Glutaminolysis Promotes Collagen Translation and Stability via α -Ketoglutarate-mediated mTOR Activation and Proline Hydroxylation

Jing Ge^{1,2*}, Huachun Cui^{1*}, Na Xie^{1*}, Sami Banerjee¹, Sijia Guo^{1,3}, Shubham Dubey¹, Stephen Barnes⁴, and Gang Liu¹

¹Division of Pulmonary, Allergy, and Critical Care Medicine, Department of Medicine, and ⁴Department of Pharmacology and Toxicology, University of Alabama at Birmingham, Birmingham, Alabama; ²Department of Geriatrics and Institute of Geriatrics, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China; and ³Department of Pulmonary, Allergy, and Critical Care Medicine, The Second Affiliated Hospital, Tianjin University of Traditional Chinese Medicine, Tianjin, China

ORCID ID: 0000-0003-2615-131X (G.L.).

Abstract

Glutaminolysis is the metabolic process of glutamine, aberration of which has been implicated in several pathogenesises. Although we and others recently found a diversity of metabolic dysregulation in organ fibrosis, it is unknown if glutaminolysis regulates the profibrotic activities of myofibroblasts, the primary effector in this pathology. In this study, we found that lung myofibroblasts demonstrated significantly augmented glutaminolysis that was mediated by elevated glutaminase 1 (Gls1). Inhibition of glutaminolysis by specific Glsl inhibitors CB-839 and BPTES as well as Glsl siRNA blunted the expression of collagens but not that of fibronectin, elastin, or myofibroblastic marker smooth muscle actin- α . We found that glutaminolysis enhanced collagen translation and stability, which were mediated by glutaminolysis-dependent mTOR complex 1 activation and collagen proline hydroxylation, respectively. Furthermore, we found that the amount of the glutaminolytic end product α -ketoglutarate (α -KG) was increased in myofibroblasts. Similar to glutaminolysis, α -KG activated mTOR complex 1 and promoted the expression of collagens but not of fibronectin, elastin, or smooth muscle actin- α . α -KG also remarkably inhibited collagen degradation in fibroblasts. Taken together, our studies identified a previously

unrecognized mechanism by which a major metabolic program regulates the exuberant production of collagens in myofibroblasts and suggest that glutaminolysis is a novel therapeutic target for treating organ fibrosis, including idiopathic pulmonary fibrosis.

Keywords: glutaminolysis; collagen; α -ketoglutarate; mTORC1; fibrosis

Clinical Relevance

We identified a previously unrecognized mechanism by which a key metabolic program, glutaminolysis, regulates the production of collagens, the principal components of fibrotic matrix. We provide solid evidence that augmented glutaminolysis promotes the expression of collagens by enhancing their translation and stability through α -ketoglutarate-mediated mTOR activation and proline hydroxylation. Our findings suggest that glutamine metabolism may represent a novel therapeutic target for treating tissue fibrosis.

Myofibroblasts are the putative effectors in organ fibrosis in that they are the primary cell populations that produce fibrotic matrix composed of disorganized collagens,

fibronectin, and elastin (1–5). It has long been recognized that targeting these overly active cells represents one of the best strategies for treating organ fibrosis (5).

However, incomplete understanding of the regulation of pathological matrix production by myofibroblasts hampers the development of effective therapies.

(Received in original form July 3, 2017; accepted in final form October 11, 2017)

*These authors contributed equally.

Supported by National Institutes of Health grants HL135830 and HL114470 (G.L.).

Author Contributions: G.L.: designed the study; J.G., H.C., N.X., S. Banerjee, S.G., S.D., S. Barnes, and G.L.: performed the experiments and/or analyzed the data; and G.L.: wrote the manuscript.

Correspondence and requests for reprints should be addressed to Gang Liu, M.D., Ph.D., Division of Pulmonary, Allergy, and Critical Care Medicine, Department of Medicine, University of Alabama at Birmingham, 901 19th Street South, BMR II 233, Birmingham, AL 35294. E-mail: gangliu@uabmc.edu.

This article has a data supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

Am J Respir Cell Mol Biol Vol 58, Iss 3, pp 378–390, Mar 2018

Copyright © 2018 by the American Thoracic Society

Originally Published in Press as DOI: 10.1165/rcmb.2017-0238OC on October 11, 2017

Internet address: www.atsjournals.org

Aberrant metabolism is a prominent feature of diabetes and cardiovascular diseases (6). There is also rapidly growing evidence showing that metabolic dysregulation plays critical roles in other pathologies (7, 8). Previous studies by our and other groups showed that myofibroblasts demonstrated elevated glycolysis (9–11). It was also found that glycolytic inhibition suppressed myofibroblastic differentiation and was effective in blunting experimental lung fibrosis in mice (9). These findings suggest that metabolic reprogramming is an integral step of myofibroblastic differentiation and the tissue fibrotic process. In our continuous endeavor to delineate the role of aberrant core metabolic pathways in the pathogenesis of lung fibrosis, we interrogated glutaminolysis, a major program of cellular metabolism (12), in lung myofibroblasts.

Glutaminolysis is the metabolic process of glutamine that is used for production of biological energy; synthesis of proteins, lipids, and nucleotides; and maintenance of redox homeostasis (13–17). Glutaminolysis involves conversion of glutamine to glutamate by glutaminase (Gls) and constitutes a key anaplerotic reaction that replenishes the tricarboxylic acid (TCA) cycle via α -ketoglutarate (α -KG) generation (13–16). In addition, as the metabolic program of one of the most abundant nutrients, glutaminolysis intricately interacts with the mTOR complex (mTORC), a central integrator of nutrient and growth factor sensing and cell growth, survival, and metabolism (16). mTORC1 regulates protein synthesis, nutrient uptake, and autophagy in response to growth factors and amino acids (18). Two of the best characterized substrates of mTORC1 are p70-S6 kinase (p70-S6K) and 4E-BP1, whereas mTORC2 phosphorylates several AGC kinases, such as AKT and SGK1, to regulate cellular metabolism (19).

As one of the most defining pathological events in tissue fibrosis, collagen production is subject to regulation at multiple levels. Newly synthesized collagen peptides undergo several post-translational modifications (PTMs), including proline and lysine hydroxylation (20). These PTMs are essential to the formation of proper conformation of the collagen peptides, which is required for

the stabilization of the triple-helix structure of collagens (20, 21). Collagens with inadequate proline hydroxylation are readily degraded before secretion, and such a process is recognized to be a very important intracellular mechanism for the quality control of the extracellular matrix (ECM) (22, 23). Hydroxylation of prolines and lysines in collagens is mediated by collagen hydroxylases, such as C-3 and C-4 prolyl hydroxylases and C-5 lysyl hydroxylases, which belong to the dioxygenase family (20). Enzyme-dependent collagen hydroxylation has been shown to require α -KG as a substrate.

In this study, we found that myofibroblasts demonstrated remarkably augmented glutaminolysis. We discovered unique mechanisms by which this metabolic process regulated the production of fibrotic matrices, particularly collagens. Our data establish a solid foundation for developing glutaminolysis-based therapeutics to treat organ fibrosis.

Methods

Reagents

Human recombinant transforming growth factor- β 1 (TGF- β 1) was obtained from PeproTech. Bleomycin was purchased from Besse Medical. CB-839 was obtained from MedchemExpress. Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide (BPTES) was purchased from R&D Systems. Torin 1 was obtained from Cayman Chemical. Chloroquine, colchicine, MG-132, cycloheximide, and dimethyl α -KG were purchased from Sigma-Aldrich. [35 S]methionine was obtained from PerkinElmer. L-Glutamine solution, L-glutamine-free RPMI 1640, and Eagle's minimal essential medium were purchased from Corning. Gls1, Smad2, and Smad3 siRNAs were obtained from Dharmacon. MitoTracker Deep Red FM was purchased from Thermo Fisher Scientific.

Experimental Pulmonary Fibrosis Model

C57BL/6 mice were purchased from The Jackson Laboratory. The bleomycin-induced lung fibrosis model was described in detail previously (9). The animal protocol was approved by the institutional animal care and use committee at the University of Alabama at Birmingham (UAB).

Isolation of Primary Human Lung Fibroblasts

Primary human lung fibroblasts were established as previously described (9). The protocol was approved by the UAB Institutional Review Board. Normal lung fibroblasts were derived from failed donor lungs. Fibroblasts were maintained in Eagle's minimal essential medium supplemented with 10% FBS. At least two independent lines at passages 3–6 were used in experiments.

Immunohistochemistry

Immunohistochemistry was performed as described in our previous studies (24).

Immunofluorescence Microscopy

Immunofluorescence assays were performed as previously described (25).

Mass Spectrometry

Mass spectrometry to analyze TCA cycle metabolites was performed by the Targeted Metabolomics and Proteomics Laboratory of UAB. Briefly, cells were harvested with ice-cold pure methanol. A quantity of 1 ml of each sample was transferred to a glass tube and dried under a gentle stream of N_2 . Standards and samples were resuspended in 50 ml of 5% acetic acid and vortexed for 15 seconds. A quantity of 50 ml of Amplifex Keto Reagent (SCIEX) was added to each sample and allowed to react for 1 hour at room temperature. Standards and samples were then dried under a gentle stream of N_2 and finally resuspended in 1 ml of 0.1% formic acid (FA). Samples were analyzed by liquid chromatography multiple reaction ion monitoring mass spectrometry. Liquid chromatography was performed using an LC20AC HPLC system (Shimadzu) with a Synergi Hydro-RP 4 mm 80A 250 $\times$ 2-mm internal diameter column (Phenomenex). Mobile phases were 1) 0.1% FA and 2) MeOH 0.1% FA. Compounds were eluted using a 5 to 40% B linear gradient from 1 to 7 minutes, followed by a column wash 40 to 100% B from 7 to 10 minutes, and reequilibrated at 5% B from 10.5 to 15 minutes. Column eluents were passed through an electrospray ionization interface of an API 4000 triple-quadrupole mass spectrometer (SCIEX). Peak areas of metabolites in the sample extracts were compared using MultiQuant software (SCIEX) with those of the known standards to calculate metabolite concentrations.

[³⁵S]Methionine Incorporation Assay

After indicated treatments, cell media were replaced with methionine-free RPMI 1640 that contained 50 μ Ci/ml [³⁵S]methionine. Two hours after the incubation, cellular extracts were prepared and resolved by SDS-PAGE. Proteins were transferred to polyvinylidene fluoride membranes, followed by exposure to X-ray film for 2 hours.

siRNA Transfection

Lung fibroblasts were transfected with siRNAs using Lipofectamine RNAiMAX (Thermo Fisher Scientific) according to the manufacturer's instructions.

RT-PCR

mRNA amounts in cells were determined by RT-PCR using the LightCycler 480 SYBR Green I Master (Roche). Primer sequences are listed in Table 1.

Western Blotting

Western blotting was performed as previously described (24). Mouse antitubulin, mouse anti-smooth muscle actin- α (anti-SMA- α), and mouse antifibronectin antibodies were purchased from Sigma-Aldrich. Rabbit anti-collagen I antibody was obtained from Abcam. Rabbit anti-phospho-p70 S6 kinase (T389), rabbit anti-phospho-4E-BP1 (T37/46), rabbit anti-phospho-AKT (S473), and rabbit anti-p62 antibodies were purchased from Cell Signaling Technology. Rabbit anti-Gls1, rabbit anti-collagen III, and rabbit antielastin antibodies were obtained from Proteintech. Rabbit anti-PAI-1 antibody was purchased from Santa Cruz Biotechnology. Densitometric assays of protein band intensities were performed with ImageJ software, and bar graphs for applicable Western blots are

presented in Figure E2 in the data supplement.

Trichloroacetic Acid/Acetone Precipitation of Soluble Protein in Cell Supernatants

Cell media were collected and centrifuged to remove dead cells and debris. Three volumes of supernatants were added to 1 vol of 100% trichloroacetic acid solution and kept on ice for 30 minutes. The samples were centrifuged at 13,000 rpm for 10 minutes. The pellets were washed with ice-cold acetone, followed by being lysed with 1 $\times$ SDS sample buffer.

IP

Human normal lung fibroblasts were lysed in radioimmunoprecipitation assay buffer. The amounts of samples of each group entering IP were normalized to ensure that there were the same amounts of collagens. IP was performed with antihydroxyproline antibody (Biorbyt). The same amount of rabbit IgG was used as a nonspecific binding control. Immunocomplexes were pulled down using protein A beads (GE Healthcare Life Sciences), followed by Western blotting with anti-collagen I antibody.

Glutamine and Glutamate Assays

Intracellular and supernatant amounts of glutamine and glutamate were determined with a glutamine and glutamate assay kit (BioVision), respectively, 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 comparisons between two groups. *P* < 0.05 was considered statistically significant.

Results**Glutamine Metabolism Is Augmented in Myofibroblasts**

To investigate the metabolism of glutamine, we initially compared its consumption by normal human lung fibroblasts and lung myofibroblasts that were induced by TGF- β 1, one of the most important profibrotic mediators (26). As shown in Figures 1A and 1B, whereas lung myofibroblasts consumed significantly more extracellular glutamine than fibroblasts did, they had less intracellular glutamine. Consistently, myofibroblasts contained greater amounts of glutamate (Figure 1C). These findings demonstrated an evident increase in glutaminolysis, the metabolic process of glutamine, in lung myofibroblasts. Glutaminolysis is initiated by Gls that is known to include the kidney/brain-type Gls1 and the liver-type Gls2 (13). We found that the mRNA level of Gls1 was significantly increased in TGF- β 1-treated fibroblasts, whereas Gls2 mRNA was barely detectable (Figure 1D), suggesting Gls1 as the primary Gls isoform in lung fibroblasts. Consistent with the elevated transcription, Gls1 protein was markedly upregulated in TGF- β 1-treated fibroblasts, along with myofibroblastic marker SMA- α and ECM components collagen I, collagen III, fibronectin, and elastin (Figure 1E). Together, these data suggest that Gls1-mediated upregulation of glutaminolysis may play a critical role in myofibroblastic differentiation of fibroblasts. We found that TGF- β 1-induced Gls1 was partially Smad2/3 dependent because their knockdown attenuated the elevation of this enzyme (Figure 1F). In addition, both the basal and TGF- β 1-enhanced Gls1 were located primarily in mitochondria (Figure 1G). Characterizing Gls1 expression

Table 1. List of Sequences of RT-PCR Primers

Genes	Sense	Antisense
Human tubulin- β 1	TGGACTCTGTTTCGCTCAGGTCCTT	AGTGGCCTTTGGCCCAGTTGTTAC
Human SMA- α	CATCACCAACTGGGACGACATGGAA	GCATAGCCCTCATAGATGGGGACATTG
Human elastin	GATAAAACGAGGTGCGGAGAGCG	GGATGGACAGCAGGAGCAGGAGG
Human Gls1	AGTTGCTGGGGGCATTCTTTTAGTT	CCTTTGATCACCACCTTCTCTTCGA
Human Gls2	GCTGCCTATAGTGGCGATGTCTCAG	TGAAGCAGTTTGACCACCTCCAGAT
Human fibronectin	TCTGGGAAATGGAAAAGGGGAATGG	CACTGAAGCAGGTTTCCTCGGTTGT
Human Col1A1	AGGGCGACAGAGGCATAAAGGG	GGGACCAACAGGACCAGCATCAC
Human Col1A2	GTACAGTTGGACCTGCTGGCATTCG	CATCCTTGGTTAGGGTCAATCCAGTAGTA

Definition of abbreviations: Col1A1 = collagen, type I, α 1; Col1A2 = collagen, type I, α 2; Gls1 = glutaminase 1; Gls2 = glutaminase 2; SMA = smooth muscle actin.

Figure 1. Glutamine metabolism is augmented in myofibroblasts. (A–C) Human normal lung fibroblasts were starved for 24 hours in Eagle's minimal essential medium (EMEM) that contained 0.1% FBS. The cells were then treated with or without 2 ng/ml transforming growth factor- β 1 (TGF- β 1) for 48 hours. Amounts of (A) supernatant and (B) intracellular glutamine and (C) intracellular glutamate were determined. Glutamine consumption was the difference between glutamine amounts in the conditioned media and those in the original media. $n = 3$. Data are expressed as mean $\pm$ SD. * $P < 0.05$;

in fibrotic lungs, we found that this enzyme was markedly increased in dense fibrotic areas in the lungs of mice with bleomycin-induced pulmonary fibrosis (Figure 1H). More significantly, myofibroblastic foci, the defining pathological feature of idiopathic pulmonary fibrosis, demonstrated remarkable Gls1 expression (Figure 1I). Together, these data suggest that elevated glutaminolysis is a common phenotype of myofibroblasts, both *in vitro* and *in vivo*.

Expression of Collagens Requires Glutaminolysis

We showed that glutaminolysis mediating Gls1 was induced by TGF- β 1. We next examined the role of this metabolic pathway in myofibroblastic differentiation. Cells were treated with the specific Gls1 inhibitor CB-839, a compound that is being tested in anticancer clinical trials, to block glutaminolysis (27, 28). We verified the intended effects of CB-839 in that it reduced glutamine consumption and increased intracellular glutamine in fibroblasts and TGF- β 1-induced myofibroblasts (Figure 2A). We found that CB-839 remarkably inhibited the expression of collagens I and III in myofibroblasts (Figure 2B). However, CB-839 had few effects on the amounts of fibronectin, elastin, and SMA- α (Figure 2B). To validate the specificity of the effects of glutaminolytic inhibition, we examined another Gls1 inhibitor, BPTES (29). Consistent with CB-839, BPTES diminished the expression of collagen I and collagen III, but not that of fibronectin, elastin, or SMA- α , in myofibroblasts (Figure 2C). Both CB-839 and BPTES could reverse the elevated collagen I in TGF- β 1-induced myofibroblasts (Figures 2D and 2E). In addition, we applied glutamine deprivation to dampen glutaminolysis and found that, similarly to CB-839 and BPTES, this procedure decreased only collagen I

and collagen III in myofibroblasts (Figure 2F). Taken together, these bodies of evidence suggest that glutaminolysis selectively regulates collagen expression in myofibroblasts. To genetically determine the role of glutaminolysis, we employed siRNAs and lentiviruses to modify Gls1 expression. We found that Gls1 knockdown significantly diminished the expression of collagens I and III (Figure 2G). In contrast, Gls1 overexpression upregulated collagens in lung fibroblasts (Figure 2H). Neither Gls1 knockdown nor overexpression had an effect on the expression of fibronectin, elastin, or SMA- α (Figures 2G and 2H). Of note, like the glutaminolytic inhibitor CB-839, Gls1 knockdown also reduced glutamine consumption in fibroblasts and TGF- β 1-induced myofibroblasts (Figure E1).

Collagens are the key ingredients of the ECM, the primary sources of which are myofibroblasts. To determine if myofibroblast-secreted collagens were also subject to glutaminolytic regulation, we examined the amounts of these proteins in cell supernatants. As shown in Figure 2I, TGF- β 1-induced myofibroblasts released strikingly more of collagens I and III than lung fibroblasts did. Whereas the increase in collagen secretion in myofibroblasts was completely blocked when Gls1 was knocked down, collagen secretion in control lung fibroblasts was not affected by Gls1 downregulation (Figure 2I). In addition, secretion of fibronectin and PAI-1 by these cells was independent of Gls1 (Figure 2I). Taken together, these findings suggest that glutaminolysis participates in ECM production by controlling the expression of collagens but not the secretion mechanism of fibroblasts. To shed light on the role of glutaminolysis in the context of fibrotic lung diseases, we used CB-839 to treat lung myofibroblasts from patients with idiopathic pulmonary fibrosis and found that glutaminolytic inhibition decreased the expression of collagens I

and III, but not fibronectin, in these cells (Figure 2J).

Glutaminolysis Regulates Collagen Expression at the Post-Transcriptional Level

We investigated if the decrease in collagen protein levels was due to a diminished transcription in myofibroblasts on glutaminolytic inhibition. To our surprise, we found that neither basal nor TGF- β 1-induced transcription of *Col1A1* and *Col1A2*, two genes that produce collagen I, was affected by CB-839 (Figure 3A). Limited supply of glutamine had no effect on the transcription of *Col1* either (Figure 3B). Consistent with the negligible impact on the protein levels of fibronectin, elastin, and SMA- α , CB-839 or glutamine limitation did not affect the transcription of these genes (Figures 3A and 3B). Collectively, these findings suggest that regulation of collagen expression by glutaminolysis does not occur at the transcriptional level. On discovering this, we next investigated protein translational activity in fibroblasts and myofibroblasts using a [35 S]methionine incorporation assay. We found that myofibroblasts demonstrated markedly increased cellular translation (Figure 3C). More importantly, the elevated translation was attenuated, although partially, by glutaminolytic inhibition (Figure 3D).

Perplexingly, the finding that the expression of collagens, but not of fibronectin, elastin, or SMA- α , was dependent on glutaminolysis appears to be indicative of selective translational regulation. However, we came to realize that this was a minor likelihood, given the uniform control of these proteins' expression in settings of organ fibrosis. We hypothesized that the differential effects of glutaminolysis were due to divergent stabilities of these profibrotic mediators in fibroblasts. We reasoned that proteins with

Figure 1. (Continued). *** $P < 0.001$ compared with the untreated control (con) group. (D) Human normal lung fibroblasts were treated as in A. mRNA amounts of glutaminase 1 (Gls1), Gls2, and tubulin- α were determined by RT-PCR. The y-axis represents amounts relative to that of tubulin- α . $n = 3$. Data are expressed as mean $\pm$ SD. * $P < 0.05$ compared with the untreated control group. (E) Human normal lung fibroblasts were treated as in A. Amounts of the indicated proteins were determined by Western blotting. (F) Human normal lung fibroblasts were transfected with control siRNA or Smad2/3 siRNA, followed by treatment with or without TGF- β 1. Amounts of the indicated proteins were determined by Western blotting. (G) Human normal lung fibroblasts were treated as in A. The expression of Gls1 was determined by immunofluorescence microscopy. Mitochondria were visualized using MitoTracker Deep Red. Nuclei were visualized using DAPI. Original magnification, $\times 40$. (H) The expression and localization of Gls1 in normal mouse lung and lungs of mice with bleomycin-induced pulmonary fibrosis were determined by immunohistochemistry. Rabbit IgG was used as a negative control. Original magnification, $\times 40$. (I) The expression and localization of Gls1 in normal human lung and idiopathic pulmonary fibrosis (IPF) lung were determined by immunohistochemistry. Original magnification, $\times 40$. A–D are representative of two or three independent experiments. BLM = bleomycin; con = control; si = siRNA; SMA = smooth muscle actin.

Figure 2. Expression of collagens requires glutaminolysis. (A) Human normal lung fibroblasts were starved for 24 hours in EMEM that contained 0.1% FBS. The cells were then pretreated with or without 1 μ M CB-839 for 1 hour, followed by treatment with or without 2 ng/ml TGF- β 1 for 48 hours. Supernatant and intracellular glutamine (Gln) amounts were determined. Gln consumption was calculated as in Figure 1A. $n = 3$. Data are presented as mean $\pm$ SD. * $P < 0.05$ compared with the untreated control group; ### $P < 0.001$ compared with the TGF- β 1-treated control group. (B) Human normal lung fibroblasts were treated as in A. Amounts of the indicated proteins were determined by Western blotting. (C) Human normal lung fibroblasts

more rapid turnover were those more susceptible to translational alterations. To test this, we blocked cellular translation and chased these proteins in fibroblasts. As shown in Figure 3E, both collagen I and collagen III nearly disappeared 4 hours after cellular translation was terminated, whereas amounts of fibronectin, elastin, and SMA- α remained largely unchanged, indicating much less stability in intracellular collagens. In aggregate, these findings suggest that glutaminolysis, via regulating general cellular translation even at a modest level, has a relatively greater impact on the expression of the unstable collagens in myofibroblasts.

Glutaminolysis Promotes mTORC1 Activation

The implication of glutaminolysis in controlling cellular translation prompted us to ask if mTORC was involved in the process, because this complex is known to be a key regulator that promotes protein synthesis (30, 31). Furthermore, mTORC is a master integrator of nutrient sensing and cell metabolism. mTORC1 activation leads to phosphorylation of p70-S6K and 4E-BP1, two molecules that keep tight control of translational initiation events (30, 31). We found that TGF- β 1 did induce mTORC1 activation in lung fibroblasts, as evidenced by increased phosphorylation of the two substrates (Figure 4A). More importantly, TGF- β 1-activated mTORC1 was blunted by glutaminolytic inhibition with CB-839 or glutamine limitation (Figures 4B and 4C). Similarly, Gls1 knockdown could also downregulate mTORC1 activation (Figure 4D). On the contrary, augmented glutaminolysis activated mTORC1, as reflected by increased phosphorylation of p70-S6K and 4E-BP1 in lung fibroblasts with Gls1 overexpression (Figure 4E). In all, these data suggest that TGF- β 1-induced

mTORC1 activation is dependent on glutaminolysis. Notably, whereas TGF- β 1 also induced Akt phosphorylation, this event was not affected by glutaminolytic inhibition (Figures 4A and 4B), suggesting that glutaminolysis is not involved in mTORC2 activation in myofibroblasts.

The finding of mTORC1 activation by glutaminolysis compelled us to investigate the effects of mTORC1 inhibition on the expression of these profibrotic mediators. As shown in Figure 4F, the mTOR-specific inhibitor Torin 1 completely inhibited the activity of this complex. More importantly, mTOR inhibition markedly reduced the expression of collagen I and collagen III, but not that of fibronectin, elastin, or SMA- α (Figure 4G), consistent with findings with glutaminolytic blockade. Together, these data suggest that glutaminolysis-dependent mTORC1 activation mediates glutaminolytic regulation of collagen expression.

α -KG Mediates Glutaminolysis Regulation of Collagen Translation by Activating mTORC1

The end product of glutaminolysis is α -KG, a metabolic intermediate of the TCA cycle (17, 32, 33). Previous studies have implicated both α -KG elevation and mTOR activation in the malignant phenotype of various cancers (34). Given the indication that glutaminolysis-dependent mTORC1 activation regulates collagen expression, we hypothesized that α -KG might be a mediator of this process. We found by mass spectrometry that amounts of α -KG were significantly increased in lung fibroblasts that were treated with TGF- β 1 (Figure 5A). More importantly, α -KG upregulation in these cells was abrogated by glutaminolytic inhibition (Figure 5B), suggesting a glutamine derivation of the elevated α -KG. We found that a cell-permeable α -KG

activated mTORC1, which indicates that it may promote cellular translation (Figure 5C). Consistent with this hypothesis, α -KG increased the protein but not the mRNA level of *Col1* (Figures 5D and 5E). α -KG had no effect on the expression of fibronectin, elastin, or SMA- α (Figures 5D and 5E). In sum, these findings suggest that glutamine-derived α -KG mediates the translational regulation by glutaminolysis through activating mTORC1.

Glutaminolysis Stabilizes Intracellular Collagens by Promoting Proline Hydroxylation

Our data indicate that collagen expression is more readily subject to glutaminolysis-mediated cellular translation than other fibrotic matrix components because of the lesser stability of collagens. We next asked if glutaminolysis also controlled collagens' production by regulating their stabilization. This hypothesis was formed on the basis of previous studies showing that proline hydroxylation, a reaction that was dependent on α -KG, the anaplerotic end product of glutaminolysis, was critical to intracellular collagen stabilization (20, 21). To test this, we first investigated channels of intracellular collagen degradation. As shown in Figure 6A, the lysosome inhibitor chloroquine remarkably decreased the degradation of collagens I and III in lung fibroblasts 4 hours after protein synthesis blockade. In contrast, proteasome inhibitor MG-132 had no effect on this event (Figure 6B). Of note, p62 level was increased as a result of lysosome inhibition (Figure 6B). These data suggest that intracellular collagen degradation is lysosome dependent but not proteasome dependent. In addition, we evaluated collagen secretion and found that the protein secretion inhibitor colchicine apparently did not affect intracellular collagen amounts

Figure 2. (Continued). were treated as in A, except that 2.5 μ M bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide (BPTES) replaced CB-839. Amounts of the indicated proteins were determined by Western blotting. (D and E) Human normal lung fibroblasts were starved for 24 hours in EMEM that contained 0.1% FBS. The cells were then treated with or without 2 ng/ml TGF- β 1 for 48 hours, followed by culture for an additional 48 hours with fresh media that contained vehicle, (D) 1 μ M CB-839, or (E) 2.5 μ M BPTES. Amounts of the indicated proteins were determined by Western blotting. (F) Human normal lung fibroblasts were starved for 24 hours in EMEM that contained 0.1% FBS. The cells were then cultured for an additional 48 hours with fresh media that contained 0 or 2 mM Gln and 0 or 2 ng/ml TGF- β 1. Amounts of the indicated proteins were determined by Western blotting. (G) Human normal lung fibroblasts were transfected with control siRNA or Gls1 siRNA. The cells were then starved for 24 hours in media that contained 0.1% FBS, followed by treatment with or without 2 ng/ml TGF- β 1 in media supplemented with 0.1% FBS and 0.2 mM Gln for 48 hours. Amounts of the indicated proteins were determined by Western blotting. (H) Human normal lung fibroblasts were infected with control lentiviruses or lentiviruses that express Gls1. Amounts of the indicated proteins were determined by Western blotting. (I) The experiments were performed as in G. Soluble proteins in cell supernatants were precipitated, and amounts of indicated proteins in the supernatants were determined by Western blotting. (J) Idiopathic pulmonary fibrosis lung fibroblasts were treated with or without 1 μ M CB-839 for 48 hours. The amounts of indicated proteins were determined by Western blotting. A–H are representative of two or three independent experiments.

Figure 3. Glutaminolysis regulates collagen (Col1A1 and Col1A2) expression at the post-transcriptional level. (A) Human normal lung fibroblasts were starved for 24 hours in EMEM that contained 0.1% FBS. The cells were then pretreated with or without 1 μ M CB-839 for 1 hour, followed by treatment with or without 2 ng/ml TGF- β 1 for 48 hours. mRNA levels of the indicated genes were determined by RT-PCR. $n = 3$. Data are presented as mean $\pm$ SD. (B) Human normal lung fibroblasts were starved for 24 hours in EMEM that contained 0.1% FBS. The cells were then cultured for an additional 48 hours with fresh media that contained 0.2 or 2 mM glutamine and 0 or 2 ng/ml TGF- β 1. mRNA levels of the indicated genes were determined by RT-PCR. $n = 3$. Data are presented as mean $\pm$ SD. (C) Human normal lung fibroblasts were starved for 24 hours in EMEM that contained 0.1% FBS. The cells were then treated with or without 2 ng/ml TGF- β 1 for 48 hours, followed by culture for 2 hours with methionine-free media that were supplemented with [35 S]methionine. Cellular extracts were prepared, resolved, and transferred to polyvinylidene fluoride membranes. [35 S]methionine incorporation was determined by autoradiography. (D) Human normal lung fibroblasts were starved for 24 hours in EMEM that contained 0.1% FBS. The cells were then pretreated with or without 1 μ M CB-839 for 1 hour, followed by treatment with or without 2 ng/ml TGF- β 1. Forty-eight hours later, the cells were cultured for 2 hours with methionine-free media that were supplemented with [35 S]methionine. [35 S]methionine incorporation was determined by autoradiography. (E) Human normal lung fibroblasts were treated with 5 μ g/ml protein translation inhibitor cycloheximide (CHX) for 0, 1, 2, or 4 hours. Amounts of the indicated proteins were determined by Western blotting. Data are representative of two independent experiments. K.D. = kilodalton.

Figure 4. Glutaminolysis promotes mTOR complex 1 activation. (A) Human normal lung fibroblasts were starved for 24 hours in EMEM that contained 0.1% FBS. The cells were then treated with 2 ng/ml TGF- β 1 for 0, 24, or 48 h. Amounts of the indicated proteins were determined by Western blotting. (B) Human normal lung fibroblasts were starved for 24 hours in EMEM that contained 0.1% FBS. The cells were then pretreated with or without 1 μ M CB-839 for 1 hour, followed by treatment with or without 2 ng/ml TGF- β 1 for 48 hours. Amounts of the indicated proteins were determined by Western blotting. (C) Human normal lung fibroblasts were starved for 24 hours in EMEM media that contained 0.1% FBS. The cells were then cultured for an additional 48 hours with fresh media that contained 0.2 or 2 mM glutamine and 0 or 2 ng/ml TGF- β 1. Amounts of the indicated proteins were determined by Western blotting. The blot for p-p70-S6K was made from the same membrane but the membrane was cut at the superimposed dashed line, to facilitate blotting with other antibodies. (D) Human normal lung fibroblasts were transfected with control siRNA or Gls1 siRNA, followed by treatment with or without TGF- β 1 for 6 hours. Amounts of the indicated proteins were determined by Western blotting. (E) Human normal lung fibroblasts were infected with control lentiviruses or lentiviruses that express Gls1. Amounts of the indicated proteins were determined by Western blotting. (F) Human normal lung fibroblasts were starved for 24 hours in EMEM that contained 0.1% FBS. The cells were then pretreated with or without 20 nM Torin 1 for 1 hour, followed by treatment with or without 2 ng/ml TGF- β 1 for 48 hours. Amounts of the indicated proteins were determined by Western blotting. (G) Human normal lung fibroblasts were treated as in F. Amounts of the indicated proteins were determined by Western blotting. A–E are representative of two independent experiments.

with 5-hour treatment (Figure 6C). These data ruled out the possibility that the remarkable decreases in intracellular collagens at 4 hours after protein synthesis termination were caused by significant secretion within the same time frame.

After discovering this, we examined the effect of glutaminolysis on intracellular collagen stability. We treated lung

fibroblasts with either vehicle or glutaminolytic inhibitor CB-839 or BPTES for 24 hours, followed by tracing collagen expression on addition of cycloheximide. As shown in Figures 6D and 6E, both CB-839 and BPTES markedly accelerated the degradation of collagen I and collagen III, suggesting that glutaminolysis is required for

intracellular collagen stabilization. More significantly, we found that α -KG dramatically slowed down collagen degradation (Figure 6F). Together with the finding that CB-839 significantly decreased the amount of α -KG in lung fibroblasts, these data suggest that glutaminolysis promotes intracellular collagen stability through α -KG and thereby enhances collagen expression in lung fibroblasts. Because it has previously been demonstrated that defective proline hydroxylation promotes collagen degradation (22, 23), we next investigated if glutaminolysis regulates collagen hydroxylation and found that blocking glutaminolysis with CB-839 evidently reduced the amount of hydroxyproline in collagen I (Figure 6G). In all, these studies indicate that glutaminolysis is required for intracellular collagen hydroxylation, which is necessary for the stabilization of these proteins.

Discussion

We previously showed that lung myofibroblasts undertook metabolic reprogramming to carry out augmented glycolysis (9). Furthermore, glycolytic inhibition diminished the myofibroblastic phenotype and alleviated experimental lung fibrosis (9). In the present study, we found that enhanced glutaminolysis was required for the translation and stabilization of collagens in these cells (Figure 7). Our studies collectively indicate that metabolic dysregulation is a frequent event in myofibroblasts and may play critical roles in the development of the profibrotic activities of the cells. These studies also suggest that aberrations in key cellular metabolisms are becoming a distinct set of a defining phenotype for these profibrotic effectors. Our findings provide novel insight into the pathogenesis of organ fibrosis and open new avenues for developing metabolism-based strategies to treat this disease.

We have demonstrated that there was an augmented cellular translation in myofibroblasts that was likely dependent on mTOR activation mediated by glutaminolysis-derived α -KG. In theory, the robust translation in these cells should have been coupled with enhanced anabolic events, presumably including collagen production and the resulting profibrotic

Figure 5. α -Ketoglutarate (α -KG) mediates glutaminolysis regulation of collagen translation by activating mTOR complex 1. (A) Human normal lung fibroblasts were starved for 24 hours in EMEM that contained 0.1% FBS. The cells were then treated with or without 2 ng/ml TGF- β 1 for 48 hours. Amounts of intracellular α -KG and glutamine were determined by mass spectrometric analysis. $n = 3$. Data are presented as mean $\pm$ SD. $**P < 0.01$; $***P < 0.001$ compared with the untreated control group. (B) Human normal lung fibroblasts were starved for 24 hours in EMEM that contained 0.1% FBS. The cells were then pretreated with or without 1 μ M CB-839 for 1 hour, followed by treatment with or without 2 ng/ml TGF- β 1 for 48 hours. Amounts of intracellular α -KG and glutamine were determined by mass spectrometric analysis. $n = 3$. Data are presented as mean $\pm$ SD. $**P < 0.01$; $***P < 0.001$ compared with the untreated control group; $###P < 0.001$ compared with the TGF- β 1-treated control group. (C) Human normal lung fibroblasts were starved for 24 hours in EMEM that contained 0.1% FBS. The cells were then treated with or without 2 mM cell-permeable dimethyl- α -KG for 48 hours. Amounts of the indicated proteins were determined by Western blotting. The blot for p-p70-S6K was made from the same membrane but the membrane was cut at the superimposed dashed line, to facilitate blotting with other antibodies. Images separated by vertical space were from the same blots with nonrelated lanes removed. (D) Human normal lung fibroblasts were treated as in C, and amounts of the indicated proteins were determined by Western blotting. (E) Human normal lung fibroblasts were treated as in C, and amounts of the indicated genes were determined by RT-PCR. $n = 3$. Data are presented as mean $\pm$ SD. C and D are representative of two independent experiments.

activities. The TCA intermediates are well known to be extracted from the cycle as precursors for production of many macromolecules, which constitutes the necessary steps of cellular anabolism that is essential to cell growth (16). As a matter of fact, glutamine-derived α -KG anaplerotically fuels the TCA cycle (16). There is also another possibility, which is that α -KG may not be the sole metabolite derived from glutaminolysis that contributes to the anabolic events in lung myofibroblasts. Indeed, there is plenty of evidence showing that glutaminolysis-associated anabolism also involves amino acid intermediates, such as aspartate, and is essential to the proliferation of cancer cells (35, 36). Therefore, glutaminolysis-

dependent anabolism may also play a critical role in the development of other profibrotic phenotypes, such as the increased proliferative activity of myofibroblasts, the effectors that adopt augmented glutamine consumption in organ fibrosis.

Although abnormal activation of mTOR has previously been found in fibrotic lungs, it is not very clear what leads to this pathological event (37–39). In this study, we provide evidence that augmented glutaminolysis was required for mTORC1 activation in myofibroblasts. To our knowledge, this is the first time a mechanistic link between an abnormal primary metabolic program and a major pathological signaling event in lung

myofibroblasts has been identified. Furthermore, given the versatile functions of mTOR in governing cell growth and survival, as well as energy production and expenditure, the role of glutaminolysis-associated mTOR activation in organ fibrosis is sure to go beyond just the regulation of collagen expression. mTOR has been well recognized to control amounts of autophagy/mitophagy that were found to be defective in myofibroblasts (40). Thus, it is of particular interest to determine if glutaminolysis participates in lung fibrosis by regulating autophagy/mitophagy via mTOR activation.

There are a number of elegant studies that have delineated how enhanced

Figure 6. Glutaminolysis stabilizes intracellular collagens by promoting proline hydroxylation. (A) Human normal lung fibroblasts were cultured for 24 hours in EMEM that contained 10% FBS. The cells were then pretreated with or without 100 μ M lysosome inhibitor chloroquine for 1 hour, followed by treatment with or without 5 μ g/ml CHX for 4 hours. Amounts of the indicated proteins were determined by Western blotting. Images separated by vertical space were from the same blots with nonrelated lanes removed. (B) Human normal lung fibroblasts were cultured for 24 hours in EMEM that contained 10% FBS. The cells were then pretreated with or without chloroquine or proteasome inhibitor MG-132 for 1 hour, followed by treatment with 5 μ g/ml CHX for 4 hours. Amounts of the indicated proteins were determined by Western blotting. (C) Human normal lung fibroblasts were cultured for 24 hours in EMEM that contained 10% FBS. The cells were then treated with 10 μ M protein secretion inhibitor colchicine for the indicated time. Amounts of the indicated proteins were determined by Western blotting. (D) Human normal lung fibroblasts were cultured for 24 hours in EMEM that contained 10% FBS. The cells were then pretreated with or without 1 μ M CB-839 for 24 hours, followed by treatment with 5 μ g/ml CHX for 0, 1.5, and 3 hours. The samples loaded for the control and CB-839 groups at CHX time 0 were normalized to ensure that the same amounts of collagen were present. The amounts of samples loaded for the other two time points were the same as that for time 0 in their respective groups. Amounts of the indicated proteins were determined by Western blotting. (E) The experiments were done as in D, except that CB-839 was replaced with 2.5 μ M BPTES. (F) Human normal lung fibroblasts were cultured for 24 hours in EMEM that contained 10% FBS. The cells were then treated with 2 mM dimethyl- α -KG for 24 hours, followed by treatment with 5 μ g/ml CHX for 0, 3, and 6 hours. The samples loaded for the con and α -KG groups at CHX time 0 were normalized to ensure that the same amounts of collagen were present. The amounts of samples loaded for the other two time points were the same as that for time 0 in their respective groups. Amounts of the indicated proteins were determined by Western blotting. (G) Human normal lung fibroblasts were cultured for 24 hours in EMEM that contained 10% FBS. The cells were then pretreated with or without 1 μ M CB-839 for 24 hours. Cells were lysed in radioimmunoprecipitation assay buffer, and an IP assay with antihydroxyproline antibody was performed. The amounts of samples of each group entering IP were normalized to ensure that the same amounts of collagen were present. The same amount of rabbit IgG was used as a nonspecific binding control. Western blotting with anti-collagen I was performed. A–F are representative of two or three independent experiments.

glutaminolysis contributes to cancer cell proliferation, resistance to apoptosis, and metastasis (17). Our present work also sheds new light on the role of glutamine metabolism in cancer pathogenesis. Because the phenotype of fibrotic myofibroblasts has been shown to be reminiscent of that of cancer-associated fibroblasts in many aspects, it is plausible that glutaminolysis-promoted collagen production that was found in lung myofibroblasts may also take place in cancer-associated fibroblasts and aid the establishment of an optimal cancer interstitium that can facilitate rapid tumor growth and dissemination.

It is well known that hydroxylation of prolines in hypoxia-inducible factor 1 is a prerequisite for ubiquitin-mediated degradation of this protein in proteasomes (41). To the contrary, this PTM appears to protect intracellular collagens from being degraded in lung myofibroblasts, which indicates that proline or lysine hydroxylation in collagens does not create a docking site for ubiquitin binding. This notion is in line with our finding that collagens are degraded primarily in lysosomes but not proteasomes. In fact, proline or lysine hydroxylation was found to be critical to the formation of proper conformations of individual collagen peptides, which were necessary for the stable triple-helix structure of intracellular procollagens (20, 21). Therefore, our finding that glutaminolysis was required for proline hydroxylation in collagens suggests that the augmentation of this metabolic program in myofibroblasts is an important mechanism employed by these cells to rapidly produce collagens with improved qualities that confer greater resistance to degradation.

The finding that glutaminolysis promoted translation and stability of collagens in myofibroblasts suggests a potential therapeutic benefit of glutaminolytic inhibition in treating lung fibrosis. However, the study was done primarily in TGF- β 1-induced lung myofibroblasts. TGF- β 1 is also a critical mediator in normal lung injury repair, and thus TGF- β 1-augmented glutaminolysis may be essential to the normal wound-healing process (42). These concerns raise an important question regarding the timing of glutaminolytic inhibition during therapeutic interventions for lung injury. In addition, one may also caution against a hasty translation of this prospect into

Figure 7. Schematic illustration of the proposed mechanism by which glutaminolysis regulates collagen expression. ECM = extracellular matrix; mTORC1 = mTOR complex 1.

therapies until better understanding is gained regarding the effects of glutaminolytic inhibition on other cell populations, such as pulmonary epithelial and endothelial cells. There is a possibility that glutamine is nevertheless essential to the regeneration of these cells after lung injury, which indicates that undifferentiated

inhibition of pulmonary glutamine metabolism may not be beneficial in treating lung fibrosis. Regardless of these considerations, we are currently taking the first step to conditionally knock out Gls1 in mouse fibroblasts and determine its effect on the pathogenesis of lung fibrosis in mice. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: We thank Taylor Berryhill at the UAB Targeted Metabolomics and Proteomics Laboratory, which is supported in part by the UAB Health Services Foundation General Endowment Fund, for helping with the mass spectrometry assay.

References

- Hardie WD, Glasser SW, Hagood JS. Emerging concepts in the pathogenesis of lung fibrosis. *Am J Pathol* 2009;175:3–16.
- Kis K, Liu X, Hagood JS. Myofibroblast differentiation and survival in fibrotic disease. *Expert Rev Mol Med* 2011;13:e27.
- Noble PW, Barkauskas CE, Jiang D. Pulmonary fibrosis: patterns and perpetrators. *J Clin Invest* 2012;122:2756–2762.
- Eickelberg O, Laurent GJ. The quest for the initial lesion in idiopathic pulmonary fibrosis: gene expression differences in IPF fibroblasts. *Am J Respir Cell Mol Biol* 2010;42:1–2.
- Hinz B, Phan SH, Thannickal VJ, Prunotto M, Desmoulière A, Varga J, et al. Recent developments in myofibroblast biology: paradigms for connective tissue remodeling. *Am J Pathol* 2012;180:1340–1355.
- Musso G, Gambino R, Cassader M. Interactions between gut microbiota and host metabolism predisposing to obesity and diabetes. *Annu Rev Med* 2011;62:361–380.
- DeBerardinis RJ, Lum JJ, Hatzivassiliou G, Thompson CB. The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. *Cell Metab* 2008;7:11–20.
- Ward PS, Thompson CB. Metabolic reprogramming: a cancer hallmark even Warburg did not anticipate. *Cancer Cell* 2012;21:297–308.
- Xie N, Tan Z, Banerjee S, Cui H, Ge J, Liu RM, et al. Glycolytic reprogramming in myofibroblast differentiation and lung fibrosis. *Am J Respir Crit Care Med* 2015;192:1462–1474.
- Kottmann RM, Kulkarni AA, Smolnycki KA, Lyda E, Dahanayake T, Salibi R, et al. Lactic acid is elevated in idiopathic pulmonary fibrosis and induces myofibroblast differentiation via pH-dependent activation of transforming growth factor-β. *Am J Respir Crit Care Med* 2012;186:740–751.
- Cho SJ, Moon JS, Lee CM, Choi AM, Stout-Delgado HW. Glucose transporter 1-dependent glycolysis is increased during aging-related lung fibrosis, and phloretin inhibits lung fibrosis. *Am J Respir Cell Mol Biol* 2017;56:521–531.
- Arts RJ, Novakovic B, Ter Horst R, Carvalho A, Bekkering S, Lachmandas E, et al. Glutaminolysis and fumarate accumulation integrate immunometabolic and epigenetic programs in trained immunity. *Cell Metab* 2016;24:807–819.
- Jin L, Alesi GN, Kang S. Glutaminolysis as a target for cancer therapy. *Oncogene* 2016;35:3619–3625.
- Yang C, Ko B, Hensley CT, Jiang L, Wasti AT, Kim J, et al. Glutamine oxidation maintains the TCA cycle and cell survival during impaired mitochondrial pyruvate transport. *Mol Cell* 2014;56:414–424.
- Tanaka K, Sasayama T, Irino Y, Takata K, Nagashima H, Satoh N, et al. Compensatory glutamine metabolism promotes glioblastoma resistance to mTOR inhibitor treatment. *J Clin Invest* 2015;125:1591–1602.
- Durán RV, Oppiger W, Robitaille AM, Heiserich L, Skendaj R, Gottlieb E, et al. Glutaminolysis activates Rag-mTORC1 signaling. *Mol Cell* 2012;47:349–358.
- Altman BJ, Stine ZE, Dang CV. From Krebs to clinic: glutamine metabolism to cancer therapy. *Nat Rev Cancer* 2016;16:619–634.
- Kim J, Guan KL. Amino acid signaling in TOR activation. *Annu Rev Biochem* 2011;80:1001–1032.
- Zinzalla V, Stracka D, Oppiger W, Hall MN. Activation of mTORC2 by association with the ribosome. *Cell* 2011;144:757–768.
- Markolovic S, Wilkins SE, Schofield CJ. Protein hydroxylation catalyzed by 2-oxoglutarate-dependent oxygenases. *J Biol Chem* 2015;290:20712–20722.

21. Schwarz RI. Collagen I and the fibroblast: high protein expression requires a new paradigm of post-transcriptional, feedback regulation. *Biochem Biophys Res* 2015;3:38–44.
22. Berg RA, Schwartz ML, Crystal RG. Regulation of the production of secretory proteins: intracellular degradation of newly synthesized “defective” collagen. *Proc Natl Acad Sci USA* 1980;77:4746–4750.
23. Ripley CR, Fant J, Bienkowski RS. Brefeldin A inhibits degradation as well as production and secretion of collagen in human lung fibroblasts. *J Biol Chem* 1993;268:3677–3682.
24. Yang S, Banerjee S, de Freitas A, Sanders YY, Ding Q, Matalon S, *et al.* Participation of miR-200 in pulmonary fibrosis. *Am J Pathol* 2012; 180:484–493.
25. Yang S, Cui H, Xie N, Icyuz M, Banerjee S, Antony VB, *et al.* miR-145 regulates myofibroblast differentiation and lung fibrosis. *FASEB J* 2013;27:2382–2391.
26. Sheppard D. Epithelial-mesenchymal interactions in fibrosis and repair: transforming growth factor- β activation by epithelial cells and fibroblasts. *Ann Am Thorac Soc* 2015;12(Suppl 1):S21–S23.
27. Gross MI, Demo SD, Dennison JB, Chen L, Chernov-Rogan T, Goyal B, *et al.* Antitumor activity of the glutaminase inhibitor CB-839 in triple-negative breast cancer. *Mol Cancer Ther* 2014;13:890–901.
28. Momcilovic M, Bailey ST, Lee JT, Fishbein MC, Magyar C, Braas D, *et al.* Targeted inhibition of EGFR and glutaminase induces metabolic crisis in EGFR mutant lung cancer. *Cell Reports* 2017;18:601–610.
29. Elgogary A, Xu Q, Poore B, Alt J, Zimmermann SC, Zhao L, *et al.* Combination therapy with BPTES nanoparticles and metformin targets the metabolic heterogeneity of pancreatic cancer. *Proc Natl Acad Sci USA* 2016;113:E5328–E5336.
30. Zoncu R, Efeyan A, Sabatini DM. mTOR: from growth signal integration to cancer, diabetes and ageing. *Nat Rev Mol Cell Biol* 2011;12:21–35.
31. Sengupta S, Peterson TR, Sabatini DM. Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress. *Mol Cell* 2010;40:310–322.
32. Carey BW, Finley LW, Cross JR, Allis CD, Thompson CB. Intracellular α -ketoglutarate maintains the pluripotency of embryonic stem cells. *Nature* 2015;518:413–416.
33. Csibi A, Fendt SM, Li C, Poulogiannis G, Choo AY, Chapski DJ, *et al.* The mTORC1 pathway stimulates glutamine metabolism and cell proliferation by repressing SIRT4. *Cell* 2013;153: 840–854.
34. Carbonneau M, Gagné LM, Lalonde ME, Germain MA, Motorina A, Guiot MC, *et al.* The oncometabolite 2-hydroxyglutarate activates the mTOR signalling pathway. *Nat Commun* 2016;7: 12700.
35. Son J, Lyssiotis CA, Ying H, Wang X, Hua S, Ligorio M, *et al.* Glutamine supports pancreatic cancer growth through a KRAS-regulated metabolic pathway. *Nature* 2013;496:101–105.
36. Bertero T, Oldham WM, Cottrill KA, Pisano S, Vanderpool RR, Yu Q, *et al.* Vascular stiffness mechanoactivates YAP/TAZ-dependent glutaminolysis to drive pulmonary hypertension. *J Clin Invest* 2016; 126:3313–3335.
37. Romero Y, Bueno M, Ramirez R, Álvarez D, Sembrat JC, Goncharova EA, *et al.* mTORC1 activation decreases autophagy in aging and idiopathic pulmonary fibrosis and contributes to apoptosis resistance in IPF fibroblasts. *Aging Cell* 2016;15:1103–1112.
38. Walker NM, Belloli EA, Stuckey L, Chan KM, Lin J, Lynch W, *et al.* Mechanistic target of rapamycin complex 1 (mTORC1) and mTORC2 as key signaling intermediates in mesenchymal cell activation. *J Biol Chem* 2016;291:6262–6271.
39. Mercer PF, Woodcock HV, Eley JD, Platé M, Sulikowski MG, Durrenberger PF, *et al.* Exploration of a potent PI3 kinase/mTOR inhibitor as a novel anti-fibrotic agent in IPF. *Thorax* 2016;71: 701–711.
40. Sosulski ML, Gongora R, Danchuk S, Dong C, Luo F, Sanchez CG. Deregulation of selective autophagy during aging and pulmonary fibrosis: the role of TGF β 1. *Aging Cell* 2015;14: 774–783.
41. Bishop T, Ratcliffe PJ. HIF hydroxylase pathways in cardiovascular physiology and medicine. *Circ Res* 2015;117:65–79.
42. Morikawa M, Derynck R, Miyazono K. TGF- β and the TGF- β family: context-dependent roles in cell and tissue physiology. *Cold Spring Harb Perspect Biol* 2016;8:a021873.