

Metformin prevents PFKFB3-related aerobic glycolysis from enhancing collagen synthesis in lung fibroblasts by regulating AMPK/mTOR pathway

CI-JUN TANG^{1*}, JI XU^{2*}, HAI-YAN YE¹ and XUE-BIN WANG¹

¹Department of Critical Care Medicine, Shanghai East Hospital, Tongji University School of Medicine, Shanghai 200120;

²Department of Emergency, Huashan Hospital, School of Medicine, Fudan University, Shanghai 200040, P.R. China

Received September 10, 2020; Accepted February 22, 2021

DOI: 10.3892/etm.2021.10013

Abstract. Aerobic glycolysis has been shown to contribute to the abnormal activation of lung fibroblasts with excessive collagen deposition in lipopolysaccharide (LPS)-induced pulmonary fibrosis. Targeting aerobic glycolysis in lung fibroblasts might therefore be considered as a promising therapeutic approach for LPS-induced pulmonary fibrosis. In the present study, the aim was to investigate whether metformin, a widely used agent for treating type 2 diabetes, could alleviate LPS-induced lung fibroblast collagen synthesis and its potential underlying mechanisms. Different concentrations of metformin were used to treat the human lung fibroblast MRC-5 cells after LPS challenge. Indicators of aerobic glycolysis in MRC-5 cells were detected by measuring glucose consumption and lactate levels in culture medium in addition to lactate dehydrogenase activity in cellular lysates. The glucose consumption, lactate levels and the lactate dehydrogenase activity were measured respectively using colorimetric/fluorometric and ELISA kits. The effects of metformin in AMP-activated protein kinase (AMPK) activation was assessed by mitochondrial complex I activity kits. Collagen I, α -smooth muscle actin (α -SMA) and collagen III were used as markers of collagen synthesis, which was measured using western blotting, whereas phosphorylated (p-) AMPK, AMPK, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) and mTOR were detected by western blotting. Metformin significantly decreased mitochondrial complex I activity and upregulated the expression of p-AMPK/AMPK

protein in a concentration-dependent manner. Furthermore, the aerobic glycolysis mediated by PFKFB3 and collagen synthesis in LPS-treated MRC-5 cells was gradually inhibited with increasing concentrations of metformin. However, this inhibitory role of metformin on PFKFB3-mediated aerobic glycolysis and collagen synthesis was prevented by treatments with 3BDO and compound C, which are specific mTOR activator and AMPK inhibitor, respectively. Taken together, the findings from this study suggested that metformin may prevent PFKFB3-associated aerobic glycolysis from enhancing collagen synthesis in lung fibroblasts via regulating the AMPK/mTOR pathway.

Introduction

Lipopolysaccharide (LPS) has been reported to induce sepsis-associated pulmonary fibrosis by directly activating the lung fibroblasts and promoting their proliferation, transformation and subsequent collagen synthesis (1-3). Pulmonary fibrosis contributes significantly to the high mortality rates in patients with respiratory distress syndrome (ARDS) (4). Previous studies have demonstrated that sepsis-related pulmonary fibrosis and its severe form, ARDS, are closely associated with metabolic alterations (5,6). Aerobic glycolysis and the Warburg effect have been proposed as an important pathogenic mechanism in the development of pulmonary fibrosis (7,8). Notably, it has been reported that pulmonary aerobic glycolysis in the LPS-treated mouse model contributes to the abnormal activation of lung fibroblasts with excessive collagen deposition (9). Furthermore, phosphofructokinase-2/fructose-2,6-bisphosphatase 3 (PFKFB3), which is a critical regulatory enzyme for glycolysis, could modulate fructose-2,6-bisphosphate (Fru-2,6-BP) synthesis, enhance glucose absorption and promote lactate production in a positive manner (10). In addition, PFKFB3-driven aerobic glycolysis has been reported to promote pulmonary collagen synthesis (5). Targeting PFKFB3-mediated aerobic glycolysis in lung fibroblasts may therefore serve as a potentially effective therapy for LPS-induced pulmonary fibrosis.

Metformin, which is a biguanide anti-hyperglycemic agent (11), has been demonstrated to alleviate pulmonary fibrosis *in vivo* and *in vitro*. For example, Gamad *et al* (12) demonstrated that metformin could attenuate bleomycin-induced pulmonary fibrosis in rats. Wang *et al* (13) demonstrated that

Correspondence to: Professor Xue-Bin Wang or Dr Hai-Yan Ye, Department of Critical Care Medicine, Shanghai East Hospital, Tongji University School of Medicine, 150 Jimo Road, Shanghai 200120, P.R. China
E-mail: wangxb0209@126.com
E-mail: eesnowye@sina.com

*Contributed equally

Key words: metformin, lipopolysaccharide, pulmonary fibrosis, lung fibroblast

metformin can protect lungs in radiation-induced pulmonary fibrosis by ameliorating collagen formation. The present study hypothesized therefore that metformin may serve a crucial role in reducing LPS-mediated pulmonary fibrosis. However, the underlying mechanism remains largely unclear.

Previous studies demonstrated that metformin could modulate glycolysis by activating the AMP-activated protein kinase (AMPK) (14). Briefly, metformin has been shown to directly prohibit respiratory-chain complex 1 of the mitochondrial electron transport chain, resulting in the decrease in adenosine triphosphate (ATP) synthesis and subsequent increased adenosine monophosphate (AMP)/ATP or adenosine diphosphate (ADP)/ATP ratio, leading to AMPK activation by binding to either AMP or ADP (15,16). Mammalian target of rapamycin (mTOR), a downstream target of AMPK, can regulate protein synthesis and cell proliferation via the phosphorylation of eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) (17-19). However, enhanced AMPK activity can inhibit rat mesangial cell proliferation and extracellular matrix deposition in high glucose condition with phosphorylation of mTOR and 4E-BP1 (20). A previous study demonstrated that mTOR activation can promote collagen synthesis in LPS-challenged lung fibroblasts by upregulating the PFKFB3-mediated aerobic glycolysis (9). The present study therefore hypothesized that metformin, an AMPK agonist, may alleviate the LPS-induced pulmonary fibrosis by regulating PFKFB3-mediated aerobic glycolysis.

The present study aimed to verify the hypothesis that pharmacological interventions on the AMPK/mTOR pathway to alleviate PFKFB3-related aerobic glycolysis by metformin could reduce collagen synthesis in an *in vitro* cellular model of LPS-treated lung fibroblasts.

Materials and methods

Reagents and antibodies. LPS from *Escherichia coli* O127:B8 was purchased from Sigma-Aldrich; Merck KGaA. The mTOR activator 3-benzyl-5-((2-nitrophenoxy) methyl)-dihydrofuran-2(3H)-one (3BDO; cat. no. S8317) and the AMPK inhibitor compound C (cat. no. S7306) were obtained from Selleck Chemicals. The primary antibodies used for western blotting were the following: Anti-AMPK α (1:1,000; cat. no. ab32047; Abcam), anti-phosphorylated-AMPK α^{Thr172} (p-AMPK α ; 1:1,000; cat. no. ab133448; Abcam), anti-4E-BP1 (1:2,000; cat. no. ab32024; Abcam), anti-phosphorylated-4E-BP1 (p-4E-BP1; 1:1,000; cat. no. ab278686; Abcam), anti-PFKFB3 (1:2,000; cat. no. ab218121; Abcam), anti-mTOR (1:1,000, cat. no. 2983; Cell Signaling Technology, Inc.), anti-phosphorylated-mTOR (p-mTOR, 1:1,000; cat. no. 2971; Cell Signaling Technology, Inc.), anti-collagen I (1:1,000; cat. no. ab260043; Abcam), anti- α -smooth muscle actin (α -SMA, 1:1,000; cat. no. 19245; Cell Signaling Technology, Inc.), anti-collagen III (1:1,000; cat. no. ab184993; Abcam) and anti-GAPDH (1:2,000; cat. no. 5174; Cell Signaling Technology, Inc.). Horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (1:2,000; cat. no. A0208; Beyotime Institute of Biotechnology) were used in this study.

Experimental design and treatment. The human lung fibroblast MRC-5 cell line was obtained from The Cell Bank of Type

Culture Collection of The Chinese Academy of Sciences. Cells were cultured in minimum essential medium (MEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μ g/ml streptomycin (Thermo Fisher Scientific, Inc.) and placed at 37°C in a humidified incubator containing 5% CO₂. MRC-5 cells were seeded into 6-well culture plates at the density of 3x10⁵ cells/well. The medium was changed for serum-free MEM for starvation overnight after reaching 70% confluence, and for complete medium containing 10% FBS. Cells were then treated with a concentration gradient of metformin (1, 5 and 10 mM; Selleck Chemicals) for 30 min and incubated with LPS (1 μ g/ml) for 48 h. To further investigate the relationship between AMPK and mTOR, MRC-5 cells were treated with 3BDO (60 μ M; Selleck Chemicals) or the AMPK-inhibitor compound C (50 μ M; Selleck Chemicals) for 30 min, followed by metformin (10 mM) for 4 h and LPS (1 μ g/ml) for 48 h as reported by Wu *et al.* (21). The IC₅₀ of lactate levels was 6.0 mM, the IC₅₀ of LDH activation was 4.7 mM and the IC₅₀ of glucose consumption was 6.3 mM (Fig. S1).

Lactate dehydrogenase (LDH) activity assay. The activity of LDH, which is a key enzyme in the last step of glycolytic pathway (9). LDH activity in the MRC-5 cell lysate was measured using the LDH Enzymatic Assay Kit (cat. no. A020-2-2; Nanjing Jiancheng Bioengineering Institute) according to the manufacturers' instructions.

Measurement of glucose consumption and lactate in culture medium. Glucose and lactate concentration in culture medium were measured with the Glucose and Lactate Colorimetric/Fluorometric Assay Kits (cat. nos. K606 and K607, respectively; both from BioVision, Inc.), respectively, according to the manufacturers' instructions.

Mitochondrial complex I activity assay. Mitochondrial complex I activity was measured with the Complex I Activity Assay kit (cat. no. ab109721; Abcam) according to the manufacturers' instructions. The extracted cell proteins were loaded onto 96-well plates and incubated with mitochondrial complex I antibody for 3 h at room temperature. Each experiment was performed in five parallel wells and the mean optical density (OD 450 nm) values were measured using the Nano Drop spectrophotometer.

Western blotting. Cells were lysed using RIPA lysis buffer at 4°C (cat. no. P0013C; Beyotime Institute of Biotechnology) supplemented with protease and phosphatase inhibitors (Roche Diagnostics) and then centrifuged at 12,000 x g for 15 min at 4°C. Protein concentration was determined with the bicinchoninic acid assay (Thermo Fisher Scientific, Inc.). Proteins (50 μ g) were separated by 6-12% SDS-PAGE, transferred onto PVDF membranes, membranes were blocked with 5% bovine serum albumin (cat. no. ST025; Beyotime Institute of Biotechnology) in TBS with 0.1% Tween-20 for 1 h at room temperature and then incubated with primary antibodies at 4°C for 15 h. Membranes were then incubated with secondary antibodies at room temperature for 1 h. Bands were detected using enhanced chemiluminescence substrate (Beyotime

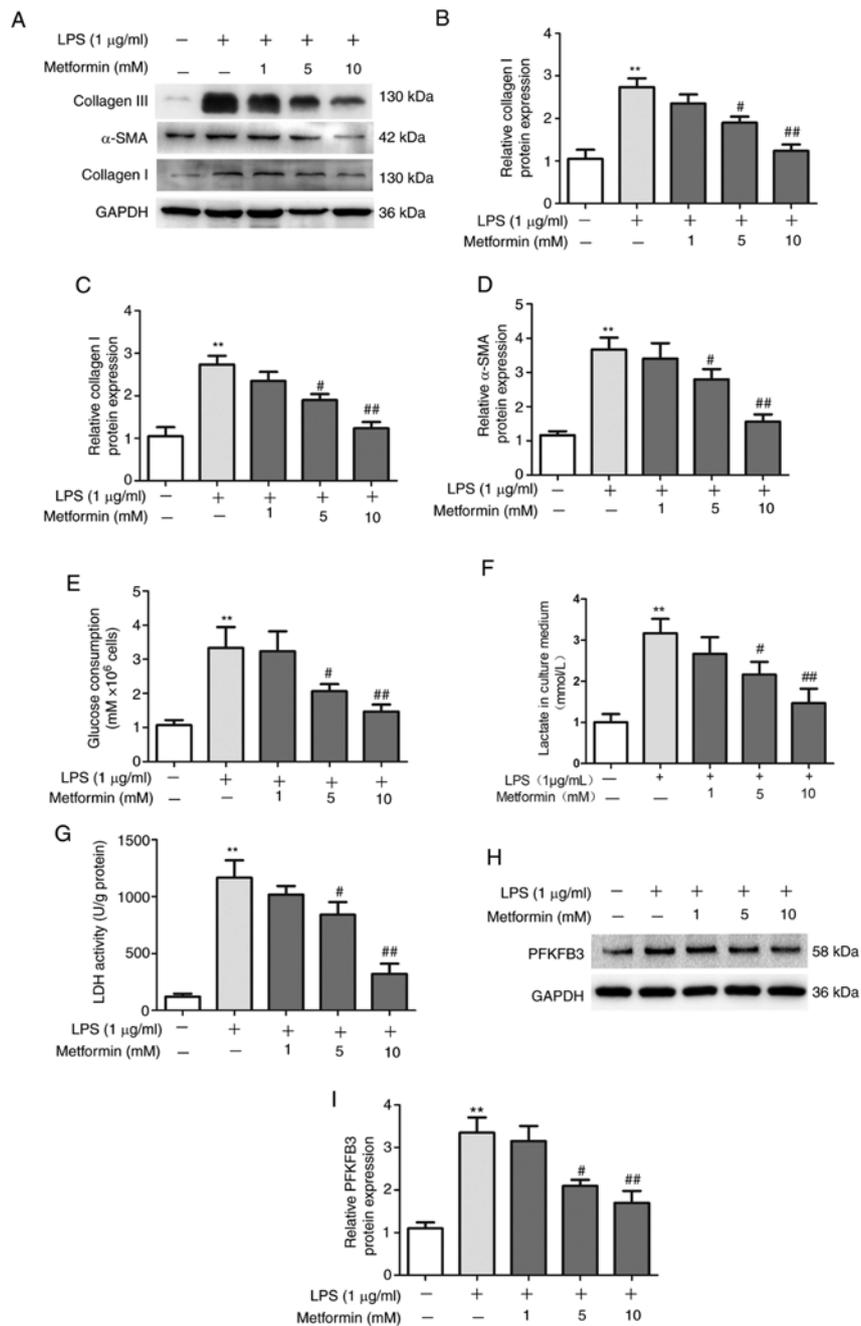


Figure 1. Metformin inhibits collagen synthesis in LPS-treated lung fibroblasts by regulating PFKFB3-mediated aerobic glycolysis. Lung fibroblasts were pre-treated with metformin at different concentrations (1, 5 and 10 mM) for 4 h and incubated with LPS (1 μg/ml) for 48 h. (A) Representative western blotting image and quantitative analysis showing the expression of (B) collagen I, (C) α-SMA and (D) collagen III in lung fibroblasts. Changes in (E) glucose consumption, (F) lactate in cell culture medium and (G) intracellular LDH activity following treatment with different concentrations of metformin. (H) Representative western blotting images and (I) quantitative analysis showing the expression of PFKFB3 in lung fibroblasts. Data were expressed as the means ± standard error of the mean (n=3). **P<0.01 vs. Control group. #P<0.05 and ##P<0.01 vs. LPS group. PFKFB3, 6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase 3; LPS, lipopolysaccharide; α-SMA, α-smooth muscle actin; LDH, lactate dehydrogenase.

Institute of Biotechnology). Relative expression levels were normalized to endogenous control using ImageLab™ software version 2.0.0.27 (Bio-Rad Laboratories, Inc.).

Statistical analysis. Data were presented as the means ± standard deviation and analyzed using GraphPad Prism 7 software (GraphPad Software, Inc.). One-way ANOVA followed by Tukey's post hoc test was used for comparison of three groups or more. P<0.05 was considered to indicate a statistically significant difference.

Results

Metformin inhibits collagen synthesis in LPS-treated lung fibroblasts by regulating PFKFB3-mediated aerobic glycolysis. In the present study, metformin at the concentration of 10 mM could successfully decrease the expression of α-SMA, collagen III and collagen I in lung fibroblasts following LPS treatment, which indicated the inhibition of collagen synthesis (Fig. 1A-D). Furthermore, LPS treatment significantly increased cell glucose consumption (Fig. 1E),

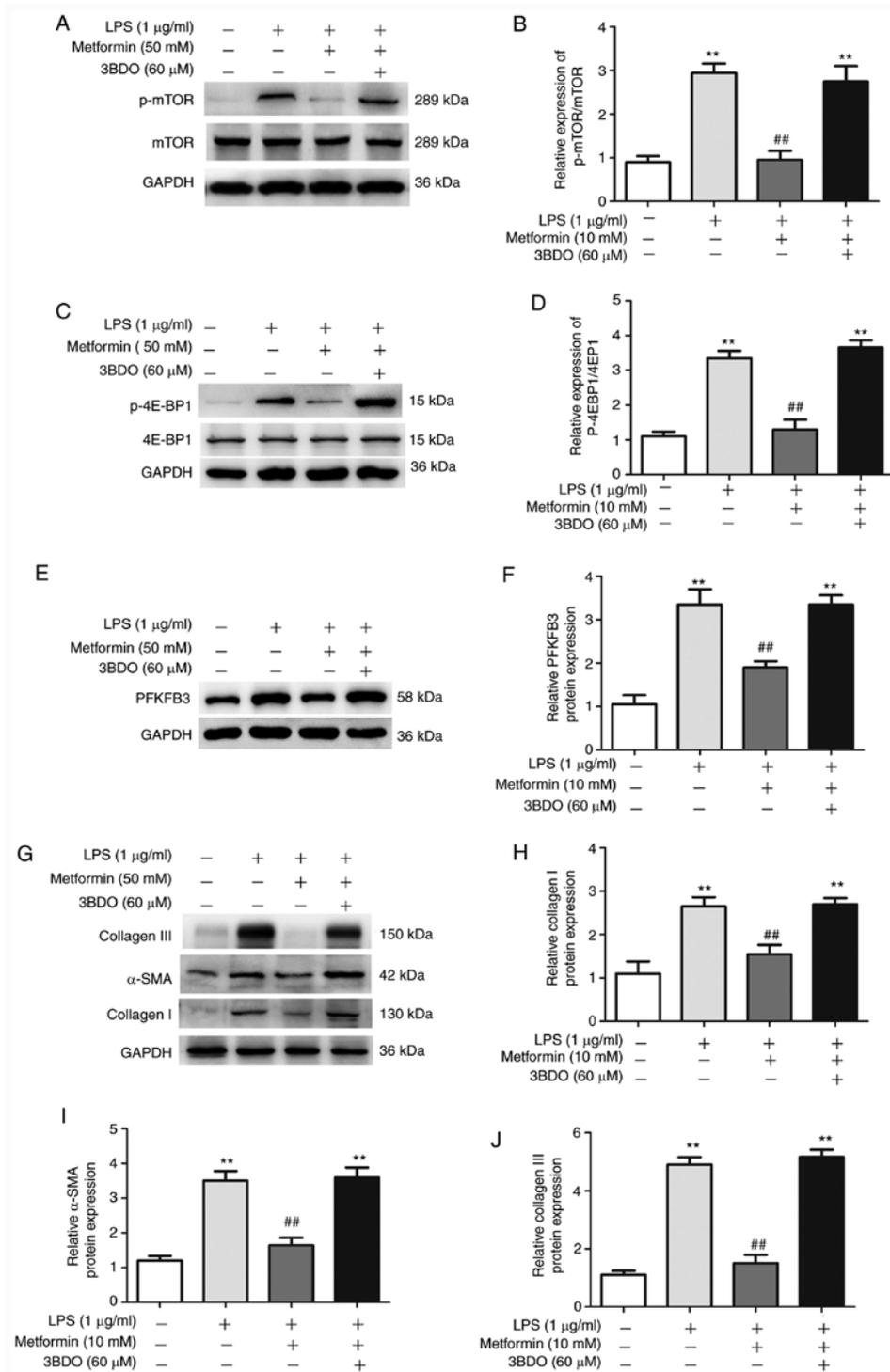


Figure 2. Metformin inhibits collagen synthesis in LPS-treated lung fibroblasts by blocking the activation of the mTOR/PFKFB3 signaling pathway. Lung fibroblasts were pre-treated with mTOR activator 3BDO at a concentration of 60 μ M for 30 min, followed by metformin treatment (10 mM) for 4 h and LPS (1 μ g/ml) for 48 h. (A) Representative western blotting images and (B) quantitative analysis showing the expression of (B) p-mTOR/mTOR in lung fibroblasts. (C) Representative western blotting images and (D) quantitative analysis showing the expression of p-4E-BP1/4EBP1 protein in lung fibroblasts. (E) Representative western blotting images and (F) quantitative analysis showing the expression of PFKFB3 protein. (G) Representative western blotting images and quantitative analysis showing the expression of (H) collagen I, (I) α -SMA, (J) collagen III in lung fibroblasts. Data were expressed as the means \pm standard error of the mean ($n=3$). ** $P<0.01$ vs. Control group. ## $P<0.01$ vs. LPS group. mTOR, mammalian target of rapamycin; PFKFB3, 6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase 3; α -SMA, α -smooth muscle actin; LPS, lipopolysaccharide; p-, phosphorylated.

enhanced lactate production (Fig. 1F) and promoted LDH activation (Fig. 1G), all of which could be reversed with metformin treatment at the concentration of 10 mM. This finding suggested that metformin may inhibit collagen synthesis by regulating aerobic glycolysis in LPS-treated

lung fibroblasts. Furthermore, LPS significantly upregulated PFKFB3 expression in MRC-5 cells compared with negative controls. However, PFKFB3 expression in lung fibroblasts was decreased and returned to the baseline level following treatment with 10 mM metformin (Fig. 1H and I). These results

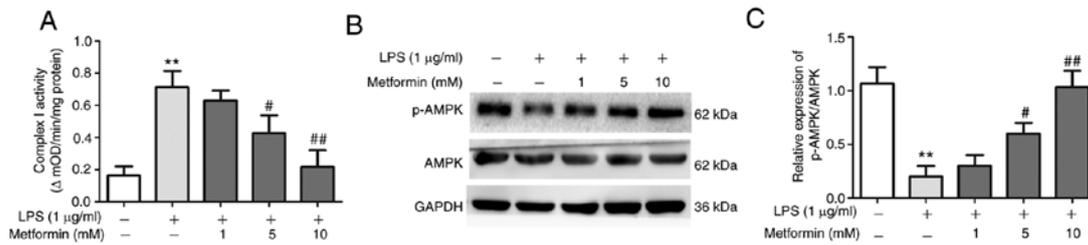


Figure 3. Metformin activates mitochondrial complex I/p-AMPK expression in LPS-treated lung fibroblasts. Lung fibroblasts were pre-treated with metformin at different concentrations (1, 5 and 10 mM) for 4 h and incubated with LPS (1 μg/ml) for 48 h. (A) Mitochondrial complex I activity at different concentrations of metformin. (B) Representative western blotting images and (C) quantitative analysis showing the expression of p-AMPK and AMPK protein in lung fibroblasts. Data were expressed as the means ± standard error of the mean (n=3). **P<0.01 vs. Control group. #P<0.05 and ##P<0.01 vs. LPS group. LPS, lipopolysaccharide; p, phosphorylated.

indicated that metformin may successfully inhibit collagen synthesis in LPS-treated lung fibroblasts by regulating PFKFB3-mediated aerobic glycolysis.

Metformin suppresses collagen synthesis in LPS-treated lung fibroblasts by blocking the activation of the mTOR/PFKFB3 signaling pathway. Activation of the mTOR signaling pathway promotes aerobic glycolysis in the lung fibroblasts by upregulating PFKFB3 expression (9). In the present study, expression of mTOR downstream pathway proteins, including p-mTOR (mTOR activation) and p-4E-BP1 (4E-BP1 activation), was significantly increased after LPS treatment; however, treatment with metformin reversed these effects (Fig. 2A-D). The mTOR activator 3BDO was subsequently used to better characterize the essential role of mTOR in LPS-induced PFKFB3 overexpression. The results demonstrated that 3BDO could inhibit the function of metformin to decrease the expression of p-mTOR, p-4E-BP1 and PFKFB3 (Fig. 2A-D), indicating that mTOR activation may upregulate PFKFB3 expression in LPS-treated lung fibroblasts. In addition, 3BDO could also reverse the effects of metformin to decrease collagen synthesis (Fig. 2G-J). These results suggested that metformin may inhibit collagen synthesis in LPS-treated lung fibroblasts by regulating the mTOR/PFKFB3 pathway.

Metformin activates mitochondrial complex I/p-AMPK expression in LPS-treated lung fibroblasts. Metformin promotes AMPK phosphorylation by decreasing the activity of mitochondrial complex I (22). In the present study, the activity of mitochondrial complex I was increased in LPS-treated lung fibroblasts, whereas it was decreased after treatment with metformin for 48 h in a concentration-dependent manner (Fig. 3A). In addition, p-AMPK protein expression was significantly decreased in LPS-treated lung fibroblasts; however, its expression gradually increased after treatment with increasing concentrations of metformin. Treatment with 10 mM metformin could stimulate p-AMPK expression back to normal baseline level (Fig. 3B and C). Taken together, these findings indicated that 10 mM metformin could successfully activate p-AMPK expression in LPS-treated lung fibroblasts.

Metformin inhibits collagen synthesis in lung fibroblasts by regulating AMPK/mTOR pathway. To better investigate the regulatory role of metformin on aerobic glycolysis in LPS-treated lung fibroblasts, the AMPK inhibitor compound C

was administered with metformin to LPS-treated lung fibroblasts. The results demonstrated that pre-treatment with compound C could reverse the inhibitory effect of metformin on the PFKFB3-mediated aerobic glycolysis in lung fibroblasts, as indicated by the higher levels of glucose consumption (Fig. 4A), increased lactate production (Fig. 4B) and enhanced LDH activation (Fig. 4C) as well as the increase in PFKFB3 expression (Fig. 4D and E). In addition, compound C successfully prevented metformin from upregulating p-AMPK (Fig. 4F and G) and downregulating p-mTOR (Fig. 4H and I) and p-4E-BP1 (Fig. 4J and K) in LPS-treated lung fibroblasts. Furthermore, the decrease in collagen synthesis, as indicated by reduced α-SMA, collagen I and collagen III expression due to metformin treatment of LPS-induced lung fibroblasts, was abolished after treatment with compound C (Fig. 4L-O). These results suggested that metformin may prevent PFKFB3-related aerobic glycolysis from enhancing collagen synthesis in lung fibroblasts by regulating AMPK/mTOR pathway.

Discussion

Previous studies have reported that metformin can alleviate bleomycin-induced idiopathic pulmonary fibrosis both *in vivo* and *in vitro* (12,23). However, whether metformin could have the same protective effect in inhibiting LPS-associated pulmonary fibrosis are still unknown and the underlying mechanism remains unclear. The results from the present study demonstrated that 10 mM metformin could successfully inhibit collagen synthesis in LPS-treated lung fibroblasts, which was similar to a previous studies reporting that 10 mM metformin can inhibit TGF-β-induced myofibroblast collagen synthesis (24). Abnormality in glucose metabolism, especially aerobic glycolysis, has been considered to serve a crucial role in the aberrant activation of lung fibroblasts and to contribute to the development and deterioration of pulmonary fibrosis (25,26). PFKFB3 protein initiates the aerobic glycolysis by improving glucose uptake, enhancing LDH activity and increasing lactate production (27). In addition, the PFKFB3-mediated glycolytic reprogramming has been reported to promote the extracellular matrix production by lung fibroblasts (9,28). In the present study, 10 mM metformin inhibited the PFKFB3-mediated aerobic glycolysis, as evidenced by the decreased glucose uptake, inhibited LDH activity and decreased lactate production in lung fibroblasts following LPS treatment. Taken together, these results

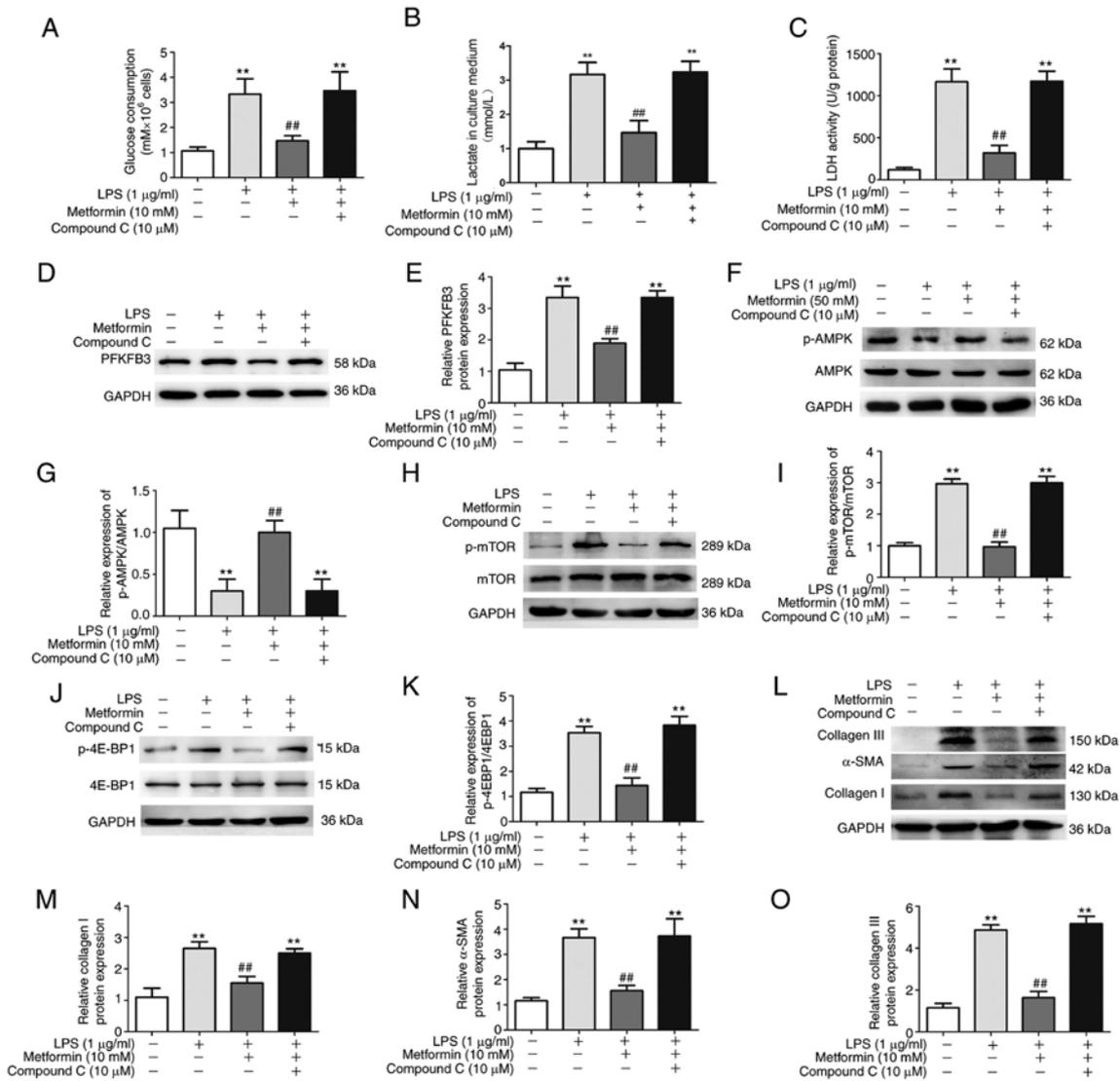


Figure 4. Metformin prevents collagen synthesis in lung fibroblasts by regulating AMPK/mTOR pathway. Lung fibroblasts were pre-treated with the AMPK inhibitor compound C at a concentration of 50 μM for 30 min, followed by metformin treatment (10 mM) for 4 h and LPS (1 μg/ml) for 48 h. (A) Changes in glucose consumption, (B) lactate in cell culture medium and (C) intracellular LDH activity. (D) Representative western blotting images and (E) quantitative analysis showing the expression of PFKFB3 protein. (F) Representative western blotting images and (G) quantitative analysis showing the expression of p-AMPK/AMPK. (H) Representative western blotting images and (I) quantitative analysis showing the expression of p-mTOR/mTOR. (J) Representative western blotting images and (K) quantitative analysis showing the expression of p-4E-BP1/4E-BP1 in lung fibroblasts. (L) Representative western blotting images and quantitative analysis showing the expression of (M) collagen I, (N) α-SMA and (O) collagen III protein in lung fibroblasts. Data were expressed as the means ± standard error of the mean (n=3). **P<0.01 vs. Control group. ##P<0.01 vs. LPS group. mTOR, mammalian target of rapamycin; LPS, lipopolysaccharide; p, phosphorylated; LDH, lactate dehydrogenase; PFKFB3, 6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase 3; α-SMA, α-smooth muscle actin.

provide direct evidence that metformin may prevent collagen synthesis by lung fibroblasts via regulating PFKFB3-mediated glycolysis, suggesting that a pharmacological intervention of metformin may have some potential therapeutic significance for LPS-induced pulmonary fibrosis.

Emerging evidence has demonstrated that the mTOR signaling pathway is vital in both the aerobic glycolysis (29,30) and collagen synthesis of lung fibroblasts (31,32). Previous studies confirmed that mTOR activation promotes aerobic glycolysis, mostly by upregulating PFKFB3 expression (33,34). In addition, active 4E-BP1, as the mTOR downstream target, is negatively associated with glycolysis (35). In the present study, 10 mM metformin successfully inhibited mTOR and 4-EBP1 activation. However, the mTOR activator 3BDO prevented metformin from decreasing PFKFB3 expression and inhibiting

collagen synthesis in LPS-treated lung fibroblasts. These findings indicated that metformin may reverse PFKFB3-mediated collagen synthesis in LPS-treated lung fibroblasts through regulating the mTOR pathway.

Metformin affects cell aerobic glycolysis metabolism by mainly regulating the AMPK activation (36). Mitochondrial complex I as the direct target of metformin can negatively regulate the phosphorylation of AMPK (23), and AMPK activation is frequently downregulated in pulmonary fibrosis (37). These results were also observed in the present study where AMPK expression was decreased in LPS-associated lung fibroblasts. In the present study, 10 mM metformin successfully activated the AMPK signaling pathway in LPS-treated lung fibroblasts by inhibiting the complex I activity and promoting p-AMPK expression. Subsequently, AMPK, which is cell energy regulator, may

inhibit aerobic glycolysis by regulating the mTOR pathway (38). Furthermore, AMPK activation by metformin leads to the phosphorylation and activation of the tuberous sclerosis complex 1/2, which in turn inhibits mTOR phosphorylation and activation (39). In the present study, after blocking AMPK expression with compound C with resultant activation of mTOR pathway, metformin unexpectedly failed to inhibit collagen synthesis and PFKFB3 expression in LPS-treated lung fibroblasts. In addition, the PFKFB3-mediated aerobic glycolysis (indicated by glucose uptake and lactate production) in LPS-treated lung fibroblasts was also not inhibited with the co-administration of compound C and metformin. Taken together, these findings suggested that metformin efficacy to attenuate collagen synthesis in LPS-treated lung fibroblasts may be mediated by aerobic glycolysis inhibition and AMPK activation. This result was consistent with a previous study reporting that metformin can alleviate sepsis-induced acute lung injury via restoring AMPK-dependent suppression of mTOR (21). The results from the present study demonstrated that metformin could prevent PFKFB3-related aerobic glycolysis from enhancing collagen synthesis in lung fibroblasts by regulating AMPK/mTOR pathway.

In the present study, MRC-5 cells were treated with serum-free MEM for overnight starvation prior to LPS treatment in order to synchronize cultured cells in the G1 phase and improve cell sensitivity to LPS treatment. A previous study reported that autophagy is enhanced and becomes a driving force for oxidative phosphorylation and ATP production when glycolysis is inhibited (40). In addition, LPS promotes lung fibroblast proliferation through autophagy inhibition via activation of the PI3K-Akt-mTOR pathway (41), which in turn mediates lung fibroblast aerobic glycolysis and collagen synthesis in LPS-induced pulmonary fibrosis (9). Subsequently, LPS promotes the aerobic glycolysis by suppressing lung fibroblast autophagy that contributes to pulmonary fibrosis.

The present study had some limitations. Firstly, pulmonary fibrosis is not limited to fibroblast activation with collagen fiber secretion and deposition (42). Previous studies have reported that numerous types of cell, including alveolar epithelium and macrophages, are also actively implicated in the etiopathogenesis of pulmonary fibrosis (43). The present study mainly focused on the regulation of lung fibroblasts by metformin following LPS exposure and failed to investigate the potential effect of metformin in other types of cell. Secondly, it takes at least 30 days for LPS to induce typical pulmonary fibrosis in mice (32). However, we observed the effect of metformin on aerobic glycolysis in lung fibroblasts after LPS exposure for only 48 h, which may not mirror the effect of a single dose of metformin on pulmonary fibrosis for a longer time *in vivo*. It remains unclear whether the effects of metformin on pulmonary fibrosis is transient or long-term. Although Wang *et al* (44) reported that metformin inhibits collagen synthesis in TGF- β 1-stimulated HFL-1 cells for 72 h, how long the effect of metformin on LPS-treated lung fibroblasts lasts for remains unknown. In addition, only cell experiments were performed in the present study, which may not reflect the mechanism in animal models. The effect and duration of metformin on animal models of pulmonary fibrosis should therefore be further investigated.

In conclusion, the present study demonstrated that metformin may decrease collagen synthesis in LPS-treated

lung fibroblasts via inhibiting PFKFB3-related aerobic glycolysis and regulating AMPK/mTOR pathway.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Top-level Clinical Discipline Project of Shanghai Pudong (grant no. PWYgf2018-05).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CJT and JX wrote the first draft of manuscript. HYY and XBW contributed to the conception and design of the research. CJT, JX, HYY and XBW contributed to the experiments and analysis of the data. CJT, JX, HYY and XBW confirm the authenticity of all the raw data. All authors critically revised the manuscript and agreed to be fully accountable for ensuring the integrity and accuracy of the work, and read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Xie T, Xu Q, Wan H, Xing S, Shang C, Gao Y and He Z: Lipopolysaccharide promotes lung fibroblast proliferation through autophagy inhibition via activation of the PI3K-Akt-mTOR pathway. *Lab Invest* 99: 625-633, 2019.
- Gu N, Xing S, Chen S, Zhou Y, Jiang T, Jiao Y, Gao Y, Yu W, He Z and Wen D: Lipopolysaccharide induced the proliferation of mouse lung fibroblasts by suppressing FoxO3a/p27 pathway. *Cell Biol Int* 42: 1311-1320, 2018.
- Zhou WQ, Wang P, Shao QP and Wang J: Lipopolysaccharide promotes pulmonary fibrosis in acute respiratory distress syndrome (ARDS) via lincRNA-p21 induced inhibition of Thy-1 expression. *Mol Cell Biochem* 419: 19-28, 2016.
- Martin C, Papazian L, Payan MJ, Saux P and Gouin F: Pulmonary fibrosis correlates with outcome in adult respiratory distress syndrome. A study in mechanically ventilated patients. *Chest* 107: 196-200, 1995.
- Xie N, Tan Z, Banerjee S, Cui H, Ge J, Liu RM, Bernard K, Thannickal VJ and Liu G: Glycolytic reprogramming in myofibroblast differentiation and lung fibrosis. *Am J Respir Crit Care Med* 192: 1462-1474, 2015.
- Zhong WJ, Yang HH, Guan XX, Xiong JB, Sun CC, Zhang CY, Luo XQ, Zhang YF, Zhang J, Duan JX, *et al*: Inhibition of glycolysis alleviates lipopolysaccharide-induced acute lung injury in a mouse model. *J Cell Physiol* 234: 4641-4654, 2019.

7. Schruf E, Schroeder V, Kuttruff CA, Weigle S, Krell M, Benz M, Bretschneider T, Holweg A, Schuler M, Frick M, *et al*: Human lung fibroblast-to-myofibroblast transformation is not driven by an LDH5-dependent metabolic shift towards aerobic glycolysis. *Respir Res* 20: 87, 2019.
8. Kang YP, Lee SB, Lee JM, Kim HM, Hong JY, Lee WJ, Choi CW, Shin HK, Kim DJ, Koh ES, *et al*: Metabolic profiling regarding pathogenesis of idiopathic pulmonary fibrosis. *J Proteome Res* 15: 1717-1724, 2016.
9. Hu X, Xu Q, Wan H, Hu Y, Xing S, Yang H, Gao Y and He Z: PI3K-Akt-mTOR/PFKFB3 pathway mediated lung fibroblast aerobic glycolysis and collagen synthesis in lipopolysaccharide-induced pulmonary fibrosis. *Lab Invest* 100: 801-811, 2020.
10. Cao Y, Zhang X, Wang L, Yang Q, Ma Q, Xu J, Wang J, Kovacs L, Ayon RJ, Liu Z, *et al*: PFKFB3-mediated endothelial glycolysis promotes pulmonary hypertension. *Proc Natl Acad Sci USA* 116: 13394-13403, 2019.
11. Kheirollahi V, Wasnick RM, Biasin V, Vazquez-Armendariz AI, Chu X, Moiseenko A, Weiss A, Wilhelm J, Zhang JS, Kwapiszewska G, *et al*: Metformin induces lipogenic differentiation in myofibroblasts to reverse lung fibrosis. *Nat Commun* 10: 2987, 2019.
12. Gamad N, Malik S, Suchal K, Vasisht S, Tomar A, Arava S, Arya DS and Bhatia J: Metformin alleviates bleomycin-induced pulmonary fibrosis in rats: Pharmacological effects and molecular mechanisms. *Biomed Pharmacother* 97: 1544-1553, 2018.
13. Wang J, Wang Y, Han J, Mei H, Yu D, Ding Q, Zhang T, Wu G, Peng G and Lin Z: Metformin attenuates radiation-induced pulmonary fibrosis in a murine model. *Radiat Res* 188: 105-113, 2017.
14. Liu Y, Bai F, Liu N, Zhang B, Qin F, Tu T, Li B, Li J, Ma Y, Ouyang F and Liu Q: Metformin improves lipid metabolism and reverses the Warburg effect in a canine model of chronic atrial fibrillation. *BMC Cardiovasc Disord* 20: 50, 2020.
15. Wang Y, An H, Liu T, Qin C, Sesaki H, Guo S, Radovick S, Hussain M, Maheshwari A, Wondisford FE, *et al*: Metformin improves mitochondrial respiratory activity through activation of AMPK. *Cell Rep* 29: 1511-1523.e1515, 2019.
16. Hawley SA, Ross FA, Chevzoff C, Green KA, Evans A, Fogarty S, Towler MC, Brown LJ, Ogunbayo OA, Evans AM and Hardie DG: Use of cells expressing gamma subunit variants to identify diverse mechanisms of AMPK activation. *Cell Metab* 11: 554-565, 2010.
17. Mossmann D, Park S and Hall MN: mTOR signalling and cellular metabolism are mutual determinants in cancer. *Nat Rev Cancer* 18: 744-757, 2018.
18. Mousavizadeh R, Hojabrpour P, Eltit F, McDonald PC, Dedhar S, McCormack RG, Duronio V, Jafarnejad SM and Scott A: $\beta 1$ integrin, ILK and mTOR regulate collagen synthesis in mechanically loaded tendon cells. *Sci Rep* 10: 12644, 2020.
19. Ma XM and Blenis J: Molecular mechanisms of mTOR-mediated translational control. *Nat Rev Mol Cell Biol* 10: 307-318, 2009.
20. Lv C, Wu C, Zhou YH, Shao Y, Wang G and Wang QY: Alpha lipoid acid modulated high glucose-induced rat mesangial cell dysfunction via mTOR/p70S6K/4E-BP1 pathway. *Int J Endocrinol* 2014: 658589, 2014.
21. Wu K, Tian R, Huang J, Yang Y, Dai J, Jiang R and Zhang L: Metformin alleviated endotoxemia-induced acute lung injury via restoring AMPK-dependent suppression of mTOR. *Chem Biol Interact* 291: 1-6, 2018.
22. Owen MR, Doran E and Halestrap AP: Evidence that metformin exerts its anti-diabetic effects through inhibition of complex I of the mitochondrial respiratory chain. *Biochem J* 348: 607-614, 2000.
23. Xiao H, Huang X, Wang S, Liu Z, Dong R, Song D and Dai H: Metformin ameliorates bleomycin-induced pulmonary fibrosis in mice by suppressing IGF-1. *Am J Transl Res* 12: 940-949, 2020.
24. Sato N, Takasaka N, Yoshida M, Tsubouchi K, Minagawa S, Araya J, Saito N, Fujita Y, Kurita Y, Kobayashi K, *et al*: Metformin attenuates lung fibrosis development via NOX4 suppression. *Respir Res* 17: 107, 2016.
25. Xu JN, Li JY, Yu ZH, Rao HW, Wang S and Lan HB: HMGB1 promotes HLF-1 proliferation and ECM production through activating HIF1- α -regulated aerobic glycolysis. *Pulm Pharmacol Ther* 45: 136-141, 2017.
26. Cho SJ, Moon JS, Lee CM, Choi AM and 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* 56: 521-531, 2017.
27. Gong Y, Lan H, Yu Z, Wang M, Wang S, Chen Y, Rao H, Li J, Sheng Z and Shao J: Blockage of glycolysis by targeting PFKFB3 alleviates sepsis-related acute lung injury via suppressing inflammation and apoptosis of alveolar epithelial cells. *Biochem Biophys Res Commun* 491: 522-529, 2017.
28. Kottmann RM, Trawick E, Judge JL, Wahl LA, Epa AP, Owens KM, Thatcher TH, Phipps RP and Sime PJ: Pharmacologic inhibition of lactate production prevents myofibroblast differentiation. *Am J Physiol Lung Cell Mol Physiol* 309: L1305-L1312, 2015.
29. Cheng SC, Quintin J, Cramer RA, Shepardson KM, Saeed S, Kumar V, Giamarellos-Bourboulis EJ, Martens JH, Rao NA, Aghajani-Refah A, *et al*: mTOR- and HIF-1 α -mediated aerobic glycolysis as metabolic basis for trained immunity. *Science* 345: 1250684, 2014.
30. Wu F, Gao P, Wu W, Wang Z, Yang J, Di J, Jiang B and Su X: STK25-induced inhibition of aerobic glycolysis via GOLPH3-mTOR pathway suppresses cell proliferation in colorectal cancer. *J Exp Clin Cancer Res* 37: 144, 2018.
31. Liu MW, Su MX, Tang DY, Hao L, Xun XH and Huang YQ: Ligustrazin increases lung cell autophagy and ameliorates paraquat-induced pulmonary fibrosis by inhibiting PI3K/Akt/mTOR and hedgehog signalling via increasing miR-193a expression. *BMC Pulm Med* 19: 35, 2019.
32. Wan H, Xie T, Xu Q, Hu X, Xing S, Yang H, Gao Y and He Z: Thy-1 depletion and integrin $\beta 3$ upregulation-mediated PI3K-Akt-mTOR pathway activation inhibits lung fibroblast autophagy in lipopolysaccharide-induced pulmonary fibrosis. *Lab Invest* 99: 1636-1649, 2019.
33. Feng Y and Wu L: mTOR up-regulation of PFKFB3 is essential for acute myeloid leukemia cell survival. *Biochem Biophys Res Commun* 483: 897-903, 2017.
34. Li Z, Liu J, Que L and Tang X: The immunoregulatory protein B7-H3 promotes aerobic glycolysis in oral squamous carcinoma via PI3K/Akt/mTOR pathway. *J Cancer* 10: 5770-5784, 2019.
35. Suginozono T, Wakabayashi K, Ato S and Ogasawara R: Effect of 2-deoxyglucose-mediated inhibition of glycolysis on the regulation of mTOR signaling and protein synthesis before and after high-intensity muscle contraction. *Metabolism* 114: 154419, 2021.
36. Duca FA, Cote CD, Rasmussen BA, Zadeh-Tahmasebi M, Rutter GA, Filippi BM and Lam TK: Metformin activates a duodenal Ampk-dependent pathway to lower hepatic glucose production in rats. *Nat Med* 21: 506-511, 2015.
37. Cho SJ, Moon JS, Lee CM, Choi AM and 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* 56: 521-531, 2017.
38. Zhang L, Yang H, Zhang W, Liang Z, Huang Q, Xu G, Zhen X and Zheng LT: Clk1-regulated aerobic glycolysis is involved in glioma chemoresistance. *J Neurochem* 142: 574-588, 2017.
39. Kim J, Kundu M, Viollet B and Guan KL: AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat Cell Biol* 13: 132-141, 2011.
40. Kawaguchi M, Aoki S, Hirao T, Morita M and Ito K: Autophagy is an important metabolic pathway to determine leukemia cell survival following suppression of the glycolytic pathway. *Biochem Biophys Res Commun* 474: 188-192, 2016.
41. Xie T, Xu Q, Wan H, Xing S, Shang C, Gao Y and He Z: Lipopolysaccharide promotes lung fibroblast proliferation through autophagy inhibition via activation of the PI3K-Akt-mTOR pathway. *Lab Invest* 99: 625-633, 2019.
42. Thannickal VJ, Toews GB, White ES, Lynch JP III and Martinez FJ: Mechanisms of pulmonary fibrosis. *Annu Rev Med* 55: 395-417, 2004.
43. Hou J, Ji J, Chen X, Cao H, Tan Y, Cui Y, Xiang Z and Han X: Alveolar epithelial cell-derived Sonic hedgehog promotes pulmonary fibrosis through OPN-dependent alternative macrophage activation. *FEBS J*: Dec 13, 2020 (Epub ahead of print). doi: <https://doi.org/10.1111/febs.15669>.
44. Wang Y, Lin C, Han R, Lu C, Li L, Hu C, Feng M, Chen H and He Y: Metformin attenuates TGF- $\beta 1$ -induced pulmonary fibrosis through inhibition of transglutaminase 2 and subsequent TGF- β pathways. *J Biotech* 10: 287, 2020.

