# Inhibitory SMAD6 interferes with BMP-dependent generation of muscle progenitor cells and perturbs proximodistal pattern of murine limb muscles

Hasan Asfour, Estelle Hirsinger, Raquel Rouco, Faouzi Zarrouki, Shinichiro Hayashi, Sandra Swist, Thomas Braun, Ketan Patel, Frederic Relaix, Guillaume Andrey, Sigmar Stricker, Delphine Duprez, Amalia Stantzou and Helge Amthor
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**Original submission**

First decision letter

MS ID#: DEVELOP/2022/201504

MS TITLE: Inhibitory SMAD6 interferes with BMP dependent generation of muscle progenitor cells and perturbs proximodistal pattern of murine limb muscles.

AUTHORS: Hasan Asfour, Estelle Hirsinger, Raquel Rouco, Faouzi Zarrouki, Shinichiro Hayashi, Sandra Swist, Thomas Braun, Ketan Patel, Frederic Relaix, Guillaume Andrey, Sigmar Stricker, Delphine Duprez, Amalia Stantzou, and Helge Amthor

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 BenchPressand 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. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the refereeâ€™s comments, and we will look over this and provide further guidance.

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*

An interesting, informative manuscript with a lot of data, though some correlational (with supporting data). Indicates BMP signalling is involved in regulating MPC fate through Hox gene expression and signalling and supports the MCT prepattern hypothesis of MPC differentiation. How muscle patterns are set up and maintained remains controversial and this manuscript provides evidence that advances the field and elegantly shows the important role of BMP signalling in muscle cell fate and differentiation and proposes how Hox genes may be involved in determining muscle identity.

*Comments for the author*

Might be nice to discuss how this work complements or differs from the Authors 2017 paper also using Smad6 overexpression to study role of BMPs in postnatal muscle development. There may be some overlap re: Figs5 and 6 and would be nice to discuss this in more detail.

Supp Fig 2 needs to be considered as a full Figure as an entire section of experiments relying on and referring to Supp Fig 2 is in the Manuscript.

Line 142 – Abrogation of BMP signalling dampens limb MPC proliferation… could this not be due to an induction of cell death?

Line 161 – what was the cause of the cell loss? Cell death or decreased cell proliferation? Line 173 says data not shown for apoptosis analysis which was negative, which lends weight to the idea of decreased cell proliferation. Please can this be incorporated into the Figures.

The Hox gene analyses suggests a strong correlation of HoxA and D expression with MPC fate. Is it possible to study muscle differentiation and BMP signalling in HoxA and HoxD mutant mouse limbs to confirm these correlations? Perhaps this is out of the remit of the current manuscript, but would certainly help support the later conclusions made.

Hox gene expression levels are changed in the Smad6 overexpression mouse limb experiments. If Hox gene levels are overexpressed in MPCs does this force early muscle differentiation and muscle splitting?

Line 294 – it would be helpful to see the Authors model for how their data fits into and expands the MCT prepattern model.

Line 308 – the Conclusion is interesting but not fully convinced the Authors have demonstrated that by the cells acquiring positional identity by expressing Hox codes this establishes muscle pattern prior to separation of premuscle masses into individual muscles. I think there is certainly a strong correlation but work looking at Hox mutants and muscle patterns will be needed to make this claim.

However, from Figure 5 and 6 its clear BMP signalling involved in muscle pattern and its loss leads to loss of some of the individual muscles or a failure of splitting or separation of premuscle masses into individual muscles.

Reviewer 2

*Advance summary and potential significance to field*

Asfour et al. investigates a potential cell autonomous role for BMP signalling in the developing limb muscles in mice. Their manuscript provides evidence that limb muscle progenitor cells are responsive to BMP signalling from the onset of their migration, and reports on the use of a genetic approach to overexpress Smad6 in limb muscle progenitor cells and inhibit BMP signalling. Blocking BMP signalling in limb muscle progenitors, but not in limb myocytes, impairs the distal migration of

muscle progenitor cells, reduces their proliferation, and accelerate their differentiation resulting in the specific loss of some autopod and zygopod muscles. The muscle patterning defect observed in Smad6- overexpressing muscles is reminiscent of that of Hoxa11/d11 knockout. The authors propose that BMP signalling acts partly through the control of Hox gene expression to provide positional information in muscle progenitor cells. They show that Hoxa11 and Pax3 are co-expressed in migrating muscle progenitor cells and further supported this observation by analysing sc-RNAseq data from developing chick and mouse limbs.

Overall, this study provides strong evidence for a cell-autonomous requirement for BMP signalling during mouse limb muscle development, a finding that confirms previous studies in the chick embryo and offers an additional control mechanism in the complex control of limb muscle development. The data presented is of very good quality and generally support the conclusions, although the evidence linking the defect observed in Smad6-overexpressing limbs to Hoxa/d gene expression and function is weaker. The manuscript would be strengthened if the Discussion elaborated further on the similarities and differences observed between the previously reported studies on BMP signalling and Hox gene function in limb muscles.

*Comments for the author*

Major comments:
1) In Fig. 1, quantification of the number of MyoD+ expressing pSmad at E11.5 should be provided, and triple immunofluorescence at E11.5 should be performed to determine whether the pSmad/MyoD population is differentiating (Myogenin positive). The expression of pSmad in the progress zone should be mentioned here. Also, the images presented in Fig.1 for Pax3, Pax7 and MyoD do not match the control images presented in Fig.4, making it difficult to compare control and Lbx1cre;RS6 limbs. In Fig. 4b, enlarged areas should be taken at the same PD level, so the images match the text description.

2) Effect of Smad6 overexpression on the migration of muscle progenitor cells: given that there are fewer Pax3+ cells in Lbx1cre;RS6 limbs, the graph in Fig. 3e should be presented as percentage of positive cells (not number). In addition, quantification comparing dorsal and ventral muscle masses should be provided. Figures 3a, 3d, and 7c all suggest a rerouting of MPCs from the dorsal to ventral muscle masses.

3) Effect of Smad6 overexpression on muscle patterning: The patterning defect reported at E14.5 and E18.5 is very specific, resulting in the loss of a few muscles of both ventral and dorsal muscle mass origin. These defects, although similar to the Hoxa11/d11 KO, were not reported in the Hoxa13 KO nor in the chick, suggesting that loss of BMP signalling in the mouse limb is not really similar to loss of Hoxa13 expression and may present differences with the avian system. This should be further discussed in the Discussion (see also point 6 below).

4) Cell autonomous versus non cell autonomous role of BMP signalling : In a previous publication Esteves de Lima et al. indicated that BMP signalling may operate on a subset of Pax7-positive, Pax3-independent fibroblasts with myogenic potential. The data presented in this study (Fig. 4) could be further discussed in light of this article.

5) Page 10, line 204-205: The data observed in the HSACre;RS6 limbs only demonstrate that BMP is required prior to muscle progenitor cell differentiation. It does not demonstrate that muscle patterning is pre-established as the authors suggest. Alternative explanations may be that sufficient progenitor cells reach their destination (migration and/or proliferation not affected), allowing them to be exposed to patterning cues.

6) Page 13, line 266: Again, I do not think that the current data strongly support the hypothesis that muscle progenitor cells are pre-patterned at an early stage of limb development: a) the scRNAseq data appear to show a closer correlation between Hoxa13 and Pax3, and Hoxa9-11 and Pax7; b) The Relationship between Pax3 and Hoxa11 is not convincing given that at E11.5 most cells that express Pax3 do not express Hoxa11 (fig. 7). c) previous publications are consistent with a PD pattern of Hoxa gene expression in the early stages of limb development with Hoxa13 expressed in the distal limb and Hoxa9-11 in the proximal limb. d) Swinehart et al showed that Hoxa11 was excluded from

muscle cells and only present in their surrounding connective tissue at E14.5. The authors would need to show that Pax3+ cells express distinct combinations of Hoxa genes at E10.5 to support the pre-pattern hypothesis.

Minor comments:
1) Fig. 5b and 5c are not needed.
2) Legend Fig. 3b: explain how counting is carried out.

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**First revision**

Author response to reviewers' comments

**Detailed responses to referees' comments**:

***Reviewer 1***
**Comments on Significance: "An interesting, informative manuscript with a lot of data, though some correlational (with supporting data). Indicates BMP signalling is involved in regulating MPC fate through Hox gene expression and signalling and supports the MCT prepattern hypothesis of MPC differentiation. How muscle patterns are set up and main- tained remains controversial and this manuscript provides evidence that advances the field and elegantly shows the important role of BMP signalling in muscle cell fate and differentiation and proposes how Hox genes may be involved in determining muscle identity."**

We thank the reviewer for this comment, reassuring us that our data are suitable for publication.

**Comment: "Might be nice to discuss how this work complements or differs from the Au- thors 2017 paper also using Smad6 overexpression to study role of BMPs in postnatal muscle development. There may be some overlap re: Figs5 and 6 and would be nice to discuss this in more detail."**

We thank the reviewer for his very helpful comment. Indeed, in Stantzou *et al*. 2017 we studied the cell-autonomous effect of BMP signaling during postnatal muscle growth using *Pax7*$^{CreERT2}$ as driver to overexpress *SMAD6* in muscle satellite cells. In- deed, the effect of BMP signaling on muscle precursors seems to be conserved when comparing herein prenatal work to previously published postnatal work.
We have included the following paragraph in the discussion:

*"In previous work, we showed that satellite cell-specific overexpression of SMAD6 or knockout of Alk3, or overexpression of the BMP antagonist Nog in postnatal mice de- creased proliferation of satellite cells, diminished their accretion during myofiber growth and retarded muscle growth, whereas overexpression of SMAD6 exclusively in terminally differentiated myofibers did not affect satellite cell dependent muscle growth (Stantzou et al., 2017). Together with herein presented results, this confirms that BMP signaling acts in a similar cell-autonomous manner in MPCs during prenatal and postnatal development."*

**Comment: "There may be some overlap re: Figs5 and 6 and would be nice to discuss this in more detail."**

Yes, the effect of BMP signaling on muscle patterning, when examining at the end of embryonic development, E14.5, thus at the end of primary fiber development (Fig. 5 [now being Fig. 6]) is identical to the end of fetal development, E18.5, thus et the end of secondary fiber development.
We have added a paragraph in the discussion:

*"We found that the patterning defect in Lbx1*$^{Cre}$*;RS6 limbs persisted from embryonic to fetal stages, showing i) that secondary myogenesis cannot compensate for embry- onic muscle defects, and ii) remaining muscles continue to grow despite persistent inhibition of BMP signaling."*

**Comment: "Supp Fig 2 needs to be considered as a full Figure as an entire section of experiments relying on and referring to Supp Fig 2 is in the Manuscript."**

Thank you very much for this comment. We have now included previous Fig. S2 as the new Fig. 2 into the manuscript.

**Comment: "Line 142 – Abrogation of BMP signalling dampens limb MPC proliferation…could this not be due to an induction of cell death?"**

We thank the reviewer for this comment. In fact, we had already performed cell death experiments, however, data were not included in the first submission. We would like to apologize for this shortcoming. We have now included data of immunostaining for cleaved Caspase-3 on E10.5 limb buds which did not reveal any signs of apoptosis in the limb mesenchyme (novel Fig. S2a). The latter results allowed us to conclude that the effect of BMP signaling is caused by decreased proliferation.
We have added the following sentence in the results part:

*"The cell death marker cleaved Caspase-3 was absent in E10.5 limb mesenchyme in both genotypes, whereas it was present at trunk level and, as expected, at interdigital positions of E12.5 autopods **(Fig. S2a)**."*

**Comment: "Line 161 – what was the cause of the cell loss? Cell death or decreased cell proliferation? Line 173 says data not shown for apoptosis analysis which was negative, which lends weight to the idea of decreased cell proliferation. Please can this be incor- porated into the Figures."**

We agree with the reviewer, please see also above. In addition: When looking at cell numbers, it becomes clear that PAX7$^+$ and MYOD$^+$ cells increase in number at the expense of PAX3$^+$ cells at E11.5, meaning that MPC number remains stable, arguing against cell death, and in agreement with findings at E10.5.
We have specified this with the following sentence:

*"We found a precocious conversion of PAX3$^+$ cells towards PAX7$^+$ and MYOD$^+$ cells in E11.5 Lbx1$^{Cre}$;RS6 limbs: the total number of PAX3$^+$ cells decreased by 85%, whereas the total number of PAX7$^+$ cells increased by 64% and the MYOD$^+$ cells by 46% **(Fig. 5a-e)**, thus the total PAX3/PAX7/MYOD population remained stable."*

**Comment: "The Hox gene analyses suggests a strong correlation of HoxA and D expres- sion with MPC fate. Is it possible to study muscle differentiation and BMP signalling in HoxA and HoxD mutant mouse limbs to confirm these correlations? Perhaps this is out of the remit of the current manuscript, but would certainly help support the later conclu- sions made."**

We agree with the reviewer. Some conditional *Hox* mouse models are available and could be used for such experiments, and yes, this would be extremely helpful to sup- port our conclusion. However, it will not be feasible for this study. We have therefore tuned down our conclusion at the end of the discussion:

*"The expression of the HOX code in MPCs may indicate that positional identity is established prior to the splitting of premuscle masses into individual muscles. Future loss- and gain-of-function experiments are required to directly test the function of Hox gene expression in MPCs."*

**Comment: "Hox gene expression levels are changed in the Smad6 overexpression mouse limb experiments. If Hox gene levels are overexpressed in MPCs does this force early muscle differentiation and muscle splitting?"**

Such GoF experiments, sadly, are currently out of our reach for this study and would remain for another study in the future. However, *Hox* GoF experiments were previ- ously performed in chick limbs.
We have added a sentence in the discussion:

*"Previous work on chick limb MPCs showed that Hoxa11 and Hoxd13 blocked expres- sion of*

*MyoD, and that Hox gain-of-function experiments resulted in distorted limb muscle patterning (Yamamoto and Kuroiwa, 2003).”*

**Comment: “Line 294 – it would be helpful to see the Authors model for how their data fits into and expands the MCT prepattern model.”**

We thank the reviewer for this comment. We have included a model **(Fig. S9)**.

**Comment: “Line 308 – the Conclusion is interesting but not fully convinced the Authors have demonstrated that by the cells acquiring positional identity by expressing Hox codes this establishes muscle pattern prior to separation of premuscle masses into individual muscles. I think there is certainly a strong correlation but work looking at Hox mutants and muscle patterns will be needed to make this claim. However, from Figure 5 and 6 its clear BMP signalling involved in muscle pattern and its loss leads to loss of some of the individual muscles or a failure of splitting or separation of premuscle masses into individ- ual muscles.”**

We agree with the reviewer’s comment. We have rephrased the conclusion and parts of the discussion in this respect. See above.

*Reviewer 2*

**Comments on Significance: ...Overall, this study provides strong evidence for a cell-autonomous requirement for BMP signalling during mouse limb muscle development, a fin- ding that confirms previous studies in the chick embryo and offers an additional control mechanism in the complex control of limb muscle development. The data presented is of very good quality and generally support the conclusions, although the evidence linking the defect observed in Smad6-overexpressing limbs to Hoxa/d gene expression and func- tion is weaker. The manuscript would be strengthened if the Discussion elaborated further on the similarities and differences observed between the previously reported stu- dies on BMP signalling and Hox gene function in limb muscles.“**

We thank the reviewer for their encouraging comment. We have reworked the dis- cussion. Please see also below.

**Comment 1: “In Fig. 1, quantification of the number of MyoD+ expressing pSmad at E11.5 should be provided….”**

We profoundly thank the reviewer for this comment. We tried to quantify the number of $MYOD^+/pSMAD^+$ at E11.5. However, this proved to be difficult, as there is an expression range from very weak to more profoundly positive in pSMAD staining. We are afraid that such quantification results will be influenced by experimenter subjectivity.

We have rephrased the results accordingly and pointed at examples of different $pSMAD^+$ levels using different arrows in Fig. 1:

*“Emerging $MYOD^+$ cells showed pSMADs in varying levels, some were negative and others showed a continuum from faintly to strongly positive.”*

**Comment 1: “In Fig. 1, …, and triple immunofluorescence at E11.5 should be performed to determine whether the pSmad/MyoD population is differentiating (Myogenin positive).”**

We agree with the reviewer. We tried such triple staining several times over the years, as the primary antibodies could in theory be combined since they were purified from different species (mouse, rat and rabbit). However, our results were never clear, because of antibody interference, and images could not be reliably interpreted. We would like to apologize that we cannot answer the question of the reviewer.

**Comment 1: „In Fig. 1, ... The expression of pSmad in the progress zone should be mentioned here.“**

We thank the reviewer for this comment. We pointed this out in figure 1 and in- cluded a phrase in the result section:

*“Of note, $pSMAD^+$ non-myogenic cells were also found in the progress zone of E11.5 limb buds **(Fig. 1)**.”*

**Comment 1: „In Fig. 1, ... Also, the images presented in Fig.1 for Pax3, Pax7 and MyoD do not match the control images presented in Fig.4, making it difficult to compare control and Lbx1cre;RS6 limbs."**

We thank the reviewer for this comment. Indeed, E11.5 mouse embryos from Fig. 1 were maybe slightly more advanced than those of Fig. 5 (previous Fig. 4). Embryo development at these stages is very fast and slight differences cannot be prevented when using different time matings for experiments. However, we can assure that sacrifice was always at noon. In Fig. 4, *Lbx1$^{Cre}$;RS6 and RS6* controls had a similar development and can be directly compared. Further, when analyzing animals of same developmental stages, we took great care to obtain similar sectioning angles and sectioning levels throughout the experiments of this study. However, sections from different animals may not always match perfectly, which may cause some dif- ferences.

**Comment 1: „In Fig. 4b, enlarged areas should be taken at the same PD level, so the images match the text description."**

We would like to thank the reviewer for this important comment. We have modified the position of the enlarged area to consistently highlight the same area, allowing a better comparison of different genotypes.

**Comment 2: „Effect of Smad6 overexpression on the migration of muscle progenitor cells: given that there are fewer Pax3+ cells in Lbx1cre;RS6 limbs, the graph in Fig. 3e should be presented as percentage of positive cells (not number). In addition, quantification comparing dorsal and ventral muscle masses should be provided. Figures 3a, 3d, and 7c all suggest a rerouting of MPCs from the dorsal to ventral muscle masses."**

We thank the reviewer for these remarks. We believe that depicting total cell num- ber is a powerful mean to show that proximal cell number in *Lbx1$^{Cre}$;RS6* limbs is equal to controls, and that cell number decreases towards distally. However, we also appreciate the comment of the reviewer and included an additional graph in the novel Fig. S2 that also shows the percentages. Indeed, there is a smaller per- centage of cells in the distal parts and a tendency towards more cells in the proximal part when comparing *Lbx1$^{Cre}$;RS6* limbs and controls. This supports the argumenta- tion of a migration defect in *Lbx1$^{Cre}$;RS6* limbs.

We have added the following paragraph in the results section:

*"As total PAX3$^{+}$ cell number in Lbx1$^{Cre}$;RS6 limbs was lower than in RS6 limbs, we also analyzed the normalized distribution of PAX3$^{+}$ cells along the proximo-distal axis. Such analysis revealed a decreased presence of normalized PAX3$^{+}$ cell numbers in the distal parts of the limb, whereas there was a tendency towards increased cell numbers in the proximal parts **(Fig. S2b)**."*

We agree with the reviewer that number of cells in Figures 3a, 3d and 7c (now being Figures 4a, 4d and 8c) differ between dorsal and ventral premuscle masses, which could evoke rerouting of migration. However, even with great care during embed- ding of embryos/limbs, slight variations in sectioning level/angle can occur. Small cell clusters, such as premuscle masses at early limb bud stages, are particularly sensible to such "technical distortion". As requested by the reviewer, we have quan- tified MPCs number (average of three consecutive sections) in dorsal and ventral premuscle masses and found no difference.

We have added the following paragraph in the results section:

*"Next we determined the distribution of PAX3$^{+}$ cell in dorsal and ventral premuscle masses. We found a ~ 40% reduction in cell numbers within the dorsal and ventral premuscle masses when comparing Lbx1$^{Cre}$;RS6 limbs with RS6 limbs **(Fig. S2c)**, which accords with the loss in total PAX3$^{+}$ cell number in Lbx1$^{Cre}$;RS6 limbs **(compare with Fig. 4b)**. Cell numbers, however, were similar when comparing dorsal and ven- tral muscle masses of the same genotype **(Fig. S2c)**. Together, these data suggest that the*

*lack of BMP signaling in MPCs attenuated their proliferation and distal mi- gration, and data argue against a loss of MPCs by apoptosis or a rerouting of migra- tion.“*

**Comment 3: „Effect of Smad6 overexpression on muscle patterning: The patterning de- fect reported at E14.5 and E18.5 is very specific, resulting in the loss of a few muscles of both ventral and dorsal muscle mass origin. These defects, although similar to the Hoxa11/d11 KO, were not reported in the Hoxa13 KO nor in the chick, suggesting that loss of BMP signalling in the mouse limb is not really similar to loss of Hoxa13 expression and may present differences with the avian system. This should be further discussed in the Discussion (see also point 6 below).“**

We again thank the reviewer for this very important point. We have discussed this in more detail:

*“Swinehart et al. showed that Hoxa11 was not expressed by differentiated muscle cells at E14.5, but in cells surrounding primary muscle fibers, such as TCF4$^{+}$ connec- tive tissue cells. Whether HOXA11 colocalizes with MPCs (which also surround pri- mary muscle fibers), however, was not investigated (Swinehart et al., 2013). It has also been shown that Hoxa13$^{-/-}$ KO and Hoxa13$^{-/-}$/d13$^{-/-}$ dKO disturb autopod deve- lopment (Fromental-Ramain et al., 1996). We here found both, Hoxa13 and Hoxd13, being expressed by MPCs. However, we here we examined scRNAseq data sets from whole limb buds, which did not allow us to specify which MPC subpopulation (e.g. autopod MPCs) expressed which HOX code. Thus, it remains to be determined whe- ther Hox gene expression in MPC follows the collinearity in the developing limb. We can therefore only speculate about the exact role of Hox genes in developing muscle, and how their expression relates to BMP signaling. Previous work on chick limb MPCs showed that Hoxa11 and Hoxd13 blocked expression of MyoD, and that Hox gain- of-function experiments resulted in distorted limb muscle patterning (Yamamoto and Kuroiwa, 2003). Many questions, however, remain unresolved: does the HOX code control MPC proliferation, myogenic lineage progression and muscle splitting? Do MPCs, through HOX code, acquire positional identity and establish a muscle pre- pattern? Alternatively, herein observed loss in specific muscles may simply result from a tissue default that is caused by insufficiently generated precursors.“*

**Comment 4: „Cell autonomous versus non cell autonomous role of BMP signalling : In a previous publication Esteves de Lima et al. indicated that BMP signalling may operate on a subset of Pax7-positive, Pax3-independent fibroblasts with myogenic potential. The data presented in this study (Fig. 4) could be further discussed in light of this article.“**

We would like to thank the reviewer for this comment.

We have devoted a new paragraph in the discussion to this point:

*“The majority of MPCs is derived from migratory and Pax-3 dependent MPCs of so- mite origin. However, recent work demonstrated a dual origin of MPCs in the deve- loping limb: a small population of MCT cells are integrated into myotybes at muscle tips close to tendons in chicken and mouse muscles (Esteves de Lima et al., 2021, Yaseen et al., 2021) , a process being promoted by BMP signaling (Esteves de Lima et al., 2021). BMP gain- and loss-of-function experiments in chicken embryos de- monstrated that BMP signaling balances the fibroblast-myoblast conversion and consequently the muscle pattern (Esteves de Lima et al., 2021). We show here, that BMP signaling also regulates the somite-derived Pax-3 dependent MPC lineage in mouse limbs. Cell-autonomous inhibition of BMP signaling in somite-derived MPCs caused absence of entire muscles. Therefore, MCT depends on the presence of so- mite-derived MPCs and are lost secondarily when muscle fails to develop. Further, the generation of the somite-independent muscle lineage depends on the presence of somite-derived muscle.“*

We have also added a sentence in the results part of figure 1:

*„This confirms, in mouse, the presence of BMP-responsive myonuclei and MPCs at the tips of primary myofibers facing tendons, reminiscent to previous work in chick (Esteves de Lima et al., 2021).“*

**Comment 5: „Page 10, line 204-205: The data observed in the HSACre;RS6 limbs only de-**

**monstrate that BMP is required prior to muscle progenitor cell differentiation. It does not demonstrate that muscle patterning is pre-established as the authors suggest. Alter- native explanations may be that sufficient progenitor cells reach their destination (mig- ration and/or proliferation not affected), allowing them to be exposed to patterning cues."**

We agree with the reviewer. We have tuned down our "too ambitious" interpretation of our data.

We have changed the text and added a sentence in the corresponding results section:

*" These results may indicate that the information for the future muscle pattern is already present in MPCs before their differentiation. An alternative explanation may be that sufficient MPCs reached their destination (as migration and/or proliferation were not affected), allowing them to be exposed to patterning cues."*

We added to the discussion:

*"Alternatively, herein observed loss in specific muscles may simply result from a tissue default that is caused by insufficiently generated precursors."*

**Comment 6: „Page 13, line 266: Again, I do not think that the current data strongly sup- port the hypothesis that muscle progenitor cells are pre-patterned at an early stage of limb development: a) the scRNAseq data appear to show a closer correlation between Hoxa13 and Pax3, and Hoxa9-11 and Pax7; b) The Relationship between Pax3 and Hoxa11 is not convincing given that at E11.5 most cells that express Pax3 do not express Hoxa11 (fig. 7). c) previous publications are consistent with a PD pattern of Hoxa gene expression in the early stages of limb development with Hoxa13 expressed in the distal limb and Hoxa9-11 in the proximal limb. d) Swinehart et al showed that Hoxa11 was excluded from muscle cells and only present in their surrounding connective tissue at E14.5. The authors would need to show that Pax3+ cells express distinct combinations of Hoxa genes at E10.5 to support the pre-pattern hypothesis."**

We are grateful to the reviewer for their comments. We reworked the discussion to address these points. See also above, as similar comments were also raised by re- viewer 1.

We discussed in addition the specific concerns of reviewer 2:

*"Swinehart et al. showed that Hoxa11 was not expressed by differentiated muscle cells at E14.5, but in cells surrounding primary muscle fibers, such as TCF4$^{+}$ connec- tive tissue cells. Whether HOXA11 colocalizes with MPCs (which also surround pri- mary muscle fibers), however, was not investigated (Swinehart et al., 2013). It has also been shown that Hoxa13$^{-/-}$ KO and Hoxa13$^{-/-}$/d13$^{-/-}$ dKO disturb autopod deve- lopment (Fromental-Ramain et al., 1996). We here found both, Hoxa13 and Hoxd13, being expressed by MPCs. However, here we examined scRNAseq data sets from whole limb buds, which did not allow us to specify which MPC subpopulation (e.g. autopod MPCs) expressed which HOX code. Thus, it remains to be determined whe- ther Hox gene expression in MPC follows the collinearity in the developing limb."*

**Minor comment 1: „Fig. 5b and 5c are not needed."**

We agree and have removed them.

**Minor comment 2: „Legend Fig. 3b: explain how counting is carried out."**

We have added the following explanation in the figure legends of Fig. 3b (new Fig. 4b):

*"Total number of cells was counted on three consecutive longitudinal sections."*

Second decision letter

MS ID#: DEVELOP/2022/201504

MS TITLE: Inhibitory SMAD6 interferes with BMP dependent generation of muscle progenitor cells and perturbs proximodistal pattern of murine limb muscles.

AUTHORS: Hasan Asfour, Estelle Hirsinger, Raquel Rouco, Faouzi Zarrouki, Shinichiro Hayashi, Sandra Swist, Thomas Braun, Ketan Patel, Frederic Relaix, Guillaume Andrey, Sigmar Stricker, Delphine Duprez, Amalia Stantzou, and Helge Amthor
ARTICLE TYPE: Research Article

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

Reviewer 2

*Advance summary and potential significance to field*

This manuscript details a genetic approach to impair BMP signalling using overexpression of inhibitory Smad6, which demonstrates the requirement for BMP signalling in the production of limb muscle progenitor cells, their migration and differentiation. Misexpression of Smad6 results in specific patterning defects of limb skeletal muscles indicating that dosage of BMP signalling is important for limb muscle patterning.

*Comments for the author*

I appreciate the detailed and honest reply to this reviewer's comments and consider that the additional graphs and comments added to the text clarifies and opens the interpretation of data to alternative possibilities.