# ORIGINAL ARTICLE

# Glycolytic Reprogramming in Myofibroblast Differentiation and Lung Fibrosis

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## Abstract

**Rationale:** Dysregulation of cellular metabolism has been shown to participate in several pathologic processes. However, the role of metabolic reprogramming is not well appreciated in the pathogenesis of organ fibrosis.

**Objectives:** To determine if glycolytic reprogramming participates in the pathogenesis of lung fibrosis and assess the therapeutic potential of glycolytic inhibition in treating lung fibrosis.

**Methods:** A cell metabolism assay was performed to determine glycolytic flux and mitochondrial respiration. Lactate levels were measured to assess glycolysis in fibroblasts and lungs. Glycolytic inhibition by genetic and pharmacologic approaches was used to demonstrate the critical role of glycolysis in lung fibrosis.

**Measurements and Main Results:** Augmentation of glycolysis is an early and sustained event during myofibroblast differentiation, which is dependent on the increased expression of critical glycolytic enzymes, in particular, 6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase 3 (PFKFB3). Augmented glycolysis contributes to the stabilization of hypoxia-inducible factor 1-$\alpha$, a master regulator of glycolytic enzymes implicated in organ fibrosis, by increasing cellular levels of tricarboxylic acid cycle intermediate succinate in lung myofibroblasts. Inhibition of glycolysis by the PFKFB3 inhibitor 3PO or genomic disruption of the *PFKFB3* gene blunted the differentiation of lung fibroblasts into myofibroblasts, and attenuated profibrotic phenotypes in myofibroblasts isolated from the lungs of patients with idiopathic pulmonary fibrosis. Inhibition of glycolysis by 3PO demonstrates therapeutic benefit in bleomycin-induced and transforming growth factor-$\beta$1–induced lung fibrosis in mice.

**Conclusions:** Our data support the novel concept of glycolytic reprogramming in the pathogenesis of lung fibrosis and provide proof-of-concept that targeting this pathway may be efficacious in treating fibrotic disorders, such as idiopathic pulmonary fibrosis.

**Keywords:** lung fibrosis; glycolysis; myofibroblast; PFKFB3; HIF-1$\alpha$

Glucose metabolism begins with its transportation via glucose transporters into cytosol, where it is converted into pyruvate through a sequence of 10 biochemical reactions termed glycolysis (1–6). Three reactions in the glycolytic pathway that are catalyzed by hexokinase (HK), phosphofructokinase (PFK), and pyruvate kinase are irreversible and often subject to regulation for the control of the glycolytic flux rate (1–6). With sufficient oxygen, most of pyruvate is carried into mitochondria to be converted to acetyl coenzyme A, which enters the tricarboxylic acid (TCA) cycle to produce nicotinamide adenine dinucleotide reduced that then flows through oxidative phosphorylation (1–3). Under oxygen deprivation, pyruvate is broken down to create lactate in the cytosol (1–3). Augmented glycolysis takes place not only in the absence of oxygen, but also under aerobic conditions, a phenomenon dubbed as "Warburg effect" (1–5, 7).

## At a Glance Commentary

**Scientific Knowledge on the Subject:** Although aberrant cellular metabolism has been shown to participate in many pathologic processes, there are few attempts to identify metabolic dysregulations associated with organ fibrosis, including idiopathic pulmonary fibrosis. Metabolic programs in fibrotic lungs remain uncharacterized. Metabolism-based therapeutics for treating fibrotic disorders is lacking.

**What This Study Adds to the Field:** In this study, we found that glycolytic reprogramming is critical to lung myofibroblast differentiation and pulmonary fibrosis. We identified glycolytic enzymes that mediate aerobic glycolysis in myofibroblasts. We demonstrated a unique mechanism of lung fibrosis in that augmented glycolysis leads to up-regulation of the tricarboxylic acid cycle intermediate succinate, which stabilizes hypoxia-inducible factor 1-α to directly promote myofibroblast differentiation. More importantly, we provided the first evidence of treating pulmonary fibrosis through glycolytic inhibition.

A defining feature of idiopathic pulmonary fibrosis (IPF) and other progressive fibrotic pathologies is the activation and persevering presence of tissue myofibroblasts (myo-Fbs) (8–13). Myo-Fbs are characterized by *de novo* expression of smooth muscle actin (SMA)-α; formation of stress fibers; and enhanced abilities to migrate, contract, and produce extracellular matrix (14–16). These features have led to the consensus that targeting myo-Fbs represents an effective therapeutic strategy in the treatment of these clinically refractory disorders, including IPF (12, 14–16).

Metabolic perturbation is implicated in the pathogenesis of several diseases, such as diabetes and cancer (2, 5, 17). However, there is a paucity of information regarding the role of cellular metabolism in tissue fibrosis. More pertinently, it is unknown what glycolytic program lung myo-Fbs use, whether glycolysis is required for lung myo-Fb differentiation and

sustainment of their distinctive phenotypes, or whether modulation of glycolysis affects the progression of lung fibrosis *in vivo*.

In this study, we found that lung myo-Fbs adopt a unique glycolytic program (i.e., aerobic glycolysis). We found that key enzymes that catalyze glycolytic reactions are up-regulated in *in vitro* differentiated myo-Fbs and myo-Fbs from patients with IPF. Furthermore, we found that glycolysis is required for the establishment and sustainment of profibrotic phenotypes. Lastly, we demonstrated that inhibition of glycolysis is an effective approach to treat pulmonary fibrosis. Our study has thus established a solid foundation for developing glycolysis-based therapies in treating fibrotic disorders. Some of the results of this study have been previously reported in the form of an abstract (18).

## Methods

### Reagents
Rat tail collagen was obtained from Invitrogen (Carlsbad, CA). Human recombinant transforming growth factor (TGF)-β1 was obtained from Peprotech (Rocky Hill, NJ). Sircol collagen assay kit was obtained from Biocolor (Carrickfergus, UK). Bleomycin was obtained from Besse Medical (West Chester, OH). 3-(3-Pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) was obtained from Axon Medchem (Reston, VA).

### Experimental Pulmonary Fibrosis Model
C57BL/6 mice were purchased from Charles River (Wilmington, MA). The bleomycin and TGF-β1–induced lung fibrosis models were described previously (19, 20) and detailed in the online supplement METHODS. The animal protocol was approved by the University of Alabama Birmingham Institutional Animal Care and Use Committee.

### Cell Lines
Human lung Fb line MRC-5 was purchased from American Type Culture Collection (Manassas, VA).

### Human Lung Tissues
IPF and failed donor control tissues were obtained from the University of Alabama Birmingham Tissue Procurement and Cell Culture Core. The protocol was

approved by the institutional review board at the University of Alabama at Birmingham.

### Isolation of Primary Human Lung Fbs
Primary human lung Fbs were established as previously described (21) and detailed in the online supplement METHODS.

### Collagen Content Determination (Sircol Assay)
Sircol assay was performed as previously described (22).

### Masson Trichrome Assay
The assay was performed using trichrome stain (Masson) kit from Sigma-Aldrich (St. Louis, MO).

### Immunohistochemistry
Immunohistochemistry was performed as described in our previous studies (23).

### Real-Time Polymerase Chain Reaction
RNA levels of SMA-α, hypoxia-inducible factor 1-α (HIF-1α), fibronectin (Fn), collagen 1A1 (Col1A1), and tubulin were determined by real-time polymerase chain reaction using SYBR Green Master Mix Kit (Roche, Indianapolis, IN). Primer sequences were provided in the online supplement METHODS.

### CRISPR/Cas9 Lentivirus Generation
The lentiviral vector LentiCRISPRv2 that expresses Cas9 and a guide RNA was developed by Dr. Feng Zhang at Massachusetts Institute of Technology and deposited to Addgene (Cambridge, MA) (24). Detailed information was provided in the online supplement METHODS.

### Western Blotting
Western blotting was performed as previously described (23). Mouse anti–SMA-α and -tubulin antibodies were from Sigma. Anti–p-Smad2, -Smad2, and –HIF-1α antibodies were from Cell Signaling (Danvers, MA). Anti–6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) antibody was from Proteintech (Chicago, IL) and Abcam (Cambridge, MA).

### Chromatin Immunoprecipitation Assay
Chromatin immunoprecipitation assays were performed as previously described (25). Detailed information was provided in the online supplement METHODS.

### Real-Time Cell Metabolism Assay

XF-24 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA) was used for real-time analysis of extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) (26). Detailed experimental protocol was provided in the online supplement METHODS.

### Intracellular and Extracellular Lactate Assays

Intracellular and extracellular levels of lactate were determined using lactate assay kit (BioVision, Milpitas, CA) according to the manufacturer's instructions.

### Intracellular Succinate Assay

Intracellular levels of succinate were determined using succinate assay kit (BioVision) according to the manufacturer's instructions.

### Statistical Analysis

One-way analysis of variance followed by the Bonferroni test was used for multiple group comparisons. Student's $t$ test was used for comparison between two groups. $P$ less than 0.05 was considered statistically significant.

## Results

### Lung myo-Fbs and Fibrotic Lungs Demonstrate Augmented Glycolysis

To determine if the pathogenesis of lung fibrosis involves glycolytic dysregulation, we assessed the glycolytic program initially in lung myo-Fbs that were differentiated with TGF-β1, a putative profibrotic growth factor (27, 28). We determined ECAR as a measure of lactate production (a surrogate for glycolytic rate) (29), using Seahorse X24 Extracellular Flux Analyzer. As shown in Figure 1A, TGF-β1 treatment led to an early and sustained increase in ECAR in lung Fbs, indicating the occurrence of enhanced glycolysis in these cells. OCR, a representation of mitochondrial respiratory activity, also had an increase after treatment with TGF-β1, but to a lesser extent than did ECAR (Figure 1B). As a result, the ratio of ECAR/OCR was increased in TGF-β1–differentiated myo-Fbs (Figure 1C).

These data suggest that lung myo-Fbs demonstrate features of aerobic glycolysis (7). More importantly, we found that myo-Fbs from IPF lungs also adopt the aerobic

glycolytic program, as reflected by greater ratios of ECAR/OCR in these cells than those in control normal lung Fbs (Figure 1D). Together, these data suggest that aerobic glycolysis may serve as a new defining phenotype for myo-Fbs. To confirm the glycolytic alterations in lung myo-Fbs, we directly measured the levels of lactate and found that both intracellular and extracellular lactate in lung myo-Fbs were significantly increased (Figures 1E and 1F). Consistent with the augmented glycolysis in lung myo-Fbs, these cells also demonstrated greater glucose consumption (Figure 1G). Altogether, the data suggest that enhanced glycolysis may participate in myo-Fb differentiation during pathologic fibrogenesis in the lungs.

To determine if a similarly augmented glycolytic program takes place *in vivo* in fibrotic lungs, we directly measured lactate contents in lung tissues and found that there is significantly more lactate in the lungs of mice that received intratracheal instillation of bleomycin, a protocol widely used to establish experimental pulmonary fibrosis (28), than that in the control mouse lungs (Figure 1H). To further confirm that the augmented glycolysis does occur during lung fibrosis, we used another well-recognized model in that mice were intratracheally administered adenovirus that express constitutively active TGF-β1 (Ad-TGF-β1[223/225]) (19). As shown in Figure 1I, there were significantly greater levels of lactate in the lungs of mice that received TGF-β1–expressing adenovirus. More importantly, lactate levels in IPF lungs were also markedly increased compared with those in normal human lungs (Figure 1J). Altogether, these data clearly demonstrate that glycolysis is reprogrammed to a higher level during the pathogenesis of lung fibrosis.

### Key Glycolytic Enzymes Are Up-regulated in Lung myo-Fbs

To delineate the mechanisms that contribute to the augmented glycolysis observed in lung myo-Fbs, we assessed the expression of key glycolytic enzymes in these cells. We found that PFKFB3 is markedly induced by TGF-β1 in lung Fbs (Figure 2A). PFKFB3 is not a rate-limiting glycolytic enzyme; instead it catalyzes the conversion of fructose-6-phosphate to fructose-2,6-bisphosphate, an allosteric activator of PFK1 and a potent stimulator of glycolysis (30–32). PFK1 and HK2,

rate-limiting enzymes in the glycolytic pathway (33, 34), were also significantly up-regulated in TGF-β1–induced lung myo-Fbs (Figures 2B and 2C). These data suggest that the augmented glycolytic program in lung myo-Fbs is caused by increased expression of key enzymes that catalyze this process. As expected, the myo-Fb marker SMA-α was up-regulated in TGF-β1–induced lung myo-Fbs (Figure 2D).

To determine if the similar mechanism that contributes to the augmented glycolysis in TGF-β1–differentiated lung myo-Fbs is also involved in IPF myo-Fbs, we examined the expression of these glycolytic enzymes in the cells. As shown in Figures 2E–2G, the expression of PFKFB3, PFK1, and HK2 was also significantly enhanced in IPF myo-Fbs, compared with that in human normal lung Fbs. Of note, there was increased expression of SMA-α in IPF myo-Fbs, consistent with the profibrotic phenotype of these cells (Figure 2H).

Although we identified several enzymes that are presumably responsible for the augmented glycolysis in lung myo-Fbs, we chose to primarily focus on PFKFB3. We reasoned that inhibition of enzymes mediating the key rate-limiting glycolytic steps, such as PFK1 and HK2, may result in drastic and irreversible plunge in glycolytic flux and potentially cause unwanted adverse effects, particularly during *in vivo* applications. However, inhibition of the non-rate-limiting PFKFB3 may have milder impact on glycolysis and thus avoid this situation (31).

During the detailed characterization of PFKFB3 expression, we found that the protein levels of this enzyme were increased rapidly and peaked at 8 hours in lung Fbs after TGF-β1 stimulation (Figure 2I), in accordance with the early increased glycolysis in these cells. The up-regulation of PFKFB3 took place at the transcriptional level because inhibition of cellular transcription by actinomycin blunted the elevated expression (Figure 2J). PFKFB3 induction was also mediated by Smad2/3-dependent TGF-β1 signaling events because either the inhibitor against TGF-β1 type I receptor or small interfering RNAs targeting Smad2/3 abolished TGF-β1–induced PFKFB3 in lung Fbs (Figures 2K and 2L).

IPF myo-Fbs are known to be heterogeneous, with cells being at different stages of differentiation (35). To determine the correlation of PFKFB3 expression and
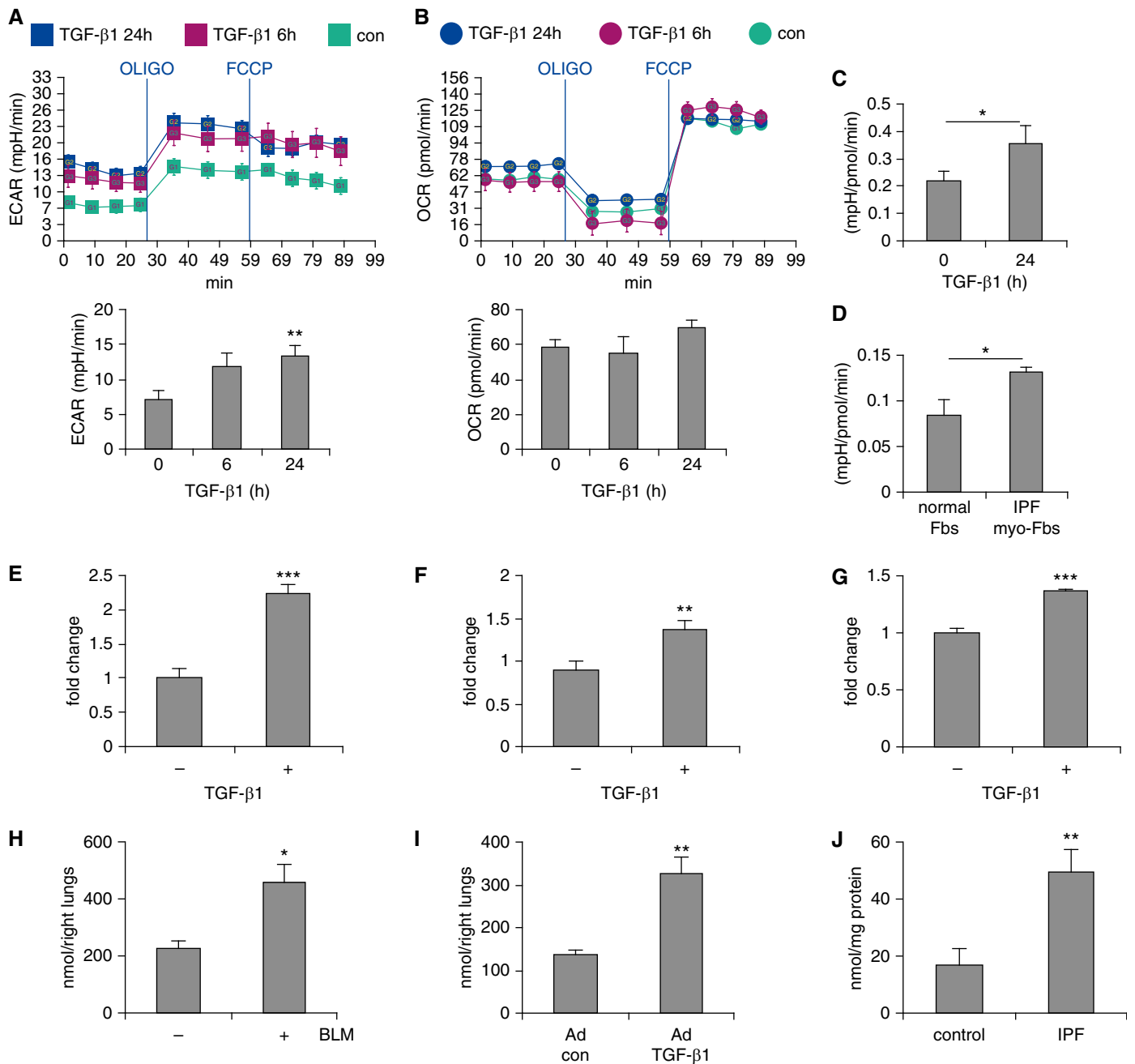
**Figure 1.** Lung myofibroblasts (myo-Fbs) and fibrotic lungs demonstrate augmented glycolysis. (*A* and *B*) Human normal lung Fbs MRC-5 were seeded in Seahorse XF-24 cell culture microplates. The cells were treated with 2 ng/ml transforming growth factor (TGF)-β1 for 0, 6, or 24 hours, followed by sequential treatments with oligomycin (OLIGO) and carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP). Real-time extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were recorded. *Bottom rows* show the basal levels of ECAR and OCR. n = 5. **$P < 0.01$ compared with time 0 by unpaired Student's *t* test. (*C*) Three independent normal lung Fb lines were treated with 2 ng/ml TGF-β1 for 0 or 24 hours, followed by sequential treatments with OLIGO and FCCP. Real-time ECAR and OCR were recorded. Mean ratios of ECAR/OCR were calculated. *$P < 0.05$ compared with time 0 by unpaired Student's *t* test. (*D*) Human normal lung Fbs and idiopathic pulmonary fibrosis (IPF) lung myo-Fbs were seeded in Seahorse microplates. Real-time ECAR and OCR were recorded. Mean ratios of ECAR/OCR were calculated. n = 4 individual lung Fb lines for each group. (*E* and *F*) Lung Fbs were treated without or with 2 ng/ml TGF-β1 for 24 hours. The cells were lysed and lactate contents in the cellular lysates (*E*) and culture media (*F*) determined. The data are presented as fold change relative to the levels of the untreated control group. (*G*) Experiments were performed as in *F*. Glucose contents in culture media were determined. (*H*) Mice were intratracheally instilled with saline or bleomycin (BLM; 1.5 U/kg). Lungs were harvested 3 weeks later, tissue extracts prepared, and lactate contents and protein concentrations determined. n = 3–4. (*I*) Mice were intratracheally instilled with control or TGF-β1–expressing adenoviruses (∼6 × 10^9 pfu/kg). Lungs were harvested 3 weeks later and lactate contents determined. n = 4. (*J*) Lactate contents in control normal and IPF lungs were determined. n = 7–10. *A–C* and *G–I*, mean ± SEM. *D–F*, mean ± SD. *C–J*, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by unpaired Student's *t* test. Experiments in *A–G* were performed two to three times. con = control.
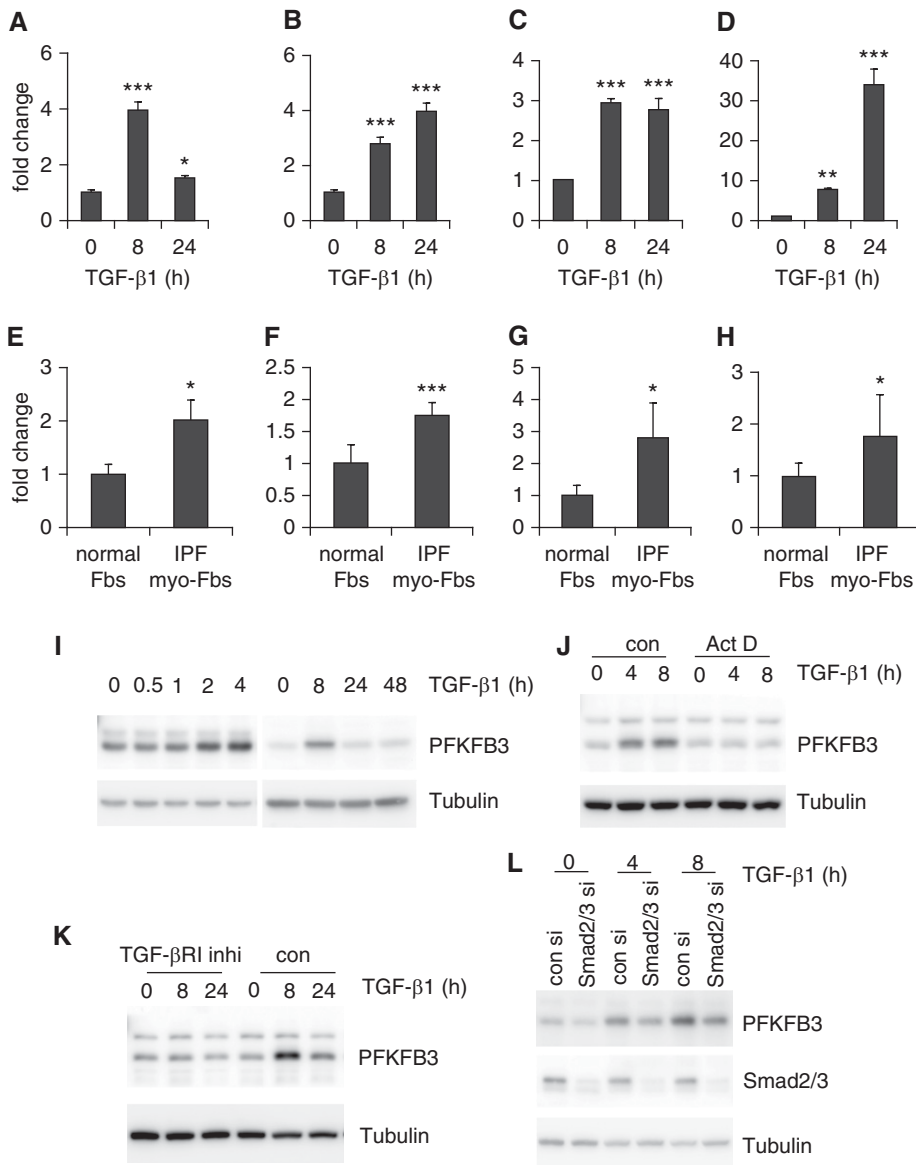
**Figure 2.** Key glycolytic enzymes are up-regulated in lung myofibroblasts (myo-Fbs). (*A–D*) Normal lung Fbs were treated with 2 ng/ml transforming growth factor (TGF)-β1 for 0, 8, or 24 hours. RNA was purified and mRNA levels of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) (*A*), phosphofructokinase-1 (PFK-1) (*B*), hexokinase 2 (HK2) (*C*), and smooth muscle actin (SMA)-α (*D*) determined by real-time polymerase chain reaction. n = 3, mean ± SD, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with time 0 by one-way analysis of variance. (*E–H*) mRNA levels of PFKFB3 (*E*), PFK-1 (*F*), HK2 (*G*), and SMA-α (*H*) in control normal lung Fbs and idiopathic pulmonary fibrosis (IPF) lung myo-Fbs were determined by real-time polymerase chain reaction. n = 5–6, mean ± SEM, *$P < 0.05$, ***$P < 0.001$ by Student's *t* test. (*I*) Normal lung Fbs were treated with 2 ng/ml TGF-β1 for the indicated periods of time and protein levels of PFKFB3 and tubulin determined by Western blotting. (*J*) Normal lung Fbs were pretreated with actinomycin D (Act D; 5 μg/ml) for 1 hour, followed by TGF-β1 treatment for 0, 4, or 8 hours. Protein levels of PFKFB3 and tubulin were determined. (*K*) Normal lung Fbs were pretreated with type I TGF-β1 receptor inhibitor SB-431542 (10 μM) for 1 hour, followed by TGF-β1 treatment for 0, 8, or 24 hours. Protein levels of PFKFB3 and tubulin were determined. (*L*) Normal lung Fbs were transfected with 20 μM control or Smad2/3 small interfering RNAs (si). Two days after transfection, cells were treated with TGF-β1 for 0, 4, or 8 hours. Protein levels of PFKFB3, Smad2/3, and tubulin were determined. Experiments in *A–D* and *I–L* were performed two to three times. con = control; inhi = inhibitor.

myo-Fb differentiation in single cells, we performed flow cytometry analysis and found that both TGF-β1–induced myo-Fbs (*see* Figure E1A in the online supplement) and IPF Fbs (*see* Figure E1B) with lower PFKFB3 expression demonstrate less expression of differentiation marker SMA-α than those with greater PFKFB3 expression. These data suggest that PFKFB3 promotes myo-Fb differentiation.

## PFKFB3 Is Up-regulated in Fibrotic Lungs

We have shown that PFKFB3 is significantly up-regulated in lung myo-Fbs. We next examined PFKFB3 expression *in vivo* in the lungs. As shown in Figures 3A and 3B, there was markedly increased expression of PFKFB3 in the lungs of mice that were treated with bleomycin or adenovirus that deliver active TGF-β1. To thoroughly characterize the localization of PFKFB3 expression in the lungs, we performed immunofluorescence microscopy and found that PFKFB3 is ubiquitously expressed in the lungs of normal mouse, including lung epithelial cells, stromal cells, and macrophages (Figure 3C, *top row*).

The expression of myo-Fb marker SMA-α was observed along the walls of blood vascular and bronchial structures in normal and fibrotic mouse lungs and also present in the fibrotic areas of fibrotic mouse lungs (Figure 3C, *top two rows*). There was increased expression of PFKFB3 in the dense fibrotic areas of fibrotic mouse lungs, with some of the elevated PFKFB3 colocalized with SMA-α (Figure 3C, *second row from top*), suggesting that augmented glycolysis caused by PFKFB3 up-regulation may contribute to myo-Fb differentiation during lung fibrogenesis. There was also low and ubiquitous expression of PFKFB3 in normal human lungs (Figure 3C, *third row from top*). It needs to be specified that the small rounded bright green spots in the normal human lungs are autofluorescence derived from red blood cells. More importantly, PFKFB3 elevation was evident in typical myofibroblastic foci in IPF lungs that was colocalized with SMA-α (Figure 3C, *bottom row*). Together, these data suggest that PFKFB3 participates in myo-Fb differentiation in fibrotic human and mouse lungs. However, it should be noted that PFKFB3 elevation
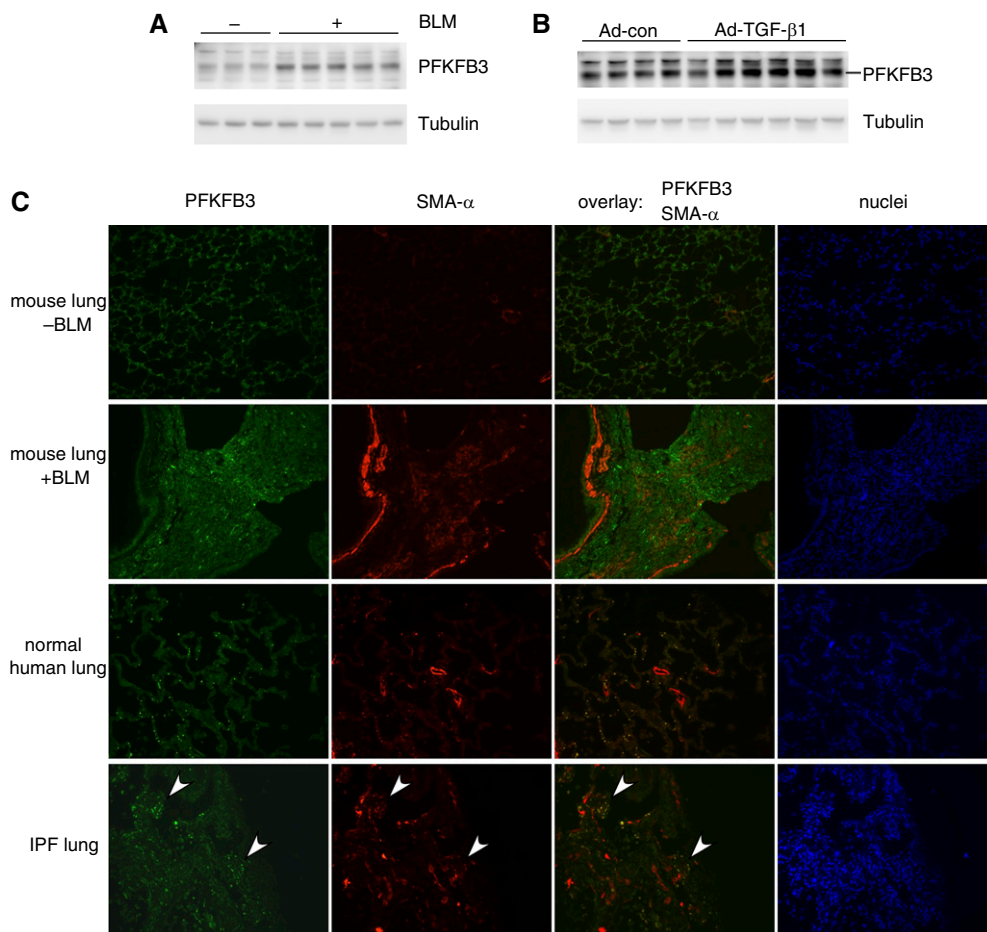
**Figure 3.** 6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) is up-regulated in fibrotic lungs. (*A* and *B*) Protein levels of PFKFB3 and tubulin in the lungs of mice that were treated intratracheally with saline (*A*), bleomycin (BLM) (*A*), control adenovirus (Ad-con) (*B*), or transforming growth factor (TGF)-β1–expressing adenovirus (Ad-TGF-β1) (*B*) for 3 weeks were determined by Western blotting. (*C*) Expression *in situ* of PFKFB3 and smooth muscle actin (SMA)-α in the lungs of mice that were treated intratracheally with saline or BLM (*top two rows*) and in human normal or idiopathic pulmonary fibrosis (IPF) lungs (*bottom two rows*) was determined by immunofluorescence microscopy. *Arrowheads* in the *bottom row* point to two myofibroblastic foci in the IPF lung. Original magnification, ×20.

was also present in lung epithelial cells, macrophages, and so forth. Therefore, the role of the induced PFKFB3 in these cell populations in the pathogenesis of lung fibrosis warrants further investigations.
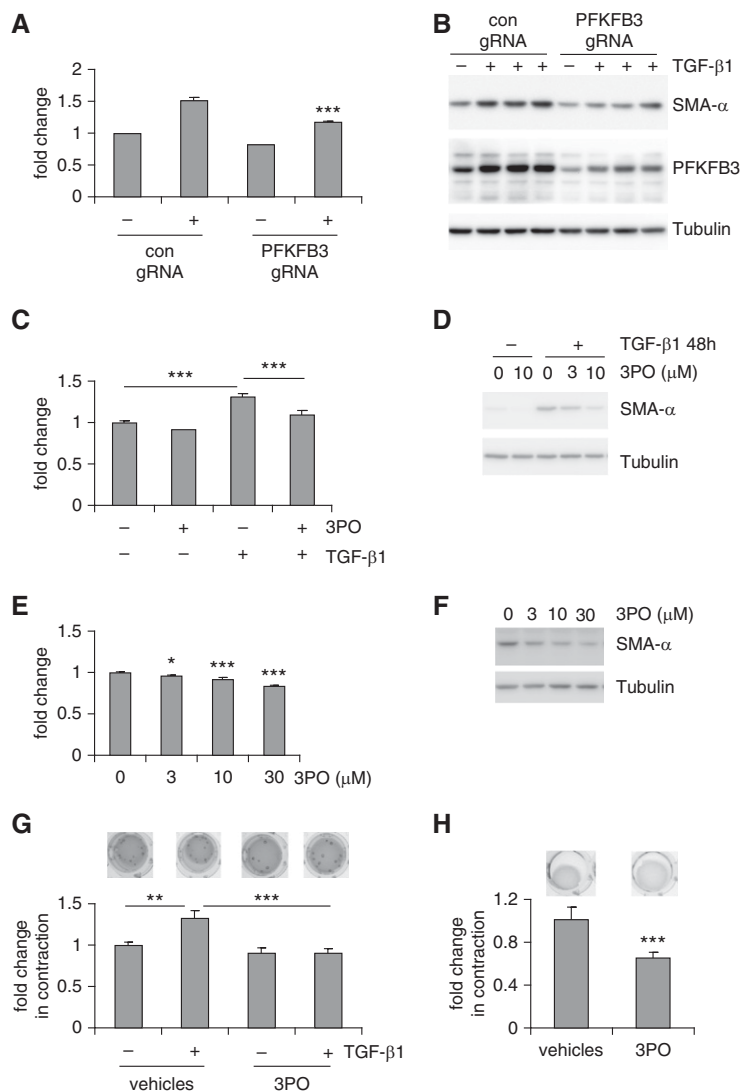
**Inhibition of Glycolysis Attenuates the Profibrotic Phenotypes of Lung myo-Fbs**

We demonstrated that lung myo-Fbs adopt enhanced glycolysis. We next asked if glycolysis is necessary for the differentiation of lung Fbs into myo-Fbs. To test this hypothesis, we used the powerful CRISPR-Cas9 system to disrupt the genomic locus of the *PFKFB3* gene, which would lead to decreased protein expression in lung Fbs. We confirmed that *PFKFB3* disruption down-regulates PFKFB3 protein levels and

dampens the augmented glycolysis in TGF-β1–induced lung myo-Fbs (Figures 4A and 4B). More importantly, TGF-β1–induced myo-Fb differentiation was also inhibited when PFKFB3 was knocked down (Figure 4B). To further characterize the role of the augmented glycolysis in myo-Fb differentiation, we used a well-defined PFKFB3 inhibitor 3PO to suppress glycolysis (31, 36). As shown in Figure 4C, 3PO dampened glycolysis in TGF-β1–induced myo-Fbs. Consistently, 3PO also effectively inhibited TGF-β1–induced myo-Fb differentiation (Figure 4D). Furthermore, 3PO diminished the glycolytic program in IPF lung myo-Fbs and reversed the differentiated state of these cells (Figures 4E and 4F). We also examined if glycolysis is required for

myo-Fb contraction, a property that has been recognized to significantly contribute to many fibrotic pathologies (37). As shown in Figure 4G, inhibition of glycolysis by 3PO remarkably decreased the contractile activity of TGF-β1–induced myo-Fbs. Furthermore, 3PO was able to attenuate the contractility of IPF myo-Fbs (Figure 4H). These data suggest that glycolysis is essential to the acquirement of the characteristic phenotypes of myo-Fbs.

Although we were concerned that inhibition of key rate-limiting glycolytic enzymes may cause deleterious side effects *in vivo*, we remained intrigued to know if it would produce similar effects to those observed with suppression of PFKFB3 *in vitro*. To do this, we pretreated lung Fbs with 2-deoxyglucose (2-DG), a glucose

**Figure 4.** Inhibition of glycolysis attenuates the profibrotic phenotypes of lung myofibroblasts (myo-Fbs). (*A*) Normal lung Fbs were infected with control Cas9 lentivirus (con) or Cas9 lentivirus expressing 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) guide RNA (gRNA). Two days after infection, the cells were starved for 1 day, followed by transforming growth factor (TGF)-β1 treatment for 0 or 24 hours. Lactate contents in culture media were determined. n = 3, mean ± SD, ***P < 0.001 compared with con+ by Student's *t* test. (*B*) The experiments were performed as in *A* and protein levels of PFKFB3, smooth muscle actin (SMA)-α, and tubulin determined. (*C*) Normal lung Fbs were pretreated with the PFKFB3 inhibitor 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) (10 μM) for 1 hour, followed by TGF-β1 treatment for 0 or 24 hours. Lactate contents in culture media were determined. n = 3, mean ± SD, ***P < 0.001 by one-way analysis of variance (ANOVA). (*D*) The experiments were performed as in *C* and protein levels of SMA-α and tubulin determined. (*E*) Idiopathic pulmonary fibrosis (IPF) lung myo-Fbs were treated with 3PO at the indicated concentrations for 1 day. Lactate contents in culture media were determined. n = 3, mean ± SD, *P < 0.05, ***P < 0.001 compared with 0 by one-way ANOVA. (*F*) The experiments were performed as in *E* and protein levels of SMA-α and tubulin determined. (*G*) Normal lung Fbs were resuspended in rat tail collagen gels. The cells were then pretreated without or with 3PO (10 μM) for 1 hour, followed or not by TGF-β1 treatment. Surface areas of the gels were calculated 2 days after gel release. n = 3, mean ± SD, **P < 0.01, ***P < 0.001 by one-way ANOVA. (*H*) IPF myo-Fbs were resuspended in collagen gels, followed or not by 3PO (10 μM) treatment for 48 hours. n = 3, mean ± SD, ***P < 0.001 by Student's *t* test. Experiments in *A*–*H* were performed two to three times.

analog that inhibits glycolysis via its actions on HK2 (38). We first confirmed that 2-DG leads to a remarkably decreased glycolysis in normal lung Fb MRC-5 cells and TGF-β1–induced lung myo-Fbs (*see* Figure E2A). Consistent with its inhibition of glycolysis, 2-DG was potent in attenuating TGF-β1–induced lung myo-Fb differentiation (*see* Figure E2B). Furthermore, we found that 2-DG diminishes glycolysis in IPF lung myo-Fbs and attenuates the differentiated phenotype of these cells (*see* Figures E2C and E2D). 2-DG also significantly decreased the contractility of IPF myo-Fbs and the contractile and migratory activities of TGF-β1–induced myo-Fbs (*see* Figures E2E–E2G). Another HK2 inhibitor, 3-bromopyruvate, was able to achieve effects similar to those by 2-DG (*see* Figures E3A–E3D) (39). Altogether, these data further confirm that glycolysis is required for lung myo-Fb differentiation.

Glycolysis has a net yield of two molecules of ATP (7). To determine if the attenuation of lung myo-Fb differentiation by glycolytic inhibition is not simply caused by a drop in the intracellular ATP levels, we treated lung Fbs with 3PO for 3 or 24 hours. As shown in Figure E4A, 3PO only had a marginal effect on the ATP levels in the cells. Furthermore, TGF-β1–induced phosphorylation of Smad2 was not affected by 3PO (*see* Figure E4B). 2-DG treatment drastically decreased intracellular ATP levels (*see* Figure E4C). However, it had no effect on TGF-β1–induced Smad2 phosphorylation (*see* Figure E4D). These data suggest that the diminished myo-Fb differentiation by inhibition of glycolysis is independent of ATP generated in this process. Additionally, glycolytic suppression by PFKFB3 inhibitor 3PO had no effect on cell proliferation or viability, in contrast to that by 2-DG (*see* Figure E4E; data not shown).
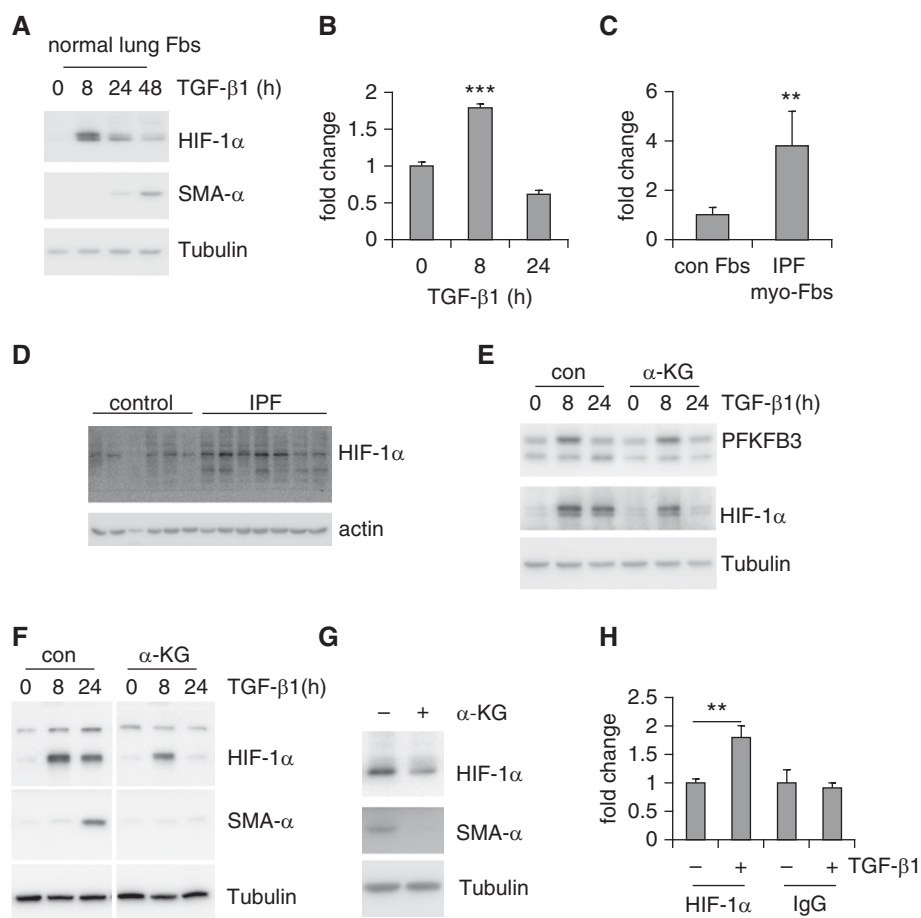
## HIF-1α Is Up-regulated in Lung myo-Fbs and Required for myo-Fb Differentiation

HIF-1α has been shown to be the master regulator of the expression of glycolytic enzymes and key transcriptional factor that drives glycolysis under hypoxic conditions (40–42). It is also well recognized that HIF-1α plays an important role in tissue fibrosis (43–47). We here asked if HIF-1α is involved in the elevated expression of PFKFB3 and myo-Fb differentiation. First,

we found that HIF-1α is rapidly up-regulated by TGF-β1 in lung Fbs at both protein and RNA levels (Figures 5A and 5B). HIF-1α was also up-regulated in IPF lung myo-Fbs and IPF lung tissues (Figures 5C and 5D). Surprisingly, we found that a cell-permeable α-ketoglutarate (α-KG) derivative, which stimulates prolyl hydroxylase activity and thus depleting HIF-1α (48), does not decrease PFKFB3 expression in lung myo-Fbs (Figure 5E). However, HIF-1α depletion by α-KG markedly diminished TGF-β1–induced myo-Fb differentiation and reversed the differentiated phenotype of IPF myo-Fbs (Figures 5F and 5G). To determine the mechanism by which HIF-1α regulates lung myo-Fb differentiation, we searched the promoter region of the *SMA-α* gene and identified a putative HIF-1α responsive element (A/GCGTG) at 184 bp upstream of the transcriptional starting site (49). We used chromatin immunoprecipitation assay to confirm that HIF-1α directly binds to this region after TGF-β1 treatment (Figure 5H). These data suggest that although HIF-1α does not participate in PFKFB3-mediated glycolytic augmentation, it is required for lung myo-Fb differentiation.

## Augmented Glycolysis Leads to Increased Succinate That Stabilizes HIF-1α and Promotes Lung myo-Fb Differentiation

Prolyl hydroxylase mediates HIF-1α hydroxylation, a process that uses α-KG as a cosubstrate and converts it into succinate (40–42). Buildup of succinate in turn inhibits HIF-1α hydroxylation, leading to HIF-1α stabilization (48, 50). Because we were aware of that, succinate is also an intermediate of the TCA cycle and dysregulated glycolysis has been shown to alter succinate levels (48). To determine if succinate plays a role in the up-regulation of HIF-1α in lung myo-Fbs, we treated lung Fbs with succinate. As shown in Figure 6A, succinate alone did not induce HIF-1α, indicating the necessity of the up-regulation of HIF-1α messenger RNAs in lung myo-Fbs. Nevertheless, succinate markedly enhanced TGF-β1–induced HIF-1α expression (Figure 6A). These data suggest that succinate stabilizes HIF-1α independently of hypoxic conditions. Next, we found that succinate is significantly up-regulated in lung myo-Fbs



Figure 5. Hypoxia-inducible factor 1-α (HIF-1α) is up-regulated in lung myofibroblasts (myo-Fbs) and required for myo-Fb differentiation. (A) Normal lung Fbs were treated with transforming growth factor (TGF)-β1 for 0, 8, 24, or 48 hours and protein levels of HIF-1α, smooth muscle actin (SMA)-α, and tubulin determined. (B) mRNA levels of HIF-1α were determined in TGF-β1–treated MRC-5 cells. n = 3, mean ± SD, ***P < 0.001 by one-way analysis of variance. (C) mRNA levels of HIF-1α were determined in control (con) normal lung Fbs and idiopathic pulmonary fibrosis (IPF) lung myo-Fbs. n = 5–6, mean ± SEM, **P < 0.01 by Student's t test. (D) Protein levels of HIF-1α and actin were determined in control normal lungs and IPF lungs. (E) Normal lung Fbs were pretreated with α-ketoglutarate (α-KG) (0.5 mM) for 1 hour, followed by TGF-β1 treatment for 0, 8, or 24 hours. Protein levels of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), HIF-1α, and tubulin were determined. (F) Normal lung Fbs were pretreated with α-KG (0.5 mM) for 1 hour, followed by TGF-β1 treatment for 0, 8, or 24 hours. Protein levels of HIF-1α, SMA-α, and tubulin were determined. The images separated by *vertical white lines* were derived from the same blots, with unrelated lanes removed. (G) IPF lung myo-Fbs were treated with α-KG (0.5 mM) for 0 or 24 hours. Protein levels of HIF-1α, SMA-α, and tubulin were determined. (H) Normal lung Fbs were treated with TGF-β1 for 0 or 8 hours. The cells were then fixed and lysed. Immunoprecipitations with anti–HIF-1α or -rabbit IgG were performed and DNA in the immunocomplexes purified. The binding of HIF-1α to the HIF-1α responsive element within the *SMA-α* promoter was quantified by real-time polymerase chain reaction and presented as fold change relative to the untreated control group. n = 3, mean ± SD, **P < 0.01 by Student's t test. Experiments in *A–C* and *E–H* were performed two to three times.

(Figure 6B). Succinate levels were also increased in fibrotic lungs (Figure 6C). More importantly, succinate promoted TGF-β1–induced myo-Fb differentiation (Figure 6D). These findings were consistent with the elevated expression of

HIF-1α in lung myo-Fbs. The rise of succinate was dependent on the augmented glycolysis in lung myo-Fbs because inhibition of glycolysis by 3PO nearly abolished such an increase in these cells (Figure 6E). Likely as the result of

attenuation of succinate elevation, HIF-1α was destabilized by 3PO in TGF-β1–induced lung myo-Fbs (Figure 6F). HIF-1α in TGF-β1 differentiated lung myo-Fbs and IPF lung myo-Fbs was also decreased by treatment with 2-DG (see Figure E5). Taken together, these data suggest that augmented glycolysis promotes myo-Fb differentiation by boosting succinate-dependent HIF-1α stabilization.

## Inhibition of Glycolysis Therapeutically Attenuates Bleomycin-induced Pulmonary Fibrosis In Vivo

The compelling evidence that glycolysis is required for lung myo-Fb differentiation in vitro intrigued us to ask if glycolytic inhibition has the potential to become a novel therapeutic to treat lung fibrosis. We chose 3PO over 2-DG for this purpose because 2-DG inhibits glycolysis high up within the glycolytic flux and invokes more complete and permanent glycolytic suppression (31). Furthermore, 2-DG needs to compete with mM levels of glucose in the blood, necessitating administration of high or toxic dosages of 2-DG (31). To determine the therapeutic value of glycolytic inhibition, we started 3PO injection 1 week after intratracheal instillation of bleomycin (Figure 7A), a time point when prominent lung inflammation recedes and active fibrogenetic process takes place (28). We first confirmed that 3PO diminishes the augmented glycolysis in fibrotic lungs, whereas it does not affect the basal glycolysis in normal mouse lungs (Figure 7B). More importantly and consistent with it inhibiting glycolysis, 3PO treatment significantly attenuated collagen deposition in the lungs of bleomycin-treated mice, compared with that in the vehicle control animals (Figure 7C). Histologic examination of the lung sections, Masson trichrome staining of the interstitial collagen, immunohistochemical assays of SMA-α, and examination of the expression of Fn and Col1A1 all confirmed that 3PO effectively attenuates bleomycin-induced lung fibrosis (Figures 7D and 7E).

In all, these data suggest that glycolytic inhibition is a promising therapeutic strategy to treat organ fibrotic disorders, such as IPF. Of note, at 10 days after bleomycin injection, there were no significant amounts of
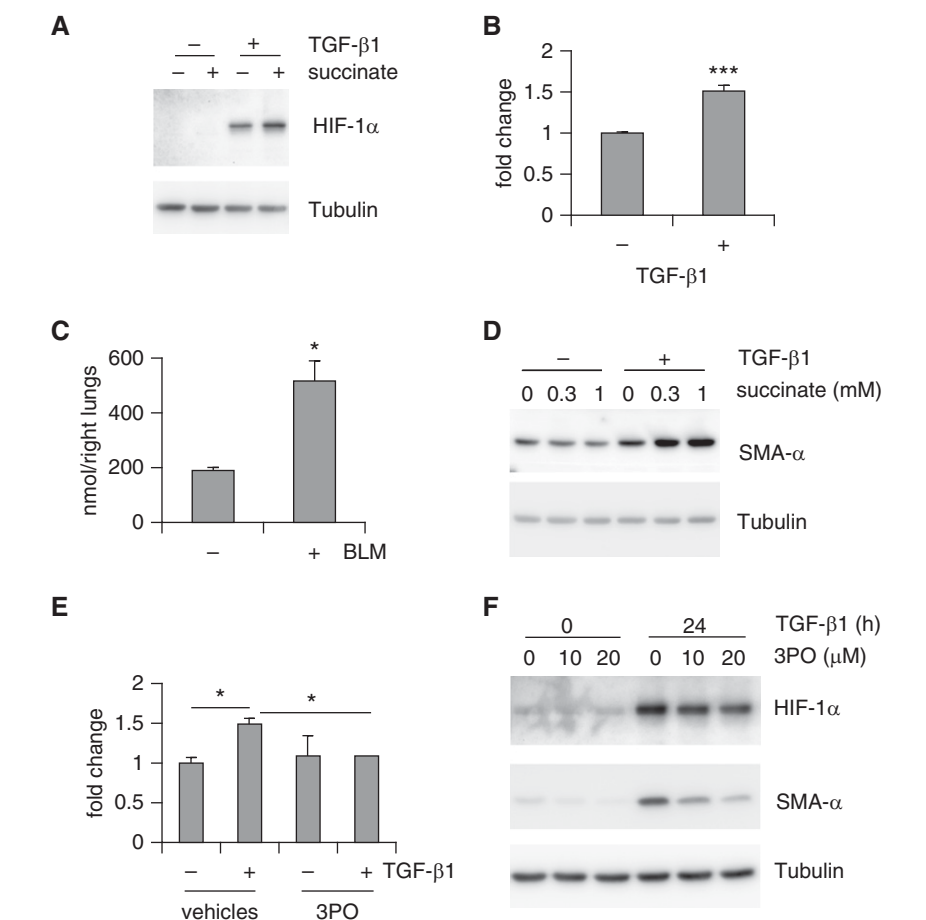


Figure 6. Augmented glycolysis leads to increased succinate that stabilizes hypoxia-inducible factor 1-α (HIF-1α) and promotes lung myofibroblasts (myo-Fbs) differentiation. (A) Normal lung Fbs were starved for 1 day, followed by pretreatment with succinate (0.5 mM) for 1 hour and transforming growth factor (TGF)-β1 treatment for 0 or 8 hours. Protein levels of HIF-1α and tubulin were determined. (B) Cellular succinate levels were determined in TGF-β1 untreated or treated MRC-5 cells. $n = 3$, mean ± SD, ***$P < 0.001$ by Student's t test. (C) Succinate levels were determined in the lungs of mice that were intratracheally treated with saline or bleomycin (BLM) for 3 weeks. $n = 3–4$, mean ± SEM, *$P < 0.05$ by Student's t test. (D) Normal lung Fbs were starved for 1 day, followed by pretreatment with succinate (0, 0.3, or 1 mM) for 1 hour and TGF-β1 treatment for 0 or 24 hours. Protein levels of smooth muscle actin (SMA)-α and tubulin were determined. (E) Normal lung Fbs were starved for 1 day, followed by pretreatment with 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) (0 or 10 μM) for 1 hour and TGF-β1 treatment for 0 or 24 hours. Cellular succinate levels were determined. $n = 3$, mean ± SD, *$P < 0.05$ by one-way analysis of variance. (F) Normal lung Fbs were starved for 1 day, followed by pretreatment with 3PO (0, 10, 20 μM) for 1 hour and TGF-β1 treatment for 0 or 24 hours. Protein levels of HIF-1α, SMA-α, and tubulin were determined. Experiments in A and B and D–F were performed two to three times.

proinflammatory cytokines in the lungs (see Figure E6B). Although there were still increased leukocytes and protein leaks in the bronchoalveolar lavage fluids from fibrotic lungs at this time point, 3PO did not affect these injury-associated parameters (see Figures E6C and E6D), suggesting that inflammatory regulation does not play a significant role in the antifibrotic effect of 3PO. Additionally, 3PO did not cause significant weight loss (see Figure E6A).

## Inhibition of Glycolysis Diminishes TGF-β1–induced Pulmonary Fibrosis In Vivo

Although bleomycin-induced lung fibrosis is the most widely used mouse model in the field, it is incompletely representative of the pathologic progression of human IPF (28). To further test the therapeutic value of glycolytic suppression by the PFKFB3 inhibitor 3PO in treating this disease, we performed a similar experiment with the
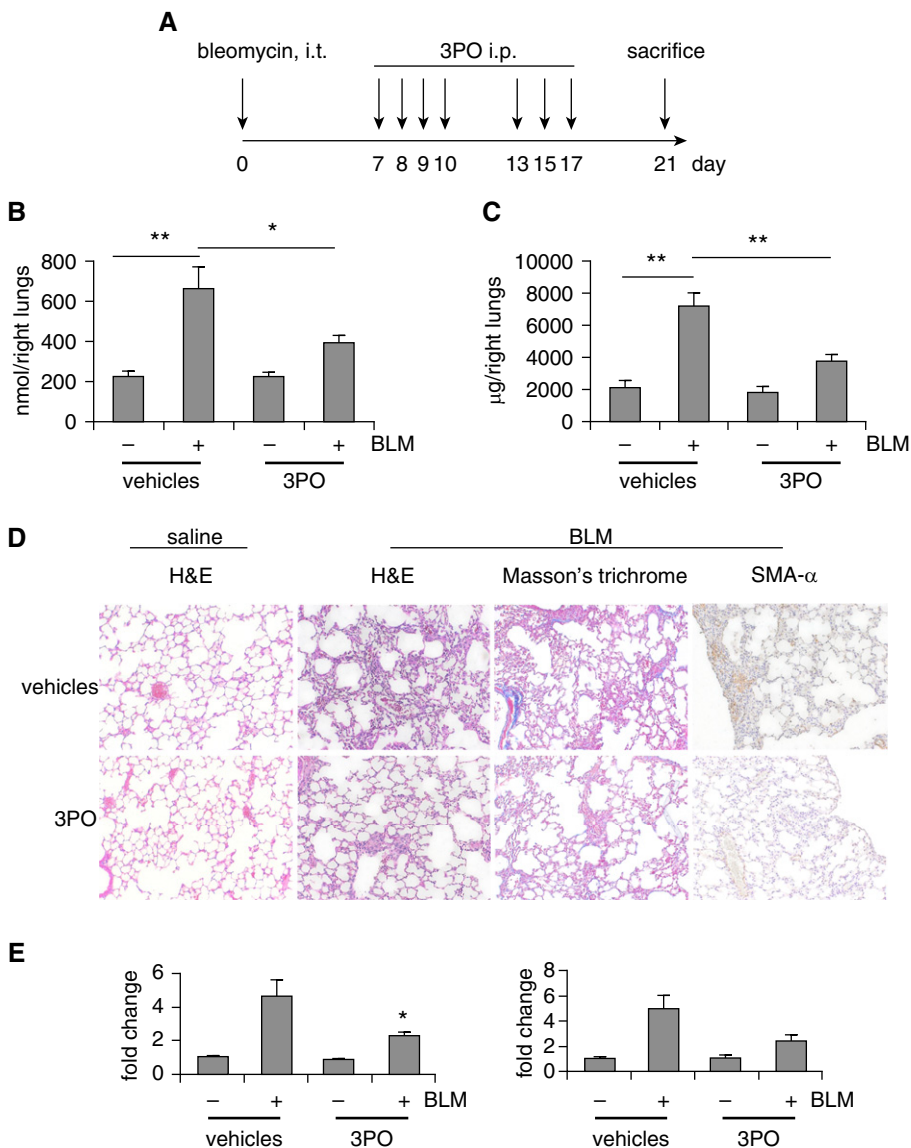
**Figure 7.** Inhibition of glycolysis therapeutically attenuates bleomycin (BLM)-induced pulmonary fibrosis *in vivo*. (*A*) Schematic illustration of the experiment design. C57BL/6 mice were intratracheally (i.t.) instilled with saline or BLM (1.5 U/kg in 50 μl saline). Seven days after the administration, the mice were intraperitoneally (i.p.) injected with 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) (50 mg/kg dissolved in 50 μl dimethyl sulfoxide) or dimethyl sulfoxide for seven times within 2 weeks. At the end of the treatment, mice were killed and lungs harvested. (*B*) Lactate contents in the lungs from the experiments were determined. n = 3, 8, 5, 10, respectively. mean ± SEM, *$P < 0.05$, **$P < 0.01$ by one-way analysis of variance (ANOVA) and Bonferroni test. (*C*) Collagen contents in right lungs were determined by Sircol assay. n = 3, 8, 5, 10, respectively. mean ± SEM, **$P < 0.01$ by one-way ANOVA and Bonferroni test. (*D*) Lung tissue sections were prepared from the above experiments. Representative images of hematoxylin–eosin (H&E) staining, Masson trichrome staining for collagen deposition, and immunohistochemistry staining for smooth muscle actin (SMA)-α are shown. Original magnification, ×20. (*E*) Messenger RNA levels of fibronectin (*left*) and collagen 1A1 (*right*) in the lungs in this experiment were determined by real-time polymerase chain reaction. n = 3–10, mean ± SEM, *$P < 0.05$ by one-way ANOVA and Bonferroni test.

TGF-β1–induced pulmonary fibrosis model (19). 3PO was given 1 week after the administration of TGF-β1–expressing adenovirus for a total of seven times (Figure 8A). As shown in Figures 8B and 8C,

3PO effectively inhibited glycolysis and fibrosis in the lungs of mice that received TGF-β1–expressing adenovirus. Taken together, we have convincingly demonstrated the therapeutic efficacy of

3PO in two well-established pulmonary fibrosis models.

## Discussion

In this study, we found that glycolytic augmentation is an early and lasting event during lung myo-Fb differentiation and an essential step for the development of lung fibrosis. More importantly, we demonstrated that glycolytic suppression using the PFKFB3 inhibitor has clear therapeutic values in two experimental pulmonary fibrosis models. Thus, we have firmly established a new concept that pulmonary fibrosis, probably other organ fibrotic pathologies as well, is a disorder of glycolytic dysregulation. This has significant implications in terms of finding new therapies for the treatment of fibrotic diseases because practical and feasible approaches to inhibit glycolysis *in vivo* have been identified and are being tested in some other metabolic disorders, such as cancer and inflammatory diseases (6, 48, 51). These new therapeutics can be readily adapted to the application of treating organ fibrotic disorders, including IPF.

We demonstrated that glycolysis is required for the initiation and sustainment of myo-Fb differentiation, which apparently accounts for the essentiality of such a metabolic reprogramming in the development of pulmonary fibrosis. However, it should be noted that other pathologic phenotypes of myo-Fbs, such as augmented proliferation and resistance to apoptosis, could be also dependent on the elevated glycolysis. In addition, highly glycolytic myo-Fbs may be capable of creating a metabolically favorable microenvironment through secreting lactate and other glycolytic intermediates to cause pathologic changes in adjacent pulmonary cells, such as cellular dysplasia, which is frequently found in the alveolar epithelia surrounding the myofibroblastic foci in IPF lungs (52). Although these hypotheses remain to be tested, there is abundant evidence alluding that such events could take place during pulmonary fibrosis. For example, ramping up of glycolysis is known to be an essential step to ensure rapid proliferation of cancer cells by supplying their synthetic demands (2). Additionally, augmented glycolysis in cancer stromal cells, including Fbs, has
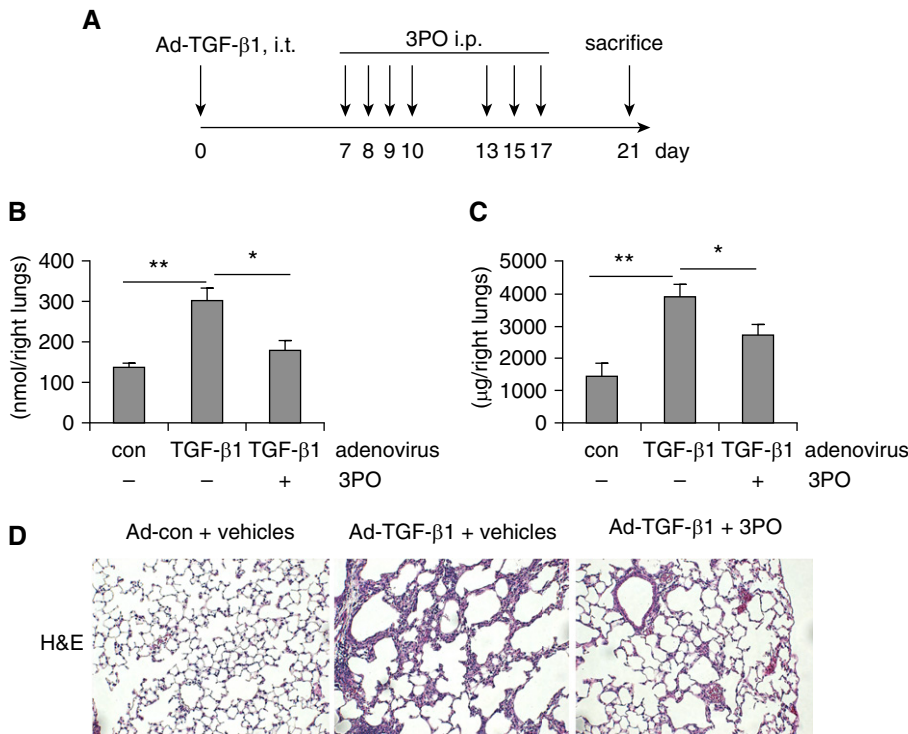
**Figure 8.** Inhibition of glycolysis diminishes transforming growth factor (TGF)-β1–induced pulmonary fibrosis *in vivo*. (*A*) Schematic illustration of the experiment design. C57BL/6 mice were intratracheally (i.t.) instilled with control adenovirus or adenovirus expressing active TGF-β1 (Ad-TGF-β1) ($6 \times 10^9$ pfu/kg). Seven days after the administration, the mice were intraperitoneally (i.p.) injected with 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) (50 mg/kg dissolved in 50 μl dimethyl sulfoxide) or dimethyl sulfoxide for seven times within 2 weeks. At the end of the treatment, mice were killed and lungs harvested. (*B*) Lactate contents in the lungs from the experiments were determined. n = 4, 7, 6, respectively; mean ± SEM, *$P < 0.05$, **$P < 0.01$ by one-way analysis of variance and Bonferroni test. (*C*) Collagen contents in right lungs were determined by Sircol assay. n = 4, 7, 6, respectively. mean ± SEM, *$P < 0.05$, **$P < 0.01$ by one-way analysis of variance and Bonferroni test. (*D*) Lung tissue sections were prepared from the above experiments. Representative images of the hematoxylin–eosin (H&E) staining are shown. Original magnification, ×20. con = control.

been shown to promote malignancy of cancer cells (53).

Although TGF-β1–induced myo-Fbs shares many profibrotic features with IPF myo-Fbs, they may not be identical in terms of mechanisms of establishment. It has been shown that myo-Fbs differentiation in IPF lungs involves several transcriptional and epigenetic mechanisms and many profibrotic mediators, including TGF-β1 (54). Therefore, it is likely that factors other than TGF-β1 could also contribute to the sustained high PFKFB3 expression in IPF myo-Fbs. This definitely remains our focus of future interests. It is also worth noting that IPF fibroblasts are known to be heterogeneous, with cells being at different stages of differentiation (35). We found that IPF fibroblasts with

lower PFKFB3 expression demonstrate less differentiation than those with greater PFKFB3 expression (*see* Figure E1B), suggesting that PFKFB3 promotes myo-Fbs differentiation. More importantly, PFKFB3 inhibition was able to reverse the profibrotic phenotype of IPF myo-Fbs (Figures 4F and 4H). Together, these data suggest that PFKFB3 is likely required for the initiation and sustainment of myo-Fbs differentiation in IPF lungs.

We found that the TCA cycle intermediate succinate is up-regulated in lung myo-Fbs. Succinate is known to originate from several sources, including from glycolytic flux via pyruvate and glutamine (55). Although it remains to be identified which source is responsible for the succinate elevation in myo-Fbs, our

data that inhibition of glycolysis decreased intracellular succinate in myo-Fbs suggest the amplified glycolytic flux as a possible explanation. Consistent with the activity of succinate in stabilizing HIF-1α, glycolytic suppression led to decreased HIF-1α in myo-Fbs.

Although HIF-1α is a master regulator that controls hypoxic induction of many glycolytic enzymes (40–42), it is unlikely to be responsible for the increased expression of PFKFB3 in lung myo-Fbs. This conclusion is based on the finding that destabilization of HIF-1α has no effect on TGF-β1–induced PFKFB3 expression in lung Fbs. Nevertheless, we found that PFKFB3 up-regulation is dependent on TGF-β1–activated Smad2/3 because either blocking TGF-β1 type I receptor or knocking down Smad2/3 abrogates the induction of PFKFB3. These findings place HIF-1α downstream of the augmented glycolysis in lung myo-Fbs. We have found that HIF-1α is required for lung myo-Fb differentiation and that binds to the HIF-1α responsive element site within the *SMA-α* promoter. Therefore, the regulation of HIF-1α by glycolysis-modulated succinate suggests the promotion of lung myo-Fb differentiation by HIF-1α as one of the direct mechanisms by which glycolysis participates in pathologic fibrogenesis.

Our data, along with previous studies from others, have identified HIF-1α as a central player in the pathogenesis of lung fibrosis (43–47). However, it should be noted that the role of the augmented glycolysis in myo-Fb differentiation involves mechanisms other than HIF-1α. A reasonable possibility involves the increased succinate in the glycolytic lung myo-Fbs. Succinate has been recently found to be capable of generating a new type of post-translational modification, named succinylation, which is able to alter protein localizations and functions (56). Therefore, identification of succinylated proteins in lung myo-Fbs could shed new light into the mechanisms by which glycolysis regulates pulmonary fibrosis.

We found that although 3PO is not as potent as 2-DG to inhibit glycolysis, it is as effective to suppress myo-Fb differentiation. These data suggest that a mild ramping down of glycolysis is sufficient to change the course of myo-Fb differentiation. Such findings are of particular importance when to harness glycolytic inhibition for treating

pulmonary fibrosis because a drastic reduction of glycolysis *in vivo*, such as that presumably caused by 2-DG, could bring serious side effects and thus becoming intolerable to patients (31). Such an implication is likely to place 3PO above 2-DG for being tested in future clinical trials for the treatment of organ fibrosis, including IPF.

Although we have found that lung myo-Fbs adopt augmented glycolysis, attenuation of bleomycin and TGF-β1–induced pulmonary fibrosis by the PFKFB3 inhibitor 3PO may not be solely attributable to the diminished glycolytic program in these cells.

It is possible that the interruption of the glycolysis in other lung cell populations, such as alveolar epithelial cells and macrophages, contributes to the *in vivo* efficacy of 3PO. Although this is not critical from the therapeutic standpoint, it is mechanistically worthwhile to consider these alternative explanations, which require targeted deletion of PFKFB3 in the various types of lung cells.

In conclusion, we demonstrated that augmentation of aerobic glycolysis is an essential step during myo-Fb differentiation. We found that PFKFB3 is up-regulated and mediates the glycolytic reprogramming in

myo-Fbs. Finally, we showed that ramping down glycolysis by inhibiting PFKFB3 is effective in diminishing myo-Fb differentiation and limiting lung fibrosis. Our findings identify a novel target for developing new therapeutic strategies to treat organ fibrosis, including IPF. ■

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