THE
EMBO
JOURNAL

Proline biosynthesis is a vent for TGFβ-induced mitochondrial redox stress

Simon Schwörer, Mirela Berisa, Sara Violante, Weige Qin, Jiajun Zhu, Ronald C. Hendrickson, Justin R. Cross, and Craig B. Thompson

Review timeline:

Submission date:	28th Aug 2019
Editorial Decision:	27th Sep 2019
Revision received:	8th Jan 2020
Editorial Decision:	26th Jan 2020
Revision received:	1st Feb 2020
Accepted:	4th Feb 2020

Editor: Daniel Klimmeck

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 27th Sep 2019

Thank you for the submission of your manuscript (EMBOJ-2019-103334) to The EMBO Journal. Also, it was helpful to discuss your study and the related context earlier this week. Your manuscript has been sent to three reviewers, and we have in the meantime received reports from all of them, which I enclose below.

As you will see, the referees acknowledge the potential interest and novelty of your results, although they also express a number of issues that will have to be addressed before they can support publication of your manuscript in The EMBO Journal. In more detail, referee #3 asks you to clarify kinetics of TGFbeta induced events, and strengthen the TGFbeta driven glucose flux into the TCA (ref#3, pts.1,2); this referee also states that cross contributions of glucose glutamine to proline/glycine as well as HIF involvement in proline production should be evaluated to substantiate the concept proposed (ref#3, pts.3,4). Both referees #1 and #2 point out that causality between mitochondrial ROS and proline synthesis needs to be corroborated. Referee #2 also asks you reanalyse pentose phosphate pathway and citrate synthesis as potential recipients of TGFbeta-induced glucose (ref#2, pts.1,2). In addition, the reviewers raise a number of issues related to presentation and appropriate discussion of the results as well as literature citations that would need to be conclusively addressed to achieve the level of robustness and clarity needed for The EMBO Journal.

I judge the comments of the referees to be generally reasonable and given their overall interest, we are happy to invite you to revise your manuscript experimentally to address the referees' comments.

REFEREE REPORTS:

Referee #1:

In this interesting, well presented paper that opens avenues for new clinical approaches to fibrosis, Thompson and

colleagues show that TGFb increases collagen synthesis in part by upregulating mitochondrial proline production, via a mechanism that includes enhancing mitochondrial ROS.
Overall, I feel the paper is a fit for EMBO without substantial additional experiments. I do have a few points for the authors to consider:
1. What is the evidence that proline biosynthesis protects fibroblasts from TGFb induced redox stress? What I found more interesting is that proline synthesis seems to be critical for collagen production and induced by a nutrient/ROS "push" mechanism. But I did not actually see a figure that nailed decreased ROS (and increased survival of exogenous ROS, e.g. H2O2, paraquat) with overexpression of proline synthesis (or the opposite with P5CS KO). This does not decrease the importance of the paper, but might argue for a different title and final sentence of the abstract.
2. What is the evidence that the PYCR reaction preferentially uses NADH? Does this depend on the compartment? I had looked at this previously and couldn't figure it out.
3. The interpretation of the Slc25a1 KO should clearly indicate that it is a citrate-malate exchanger and that this explains the differential effect on the left and right hand sides of the TCA cycle.
4. The lbNOX experiment is wonderful but the interpretation should be properly cautious, given the direct (ROS-independent) need for NADH to drive proline synthesis and the fact that the effect is seen both in the basal and the activated state.
5. If I were to recommend one additional experiment (optional), it would be metabolomics in the lbNOX conditions. This might illuminate better the mechanism and why both the cytosolic and mitochondrial lbNOX surprisingly have the same effect.
6. The mention of plants in the discussion is appropriate, but it seems that a small but meaningful literature on the role of proline in redox shuttling and other defense processes in mammals is not well covered. Also, the introduction could perhaps cover a greater diversity of labs, and much of the discussion up to the plant part is a straight rehashing of the results, which could probably be made more interesting or concise.
7. Can the authors better explain the results in figure 4f/g in terms of the specific respiratory chain complexes producing ROS and how this relates to proline? I personally found the patterns confusing. If the reasons for differential effects of the different respiratory chain inhibitors are not clear, mention this.

Referee #2:

The authors explore the mechanism by which TGF-beta couples increased uptake of glucose and glutamine to the increased production of collagen-rich matrix proteins. Using glutamine tracing, they show that TGF-beta diverts glutamine carbons towards proline biosynthesis which is then incorporated into collagens. TGF-beta up-regulates genes involved in proline biosynthesis (in a Smad4-dependent manner), most notably P5CS, whose expression level is correlated to excessive collagen deposition in fibrotic diseases. The authors demonstrate that upon TGFβ stimulation, mitochondrial ROS arises as a consequence of excessive reducing equivalent availability from increased glutamine-driven TCA cycle activities. The authors propose that proline biosynthesis dissipates excessive reducing equivalents to avoid accumulation of mitochondrial ROS, acting as a safety valve for redox stress. The authors show that increasing mitochondrial carbon load simply by blocking citrate export leads to excessive mitochondrial oxidation and further boosts the proline biosynthetic pathway. The manuscript is well written, the concept seems fresh, and also the paper is full of clever application of new genetic tools for redox biology and metabolism.

Comments/Suggestions:

1) Given the importance of the pentose phosphate pathway in ROS defense and reductive synthesis (via the supply of cytosolic NADPH), it would be important to see if TGF-beta diverted glucose carbon into PPP. This can be achieved by further analyzing the [U-13C] glucose tracer data the authors already have.

2) Similarly, do the authors observe elevated reductive biosynthesis of citrate or aspartate, which is expected? Again this data should be in their [U-13C] glutamine tracer data and it would be nice for them to report.

3) What is unclear to this reviewer if mitochondrial ROS by itself underlies elevated proline biosynthesis, as being proposed by this manuscript. Mitochondrial ROS does not appear sufficient, as proline biosynthesis is not up-regulated under mitochondrial inhibitors (Metformin, Rotenone, Myxo, Anti), which are known drivers of mitochondrial ROS.

4) For Figure 2d, it would be nice to show the pool size on top of the % labeled (either in the main figure, or in the supplemental figure), such that the readers can directly compare it to Figure 2e, which show the total pool (normalized to mock) for each metabolite. It would provide a more complete picture for the extent of metabolic up-regulation by TGFβ.

5) Legend for Figure 2d says ""Metabolic steady state was reached at this time point (data not shown)". Please show the data in the supplemental figure, or remove this sentence.

6) Most readers will not know what the citrate transporter inhibitor is, please elaborate with a reference and description.

Referee #3:

In this manuscript, the authors link the stimulation of transforming growth factor beta-1 (TGFβ) to an increase in proline biosynthesis via carbon metabolic switching and ROS production in fibroblasts. The metabolic conversion of glucose or amino acids through TGFβ signaling has been the focus of many previous studies. The authors however highlighted a novel aspect of proline biosynthesis regulation that depends on TGFβ-mediated mitochondrial redox stress. Using metabolic tracing experiment, they found that glutamine, but not glucose, was preferentially used to support proline biosynthesis rather than catabolism through the TCA cycle. Interestingly, this metabolic phenotype was correlated with the increase of mitochondrial ROS stress at the early stage upon TGFβ stimulation. Furthermore, Smad4 is identified to modulate TGFβ-induced proline biosynthesis in mitochondria.

Although some aspects of the mechanism are already known, this study still presents a novel mechanistic aspect by which TGFβ facilitates proline production through increased glutaminolysis to avoid excessive redox stress, that should attract broad interest in the field. However, there are some concerns that need to be addressed to strengthen the conclusion.

1. The promoting effects of TGFβ activation on translation rate, nutrients consumption and respiration in fibroblasts are clear in Figure 1. In those experiments, TGFβ treatment was used for 48 hours. Since it is generally accepted that TGFβ signaling plays a dual role in many biological processes, a series of time-course experiments are important here to clarify the above phenomena at either early or late time point in response to TGFβ treatment. Moreover, the authors should also indicate what concentration of TGFβ that they used in those experiments via description in figure legends.

2. In Figure 1i, the authors showed that TGFβ potently stimulated mitochondrial oxygen consumption by measuring oxygen consumption rate (OCR). However, Nigdelioglu et al. in their paper demonstrated that TGFβ enhanced glycolytic metabolism by measuring increased extracellular acidification rate (ECAR) level in fibroblasts (PMID: 27836973). To support the authors' conclusion that TGFβ facilitates glucose to the downstream TCA cycle and mitochondrial oxidation rather than secreted as lactate, they need to further test ECAR levels and compare ECAR value with OCR value to determine which is the dominate pathway - mitochondrial oxidative phosphorylation or non-mitochondrial metabolism (glycolysis)?

3. Figure 2 showed that either glucose-derived glycine or glutamine-derived proline was shown to be incorporated into collagen I. However, carbons from glucose or glutamine could contribute to both proline and glycine. The authors should use metabolic tracing experiments to show whether glucose and glutamine will also increase the fraction of proline and glycine, respectively.

4. In Figure 4, the authors showed that TGFβ stimulation will cause mitochondrial ROS generation and HIF-1α stabilization at the same time. By using the inhibitors of ETC or mitochondrial antioxidant, they demonstrated that proline synthesis was caused by TGFβ-induced mitochondrial ROS stress. However, it is well known that ROS will also stabilize HIF-1 protein in either hypoxic or non-hypoxic condition. Thus, it is possible that HIF-1 is involved in TGFβ-induced proline production. The authors should clarify whether ROS alone or ROS-mediated HIF-1 is a key factor to regulate this metabolic process.

In this interesting, well presented paper that opens avenues for new clinical approaches to fibrosis, Thompson and colleagues show that TGFβ increases collagen synthesis in part by upregulating mitochondrial proline production, via a mechanism that includes enhancing mitochondrial ROS.
Overall, I feel the paper is a fit for EMBO without substantial additional experiments. I do have a few points for the authors to consider:

1. What is the evidence that proline biosynthesis protects fibroblasts from TGFβ induced redox stress? What I found more interesting is that proline synthesis seems to be critical for collagen production and induced by a nutrient/ROS "push" mechanism. But I did not actually see a figure that nailed decreased ROS (and increased survival of exogenous ROS, e.g. H2O2, paraquat) with overexpression of proline synthesis (or the opposite with P5CS KO). This does not decrease the importance of the paper, but might argue for a different title and final sentence of the abstract.

We thank the reviewer for this excellent suggestion. To address it, we compared ROS levels after 48h stimulation with TGFβ in control vs. P5CS-ko fibroblasts in the presence of proline in the culture medium to avoid effects of proline loss on protein translation and growth that were reported recently. As observed before, TGFβ-induced ROS have returned to baseline at 48h in wildtype cells. However, P5CS-ko fibroblasts continue to display increased ROS 48h after TGFβ treatment even when cultured in proline-containing medium (Figure 5f). These data demonstrate that activation of the proline biosynthetic pathway and not proline itself protects fibroblasts from TGFβ-induced redox stress. We have also revised the title and the abstract in response to the reviewer's suggestions.

2. What is the evidence that the PYCR reaction preferentially uses NADH? Does this depend on the compartment? I had looked at this previously and couldn't figure it out.

We apologize for not providing a reference for this before. De Ingeniis et al. (2012) showed that PYCR1 and PYCR2 preferentially use NADH and are located in the mitochondria, while PYCRL preferentially uses NADPH and is located in the cytosol. We have now included this reference in the revised manuscript.

3. The interpretation of the Slc25a1 KO should clearly indicate that it is a citrate-malate exchanger and that this explains the differential effect on the left and right hand sides of the TCA cycle.

We agree with the reviewer that a lack of malate import into the TCA cycle in Slc25a1 ko cells might explain the reduced abundance of malate and aspartate. We included this possibility in the revised manuscript and also indicated that CTP is a citrate-malate exchanger.

4. The lbNOX experiment is wonderful but the interpretation should be properly cautious, given the direct (ROS-independent) need for NADH to drive proline synthesis and the fact that the effect is seen both in the basal and the activated state.

We thank the reviewer for pointing this out. Based on our experimental data, we cannot distinguish if the reduced proline abundance in cells expressing mitoLbNOX is the result of a direct effect on proline biosynthesis, which consumes NADH, or reduced levels of mitochondrial ATP generation by oxidative phosphorylation as ATP is also required for proline biosynthesis (Figure 4g). Therefore, we now state both possibilities in the revised manuscript.

5. If I were to recommend one additional experiment (optional), it would be metabolomics in the lbNOX conditions. This might illuminate better the mechanism and why both the cytosolic and mitochondrial lbNOX surprisingly have the same effect.

We apologize for not being clear about the experiments using mitochondrial LbNOX and mitochondrial TPNOX. We did not use a cytosolic LbNOX construct. As proline biosynthesis takes place in mitochondria, we focused on the effects of mitochondrial redox potential on proline levels by using either mitochondrial LbNOX (consuming NADH) or mitochondrial TPNOX (consuming NADPH).
At the suggestion of the reviewer, we performed a metabolomic analysis by GC-MS of fibroblasts expressing these constructs. This analysis largely confirmed previously published data (Titov et al., 2016; Cracan et al., 2017). For example, succinate accumulated in cells expressing either mitoLbNOX or mitoTPNOX, while the lactate/pyruvate ratio declined. We included these data in the revised manuscript (EV Figure 5b). However, since these data have been published before, we did not include the full metabolomic profile in the revised manuscript.

6. The mention of plants in the discussion is appropriate, but it seems that a small but meaningful literature on the role of proline in redox shuttling and other defense processes in mammals is not well covered. Also, the introduction could perhaps cover a greater diversity of labs, and much of the discussion up to the

plant part is a straight rehashing of the results, which could probably be made more interesting or concise.

We followed the reviewer's suggestions and made the following changes to the text:
1. We added additional references to the introduction.
2. We reduced and revised the discussion on the role of proline in redox defense.
3. We added additional discussion on the role of proline in redox defense in mammalian cells.

7. Can the authors better explain the results in figure 4f/g in terms of the specific respiratory chain complexes producing ROS and how this relates to proline? I personally found the patterns confusing. If the reasons for differential effects of the different respiratory chain inhibitors are not clear, mention this.

We thank the reviewer for the opportunity to make this clearer. Our study shows that both an elevated mitochondrial redox state as well as intact mitochondrial electron transport and ATP synthesis are required for proline biosynthesis. This explains why proline biosynthesis is impaired upon ETC inhibition regardless of which respiratory chain complex is targeted. Recently, it has been demonstrated that TGFβ induces mitochondrial ROS at complex III (Jain et al., 2013), and we confirmed the induction of mitochondrial ROS by TGFβ. As shown in the new experiment in Figure 5f, TGFβ-induced ROS is suppressed as a result of P5CS activity and proline biosynthesis.

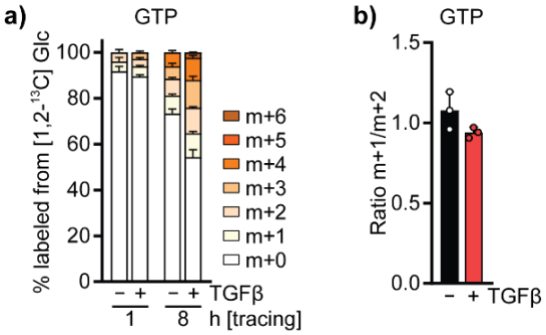
Referee #2:

The authors explore the mechanism by which TGF-beta couples increased uptake of glucose and glutamine to the increased production of collagen-rich matrix proteins. Using glutamine tracing, they show that TGF-beta diverts glutamine carbons towards proline biosynthesis which is then incorporated into collagens. TGF-beta up-regulates genes involved in proline biosynthesis (in a Smad4-dependent manner), most notably P5CS, whose expression level is correlated to excessive collagen deposition in fibrotic diseases. The authors demonstrate that upon TGFβ stimulation, mitochondrial ROS arises as a consequence of excessive reducing equivalent availability from increased glutamine-driven TCA cycle activities. The authors propose that proline biosynthesis dissipates excessive reducing equivalents to avoid accumulation of mitochondrial ROS, acting as a safety valve for redox stress. The authors show that increasing mitochondrial carbon load simply by blocking citrate export leads to excessive mitochondrial oxidation and further boosts the proline biosynthetic pathway. The manuscript is well written, the concept seems fresh, and also the paper is full of clever application of new genetic tools for redox biology and metabolism.

Comments/Suggestions:

1) Given the importance of the pentose phosphate pathway in ROS defense and reductive synthesis (via the supply of cytosolic NADPH), it would be important to see if TGF-beta diverted glucose carbon into PPP. This can be achieved by further analyzing the [U-13C] glucose tracer data the authors already have.

This was an excellent suggestion, which we addressed by performing metabolic tracing using [1,2-13C] glucose as a tracer. This tracer allows to distinguish glycolytic carbons that move directly through glycolysis (m+2 labeled) from those that are first directed through the PPP and then re-enter glycolysis (m+1 labeled). We found that PPP activity constituted only a minor proportion of the glycolytic flux in our cells and was not altered by TGFβ stimulation, as shown by the unchanged ratio of m+1/m+2 labeled lactate (EV Figure 6g).
Additionally, we found that the relative contribution of the PPP to nucleotide synthesis was larger than the backflow of PPP metabolites into glycolysis as evidenced by an increased m+1/m+2 ratio in GTP compared to lactate (1.078 +/- 0.116 vs 0.047 +/- 0.002) (Additional Figure 1). While TGFβ stimulation increases utilization of the PPP for nucleotide synthesis, it does not appear to alter the relative contribution of the oxidative vs non-oxidative PPP as indicated by an unchanged ratio of m+1/m+2 labeled GTP (Additional Figure 1). Therefore, we conclude that TGFβ does not preferentially activate the PPP for ROS defense, and that other mechanisms are used for this purpose.



Additional Figure 1: Tracing of [1,2-13C] glucose into GTP. Shown is the percentage of labeling (a) and the ratio of m+1/m+2 labeled GTP (b).

2) Similarly, do the authors observe elevated reductive biosynthesis of citrate or aspartate, which is expected? Again this data should be in their [U-13C] glutamine tracer data and it would be nice for them to report.

We are now showing all isotopomers at 8h tracing with [U-13C] glutamine (Figure 2c-h) and we included the kinetic labeling on reductive biosynthesis of citrate, malate and aspartate in the revised manuscript (EV Figure 3d). These data show that TGFβ does not alter reductive biosynthesis of citrate and aspartate from glutamine-derived alpha-ketoglutarate.

3) What is unclear to this reviewer if mitochondrial ROS by itself underlies elevated proline biosynthesis, as being proposed by this manuscript. Mitochondrial ROS does not appear sufficient, as proline biosynthesis is not up-regulated under mitochondrial inhibitors (Metformin, Rotenone, Myxo, Anti), which are known drivers of mitochondrial ROS.

We thank the reviewer for highlighting this point. Our data suggest that an excess of mitochondrial redox potential beyond which can be used for ATP production drives proline synthesis. However, under conditions of ETC inhibition, which result in accumulation of mitochondrial electrons, proline levels decline (Figure 4d), suggesting that proline biosynthesis requires both an elevated mitochondrial redox potential well as an intact mitochondrial electron transport. Thus, mitochondrial redox potential elevation is not sufficient to activate proline biosynthesis. Mitochondrial electron transport also generates an electrochemical gradient that drives ATP synthesis, and we found that uncoupling this gradient from ATP production, as well as direct inhibition of mitochondrial ATP synthesis, impairs proline biosynthesis (Figure 4f, g). Proline biosynthesis requires mitochondrial NAD(P)H and ATP (Figure 3a), and this potentially

explains why both mitochondrial redox potential as well as an intact electron transport are critical for fibroblasts to synthesize proline.

4) For Figure 2d, it would be nice to show the pool size on top of the % labeled (either in the main figure, or in the supplemental figure), such that the readers can directly compare it to Figure 2e, which show the total pool (normalized to mock) for each metabolite. It would provide a more complete picture for the extent of metabolic up-regulation by TGFβ.

We agree with the reviewer that showing the pool size will provide a more complete picture and will be helpful for future readers of our study. In the revised manuscript, we thus show both the % labeled and the pool size for each isotopomer in each metabolite we analyzed (Figure 2c-h).

5) Legend for Figure 2d says "Metabolic steady state was reached at this time point (data not shown)". Please show the data in the supplemental figure, or remove this sentence.

We followed the reviewer's suggestion and now included the kinetic labeling for the relevant isotopomers derived from glutamine in the supplement (EV Figure 3b-d), which shows that for proline, metabolic steady state was reached after 8 hours of tracing with [U-13C] glutamine.

6) Most readers will not know what the citrate transporter inhibitor is, please elaborate with a reference and description.

In the revised manuscript we included the reference, chemical name and the fact that CTPi is a competitive inhibitor is the citrate transport protein.

Referee #3:

In this manuscript, the authors link the stimulation of transforming growth factor beta-1 (TGFβ) to an increase in proline biosynthesis via carbon metabolic switching and ROS production in fibroblasts. The metabolic conversion of glucose or amino acids through TGFβ signaling has been the focus of many previous studies. The authors however highlighted a novel aspect of proline biosynthesis regulation that depends on TGFβ-mediated mitochondrial redox stress. Using metabolic tracing experiment, they found that glutamine, but not glucose, was preferentially used to support proline biosynthesis rather than catabolism through the TCA cycle. Interestingly, this metabolic phenotype was correlated with the increase of mitochondrial ROS stress at the early stage upon TGFβ stimulation. Furthermore, Smad4 is identified to modulate TGFβ-induced proline biosynthesis in mitochondria.

Although some aspects of the mechanism are already known, this study still presents a novel mechanistic aspect by which TGFβ facilitates proline production through increased glutaminolysis to avoid excessive redox stress, that should attract broad interest in the field. However, there are some concerns that need to be addressed to strengthen the conclusion.

1. The promoting effects of TGFβ activation on translation rate, nutrients consumption and respiration in fibroblasts are clear in Figure 1. In those experiments, TGFβ treatment was used for 48 hours. Since it is generally accepted that TGFβ signaling plays a dual role in many biological processes, a series of time-course experiments are important here to clarify the above phenomena at either early or late time point in response to TGFβ treatment. Moreover, the authors should also indicate what concentration of TGFβ that they used in those experiments via description in figure legends.

We followed the reviewer's suggestion and performed time course experiments on nutrient consumption/secretion, translation and respiration. These experiments helped us to better define the kinetics of TGFβ-induced metabolic reprogramming and how it supports translation and proline biosynthesis.
First, we found that stimulation of glucose uptake is an early event in response to TGFβ stimulation and plateaus after 24h (EV Figure 1g). Lactate secretion following TGFβ treatment increases in proportion to the glucose that is taken up by the cells (EV Figure 1h, i). Since the respiratory coupling is similar (EV Figure 1j), TGFβ-treated cells also have more

glucose available for biosynthesis and mitochondrial oxidation. Consistently, we find more glycolytic carbons entering into the mitochondria in TGFβ-stimulated cells (EV Figure 2d), and that citrate, which is mainly produced from glucose carbons, also increases early following TGFβ stimulation (EV Figure 5a).
Second, TGFβ-treated cells start to increase glutamine uptake slightly later than glucose uptake, however, the uptake of glutamine continuously increases following TGFβ stimulation, while glucose uptake reaches a plateau after 24h (EV Figure 1g, m). This might be explained by the high flux of glutamine carbon into proline (Figure 2i), resulting in a continuous demand for cells to take up glutamine to sustain proline biosynthesis in addition to TCA cycle anaplerosis.
Third, we found that translation increases in proportion to mitochondrial ATP production (Figure 1i, EV Figure 1k, l), suggesting that TGFβ-induced oxidative phosphorylation is coupled to protein synthesis.
Fourth, TGFβ stimulation causes a rapid increase in mitochondrial respiration (Figure 4a) which matches the timing of the increase in ROS in response to TGFβ treatment (Figure 5a), suggesting that an increase in mitochondrial oxidation underlies the TGFβ-induced ROS. While respiration quickly plateaus, mitochondrial substrate availability (measured as FCCP-induced respiration) continues to increase over time following TGFβ stimulation (Figure 4b), suggesting that TGFβ-treated cells accumulate mitochondrial substrates in excess of that needed to support mitochondrial ATP synthesis.
Taken together, the series of time course experiments shows that TGFβ stimulation rapidly increases nutrient uptake and mitochondrial oxidative metabolism, resulting in increased oxidative phosphorylation that supports the bioenergetic demand of increased translation and results in the generation of mitochondrial ROS that is relieved by the Smad4-dependent induction of proline biosynthesis. Furthermore, TCA cycle activity results in the accumulation of mitochondrial redox potential that is used to drive the flux of glutamine carbons into proline biosynthesis.
We also indicated the concentration of TGFβ that we used in our experiments (2 ng/mL) in the figure legends, in addition to the methods section.

2. In Figure 1i, the authors showed that TGFβ potently stimulated mitochondrial oxygen consumption by measuring oxygen consumption rate (OCR). However, Nigdelioglu et al. in their paper demonstrated that TGFβ enhanced glycolytic metabolism by measuring increased extracellular acidification rate (ECAR) level in

fibroblasts (PMID: 27836973). To support the authors' conclusion that TGFβ facilitates glucose to the downstream TCA cycle and mitochondrial oxidation rather than secreted as lactate, they need to further test ECAR levels and compare ECAR value with OCR value to determine which is the dominate pathway - mitochondrial oxidative phosphorylation or non-mitochondrial metabolism (glycolysis)?

We thank the reviewer for the opportunity to make this clearer. We observed that TGFβ treatment increases the uptake of glucose and secretion of lactate (Figure 1g, EV Figure 1e), as reported by others. In line with Nigdelioglu et al., we observed that TGFβ increases the glycolytic rate, and consistently, TGFβ increases the extracellular acidification rate (EV Figure 1d, f). Thus, TGFβ-treated cells display increased levels of glycolysis compared to controls. We included these data in the revised manuscript. However, when comparing the glucose uptake and lactate secretion over time, we found that TGFβ increases lactate secretion in proportion to the glucose that is taken up by the cells (EV Figure 1g-i). Thus, the respiratory coupling remains similar (EV Figure 1j), indicating that TGFβ-stimulated cells also retain more glycolytic carbon inside the cell for biosynthesis and mitochondrial oxidation, in addition to being secreted as lactate. We confirmed this by using glucose tracing experiments, which demonstrate that TGFβ increases utilization of glucose for serine/glycine biosynthesis (EV Figure 2b, c), for nucleotide biosynthesis (see Additional Figure 1 in response to reviewer #2) and for mitochondrial oxidation (EV Figure 2d-g).

3. Figure 2 showed that either glucose-derived glycine or glutamine-derived proline was shown to be incorporated into collagen I. However, carbons from glucose or glutamine could contribute to both proline and glycine. The authors should use metabolic tracing experiments to show whether glucose and glutamine will also increase the fraction of proline and glycine, respectively.

This was an excellent point that we have addressed by first tracing [U-13C] glutamine into serine and glycine, which shows that there is no contribution of glutamine carbon to serine/glycine biosynthesis (EV Figure 2h). During serine biosynthesis, the alpha-amine group of glutamate is transferred to phospho-pyruvate in a reaction catalyzed by PSAT1, yielding phospho-serine and alpha-ketoglutarate. Thus, we labeled cells with [α-15N] glutamine and found a substantial contribution of the glutamine alpha-nitrogen to serine and glycine (EV Figure 2i). In agreement with TGFβ-induced expression of PSAT1 (Hamanaka et al., 2019), TGFβ increases

glutamine [α-15N]-labeling of serine and glycine (EV Figure 2i). Thus, in addition to using glutamine carbon to support proline biosynthesis, TGFβ-stimulated fibroblasts use the glutamine alpha-nitrogen to support serine/glycine biosynthesis.
To test whether glucose contributes to proline, we traced [U-13C] glucose for up to 24h. Despite a contribution of glucose carbon to glutamate (EV Figure 2e), only a minor fraction of proline was labeled from glucose (EV Figure 3g), and this small fraction is predominantly derived from glucose carbon that has undergone two rounds of TCA cycle (m+4). While at this point we can only speculate why there is only m+4 but no m+2 proline, our data indicate that glucose carbon does not substantially contribute to proline. This is consistent with the high and stable labeling of proline from glutamine over time (EV Figure 3b).

4. In Figure 4, the authors showed that TGFβ stimulation will cause mitochondrial ROS generation and HIF-1α stabilization at the same time. By using the inhibitors of ETC or mitochondrial antioxidant, they demonstrated that proline synthesis was caused by TGFβ-induced mitochondrial ROS stress. However, it is well known that ROS will also stabilize HIF-1 protein in either hypoxic or non-hypoxic condition. Thus, it is possible that HIF-1 is involved in TGFβ-induced proline production. The authors should clarify whether ROS alone or ROS-mediated HIF-1 is a key factor to regulate this metabolic process.

We thank the reviewer for this suggestion. To address this, we used CRISPR/Cas9 to prevent the transient stabilization of HIF-1α in response to TGFβ-stimulation (EV Figure 6c). We found that proline levels were unchanged upon HIF-1α deletion both in mock and TGFβ-treated cells (EV Figure 6d), indicating that HIF-1α is not directly involved in TGFβ-induced proline biosynthesis.

2nd Editorial Decision 26th Jan 2020

Thank you for submitting your revised manuscript for consideration by The EMBO Journal. Your revised study was sent back to the three referees for re-evaluation, and we have received comments from two of them, which I enclose below. As you will see the referees find that their concerns have been sufficiently addressed and they are now broadly in favour of publication. Please note that while referee #2 was not able to look back into your complemented work at this time, we have editorially assessed your rebuttal and found his/her concerns to be appropriately addressed.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal, pending some minor issues related to formatting and data representation points listed below, which need to be adjusted at re-submission.

REFEREE REPORTS:

Referee #1:

Good revision.

Referee #3:

The authors have adequately addressed all of my previous concerns by performing additional experiments as well as further analyzing their metabolic tracing data in their revised manuscript. Now, the new evidence enhances the conclusion that TGFβ facilitates proline production by increased glutaminolysis to avoid excessive redox stress in fibroblasts.

2nd Revision - authors' response 1st Feb 2020

The authors made the requested editorial changes.

3rd Editorial Decision 4th Feb 2020

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended manuscript and concluded that the remaining minor concerns have been sufficiently addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name:	
Journal Submitted to:	
Manuscript Number:	

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	The sample size was chosen based on previous experience with the assays performed in this study as previously published by our laboratory and others.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	For Seahorse analysis, data from the top and bottom row of the plate was excluded due to medium evaporation. One sample in EV Figure 4b for sgPycr2-2 was excluded due to low signal of the internal standard.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	For each figure involving comparison of 2 groups, a Student's t-test was used with Welch's correction to account for unequal variances. For comparison of three or more groups, one-way ANOVA was used and corrected for multiple comparison using the Holm-Sidak method. For comparison of two ore or more groups with two different treatments, two-way ANOVA was used and corrected for multiple comparison using the Holm-Sidak method. For analysis of gene expression datasets, moderate t-statistics was used and corrected for multiple comparison using the Benjamini & Hochberg false discovery rate method with FDR<1%. These were the most approriate tests for the different group sizes and experimental set-ups.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Data were normally distributed based on D'Agostino-Pearson normality test.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-report

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is there an estimate of variation within each group of data?	For each figure, the standard deviation is shown. In most figures individual data points are shown.
Is the variance similar between the groups that are being statistically compared?	The variance was not always similar between groups. To account for this, Welch's correction was used.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Vinculin (Sigma, V9131), β-Actin (Sigma, A5441), α-Tubulin (Sigma, T9026), Collagen I (Abcam, ab21286), Collagen IV (Proteintech, 55131-1-AP), Fibronectin (Abcam, ab2413), Puromycin (EMD Millipore, MABE343), GLS1 (Abcam, ab156876), P5CS (Sigma, HPA008333), PYCR1 (Proteintech, 20962-1-AP), PYCR2 (Proteintech, 17146-1-AP), HA-tag (Sigma, SAB4300603), FLAG-tag (Sigma, F1804), Smad4 (Santa Cruz, sc-7966), HIF-1α (Cayman Chemical, 10006421), Smad2 phospho-S465/467 (Cell Signaling, 3108S), Slc25a1 (Proteintech, 15235-1-AP), anti-rabbit HRP (GE, NA934V), anti-mouse HRP (Sigma, NA931).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NIH-3T3 cells and IMR90 cells were obtained from ATCC and were found mycoplasma-free by the MycoAlert Mycoplasma Detection Kit (Lonza).

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA