Transcriptome analysis reveals key genes modulated by ALK5 inhibition in a bleomycin model of systemic sclerosis

Benjamin E. Decato1,*, Ron Ammar1,*, Lauren Reinke-Breen1,*, John R. Thompson1, Anthony V. Azzara1,2
*These authors contributed equally.

1Research & Early Development, Bristol-Myers Squibb Company. Route 206 & Province Line Rd, Lawrenceville, NJ 08543 USA.

2Address correspondence to Anthony V. Azzara, Ph.D.
E-mail: anthony.azzara@bms.com.
Postal address: Anthony V. Azzara, Mail Stop M.4880G, 3551 Lawrenceville Road, Princeton, NJ 08540

Running Head
Gene expression profiling of bleomycin-induced model of systemic sclerosis

Abstract

Objective: Systemic sclerosis (SSc) is a rheumatic autoimmune disease affecting roughly 20,000 people worldwide and characterized by excessive collagen accumulation in the skin and internal organs. Despite the high morbidity and mortality associated with SSc, there are no approved disease-modifying agents. Our objective in this study was to explore transcriptomic and model-based drug discovery approaches for systemic sclerosis.

Methods: In this study, we explored the molecular basis for SSc pathogenesis in a well-studied mouse model of scleroderma. We profiled the skin and lung transcriptomes of mice at multiple timepoints, analyzing the differential gene expression that underscores the development and resolution of bleomycin-induced fibrosis.

Results: We observed shared expression signatures of upregulation and downregulation in fibrotic skin and lung tissue, and observed significant upregulation of
key pro-fibrotic genes including GDF15, Saa3, Cxcl10, Spp1, and Timp1. To identify changes in gene expression in responses to anti-fibrotic therapy, we assessed the effect of TGF-β pathway inhibition via oral ALK5 (TGF-β receptor I) inhibitor SB525334 and observed a time-lagged response in the lung relative to skin. We also implemented a machine learning algorithm that showed promise at predicting lung function using transcriptome data from both skin and lung biopsies.

**Conclusion:** This study provides the most comprehensive look at the gene expression dynamics of an animal model of systemic sclerosis to date, provides a rich dataset for future comparative fibrotic disease research, and helps refine our understanding of pathways at work during SSc pathogenesis and intervention.

**Keywords:** Systemic sclerosis, scleroderma; RNA-seq; Fibrosis; Bleomycin; ALK5 inhibitor

**Key messages**

Subcutaneous bleomycin injections induced coordinated pro-fibrotic gene expression changes in skin and lung tissue.

Transcriptomic response to ALK5 inhibition was robust but time-lagged in lung relative to skin.

Transcriptomic profiling from the skin and lung accurately predicted markers of lung function.

**Introduction**

Systemic sclerosis (SSc, the systemic form of scleroderma) is a chronic progressive disease characterized by three main features: vascular injury, immunological abnormalities, and fibrosis of the skin and various internal organs, including the lung.

While skin fibrosis is the hallmark feature of SSc, scleroderma interstitial lung fibrosis is responsible for much of the morbidity and mortality associated with this disease.(1) As of 2018, an estimated 19,390 people were living with SSc with significant predicted growth in new cases through 2038.(2) SSc patients with significant internal organ
involvement have a 10-year survival rate of only 38%. (3) The mechanism underlying the development of fibrosis remains unclear, and current therapeutic options are limited and provide only a modest benefit to patients. (4, 5)

A well-characterized mouse model of scleroderma involves daily subcutaneous injections of the antitumor antibiotic bleomycin (BLM), which leads to localized dermal fibrosis as well as pulmonary fibrosis. (6) In the present study, we utilized this model, which mimics several key features of human SSc, to examine the pathological mechanisms underlying the development and resolution of fibrosis in SSc. It is well established that the TGF-β signaling pathway is required for bleomycin-induced fibrosis in this model and that genetic or pharmacological inhibition of this pathway causes resolution of dermal and pulmonary fibrosis. (7) Thus, we also used the ALK5 (TGF-β receptor I) inhibitor SB525334 to investigate genome-wide changes that occur during TGF-β inhibitor-mediated resolution.

We characterized the development of dermal and pulmonary fibrosis in the bleomycin-induced mouse model of scleroderma. We compared skin and lung signatures, noting significant overlap between the time-course trajectories of increased and decreased expression, as well as produced a proof-of-concept model for predicting lung function outcomes from lung and skin gene expression data, and report the expression changes in well-known collagen formation and degradation genes in response to the ALK5 inhibitor. Taken together, the data generated in this study and our results provide a trove of resources from which the scientific community can build to better understand the development, progression, and treatment of scleroderma.
Results

Experimental design and phenotypic profiling of a bleomycin-induced mouse model of scleroderma

To induce skin and lung fibrosis, female C57BL/6NTac mice were subjected to daily subcutaneous injections of either bleomycin or PBS five times per week for two weeks. Mice were sacrificed on days 7, 14, 21, 28, or 42 following the first bleomycin injections (Figure 1). For the mice that were sacrificed on days 21 or 28 (Groups #5-10), oral dosing of the ALK5 inhibitor SB525334 was initiated one day after the last bleomycin injection. BID dosing in these groups continued through the day prior to sacrifice. To assess the extent of fibrosis, hydroxyproline analysis was conducted on skin and lung tissues from animals sacrificed at all timepoints. Additionally, flexiVent lung function analysis was performed on mice that were sacrificed on Day 21, 28, or 42 (see Methods for details).

Bleomycin induced an increase in collagen deposition in the skin and lungs, indicated by increased hydroxyproline content in both tissues (Figure 2A, B). Skin fibrosis was most prominent at days 14 and 21 and showed evidence of resolution at day 42 (Figure 2A). Treatment with the ALK5 inhibitor caused a decrease in skin fibrosis at day 21. Lung fibrosis was evident at Day 21 and peaked at day 28 (Figure 2B). Treatment with the ALK5 inhibitor caused a decrease in lung fibrosis at Days 21 and 28. Results from flexiVent analysis indicate that lung function was significantly impaired at days 21 and 28 (Figure 2C). Treatment with the ALK5 inhibitor promoted a modest improvement in lung function at both time points. Representative histopathology images at each stage
of the experiment for lung and skin are available in Supplementary Figure S1, available at *Rheumatology* online.

RNA was isolated from skin and lung tissue harvested from mice that were sacrificed at all timepoints, and bulk RNA-seq was conducted on all samples. The resulting gene expression libraries allowed us to investigate transcriptional changes underlying the development and regression of bleomycin-induced fibrosis, as well as the effects of TGF-β pathway inhibition on both tissues. Dimensionality reduction via t-SNE(8) revealed stronger separation of gene expression profiles by tissue type than by timepoint, bleomycin/PBS treatment, or ALK5 intervention (Supplementary Figure S2A, available at *Rheumatology* online). Principal components analysis of individual tissues showed substantial variation by treatment and tissue in the first two principal components for both tissues (Supplementary figure S2B, available at *Rheumatology* online).

**Coordinated skin and lung gene expression changes in response to bleomycin injury**

We first sought to understand the transcriptional changes underlying bleomycin-induced fibrosis. For each of the five timepoints (days 7, 14, 21, 28, and 42), we compared PBS- and bleomycin- treated mice, and summarized the number of differentially expressed genes identified by limma(9) for each tissue in Figure 3A. The number of differentially expressed genes increased from day 7 to a maximum at day 14, just after cessation of the bleomycin treatment, and we observed substantial reduction from that maximum by day 42. This pattern of differential gene expression gave us confidence that the
observed changes were driven primarily by the bleomycin treatment. For each of these
differentially expressed gene sets, we used GSEA to compute enrichment of pathways
in MSigDB including KEGG, Biocarta, and Reactome (Supplementary Figures S3 & S4,
available at Rheumatology online). We observed significant upregulation of cell cycle,
KEGG disease, and biosynthesis pathways through early timepoints, giving way to
upregulated extracellular matrix formation and degradation pathways in both tissues at
days 28 and 42.

A key objective of our study was to assess the degree and temporal trajectory of
change in the transcriptional profiles in skin versus lung. To do this, we performed
unsupervised clustering on the differentially expressed genes and explored whether
gene expression changes were occurring in the same direction over time for both skin
and lung. The clusters that resulted are shown in Figure 3B. In skin, these clusters
roughly represented four unique trajectories, which we have named Skin1 – Skin4:
persistent upregulation in response to bleomycin, followed by downregulation post-bleo
(Skin1); increasing downregulation in response to bleomycin, followed by upregulation
post-bleo (Skin2); persistent downregulation in response to bleomycin, followed by
upregulation post-bleo (Skin3); and increasing upregulation in response to bleomycin,
followed by downregulation post-bleo (Skin4).

Lung clusters were produced and named arbitrarily, and then re-ordered in Figure 3B
according to their closest matching Skin cluster as identified in the UpSet plot in Figure
3C. The largest overlap occurred between Skin1 and Lung3, meaning that most genes
with a persistent upregulation in response to bleomycin followed by down-regulation
post-bleo shared that pattern in both lung and skin. Lung cluster trajectories matched
skin cluster trajectories in all four cases, indicating that similar sets of genes are being activated or repressed in response to bleomycin in both tissues. A full list of cluster membership is available in Supplementary Table S1 (available at Rheumatology online), and cluster probability distributions are shown in Supplementary Figure S5B (available at Rheumatology online).

To get a more detailed view of which genes showed the largest coordinated bleomycin-induced changes in both skin and lung, we generated volcano plots of differential expression for skin and lung at day 14, when bleo-induced changes reached an apex in both tissues (Figure 3D). We observed 261 and 59 genes that displayed differential expression between bleomycin and PBS in skin and lung, respectively (colored blue, absolute log fold change > 3 and FDR-corrected p-value < 0.1; Supplemental Table S2, available at Rheumatology online). Exploration of overlap yielded 8 genes differentially expressed in both tissues (labeled on both volcano plots; Timp1 log fold change 2.913 in skin). This provided us with a short list of large, coordinated gene expression changes across both tissues. Notably, many of these genes have been previously shown to be increased in SSc patients (see Discussion for a complete list). Taken together, these results point to shared pathophysiology between the bleomycin-induced mouse model of scleroderma and human SSc.

**Time-lagged differential gene expression of ALK5 inhibitor driven intervention**

Next, we sought to understand the effect of the ALK5 inhibitor SB525334 on resolution of bleomycin-induced fibrosis by exploring differential expression between SB525334-
treated and vehicle-treated mice in both lung and skin at days 21 and 28. Figure 4A shows that at day 21, there are substantial expression differences in the SB525334-treated mice in skin, but no subsequent changes in lung. By day 28, a comparable number of differentially expressed genes are observable in both tissues, suggesting that systemic effects of oral SB525334 may not be temporally consistent across tissues.

Of the genes showing differential expression in mice treated with SB525334, 1,055 were differential at both timepoints in the skin, and 750 were shared between skin and lung on day 28 (Figure 4B). Analysis of the top differentially expressed genes at Day 28 between SB525334 and vehicle treated samples revealed downregulation of several keratin associated genes in skin and one, Krt4, in lung (Figure 4C). Several collagen genes including Col1a1, Col1a2, Col3a1, Col5a1, Col5a2, and Col12a1 also displayed time-lagged differential expression between bleomycin- and PBS treated mice, and reductions in response to SB525334 (Supplemental Figure S6, available at Rheumatology online). GSEA pathway analysis (see Methods) for genes significantly differentially expressed in skin and lung at day 28 revealed downregulation of the Naba collagen pathway in SB525334-treated skin but not lung, suggesting a potential for continued lag in response (Figure 4D). One gene, Bgn, codes for biglycan and was significantly downregulated in the SB525334 group in all four tissues/timepoints pairs. Biglycan has been previously associated with type I and type II collagens(10), and its synthesis is stimulated by ALK5.(11)

**Predicting lung function with lung and skin transcriptional profiles**
Of the measurements available for mouse lung function, inspiratory capacity (IC) is considered to be the most analogous to forced vital capacity (FVC). In general, we observed that IC was higher for PBS-treated mice than for bleomycin-treated mice, indicating that bleomycin treatment caused impairment of lung function.

Trained on the lung and skin transcriptional data, we constructed models of IC (see Methods) in order to determine if lung or skin biopsies could function as surrogate biomarkers of lung function. Due to limited availability of validation data sets, we used repeated cross-validated performance to assess the models.

We observed that $M_1$ and $M_2$ models were equally informative for predicting IC ($\text{RMSE}(\mu_1) \approx 0.8$ and $\text{RMSE}(\mu_2) \approx 0.8$; Figure 5). While our sample numbers were limited, these results are encouraging and suggest that RNA-seq from lung or skin could serve as a biomarker of lung function.

**Discussion**

The subcutaneous bleomycin mouse model is widely used to support efforts to develop novel therapies for scleroderma, including *in vivo* efficacy studies and biomarker identification and validation. The transcriptomics data presented here represents a novel resource that can be utilized to support ongoing and future studies using this model. The novel design of the present study allows for the comparison of gene expression changes in both skin and lung in response to bleomycin. Our results demonstrate a coordinated response to bleomycin across tissue types, highlighted by the trajectory clusters in Figure 3B. These findings reflect one of the hallmark features
seen in SSc patients: the relatively concurrent development of fibrosis across multiple organs. (12) Thus, our datasets represent a novel resource that can be used to further investigate the systemic development and progression of tissue fibrosis. Further, these data represent the possibility that genetic signatures from peripherally accessible skin biopsies could reflect and/or predict the disease status of the lung. This may represent a significant enhancement over current lung evaluations, which are limited to function imaging-based morphology, due to the risk associated with taking lung tissue samples or lavage.

Additionally, as highlighted in Figure 3D, a number of genes that exhibited differential expression in both skin and lung in response to bleo have been shown to be increased in SSc patients:

GDF15 – GDF15 (growth differentiation factor 15) is a distant member of the TGF-β family. GDF15 is increased in the serum of SSc patients and is correlated with disease severity and extent of organ involvement, particularly with lung fibrosis. (13, 14)

CXCL10 – CXCL10 (also known as IP-10) is a Th1 chemokine that is induced by IFN-γ. (15) CXCL10 is increased in the skin and serum of SSc patients, and increased serum levels of CXCL10 are associated with disease severity and increased internal organ involvement. (16-18)

MMP10 – MMP10 (also known as stromelysin 2) is a matrix metalloproteinase that plays a critical role in ECM degradation and remodeling during wound healing and vascular remodeling. (19) MMP10 is increased in the serum and pulmonary arteries of SSc patients with pulmonary hypertension. (20)
TIMP-1 – TIMP-1 (tissue inhibitor of matrix metalloproteinase-1) levels are increased in the serum and lesional skin of SSc patients. (21-23) Interestingly, TIMP-1 is one of three soluble proteins comprising the ELF (enhanced liver fibrosis) test. The ELF test was recently validated as an SSc biomarker that correlates with both skin and lung involvement. (24)

Saa3 – Saa3 is a member of the serum amyloid A (SAA) family, which consist of early-phase proteins known to play a key role in inflammation. (25) Serum SAA levels are increased in SSc patients and correlated with the extent of pulmonary involvement. (25, 26)

Retnla – Retnla (resistin-like alpha, also known as RELM-α or FIZZ1) has been identified as an M2 macrophage marker in mice. (27) The closest human homolog, RETLN-β, is increased in the lungs of SSc patients with pulmonary hypertension. (28)

SPP1 – SPP1 (secreted phosphoprotein 1, also known as osteopontin) is a matricellular protein that exhibits proinflammatory and profibrotic properties. (29) Osteopontin is increased in both the serum and lesional skin of SSc patients. (29, 30)

These findings support the utility of this model to help identify novel targets and/or biomarkers for SSc. Our data showing that Alk5 inhibition exhibits a time-lagged response in lung versus skin suggests that in vivo compound efficacy studies may need to be designed to allow for analysis of different tissues at multiple timepoints to thoroughly assess systemic treatment effects.
Our preliminary analyses using novel modeling techniques to predict lung function based on gene expression have laid the groundwork for future translational studies. Our findings suggest that there are similarities in gene expression patterns in skin and lung that can be taken advantage of to allow for an increased ability to monitor the fibrotic state of the lungs of SSc patients by assessing gene expression changes in skin biopsies. Analysis of gene expression patterns in the skin may potentially offer insight into which patients will develop SSc-ILD, an aspect of the disease that accounts for a considerable proportion of its morbidity and mortality. The ability to better identify these at-risk patients would allow for earlier treatment and improved patient outcomes.

**Methods**

**Study design**

Twelve-week-old female C57BL/6NTac mice (Taconic) were given daily subcutaneous injections of either bleomycin (Hospira; 10 mg/kg per day) or PBS (vehicle control) in a shaved interscapular region. Injections were done using a 27-gauge needle, five times per week for two consecutive weeks (Days 0-4 and 7-11, inclusive). Mice were re-shaved as necessary, and circles were re-drawn every 1-2 days throughout the study.(31, 32)

Beginning on Day 12, the ALK5 inhibitor SB525334 (30mpk) or vehicle was delivered (PO, BID dosing). Note that Groups 1-2 and Groups 3-4 were sacrificed on Day 7 and Day 14, respectively, and thus did not receive oral dosing. Groups 5-7 were sacrificed on Day 21. The last oral doses were administered on Day 27. Groups 8-10 were
sacrificed on Day 28. Groups 11-12 (which specifically serve to assess resolution in this
model, and do not examine the effect of SB525334) were sacrificed on Day 42.

Supplementary figure S7A (available at *Rheumatology* online) provides additional
information on groups (12 groups, n = 8-10 per group at onset of the study; a total of
seven animals died during the study).

**Lung function analysis**

The mechanical properties of the mouse lungs were determined using the flexiVent
apparatus (Scireq, Montreal, QC, Canada). In brief, mice were anesthetized by
intraperitoneal administration of Ketamine (91 mg/kg; VedCo) and Xylazine (9.1 mg/kg;
Akorn Animal Health), a tracheotomy was performed, and an 18-gauge cannula was
inserted into a slit in the trachea and connected to the flexiVent computer-controlled
rodent ventilator. After an initial period of ventilation, measurement of lung mechanical
properties was initiated by a computer-generated program to measure inspiratory
capacity, compliance, lung resistance, tissue elastance, and tissue damping. These
measurements were repeated 3 times for each animal.

**Analysis of hydroxyproline content**

Hydroxyproline analysis was conducted on half of an 8mm skin biopsy, or on the
middle, inferior, and post-caval lung lobes. The QuickZyme Total Collagen Assay kit
(QuickZyme Biosciences, The Netherlands) was used according to the manufacturer’s
instructions. For skin, hydroxyproline levels were normalized to tissue weights to
account for potential variability in tissue procurement.
RNA-seq library preparation and pre-processing

Small pieces of tissue (~5mg) were added to a 2ml tube with one 5mm diameter stainless steel bead (Cat # 69989, Qiagen, Valencia, CA) and 1.2ml Trizol® (Life Technologies, Cat # 15596018, Grand Island, NY). Tissue was homogenized by TissueLyzerII (Qiagen, Valencia, CA) (lung for 1 minute, skin for 3 minutes). 500ul chloroform was then added to the homogenate. After vortexing for 30 seconds, samples were centrifuged at 14,000 rpm for 15 minutes at 4°C. The supernatant layer was collected into a deep 96-well plate, mixed with 50ul 70% ethanol. The mixtures were transferred to the RNaseasy 96 well plate, (RNaseasy® 96 Kit, Cat # 74881, Qiagen, Valencia, CA 91355). RNA was isolated by following the manufacturer’s protocol. RNA was eluted in 90ul H2O then treated with DNaseI by adding 10ul DNaseI 10X buffer + 2ul DNaseI (ThermoFisher, Cat# AM2222) for 15 min at room temperature. Treated RNA was cleaned up by using RNaseasy® 96 Kit following the manufacturer’s protocol. The quality and quantity of the isolated total RNA were evaluated with a NanoDrop 8000 spectrophotometer (Thermo Scientific, Wilmington, DE). RNA was then normalized to 50ng/ul, randomized with DOE, prepared using the Illumina TruSeq Total RNA-Gold kit and sequenced with Illumina HiSeq sequencing.

Raw sequence files were processed by the bcl2fastq software in BaseSpace to generate FASTQ files for each sample. Sequence reads in the FASTQ files were then aligned to the Mouse.B38 genome build using Omicsoft ArrayStudio with the OSA alignment algorithm.(33) Ensembl gene models (version R86) were used by Omicsoft’s reimplementation of the RSEM algorithm to generate gene-level counts for each
The resulting gene by sample count matrix was further processed in R using \textit{voomWithQualityWeights} together with a \textit{limma} linear modeling workflow to assess differential gene expression.\footnote{}\footnote{} Quality control statistics including mapping rate, mitochondrial DNA rate, and more are available in Supplemental table S3.

RNA quality as measured through RIN score is available in Supplementary figure S7B (available at \textit{Rheumatology} online). Multiple plates were used for RNA-seq library preparation: we used \textit{ComBat}\footnote{} to correct for this confounding variable and demonstrate the reduction of variance explained by plate pre- and post-run using Variance Partition Analysis (Supplementary figure S7C & D, available at \textit{Rheumatology} online).

\textbf{Differential gene expression analysis}

Differential expression analysis was performed independently for lung and skin samples using \textit{limma} with plate and RIN score included as covariates. Genes were identified as differentially expressed if their FDR-corrected p-value was less than 0.1; p-value distributions are available in Supplementary figures S5A and S5C (available at \textit{Rheumatology} online) for all contrasts studied. All differential expression performed for every tissue and contrast, with gene names, log fold changes, and adjusted p-values is summarized in Supplementary Table S4 (available at \textit{Rheumatology} online).

Time-course clusters were identified using \textit{TCseq} and UpSet plots were generated using UpSetR.\footnote{...}
We used the Molecular Signatures Database (MSigDB)(43) and computed Gene Set Enrichment Analysis as described previously.(44) We used the fast implementation fgsea.(45)

The GSEA enrichment score (ES) represents the degree to which a gene set is overrepresented at the top or bottom of a ranked list of genes. A positive ES indicates gene set enrichment at the top of the ranked list, and a negative ES indicates gene set enrichment at the bottom of the ranked list. The ES is a function of gene set size, and, therefore, ESs cannot be directly compared across gene sets. Cross-gene set comparisons are facilitated by the normalized enrichment score (NES).

**Model construction and optimization**

Inspiratory Capacity (IC) was modeled as a continuous response by fitting a linear regression model to the RNA-Seq expression data, computed with an elastic net regularization path. We modeled lung and skin tissues separately yielding two models of IC, $\mathcal{M}_{\text{lung}}$ and $\mathcal{M}_{\text{skin}}$. Linear regression can be unreliable when $\hat{\sigma} > \sigma$ (relatively few samples with many transcript observations). By linearly combining both $\hat{\sigma}_1$ and $\hat{\sigma}_2$ penalties of the lasso and ridge regression methods, respectively, elastic net regularization improves model performance.(46-48)

Elastic net training requires the selection of both a lasso and ridge mixing parameter, $\alpha$, and a penalty strength parameter, $\lambda$. To identify the optimal combination with the highest performance, we conducted 10-fold balanced cross-validation for each $(\alpha, \lambda)$ pair in a grid search on each training set. We chose $\lambda = 0.95$ based on the suggestion in the glmnet documentation to set $\hat{\sigma} = 1 - \sigma$ for some small $\sigma > 0$.46 The rationale is to
improve numerical stability and reduce the degeneracies caused by high correlations between covariates.

We performed 100 repeats of 10-fold cross-validation in caret to select the \( \theta \) that yielded the highest performing final model (with the lowest mean-squared error).(49)

**Disclosures**
BED, RA, LR-B, JRT, and AVA were employees of Bristol Myers Squibb when the work was done and may hold stock in the company.

**Funding Source**
All work described in this manuscript was funded by Bristol Myers Squibb.

**Ethics approval**
This study was conducted with the approval of the Bristol Myers Squibb Internal Animal Care and Use Committee. All protocols and procedures were reviewed and approved prior to the start of the animal experiments.

**Data and code availability**
All RNA-seq data analyzed in this manuscript was deposited in GEO under accession number GSE132869. Code associated with this analysis can be found at www.github.com/bdecato/SSc-Transcriptome-Manuscript-Code.

**Acknowledgements**
The authors would like to thank L. Burns for *in vivo* support, Yan Zhang for isolating RNA from skin and lung tissue, Manling Ma-Edmonds for conducting the RNA-seq library prep and running the RNA-seq analysis, and anonymous reviewers for their helpful feedback.

**Figure Captions**

**Figure 1:** Overview of study design and animal groups. Bleomycin was dosed at 10 mg/kg/day, and SB525334 was dosed at 30 mg/kg. BLM: bleomycin, BID: Twice a day; PO: Oral administration; SC: subcutaneous

**Figure 2:** Effects of bleomycin treatment and ALK5 inhibition on markers of collagen formation and lung function. (A) Hydroxyproline content of skin tissue. (B) Hydroxyproline content of lung tissue (ml, middle lobe; il, inferior lobe; pcl, postcaval lobe). (C) Lung function as assessed by flexiVent analysis. (BLM, bleomycin; ALK5i, the ALK5 inhibitor SB525334). Error bars represent SEM. Statistical significance was assessed using One-way ANOVA with Bonferroni’s correction.

**Figure 3:** Integrative differential gene expression analysis between bleomycin-treated mice and control across tissues. (A) Number of differentially expressed genes in lung and skin between bleomycin (BLM) and PBS treated mice. (B) Cluster-matched patterns of z-score normalized differential expression in skin (top) and skin (bottom). (C) UpSet plot showing the number of genes in each cluster (left) and magnitude of overlaps between clusters (top). (D) Volcano plots of differential
expression in skin and lung, colored by FDR-corrected significance in skin and an absolute log-fold-change >=3, with shared genes labeled on the lung volcano plot.

**Figure 4**: Differential gene expression and pathway enrichment in response to ALK5i treatment. (A) Number of differentially expressed genes in lung and skin between ALK5 inhibitor treated and PBS treated mice. (B) UpSet plot showing the overlap of differentially expressed gene sets in the four time/tissue pairs for ALK5 inhibitor vs PBS treated mice. (C) Pathway enrichment UpDown plots showing the fraction and number of genes in each pathway upregulated/downregulated in the SB525334 arm compared to vehicle on day 28 for skin and lung.

**Figure 5**: Boxplots of R² predictive power for elastic net models predicting inspiratory capacity from expression data.

**References**


Figure 1: Overview of study design and animal groups. Bleomycin was dosed at 10 mg/kg/day, and SB525334 was dosed at 30 mg/kg. (BLM, bleomycin)

203x190mm (600 x 600 DPI)
Figure 2: (A) Hydroxyproline content of skin tissue. (B) Hydroxyproline content of lung tissue (ml, middle lobe; il, inferior lobe; pcl, postcaval lobe). (C) Lung function as assessed by flexiVent analysis. (BLM, bleomycin; ALK5i, the ALK5 inhibitor SB525334). Error bars represent SEM. Statistical significance was assessed using One-way ANOVA with Bonferroni’s correction.

203x228mm (600 x 600 DPI)
Figure 3: (A) Number of differentially expressed genes in lung and skin between bleomycin (BLM) and PBS treated mice. (B) Cluster-matched patterns of z-score normalized differential expression in skin (top) and skin (bottom). (C) UpSet plot showing the number of genes in each cluster (left) and magnitude of overlaps between clusters (top). (D) Volcano plots of differential expression in skin and lung, colored by FDR-corrected significance in skin and an absolute log-fold-change $\geq 3$, with shared genes labeled on the lung volcano plot.
Figure 4: (A) Number of differentially expressed genes in lung and skin between ALK5 inhibitor treated and PBS treated mice. (B) UpSet plot showing the overlap of differentially expressed gene sets in the four time/tissue pairs for ALK5 inhibitor vs PBS treated mice. (C) Pathway enrichment UpDown plots showing the fraction and number of genes in each pathway upregulated/downregulated in the SB525334 arm compared to vehicle on day 28 for skin and lung.
Figure 5: Boxplots of $R^2$ predictive power for elastic net models predicting inspiratory capacity trained on expression data from lung and skin.

148x97mm (600 x 600 DPI)