EMBO reports

Orphan nuclear receptor COUP-TFII enhances myofibroblasts glycolysis leading to kidney fibrosis

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1st Editorial Decision 30th Jun 2020

Dear Dr. Li,

Thank you for transferring your manuscript to EMBO Reports, which was previously reviewed at The EMBO Journal. I have now taken a detailed look at the manuscript and the referee reports.

Referees appreciate the proposed role of COUP-TFII in fibrosis. However, they also raise some concerns that need to be addressed for publication here. In particular,

1. more insight into the upstream regulators of COUP-TFII upon kidney injury (interleukins? - ref #3, general comment a) and targets of COUP-TFII is required (ref #3, general point b).
2. stronger support for the interplay between COUP-TFII and PGC-1 needs to be provided (ref #2 general comments, ref #3, general point b)
3. data from C3H10T1/2 cells need to be supported by an independent model (ref #2 general comments, ref #3, general comment c).
4. the effect of COUP-TFII depletion on metabolism needs stronger support (ref #1, paragraph starting as 'Regarding the Seahorse experiments' and ref #2, general remarks)

Referees mention that how COUP-TFII regulates PGC-1a expression and how exactly PGC-1a affects fibrosis remain unclear. Addressing these points, at least partially, would significantly strengthen the manuscript. However, not being able to pinpoint the mechanism will not preclude from publication. If this is the case, it is essential to discuss possible mechanisms. Moreover, the epistasis between these players will need to be strengthened (as in point 2 above).

Please let me know if you would like to discuss any point further.

I find the reports informed and constructive, and believe that addressing the concerns raised will significantly strengthen the manuscript.

Given these positive recommendations, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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plan should you need additional time, and also if you see a paper with related content published elsewhere.***

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2. Your manuscript contains statistics and error bars based on n=2 or on technical replicates. Please use scatter plots in these cases.

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1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14693178/authorguide#transparentprocess
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4) a complete author checklist, which you can download from our author guidelines (<http://embor.embopress.org/authorguide>). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

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6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <http://embor.embopress.org/authorguide#expandedview>.

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) We would also encourage you to include the source data for figure panels that show essential data.

Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available <http://embor.embopress.org/authorguide#sourcedata>.

8) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at <http://embor.embopress.org/authorguide#datacitation>.

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Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Method) that follows the model below. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843
(https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

10) Regarding data quantification, please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
Please note that error bars and statistical comparisons may only be applied to data obtained from at least three independent biological replicates.
Please also include scale bars in all microscopy images.

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely,

Deniz Senyilmaz Tiebe

Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

The manuscript from Li and colleagues shows that COUP-TFII is a driver of fibrosis using both cell and mouse models. Mechanistically, they suggest that this is due to a metabolic shift-potentially driven by PGC-1a.
The authors have made some novel observations worthy of publication and have identified a potential new therapeutic target to counteract diseases characterized by fibrosis. One limitation is that mechanistically it is still unclear how COUP-TFII regulates the observed metabolic shift. Authors show a relationship between COUP-TFII and PGC-1a expression, however, it is still not clear to me how COUP-TFII impacts on PGC-1a expression and ultimately how PGC-1a may impact on fibrosis.
Throughout the manuscript I found some issues with the way data is represented and analyzed (particularly the mouse experiments). Some essential controls are missing. Legend description lacks detail and is confusing in some instances. It is not clear how statistical analysis was performed (more detail is necessary). I think that discussion needs to better explain possible mechanisms by which PGC-1a impacts on fibrosis development.

Response: We appreciate reviewer's comments. Many changed were made in our figures per reviewer's comments, including a new Fig7 to explain the role of PGC1α in myoblasts differentiation.

Specific comments.
Figure 1- With regards to the increased COUP-TFII in CDK patients- I wonder if expression correlates with severity of disease and fibrosis. Additionally have the results been normalized by age- which is a major factor in development of fibrosis?

Response: We analyzed the previously published microarray data (Nakagawa, Nishihara et al, 2015) and found the elevated COUP-TFII mRNA in CKD patients (new Fig1a, revised former Fig1c). We don't have the complete detailed patients' information. The slides we obtained from patients with TMA and DN were de-identified (new Fig1b, revised former Fig1a). Therefore we are not able to correlate with severity of disease or normalize by age.

Authors have shown increased COUP-TFII in IPF derived fibroblasts. It is not clear in the text how many fibroblast-derived patients were analyzed and no quantification is shown.

Response: These are images from IPF patients, not IPF derived fibroblasts. We have clarified this in the text and added the quantification (new Fig1c, revised former Fig1b).

Authors comment about the co-localization between a-SMA and expression of COUP-TFII-however no quantification is provided.

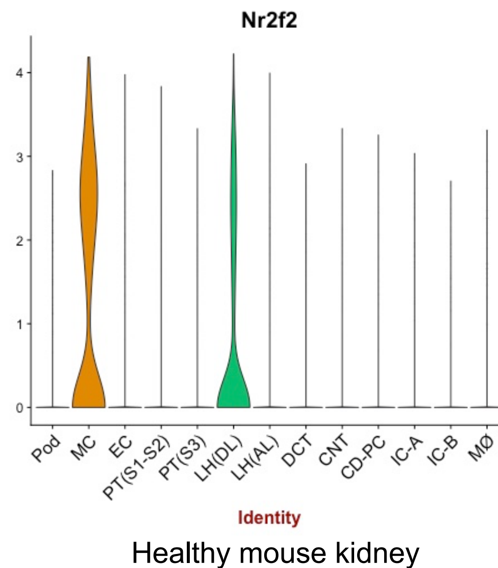
Response: We added the quantification of co-localization (new Fig1b, revised former Fig1a).

Figure 2- Authors show that COUP-TFII positive cells derive from Foxd1 population- using adult kidneys form mice expressing a Foxd1- tomato reporter. The images are convincing- but no quantification is provided- how many animals were analyzed?

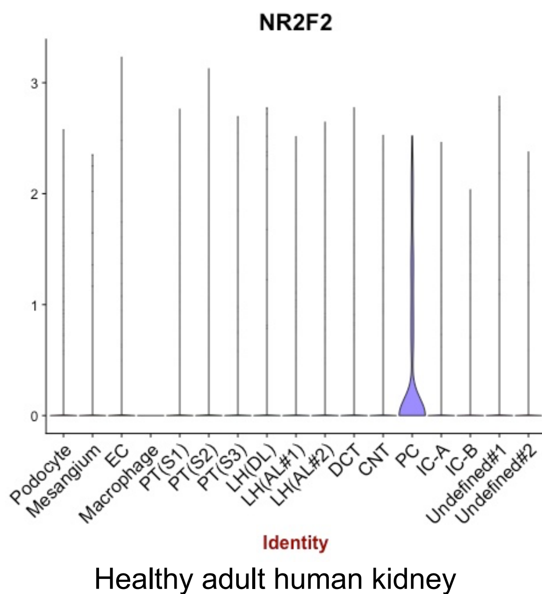
Response: We added the quantification from 2 animals (new Fig1c, revision of former Fig1c).

Single cell RNA-seq form pre-existing databases supports the findings. Out of curiosity- in the mouse or human databases did you find also enrichment of COUP-TFII in non-injured kidneys (controls)?

Response: There is enrichment of COUP-TFII in principal cells in healthy human adult kidneys, but most the COUP-TFII expression is found in mesenchyme and Loop of Henle (descending limb) in healthy mouse kidneys (see the figure in below, http://humphreyslab.com/SingleCell/). Consistent with these data, we found scattered COUP-TFII expression by immunofluorescent staining in control 'healthy' kidney, which was obtained from the non-tumor portions of total nephrectomy samples in patients with renal cell carcinoma (new Fig1b), or control mouse kidney (new Fig1a & 1c).



Healthy mouse kidney



Healthy adult human kidney

Figure 3- Regarding the quantification in 3a- I understand that n=3 mice were analyzed per

group- so it is not clear to me to what the individual data points refer to. Data should be shown as mean value per mouse (so that mouse-to-mouse variability is properly accounted for).

Response: Thanks for your advice. We agree. We re-analyzed the data and changed the figures per your suggestion (new Fig3a, revision from former Fig3a). The individual data points refer to each animal.

I have similar concerns with e and f- however I could not find details describing these data in the figure legend (how many animals?).

Response: We changed the figure to reflect n=3 animals (new Fig3f, revised former Fig3f). The details describing these data are in the Fig3 legend.

Authors should try to be consistent with the way data is presented - dot-plots are probably the best way- but types of graphs vary within this figure.

Response: We agree. We changed all the figures in this manuscript to dot-plots.

Statistics need to be clarified- in Figure legend t test is mentioned (what type?)- However, a One-Way ANOVA followed by the appropriate Post-hoc test would be the required for multiple comparisons.

Response: In the Figure legend, we clarified the statistics and highlight as yellow. The t test is paired t test. We are using one-way ANOVA for multiple comparisons.

Figure 4- Images are convincing in showing reduced fibrosis following genetic ablation of COUP-TFII- however, again it is not clear how analyses of tissues staining (for COUP-TFII, a-SMA and Collagen1) was performed. Figure legend mentions that "8-10 images were taken and quantified for each animal (represented by each dot)"- To me- this reads as if each dot is an animal (which is the correct way to represent the data) but does not match with n=4 for WT group and n=6 for KO. I do not think it is acceptable to do statistical analysis based on quantification of individual images and not take into account mouse to mouse variability.

Response: Thanks for your advice. We agree. 8-10 images were taken and quantified for each animal. The mean of quantifications from these 8-10 images per animal was represented by each dot. We re-analyzed the data and changed the figures per your suggestion. The individual data points refer to each animal (n=4 for WT group and n=6 for KO) (new Fig4d, revised former Fig4d).

Figure 5- Quantification of a-SMA is not shown.

Response: We added the quantification of αSMA (new Fig5d, revised former Fig5d).

Figure 6- the organization of the Figure could be improved- not easy to follow the data and description. For instance (e) refers to separate pieces of data. I would suggest separating it to improve clarity.

Response: Thanks for your suggestion. We rearranged Fig6.

Regarding the Seahorse experiments- authors treated cells with 10ng/ml of TGFb-1 and

measured ECAR and OCR. What about the untreated controls? These need to be shown. How does COUP-TFII KO impact on respiration in the absence of TGFb-1 stimulus? Additionally, out of curiosity, does overexpression of COUP-TFII impact on respiration- as you have shown to enhance expression of a-SMA, collagen and reduce expression of PGC-1 (even in the absence of TGFB-1)?

Response: Thanks for your suggestion. We added the result of seahorse experiments using untreated controls and COUP-TFII OE cells (new Fig6d & 6e, revised former Fig6e). As shown in new Fig 6e, COUP-TFII OE significantly increased, while COUP-TFII KO decreased, the baseline, rate of glucose driven glycolysis, glycolytic capacity, and glycolytic reserve when compared to WT cells in response to TGFβ1 stimulation. There are no significant differences among WT, OE, and KO cells at baseline or with glucose driven glycolysis without TGFβ1 treatment. We added our interpretation of these results in the Discussion session (highlight in yellow). Although we acquired OCR data with ECAR in the glycolysis stress test, the glycolysis stress test is not designed to test OCR after mitochondria challenge. Therefore, we decided not to show the OCR data in the glycolysis stress test. We will do the proper mitochondria stress test using these 3 cell lines in the future. We are focusing on glycolysis in this manuscript.

In Figure f- can you better represent the statistical comparisons?- the 2 asterisks on top of KO with TGFB-1 refer to what comparison?

Response: We clarified the statistical comparisons in the new Fig6c (revised former Fig6f).

In the Chip-qPCR experiment what does the N refer to- IgG control?

Response: In the Chip-qPCR experiment, yes, the N refers to IgG control. We clarified this in the legend, which is now Fig 7C (revised former Fig6h).

We thank the Reviewer for the very helpful comments.

Referee #2:
EMBOJ-2020-105756, corr. author Dr. Li
"Orphan nuclear receptor COUP-TFII drives the myofibroblast metabolic shift leading to fibrosis"
Summary:
The issue at hand is very interesting and timely, since the authors describe the function of an orphan member of the nuclear receptor family with unknown endogenous ligands as central for development of fibrosis in the context of increased glycolysis. For this they use several very different models of inducing fibrosis and show that the mechanisms at hand is operative in all models so far tested. Finally by preventing glycolysis they tempt to proof that this mechanism is indeed causing at least in part fibrosis and fibrosis associated changes in gene expression.

General comments:
There is a lack of depth in this manuscript, since the authors have not shown that glycolysis is increased in all models in vivo as well as in the tissue culture experiments performed. Gene expression studies, without flux analysis is not sufficient, since glycolysis is not primarily regulated on the mRNA level.

Response: We appreciate your concern about in vivo evidence of glycolysis. The glycolysis profile is heterogeneous even in the non-injured kidney, given different cell types and oxygen

availability. Kidney proximal tubules are known to mostly depend on oxidative phosphorylation, versus distal nephron segments, podocytes and fibroblasts are much more glycolytic (Brinkkoetter, Bork et al., 2019, Ding, Jiang et al., 2017, Ghazi, Polesel et al., 2019, Kang, Ahn et al., 2015). Therefore, the glycolysis profile in the whole kidney, especially in different injury models, is largely unknown. New technology, such as high-resolution live imaging like multiphoton microscopy, might provide new insight to study metabolism in living animals. We found that expression of COUP-TFII was significantly increased, predominantly located in αSMA+ interstitial areas (new Fig1b, 3a). Giving the evidence of increased glycolysis of proliferating fibroblasts in fibrosis (Ding et al., 2017, Hou & Syn, 2018, Lemos, McMurdo et al., 2018), we hypothesized that COUP-TFII contributed to organ fibrosis via a regulatory role in the metabolism of the myofibroblast. Indeed, we demonstrated increased lactate production in COUP-TFII overexpressing cells (OE) with or without TGFβ1 stimulation (new Fig6c). Our proteomic data, derived from COUP-TFII OE and WT cells, also revealed that COUP-TFII promotes the expression of proteins enriched in metabolic processes critical for myofibroblasts, in particular, suppression of FAO and enhancement of glycolysis (new Fig6a&b). Results from lactate production and proteomic experiments demonstrated enhanced glycolysis in fibroblasts, in addition to mRNA level.

Furthermore, the role of PGC1alpha has not been finally proven in all models and functionally in the cell tested, the same holds true for the role of changes in fatty acid oxidation. One would expect, that in all models at least some metabolic pathways would be examined on the level of the metabolite itself, to show consistence with the hypothesis. Comparability of in vivo models and cell models and proof of functionality are mandatory.

Response: Thanks for your comments. Recently, there are several papers where the authors examined the role of PGC1α in kidney injury models (Dumesic, Egan et al., 2019, Han, Wu et al., 2017, Tran, Zsengeller et al., 2016). The expression of PGC1α was decreased after injury in these reports. Overexpression of PGC1α in proximal tubules ameliorated renal fibrosis. As we stated above, the metabolic profile in the whole kidney is heterogeneous. In the future, a comprehensive metabolic profile in a whole kidney using live imaging or proteomics will help us understand the metabolic changes after kidney injury. This manuscript is focused on the role of COUP-TFII in injury-induced kidney fibrosis, particularly in the fibroblast/myofibroblast compartment. Acknowledging the reported role of PGC1α in kidney injury and repair, we explored the relationship between COUP-TFII and PGC1α in our cell culture system. Following your suggestion, we added serial experiments using PGC1α adenovirus to overexpress PGC1a in fibroblasts. We further characterize the role of PGC1α in FAO and the COUP-TFII effect on myofibroblast differentiation. The data are summarized in Fig 7. In response to your concerns we have modified the title to focus on glycolysis since we agree that much of the evidence for decreased FAO is related to downregulation of proteins involved in FAO. The new title is now: “Orphan nuclear receptor COUP-TFII enhances myofibroblasts glycolysis leading to fibrosis".

Minor comments:
I am not necessarily convinced by their time-course study reportedly showing that increase in NR2F2 directly proceeds the increase in SMA expression. This is important because the later argumentation is built on that finding. A similar timecourse could also be done in the animal models used.

Response: We demonstrated that COUP-TFII expression was upregulated as early as day 2 after injury, which is well before up-regulation of αSMA and any histologic evidence of fibrosis

was evident in our UUO model (Fig3b). Overexpression of COUP-TFII in C3H/10T1/2 cells alone increased αSMA expression without TGFβ stimulation (Fig5d). These data support our hypothesis that COUP-TFII drives myofibroblast differentiation.

The proteomic analysis in Fig 6 is ok, but is validated by measuring mRNA which seems a little strange when you consider that there are perfectly good antibodies for each of the selected targets/genes. Furthermore, there should have been validation of increased activity; again activity assays for HK and LDH are available and relatively straight-forward to perform. Glut1 activity or general glucose uptake could also be easily assessed by non-radioactive means.

Response: Thanks for your comments. As you pointed out, proteomic analysis demonstrated enhanced glycolysis and suppressed FAO by COUP-TFII. We validated these data by measuring lactate production (new Fig6c) and Seahorse glycolysis stress test (new Fig6d&e). We agree that measuring activity of HK and LDH with glucose uptake will be additional evidence of enhanced glycolysis. The enzymatic activity experiments, however, will not change our conclusion derived from the lactate assay and Seahorse study.

With respect to the experiments in which cells were co-stimulated with TGF-ß1 and 2DG as a means of inhibiting glycolysis, the author's neither state the concentration of 2DG used or have a 2DG alone control. This is important because 2DG is very potent and it could induce apoptosis as consequence of inhibiting metabolism. The authors must show, that the concentration of 2DG "normalizes" glycolysis, but not totally suppresses it, because totally suppressing glycolysis will affect many other cell functions too. In addition, there is in many systems a relation of cell proliferation to the expression of growth factors. This needs to be controlled for and an artefact needs to be excluded.

Response: We agree with your comments that 2DG could induce apoptosis as a consequence of inhibiting metabolism. The concentration of 2DG used in our cell experiments is 10mM, which has been widely used in previous studies for inhibiting glycolysis (Ding et al., 2017, Henderson, Duffy et al., 2020, Xie, Tan et al., 2015). These studies consistently reported that 10mM 2DG was adequate to inhibit glycolysis without affect cell viability. Using this concentration of 2DG from these studies, we tested 2DG in COUP-TFII OE cells treated with TGFβ1. As showed in our new Fig6f that 2DG was able to “normalize” TGFβ1-induced gene expression involved in glycolysis (the mRNA level in the 2DG+TGFb1 group is similar to the control group), and then decreased the TGFb1-induced fibrosis marker (αSMA and collagen1). There was abundant COUP-TFII protein in the 2DG group (new Fig6g), which reflects cell viability.

The seahorse data show functionality, but that data is problematic. Such analysis produces a lot of parameters in addition to the profiles that are shown in the figure. This include measures of non-glycolytic acidification, glycolysis, glycolytic capacity and glycolytic reserve, but none of these parameters are shown; the author's mention a significant decrease in glycolysis but they do not actually show the hard data other than the profiles which are meaningless to the non-expert. The also talk about observing a decreased mitochondrial oxygen consumption in the KO cells in the same experiment. This could be interpreted as such, but they should have performed the mitochondria test to really confirm whether that is the case. This is because the glycolysis stress is performed slightly differently to the mito stress test in that the media you pre-incubate the cells in, contains no glucose and no pyruvate but glutamate. For the mito stress test, the media include all three energy metabolites, so the initial metabolic stress on the cells is complete different in the respective assays. Furthermore, the author's keep referring to switching in metabolism without any solid evidence, but there are seahorse assays available

which can measure the dependency of a cell on a given energy substrate which could show whether there is change in the metabolic capacities of the KO-cells. One other technical point, the seahorse data does not seem to be normalized to anything i.e. cell number, protein content, cell proliferation rate etc. Given that the KO-cells have a lower proliferation, it might be that the wells were not as confluent as the wild-type cells and this could give rise to the differences observed.

Response: Thanks for your detailed and knowledgeable comments. We normalized our seahorse data with protein content. We also analyzed and present the data with non-glycolytic acidification, glycolysis, glycolytic capacity and glycolytic reserve (new Fig 6e). We agreed with your comments on the mito stress test. Although we acquired OCR data with ECAR in the glycolysis stress test, the glycolysis stress test is not designed to test OCR after mitochondria challenge. Therefore, we decide not to show the OCR data obtained in the glycolysis stress test. We are focusing on glycolysis in this manuscript. We will do the proper mito stress test using these 3 cell lines in the future. We will also perform the cell energy phenotype test by seahorse assay to characterize the metabolic profile in these 3 cell lines in the future.

Lactate measurements in the media are shown as a means of confirming that there are changes in glycolysis. This is an example of the back-to-front nature of this study. In that such measurements would generally be performed first as to give some initial evidence that they are changes in glycolysis which would then lead you onto performing the seahorse as a means of characterizing and describing those differences. At the end of the day, the extracellular acidifications measurements performed by the seahorse are essentially measuring changes in lactate in the media, so it is not surprising that they see the same result. However, the lactate measurements are only useful if they are expressed as a rate of change and shown in parallel with the glucose consumption from the media. Furthermore, the lactate measurements are performed not only in the KO but also the OE cells in the presence and absence of TGFß1. The same cells and experimental conditions should have also been included in the seahorse experiments, although it is stated in the text the KO were stimulated with TGFß1 in the seahorse experiments, but I cannot see the data in the figure. In addition, as stated above, interpretations about metabolisms without precise determination of metabolites and their flux is prone to give unsatisfactory results and potentially interpretations not covered by data.

Response: We appreciate the Reviewer's helpful comments. Fig6 is re-arranged to show the lactate measurements as new Fig6c, followed by the seahorse data (new Fig 6d&e). To be consistent with lactate measurement experiments, we performed seahorse experiments using COUP OE cells, in addition to KO and WT cells, treated with and without TGFβ1 (new Fig6d&e). We found that COUP-TFII OE cells significantly increased, while COUP-TFII KO decreased, at baseline, the rate of glycolysis, glycolytic capacity, and glycolytic reserve when compared to WT cells treated with TGFβ1 (Fig 6e). Interestingly, unlike the lactate assay, COUP-TFII OE cells did not increase glycolysis without TGFβ1 stimulation (Fig 6e). This might be due to the base medium used in Seahorse experiments, which did not have glucose and pyruvate. Collectively, these data demonstrate increased glycolysis in COUP-TF OE cells in response to TGFβ1 stimulation.

We thank the Reviewer for the very helpful comments.

Referee #3:

Summary

The current manuscript by Li et al. explores the role of nuclear receptor COUP-TFII in organ

fibrosis. In particular, the authors demonstrate that COUP-TFII expression levels were up-regulated in various conditions of human organ fibrosis, including chronic kidney disease, diabetic nephropathy, and idiopathic pulmonary fibrosis. Upon interleukin 1beta exposure, COUP-TFII levels also increased in human kidney organoids, co-localizing with aSMA expression. Lineage tracing studies showed that COUP-TFII was mostly expressed in kidney stromal cells and was induced upon experimental kidney injury in aSMA-positive cells, overall preceding the induction of fibrotic markers upon disease progression. KO of COUP-TFII ameliorated renal fibrosis in mice, and genetic manipulation of COUP-TFII expression levels in cultured pericytes altered the levels of fibrotic markers, aSMA and collagen, even in the absence of pro-fibrotic TGFbeta signaling. Cellular studies demonstrated that COUP-TFII overexpression in pericytes triggered a metabolic shift from fatty acid oxidation to glycolysis, thereby enhancing a pro-fibrotic phenotype. Inhibition of TGFbeta-dependent glycolysis in COUP-TFII overexpressing cells attenuated fibrosis marker gene expression, while downregulating levels of PGC-1 transcriptional co-factor as a key component in mitochondrial biogenesis. Overall, the authors conclude that COUP-TFII represents a potential target in the treatment of fibrotic kidney disease.

General comments
Fibrosis still represents a non-reversible process in many disease entities, thereby imposing a major clinical challenge on affected patients. In this respect, the current manuscript by Li et al. addresses an important and clinically relevant topic. A particular strength of the manuscript resides in the use of multiple animal models, elaborate lineage tracing studies and the use of state-of-the-art model systems, including human organoids. The manuscript is concise, well written and structured.

Response: Thanks for your supportive comments.

However, the following main issues require additional attention by the authors:

a) While the regulatory function of COUP-TFII for the fibrotic response in pericytic cells has been nicely demonstrated, the upstream events leading to enhanced COUP-TFII levels upon kidney injury remain elusive. As demonstrated in the organoid system, interleukins may play a role here. The authors should attempt to define the upstream signaling pathways leading to COUP-TFII induction in more detail. Ideally, a screen for upstream regulators should be performed in the organoid system.

Response: This is an excellent suggestion. COUP-TFII remains an orphan nuclear receptor with unknown ligand since it was cloned in 1986. As you pointed out, IL-1β increased the expression of COUP-TFII and αSMA, mostly in the stoma region in the organoid system. In an attempt to screen for upstream regulators on COUP-TFII, we treated organoids with PDGF, TGFb1 or cobalt chloride (CoCl2, induces hypoxia inducible factor (HIF) signaling, mimicking the response to hypoxia) in addition to IL-1 in vitro. As shown in Fig1e, COUP-TFII expression increased significantly in organoids treated with TGFβ1 and cobalt chloride, but not PDGF. These results suggested that inflammation, hypoxia, and TGFβ1 might be the upstream regulators to increase COUP-TFI expression.

b) The mechanistic underpinnings of the pro-fibrotic role of COUP-TFII in pericytes are only vaguely described: Does genetic reconstitution of PGC-1 in COUP-TFII OE cells rescue the glycolytic and pro-fibrotic phenotype or -conversely- does PGC-1 knockdown in COUP-TFII KO cells reverse corresponding effects on FA Ox and glycolysis? Please also define the global COUP-TFII targets in pericytes by performing CHIP Seq analysis under basal and TGFbeta-

induced conditions.

Response: Thanks for your suggestions. We successfully overexpressed PGC1α through adenovirus transduction in C3H/10T1/2 cells (WT) (new Fig7d). PGC1α OE significantly increased expression of Cpt1 and PDK4, two key contributors to FAO (new Fig7e). However, overexpression of PGC1α did not abrogate the TGFβ1 up-regulated genes in glycolysis and αSMA (new Fig7e). Furthermore, we infected COUP-TFII OE cells with the PGC1α adenovirus (Fig 7f). PGC1α OE did not decrease αSMA protein neither in the absence or presence of TGFβ1. These data are consistent with the concepts that fibroblasts/myofibroblasts primarily rely on glycolysis (Rabelink & Carmeliet, 2018, Xie et al., 2015). Our Chip-qPCR results revealed that COUP-TFII directly binds to the PGC1α promoter (new Fig7c). Therefore, overexpression of COUP-TFII suppressed FAO and this was associated with decreased PGC1α myofibroblast. However, activation of FAO by overexpressing PGC1α was insufficient along to abrogate glycolysis, either induced by TGFβ or COUP-TFII overexpression, in myofibroblasts. The lack of effect of PGC1α on myofibroblast suggests that there are other factors modified by COUP-TFII that may play important roles in driving glycolysis and reducing FAO. It might also be due to the dominant reliance of fibroblast/myofibroblast on glycolysis. All the results are summarized in Fig 7. Ultimately, we will perform CHIP Seq analysis as you suggest in the future, identifying more targets of COUP-TFII.

c) Key cell culture data from C3H10T1/2 cells should be supported by an independent model, ideally the human organoid system (i.g. does COUP-TFII OE or KO/KD also regulate glycolsis etc in an independent setting?
Addition of respective experimental data will significantly strengthen the case for publication.

Response: Human kidney organoids provide advantages of 3D human nephron structures with multiple kidney cell types, nephron structures and a rich stroma. It is a challenge to genetically manipulate COUP-TFII in the human organoid system giving the critical role of COUP-TFII in developmental biology (Pereira, Qiu et al., 1995, Xie, Qin et al., 2011). If we knock out COUP-TFII in the embryonic stem cells, there is a high chance of affecting the organoid maturation. Furthermore, organoids have multiple human kidney cell types, which all harbor different metabolic profiles similar to the variability in vivo. Global overexpression or KO/KD of COUP-TFII in organoids might produce complex metabolic effects. Nonetheless, organoids are idea ex vivo human cell system mimic in vivo situation. We will try to do the COUP-TFII OE or KO/KD in organoids in the future. The results may prove valuable as the Reviewer suggests.

Specific comments
The discussion section in large parts recapitulates the results section. Please avoid duplications here and rather focus on relevant literature citations.

Response: Thanks for your comments. We made several changes in the discussion highlighted in yellow.

We thank the Reviewer for the very helpful comments.

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1st Revision - Editorial Decision 18th Mar 2021

Dear Li,

Thank you for submitting your revised manuscript. It has now been seen by two of the original referees. As you can see, the referees finds that the study is significantly improved during revision. However, referee #1 (former referee #3 of the first round) has one remaining concern. As we discussed during our recent video chat, to address this point, please make sure to label the PGC1α rescue panel more clearly and discuss its results in more depth in the text.

Before I can accept the manuscript, I need you to address the additional points below:

• Please provide 3-5 keywords for your study. These will be visible in the html version of the paper and on PubMed and will help increase the discoverability of your work.
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• In addition, please provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels.

Thank you again for giving us to consider your manuscript for EMBO Reports, I look forward to your minor revision.

Kind regards,

Deniz

--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

The authors have improved the manuscript by adding new data. With respect to the original major issues, the authors have provided new data on the upstream regulatory events governing COUPTF expression which is strengthening the manuscript.
However, the authors do not provide any new data on the functional importance of PGC1 in the COUPTF pathway as no reconstitution experiments (rescue experiments) were performed as originally requested. A functional rescue of COUPTF OE by PGC OE or vice versa seems mandatory to establish the functional link.

Referee #2:

Authors have adequately responded to my concerns by adding additional quantifications, data and clarifying the text. Overall, the manuscript still has some weaknesses in terms of mechanism, but the findings are of interest and should be published.

March 8, 2021

Deniz Senyilmaz Tiebe, PhD
Editor, EMBO Reports
Meyerhofstrasse 1
D-69117 Heidelberg
Germany

Dear Dr. Tiebe,

Thank you for giving us another chance to address the remaining concern of Reviewer 1 (former reviewer #3 of the first round). As you pointed out, all the suggestions from the reviewers significantly improved our manuscript. In particular to address this reviewer's concern in his/her initial review, we did the recommended rescue experiments to overexpress PGC1α in COUP-TFII OE cells treated with or without TGFβ1 (Fig 7F of our revised submission). PGC1α OE did not decrease COUP-TFII induced αSMA protein production, either in the absence or presence of TGFβ1. We did not label the Fig 7F clearly, which might have led to the perception that we initially had not performed this important experiment as the reviewer requested. We have now relabeled Fig 7F by adding a line with COUP-TFII OE+ to indicate that they are COUP-TFII OE cells. We also made change to the Fig 7 legend to clarify the results.

Below is a detailed response to Reviewer #1 (former Reviewer #3). We have included a new version of the manuscript with changes highlighted as yellow. We hope you and the Reviewer are satisfied with our response to this concern, and we hope you find our additional experiments responsive so that our manuscript is deemed acceptable for publication in EMBO Reports.

Sincerely yours

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Referee #1 (Former **Referee #3** of the initial review rounds)
The authors have improved the manuscript by adding new data. With respect to the original major issues, the authors have provided new data on the upstream regulatory events governing COUPTF expression which is strengthening the manuscript.
However, the authors do not provide any new data on the functional importance of PGC1 in the COUPTF pathway as no reconstitution experiments (rescue experiments) were performed as originally requested. A functional rescue of COUPTF OE by PGC OE or vice versa seems mandatory to establish the functional link.

Response: Thanks for your suggestions. We agree with you that this experiment is very important. We did the rescue experiments as you suggested and included the results in our revised submission, but we did not label Figure 7F clearly enough to convey this adequately. We overexpressed PGC1α in COUP-TFII OE cells treated with or without TGFβ1. PGC1α OE did not decrease COUP-TFII induced αSMA protein production, either in the absence or presence of TGFβ1. The result is presented in Fig 7F in the revised manuscript. We did not label it clearly which might lead to the perception that we did not perform this experiment as you requested initially. These data suggest that overexpress PGC1α did not rescue the COUP-TFII effect on αSMA production. This inability to rescue the effect of COUP-TFII on αSMA production might be due to the dominant reliance of fibroblast/myofibroblast on glycolysis. Although COUP-TFII decreases PGC-1α expression, overexpression of PGC1α alone was insufficient to abrogate the enhanced glycolysis. The result indicates that there are other factors modified by COUP-TFII that play important roles in driving glycolysis. We relabeled the Fig 7F by adding a line with COUP-TFII OE+ to clarify the result. We also made changes to the Fig 7 legend to clarify the results.

The authors have addressed all minor editorial requests.

2nd Revision - Editorial Decision 1st Apr 2021

Dear Li,

Thank you for submitting your revised manuscript. I have now looked at everything and all is fine. Therefore, I am very pleased to accept your manuscript for publication in EMBO Reports.

Congratulations on a nice work!

Kind regards,

Deniz
--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

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- the assay(s) and method(s) used to carry out the reported observations and measurements
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- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
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  - are tests one-sided or two-sided?
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  - 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.

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7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	YES

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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	YES
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