Transforming Growth Factor (TGF)-\(\beta\) Promotes de Novo Serine Synthesis for Collagen Production*

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TGF-\(\beta\) promotes excessive collagen deposition in fibrotic diseases such as idiopathic pulmonary fibrosis (IPF). The amino acid composition of collagen is unique due to its high (33%) glycine content. Here, we report that TGF-\(\beta\) induces expression of glycogenic genes and increases glycolytic flux. TGF-\(\beta\) also induces the expression of the enzymes of the de novo serine synthesis pathway (phosphoglycerate dehydrogenase (PHGDH), phosphoserine aminotransferase 1 (PSAT1), and phosphoserine phosphatase (PSPH)) and de novo glycine synthesis (serine hydroxymethyltransferase 2 (SHMT2)). Studies in fibroblasts with genetic attenuation of PHGDH or SHMT2 and pharmacologic inhibition of PHGDH showed that these enzymes are required for collagen synthesis. Furthermore, metabolic labeling experiments demonstrated carbon from glucose incorporated into collagen. Lungs from humans with IPF demonstrated increased expression of PHGDH and SHMT2. These results indicate that the de novo serine synthesis pathway is necessary for TGF-\(\beta\)-induced collagen production and suggest that this pathway may be a therapeutic target for treatment of fibrotic diseases including IPF.

Organ injury triggers a complex cascade of responses that results in fibrosis (1). Although the fibrogenic response may be required for the healing process, if it persists, it may lead to progressive organ scarring, dysfunction, and consequently failure (1, 2). Organ fibrosis and resultant organ failure are common and account for at least one-third of deaths worldwide (1, 2). For example, idiopathic pulmonary fibrosis (IPF) is a progressive disease associated with significant morbidity and mortality. It affects ~89,000 people in the United States with a median survival of 3.5 years (3, 4). IPF is characterized by excessive deposition of extracellular matrix, which leads to progressive impairment of gas exchange due to replacement of alveoli with fibrotic tissue. Despite several decades of research and advances, the impact of currently approved therapies is very limited (5, 6).

A defining feature of IPF is fibroblast to myofibroblast differentiation, and excess collagen deposition (7). Fibroblasts, under stimulation by transforming growth factor-\(\beta\) (TGF-\(\beta\)), a multifunctional cytokine that regulates cellular proliferation, differentiation, and extracellular matrix deposition, respond by altering their gene expression profile with de novo expression of cytoskeletal and contractile proteins normally found within smooth muscle cells, and components of the extracellular matrix including collagen (8–12).

Collagen is the main structural protein in the extracellular space and is produced in excess in patients with IPF and those with other organ fibrosis (13). Over 90% of the collagen in the human body is type I collagen (13). The amino acid composition of collagen is unique compared with other proteins due to its high glycine content, which constitutes approximately one-third of the amino acids in collagen (14). The high glycine content is critical for stabilization of collagen helix by facilitating hydrogen bonding and the formation of intermolecular cross-links (14).

Glycine is the smallest of the 20 amino acids found in proteins. It is a non-essential amino acid as it is synthesized from serine, which is another non-essential amino acid that can be produced through the serine biosynthetic pathway. De novo synthesis of serine through the serine biosynthetic pathway diverges from glycolysis via a glycolytic intermediate 3-phosphoglycerate, which is converted to serine in a three-step enzymatic reaction including the following enzymes in order: phosphoglycerate dehydrogenase (PHGDH), phosphoserine aminotransferase 1 (PSAT1), and phosphoserine phosphatase (PSPH). Serine then fuels glycine synthesis via two serine hydroxymethyltransferases: \(\alpha\)-SMA, \(\alpha\)-smooth muscle actin; HLF, human lung fibroblasts; ECAR, extracellular acidification rate; qRT, quantitative RT; 2-DG, 2-deoxy-D-glucose; LDH, lactate dehydrogenase; ANOVA, analysis of variance; MEM, minimal essential medium.

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† The abbreviations used are: IPF, idiopathic pulmonary fibrosis; PHGDH, phosphoglycerate dehydrogenase; PSAT1, phosphoserine aminotransferase 1; PSPH, phosphoserine phosphatase; SHMT, serine hydroxymethyltransferase; \(\alpha\)-SMA, \(\alpha\)-smooth muscle actin; HLF, human lung fibroblasts; ECAR, extracellular acidification rate; qRT, quantitative RT; 2-DG, 2-deoxy-D-glucose; LDH, lactate dehydrogenase; ANOVA, analysis of variance; MEM, minimal essential medium.
De Novo Serine Synthesis and Collagen

A

Collagen I
α-SMA
α-Tubulin

TGF-β (hours) 0 24 48

B

ECAR (mM/min)

Glucose
Oligomycin
2-DG

Time (minutes) 0 20 40 60 80 100

C

Glycolysis

Glycolytic Capacity

ECAR (mM/min)

TGF-β (hours) 0 24 48

D

Fibroblast subject 1
Fibroblast subject 2

Relative Normalized Expression

E

GLUT1 mRNA expression (fold change)

PKM2 mRNA expression (fold change)

LDHA mRNA expression (fold change)

Control TGF-β
Control TGF-β
Control TGF-β

F

ECAR (mM/min)

TGF-β + SB-431542
Oligomycin
2-DG

Glucose

Time (minutes) 0 20 40 60 80 100

G

PKM2 mRNA expression (fold change)

LDHA mRNA expression (fold change)

Control TGF-β
Control TGF-β
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droxymethyltransferase (SHMT) genes; *SHMT1*, encoding the cytoplasmic isozyme (*SHMT1*), and *SHMT2*, encoding the mitochondrial one (*SHMT2*) (15–17).

Here, we show that TGF-β up-regulates glycolytic genes and flux in human fibroblasts. Inhibition of glycolysis resulted in the inhibition of myofibroblast differentiation and production of collagen protein without a significant change in transcription of α-1 type collagen and other TGF-β target genes. TGF-β induced the mRNA expression of most of the enzymes of the glycolytic pathway as well as mRNA and protein expression of enzymes that play a role in de novo synthesis of serine and glycine, including *PHGDH* and *SHMT2*. Genetic deletion of *PHGDH* or *SHMT2* and pharmacologic inhibition of PHGDH attenuated TGF-β-induced collagen protein production. Metabolic labeling studies showed incorporation of glucose-derived carbon into collagen protein in human fibroblasts. Furthermore, the expressions of both PHGDH and SHMT2 were increased in fibroblastic foci in lungs obtained from patients with IPF. Collectively, our results suggest that de novo synthesis of serine and glycine is required for collagen synthesis. This pathway may be a therapeutic target for treatment of organ fibrosis including IPF.

**Results**

**TGF-β Up-regulates Glycolytic Enzyme Expression and Glycolytic Activity in Primary Human Lung Fibroblasts**—Treatment of primary human lung fibroblasts (HLFs) (Lonza, Allendale, NJ) with active TGF-β (1 ng/ml) for 48 h results in increasing amounts of α-smooth muscle actin (α-SMA, a marker for myofibroblast differentiation) and type I collagen in a time-dependent manner (Fig. 1A). To determine the effect of TGF-β on glycolysis, we treated HLFs with active TGF-β (1 ng/ml) or left untreated, and measured the extracellular acidification rate (ECAR) as a surrogate for glycolytic lactate production using the Seahorse XF®24 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA). ECAR was measured using the XF Glycolysis Stress Test Kit at 24 and 48 h after TGF-β treatment (Fig. 1B). Compared with the control-treated group, TGF-β caused a time-dependent increase in glycolysis and glycolytic capacity (Fig. 1, B and C). To determine whether the increased lactate production induced by TGF-β was due to up-regulation of specific glycolytic enzymes, we performed quantitative reverse transcription-polymerase chain reaction (qRT-PCR) from HLFs 24 h after treatment of TGF-β. Heat map of differentially expressed genes after TGF-β treatment of 2 different primary cultures of HLFs are shown in Fig. 1D. Remarkably, compared with control, TGF-β caused increased expression of the majority of glycolytic enzymes including *HK2*, *Aldolase A*, *Enolase 1*, *PKM2*, and *LDHA*. The TGF-β-induced up-regulation of glycolytic enzyme gene expression (*GLUT1* and *PKM2* and *LDHA*) was confirmed by qRT-PCR (Fig. 1E). Collectively, these results suggest that TGF-β up-regulates the expression of glycolytic enzymes and glycolytic activity in HLFs.

TGF-β signals through a receptor complex comprised of type I and type II receptors. To determine whether the effect of TGF-β on glycolysis was mediated via TGF-β receptor, we treated HLFs with TGF-β in the absence or presence of a TGF-β receptor I inhibitor, SB-431542 (10 μM), and measured ECAR and gene expression of glycolytic enzymes. Treatment with SB-431542 completely inhibited the TGF-β-induced increase in lactate production (Fig. 1F) and glycolytic enzyme gene expression (Fig. 1G).

**Glycolysis Is Required for Myofibroblast Differentiation and Collagen Production**—Because TGF-β up-regulated the expression of glycolytic enzymes and increased glycolytic activity, we sought to determine whether up-regulation of glycolysis was required for TGF-β-induced myofibroblast differentiation and collagen production. To evaluate the effect of glycolysis on the TGF-β-induced increase in lactate production, we treated HLFs with inhibitors of glycolysis including 2-deoxy-D-glucose (2-DG) (5 mM) and sodium oxamate (25 mM), which inhibit hexokinase and LDH, respectively, and measured ECAR. In another experiment, we substituted galactose for glucose to reduce glycolysis activity, because galactose enters glycolysis through the Leloir pathway, which occurs at a significantly lower rate than glucose entry into glycolysis (18, 19). Inhibition of glycolysis with 2-DG or sodium oxamate or by substitution of glucose with galactose attenuated lactate production in HLFs (Fig. 2, A–C).

To evaluate the effect of glycolysis on TGF-β-induced myofibroblast differentiation and collagen production, we treated HLFs with TGF-β in the absence or presence of 2-DG (1 and 5 mM), or sodium oxamate (10 and 25 mM) or galactose (25 mM), which was substituted with glucose to inhibit glycolysis. We then assessed α-SMA (myofibroblast differentiation marker) and collagen 1 protein expression at 24 and 48 h. Inhibition of
glycolysis with 2-DG, sodium oxamate, or galactose substitution attenuated TGF-β-induced myofibroblast differentiation and collagen production (Fig. 2, D–F). To genetically confirm the effect of glycolysis on TGF-β-induced myofibroblast differentiation and collagen production, we generated HLFs with knockdown of LDHA using siRNA. Compared with control siRNA, HLFs with knockdown of LDHA had attenuation of TGF-β-induced collagen production and myofibroblast differentiation (Fig. 2G).

We next sought to determine whether glycolysis was required for the transcriptional response to TGF-β. Binding of TGF-β to the type II receptor leads to recruitment and phosphorylation of the type I receptor. The type I receptor then phosphorylates receptor-regulated SMADs (R-SMADs), such as SMAD2 and SMAD3, which bind the coSMAD, SMAD4. The R-SMAD/coSMAD complexes accumulate in the nucleus where they act as transcription factors and participate in the regulation of target gene expression. To determine whether glycolysis affects signaling and gene transcription downstream of TGF-β, we treated HLFs with TGF-β in the absence or presence of 2-DG, sodium oxamate, or galactose substitution, and performed Western blotting for SMAD3 and phosphorylated SMAD3 (phospho-SMAD3). Intriguingly, TGF-β-induced phosphorylation of SMAD3 was not affected by inhibition of glycolysis by 2-DG, sodium oxamate (Fig. 2H), or galactose substitution (Fig. 2I). We then evaluated whether glycolysis regulated the response to TGF-β at the gene transcription level. We treated HLFs with TGF-β in the absence or presence of 2-DG (5 mM) and assessed TGF-β-induced gene transcription at 24 h. Inhibition of glycolysis did not cause a significant change in expression of TGF-β target genes such as COL1A1, CTGF, or PAI-1 (Fig. 2I). Collectively, these results suggest that glycolysis is required for TGF-β-induced myofibroblast differentiation and collagen production without an effect on TGF-β-induced SMAD activation or gene transcription.

The de novo Serine Synthesis Pathway Is Up-regulated and Required for TGF-β-induced Collagen Production—Because the transcriptional response to TGF-β was unaffected by the inhibition of glycolysis, we hypothesized that biosynthetic reactions using glycolytic intermediates may promote the synthesis of TGF-β-induced proteins including collagen, the main structural protein in the extracellular matrix. Organ fibrosis including IPF is characterized by increased deposition of type I collagen, which makes up the majority (90%) of collagen in the human body. One-third of amino acids present in collagen are glycine. Because glycine is so abundant in collagen, we sought to determine whether de novo synthesis of glycine through the de novo serine synthesis pathway was up-regulated after TGF-β treatment (Fig. 3A). We treated HLFs with TGF-β and evaluated the expression of enzymes that regulate the de novo serine synthesis pathway. Compared with control, TGF-β up-regulated the gene expression of enzymes in the de novo serine synthesis pathway including 3-phosphoglycerate dehydrogenase (PHGDH), phosphoserine aminotransferase 1 (PSAT1), phosphoserine phosphatase (PSPH), and serine hydroxymethyltransferase 2 (mitochondrial) (SHMT2) (Fig. 3B). In contrast, serine hydroxymethyltransferase 1 (cytoplasmic) (SHMT1) gene expression was reduced following treatment with TGF-β (Fig. 3B).

Complementing the transcriptional regulation of the de novo serine synthesis pathway enzymes, TGF-β stimulated up-regulation of the protein expression of these enzymes in a time-dependent manner (Fig. 3C). In contrast to up-regulation of PHGDH and SHMT2, protein expression of the SHMT1 protein was reduced after TGF-β treatment similar to its mRNA expression (Fig. 3C). TGF-β-induced up-regulation of PHGDH and SHMT2 mRNA and protein expression was attenuated in HLFs treated with the TGF-β receptor 1 inhibitor, SB-431542 (10 μM) (Fig. 3, D and E). HLFs with SMAD3 knockdown displayed reduced levels of PHGDH and SHMT2 proteins, suggesting that the stimulatory effect of TGF-β on the expression of these enzymes was SMAD-dependent (Fig. 4).

Because culture media contains a significant amount of serine and glycine, we formulated culture media that lacks serine, glycine, or both serine and glycine to determine the specific contribution of the de novo synthesis of glycine through the de novo serine synthesis pathway to collagen production. We then cultured HLFs in the absence of either serine, glycine, or both serine and glycine and treated them with TGF-β before we assessed the type I collagen expression. Removal of exogenous serine or glycine did not affect collagen production suggesting that de novo glycine synthesis is sufficient for the production of collagen (Fig. 5A). In other words, de novo glycine synthesis is sufficient to provide the glycine necessary for collagen production.

To investigate whether the de novo serine synthesis pathway is required for TGF-β-induced collagen production, we generated HLFs with knockdown of PHGDH or SHMT2 using siRNA (Fig. 5, B and C). Compared with control siRNA-treated cells, HLFs with knockdown of PHGDH or SHMT2 had attenuation of TGF-β-induced collagen production when the HLFs were cultured in media with serine and glycine (Fig. 5, B and C). When the HLFs with knockdown of PHGDH or SHMT2 were treated with TGF-β, we observed a further reduction in collagen production, indicating that the de novo serine synthesis pathway was responsible for the increased synthesis of collagen. These results suggest that de novo synthesis of glycine is required for TGF-β-induced collagen production.

**FIGURE 2. Elevated glycolytic rates are required for collagen protein production without an effect on transcriptional responses.** HLFs were plated on an XF24 culture plate and treated with 2-deoxyglucose (A), sodium oxamate (B), or cultured in the presence of galactose (C) for 1 h before glycolysis stress test. HLFs were treated with TGF-β for the indicated intervals in the presence or absence of 2-deoxyglucose (D), sodium oxamate (E), or galactose (F) before cellular lysates were resolved by SDS-PAGE and membranes were blotted for type I collagen, α-SMA, or α-Tubulin. G, we generated HLFs with knockdown of LDHA using RNAi technology. We treated HLFs with LDHA knockdown and control siRNA-treated cells with TGF-β for the indicated intervals. Cellular lysates were resolved by SDS-PAGE and membranes were blotted for LDHA, type I collagen, α-SMA, or α-Tubulin. HLFs were pretreated with 2-deoxyglucose (H) or sodium oxamate or cultured in the presence of galactose or glucose (I) followed by treatment with TGF-β for the indicated intervals. Cellular lysates were resolved by SDS-PAGE and membranes were blotted for SMAD3 or SMAD3 phosphorylated at Ser-423/425. J, HLFs were treated with TGF-β for 24 h in the presence or absence of 2-deoxyglucose. mRNA was isolated and expression of SMAD target genes were analyzed by qRT-PCR. Col1A1, collagen type 1 α1; CTGF, connective tissue growth factor; PAI-1, plasminogen activator inhibitor-1. Expression was normalized to RPL19 levels. Scatter dot plot shows mean ± S.D. (n = 3, biologic replicate). We subjected the data to one-way ANOVA. When ANOVA indicated a significant difference, individual differences with two-tailed Student’s t test using Bonferroni’s correction for multiple comparisons. * p < 0.05. Immunoblot and Seahorse data are representative of 3 or more independent experiments.
cultured in media lacking serine and glycine, TGF-β production was completely abolished (Fig. 5, D and E).

To determine how pharmacologic inhibition of the de novo serine synthesis pathway affects collagen production, we treated HLFs with TGF-β in the presence or absence of a recently identified small molecule inhibitor of PHGDH (CBR-5884) (20). Treatment of HLFs with CBR-5884 attenuated TGF-β-induced collagen production (Fig. 6).

Taken together, these results support the importance of the de novo serine synthesis pathway in collagen production. To confirm that glucose-derived carbon is incorporated into collagen, we cultured HLFs with media that has either [12C]glucose or uniformly labeled [13C]glucose and treated the cells with TGF-β. Twenty-four hours after TGF-β treatment, lysates were resolved by electrophoresis and bands corresponding to collagen were isolated for analysis by liquid chromatography tandem mass spectrometry. As seen in Fig. 7, collagen peptides from cells cultured in [13C]glucose showed increased peptide mass, consistent with incorporation of carbon from glucose into collagen. Collectively, these results suggest that de novo synthesis of glycine is sufficient and required for collagen production in HLFs.

FIGURE 3. De novo serine synthesis pathway is up-regulated by TGF-β. A, schematic showing the de novo serine synthesis pathway. The glycolytic intermediate 3-phosphoglycerate is converted to serine in a three-step enzymatic reaction including the enzymes in the order: PHGDH, PSAT1, and PSPH. Serine then fuels glycine synthesis via two SHMT genes; SHMT1, encoding the cytoplasmic isozyme (SHMT1), and SHMT2, encoding the mitochondrial isoenzyme (SHMT2). HLFs were exposed to TGF-β for 24 h. B, mRNA was isolated and expression of the enzymes of the de novo serine and glycine synthesis were analyzed by qRT-PCR. Expression was normalized to RPL19 levels. Scatter dot plot shows mean ± S.D. (n = 3, biologic replicate). Significance was determined by two-tailed Student’s t test. *, p < 0.05. C, cellular lysates were resolved by SDS-PAGE and membranes were blotted for PHGDH, SHMT2 and SHMT1, or α-Tubulin. We treated HLFs with TGF-β and in the absence or presence of a TGF-β receptor I inhibitor, SB-431542 (10 μM), and measured (D) the expression of enzymes of the de novo serine and glycine synthesis by qRT-PCR, as well as (E) the protein expression of PHGDH and SHMT2. Gene expression was normalized to RPL19 levels. Scatter dot plot shows mean ± S.D. (n = 3, biologic replicate). We subjected the data to one-way ANOVA. When ANOVA indicated a significant difference, individual differences between groups were determined with two-tailed Student’s t test using Bonferroni’s correction for multiple comparisons. *, p < 0.05. Immunoblot data are representative of 3 or more independent experiments.
The conversion of serine to glycine by SHMT2 is an important source of 1-carbon units for the tetrahydrofolate (THF) cycle (21). Exogenous formate can provide 1-carbon units directly to the THF cycle and can rescue cellular growth defects caused by loss of serine homeostasis (16, 22–24). To determine whether the effect of SHMT2 inhibition on collagen production was due to loss of mitochondrial 1-carbon units, we cultured HLFs with SHMT2 knockdown in the presence or absence of exogenous formate both in the presence (Fig. 8A) and absence (Fig. 8B) of exogenous serine and glycine, and treated with TGF-β. Addition of formate failed to reverse the inhibition of collagen expression suggesting that it was not the loss of mitochondrial 1-carbon units that was responsible for the reduced collagen production we observed in SHMT2 knockdown cells (Fig. 8).

Although we found reduced expression levels of the cytosolic SHMT1 in HLFs after TGF-β stimulation (Fig. 3, B and C), we sought to determine whether SHMT1 might still be required for collagen production. Knockdown of SHMT1 did not prevent induction of collagen synthesis downstream of TGF-β either in the presence or absence of exogenous serine and glycine (Fig. 9, A and B). This result is consistent with recent reports, which demonstrate that under most circumstances, SHMT1 catalyzes production of serine from glycine (16, 22). Only when PHGDH or the mitochondrial THF cycle is inhibited does SHMT1 switch flux direction to produce glycine.

De Novo Serine Synthesis Pathway Is Up-regulated during Fibrosis in Vivo

To determine whether the expression of the de novo serine synthesis pathway enzymes is up-regulated during fibrosis in vivo, we performed Western blotting and immunohistochemistry staining for PHGDH and SHMT2 in lung tissues obtained from patients with IPF during lung transplantation and from healthy donors through an organ procurement organization. The expression of both PHGDH and SHMT2 was increased in lung homogenates from four patients with IPF compared with expression in four healthy donors (Fig. 10, A and B). Fibroblastic foci, which are dense collections of myofibroblasts and scar tissue, the main pathological feature of IPF, also demonstrated increased expression of both PHGDH and SHMT2 in fibroblasts (Fig. 10C). These results suggest the importance of the de novo serine synthesis pathway in fibrosis in vivo.

Discussion

Changes in cellular phenotype are accompanied by changes in cellular metabolite usage. Metabolic reprogramming has been studied extensively in cancer biology, and multiple therapeutics are currently in development in an attempt to target the metabolic dependences of cancer cells. The metabolic changes associated with other diseases, including fibrotic diseases have been less well studied.

Tissue injury is followed either by appropriate wound healing or fibrosis characterized by excessive collagen deposition. For example, persistent lung injury causes fibrosis that is thought to result from an abnormal healing process that leads to increased deposition of extracellular matrix, which then causes progressive impairment of gas exchange due to replacement of alveoli with fibrotic tissue (3, 4). In pulmonary fibrosis, fibroblasts differentiate into myofibroblasts, which are the primary cells that secrete collagen into the extracellular space (7). TGF-β1, the prototypical cytokine, which regulates the transcription of profibrotic genes is required and sufficient for the development of pulmonary fibrosis (8–12). However, the mechanisms that result in excess collagen deposition are not completely understood.

The amino acid composition of collagen is unique compared with other proteins because glycine, a non-essential amino acid, makes up approximately one-third of the amino acids found in collagen (14). De novo synthesis of glycine is mediated via the de novo serine synthesis pathway, which includes the following enzymes in order: PHGDH, PSAT1, and PSPH to synthesize serine, which is then converted to glycine via SHMT1s (15–17). Our study shows that this pathway is up-regulated in fibroblasts in response to TGF-β in vitro and in lung tissues from humans with IPF in vivo. Specifically, we demonstrated that TGF-β increases the gene and protein expression of PHGDH, the key regulating enzyme in de novo serine synthesis, and SHMT2, the enzyme that converts serine into glycine in the mitochondria. Complete inhibition of collagen protein expression when serine and glycine are removed from the media in HLFs with PHGDH or SHMT2 knocked down suggest that inhibition of the de novo serine synthesis pathway may be more effective when combined with a serine/glycine-deficient diet (25).
In addition, we demonstrated that in response to TGF-β/H9252, lung fibroblasts up-regulate glycolysis, which produces the glycolytic intermediate metabolite that is the substrate for PHGDH. These results are consistent with two recent studies that show a TGF-β/H9252-induced metabolic reprogramming consistent with increased glycolysis in fibroblasts (26, 27). Interestingly, we found that although knockdown of SHMT2 inhibited collagen production in HLFs, knockdown of SHMT1 had no effect on collagen accumulation downstream of TGF-β. Although seemingly paradoxical that the mitochondrial, and not the cytosolic enzyme, would be required for production of collagen protein, recent studies have demonstrated that under most circumstances SHMT1 consumes glycine, producing serine (16, 22). The cytosolic pathway may be activated with inhibition of the mitochondrial pathway due to depletion of 10-formyl-tetrahydrofolate as a result of reduced production of formate, which is excreted from the mitochondria. To determine whether a similar mechanism may be responsible for the reduced collagen production in SHMT2 knockdowns, we added formate when we cultured HLFs with SHMT2 knockdown. Because the formate failed to reverse the effects of SHMT2 knockdown, our results suggest that the effect of SHMT2 is upstream of formate formation and provide further support for de novo glycine production being the main mechanism for the observed effect. Our results that show a down-regulation of SHMT1 expression also correlates with findings from a recent report showing that up-regulation of the de novo serine synthesis leads to inhibition of SHMT1 activity (16).

Last, our proteomics data provided clear evidence and support for glucose-derived glycine being incorporated into collagen. Collectively, our results suggest that de novo synthesis of glycine from glucose through the de novo serine synthesis pathway is required for collagen synthesis. This pathway may be a therapeutic target for treatment of organ fibrosis including IPF.

**Experimental Procedures**

**Primary Culture of Human Lung Fibroblasts**

Normal human lung fibroblasts purchased from Lonza (catalog number CC-2512) and obtained from explanted lungs technology. Cells were then exposed to TGF-β for the indicated time intervals in the presence (B and C) or absence (D and E) of extracellular serine and glycine as indicated. Cellular lysates were resolved by SDS-PAGE and membranes were blotted for type I collagen, α-SMA, or α-Tubulin. We generated HLFs with PHGDH (B and D) or SHMT2 (C and E) knockdowns using siRNA technology.
from patients undergoing lung transplantation were used for our studies. For experiments, cells were grown in DMEM (Corning, catalog number 10-013-CV) supplemented with 2 mM glutamine (Gibco, catalog number 25030081), 10% FBS (Gemini, catalog number 100-106), 1% penicillin-streptomycin (Gemini, catalog number 400-109) on 12-well plates at a density of \(1 \times 10^5\) cells/well for 24 h. Cells were serum starved in DMEM containing 0.1% bovine serum albumin (BSA, Dot Scientific, catalog number 9048-46-86) and 2 mM glutamine for 24 h prior to treatment with TGF-\(\beta\) (1 ng/ml; Millipore-Calbiochem, catalog number 16450). For galactose substitution experiments, glucose-free DMEM (Gibco, catalog number 11966) was supplemented with either 25 mM D-glucose (Fisher, catalog number BP350) or 25 mM D-galactose (Fisher, catalog number BP656). For serine- or glycine-free medium, minimal essential medium (MEM) (Corning, catalog number 11090-081) was used and supplemented with 4 mM glutamine (Gibco, catalog number 25030), 2 mM sodium pyruvate (Gibco, catalog number 11360-070), d-glucose (Fisher, catalog number BP350; final concentration 25 mM), and MEM vitamins (Gibco, catalog number 11120). MEM lacking glycine was supplemented with 0.4 mM L-serine (MP, catalog number 194737) and MEM lacking serine was supplemented with 0.4 mM glycine (MP, catalog number 194681). 2-Deoxy-D-glucose (catalog number 154-17-6), sodium oxamate (catalog number O2751), sodium formate (catalog number 141-53-7), and SB-431542 (catalog number S4317) were purchased from Sigma. All primary cultures were used from passage 3 to 5. The inhibitor used for pharmacological inhibition of the PHGDH enzyme was CBR 5884 (Tocris, catalog number: 5836, lot number 1A/182684).

**Cell Lysis and Western Blotting**

Cells were scraped into buffer containing 25 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate, 2 mM EDTA, 2 mM EGTA, 10% glycerol, 1 mM NaF, 200 mM sodium orthovanadate, and protease and phosphatase inhibitors mixture (Thermo-Scientific, catalog number 1861284), sonicated for 10 s on a Fisher Scientific 100 model at speed setting 2. Samples were resolved on 4–15% Criterion gels (Bio-Rad, catalog number 5671083) and transferred to nitrocellulose (Bio-Rad, catalog number 1620167). Primary antibodies used were rabbit Collagen 1 (Cedarlane, catalog number CL50111AP-1, lot number 323-N; 1:3000), mouse anti-\(\alpha\)-smooth muscle monoclonal antibody (Sigma, catalog number A2547: 1:1,000), mouse anti-\(\alpha\)-tubulin monoclonal antibody (Sigma, catalog number T6074, lot number 034K488; 1:20,000), mouse anti-\(\beta\)-actin monoclonal antibody (Sigma, catalog number A5441; lot number 037K488; 1:20,000), rabbit LDHA (Cell Signaling, catalog number 2238S), rabbit PHGDH (Cell Signaling, catalog number 13428, lot number 1; 1:1,000), rabbit SHMT1 (Cell Signaling, catalog number 12612, lot number 1; 1:1,000), rabbit SHMT2 (Cell Signaling, catalog number 12762, lot number 1; 1:1,000), rabbit pSMAD3 monoclonal antibody (Cell Signaling, catalog number 9520, lot number 13; 1:1,000), rabbit SMAD3 monoclonal anti-

**FIGURE 7. Glucose-derived glycine is incorporated into collagen.** HLFs were exposed to TGF-\(\beta\) in the presence of either \(^{12}\)C-glucose or \(^{13}\)C-glucose. After 24 h, cellular lysates were resolved by SDS-PAGE and bands corresponding to collagen 1 were excised. Protein was digested with trypsin and the resulting peptides were analyzed by LC-MS/MS.
De Novo Serine Synthesis and Collagen

The following primer sequences were used: 5'-AGATGCTCAG-GTTCAGAGA-3', 5'-CATGGTCTCATGGGTCTTAC-3', CTGF, 5'-GGTCAACGAGTGGAG-3', 5'-AGGAGGCTTGCATTTT-3'; LDHA 5'-CGATTCGGAGATCTTGGCC-3', 5'-TGGAAACAAAGGATC-3', PHGDH 5'-GGGAGGATCTCCTTCT-3', 5'-GTCATTGGAGAGGGTCTG-3', PSPH 5'-GGGTTCTCCAACAT-3', 5'-TTCCCCAGGAGGTGAGC-3', PSAT1 5'-GGGAGACCTCAGAGAGTT-3', 5'-ATGCTTCCACAGACAG-3', SHMT1 5'-GATGACCCACACCACCTCA-3', 5'-ACAGCAACCCCCCTTTCTGTGA-3', SHMT2 5'-GCTGCTCTAGACACGAGATTCG-3', 5'-GCGAGGAGGCGC-3', COL1A1 5'-GGTCAATGGCCTCCCAGCT-3', 5'-GGCACCATCTACCTTTCTTT-3', PAI-1 5'-GGGTTCTCCAACAGAAGT-3', 5'-GCGAGGCTGACTTCACGAGGT-3', GLUT1 (CTGGCAATACGTCGCTTTCTG-3'), and GLT1 (CTGGCAATACGTCGCTTTCTG-3').

Quantitative PCR

RNA as isolated form cells using an Aurum Total RNA Mini Kit (Bio-Rad, catalog number 732-6820) and reverse transcribed using iScript Reverse Transcription Supermix (Bio-Rad, catalog number 1708841). Quantitative mRNA expression was determined by real-time RT-PCR using iTaq Universal SYBR Green Supermix (Bio-Rad, catalog number 172-5121). The following primer sequences were used: α-SMA (5'-GGCGGTGCTTCTCTCCTAT-3', 5'-CCAGATCCAGCCATA-3'), RPL19 (control), (5'-AGATGCTCAG-GTTCAGAGA-3', 5'-CATGGTCTCATGGGTCTTAC-3', CTGF, 5'-GGTCAACGAGTGGAG-3', 5'-AGGAGGCTTGCATTTT-3'; LDHA 5'-CGATTCGGAGATCTTGGCC-3', 5'-TGGAAACAAAGGATC-3', PHGDH 5'-GGGAGGATCTCCTTCT-3', 5'-GTCATTGGAGAGGGTCTG-3', PSPH 5'-GGGTTCTCCAACAT-3', 5'-TTCCCCAGGAGGTGAGC-3', PSAT1 5'-GGGAGACCTCAGAGAGTT-3', 5'-ATGCTTCCACAGACAG-3', SHMT1 5'-GATGACCCACACCACCTCA-3', 5'-ACAGCAACCCCCCTTTCTGTGA-3', SHMT2 5'-GCTGCTCTAGACACGAGATTCG-3', 5'-GCGAGGAGGCGC-3', COL1A1 5'-GGTCAATGGCCTCCCAGCT-3', 5'-GGCACCATCTACCTTTCTTT-3', PAI-1 5'-GGGTTCTCCAACAGAAGT-3', 5'-GCGAGGCTGACTTCACGAGGT-3', GLUT1 (CTGGCAATACGTCGCTTTCTG-3'), and GLT1 (CTGGCAATACGTCGCTTTCTG-3').

Glycolysis Stress Test and Basal Oxygen Consumption

Glycolytic rates were measured using the XF24 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA). HLFs were seeded at a density of 3 x 10^6 cells on a Seahorse plate coated with Cell-Tak (BD Biosciences, catalog number 354240). Cells were equilibrated with XF Base media (Seahorse, catalog number 102353-100) at 37 °C for 1 h in an incubator lacking CO₂. Glycolysis was measured using Seahorse XF Glycolysis Stress Test Kit (Seahorse, catalog number 103020-100) according to the manufacturer’s protocol followed by sequential treatments with glucose (10 mM), oligomycin (1.0 μM), and 2-DG (100 mM). For inhibitor experiments, drugs were added during the 1-h equilibration period.

HLFs were plated at a density of 3 x 10^4 and glycolysis rates were measured following a 1-h pretreatment with 25 mM sodium oxamate or 5 mM 2-DG by sequential treatments with glucose (25 mM), oligomycin (1.0 μM), and 2-DG (100 mM). Glycolysis stress test measurements were repeated in at least three independent experiments.

siRNA Knockdowns

Primary HLFs (1 x 10^6 cells) were transfected with an siRNA (250 pmol) using a Nucleofector 2b device (Lonza) and protocol A024 as described by the manufacturer. Cells were transferred to a 10-cm tissue culture dish and siRNA containing media was removed 1 day later. siRNA products are obtained from Dharmacon and the siRNAs used are as follows: non-targeting siRNA, target sequence: UGGUUUAACUGUCATACCATGATG-3', PHGDH siRNA1, target sequence: CGAAUUGCGUUGU-AAGACUAU (Dharmacon catalog number J-008201-06-0002); PSAT1 siRNA1, target sequence: UGGUUUACAUGUCATTGC-3', 5'-GGGAGACCTCAGAGAGTT-3', 5'-ATGCTTCCACAGACAG-3', SHMT1 5'-GATGACCCACACCACCTCA-3', 5'-ACAGCAACCCCCCTTTCTGTGA-3', SHMT2 5'-GCTGCTCTAGACACGAGATTCG-3', 5'-GCGAGGAGGCGC-3', COL1A1 5'-GGTCAATGGCCTCCCAGCT-3', 5'-GGCACCATCTACCTTTCTTT-3', PAI-1 5'-GGGTTCTCCAACAGAAGT-3', 5'-GCGAGGCTGACTTCACGAGGT-3', GLUT1 (CTGGCAATACGTCGCTTTCTG-3'), and GLT1 (CTGGCAATACGTCGCTTTCTG-3').
CAGUUC (Dharmacon, catalog number J-009518-05-0002); PHGDH siRNA2, target sequence: GACCCUUGCUGCCGAAGA (Dharmacon, catalog number J-009518-07-0002); SHMT1 siRNA1, target sequence: CCUAGGCUCUUGC-UUAAAU (Dharmacon, catalog number J-004617-06-0002); SHMT1 siRNA2, target sequence: GAGCUGGCAUGAUC-UUCUA (Dharmacon, catalog number J-004906-05-02); SHMT2 siRNA1, target sequence: GGAGAUCCCUUAC-ACAUUU (Dharmacon, catalog number J-004906-06-02); SHMT2 siRNA2, target sequence: ACAAGUACUCG-GAGGGUUA (Dharmacon, catalog number J-004906-05-02); SMAD3 siRNA1, target sequence: CAACAGGAAUGCAGCAGUG (Dharmacon, catalog number J-020067-05-0002); SMAD3 siRNA2, target sequence: GAGUUCGCCUUCAAUAGA (Dharmacon, catalog number J-020067-06-0002).

**Metabolic Labeling and Mass Spectrometry**

Labeling and Peptide Preparation—HLFs were serum starved in glucose-free medium supplemented with glucose or [13C]glucose (Cambridge Isotope Laboratories, δ-glucose U-13C6, 99%, catalog number CLM-1396, lot number PR-25681). Twenty-four hours after TGF-β treatment, cellular lysates were resolved on 4–15% gels. The gel was stained with Imperial Stain (Pierce) at room temperature, and destained overnight in dH2O at 4 °C. For each lane, gel sections corresponding to collagen protein were excised by a sterile razor blade and sent for proteomic analysis at the Taplin Mass Spectrometry Facility, Harvard University. Gel pieces were washed with acetonitrile for 10 min, then dried in a SpeedVac. Pieces were rehydrated in 50 mM ammonium bicarbonate solution containing 12.5 ng/μl of sequencing grade trypsin (Promega; 45 min, 4 °C). Trypsin solution was replaced with 50 mM ammonium bicarbonate solution and samples were incubated at 37 °C overnight. Peptides were extracted by removing the ammonium bicarbonate solution, followed by one wash with a solution containing 50% acetonitrile and 1% formic acid. The extracts were then dried in a SpeedVac.

**LC-MS/MS**—Samples were reconstituted in 5–10 μl of HPLC solvent A (2.5% acetonitrile, 0.1% formic acid). A nanoscale reverse-phase HPLC capillary column was created by packing 2.6-μm C18 spherical silica beads into a fused silica capillary (100 μm inner diameter × ~30 cm length) with a flame-drawn tip. Each sample was loaded via a Famos auto sampler (LC Packings). A gradient was formed and peptides were eluted with increasing concentrations of solvent B (97.5% acetonitrile, 0.1% formic acid). As peptides eluted were subjected to electrospray ionization and then entered into an LTQ Orbitrap Velos Pro ion-trap mass spectrometer (ThermoFisher Scientific). Peptides were detected, isolated, and fragmented to produce a tandem mass spectrum of specific fragment ions for each peptide. Peptide sequences (and hence protein identity) were determined by matching protein databases with the acquired fragmentation pattern by the software program.
Sequest (Thermo Fisher Scientific). Peptides matching to Collagen/1(I) chain protein (Uniprot P02452) were assessed for their metabolic incorporation from heavy glucose samples (13C-isotope incorporation). Peptides were manually validated (MS2) and their MS peak isotopic envelope was examined for heavy 13C incorporation, showing up as more isotopologue peaks.

**Human Samples**

The collection and use of human lung specimens were approved by the University of Chicago Institutional Review Board. Lung tissues from 4 patients with IPF who underwent lung transplantation and lung tissues from 4 healthy donors were used for immunohistochemistry and Western blotting for PHGH and SHMT2.

**Histological Analysis**

After deparaffinization and rehydration, tissue sections were treated with antigen retrieval buffer (S1699, DAKO) in a steamer for 20 min. Rabbit polyclonal anti-PHGDH antibody (Proteintech, catalog number 14719-1-AP; 1:1000) or rabbit polyclonal anti-SHMT2 antibody (Proteintech, catalog number 10099-1-AP; 1:800) was applied on tissue sections for a 1-h incubation at room temperature in a humidity chamber. Following a TBS wash, tissue sections were incubated with biotinylated anti-rabbit IgG (1:200, BA-1000, Vector Laboratories) for 30 min at room temperature. The antigen-antibody binding was detected by Elite kit (PK-6100, Vector Laboratories) and DAB (DAKO, K3468) system. Tissue sections were briefly immersed in hematoxylin for counterstaining and were covered with cover glasses.

**Statistical Analysis**

The data were analyzed in Prism 4 (GraphPad Software Inc., La Jolla, CA). All data are shown as mean ± S.D. We subjected all data greater than two groups to one-way ANOVA. When ANOVA indicated a significant difference, we explored individual differences with two-tailed Student’s t test using Bonferroni’s correction for multiple comparisons. Statistical significance was defined as p < 0.05.
Author Contributions—R. B. H. and G. M. M. conceived the project, designed the experiments, interpreted the results, and wrote the manuscript; R. N., A. Y. M., E. O., L. J. W., K. S., D. Wu, D. Wo., T. C., P. S. W., and R. B. H. performed the experiments, data acquisition, and analysis; A. N. H. performed lung histological and pathological analyses and interpretation; C. B. provided lung tissue samples and assisted with histopathological analysis; N. O. D. and N. S. C. contributed reagents, interpreted results, and assisted with manuscript writing and editing.

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