TGF-β-driven downregulation of the transcription factor TCF7L2 affects Wnt/β-catenin signaling in PDGFRα+ fibroblasts

Osvaldo Contreras, Hesham Soliman, Marine Theret, Fabio M. V. Rossi and Enrique Brandan
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First decision letter

MS ID#: JOCES/2019/242297

MS TITLE: Extracellular TGF-β downregulates the expression of Wnt transcription factor TCF7L2/TCF4 in mesenchymal stromal cells and fibroblasts

AUTHORS: Osvaldo Contreras, Hesham Soliman, Theret Marine, Fabio Rossi, and Enrique Brandan
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The paper by Contreras et al. investigates the regulation of TCF7L2 by TGF-β in mesenchymal stromal cells and fibroblasts. The authors show that TCF7L2 is downregulated in both of these cell types by TGF-β at the protein and RNA level.
They conclude that the inhibition of transcription is via the activity of HDACs, and that the inhibition of protein levels is via TGF-β-induced protein degradation via the proteasome. They show that this regulation is specific to these cell types and not evident in myoblasts. They conclude that TGF-β-induced TCF72 downregulation could be a useful target in tissue fibrosis. These results are novel and demonstrate interplay between TGF-β and Wnt signalling in mesenchymal stem stromal cells.

Comments for the author

Overall, I think the study is of good quality and the data for the most part support the conclusions.

There are a number of small issues with the data that need to be attended to as outlined below.

1. In Figures 1C and S1A the data are not in agreement. The RT-PCR data in Fig S1A shows readily detectable levels of Lef1 in FAPs, but this is not so in the qPCR data in Fig 1C. Why is this?

2. In Figure 1E it would be good to show the separate panels (i.e. the TCF7L2, the LN-α2 and the nuclei) so that it was clear which cells express TCF7L2.

3. In Figure S1F it is very hard to work out which groups of cells correspond to which tissue as the colors in the key are not clear. This needs to be improved.

4. The authors show the bulk levels of TCF7L2 before and after injury for the skeletal muscle but not for the diaphragm. It would be good to show this for the diaphragm too.

5. The pSmad3 staining comes a bit out of the blue. The authors need to show pSmad3 staining in WT diaphragm as well as in the injured.

6. In Figure 4G there is a curious result. Why after 48 hr does the SB525334 not inhibit TGF-β's effects?

7. In Figure 5, the authors see no effect of the Smad3 inhibitor and conclude that the TGF-β effect is via a semi-independent pathway. This is possible, but it is also possible that Smad2 and Smad3 are acting redundantly, as is often the case. CRISPR-induced knockout of the Smads would be the best way to test this.

8. TSA is not a very specific HDAC inhibitor. The authors should use a more specific inhibitor and also show that the effect in their cells is really at the level of transcription.

9. The diagram in Figure 8 is a bit confusing as they should make it clear that the downregulation of TCF7L2 targets by TGF-β is via the downregulation of TCF7L2 levels.

Reviewer 2

Advance summary and potential significance to field

The present manuscript shows new and interesting data about an interplay between Wnt and TGFβ signalling pathways in fibrotic diseases. The authors demonstrate in a consistent and well arranged set of experiments the role TCF7l2 or TCF4 in the fibrotic remodeling of dystrophic muscles using a dystrophic mouse model. Subsequently, the authors exemplify that TGFβ1 as well as TGFβ2+3 are involved in reduction of the cellular amount of the transcription factor TCF7l2, which is specific for

mesenchymal cells in contrast to myoblasts. The necessity of the TGFβ receptor proteins in this process is proved by using a pharmacological inhibitor. Further studies give evidence for the involvement of protein modifications such as acetylation/deacetylation and ubiquitination in the TGFβ-induced reduction of TCF7l2 protein. The discussion is detailed and includes a critical view about experimental limitations.

In summary, this manuscript contains interesting new data about the interplay between two important signaling pathways in fibrosis and is very relevant for researchers working in the field of fibrotic diseases.
The main body of data is convincing and well controlled. The experiments regarding the mechanisms involved in the molecular regulation of TCF7l2 concentration are not so excessive, mainly the use of limited experimental setups, namely the use of one pharmacological inhibitor for each target (Smad3, histone deacetylase and the ubiquitin-proteasome system) restricts the informational value.
Alltogether the manuscript is sound and of high interest. I support its publication after addressing the following points:

Comments for the author

- To analyse the TGFβ influence on the nuclear localization of TCF7l2, the authors show in Fig. 4 immunhistochemical experiments. To my feeling, the extension of the biochemical protein studies using cytoplasmic and nuclear protein fractions on cells stimulated with TGFβ would be more convincing, as shown in Fig. 3 for unstimulated cells.
Furthermore, it is not clear to me, what is meant by the statement in lines 349/350: 'TGF-β signaling did not alter TCF7L2 protein subcellular distribution or nuclei-cytoplasm shuttle (Fig. 4D,E).'
I can not see an analysis of the cytoplasm/nucleus shuttling of TCF7l2.

- The influence of Smad signaling on TCF7l2, described from line 365 onward is not convincing. The authors treated the cells only with the inhibitor SIS3 which inhibits Smad3 only. A possible role of Smad2 phosphorylation and signalling, which is present in most cell types, is not ruled out. The possible part of TGFβ signaling by Smad 2 and 3 after binding to Smad4 may be studied more convincing in cells, after Smad4 knock-out (cell lines with mutated Smad4 or after CRIPR/Cas) or knock down (siRNA), which is necessary for both Smad2 and 3 mediated modulation of gene expression.

- Some literature which deals with TGFβ and Wnt signalling is not cited in the manuscript:
such as:
Requirement of TCF7L2 for TGF-beta-dependent transcriptional activation of the TMEPAI gene. Nakano N1, Itoh S, Watanabe Y, Maeyama K, Itoh F, Kato M. J Biol Chem. 2010 Dec 3;285(49):38023-33. doi: 10.1074/jbc.M110.132209.

TGFbeta3 inhibits E-cadherin gene expression in palate medial-edge epithelial cells through a Smad2-Smad4-LEF1 transcription complex Nawshad A, Medici D, Liu CC, Hay ED. J Cell Sci 2007;120:

Minor points:

In Fig2, the description of part E is missing.

In line 291, the authors describe results obtained with C3H10/1/2 cells and give Fig. 2SA,B as reference which shows results of NIH3T3 cells.

In lines384-387, a mistake in text editing occured.

In line 468, an error in sentence structure occurred.

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:

The paper by Contreras et al. investigates the regulation of TCF7L2 by TGF-β in mesenchymal stromal cells and fibroblasts. The authors show that TCF7L2 is downregulated in both of these cell types by TGF-β at the protein and RNA levels. They conclude that the inhibition of transcription is via the activity of HDACs, and that the inhibition of protein levels is via TGF-β- induced protein degradation via the proteasome. They show that this regulation is specific to these cell types and not evident in myoblasts. They conclude that TGF-β-induced TCF72 downregulation could be a useful target in tissue fibrosis. These results are novel and demonstrate the interplay between TGF-β and Wnt signaling in mesenchymal stem stromal cells.

Reviewer 1 Comments for the Author:

Overall, I think the study is of good quality and the data, for the most part, support the conclusions. There are a number of small issues with the data that need to be attended to as outlined below:

1. In Figures 1C and S1A the data are not in agreement. The RT-PCR data in Fig S1A shows readily detectable levels of Lef1 in FAPs, but this is not so in the qPCR data in Fig 1C. Why is this?

This is because the RT-PCR data presented in Fig. S1A shows the amplicons of each Tcf/Lef paralog after 35 cycles. Therefore, Fig. 1C and Fig. S1A data seems not to agree because the qPCR data (Fig. 1C) is relative to the Ct (or Cq) values of each gene in order to quantify the absolute amount of a target sequence. We have now specified in the figure legend the fact that the images represent the end product of the PCR after 35 cycles. The correct phrase is now presented in the legend to figure (Fig. S1A), in the first paragraph. Nevertheless, we expanded our Tcf/Lef expression analyses to further corroborate their differential expression. Therefore, we have accordingly included public RNAseq data (skeletal muscle tissue from Scott et al., 2019 and heart from Schafer et al., 2017) in our study. Please, see Fig. 1D. Also, new heatmaps are now provided in Fig. 1E, K. Please, refer to the *Results* section, Pages 10 and 11.

2. In Figure 1E it would be good to show the separate panels (i.e. the TCF7L2, the LN-α2 and the nuclei) so that it was clear which cells express TCF7L2.

We have followed the recommendation of the reviewer. Thus, we included nuclei staining in the LN-α2 panel to clarify which cells are expressing the TCF7L2 transcription factor within the skeletal muscle interstitial space. Therefore, we present an updated version of Fig. 2C.

3. In Figure S1F it is very hard to work out which groups of cells correspond to which tissue as the colors in the key are not clear. This needs to be improved.

We have improved the quality of the t-SNE plot of all cells collected by FACS and also added an overlay with the predominant cell type composing each cluster. We hope that the group of cells that correspond to each tissue is now more clear.

4. The authors show the bulk levels of TCF7L2 before and after injury for the skeletal muscle but not for the diaphragm. It would be good to show this for the diaphragm too.

We do not have an equivalent model of acute injury for the diaphragm similar to the glycerol injection in the *tibialis anterior* muscle. However, we previously showed that TCF7L2+ cells expand within the diaphragm of the *mdx* mice, and as a consequence, the bulk protein levels of TCF7L2 increased in muscles of the murine model for Duchenne Muscular Dystrophy (Contreras et al., 2016). Also, the total increase of TCF7L2 protein in muscle samples correlates with the amount of inflammation, damage, and fibrosis due to skeletal muscle chronic damage. Furthermore, we corroborated our findings related to the expansion of TCF7L2+ stromal progenitors in a mild-inflammatory model of neuromuscular disease using the ALS (hSOD1G93A)

murine model (Gonzalez et al., 2017). We hope that our answer to the limitations of acutely damaging the diaphragm is now clear.

5. The pSmad3 staining comes a bit out of the blue. The authors need to show pSmad3 staining in WT diaphragm as well as in the injured.

We have followed the recommendation of the reviewer. Thus, we show a new image with phospho-Smad3 staining in the diaphragm of both wild-type and *mdx* mice. Please, refer to Fig. 2F. Also, we have included a new quantification of the percentage of phospho-Smad3-positive cells in fibers and stromal cells in WT and *mdx* (Fig. 2G). Also, an updated version of the Fig. 2 legend is now presented.

6. In Figure 4G there is a curious result. Why after 48 hr does the SB525334 not inhibit TGF-ß's effects?

The reviewer raises a good point. Although there is a partial inhibition of about 20-25%, we do not know why the ALK5 inhibitor (SB525334) does not completely inhibit TGF-β-mediated repression of TCF7L2 after 48 h of co-treatment as it does at 24 h. In order to clarify this point, we added "relative % of TCF7L2" to the western blot (Fig. 4G). Nevertheless, we could speculate that TGF-β ligand is more stable and stays longer in culture compared to SB525334, or that TGF-β has a priming effect over the expression of TCF7L2 that is more stable and lasts longer than the inhibitory effect of SB525334 on ALK5 phosphorylation.

7. In Figure 5, the authors see no effect of the Smad3 inhibitor and conclude that the TGF-β effect is via a Smad-independent pathway. This is possible, but it is also possible that Smad2 and Smad3 are acting redundantly, as is often the case. CRISPR-induced knockout of the Smads would be the best way to test this.

We agree with the reviewer's comment. We were working to address this point with our collaborators, Fabio Rossi's lab from the University of British Columbia. However, because of the circumstances relating to the coronavirus pandemic not only our University but also theirs are closed until further notice. Hence, we think it will be hard, almost impossible, to perform CRISPR-induced knockout of the Smad2/4, and therefore, to further address this point in the foreseeable future. We believe that the requested experiment does not fundamentally change the major conclusions of our work. However, we have improved our statement in order to make clear to the audience the possibility raised by the reviewer. The new phrase is as follows: "*Overall, these results suggest that another, not yet identified, Smad-independent pathway may participate in TGF-β-mediated downregulation of TCF7L2 expression; or that rather than Smad3 activation, Smad2/Smad4 cofactors could be participating in TGF-β-mediated TCF7L2 downregulation.*" Please, refer to the *Results* section, Page 16, first paragraph.

8. TSA is not a very specific HDAC inhibitor. The authors should use a more specific inhibitor and also show that the effect in their cells is really at the level of transcription.

Trichostatin A (TSA) is a hydroxamic acid produced by the bacteria *S. hygroscopicus*. This compound is known to be a reversible inhibitor of histone deacetylases (HDACs), specifically class I and II but not class III, and therefore, it is used as a chromatin remodeling agent that enhances the pool of acetylated histones. We decided to use TSA because is one of the best known and validated targets for the development of antineoplastic agents.
To corroborate our results we performed analyses of the expression of *Tcf7l2*, including other genes, using an available public source of RNA sequencing data from recent work in lung fibroblasts that used both TSA and pracinostat (another validated pan HDAC inhibitor) (Fig. 5G) (Jones et al., 2019). Both TSA and pracinostat attenuate TGF-B- induced repression of *Tcf7l2* gene expression (Fig. 5E-G). Also, HDAC inhibition also blocked TGF-B-induced changes in TCF7L2-Wnt gene expression (Fig. S7). Interestingly, TSA and pracinostat also attenuate the TGF-B-mediated increase expression of extracellular matrix genes (Jones et al., 2019) (Fig. 5G). We present the results as a heat map (Fig. 5G). Please, refer to page 14, last paragraph, lines 381-384.
Also, histone deacetylase inhibitors (HDACi) have emerged as potential drugs or compounds to

be tried in pre-clinical and clinical studies to improve tissue regeneration and repair in muscular Dystrophies. Central to this idea is that PDGFRa+ cells are targeted by HDACi in muscular dystrophies. This is a pharmacological intervention (used in pre- clinical studies and clinical trials) that counters DMD progression by increasing regeneration through the inhibition of fibro-adipogenic degeneration while favoring FAPs and myogenic progenitors' intercellular communication. Accordingly, we have decided to include these studies in the reference section. Please, also refer to the *Discussion* section, Page 21, second paragraph.

9. The diagram in Figure 8 is a bit confusing as they should make it clear that the downregulation of TCF7L2 targets by TGF-ß is via the downregulation of TCF7L2 levels.

We have followed the recommendation of the reviewer. Accordingly, we present new data on gene expression of several validated Wnt/TCF7L2-targets following TGF-β treatment (Fig. 3K,M). Altogether, TGF-β induces both up-regulation and down-regulation of Wnt/TCF7L2-target genes in lung and cardiac fibroblasts (Fig. 3K,L). This data (heatmap) were generated from the work of Jones et al., 2019 and Schafer et al., 2017. Also, Fig. S2H shows the relative mRNA levels of several TCF7L2-validated target genes following TGF-β stimulation in MSCs. TGF-β stimulation increases gene expression of Nfatc1, Lef1, and Tcf7. However, TGF-β treatment reduces the expression of Tcf7l2, Tcf7l1, Sox9, and Axin2. That means that TGF-β differentially modulates the expression of TCF7L2-target genes. However, we agree that the model presented in Fig. 8 might not accurately represent these results, and therefore, a summary figure with an improved proposed model is now presented in Fig. 8. In this model most, of our observations are recapitulated and integrated. Moreover, we have revised the writing to make our statements clearer. These models are described on page 17, second paragraph, in the *Discussion* section.

Reviewer 2 Advance Summary and Potential Significance to Field:

The present manuscript shows new and interesting data about an interplay between Wnt and TGFβ signaling pathways in fibrotic diseases. The authors demonstrate in a consistent and well- arranged set of experiments the role TCF7l2 or TCF4 in the fibrotic remodeling of dystrophic muscles using a dystrophic mouse model. Subsequently, the authors exemplify that TGFβ1, as well as TGFβ2+3, are involved in the reduction of the cellular amount of the transcription factor TCF7l2, which is specific for mesenchymal cells in contrast to myoblasts. The necessity of the TGFβ receptor proteins in this process is proved by using a pharmacological inhibitor. Further studies give evidence for the involvement of protein modifications such as acetylation/deacetylation and ubiquitination in the TGFβ-induced reduction of TCF7l2 protein. The discussion is detailed and includes a critical view about experimental limitations.

In summary, this manuscript contains interesting new data about the interplay between two important signaling pathways in fibrosis and is very relevant for researchers working in the field of fibrotic diseases. The main body of data is convincing and well-controlled. The experiments regarding the mechanisms involved in the molecular regulation of TCF7l2 concentration are not so excessive, mainly the use of limited experimental setups, namely the use of one pharmacological inhibitor for each target (Smad3, histone deacetylase and the ubiquitin- proteasome system) restricts the informational value. Altogether, the manuscript is sound and of high interest. I support its publication after addressing the following points:

Reviewer 2 Comments for the Author:

- To analyze the TGFβ influence on the nuclear localization of TCF7l2, the authors show in Fig. 4 immunohistochemical experiments. To my feeling, the extension of the biochemical protein studies using cytoplasmic and nuclear protein fractions on cells stimulated with TGFβ would be more convincing, as shown in Fig. 3 for unstimulated cells. Furthermore, it is not clear to me, what is meant by the statement in lines 349/350: 'TGF-β signaling did not alter TCF7L2 protein subcellular distribution or nuclei-cytoplasm shuttle (Fig. 4D,E)'? I can not see an analysis of the cytoplasm/nucleus shuttling of TCF7l2.

We agree with the reviewer's comment. Therefore, we have improved the results of Fig. 4A using

subcellular fractionation of cytoplasm and nuclei, which shows that TCF7L2 expression is decreased in the cytoplasm and nucleus following TGF-β treatment. We have also deleted the following unclear phrase: "*or nuclei-cytoplasm shuttle*". Please, refer to line 343, second paragraph, page 13.

- The influence of Smad signaling on TCF7l2, described from line 365 onward is not convincing. The authors treated the cells only with the inhibitor SIS3 which inhibits Smad3 only. A possible role of Smad2 phosphorylation and signaling, which is present in most cell types, is not ruled out. The possible part of TGFβ signaling by Smad 2 and 3 after binding to Smad4 may be studied more convincing in cells, after Smad4 knock-out (cell lines with mutated Smad4 or after CRISPR/Cas) or knockdown (siRNA), which is necessary for both Smad2 and 3 mediated modulation of gene expression.

As well pointed out by the Reviewer, we agree that we have not addressed this question and acknowledge the reviewer's suggestions. In fact, we were working to address this point with our collaborators, Fabio Rossi's lab from the University of British Columbia. However, because of the circumstances relating to the coronavirus pandemic not only our University but also theirs are closed until further notice. Hence, it will be almost impossible to perform the suggested experiments and address this question in the foreseeable future. We believe that the requested experiment does not fundamentally change the major conclusions of our work. Nevertheless, we have improved our statement in order to make clear to the audience the possibility raised by the reviewer. The new phrase is as follows: "*Overall*, *these results suggest that another*, *not yet identified*, *Smad-independent pathway may participate in TGF-β-mediated downregulation of TCF7L2 expression*; *or that rather than Smad3 activation*, *Smad2/Smad4 cofactors could be participating in TGF-β-mediated TCF7L2 downregulation*." Please, refer to the *Results* section, Page 16, first paragraph.

- Some literature which deals with TGFβ and Wnt signaling is not cited in the manuscript: such as: Requirement of TCF7L2 for TGF-beta-dependent transcriptional activation of the TMEPAI gene. Nakano N, Itoh S, Watanabe Y, Maeyama K, Itoh F, Kato M. J Biol Chem. 2010 Dec 3;285(49):38023-33. doi: 10.1074/jbc.M110.132209.

TGFbeta3 inhibits E-cadherin gene expression in palate medial-edge epithelial cells through a Smad2-Smad4-LEF1 transcription complex. Nawshad A, Medici D, Liu CC, Hay ED.
J Cell Sci 2007;120:

The two references mentioned above were included and are described on page 7, second paragraph, (Nakano et al., 2010) and on page 8, first paragraph, (Nashaw et al., 2007); and accordingly cited in the *References* section.

The reviewer comments helped us to expand our search of the literature on the Wnt/TGF-β crosstalk, and therefore, we decided to add the following reference:

Association of β-catenin with P-Smad3 but not LEF-1 dissociates *in vitro* profibrotic from anti-inflammatory effects of TGF-β1. Xinrui Tian, Jianlin Zhang, Thian Kui Tan, J. Guy Lyons, Hong Zhao, Bo Niu, So Ra Lee, Tania Tsatralis, Ye Zhao, Ya Wang, Qi Cao, Changqi Wang, Yiping Wang, Vincent W. S. Lee, Michael Kahn, Guoping Zheng, and David C. H. Harris.

The above reference was included and is described on page 8, first paragraph, (Tian et al., 2013) and accordingly cited in the *References* section.

Minor points:

In Fig 2, the description of part E is missing.
We have added the missing description of Fig. 2E.

In line 291, the authors describe results obtained with C3H10/1/2 cells and give Fig. 2SA,B as a reference, which shows results of NIH3T3 cells.
The correct phrase is now presented in lines 291-292, first paragraph.

In lines 384-387, a mistake in text editing occurred.
We have fixed the mistake. Please refer to lines 384-387, first paragraph.

In line 468, an error in sentence structure occurred.
We have fixed the mistake. Please refer to lines 468-469, last paragraph.

Second decision letter

MS ID#: JOCES/2019/242297

MS TITLE: TGF-β-driven downregulation of the Wnt/β-Catenin transcription factor TCF7L2/TCF4 in PDGFRα+ fibroblasts

AUTHORS: Osvaldo Contreras, Hesham Soliman, Theret Marine, Fabio M.V. Rossi, and Enrique Brandan
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The paper by Contreras et al. investigates the regulation of TCF7L2 by TGF-β in mesenchymal stromal cells and fibroblasts. The authors show that TCF7L2 is downregulated in both of these cell types by damage-induced TGF-β at the protein and RNA level. They identify the mechanism. The results are novel and demonstrate interesting interplay between TGF-β and Wnt signalling in mesenchymal stem cells. They conclude that TGF-β-induced TCF72 downregulation could be a useful target in tissue repair and regeneration.

Comments for the author

The authors have substantially improved the manuscript with their revisions. They have answered my questions and queries with new data and have clarified the text.

I have no further concerns and consider that the paper is now suitable for publication in JCS.

Reviewer 2

Advance summary and potential significance to field

The revised manuscript entitled: “Extracellular TGF-β downregulates the expression of Wnt transcription factor TCF7L2/TCF4 in mesenchymal stromal cells and fibroblasts” by Contreras, Soliman, Theret, Rossi and Brandan does not meet completely the suggestions and my expectations, but the weighting of the molecular mechanisms by which TGFβ modulates TCF7L2 has been adopted according to the presented data.

Comments for the author

I would recommend the publication of the manuscript in its present form.