Original Article

MOLECULAR METABOLISM

Epigenetic regulation of IPF fibroblast phenotype by glutaminolysis

Zheyi Xiang [1,3], Le Bai [1,3], Jennifer Q. Zhou [1], Ricardo R. Cevallos [2], Jonathan R. Sanders [2], Gang Liu [1], Karen Bernard [1], Yan Y. Sanders [1,*]

ABSTRACT

Objective: Excessive extra-cellular-matrix production and uncontrolled proliferation of the fibroblasts are characteristics of many fibrotic diseases, including idiopathic pulmonary fibrosis (IPF). The fibroblasts have enhanced glutaminolysis with up-regulated glutaminase, GLS1, which converts glutamine to glutamate. Here, we investigated the role of glutaminolysis and glutaminolysis-derived metabolite α-ketoglutarate (α-KG) on IPF fibroblast phenotype and gene expression.

Methods: Reduced glutamine conditions were carried out either using glutamine-free culture medium or silencing the expression of GLS1 with siRNA, with or without α-KG compensation. Cell phenotype has been characterized under these different conditions, and gene expression profile was examined by RNA-Seq. Specific profibrotic genes (Col3A1 and PLK1) expression were examined by real-time PCR and western blots. The levels of repressive histone H3K27me3, which demethylase activity is affected by glutaminolysis, were examined and H3K27me3 association with promoter region of Col3A1 and PLK1 were checked by ChIP assays. Effects of reduced glutaminolysis on fibrosis markers were checked in an animal model of lung fibrosis.

Results: The lack of glutamine in the culture medium alters the profibrotic phenotype of activated fibroblasts. The addition of exogenous and glutaminolysis-derived metabolite α-KG to glutamine-free media barely restores the pro-fibrotic phenotype of activated fibroblasts. Many genes are down-regulated in glutamine-free medium, α-KG supplementation only rescues a limited number of genes. As α-KG is a cofactor for histone demethylases of H3K27me3, the reduced glutaminolysis alters H3K27me3 levels, and enriches H3K27me3 association with Col3A1 and PLK1 promoter region. Adding α-KG in glutamine-free medium depleted H3K27me3 association with Col3A1 promoter region but not that of PLK1. In a murine model of lung fibrosis, mice with reduced glutaminolysis showed markedly reduced fibrotic markers.

Conclusions: This study indicates that glutamine is critical for supporting pro-fibrotic fibroblast phenotype in lung fibrosis, partially through α-KG-dependent and −independent mechanisms, and supports targeting fibroblast metabolism as a therapeutic method for fibrotic diseases.

Keywords Glutamine; α-KG; H3K27me3; Col3A1; PLK1; Lung fibrosis; Running head

1. INTRODUCTION

Glutamine (Gln) is the most abundant amino acid in human circulation and intracellular pools; glutamine is also involved in many cellular processes [1,2]. Although it is a non-essential amino acid, it becomes conditionally essential during catabolic stress, such as during states of pregnancy, or critical illness [3,4]. Glutamine is converted to glutamate by glutaminase (GLS), and then to α-ketoglutarate (α-KG) by glutamate dehydrogenase to enter the tricarboxylic acid (TCA) cycle [5]. GLS is the initial enzyme in the glutaminolysis pathway. GLS was reported as a key player in cancer cell metabolism that promotes tumor growth, and therefore targeting GLS has been explored as a potential therapy for cancer treatment [6,7]. GLS isoform GLS1 is increased in differentiated fibroblasts and fibrotic lung tissues [8].

Many studies have established the link between metabolism and the cell epigenetic status [9,10]. A lot of molecules derived from intermediary metabolism are key regulators of chromatin enzymatic activities [11]. α-KG is an important metabolite derived from glutaminolysis and has a critical role in different metabolic and cellular pathways. α-KG serves as a cofactor for multiple dioxygenases [12,13]. It regulates the activity of prolyl-4 hydroxylase, which controls the biosynthesis of collagen [14]. It is also a cofactor for epigenetic enzymes [15], such as ten-eleven translocation hydroxylases involved in DNA methylation [16] and Jumonji C domain containing (JMJC) lysine demethylases which are major histone demethylases [12]. One hypothesis is that altered glutaminolysis may affect cellular histone methylation via providing intracellular α-KG, which in turn regulates the transcriptional and epigenetic state of cells [13]. In our previous studies, we demonstrated that decreased histone demethylase activity in glutamine-free medium can be rescued by adding exogenous α-KG to the medium, which affects the histone demethylase substrate H3K27me3 [17]. The changes in H3K27me3 levels likely alter the

[1]Division of Pulmonary, Allergy, and Critical Care Medicine, Department of Medicine, University of Alabama at Birmingham, Birmingham, AL 35294, USA [2]Department of Biochemistry and Molecular Genetics, Birmingham, AL 35255, USA

[3] These authors contributed equally: Zheyi Xiang, Le Bai.

*Corresponding author: University of Alabama at Birmingham, 901 19th Street South, BMRII Room 408, Birmingham, AL 35294, USA. E-mail: yans@uab.edu (Y.Y. Sanders).

Received August 30, 2022 • Revision received December 6, 2022 • Accepted December 7, 2022 • Available online 13 December 2022

expression of the genes that are associated with H3K27me3 thus affecting the cell phenotype.

Profibrotic cell phenotype is characteristics of IPF fibroblast, which exhibits increased collagen production, uncontrolled proliferation [18], and enhanced glutaminolysis with upregulated GLS1 [19]. Glutamine controls the synthesis of fibrotic markers such as collagen at both the transcriptional and translational levels [20,21]. A study in pulmonary fibrosis indicated that glutaminolysis promotes collagen translation and stability through α-KG mediated mTOR activation and proline hydroxylation [8]. Collagen type I and III are the main components of extracellular matrix in pulmonary fibrosis [22]. Previously, we have showed that Col3A1 RNA expression is associated with histone modification H3K27me3 [23], which histone mark could be affected by changes of glutamine levels in IPF fibroblasts [17]. In the current study, we explored the role of glutaminolysis and its metabolite α-KG on IPF fibroblast phenotype and on gene expression profile. We examined whether glutaminolysis regulates gene expression through altered histone modification in response to metabolic changes. Our study showed that metabolite levels provide an additional layer of epigenetic control and transcriptional regulation in lung fibroblasts.

2. MATERIALS AND METHODS

2.1. Cell culture and treatments

University of Alabama at Birmingham (UAB) Tissue Procurement Facility provided human primary IPF lung culture of fibroblasts, which were derived from de-identified tissues (Table 1S online). The protocol is approved by the UAB Institutional Review Board. IPF was diagnosed by a multidisciplinary approach according to the guidelines from American Thoracic Society/European Respiratory Society [24]. The fibroblasts were used before passage 5, and were kept in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Grand Island, NY) with 10% fetal bovine serum (FBS, Life Technologies), 100 units/ml penicillin, 100 μg/ml streptomycin, 1.25 μg/ml amphotericin B, and 2 mm L-glutamine (G1251, Sigma) at 5% CO_2 at 37 °C. For glutamine-free condition, the culture medium was changed to glutamine-free (0 mM of L-glutamine) while all the other components remain the same when the cells reached 80% confluent. For the condition with added α-KG, the cell permeable α-ketoglutarate (AK126628, Ark Pharm Inc., Arlington Heights, IL) was added to L-glutamine free medium at 2 mM. The cells were harvested for various assays after 48 h or as indicated.

2.2. siRNA transfection

GLS1 siRNA (NM_0149,054) and Non-targeting (NT) siRNA sequences are listed in Table 2S (online supplementary data). siRNA transfections were carried out according to manufacturer's instructions with Opti-MEM (Thermo Fisher Scientific) using lipofectamine RNAi/MAX (Invitrogen). The GLS1 siRNA transfection in IPF lung fibroblasts were done in full medium (2 mM L-glutamine) as indicated above, cells were subjected to assays after 48 h of transfection.

2.3. Proliferation assay

The primary IPF fibroblasts were seeded into a 12-well plate at a density of 10,000 cells/well in full medium for overnight. Then the culture medium was changed to fresh full medium, or glutamine-free medium, or glutamine-free medium with 2 mM α-KG. The cells were counted using a conventional hemocytometer counting chamber at 24 h, 48 h and 72 h following published protocol [25].

2.4. Cell migration assay

When the cells are confluent as a monolayer in full medium, the medium was discarded, and a scratch was created with a sterile P20 pipette tip. After rinsed the plate with PBS to remove suspended cells, the cells were cultured in fresh medium of control (full medium with 2 mM glutamine), or glutamine-free (full medium without glutamine), or glutamine-free with 2 mM of α-KG. The wound closure was monitored and photographed with a Keyence microscope (Itasca, IL) at indicated time points. The distance of the edges of the scratch were measured with the obtained image.

2.5. Immunofluorescence

Immunofluorescence staining was performed as descried previously [26]. Rabbit anti-Col3A1 was purchased from Abclonal (cat# A3795, Woburn, MA), H3K27me3 (Cat#9733) was from Cell Signaling. DAPI (Cat#D1306, Thermo Fisher) was used for nuclear staining. Images were acquired with a Keyence microscope (Itasca, IL). Fluorescence signals were quantitated with ImageJ software.

2.6. RNA extraction and real-time RT-PCR

RNA was extracted with RNeasy Mini Kit (Qiagen, Valencia, CA), and transcribed into cDNA with a cDNA synthesis kit (Takara Bio, Mountain View, CA). Real-time RT-PCR was performed in triplicate and normalized to β-actin using the ΔΔCt method [27]. Primers used to examine RNA level expression are listed in Table 2S.

2.7. RNA-sequencing and data analysis

Extracted RNA from three primary IPF fibroblast cell lines under the three conditions described as above was sent to Genewiz (South Plainfield, NJ) for library preparation and RNA-Sequencing (RNA-Seq). Details of the data analyzed are described in online supplementary data.

2.8. Protein extraction and Immunoblotting

Whole cell lysates were prepared after washing the cells in cold PBS and lysed with 2x SDS reducing sample buffer containing protease inhibitors. The lysates protein concentration was quantified using a Micro BCA Protein Assay kit (Thermo Scientific). Lysates were then subjected to SDS-PAGE; western immunoblotting was performed as described previously [28]. Immunoblots were imaged with an Amersham Biosciences 600 Imager (GE Healthcare). Signals were quantitated with ImageJ software. For antibodies, anti-Col3A1 (A3795) was from Abclonal (Woburn, MA), anti-β-actin (#2128) and anti-PLK1 (#4513) were from Cell signaling (Beverly, MA), anti-GLS1 (cat #12885-1-AP) was from Proteintech (Rosemont, IL).

2.9. Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays were performed as per the manufacturer's protocol (ab500, Abcam, Cambridge, MA) with minor modifications [29]. Antibody against H3K27Me3 (cat#39155) used for ChIP assays is from Active Motif (Carlsbad, CA). For ChIP assays, primers used are listed in Table 2S. ChIP-DNA was amplified by real-time PCR, using SYBR® Green PCR Master Mix (Life Technologies). Results are normalized to input DNA.

2.10. GLS1$^{+/-}$ heterozygous mice model of bleomycin induced lung fibrosis

All animal studies were performed in accordance with UAB Institutional Animal Care and Use Committee approved protocols. GLS1$^{+/-}$ heterozygous mice (#017956) were purchase from Jackson

laboratory. At age of 8-week, both wild type (WT) and GLS1$^{+/-}$ heterozygous (Hets) mice were subjected to saline or bleomycin injury. A single dose of normal saline or bleomycin sulfate at 1.5 u/kg body weight was instilled intratracheally. All mice were sacrificed at day 28 post injury. Whole lung tissues were prepared for whole lung lysates for western blots. Some mice lung tissues were prepared for histology with H&E stains. A semiquantitative Ashcroft scale, ranging from 0 to 8, was used to grade severity of lung fibrosis after bleomycin injury [30].

2.11. Statistical analysis

Data are presented as the mean ± standard deviation (SD). All data were statistically analyzed using GraphPad Prism 5.0 (La Jolla, CA). One-way ANOVA was used to compare between multiple groups; comparison between two groups of the same mice were with Student's t test; comparison between two groups of different kinds of mice (wild type vs heterozygous) were used Kolmogorov–Smirnov test. A P value of <0.05 was considered to be statistically significant.

3. RESULTS

3.1. Glutaminolysis regulates IPF fibroblast proliferation, migration, and collagen production

The dysregulated fibroblast proliferation, excessive extracellular matrix production, increased migration and apoptosis resistance are some of the characteristics of pro-fibrotic fibroblasts in IPF [18,31]. Previously, we demonstrated that reduced glutamine levels in cell culture increases apoptotic susceptibility of IPF fibroblasts [17]. Here, we further examined the effects of glutamine withdrawal on IPF fibroblasts proliferation, migration and collagen production. At day one, the cells were plated at about 10,000 cell/well in 12-well plate overnight in control medium. The culture medium was then changed to control (full medium) or glutamine-free medium. Cell count was determined at day 2 and day 3 after changing the media. The cells in control medium reached near 40,000 cell/well at day 3, whereas the cells in glutamine-free medium hardly doubled (Figure 1A). Under similar culture condition, i.e. after cells formed a monolayer, scratch assays were carried out to monitor fibroblast migration with or without glutamine in the culture medium. The cells in control medium migrated much faster than the ones in glutamine-free medium, and filled the scratch after 24 h. In contrast, the gap of the scratch was still visible after 24 h in glutamine-free medium (Figure 1B top two panels). By 48 h, the gaps were filled under both conditions, but the glutamine-free condition seemed with fewer cells.

We then examined if the collagen deposition is affected by these conditions. Collagen III is increased in IPF lung fibroblasts [23], collagen III presence was shown by immune-fluorescent studies after 48 h of culturing the cells in control or glutamine-free medium (Figure 1C, left and middle panels). Noticeably less staining of collagen III in the glutamine-free medium was observed when compared to the

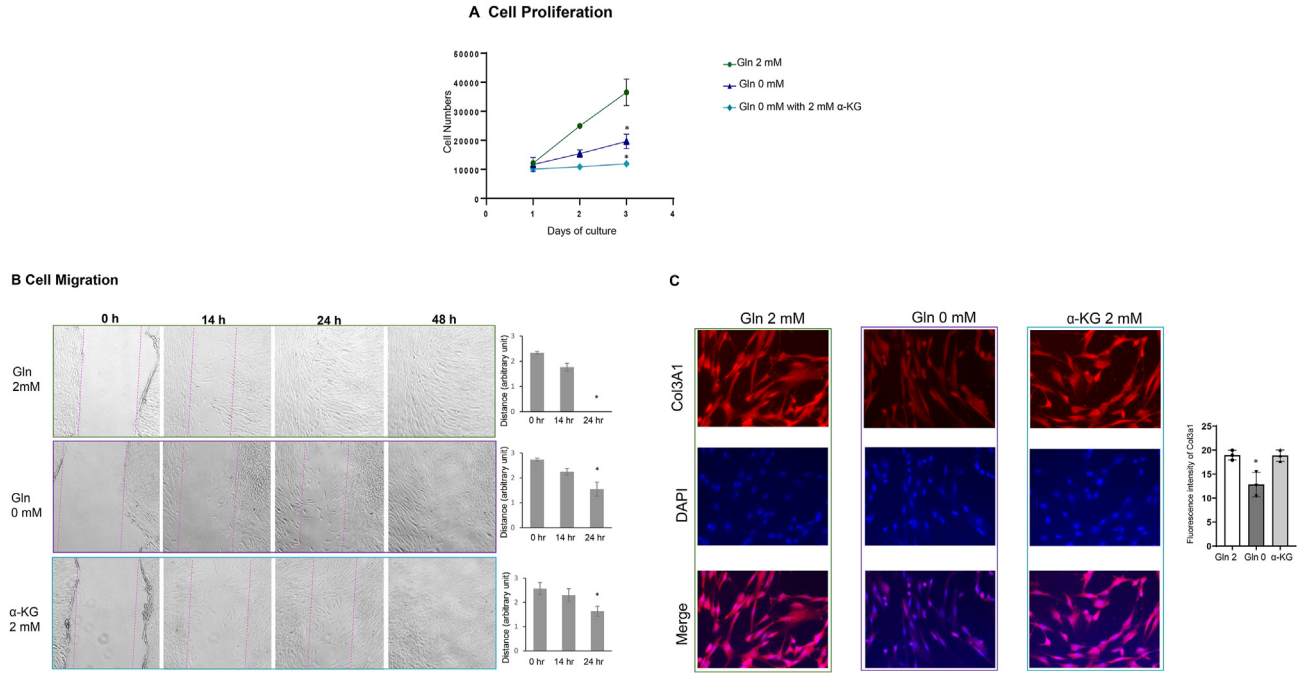


Figure 1: Cell proliferation, migration and collagen III deposition in IPF lung fibroblasts cultured in medium containing 2 mM glutamine (control), 0 mM glutamine and 0 mM glutamine plus 2 mM α-KG. The media of cultured cells under control condition, was changed to fresh medium with 2 mM glutamine, 0 mM glutamine, or 0 mM glutamine with 2 mM α-KG when cells reached 80% confluency. A. Cell numbers were counted each day during 3-day period under these different culture conditions to assess cell proliferation (see methods for detailed protocols). Growth curve were plotted as average ± standard error of cell numbers from three different IPF cell lines of each day. The difference was significant on day 3 under each condition (*$p < 0.05$). B. Cell migration was examined after scratching the cell monolayer with a sterile pipette tip, the migration of the cells were recorded by imaging at different time points at 4×. The edges of the scratches were marked with dashed lines, which were shown by the distance bar with arbitrary unit under 0, 14, or 24 h after the scratches were created. The histogram of scratch distance under each condition was made with average ± standard error of three cell lines. *$p < 0.05$ at 24 h vs 0 h. C. Representative images of immunofluorescence stain of collagen III in IPF lung fibroblasts under different culture conditions after 48 h. Cell were stained with antibodies against Col3A1 (red), and DAPI (blue). Images were also merged to visualize both stains in one image. Pictures were taken at 20× with a Keyence microscope. Histogram is the average ± standard error of at least three sildes of immunofluorescence intensity of Col3A1 stains measured by ImageJ program. *$p < 0.05$, Gln 0 mM vs Gln 2 mM, or α-KG 2 mM.

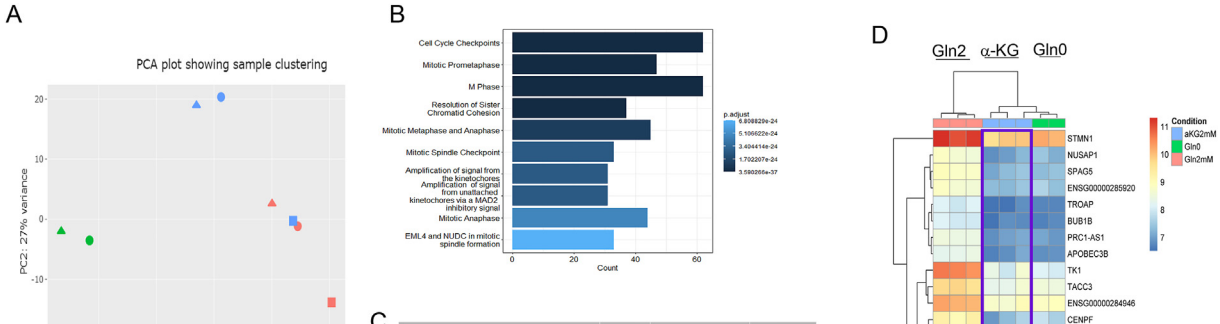
control medium condition. These data indicate that glutamine withdrawal from the culture medium slows IPF fibroblasts proliferation, migration and collagen production.

3.2. α-KG restores IPF fibroblasts collagen production in the absence of glutamine, but not affecting cell proliferation and migration

Since glutaminolysis-derived α-KG enters the tricarboxylic acid (TCA) cycle [32], we reasoned that the addition of exogenous α-KG may reverse some of the effects of reduced glutaminolysis on IPF fibroblast phenotype. To test this hypothesis, we cultured IPF fibroblasts under the glutamine-free condition, in the presence or absence of 2 mM α-KG. With exogenous α-KG in the glutamine-free medium, we did not observe increased cell proliferation. The proliferation was similar to or even slower compared to IPF fibroblasts proliferation under glutamine-free condition (Figure 1A). Similar results were observed for migration of IPF fibroblasts cultured in glutamine-free medium, in the presence or absence of α-KG. With the exogenous α-KG, by 24 h, only some cells filled the gap (Figure 1B, bottom panel), in contrast, the gap in full medium control was closed. However, immunofluorescence staining of collagen III showed that the addition of α-KG to glutamine-free medium restored IPF fibroblasts synthesis of collagen III. IPF fibroblasts with added α-KG demonstrated heightened fluorescent signal associated with collagen III compared to those cells in glutamine-free condition without α-KG, and similar to cells cultured in control media (with 2 mM glutamine) (Figure 1C, right panel). These data suggests that the addition of α-KG in glutamine-free medium has limited effects on cell proliferation or migration, but rescues the decreased collagen induced by glutamine withdrawal from the culture medium.

3.3. α-KG partially rescues IPF fibroblast gene expression profile under glutamine-free condition

We next examined the regulation of IPF fibroblast gene expression by glutaminolysis. As previously described, IPF fibroblasts were cultured in control or glutamine-free media supplemented with or without α-KG. We performed RNA-Seq with primary IPF fibroblasts derived from three different individuals under these culture conditions. When cells reached 80% confluency, the control medium was changed to fresh medium containing either 2 mM glutamine, glutamine-free (0 mM), or glutamine-free plus α-KG (0 mM glutamine with 2 mM α-KG). The cells were cultured for 48 h before collecting RNA for sequencing. Principal-components analysis (PCA) of our RNA-seq data projected a strikingly large variance between the cells cultured in glutamine-free medium compared to cells cultured in control medium. The addition of α-KG only rescued limited expression profile towards those observed under control condition (with 2 mM glutamine). Each cell line seems to cluster together under glutamine-free and α-KG conditions (Figure 2A). However, the greatest variance is observed between the donors, which reflected the heterogeneity of the IPF fibroblasts [33]. PCA also identified one sample under glutamine-free condition to be an outlier; therefore, for further analysis, we only used two samples that were obtained from cells cultured under glutamine-free condition (used IPF-B and C, under Gln 0 mM), while we used all three samples for the other two conditions (Gln 2 mM and Gln 0 mM plus α-KG 2 mM). There are many genes that are down-regulated by glutamine withdrawal (Figure 1SA, online supplementary data). However, adding α-KG to the glutamine-free medium only has limited effects on a small portion of genes that were susceptible to glutamine withdrawal from the culture media (Figure 1SB—D).



GO biological process	Counts	Fold Enrichment	FDR
cell cycle (GO:0007049)	168	6.67	8.01E-84
mitotic cell cycle (GO:0000278)	123	9.73	5.59E-75
cell cycle process (GO:0022402)	133	7.85	1.11E-71
mitotic cell cycle process (GO:1903047)	112	10.43	1.57E-70
cell division (GO:0051301)	98	9.46	2.01E-57
chromosome organization (GO:0051276)	91	10.17	2.46E-55
regulation of cell cycle process (GO:0010564)	98	6.8	6.42E-46
regulation of cell cycle (GO:0051726)	117	5.17	7.24E-45
chromosome segregation (GO:0007059)	69	11.76	1.34E-44
nuclear chromosome segregation (GO:0098813)	61	12.93	3.97E-41

Figure 2: RNA-Seq data from three primary IPF lung fibroblasts culture in the presence of 2 mM glutamine, 0 mM glutamine, or 0 mM glutamine plus α-KG 2 mM. RNAs were collected 48 h after treatment and subjected to RNA-Seq. A. PCA plot showing sample clustering. Three different samples are shown by different colors. Lung fibroblasts from IPF patient A (Red), patient B (Green), and patient C (Blue), were cultured with 2 mM glutamine (square), 0 mM glutamine (triangle), or 0 mM glutamine with 2 mM α-KG (circle). Significant variability can be observed between the different samples. Following analysis for the 0 mM glutamine condition only used samples from patients B and C (green and blue triangle), see text for details. B. Pathway enrichment analysis of altered genes under glutamine 0 mM vs 2 mM glutamine. Top 10 most significantly enriched terms are presented in the figure. C. GO term enrichment analysis of altered genes in glutamine 0 mM vs 2 mM. GO term enrichment (Biological Process database) showed that most significant terms are related to cell cycle. GO term analysis returned 168 highly down-regulated genes (fold change >1.5) associated with the term "cell cycle". Top ranked most significantly enriched terms are presented in the table. D. Heatmap showing the top 20 most differentially expressed genes when compared to 2 mM glutamine (Gln2, $q < 0.05$). More RNA-Seq analysis are in online supplementary data. The gene expression in response to the addition of α-KG in the glutamine-free medium is shown with purple box.

The RNA-Seq data from IPF cells indicated that glutamine withdrawal altered many pathways (Figure 2B,C). GO term enrichment shows the most affected pathway is cell cycle (Figure 2C). From the top 20 most differentially expressed genes, some genes, such as BIRC5, have been previously reported to modulate IPF fibroblast phenotype [17] (Figure 2D, and online Table 3S). Overall, the RNA-Seq data described above show significantly altered gene expression profile of IPF fibroblasts under condition of glutamine deficiency, however, with limited rescue following the addition of α-KG in the glutamine-free medium.

3.4. Glutaminolysis regulates Col3A1 and PLK1 expression via α-KG-dependent and -independent mechanisms

In fibrotic diseases, fibroblasts have been shown to express higher levels of Col3A1 [23,34]. Recent studies have demonstrated that glutaminolysis is increased in fibrosis [8,33,35]; and we observed glutamine-free condition would reduce Col3A1, which is rescued by adding α-KG (Figure 1C). Thus, we further examined mechanisms by which glutaminolysis regulates Col3A1 expression in IPF fibroblasts. Under glutamine free condition, Col3A1 expression is decreased at protein and mRNA levels (Figure 3A—C) suggesting a regulation of Col3A1 expression at both transcriptional and post-transcriptional levels. Cell-permeable α-KG added in the gluntamine-free medium, partially recovered Col3A1 expression both at protein and RNA levels (Figure 3A—C). This observation is in consistence of the data that we showed in Figure 1C, and suggests that glutaminolysis regulates Col3A1 expression via an α-KG-dependent mechanism.

From RNA-Seq data comparing control to glutamine-free condition, among the top 20 differentiated genes, PLK1 is one of the cell cycle related genes that was previously reported to be involved in lung fibroblasts proliferation [36]. We further examined PLK1 expression under control and glutamine deficiency conditions. Similarly to Col3A1, glutamine-free condition induced a decrease in PLK1 expression at both the protein and mRNA levels (Figure 3D—F). However, unlike Col3A1, addition of α-KG did not rescue PLK1 expression either at the protein or mRNA levels (Figure 3D—F). This indicates that glutaminolysis regulates PLK1 expression via an α-KG-independent mechanism. Taken together, these data suggest that glutaminolysis differentially regulates Col3A1 and PLK1 expression via α-KG-dependent and -independent mechanisms.

3.5. GLS1 silencing mimics the effects of glutamine-free condition on Col3A1 and PLK1 expression

GLS1 is the key enzyme that controls glutaminolysis in IPF, converts glutamine to glutamate [35]. To determine whether glutamine-mediated regulation of Col3A1 and PLK1 involves GLS1, we silenced GLS1 using siRNA in IPF fibroblasts cultured under 2 mM glutamine containing control medium. 48 h post siRNA transfection, cells were collected to examine the genes expression. In GLS1-silenced cells, the Col3A1 and PLK1 expression was significantly down-regulated at both the protein and RNA levels (Figure 4). The addition of α-KG to the control medium (with 2 mM glutamine), which bypass the blocked conversion of glutamine to glutamate, upregulated Col3A1 at both protein and mRNA levels (Figure 4 A—C); however, the addition of α-KG failed to rescue PLK1 at protein or mRNA levels (Figure 4 A,B,D). Individual differences also play a role in response to altered metabolic conditions. Here, we showed representative data of two different IPF cell lines. In response to GLS1 silencing, one cell line has modest (represented by triangles in Figure 4C) while the other one has robust

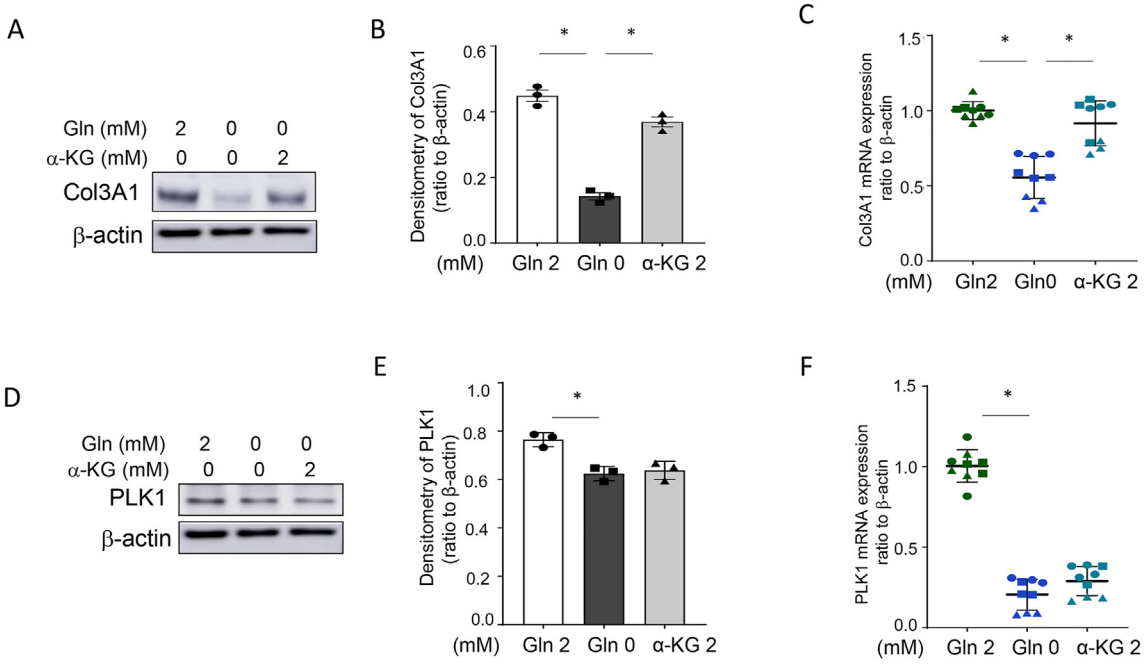


Figure 3: Expression of Col3A1 and PLK1 in IPF fibroblasts grown in media of different glutamine concentrations. Col3A1 and PLK1 expression in IPF lung fibroblasts in culture medium with 2 mM glutamine (control), 0 mM glutamine, or 0 mM glutamine with 2 mM α-KG for 48 h. A/B: (A) Representative western blotting of Col3A1 expression, with β-actin as loading control. (B) Col3A1/β-actin determined by densitometry under culture conditions described above, n = 3 experimental repeats. (C) Col3A1 mRNA levels expression was assessed by real-time PCR. Triangles, squares, or circles indicate three different patient-derived IPF fibroblast lines. Expressed values represent mean ± SD; n = 3 experimental replicates for each cell line. D/E: (D) Representative western blotting of PLK1 expression with β-actin as loading control. (E) PLK1 ratio to β-actin determined by densitometry under the same culture conditions as in D with n = 3 experimental repeats. F: PLK1 mRNA levels expression were determined by real-time PCR. Triangles, squares, or circles indicate three different patient-derived IPF cell lines. Expressed values represent mean ± SD; n = 3 experimental replicates of each cell line. *$p < 0.05$, Glutamine 0 mM (Gln 0) vs Gln 2 mM or Gln0 plus α-KG as indicated. Additional cell lines response under the same condition by western blots are shown in online data Figure 2SA.

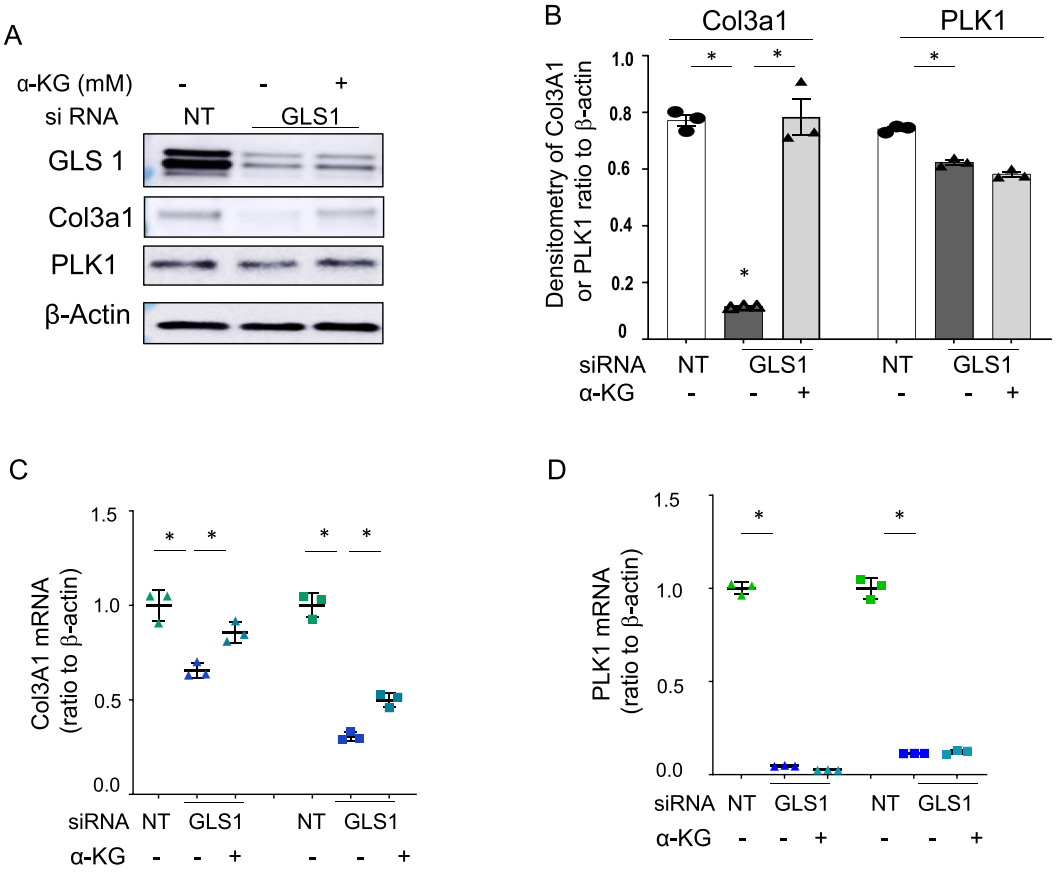


Figure 4: Expression of Col3A1 and PLK1 in IPF fibroblasts transfected with non-targeting (NT) or GLS1 siRNA. IPF cells are transfected with NT or GLS1 siRNA in the presence of 2 mM glutamine. GLS1-silenced cells were cultured in presence of glutamine 2 mM with or without adding α-KG 2 mM. The cells were collected 48 h after transfection for western blots or real-time PCR. A/B: (A) Representative western-blotting to examine the protein levels for GLS1, Col3A1 and PLK1. (B) Densitometry analysis of Col3A1 and PLK1 associated signals: ration of Col3A1 and PLK1 to β-actin, as shown in A, n = 3 experimental replicates. *$p < 0.05$, GLS1 siRNA compared to NT siRNA, or GLS1 siRNA with 2 mM Gln plus α-KG. C/D: RNA expression of Col3A1 (C) or PLK1 (D) in two IPF fibroblast cell lines transfected with NT or GLS1 siRNA cultured in the presence of 2 mM glutamine with or without added α-KG as in A. Triangles or squares represent two different IPF patient-derived primary cells. Expressed values represent mean ± SD, 3 experimental replicates of each cell line. *$p < 0.05$, siRNA GLS1 vs NT, or vs siRNA GLS1 with 2 mM glutamine plus α-KG.

reduction of Col3A1 expression (Figure 4C). Despite the heterogeneity of IPF fibroblasts, this data confirms that reduced glutaminolysis down-regulates Col3A1 and PLK1 expression, while the metabolite α-KG only rescues a subset of genes that are α-KG dependent, such as Col3A1.

3.6. Col3A1 and PLK1 association with histone H3K27me3

In previous studies, we demonstrated that α-KG availability directly affects H3K27me3 demethylases activities in lung fibroblasts [17]. Here, we checked overall baseline levels of H3K27me3 in IPF primary cell lines under different glutaminolysis conditions. We confirmed by western blotting that H3K27me3 levels are low in control medium (with 2 mM glutamine), increased in glutamine-free medium, and reduced with added α-KG in glutamine-free medium (Figure 5A,B and online Figure 2SB). Immunofluorescence staining of H3K27me3 showed most intense staining under glutamine-free condition compared to control with 2 mM glutamine, or glutamine-free with added α-KG (Figure 5C, and online data Figure 3S).

We then examined the changes of H3K27me3 at specific gene promoter regions. The association of H3K27me3 with Col3A1 and PLK1 promoter regions was examined by ChIP with primers listed in Table 2S. We observed a significant increase in H3K27me3 association with both Col3A1 and PLK1 promoters under glutamine-free condition compared to control (Figure 5D,E) likely underpinning reduced mRNA expression of Col3A1 and PLK1 under glutamine-free condition. With added α-KG, the association between the repressive mark H3K27me3 and Col3A1 promoter region is depleted, which likely drives the up-regulation of Col3A1 expression at mRNA levels (Figure 3C). However, the α-KG compensation did not reduce the association of H3K27me3 with Col3A1 to the level observed under 2 mM of glutamine, which indicates that other factors may be also involved in the regulation of Col3A1 expression by glutamine. For PLK1, the addition of α-KG did not significantly reverse the increased association between H3K27me3 and PLK1 promoter region under glutamine-free condition (Figure 5E). This data indicates that the overall baseline levels of H3K27me3 are sensitive to changes in the concentration of glutamine and its metabolite α-KG. However, the association of H3K27me3 with specific gene promoter region is regulated differentially. In summary, H3K27me3 is enriched at the promoter region of Col3A1 and PLK1 in glutamine-free condition, likely repressing their expression; however, the addition of α-KG under glutamine-free condition only depleted H3K27me3 association with Col3A1 not that with PLK1.

3.7. GLS1$^{+/-}$ heterozygous mice showed reduced fibrotic markers in response to lung injury with bleomycin

Previously, we have shown that inhibition of GLS1 attenuates lung fibrosis [19]. Since GLS1$^{-/-}$ null mice die shortly after birth [37], we

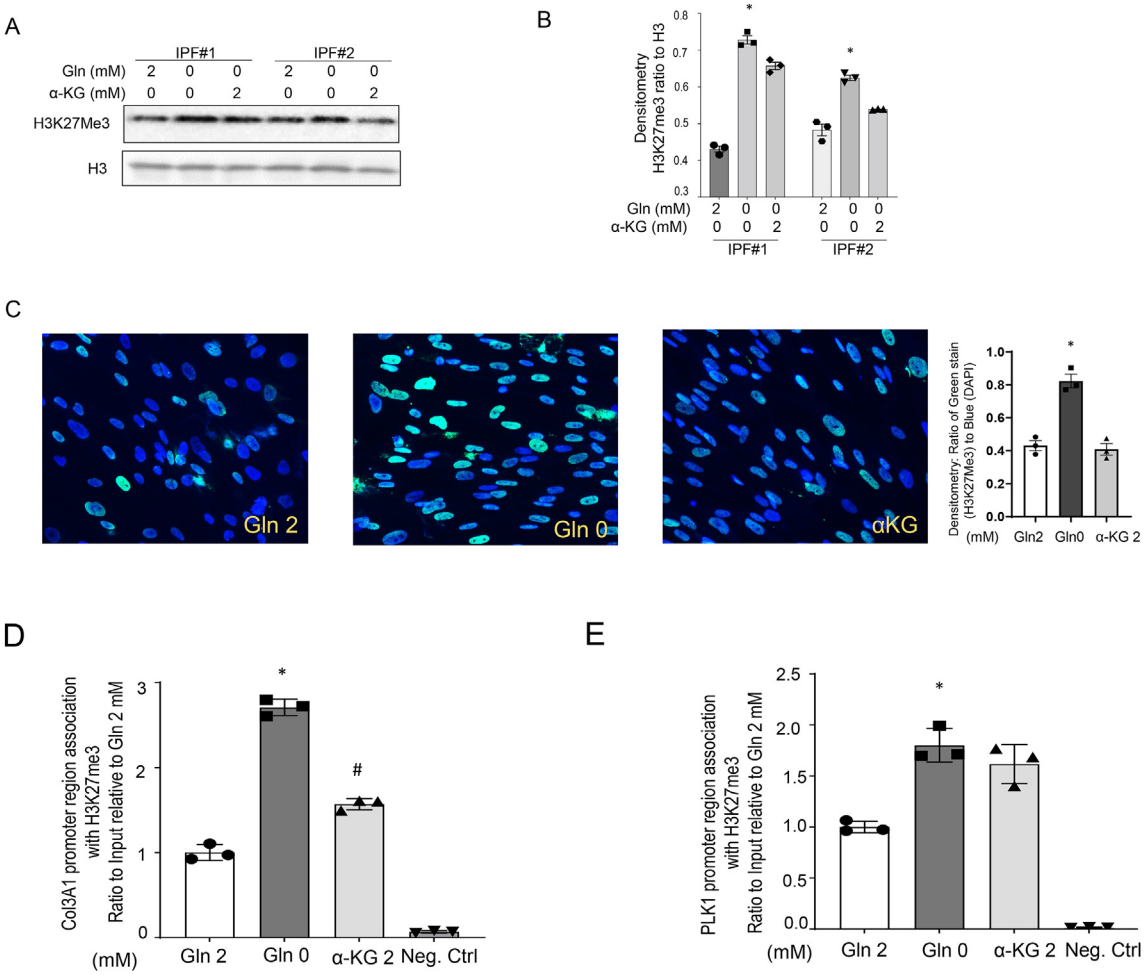


Figure 5: Histone H3K27me3 in IPF fibroblasts and its association with Col3A1 or PLK1 promoter region. IPF lung fibroblasts were kept in control medium (with 2 mM glutamine), when the cells were at 70% confluency, medium was changed to fresh medium with glutamine 2 mM, 0 mM, or 0 mM with α-KG (2 mM) for 48 h. A. Representative western blots of H3K27me3 overall baseline levels in two different IPF primary cell lines under the culture conditions described above, H3 is the loading control. Additional IPF cell lines responsed by WB are in online data Figure 2SB. B. Densitometry analysis of H3K27me3 signals with ratio to H3 as shown in A, n = 3 experimental repeats. The results are expressed as mean ± SD from three independent experiments from these two IPF cell lines. *p < 0.05, compared with glutamine 2 mM or with added 2 mM α-KG. C. Representative merged images of immunofluorescence of IPF fibroblasts stained with antibodies against H3K27me3 (green) and DAPI (blue). Note, the number of cells seeded in the control medium was half compared to the number of cells seeded for the glutamine-free and glutamine 0 mM plus 2 mM α-KG conditions to avoid overcrowding of cells. The images were taken with a Keynence microscope, at 20× magnification. Additional images with DAPI or H3K27me3 alone are in online supplementary data (Figure 3S). Right panel: densitometry of immunoflurescencent stain of green (H3K27me3) ratio to blue (DAPI). *p < 0.05, Gln 0 vs Gln 2 or α-KG. D and E. The IPF fibroblasts were collected after 48 h of each experimental condition. The association of H3K27me3 with Col3A1 (D) or PLK1 (E) was analyzed by ChIP assays. DNA was immunoprecipitated with specific antibody against H3K27me3. Primers for PCR are listed in Table 2S. Negative control represents IgG pull-down. qPCR data were analyzed by using the 2-ΔΔCt method, and results were normalized to input DNA, expressed as fold changes relative to control samples (cells culture under glutamine 2 mM). The values are expressed as mean ± SD from average of three independent experiments of one representative cell line. *p < 0.05, compared to glutamine 2 mM (Gln2), #p < 0.05, compared to glutamine-free (Gln0).

used a heterozygous (GLS1$^{+/-}$, Hets) strain to examine effects of reduced GLS1 expression on lung fibrosis and expression of collagen III and PLK1 in lung tissues. Studies showed that these GLS1$^{+/-}$ mice demonstrated significant downregulation (up to 50%) of GLS1 enzymatic activity in the brain and kidney compared to their WT (GLS1$^{+/+}$) counterparts [37,38]. We subjected 8-week old wild-type (WT) and GLS1$^{+/-}$ heterozygous mice to bleomycin-induced lung fibrosis. In this murine model of lung fibrosis, extracellular matrix accumulation has been shown to start between day 14 to day 20 after injury [39], and almost reach the max of total increase of collagen at day 28 [40]. We collected whole lung tissues at day 28 to examine collagen III and PLK1 expression levels (Figure 6A). GLS1$^{+/-}$ heterozygous mice showed less lung fibrosis compared to WT mice in response to bleomycin injury by H&E stain (Figure 6B), and by the semi-quantitative Ashcroft score (Figure 6C). Whole lung lysates from GLS1$^{+/-}$ heterozygous mice have reduced GLS1 (Figure 6D,E), and less collagen III compared to lung lysates from WT mice with bleomycin injury (Figure 6D,F) as shown by western-blot analysis. Similarly, PLK1 is also reduced in GLS1 $^{+/-}$ heterozygous mice with bleomycin injury compared to WT mice (Figure 6D,G). Taken together, our data showed that GLS1$^{+/-}$ heterozygous mice showed reduced collagen III and PLK1 expression in the lung tissues of a murine model of lung fibrosis.

4. DISCUSSION

Previously, our group demonstrated that glutaminolysis is required for fibroblast differentiation [35,41], and regulates fibroblast apoptosis [17]. In our present study, we further characterized the cell phenotype

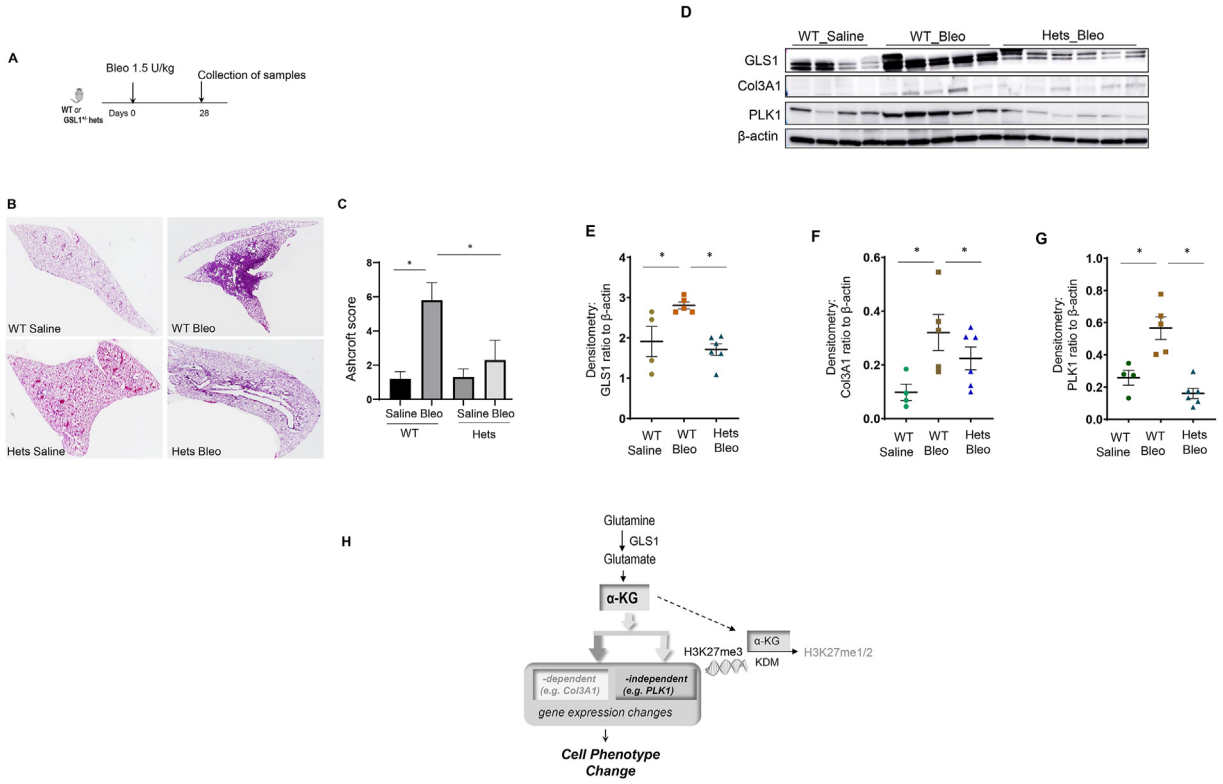


Figure 6: Reduced fibrotic markers in the lungs of bleomycin-injured GLS1+/− heterozygous mice. A. Schematic timeline of animal studies. 8-week old WT or GLS1+/− heterozygous mice were subjected to saline or bleomycin injury. All mice were sacrificed 28 days after injury, samples were collected for assays. B. H&E stain of WT or GLS1+/− hets mice lungs subjected to saline or bleomycin. C. Semiquantitative Ashcroft score of lung sections with WT or GLS1+/− heterozygous mice subjected to saline or bleomycin on day 28 after injury. *$p < 0.05$, WT saline vs Bleo, or WT vs Hets of bleomycin injured mice ($n = 3$). D. Mice lung tissues were collected and the expression of GLS1, Col3A1, and PLK1 were examined by western blots for each of the following group: WT saline ($n = 4$), WT with bleomycin injury ($n = 5$), and Hets with bleomycin injury ($n = 6$). β-actin is the loading control. E-G. Densitometry of GLS1 (E), Col3A1 (F), and PLK1 (G) relative to β-actin (mean with standard error) as shown on the western blots in D. *$p < 0.05$, by t-test (WT saline vs bleo) or Kolmogorov–Smirnov test (WT bleo vs Hets bleo). H. Schematic diagram of possible mechanisms of the findings from this study. Glutamine control cell phenotype through an α-KG dependent and independent mechanisms to regulate gene expression.

changes under different metabolic conditions. We compared the differential expression of genes with and without glutamine, and examined if α-KG could compensate for glutamine-free condition. In this study (Figure 6H), we found many genes are glutamine dependent, among those, some are α-KG dependent, like Col3A1, and some are α-KG independent, such as PLK1. We also explored if altered glutaminolysis modulating gene expression through association with histone modification H3K27me3. Our study confirmed that reduced glutaminolysis would lessen lung fibrosis. We provide the novel connection of metabolic alterations resulted gene expression change, at least partially, through histone modification in lung fibroblasts.

Although glutamine is a non-essential amino acid, most mammalian cells still require at least millimolar levels of glutamine to survive and proliferate [42]. Studies have shown those fast growing cells, like cancer cells, require more glutamine, and is currently considered as essential in cancer cells [43]. Therefore, glutamine deprivation has been considered as a therapeutic approach in cancer. IPF is a disease that has many similarity to cancer [44,45], and we have shown that glutaminolysis is required for lung fibroblast differentiation and activation [35], and reduced glutamine induces apoptosis in IPF fibroblasts [17]. In the current study, we explored the effects of glutamine on IPF fibroblast proliferation, migration and matrix deposition. Glutamine is converted to glutamate and then to α-KG to enter tricarboxylic acid cycle [43]. We thought adding α-KG in glutamine-free medium would compensate and recover many functions of the cells with glutamine withdrawal. Unexpectedly, adding α-KG to the glutamine-free medium did not improve proliferation nor migration in these IPF fibroblasts. This may due to the inability to recover certain important genes that are involved in such cellular functions, such as PLK1 that we examined closely. As the RNA-Seq data indicated, the top pathways affected by glutamine withdrew are cell cycle related. Adding α-KG only rescued few genes compared to the glutamine-free condition. Although the added α-KG did not improve the proliferation or migration in IPF lung fibroblasts, α-KG has many functions in cell growth [15]. In a study with endothelial metabolism, the addition of α-KG rescued cellular growth in glutamine-depleted media [46]. In another study with NIH3T3 and chondrocytes, the α-KG addition in standard culture media was reported to increase proliferation [47], likely due to the increased extracellular matrix. Some of these observations are consistent with our observation of the increased collagen III in the cells compensated with α-KG. Nonetheless, our data indicate that only few changes caused by glutamine-free could be rescued by adding α-KG in IPF lung fibroblasts. The RNA-Seq data showed some genes are rescued, but fewer with significant cut off criteria. Of the three primary IPF fibroblast cell lines we examined, the gene expression has huge differences at baseline among these individuals, which are larger than the culture conditions. The heterogeneity and small size of the samples are the main limitation of this study. However, despite the heterogeneity of the

different IPF cell lines, each cell line presented similar expression pattern under the glutamine-free and with added α-KG conditions.

Ours and previous studies have demonstrated that glutamine is critical for profibrotic genes in IPF lung fibroblasts, withdrew glutamine would reduce such gene expression, such as Col3A1 in this study, Col1A1, and α-SMA reported before [8,35]. In a note, besides IPF cell lines, we also examined fibroblasts from IPF and non-IPF donors for these individual gene expression, which non-IPF showed similar pattern of changes as with IPF under these conditions. Among the altered gene expression due to glutamine withdrawal, there are α-KG-dependent, and -independent genes. We examined two genes in detail. Col3A1 is involved in IPF pathogenesis [23], belongs to the collagen family, which were reported to be affected by different metabolic conditions [48]. PLK1 is reported to be involved in IPF cell proliferation [49], and is on top of the differential gene expression list from the RNA-Seq data. PLK1 remained down-regulated after adding α-KG supports the observation of no improved proliferation with added α-KG in glutamine-free medium. A prior IPF-related study showed reduced glutaminolysis down-regulates collagen in lung fibroblasts [8] through α-KG mediated mTOR activation and proline hydroxylation at translational level. These previous studies demonstrated glutamine regulate collagen expression at the post-transcriptional level [8,21]. In addition, our study showed that glutamine and its metabolite α-KG also control Col3A1 at transcriptional level through histone H3K27me3 at its promoter region.

With the established link that metabolites reprogram epigenetic status of the cells to regulate gene expression [50,51]; many metabolites are reported to be involved in histone modifications [52,53]. α-KG is involved in affecting H3K27me3 levels through regulating JMJC family histone demethylase [13]. It is well established that histone modifications are critical for gene expression [54]. Histone H3K27me3 is a repressive mark [55]. Its demethylases UTX and JMJD3 belongs to JMJC family which uses glutaminolysis metabolite α-KG as a cofactor [13]. We have confirmed that the overall H3K27me3 levels are upregulated significantly with reduced glutaminolysis [17]. For specific genes, the increased association of the repressive mark H3K27me3 with gene promoter region represses gene expression [56]. We observed robust increased H3K27me3 association at the promoter region in both Col3A1 and PLK1 in response to glutamine deficiency, likely due to the decreased demethylase activity of the reduced glutamine metabolite [17]. With the compensation of α-KG in glutamine-free media, reduced association of H3K27me3 at Col3A1 promoter region was noticed, probably due to the increased demethylase activity with added α-KG at the specific gene Col3A1. However, we did not observe such changes of H3K27me3 at PLK1 promoter region with added α-KG, which may indicate other α-KG-independent mechanisms are dominant in regulating PLK1 mRNA level expression under this condition.

As we mentioned early, besides as a co-factor for histone related enzymes [17], α-KG is also involved in regulation through mTOR activation at translational levels [8]. Thus, changes in metabolic condition resulted differences are a combined effects to each specific gene and/or protein expression changes. We also have to point out the well-known fact of IPF fibroblast heterogeneity [57], which would contribute to the different individual cell line reaction to the altered microenvironment. For example, some IPF cell lines have more robust changes than others in response to the changed culture conditions.

In our previous study we showed reduced GLS1 in lung fibroblasts attenuates bleomycin-induced lung fibrosis [19]. In this study, we used a GLS1$^{+/-}$ heterozygous [37,38] animal model of lung fibrosis further confirmed that reduced GLS1 is protective for lung fibrosis. The whole lung tissue showed reduced Col3A1 and PLK1. Since the GLS1$^{+/-}$ heterozygous mice have an overall reduced GLS1 expression, we would regard the reduced fibrosis as an overall effects. We realize that reduced GLS1 would have other effects than regulating H3K27me3, as we have demonstrated that some genes are not regulated through the GLS1-αKG-H3K27me3 axis. Therefore, we did not pursue to relate those gene expression changes with H3K27me3 levels in the whole lung tissues. Also, our previous study showed that ablation of GLS1 attenuates bleomycin induced lung fibrosis [19], thus we did not further evaluate the hydroxyproline content in bleomycin injured GLS1$^{+/-}$ hets mice, which would be the limitations of this study.

Nonetheless, our current study confirmed that reduced glutaminolysis would alter cell phenotype, and α-KG only partially compensates for glutamine-free condition regarding cellular functions and gene expression. Among the genes that are glutamine dependent, some are α-KG dependent, like Col3A1, and some are α-KG independent, such as PLK1. The altered expression by glutamine deficient is likely, at least partially, through repressive histone mark H3K27me3. Our study supports that metabolic control of cellular function is likely through epigenetic regulation of histone modification to modulate gene expression.

5. CONCLUSIONS

We found that glutaminolysis is important in maintaining IPF fibroblasts cell phenotype. Compensating the main metabolite, α-KG, to the glutamine-free medium has limited effects on the cell phenotype, such as cell proliferation and migration, but would compensate for collagen accumulation. RNA-Seq data indicate that only some of the differential expressed genes under glutamine-free condition are α-KG-dependent, while most are α-KG-independent. Col3A1 is an α-KG-dependent gene that is down-regulated at both RNA and protein levels, either by glutamine-free or siRNA GLS1 knockdown, which can be rescued by adding α-KG. While PLK1, one of the top hits of the cell cycles genes from RNA-seq data that are down-regulated in glutamine-free condition, its expression did not change with added α-KG. As α-KG is a cofactor for histone demethylase of H3K27me3, the associations of the repressive histone mark H3K27me3 with Col3A1 and PLK1 were depleted under glutamine-free condition. In an animal model of bleomycin induced lung fibrosis with reduced glutaminolysis of heterozygous GLS1 $^{+/-}$ mice, decreased fibrosis were observed with reduced fibrotic markers. This study indicates that glutamine is critical for supporting pro-fibrotic cell phenotype, partially through α-KG-depended and −independent mechanisms, and supports the new treatment approach of targeting metabolism for fibrotic diseases.

FUNDING SOURCES

This work was supported by the National Institutes of Health (R01AG050567 and R01HL151702 to YYS). The funders had no involvement in the study design, collector, analysis, interpretation of data or report writing.

AUTHORSHIP CONTRIBUTIONS

ZX, LB, JZ, KB: conducted research; ZX, LB, JZ, KB, GL, YYS: analyzed data; RC and JRS: analyzed RNA-Seq data; KB, YYS: prepared the manuscript; YYS: Conceptualized and designed the study. All authors have read and approved the final manuscript.

DATA AVAILABILITY

Data will be made available on request.

CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2022.101655.

REFERENCES

[1] Smith RJ. Glutamine metabolism and its physiologic importance. JPEN J Parenter Enteral Nutr 1990;14(4 Suppl):40S—4S.

[2] Watford M. Glutamine and glutamate: nonessential or essential amino acids? Animal Nutr 2015;1(3):119—22.

[3] Neu J, Demarco V, Freeman B, McCain M, Strauss D. Is glutamine a conditionally essential amino acid during pregnancy?177. Pediatr Res 1996;40:544.

[4] Jackson NC, Carroll PV, Russell-Jones DL, Sonksen PH, Treacher DF, Umpleby AM. The metabolic consequences of critical illness: acute effects on glutamine and protein metabolism. Am J Physiol 1999;276(1 Pt 1):E163—70.

[5] Wong CC, Qian Y, Yu J. Interplay between epigenetics and metabolism in oncogenesis: mechanisms and therapeutic approaches. Oncogene 2017;36(24):3359—74.

[6] Xiang Y, Stine ZE, Xia J, Lu Y, O'Connor RS, Altman BJ, et al. Targeted inhibition of tumor-specific glutaminase diminishes cell-autonomous tumorigenesis. J Clin Invest 2015;125(6):2293—306.

[7] Jacque N, Ronchetti AM, Larrue C, Meunier G, Birsen R, Willems L, et al. Targeting glutaminolysis has antileukemic activity in acute myeloid leukemia and synergizes with BCL-2 inhibition. Blood 2015;126(11):1346—56.

[8] Ge J, Cui H, Xie N, Banerjee S, Guo S, Dubey S, et al. Glutaminolysis promotes collagen translation and stability via alpha-Ketoglutarate-mediated mTOR activation and proline hydroxylation. Am J Respir Cell Mol Biol 2018;58(3):378—90.

[9] Hitchler MJ, Domann FE. Metabolic defects provide a spark for the epigenetic switch in cancer. Free Radic Biol Med 2009;47(2):115—27.

[10] Rajendran P, Williams DE, Ho E, Dashwood RH. Metabolism as a key to histone deacetylase inhibition. Crit Rev Biochem Mol Biol 2011;46(3):181—99.

[11] Burgio G, Onorati MC, Corona DF. Chromatin remodeling regulation by small molecules and metabolites. Biochim Biophys Acta 2010;1799(10-12):671—80.

[12] Tsukada Y, Fang J, Erdjument-Bromage H, Warren ME, Borchers CH, Tempst P, et al. Histone demethylation by a—family of JmjC domain-containing proteins. Nature 2006;439(7078):811—6.

[13] Carey BW, Finley LW, Cross JR, Allis CD, Thompson CB. Intracellular alpha-ketoglutarate maintains the pluripotency of embryonic stem cells. Nature 2015;518(7539):413—6.

[14] Kivirikko KI, Pihlajaniemi T. Collagen hydroxylases and the protein disulfide isomerase subunit of prolyl 4-hydroxylases. Adv Enzymol Relat Area Mol Biol 1998;72:325—98.

[15] Zdzisinska B, Zurek A, Kandefer-Szerszen M. Alpha-ketoglutarate as a molecule with pleiotropic activity: well-known and novel possibilities of therapeutic use. Arch Immunol Ther Exp 2017;65(1):21—36.

[16] Ito S, D'Alessio AC, Taranova OV, Hong K, Sowers LC, Zhang Y. Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. Nature 2010;466(7310):1129—33.

[17] Bai L, Bernard K, Tang X, Hu M, Horowitz JC, Thannickal VJ, et al. Glutaminolysis epigenetically regulates anti-apoptotic gene expression in IPF fibroblasts. Am J Respir Cell Mol Biol 2019 Jan;60(1):49—57.

[18] Vancheri C. Idiopathic pulmonary fibrosis and cancer: do they really look similar? BMC Med 2015;13:220.

[19] Cui H, Xie N, Jiang D, Banerjee S, Ge J, Sanders YY, et al. Inhibition of glutaminase 1 attenuates experimental pulmonary fibrosis. Am J Respir Cell Mol Biol 2019 Oct;61(4):492—500.

[20] Bellon G, Chaqour B, Wegrowski Y, Monboisse JC, Borel JP. Glutamine increases collagen gene transcription in cultured human fibroblasts. Biochim Biophys Acta 1995;1268(3):311—23.

[21] Karna E, Miltyk W, Wolczynski S, Palka JA. The potential mechanism for glutamine-induced collagen biosynthesis in cultured human skin fibroblasts. Comp Biochem Physiol B Biochem Mol Biol 2001;130(1):23—32.

[22] Broekelmann TJ, Limper AH, Colby TV, McDonald JA. Transforming growth factor beta 1 is present at sites of extracellular matrix gene expression in human pulmonary fibrosis. Proc Natl Acad Sci U S A 1991;88(15):6642—6.

[23] Zhang X, Liu H, Hock T, Thannickal VJ, Sanders YY. Histone deacetylase inhibition downregulates collagen 3A1 in fibrotic lung fibroblasts. Int J Mol Sci 2013;14(10):19605—17.

[24] Raghu G, Collard HR, Egan JJ, Martinez FJ, Behr J, Brown KK, et al. An official ATS/ERS/JRS/ALAT statement: idiopathic pulmonary fibrosis: evidence-based guidelines for diagnosis and management. Am J Respir Crit Care Med 2011;183(6):788—824.

[25] Morten BC, Scott RJ, Avery-Kiejda KA. Comparison of three different methods for determining cell proliferation in breast cancer cell lines. J Vis Exp 2016;115.

[26] Sanders YY, Lyv X, Zhou QJ, Xiang Z, Stanford D, Bodduluri S, et al. Brd4-p300 inhibition downregulates Nox4 and accelerates lung fibrosis resolution in aged mice. JCI Insight 2020;5(14).

[27] Sanders YY, Pardo A, Selman M, Nuovo GJ, Tollefsbol TO, Siegal GP, et al. Thy-1 promoter hypermethylation: a novel epigenetic pathogenic mechanism in pulmonary fibrosis. Am J Respir Cell Mol Biol 2008;39(5):610—8.

[28] Sanders YY, Kumbla P, Hagood JS. Enhanced myofibroblastic differentiation and survival in Thy-1(-) lung fibroblasts. Am J Respir Cell Mol Biol 2007;36(2):226—35.

[29] Sanders YY, Liu H, Liu G, Thannickal VJ. Epigenetic mechanisms regulate NADPH oxidase-4 expression in cellular senescence. Free Radic Biol Med 2015;79:197—205.

[30] Ashcroft T, Simpson JM, Timbrell V. Simple method of estimating severity of pulmonary fibrosis on a numerical scale. J Clin Pathol 1988;41(4):467—70.

[31] Suganuma H, Sato A, Tamura R, Chida K. Enhanced migration of fibroblasts derived from lungs with fibrotic lesions. Thorax 1995;50(9):984—9.

[32] Xiao D, Zeng L, Yao K, Kong X, Wu G, Yin Y. The glutamine-alpha-ketoglutarate (AKG) metabolism and its nutritional implications. Amino Acids 2016;48(9):2067—80.

[33] Zhao YD, Yin L, Archer S, Lu C, Zhao G, Yao Y, et al. Metabolic heterogeneity of idiopathic pulmonary fibrosis: a metabolomic study. BMJ Open Respiratory Res 2017;4(1).

[34] Nielsen MJ, Karsdal MA. Chapter 3 - type III collagen. In: Biochemistry of collagens, Laminins and Elastin. Academic Press; 2016. p. 21—30.

[35] Bernard K, Logsdon NJ, Benavides GA, Sanders Y, Zhang J, Darley-Usmar VM, et al. Glutaminolysis is required for transforming growth factor-beta1-induced myofibroblast differentiation and activation. J Biol Chem 2018;293(4):1218—28.

[36] Penke LR, Speth JM, Dommeti VL, White ES, Bergin IL, Peters-Golden M. FOXM1 is a critical driver of lung fibroblast activation and fibrogenesis. J Clin Invest 2018;128(6):2389—405.

[37] Masson J, Darmon M, Conjard A, Chuhma N, Ropert N, Thoby-Brisson M, et al. Mice lacking brain/kidney phosphate-activated glutaminase have impaired

glutamatergic synaptic transmission, altered breathing, disorganized goal-directed behavior and die shortly after birth. J Neurosci 2006;26(17):4660–71.
[38] Gaisler-Salomon I, Miller GM, Chuhma N, Lee S, Zhang H, Ghoddoussi F, et al. Glutaminase-deficient mice display hippocampal hypoactivity, insensitivity to pro-psychotic drugs and potentiated latent inhibition: relevance to schizophrenia. Neuropsychopharmacology 2009;34(10):2305–22.
[39] Moeller A, Ask K, Warburton D, Gauldie J, Kolb M. The bleomycin animal model: a useful tool to investigate treatment options for idiopathic pulmonary fibrosis? Int J Biochem Cell Biol 2008;40(3):362–82.
[40] Scotton CJ, Hayes B, Alexander R, Datta A, Forty EJ, Mercer PF, et al. Ex vivo micro-computed tomography analysis of bleomycin-induced lung fibrosis for preclinical drug evaluation. Eur Respir J 2013;42(6):1633–45.
[41] Bernard K, Logsdon NJ, Ravi S, Xie N, Persons BP, Rangarajan S, et al. Metabolic reprogramming is required for myofibroblast contractility and differentiation. J Biol Chem 2015;290(42):25427–38.
[42] Zhang J, Pavlova NN, Thompson CB. Cancer cell metabolism: the essential role of the nonessential amino acid, glutamine. EMBO J 2017;36(10):1302–15.
[43] Yoo HC, Yu YC, Sung Y, Han JM. Glutamine reliance in cell metabolism. Exp Mol Med 2020;52(9):1496–516.
[44] Ballester B, Milara J, Cortijo J. Idiopathic pulmonary fibrosis and lung cancer: mechanisms and molecular targets. Int J Mol Sci 2019;20(3):593.
[45] Duan J, Zhong B, Fan Z, Zhang H, Xu M, Zhang X, et al. DNA methylation in pulmonary fibrosis and lung cancer. Expert Rev Respir Med 2022:1–10.
[46] Kim B, Li J, Jang C, Arany Z. Glutamine fuels proliferation but not migration of endothelial cells. Embo j 2017;36(16):2321–33.
[47] Singh D, Vishnoi T, Kumar A. Effect of alpha-ketoglutarate on growth and metabolism of cells cultured on three-dimensional cryogel matrix. Int J Biol Sci 2013;9(5):521–30.
[48] Albaugh VL, Mukherjee K, Barbul A. Proline precursors and collagen synthesis: biochemical challenges of nutrient supplementation and wound healing. J Nutr 2017;147(11):2011–7.
[49] Kasam RK, Ghandikota S, Soundararajan D, Reddy GB, Huang SK, Jegga AG, et al. Inhibition of Aurora Kinase B attenuates fibroblast activation and pulmonary fibrosis. EMBO Mol Med 2020;12(9):e12131.
[50] Kinnaird A, Zhao S, Wellen KE, Michelakis ED. Metabolic control of epigenetics in cancer. Nat Rev Cancer 2016;16(11):694–707.
[51] Martinez-Pastor B, Cosentino C, Mostoslavsky R. A tale of metabolites: the cross-talk between chromatin and energy metabolism. Cancer Discov 2013;3(5):497–501.
[52] Wellen KE, Hatzivassiliou G, Sachdeva UM, Bui TV, Cross JR, Thompson CB. ATP-citrate lyase links cellular metabolism to histone acetylation. Science 2009;324(5930):1076–80.
[53] Tanno M, Sakamoto J, Miura T, Shimamoto K, Horio Y. Nucleocytoplasmic shuttling of the NAD+-dependent histone deacetylase SIRT1. J Biol Chem 2007;282(9):6823–32.
[54] Eberharter A, Becker PB. Histone acetylation: a switch between repressive and permissive chromatin. Second in review series on chromatin dynamics. EMBO Rep 2002;3(3):224–9.
[55] Cao R, Wang L, Wang H, Xia L, Erdjument-Bromage H, Tempst P, et al. Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science 2002;298(5595):1039–43.
[56] Cai Y, Zhang Y, Loh YP, Tng JQ, Lim MC, Cao Z, et al. H3K27me3-rich genomic regions can function as silencers to repress gene expression via chromatin interactions. Nat Commun 2021;12(1):719.
[57] Lyu X, Hu M, Peng J, Zhang X, Sanders YY. HDAC inhibitors as antifibrotic drugs in cardiac and pulmonary fibrosis. Therapeutic Adv Chronic Dis 2019;10. 2040622319862697-2040622319862697.

