transcriptase	Tert mutations have been associated with interstitial lung disease (90) Shortened telomeres and Tert mutations were identified in patients with sporadic IPF (91)	Telomerase is a ribonucleoprotein polymerase that maintains telomere ends by addition of the telomere repeat TTAGGG. Telomerase expression plays a role in cellular senescence, as it is normally repressed in postnatal somatic cells resulting in progressive shortening of telomeres. Studies in mouse suggest that telomerase also participates in chromosomal repair
Cxcl12/ Sdf1	Chemokine (C-X-C Motif) Ligand 12 Sdf-1	Promotes recruitment of CXCR4 fibrocytes to lung in response to intratracheal bleomycin treatment (92) Plasma and BAL levels of SDF-1/CXCL12 are increased in IPF patients (93)	This gene encodes a member of the alpha chemokine protein family. The encoded protein is secreted and functions as the ligand for the G-protein coupled receptor, chemokine (C-X-C motif) receptor 4. The encoded protein plays a role in many diverse cellular functions, including embryogenesis, immune surveillance, inflammation response, tissue homeostasis, and tumor growth and metastasis
Tgfr2	Transforming growth factor beta receptor 2	Upon binding with TGF- β , TGFBR2 recruits TGFBR1 and phosphorylates TGFBR1 which in turn initiates signal transduction (94) Treatment of hamsters with soluble Tgfr2 after intratracheal instillation of bleomycin significantly reduced BL induced lung fibrosis as shown by decreases in the lung hydroxyproline level and prolyl hydroxylase activity (70)	The encoded protein is a transmembrane protein that has a protein kinase domain, forms a heterodimeric complex with another receptor protein, and binds TGF- β . This receptor/ligand complex phosphorylates proteins, which then enter the nucleus and regulate the transcription of a subset of genes related to cell proliferation