SMAD2 promotes myogenin expression and terminal myogenic differentiation.

Émilie Lamarche, Hamood AlSudais, Rashida Rajgara, Dechen Fu, Saadeddine Omaiche and Nadine Wiper-Bergeron
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AUTHORS: Emilie Lamarche, Hamood AlSudais, Rashida Rajgara, Dechen Fu, Saadeddine Omaiche, and Nadine Wiper-Bergeron

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and 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. 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 manuscript by Lamarche et al presents novel data pertaining to the function of Smad2 during the process of myogenesis. This is an important subject as it is becoming evident that Smad2 and Smad3 are not simply relays for the TGF-b signaling pathway. This manuscript attempts to provide new data to explain how Smad2 can influence myogenesis. The authors have taken advantage of most state of the art technics to test their hypothesis. Many genes display expression patterns that do not to fit with what is believed to be coherent with their known function (e.g. Smad2 and Smad3 during myogenesis being a perfect example). A better understanding of how Smad2 can modulate myogenesis in absence of TGF-b is important to understand the biology governing myogenesis.

Comments for the author

General comments:

The paper addresses important questions regarding the role of Smad2 in relation to myogenesis and the authors present new data that indicate an independence from TGF-b. The manuscript is well structured and easy to read. The manuscript has 8 figures with data from C2C12 cells and primary myoblasts which makes for an interesting comparison. The introduction is a good and to the point. The results section is well structured and the experiments are presented in a logical sequence.

The discussion could address more in depth some of the results presented between C2C12 and primary fibroblasts.

General criticisms:

In general most of the figures have the potential to be confusing because it is not always evident if the data is for the C2C12 cells or the primary myoblasts. This should be clarified on every figure. Sometimes you have to reread the legends to make sure that you get it right.

Although the entire premise of the manuscript is the TGF-b independent role of Smad2 during myogenesis, the authors do not address the fact that the serums (both FBS and HS) used throughout the manuscript contain plenty of TGF-b. How do they go around this issue? No data is presented to confirm that Smad2 is not being Pi in the C-terminal end. Some experiments use a truncated Smad2 which is ok but still it would nice to see if there is Pi-Smad2 present.

Along the same line of questioning, how do they eliminate Smad3 from the equation here? Is there a possibility that Pi-Smad3 could interfere? Have they looked at Pi-Smad3 in these experimental settings?

In addition, they over express Smad2 in C2C12 cells but what about the endogenous levels?
One control experiment that would possibly be interesting to see is the overexpression of a phosphomimic-Smad2 where the 2 serines at the C-terminus are mutated to glu or asp to see what happens under that situation in their experimental setup. It would further strengthen the conclusion of the paper.

Some of the data does not seem to be consistent: for example in Fig 4I Myogenin positive cells are increased in absence of Smad2 which is not what you would expect from the data in the C2C12 cells which show that Smad2 increases Myogenin. There is no discussion of this issue. Since Myogenin is decreased when Smad2 is floxed why would there be an increase in Myogenin positive cells?

In figure 7 panel F is somewhat confusing. This experiment is overexpressing both Smad2 and shSmad2 in the same cells to assess myogenin, so why overexpress and knockdown at the same time? In addition this result does not seem to match that in panel D where Klf4 does not increase myogenin very much in absence of Smad2.

Details to be addressed:

Line 77: states that Smad2 and Smad3 bind the same DNA RE. This statement is not correct and in fact Smad2 and Smad3 have highly different target genes with DNA RE that are specific. Some DNA RE require possibly both together but one cannot say that they are identical.

Line 97-99: Fig1 is not referred to correctly; it should be Fig 1B and at the end of line 99 insert Fig 1C.

line 139: in Fig 3 panels D & E are not labelled properly

line 161-2: it would be nice to have the % of what is regained since it is difficult to determine this from figure 4.

Line 477-484: fig 1 panel C only Smad2 is shown, myogenin is missing?

Line 507-520L fig 3 panel D and E are not indicated in the figure itself.

In figure 6 there is a big difference between the level of klf-4 protein following Smad2 ectopic expression in panel B and the Klf4 reporter assays of panel C (are there implications of this difference?). Also different colors should be used for Smad2 ectopic expression and the Smad2 KO data to make it easier for the readers.

Line 573: (E) it should be Reporter assay instead of transcription assay since they are different technics.

Some of the figures would benefit from having a different display of the results and to specify clearly which cells were used for each analysis. In general is it difficult to determine what is a significant difference in the figures, there are letters above columns such as a, b, c or ab without details as to what exactly they represent.

Reviewer 2

Advance summary and potential significance to field

Smad2 is a downstream transcription factor of TGFb superfamily signaling which is known to inhibit myogenic differentiation. In this manuscript, the authors identified that Smad2 is essential for proper myogenic differentiation during muscle regeneration. This is confirmed by C2C12 myoblasts, purified satellite cells, and satellite cell-specific Smad2 conditional knockout mice. This myogenic promotion by Smad2 is independent of TGFb signaling and cooperated with Klf4 which is activated by Smad2. Finally, the authors identified several myogenic inhibitors which were down-regulated by Smad2, explaining the myogenic effects of Smad2 on myogenesis.

Comments for the author

Smad2 is a downstream transcription factor of TGFb superfamily signaling which is known to inhibit myogenic differentiation. In this manuscript, the authors identified that Smad2 is essential for proper myogenic differentiation during muscle regeneration. This is confirmed by C2C12 myoblasts, purified satellite cells, and satellite cell-specific Smad2 conditional knockout mice. This myogenic promotion by Smad2 is independent of TGFb signaling and cooperated with Klf4 which is activated by Smad2. Finally, the authors identified several myogenic inhibitors which were down-regulated by Smad2, explaining the myogenic effects of Smad2 on myogenesis.

Overall, this is an interesting study, and finding of Smad2 regulating myogenic differentiation is novel. However, several issues need to be addressed before publication.

1. In Figure 2C, the cell number of Dmad2-overexpressing cells seems to be higher compared with the control cells. The authors should clarify whether known down and overexpression of Smad2 affect cell proliferation and apoptosis before undergoing differentiation.

2. Smad3 is also shown to be an essential factor in myogenic differentiation, indicating that Smad3 is a complementary factor of Smad2 in myogenesis. Therefore, it is interesting to know whether the double knockdown of Smad2 and 3 can significantly reduce myogenic differentiation.
3. Please confirm the RNA expression of Smad2-downstream negative regulators (Bmp4, Fgf2, Mstn, Igf1 Igfbp3) for myogenesis in WT and Smad2-KO satellite cells.
4. It is not so clear why Npnt gene was chosen out of several Klf4-target genes. In addition, the biological function of Npnt is not descrived.
5. Primer sequences used for this manuscript should be organized in a table.

First revision

Author response to reviewers' comments

We are pleased to resubmit our manuscript entitled " *SMAD2 promotes myogenin expression and terminal myogenic differentiation* " (DEVELOP/2020/195495) following your request for revisions. We wish to thank our two reviewers for their comprehensive review of our original manuscript and for their helpful comments. We are in particular very happy that Reviewers 1 found that our work is tackling an “an important subject”, and our work “presents novel data” and takes “advantage of most state-of- the-art technics to test their hypothesis”. Reviewer 2 found “Overall, this is an interesting study, and finding of Smad2 regulating myogenic differentiation is novel.”

In revising our manuscript, we have addressed all the comments and concerns raised by our reviewers. We have completed the author’s checklist and have ensured that our manuscript is compliant with journal policies and guidelines. Please find a detailed list of the corrections below. In the resubmitted manuscript, all changed text is indicated in blue to facilitate review of the revised manuscript.

Please note that our laboratories are currently functioning at 30% due to public health measures, and with the apparent second wave of COVID-19 infections in Canada, this situation is unlikely to change soon. This has undoubtedly limited our ability to access resources necessary for experimentations and made certain experiments more challenging to address. Nonetheless, we have made our best effort to fully address our reviewers’ comments, which we hope will make the manuscript publishable in *Development*.

Details of responses and corresponding revisions below.

REVIEWER 1

General criticisms:

1. In general, most of the figures have the potential to be confusing because it is not always evident if the data is for the C2C12 cells or the primary myoblasts. This should be clarified on every figure. Sometimes you have to reread the legends to make sure that you get it right.

Thank you for this comment. The figures are now labelled clearly to indicate which cell model is being used. We have also opted to use orange bars for all gain-of-function experiments where SMAD2 is overexpressed and blue bars when we are studying knockdown models. We hope this change will improve the readability of the manuscript.

2. Although the entire premise of the manuscript is the TGF-b independent role of Smad2 during myogenesis, the authors do not address the fact that the serums (both FBS and HS) used throughout the manuscript contain plenty of TGF-b. How do they go around this issue? No data is presented to confirm that Smad2 is not being Pi in the C-terminal end. Some experiments use a truncated Smad2 which is ok but still it would nice to see if there is Pi-Smad2 present.

We also expected that the serum used for growth and differentiation media contained sufficient TGFβ

to promote activation of SMAD2/3 and investigated this specifically in our paper (Lamarche et al. (2015), *Skeletal Muscle* 18(5):8 doi: 10.1186/s13395-05-0032-z). In this paper, we examined the phosphorylation status of SMAD2 and SMAD3 in our serum-containing media and found no detectable C-terminal phosphorylation of either factor in the absence of exogenous TGFß. We further confirmed this result by investigating SMAD2 phosphorylation in a time course through growth and differentiation and found no detectable phosphorylation. This new data is included as Figure 2K.

The results section was modified as follows: (page 5, lines 120-124) "Since myoblasts can produce TGFß ligands and do express TGF receptors, we examined SMAD2 phosphorylation in proliferating and differentiating myoblasts. We found no detectable C-terminal phosphorylation in these cells in the absence of exogenous TGFß (Fig. 2K) consistent with our previous observations (Lamarche et al., 2015), where SMAD3 was also found to not be phosphorylated in untreated cells."

The Figure 2 legend was modified as follows: (page 24, lines 506-509) "**(K)** Representative western blot of C-terminally phosphorylated SMAD2 (pSMAD2), SMAD2 and MyHC expression in proliferating and differentiating C2C12 cells for the indicated time points. C2C12 cells treated for 7h in the presence of 2 ng/ml TGF□is included as a positive control. CyPB is a loading control."

3. Along the same line of questioning, how do they eliminate Smad3 from the equation here? Is there a possibility that Pi-Smad3 could interfere? Have they looked at Pi-Smad3 in these experimental settings?

Indeed, SMAD3 is still present in our models. The expression and phosphorylation of SMAD3 in the absence of exogenous TGFß was examined in our publication (Lamarche et al. (2015), *Skeletal Muscle* 18(5):8 doi: 10.1186/s13395-05-0032-z). As with SMAD2, SMAD3 was not appreciably phosphorylated in the absence of TGFß. We also investigated any potential changes to *Smad3* expression levels in response to changes in SMAD2 (both overexpression and knockdown) and found no evidence of compensatory expression. This data can be found in Figure 2G and 2J (C2C12 model) and Figure 3F (primary myoblast knockout model). To emphasize this point, we have added the following statement to the results section: (page 5, lines 120-124) "Since myoblasts can produce TGFß ligands and do express TGF receptors, we examined SMAD2 phosphorylation in proliferating and differentiating myoblasts. We found no detectable C-terminal phosphorylation in these cells in the absence of exogenous TGFß (Fig. 2K) consistent with our previous observations (Lamarche et al., 2015), where SMAD3 was also found to not be phosphorylated in untreated cells."

4. In addition, they over express Smad2 in C2C12 cells but what about the endogenous levels?

Overexpression of SMAD2 results in an ~3-fold increase in protein expression over endogenous levels. This is included in Figure 2A and 2H, I, as well as in Figure 6B. mRNA levels relative to empty vector controls are also shown in Figure 2B.

5. One control experiment that would possibly be interesting to see is the overexpression of a phosphomimic-Smad2 where the 2 serines at the C-terminus are mutated to glu or asp to see what happens under that situation in their experimental setup. It would further strengthen the conclusion of the paper.

Indeed, this would be a very interesting experiment. Unfortunately, we are not able to conduct this experiment and do not wish to incur delays in publication. Our research laboratories are functioning at 30% capacity, with some stock issues, and it has proven challenging to obtain, or in-house manufacture, the phospho-mimetic version of SMAD2. Since the critique is worded as non-essential, we hope that not including this additional model is acceptable.

6. Some of the data does not seem to be consistent: for example in Fig 4I Myogenin positive cells are increased in absence of Smad2 which is not what you would expect from the data in the C2C12 cells which show that Smad2 increases Myogenin. There is no discussion of this issue. Since Myogenin is decreased when Smad2 is floxed why would there be an increase in Myogenin positive cells?

We agree with our reviewer that this result, and the way that we presented it, appears paradoxical and is at best confusing. We apologize for the lack of clarity. While SMAD2 is an important regulator of

myogenin expression, its loss does not completely prevent myogenin expression. We observe lower *Myog* levels in primary myoblasts lacking *Smad2* (Figure 3F, G) and impairment of myogenic differentiation. *In vivo*, repair at 7 days post-injury was reduced in the Smad2$^{SC-/-}$, and so we allowed repair to progress for another week to determine if the repair was critically impaired or simply delayed. Since myogenin expression follows a predictable pattern of induction during repair followed by downregulation as repair is achieved, we scored the number of myogenin+ cells as a way to measure the progression of repair – more simply, whether repair was still ongoing, at the 14 dpi time point. We thus interpreted the higher number of myogenin+ cells in the Smad2$^{SC-/-}$ as evidence that repair was delayed but not impaired. This is also supported by the normalization of the cross-sectional area measures. Since the myogenin counts can cause confusion and does not provide support beyond the restoration of the myofiber cross-sectional area, we have opted to deleted panel I from figure 4.

7. In figure 7 panel F is somewhat confusing. This experiment is overexpressing both Smad2 and shSmad2 in the same cells to assess myogenin, so why overexpress and knockdown at the same time? In addition this result does not seem to match that in panel D where Klf4 does not increase myogenin very much in absence of Smad2.

We thank our reviewer for this criticism and wholeheartedly agree with the assessment. The purpose of this experiment presented in Figure 7F was to demonstrate that SMAD2 is required for KLF4 to stimulate *Myog* promoter activity. However, we agree that the experimental system is not ideal and that the phenomenon is described by our data presented in Figure 7D and E. As such, we have opted to remove this panel altogether. Rather, we have included an additional condition to Figure 7 panel E, where both SMAD2 and KLF4 are added to the system, demonstrating that additional SMAD2 does not further enhance Myog promoter activity over KLF4 alone.

Minor comments

8. Line 77: states that Smad2 and Smad3 bind the same DNA RE. This statement is not correct and in fact Smad2 and Smad3 have highly different target genes with DNA RE that are specific. Some DNA RE require possibly both together but one cannot say that they are identical.

We regret this oversimplification. We have made the correction to now read "SMAD2 and SMAD3 are highly conserved and are activated similarly ~~and bind the same DNA response element in target promoters~~, however there is increasing evidence that these two transcription factors have divergent roles *in vivo* with functions beyond classical TGFß signaling." (page 4, lines 75-77).

9. Line 97-99: Fig1 is not referred to correctly; it should be Fig 1B and at the end of line 99 insert Fig 1C.

We are unsure which figure reference was found to be incorrect as lines 97-99 span the description of both Figure 1 and 2. To ensure that we have not made any errors, we have verified that Figure 1C is referenced at the end of line 97.

10. line 139: in Fig 3 panels D & E are not labelled properly

We have corrected the figure to include the missing panel labels. We thank our reviewer for bringing this to our attention.

11. line 161-2: it would be nice to have the % of what is regained since it is difficult to determine this from figure 4.

The lines referenced refer to the PAX7+ cell numbers in regenerating muscle (panel D), where there is no rescue. We assume our reviewer is referring to the regain of myofiber cross-sectional area and agree that expressing this as a % regained would improve readability. We have modified the text as follows: "One week after injury, WT muscle repaired efficiently, regaining a fiber cross-sectional area of 50.5 ± 0.07% of uninjured controls (Fig. 4B, C, white bars). *Smad2*$^{SC-/-}$ muscle, however, had impaired regeneration, with fiber cross-sectional areas significantly smaller than those of injured WT mice and recovering only 26.0 ± 0.13% of the cross-sectional area of uninjured controls (Fig. 4B ,C, blue bars)." (page 7, lines 163-167). For the 14 dpi data (panel G), we modified the text as follows: "At this time point, the injured WT myofibers regained 76.9 ± 0.24% of WT uninjured TA muscle cross-

sectional area, whereas *Smad2*$^{SC-/-}$ injured muscle regained 78.1 ± 0.10% of uninjured control cross-sectional area to levels comparable to WT muscle, suggesting that loss of SMAD2 causes a delay in muscle regeneration (Fig. 4F, G).” (page 7, lines 175-178).

12. Line 477-484: fig 1 panel C only Smad2 is shown, myogenin is missing?

We apologize. Myogenin westerns were used to confirm differentiation in panel B but were not quantified in panel C in the original manuscript. We have corrected this mistake. The quantifications are included in

Figure 1 panel C. Corresponding changes were made to the figure legend as follows: (page 24, lines 489-490) “**(C)** Quantification of SMAD2 and MYOG expression from (B) as compared to LC. n=3. For panels A and C, bars are means ± s.e.m, (biological replicates).”

13. Line 507-520L fig 3 panel D and E are not indicated in the figure itself.

We apologize for this mistake. We have added the panel labels to the figure for panels D and E.

14. In figure 6 there is a big difference between the level of klf-4 protein following Smad2 ectopic expression in panel B and the Klf4 reporter assays of panel C (are there implications of this difference?). Also different colors should be used for Smad2 ectopic expression and the Smad2 KO data to make it easier for the readers.

We thank you reviewer for this comment and correction. We have changed the colour of the bars for overexpression in this figure to orange to improve readability and comprehension. For the discrepancies between the graph in panel B and the reporter assay shown in panel C, we are comparing protein levels to the activity of the *Klf4* promoter. We did, however, take a close look at the data in panel B and identified a small error in the quantification. New values are now presented, which, while not changing the overall conclusion, do demonstrate a more robust stimulation of protein expression consistent with the representative blots.

15. Line 573: (E) it should be Reporter assay instead of transcription assay since they are different technics.

We have made the correction as follows: “**(E)** Reporter assay measuring activity of the *Myog* promoter in C2C12 cells in the presence of SMAD2 and KLF4 relative to controls. n=5.” (page 27, line 587-589)

16. Some of the figures would benefit from having a different display of the results and to specify clearly which cells were used for each analysis. In general is it difficult to determine what is a significant difference in the figures, there are letters above columns such as a, b, c or ab without details as to what exactly they represent.

While remaining in accordance with the journal policy on descriptive statistics, we have added additional labels to figures to clearly indicate which model system is being used to avoid confusion. We have also further clarified the meaning of bar labels in the methods section as well as relevant figure legends. Here is a list of changes made to address this point:

Page 17, lines 462-465, Methods: “…Asterisks are used to indicate statistically significant changes from a control group as follows: *p<0.05; **p<0.01, ***p<0.001 (Student t test). For multiple comparisons (ANOVA), lower case letters are used to label means, such that bars bearing different letters are statistically different from one another with a minimum p value of <0.05.”

Page 25-26, lines 536-551, Figure legend: “…**(C)** Average cross-sectional area of muscle fibers in WT and *Smad2*$^{SC-/-}$ mice injured as in (B). Black dot data points are male mice and white dots represent female mice. Means indicated with different letters are significantly different from one another at a minimum cut-off of p<0.05, n= 8 pairs for uninjured muscle, and n=7 for cardiotoxin injured muscle. **(D)** Number of PAX7+ cells per area of uninjured and injured TA from (B), n=3. **(E)** Representative western blot of SMAD2 protein expression in isolated satellite cells from WT and *Smad2*$^{SC-/-}$ hindlimb

14 days post- injury with cardiotoxin to the left TA muscle (14 d.p.i). Cyclophilin B is a loading control. **(F)** Representative images of cardiotoxin (CTX)-injured and uninjured TA muscle sections from WT and *Smad2*$^{SC-/-}$ mice after repair for 14 days. Scale bar = 50 µm. **(G)** Average cross-sectional area of muscle fibers in WT and *Smad2*$^{SC-/-}$ mice injured as in (F). Means indicated with different letters are significantly different from one another at a minimum cut-off of $p<0.05$, n= 5 pairs. Black dot data points are male mice and white dots represent female mice. **(H)** Number of PAX7+ cells per area of uninjured and injured TA from (F). Means indicated with different letters are significantly different from one another at a minimum cut-off of $p<0.05$, n=4 for WT and n=5 for *Smad2*$^{SC-/-}$. For panels C, D, G and H, bars are means ± s.e.m (biological replicates)."

17. The discussion could address more in depth some of the results presented between C2C12 and primary fibroblasts.

We have modified the discussion as follows: (page 11, lines 283-294): "Herein, we identify SMAD2 as a powerful regulator of terminal myogenic differentiation and fusion using overexpression in C2C12 myoblasts, primary myoblasts isolated from floxed mice and *in vivo* using a conditional null model. Overexpression of SMAD2 in C2C12 cells enhanced myogenic differentiation, increased myotube size and promoted myogenin and myomaker expression, while knockout of SMAD2 decreased myotube and myofiber size and reduced myogenin expression without changes in myomaker expression. There was strong concordance between in culture and *in vivo* models, we noted that overexpression of SMAD2 enhanced the differentiation index in C2C12 myoblasts, while loss of SMAD2 in primary myoblasts did not reduce it. This discrepancy, and the results of the *in vivo* regeneration experiments lead us to conclude that SMAD2 is involved in late myogenic differentiation and fusion and that while high levels of SMAD2 can enhance differentiation, its loss does not prevent differentiation from occurring. Indeed, SMAD2 gain and loss of function experiments…"

REVIEWER 2

1. In Figure 2C, the cell number of Smad2-overexpressing cells seems to be higher compared with the control cells. The authors should clarify whether known down and overexpression of Smad2 affect cell proliferation and apoptosis before undergoing differentiation.

We thank our reviewer for this comment. We agree that cell culture density can affect the efficiency of myoblast fusion, and for this reason, we have quantified the number of nuclei in both overexpression systems and in the knockouts. In Figure 2 (overexpression system) we have added a panel (new panel F) that shows that cell numbers in our cultures are not significantly different following overexpression of SMAD2. This is further emphasized in panel P (old panel N) where cell numbers are also indicted for the SMAD2 mutant. To further support this, we now provide a supplementary figure (Supplementary Figure 1) that includes Ki67 staining for C2C12 myoblasts cultures overexpressing SMAD2 (and their controls) and primary myoblasts isolated from the Smad2$^{fl/fl}$ model. We have also added a supplementary figure legend and methods to support this figure. BrdU staining is also shown for the C2C12 overexpression model as Supplemental Figure 1B. This data, along with the cell counts in Fig. 2P (overexpression model) and Fig. 3E (primary myoblast knock-out model) confirm that changes in cell number do not underlie the effects on myogenic differentiation that we describe. We have modified the text as follows:

Results section, page 5, lines 104-107: "The enhanced differentiation and fusion was not due to variations in cell numbers as these were unchanged by SMAD2 overexpression (Fig. 2F). Further, we did not observe any differences in the percentage of Ki67+ cells or BrdU uptake in SMAD2 overexpressing cells as compared to controls (Supplemental Figure 1A, B)."

Results section, page 6, lines 143-145: "The culture density was unaffected by loss of *Smad2* expression (Fig. 3E) and the percentage of Ki67+ cells was similarly unaffected (Supplemental Figure 1C)."

Figure legend, pages 24-25, lines 499-516: "… **(F)** Total nuclei per mm^2 for cultures differentiated as in (C). n=3. **(G)** *Smad3* and myogenic marker mRNA expression in myoblasts transduced as in (A) after induction to differentiate for one day (DM1). n=3. **(H)** Representative western blot of SMAD2 and MYOG expression in myoblasts transduced as in (A) after induction to differentiate for one day (DM 1).

Cyclophilin B (CyPB) is a loading control. **(I)** Quantification of western blots represented in (H). n=3. **(J)** RTqPCR analysis of *Smad3, Pax7, Cebpb, Myod1 and Myog* expression in myoblasts transduced as in (A) and cultured in growth medium. Data for *Smad2*-overexpressing cultures is shown as the means relative to controls indicated by the red line. n=3. **(K)** Representative western blot of C-terminally phosphorylated SMAD2 (pSMAD2), SMAD2 and MyHC expression in proliferating and differentiating C2C12 cells for the indicated time points. C2C12 cells treated for 7h in the presence of 2 ng/ml TGF¤ is included as a positive control. CyPB is a loading control. **(L)** Western blot of C2C12 cells transduced to express SMAD2 or a truncated SMAD2 lacking the C-terminal SSMS motif (SMAD2ΔSSMS). **(M)** Immunostaining for MyHC (green) cells from (L) differentiated for 4 days. Scale bar = 50 μm. **(N)** Differentiation index (# nuclei in MyHC+ cells/ total nuclei) from cells differentiated as in (M). n=3. **(O)** Fusion index (# nuclei found in MyHC+ cells with 2 or more nuclei/ # myotubes) from cells differentiated as in (M). n=3. **(P)** Cell culture density expressed as nuclei/mm^2 in images used to calculate (N, O). n=3. For panels B, D-G, I, J, N-O, bars are means ± s.e.m (biological replicates)."

2. Smad3 is also shown to be an essential factor in myogenic differentiation, indicating that Smad3 is a complementary factor of Smad2 in myogenesis. Therefore, it is interesting to know whether the double knockdown of Smad2 and 3 can significantly reduce myogenic differentiation.

We agree that the double knockout would be interesting. Unfortunately, we do not have the animal model nor the constructs in laboratory to conduct this double knockout experiment in myoblasts. Given that the pandemic has reduced research intensity with current allowable occupancy to 30%, completing this experiment would lead to an important delay in publication. However, because we agree that this is an interesting comment, we have expanded the manuscript Discussion to consider the possibility that SMAD2 and SMAD3 act similarly as pro-myogenic factors in the absence of TGFß. The changes are as follows:

Discussion, pages 12-13, lines 335-357: "A pro-myogenic role has also been identified for SMAD3 (Ge et al., 2011; Ge et al., 2012). As our work focuses on the function of SMAD2 during myogenic differentiation, we cannot exclude a role for SMAD3 in our studies. Indeed, SMAD3 has some pro-myogenic functions (Ge et al., 2011; Ge et al., 2012), and thus a knockdown of both SMAD2 and SMAD3 could potentially impair myogenic differentiation to a greater extent than SMAD2 alone. Indeed, given the known collaboration of SMAD3 with master transcription factors such as MYOD and OCT4, it remains possible that transcription factors such as MYOD could also recruit both SMAD2 and SMAD3 to target genes (Mullen et al., 2011).

To influence gene expression, SMAD2 must gain entry into the nucleus, a process that in the context of TGFß signaling requires both phosphorylation of SMAD2 and its interaction with the co-SMAD, SMAD4. While TGFß has been shown to regulate the interaction of SMAD2 with SMAD4 in a phosphorylation- dependent mechanism, the transcriptional output from SMAD2-dependent genes appears to be mediated more by the retention of phosphorylated SMAD2 in the nucleus, rather than its import (Schmierer and Hill, 2005). Indeed, TGFß signaling does not appear to regulate the nuclear import rate for SMAD2, but rather decreases its export from the nucleus (Xu et al., 2002). However, phosphorylation of C-terminal serine residues by the ligand-bound TGFß receptor is believed to induce a conformational change that allows both interaction with SMAD4 and more efficient interaction with DNA response elements in target promoters, a situation that is unlikely to happen in our current model. As such, in the absence of C- terminal phosphorylation, interaction with transcription factors such as MYOD may direct SMAD2 to gene targets promoting efficient myogenic differentiation, while TGFß signalling, and downstream interaction with SMAD4 would be predicted to drive a different, anti-myogenic gene expression program."

3. Please confirm the RNA expression of Smad2-downstream negative regulators (Bmp4, Fgf2, Mstn, Igf1, Igfbp3) for myogenesis in WT and Smad2-KO satellite cells.

As indicated in Figure 8, the SMAD2 downstream regulators *Bmp4*, *Fgf2*, *Mstn*, *Igf1* and *Igfbp3* were all downregulated in the primary myoblasts lacking Smad2$^{SC-/-}$ model in our screen. In response to this critique, we have validated these findings by RT-qPCR as requested in both the C2C12 model with SMAD2 overexpression and in the WT and Smad2$^{SC-/-}$ myoblasts. This data is now included in Figure 8C. The text of the figure legend has been modified as follows: (page 28, lines 611-613) **"(C,D)** RT-qPCR analysis of *Igbp3, Fgf2, Bmp4* and *Igf1* expression relative to controls (horizontal line) in cells

transduced as in (A) and differentiated for 24 hours. n=3 for SMAD2 overexpression and n=4 for *Smad2*$^{SC-/-}$. For panels C and D, bars are means ± s.e.m (biological replicates)."

The results section was modified as follows: (pages 10-11, lines 273-279) "To validate our findings, we performed RT-qPCR analysis in SMAD2-overexpressing C2C12 myoblasts that had been differentiated for one day (Fig. 8C). While *Mstn* expression was not significantly downregulated (highly variable) with SMAD2-overexpression (data not shown), all of the other candidate genes were consistently downregulated (Fig. 8C). In differentiating *Smad2*$^{SC-/-}$-derived primary myoblasts, while variability was increased, we observed upregulation of all factors, with *Bmp4* showing the most robust result (upregulated in all trials)."

4. It is not so clear why Npnt gene was chosen out of several Klf4-target genes. In addition, the biological function of Npnt is not descrived (sic).

In assessing the literature for regulators of myogenic fusion (and not differentiation), we opted to evaluate both myomaker and KLF4 expression. Npnt expression was selected as a downstream KLF4 target and known regulator of myoblast fusion (https://pubmed.ncbi.nlm.nih.gov/21316587/). This was stated in lines 200-201 (page 8) of the manuscript (revised version).

5. Primer sequences used for this manuscript should be organized in a table.

We have organized primer sequences used into two supplementary table for ease of access. Supplementary Table 1 includes primers used for RT-qPCR and Supplementary Table 2 includes sequences used for chromatin immunoprecipitation. We thank our reviewer for this suggestion.

Additional changes include inclusion of antibody validation information in the Supplemental methods file. We thank you for the opportunity to revise our manuscript and look forward to your decision on our work. We re confident that we have addressed all of the reviewers' critiques and hope the changes make our work acceptable for publication in *Development*.

Second decision letter

MS ID#: DEVELOP/2020/195495

MS TITLE: SMAD2 promotes myogenin expression and terminal myogenic differentiation.

AUTHORS: Emilie Lamarche, Hamood AlSudais, Rashida Rajgara, Dechen Fu, Saadeddine Omaiche, and Nadine Wiper-Bergeron
ARTICLE TYPE: Research Article

Happy Holidays! I am pleased to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

This study demonstrate that Smad2 play a role during myogenesis that is independent of TGF-b. This is important as it demonstrate that we do not understand everything regarding Smad2 & 3 which are mostly considered TGF-beta mediators.

Comments for the author

The authors have adressed all the comments that I raised in my first review and I am satisfied with the revised version.

Reviewer 2

Advance summary and potential significance to field

The finding of Smad2 regulating myogenic differentiation is novel in the field of myogenesis.

Comments for the author

The authors attempted to revise this manuscript based on the reviewers' requests.
This reviewer still wants to see whether double knockdown of Smad2 and Smad3 in myoblasts to see whether the knockdown phenotypes are exaggerated.