# Hox11-expressing interstitial cells contribute to adult skeletal muscle at homeostasis

Corey G. K. Flynn, Paul R. Van Ginkel, Katharine Hubert, Qingyuan Guo, Steven M. Hrycaj, Aubrey E. McDermott, Angelo Madruga, Anna P. Miller and Deneen M. Wellik
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**Original submission**

First decision letter

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MS TITLE: Hox11-expressing interstitial cells contribute to adult skeletal muscle at homeostasis

AUTHORS: Corey G.K. Flynn, Qingyuan Guo, Paul R Van Ginkel, Steven M Hrycaj, Aubrey E McDermott, Angelo Madruga, and Deneen M. Wellik

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. Each reviewer considers the contributions of Hoxa11-expressing cells to postnatal muscle creation to be potentially important. A shared concern was the extent of analysis performed to define Hoxa11-expressing cells so they can be best considered in context of other described muscle cell populations, in particular their co-expression of other key markers like those expressed by satellite cells. Other comments requested further quantification. I think these are important to address. By contrast I don't feel it would be necessary for the study to genetically deplete Hoxa11 cells and assess muscle homeostasis (Reviewer 1, comment #7). If you are able to revise the manuscript along the lines suggested, which is likely to 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 referee’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*

In this study, using Hoxa11 reporter mice lines, the authors identify a novel population of Hoxa11-expressing cells capable of contributing to skeletal muscle fibers during adult homeostasis but are not satellite cells. This finding is potentially interesting to help the understanding of muscle development and homeostasis.

*Comments for the author*

In this study, using Hoxa11 reporter mice lines, the authors identify a novel population of Hoxa11-expressing non-satellite cells polulation that are capable of contribute to skeletal muscle fibers during adult homeostasis. The genetic approaches used in this study is solid, and the finding is potentially interesting to help the understanding of muscle development and homeostasis, but some data suggesting that a subset of Hoxa11 cells could be satellite cells. Related to this concern, the authors did not fully characterize the molecular and cellular heterogeneity of this Hoxa11-expressing population. Importantly, the functional significance of this Hoxa11-expressing population in contributing muscle homeostasis and regeneration remains unknown. Given these limitations, I do not feel this study has met the standard for publication in Development. My specific concerns are listed below.

Major
1. The CD45-/CD31-/Sca1-/ITGA7+ gating has been a common way to sort satellite cells. In Fig 2C of PMID 27826411 (by Amy Wager group), more than 90% of ITGA+ cells are positive for Pax7-zsGreen reporter. Therefore, the FACS data shown in Fig 2E indicate that about 7.6/27.5=27% of ITGA7+ cells are GFP+. The authors should collect these ITGA7+/GFP+ cells for gene expression (RNA-seq) and functional analysis (immunostaining with PAX7, MyoD; in vitro culture followed by myogenetic differentiation, etc) to confirm that the Hoxa11-expressing cells are non-satellite cells.

2. Following the above question 1, there are 0.6/4.4 = 14% of VCAM1+ satellite cells positive for EGFP reporter. This data also suggests some satellite cells express Hoxa11 reporter.

3. The FACS data shown in Fig 2E raised my concern that some satellite cells may also express Hoxa11. Thereefore, I checked our in-house scRNA-seq data of mouse muscle regeneration. As shown in the below figures [data provided in confidence to the authors], indeed, we see Hoxa11 is expressed in the FAP (PDGFRa+) and satellite cells populations. I would encourage the author to sort the Hoxa11 reporter cells for scRNA-seq analysis to reveal further details of this heterogenous population. If performing scRNA-seq analysis is not possible, the authors should at least analyze the publicly available mouse muscle scRNA-seq data to characterize the heterogeneity and gene expression signatures of the Hoxa11 cells. It is critical to determine whether a subset of satellite cell also express hoxa11.

4. Fig 2F, quantitative analysis is needed.

5. Line 207 “At this time point, very low levels of tdTomato expression can be observed in a small number myofibers (Fig 3B)” and Line 224 “only faint tdTomato signal in a few muscle fibers”. Despite weak reporter signal in a small number of fibers, these data can also be interpreted in an alternative way that some (or a small portion) of the muscle fiber nuclei also express Hoxa11, but the reporter signal is diluted within the multinucleated myofibers. To address this concern, the author should transplant the TdT or GFP cells to the control mice that do not express any reporter. This experiment will demonstrate that the reporter signal of the myofiber is indeed contributed by the transplanted cells but not expressed from host myofiber nuclei. Another way to address this concern is to isolate myofibers followed by Tmx treatment in vitro to make sure that myofiber nuclei won't express reporter gene.

6. In figure 7, the author showed that the hoxa11 and pax7 do not co-express. This analysis is convincing. However, as described in Q1-3, the FACS data and scRNA-seq data suggest some satellite cells are GFP positive. How to explain such difference?

7. What is the biological significance of these hoxa11-expressing cells? If these hoxa11 cells are depleted (via DTR or other approaches), does it affect muscle homeostasis or regeneration?

Minor:
8. Line 174, typo, FAP should be "Itga7-"

Reviewer 2

*Advance summary and potential significance to field*

The manuscript by Flynn et al. uses a Hoxa11 reporter gene and a Hoxa11-CreER transgenic construct to identify and lineage trace a population of muscle interstitial cells that contribute to adult myofibers under homeostasis. FACS analysis was used to define the molecular properties of the Hoxa11 interstitial cell population, which were shown to be non-hematopoietic and non-endothelial. Sorting with additional markers suggested subpopulations of Hoxa11 positive cells shared markers with FAPs and SMMCs, but not satellite cells. Histology confirmed the Hoxa11 interstitial cell population is heterogeneous, as the Hoxa11 reporter gene partially overlapped with Twist 2, PDGFRa, and Tcf4 expressing cells but not Pax7 or CD31 expressing cells. Functionally, the Hoxa11 lineage is restricted to interstitial cells in the embryo, and then progressively contributes to all muscle fiber types in the adult. In addition, a nuclear localized lineage reporter suggests the Hoxa11 interstitial cell population fuses with myofibers in the adult. The authors conclude that the Hoxa11 expressing cells are a distinctive progenitor cell population that contributes to muscle homeostasis.

The identification of a new myogenic progenitor cell population is novel and exciting, and this discovery would be of great interest to the skeletal muscle field. However, there are a number of issues that must be addressed prior to publication.

*Comments for the author*

Major concerns.

1. What is the relationship between Hoxa11, Tw2, PDGFRa, and Tcf4 expressing interstitial cells? The histology in Fig 2 and the fiber-type analysis in Fig 6 argue cells in the Hoxa11 lineage encompass a heterogeneous population that includes a subset of Tw2-progenitors and a subset of FAPs but not all. A full quantification of single- and double-positive cells in Fig 2F-J would be a good starting point to tease apart what the Hoxa11 lineage is or is not. The FACS analysis is equally vague. The FACS plots are not clear for the non-expert. Fig 2A-E could be moved to Supplemental Data and replaced with a set of graphs that clearly shows the percent of the Hoxa11 interstitial cell population that overlaps with hematopoietic, endothelial, FAP, and satellite cell markers. It is unclear why the authors didn’t also isolate mononucleate Hoxa11 cells by FACS, and then use scSeq to define the progenitor cell population. On this same note, the comparisons between Hoxa11 and Tw2 progenitors is difficult to understand when the fiber-type analysis was done at 8 weeks for Hoxa11 (Fig 6) and 16 weeks for Tw2 (Liu et al). The contribution to each fiber type should also be quantified (Fig 6).

2. The lineage trace with H2B-mCherry is a great experiment. But without a series of double labels, it is not possible to conclude Hoxa11 nuclei were present in myofibers. The Pax7/Hoxa11 double label in 7H could be moved to Fig 5, and a Hoxa11/Mef2c double-label (and preferably additional nuclear marker of myofibers) must be performed to conclude the Hoxa11 positive nuclei are in fact in myofibers.

3. A Hoxa11 lineage trace was most likely done in the context of a muscle injury. What was the result? Why has that not been addressed? This could be a key distinguishing factor between Hoxa11 progenitors and Tw2 progenitors.

Minor concerns.

1. Fig S8. The lack of critical “not” in the legend title nullifies the premise of the entire manuscript.

2. Is eGFP expression really maintained throughout life? Or just through 8 weeks?

3. Is Hoxa11 expressed in all forelimb muscles? Only one cross section is shown in Fig 1, which can’t possibly include all muscles. A diagram in the figure of the muscles examined would be extremely helpful.

4. Line 156. Please clarify the time point and markers used to substantiate the statement “Hoxa11 expression in muscle is only in the interstitial cells”.

5. Line 206. “images show tdTom expression in tendon, muscle, and bone”. Where is each tissue in the figure? What markers were used to distinguish the tissues?

6. Line 235. Hindlimbs were comparable to forelimbs. Where is the quantification or other details to substantiate this statement?

7. Discussion. There is a nice survey of the known interstitial cell types in the muscle. What is lacking is a discussion that synthesizes Hoxa11 progenitors with these other cell types. How do the authors predict the Hoxa11 progenitors contribute to homeostasis and regeneration? Why does Hoxa11 label so many (seemingly) non-overlapping cell populations? Is there something significant about cellular contributions to type 1 myofibers? Some discussion about mechanism would help put these discoveries in context.

Reviewer 3

*Advance summary and potential significance to field*

In the manuscript “Hox11-expressing interstitial cells contribute to adult skeletal muscle at homeostasis”, Flynn et al. claim Hoxa11-expressing cells represent a non-satellite cell source of myogenic progenitors that contribute to myofibers attached to zeugopods during homeostasis. The authors take a strong genetic approach for permanent labeling of Hoxa11-expressing cells with either a cytoplasmic or a nuclear fluorescent protein and trace the fluorescent markers into myofibers to different extents depending on the skeletal muscle group.
While the concept that interstitial cells contribute to myofiber homeostasis is certainly not new (e.g. Twist2 cells, which the authors demonstrate are largely overlapping with the Hoxa11 cells), this body of work represents an significant addition to our body of knowledge pertaining to non-canonical cell sources contributing to muscle fibers.

*Comments for the author*

Major concerns

1) The authors wish to exclude the possibility of “transport of tdTomato+ cytoplasm or tdTomato mRNA” accounting for the lineage-labeling of myofibers.
Hence, they included a nuclear Cre reporter, i.e. H2B-mCherry in addition to a cytoplasmic tomato reporter. However, this approach fails to exclude the possibility of mRNA transport. Just like mRNA enconding cytoplasmic tdTomato H2B-mCherry mRNA molecules should exist in the cytoplasm of Hoxa11-expressing cells being constitutively transcribed from the Rosa26 locus after Cre-mediated recombination. Transport of H2B-mCherry mRNA into myofibers could also result in mCherry positive myonuclei after translation in the muscle fibers and transport into the myonuclei. The authors make an additional argument (page 11, lines 263-266) but that is rather speculative without knowing differences for example in exosome production between Hoxa11 and Pdgfra expressing cells. The authors could attempt to detect the “genomic scar” left behind at the Rosa26 locus after Cre-mediated recombination specifically in myofiber nuclei if they wish to obtain definitive

evidence. Alternatively, they should soften their conclusions throughout the manuscript regarding the addition of nuclei from Hoxa11 cells into myofibers.

2) It appears that Hoxa11-expressing cells are much more prevalent during fetal and early post-natal development compared to the adult (Figure 1, panel A). This is very interesting and would benefit from additional characterization.
For this, the authors should quantify Hoxa11-expressing cells at the different timepoints they have analyzed.

3) The authors use both flow cytometry and immunofluorescence analyses to analyze, which cell types Hoxa11-expressing overlap or not overlap with. The combination of these two approaches is quite strong as it gives both spatial as well as quantitative information. For Twist2+ cells, a flow protocol is not available and the authors supply a representative image of co-immunofluorescence for Twist2 and Hoxa11-GFP. Based on these results the authors state that "most Hoxa11eGFP-expressing cells also express Twist2". This qualitative observation would strongly benefit from quantification of the percentage of dually positive cells.

Minor concerns

1) Image panels in several figures including Fig. 1, Fig. 4, Fig. 5, Fig. 7 and supplemental figures S1, S2, S4, S5, S7 and S8 are missing scale bars.

2) Page 4, line 87: Work from Doug Millay's lab has provided strong evidence that muscle hypertrophy is compromised after satellite cell ablation which is in contrast to the cited work by the Peterson group. It would be good to reference that work as well to give a more complete account of the effects of satellite cell ablation on muscle hypertrophy.

3) Page 4, lines 91-92: The authors state that "neither somite-derived muscle progenitors nor satellite cells possess intrinsic muscle patterning information". Recent work from Yusuku Ono's laboratory has demonstrated a role for Hoxa10 in mediating positional memory in the satellite cell. The authors may want to alter that statement and should include discussion of the work from the Ono group (DOI: 10.1126/sciadv.abd7924),

4) The authors claim that Hoxa11 expression is restricted to muscle groups attached to the zeugopods. Since the current manuscript uses new mouse models including lineage-labeling of Hoxa11-expressing cells it would be beneficial to include data from e.g. the stylopod for comparison.
5) Page 7, line 156: The authors should be careful and avoid equating expression of the Hoxa11-CreERT2 induced lineage label with Hoxa11 expression.

6) The manuscript is overall very well written but should be spell checked as I stumbled across a typo (page 12, lines 290-292).

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**First revision**

Author response to reviewers' comments

As the comments on our original manuscript submitted more than 6 months ago were extensive, we would like to provide first an overall introduction to the revision, outlining new experiments. We focused our major efforts over the past five months addressing the issue of the specificity of the contribution of interstitial cells to myofibers, seemingly the crux of the main concerns. In brief, we have developed or added the following experiments:

1. Newly developed *in vitro* culture experiments show that Hoxa11iTom+ cells (lineage labeled by our Hoxa11CreERT2) are able to contribute to existing myotubes in culture. Developing a rigorous and reproducible assay for this took many months. In short, we find that Hoxa11iTom+ cells, alone, cannot form myotubes under any conditions we have tried. However, when added to differentiating myotubes (i.e. from Pax7-lineage or C2C12 cells

that began forming myotubes *in vitro*), Hoxa11iTom+ interstitial cells contribute to myofibers. (Not reported in the manuscript, adding the two cell types together at the same time led to much lower contribution then adding them after visible myotube formation). This supports the idea that Hoxa11-expressing interstitial cells are not stem cells, but progenitors that can contribute to existing fibers, as observed *in vivo*. Notably, contribution in culture was only observed in some myofibers and not all myofibers, and many mononuclear Hoxa11iTom+ cells adhered in culture near non-Tom+ myofibers. These data refute the idea that some non-specific release and uptake of mRNA or protein into fibers is the cause of this contribution. Further, the addition of an EV inhibitor to this culture did not prevent Hoxa11iTom contribution to myotubes. This now forms a new Figure 8 in the manuscript and also accounts for the bulk of Ms. Flynn's experimental time over these months.

2. *In vivo* lineage labeling in response to CTX injury shows that there is very little/no contribution to injured myofibers at 7 days after injury (even though the contralateral muscle shows a high level of contribution). Hoxa11tdTom+ contribution to larger (more highly regenerated) myofibers within the injury site is observed at 14 DPI. These data further strengthens the idea that Hoxa11-expressing cells are not stem cells but contribute to existing myofibers. This forms the new Figure 9 in the manuscript.
3. We add a new portion to Figure 2 re-analyzing two biological replicates of previously reported scRNA-seq data from hindlimb muscle and show that Hox11 genes have a similar profile to PDGFRa and Twist2 – in fibroblasts, while Pax7 and MyoD show predominant expression in satellite cells/myoblasts, also as expected. Notably, these data also clearly show that all genes examined did not appear completely exclusive by these analyses – with all having low representation in other clusters. These data are further expanded upon in new Supplemental Figure 4 and addresses the gist of several comments from reviewers. The very low representation of transcripts in scRNA-seq data clearly results in some statistical mistakes in clustering (not surprising when working with <10% of transcripts) and we believe cannot be viewed with the same rigor as counting >2000 individual cells in tissue sections.
4. Flow analyses, IF and other such assays were repeated with additional replicates. We have performed the flow analyses more than 10 times, obtaining similar results to those we previously submitted in the original manuscript but also not improving on the very poor separation of signal for CD34, congruent with the previous publications in which they were used. If the antibody used does not have high specificity and strong signal, there is no way to separate negative and positive completely. Thus, there is no way to determine absolutely positive or negative signals. Likewise, the IF assays are qualitatively rigorous, but we were not able to reproduce enough of these data for full quantification without spending significant additional time (and we already extended the revision deadline once). We modified our presentation and discussion to clearly discuss this. Qualitatively, our data are sound and highly reproducible.

After months of additional work to improve this manuscript, we hope it can now move forward to publication. We believe this represents an important new area of biology that deserves attention and much more investigation. We provide a more detailed response below:

Reviewer 1 Comments

Major

1. *The CD45-/CD31-/Sca1-/ITGA7+ gating has been a common way to sort satellite cells. In Fig 2C of PMID 27826411 (by Amy Wager group), more than 90% of ITGA+ cells are positive for Pax7-zsGreen reporter. Therefore, the FACS data shown in Fig 2E indicate that about 7.6/27.5=27% of ITGA7+ cells are GFP+. The authors should collect these ITGA7+/GFP+ cells for gene expression (RNA-seq) and functional analysis (immunostaining with PAX7, MyoD; in vitro culture followed by myogenetic differentiation, etc) to confirm that the Hoxa11-expressing cells are non-satellite cells.*

The separation in FACS is much too poor to do this reliably, but we developed an *in vitro* assay that rigorously shows that Hoxa11iTom+ cells contribute to existing myofibers (see introduction above).

*2. Following the above question 1, there are 0.6/4.4 = 14% of VCAM1+ satellite cells positive for EGFP reporter. This data also suggests some satellite cells express Hoxa11 reporter.*

After repeating these flow cytometry analyses nearly a dozen additional times, we can achieve no better separation than those published, not nearly allowing for a quantitative absolute analysis of presence and absence. Because of this, we have modified the revision to show only the fibroblast sub-type specification and rely on our counting of more than 2000 individual cells to support the rigor of our finding regarding the lack of overlap of Hoxa11 and Pax7 – this was the most highly rigorous analysis performed and was absolutely ZERO.

*3. The FACS data shown in Fig 2E raised my concern that some satellite cells may also express Hoxa11. Therefore, I checked our in-house scRNA-seq data of mouse muscle regeneration. As shown in the below figures, indeed, we see Hoxa11 is expressed in the FAP (PDGFRa+) and satellite cells populations. I would encourage the author to sort the Hoxa11 reporter cells for scRNA-seq analysis to reveal further details of this heterogenous population. If performing scRNA-seq analysis is not possible, the authors should at least analyze the publicly available mouse muscle scRNA-seq data to characterize the heterogeneity and gene expression signatures of the Hoxa11 cells. It is critical to determine whether a subset of satellite cell also express hoxa11.*

We could not agree more that it was critical to determine whether Hox-expressing cells are satellite cells, in part or in whole. Being of lateral plate mesoderm origin, this seemed quite unlikely, but given we are showing a wealth of evidence that these interstitial cells (ICs) are contributing to muscle fibers, there is no reason to imagine they may not be doing this by contributing to satellite cells. It is also possible, if unexpected, that Hoxa11 expression arises in satellite cells. Frankly, either of these outcomes would have made pursuing this work much easier. I wish it had been the case, but it is not. I will highlight that, among the more than 2000 individual cells stained, imaged and counted using reporter expression, lineage expression and IF-IHC, ZERO cells showed co-expression of Hoxa11 and Pax7. I'm not sure one can be more rigorous than that.

I'm somewhat surprised that this reviewer is suggesting that scRNA-seq might be considered somehow more rigorous than our analyses counting >2000 individual cells in tissue sections. Limitations to scRNA-seq technology include bias of transcript coverage, low capture efficiency, batch effects, and sequencing coverage (both variable read depth and lengths that lead to imperfect message identification and even less perfect clustering). At best case, scRNA-seq loses ~90% of the information available in any single cell. Further, the methods used to remove dying cells, the normalization prior to integration, and the integration parameters themselves have enormous impacts on the clustering of cell types. Even with the most rigorous and repetitive iterations of the data sets, these models make statistical decisions for each cell based on much less information than the cell really contains and is prone to errors. So, while this methodology is being utilized by many (including us) and can be clarifying in some cases, and hypothesis-generating in others, I have heard or read no sound rationale that scRNA-seq data can be used to overturn rigorous *in situ* tissue examination of protein expression. In fact, quite the opposite. After months spent rigorously analyzing our scRNA-seq data for other studies, we immediately seek to validate our findings *in vivo*!

We added new data in Figure 2 and Supplemental Figure 4 showing this point as it is important. As added data for reviewers and to show this is not due to cherry picking of data for publication, we also looked at where Pax7 is expressed in other scRNA-seq data that are published with reasonably usable interfaces to the data (April Pyle's laboratory, https://aprilpylelab.com/datasets/adult-hindlimb-muscle/). Based on these data, I believe it highlights the noise in scRNA-seq information sets. One could use these data to state that Pax7 is not really a satellite cell marker, but that it is also in smooth muscle cells, Schwann cells, FAPs and endothelial cells, but I think the usefulness of Pax7 as a satellite cell marker is well established by more rigorous methods.

Likewise, Hoxa11, Hoxc11, Hoxd11, PDGFRa and Twist2 can all be found in scRNA-seq data in the exact same populations, though at different levels (from Xi, H., et al., *A Human Skeletal Muscle Atlas Identifies the Trajectories of Stem and Progenitor Cells across Development and from Human Pluripotent Stem Cells*. Cell Stem Cell, 2020. **27**(1): p. 181-185). Combined with transcription factors being among the most poorly represented transcripts in scRNA-seq data, these analyses

must be taken with a grain of salt, so to speak – and critically, verified by more rigorous methods, like the quantified IF we report in this manuscript.

Thus, scRNA-seq data not withstanding, we maintain that our *in situ* data and extensive quantification (IF-IHC, reporter and lineage expression) is much more rigorous than both sc-seq and FACS.

*4. Fig 2F, quantitative analysis is needed.*

We were not able to get back to this analysis in the five months due to the focus of our efforts stated in the introduction. We clearly state the quantitative nature of this finding, but also add an analysis of scRNA-seq data to further support the cell types and overlap of fibroblast cells. In the discussion, we call out the important need for meaningful comparisons among the many interstitial subsets to be performed in future studies.

*5. Line 207 "At this time point, very low levels of tdTomato expression can be observed in a small number myofibers (Fig 3B)" and Line 224 "only faint tdTomato signal in a few muscle fibers". Despite weak reporter signal in a small number of fibers, these data can also be interpreted in an alternative way that some (or a small portion) of the muscle fiber nuclei also express Hoxa11, but the reporter signal is diluted within the multinucleated myofibers. To address this concern, the author should transplant the TdT or GFP cells to the control mice that do not express any reporter. This experiment will demonstrate that the reporter signal of the myofiber is indeed contributed by the transplanted cells but not expressed from host myofiber nuclei. Another way to address this concern is to isolate myofibers followed by Tmx treatment in vitro to make sure that myofiber nuclei won't express reporter gene.*

We look forward to pursuing transplant studies in another study in some years (and after we achieve funding for this project), but to address this issue, we hope the *in vitro* assay described in the introduction provides additional evidence on our finding and include these data in Figure 8. The new data showing that under no conditions we tried could we get isolated Hoxa11iTom+ cells to differentiate into muscle alone further supports Hoxa11 is not expressed in myoblast populations.

*6. In figure 7, the author showed that the hoxa11 and pax7 do not co-express. This analysis is convincing. However, as described in Q1-3, the FACS data and scRNA-seq data suggest some satellite cells are GFP positive. How to explain such difference?*

Please see the introduction and response to Comment 1 for scRNA-seq discussion. This is due to the limitation of flow cytometry data and scRNA-seq data. We have modified our presentation of flow data and confine this analysis to stromal populations due to this.

7. What is the biological significance of these hoxa11-expressing cells? If these hoxa11 cells are depleted (via DTR or other approaches), does it affect muscle homeostasis or regeneration?
This an outstanding question and one which we are currently validating our genetic models to pursue. We hope to continue to report on additional questions regarding this novel set of cells in future publications.

Minor:
*Line 174, typo, FAP should be "Itga"7-"*
This has been corrected, thank you.

Reviewer 2 Comments for the Author:
Major concerns.

1. *What is the relationship between Hoxa11, Tw2, PDGFRa, and Tcf4 expressing interstitial cells? The histology in Fig 2 and the fiber-type analysis in Fig 6 argue cells in the Hoxa11 lineage encompass a heterogeneous population that includes a subset of Tw2-progenitors and a subset of FAPs but not all. A full quantification of single- and double-positive cells in Fig 2F-J would be a good starting point to tease apart what the Hoxa11 lineage is or is not. The FACS analysis is equally vague. The FACS plots are not clear for the non-expert. Fig*

*2A-E could be moved to Supplemental Data and replaced with a set of graphs that clearly shows the percent of the Hoxa11 interstitial cell population that overlaps with hematopoietic, endothelial, FAP, and satellite cell markers. It is unclear why the authors didn't also isolate mononucleate Hoxa11 cells by FACS, and then use scSeq to define the progenitor cell population. On this same note, the comparisons between Hoxa11 and Tw2 progenitors is difficult to understand when the fiber-type analysis was done at 8 weeks for Hoxa11 (Fig 6) and 16 weeks for Tw2 (Liu et al). The contribution to each fiber type should also be quantified (Fig 6).*

Please see intro and responses above. We have added scRNA-seq analyses using existing data sets to place Hox11 in the context of other these cells.

2. *The lineage trace with H2B-mCherry is a great experiment. But without a series of double labels, it is not possible to conclude Hoxa11 nuclei were present in myofibers. The Pax7/Hoxa11 double label in 7H could be moved to Fig 5, and a Hoxa11/Mef2c double-label (and preferably additional nuclear marker of myofibers) must be performed to conclude the Hoxa11 positive nuclei are in fact in myofibers.*

We have added a set of panels with dystrophin staining to show the lineage labeled nuclei are in fact in myofibers. We believe the suggested addition of these data strengthens the report.

*3. A Hoxa11 lineage trace was most likely done in the context of a muscle injury. What was the result? Why has that not been addressed? This could be a key distinguishing factor between Hoxa11 progenitors and Tw2 progenitors.*

Excellent suggestion. We add initial analyses as a new Figure 9. We plan to more fully explore this in another manuscript, but initial results certainly support the idea that ICs are not stem cells, but a progenitor population that can contribute to existing myofibers. This is very strongly additionally supported by the *in vitro*/culture experiments which we believe also addresses this reviewer's point here.

3. Fig S8. The lack of critical "not" in the legend title nullifies the premise of the entire manuscript.

Figure S8 is now Figure S10 and this is corrected.

4. *Is eGFP expression really maintained throughout life? Or just through 8 weeks?*

We have modified this statement to 'through adult stages', but also add panels to a new Supplemental Figure 1 that shows Hoxa11eGFP expression at 7 months of age.

5. *Is Hoxa11 expressed in all forelimb muscles? Only one cross section is shown in Fig 1, which can't possibly include all muscles. A diagram in the figure of the muscles examined would be extremely helpful.*

A new Supplemental Figure 1 now fully supports this statement by examining each muscle in the forelimb.

*Line 156. Please clarify the time point and markers used to substantiate the statement "Hoxa11 expression in muscle is only in the interstitial cells".*

The time point for this statement is now clarified in this section.

*Line 206. "images show tdTom expression in tendon, muscle, and bone". Where is each tissue in the figure? What markers were used to distinguish the tissues?*

These tissue types are easily distinguished histologically, and the radius, ulna and tendons are now marked in edited Figure 3.

*Line 235. Hindlimbs were comparable to forelimbs. Where is the quantification or other details to substantiate this statement?*

We have further clarified and qualified this statement with additional details: “Hindlimbs from these animals were also examined and Hoxa11iTom+ lineage contribution is observed (high levels of contribution to the Gastrocnemius, moderate contributions to the Tibialis Anterior, and low contribution to the Soleus,...”

*Discussion. There is a nice survey of the known interstitial cell types in the muscle. What is lacking is a discussion that synthesizes Hoxa11 progenitors with these other cell types. How do the authors predict the Hoxa11 progenitors contribute to homeostasis and regeneration? Why does Hoxa11 label so many (seemingly) non-overlapping cell populations? Is there something significant about cellular contributions to type 1 myofibers? Some discussion about mechanism would help put these discoveries in context.*

We have extensively modified the discussion and include this important discussion based on added data.

Reviewer 3 Comments for the Author:
Major concerns
*The authors wish to exclude the possibility of “transport of tdTomato+ cytoplasm or tdTomato mRNA” accounting for the lineage-labeling of myofibers. Hence, they included a nuclear Cre reporter, i.e. H2B-mCherry in addition to a cytoplasmic tomato reporter. However, this approach fails to exclude the possibility of mRNA transport. Just like mRNA enconding cytoplasmic tdTomato, H2B-mCherry mRNA molecules should exist in the cytoplasm of Hoxa11-expressing cells being constitutively transcribed from the Rosa26 locus after Cre-mediated recombination. Transport of H2B-mCherry mRNA into myofibers could also result in mCherry positive myonuclei after translation in the muscle fibers and transport into the myonuclei. The authors make an additional argument (page 11, lines 263-) but that is rather speculative without knowing differences for example in exosome production between Hoxa11 and Pdgfra expressing cells. The authors could attempt to detect the “genomic scar” left behind at the Rosa26 locus after Cre-mediated recombination specifically in myofiber nuclei if they wish to obtain definitive evidence. Alternatively, they should soften their conclusions throughout the manuscript regarding the addition of nuclei from Hoxa11 cells into myofibers.*

Please see introduction and answer to previous comments; additional data strongly support our conclusion.

*It appears that Hoxa11-expressing cells are much more prevalent during fetal and early post-natal development compared to the adult (Figure 1, panel A). This is very interesting and would benefit from additional characterization. For this, the authors should quantify Hoxa11-expressing cells at the different timepoints they have analyzed.*

This is an interesting point and we have definitely found that Hoxa11-expressing cells decrease in all musculoskeletal tissue with age (skeleton, tendon and muscle), a point we have made in previous reports (but not quantified) in skeleton. We add a qualitative mention of this to the manuscript, but it would be an additional project to quantify. Rigor would require we quantify all muscles and at several locations and this would take months. We add qualitative commentary on this along with an additional later time point that qualitatively supports this finding the reviewer raises.

*The authors use both flow cytometry and immunofluorescence analyses to analyze, which cell types Hoxa11-expressing overlap or not overlap with. The combination of these two approaches is quite strong as it gives both spatial as well as quantitative information. For Twist2+ cells, a flow protocol is not available and the authors supply a representative image of co-immunofluorescence for Twist2 and Hoxa11-GFP. Based on these results the authors state that “most Hoxa11eGFP-expressing cells also express Twist2”. This qualitative observation would strongly benefit from quantification of the percentage of dually positive cells.*

In the course of the five months of this revision, we were not able to gather additional data to provide quantification, but do add sc-seq data to further support overlap of populations New intriguing data in muscle injury will examine this more closely in future publications.

Minor concerns

*Image panels in several figures including Fig. 1, Fig. 4, Fig. 5, Fig. 7 and supplemental figures S1, S2, S4, S5, S7 and S8 are missing scale bars.*

Scale bars have been added throughout.

*Page 4, line 87: Work from Doug Millay's lab has provided strong evidence that muscle hypertrophy is compromised after satellite cell ablation, which is in contrast to the cited work by the Peterson group. It would be good to reference that work as well to give a more complete account of the effects of satellite cell ablation on muscle hypertrophy.*

We have added references to the introduction to more completely convey the contrasting findings on this topic, including Doug Millay's.

*Page 4, lines 91-92: The authors state that "neither somite-derived muscle progenitors nor satellite cells possess intrinsic muscle patterning information". Recent work from Yusuku Ono's laboratory has demonstrated a role for Hoxa10 in mediating positional memory in the satellite cell. The authors may want to alter that statement and should include discussion of the work from the Ono group (DOI: 10.1126/sciadv.abd7924).*

*Note: we have removed information which was provided for the referees in confidence*

*The authors claim that Hoxa11 expression is restricted to muscle groups attached to the zeugopods. Since the current manuscript uses new mouse models including lineage-labeling of Hoxa11-expressing cells it would be beneficial to include data from e.g. the stylopod for comparison.*

We have modified our statement and referred to previous publications where this was reported. (Of note, many muscles attached to the zeugopod are in the stylopod region, but expression ends there in the limb musculature).

*Page 7, line 156: The authors should be careful and avoid equating expression of the Hoxa11-CreERT2 induced lineage label with Hoxa11 expression.*

We have carefully read the manuscript and tried to pay attention to this important point throughout.

*The manuscript is overall very well written but should be spell checked as I stumbled across a typo (page 12, lines 290-292).*

We have corrected this and gone over the manuscript carefully. We have undergone such extensive modification, I hope we have caught any additional typos, but will continue to proof this prior to publication.

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Second decision letter

MS ID#: DEVELOP/2022/201026

MS TITLE: Hox11-expressing interstitial cells contribute to adult skeletal muscle at homeostasis

AUTHORS: Corey G.K. Flynn, Paul R Van Ginkel, Katharine Hubert, Qingyuan Guo, Steven M Hrycaj, Aubrey E McDermott, Angelo Madruga, Anna P Miller, and Deneen M. Wellik
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. The reviewers indicate some typos and minor editing changes, and they suggest some points you may want to address in writing in a final submitted version or at proofing stage. If you are to make changes to the manuscript, please return within the next week if possible.

Reviewer 1

*Advance summary and potential significance to field*

The authors presented solid data to demonstrate that the Hox11a-expressing cells represent a previously uncharacteristic population that can directly contribute to muscle fiber nuclei. This finding is important for the future study of muscle homeostasis and development.

*Comments for the author*

The authors did extensive analysis to address my concerns on the initial version of the manuscript. I appreciate their effort and I can see that the quality of the current version of the manuscript has been much improved. The rigorous analysis of the expression pattern of Pax7 and Hoxa11 in ~2,000 single cell is convincing. This addressed one of my biggest concerns that some satellite cells could be Hoxa11+. The authors also did a careful analysis to show that the Hox11+ cells are not FAPs but likely to be positive for Twist2 cells. The data from their new in vitro assay is also convincing.

With all that, I am convinced that the expression of Hox11 marks a non-satellite cell population can contribute to muscle. In the meantime, there are still several limitations that I'd like to highlight. One question is, what is the cellular identity of these Hox11+ cells? From the data, these cells are made up of a heterogeneous population, with only a subset of cells can contribute to muscle nuclei. For instance, the PDGFa+/Hoxa11+ double cells do not become fiber nuclei. Therefore, what are the Hoxa11 cells are myogenic? And why? Another question is, whether and how the depletion of these cells affect muscle development, homeostasis, and/or repair.

Despite these limitations, I agree with the authors that they identified a previously unknown population cells that are myogenic. The data is solid, and the work was clearly presented. I would suggest the publication of this work and hope some of the questions can be addressed in the future studies by the authors or others.

Reviewer 2

*Advance summary and potential significance to field*

The identification of a new cell population that can contribute to the myogenic lineage is novel and exciting. This manuscript will be of great interest to the skeletal muscle field.

*Comments for the author*

Overall, the manuscript is greatly improved and the new experiments (in vitro fusion and injury) are exciting. A majority of my concerns have been addressed. I suggest that all abbreviations in the figures be explained in the figure legends (e.g. FAPs and SMMCs in fig 2). Quantification of the fiber types in fig 6 would also be nice to see (is there actually a fiber type preference or not?).

Reviewer 3

*Advance summary and potential significance to field*

No changes from my initial report of the authors' first submisssion.
*Comments for the author*

The authors have in large part addressed all my major concerns and the authors' revisions significantly solidify their' conclusions and hence strengthen the manuscript. However, one major concern is still outstanding. In the initial submission the authors had made an interesting discovery of dynamic changes in Hoxa11-expressing cells with developmental time. However, there was no quantification of cell numbers, and this was a solely qualitative observation.
From my point of view, more importantly, the authors did not provide quantification of Twist2+/Hoxa11-GFP dually positive cells to support their claim that "most Hoxa11eGFP-expressing cells also express Twist2." While labor intensive, quantifications of dual immunofluorescence stainings are an accepted standard in our field. It is unclear to me how the provided time frame for the revision precluded this important additional work to solidify the authors' conclusions. Presumably, the authors already have all the dual immunofluorescent stainings completed with sufficient n number to make the claim in their original submission. Additional imaging and subsequent blinded quantification should have been possible in the provided time frame. I concur with the authors that quantifications of developmental changes in Hoxa11-expressing cells may not be absolutely necessary for this study since it wouldn't affect any of the authors' main conclusions. By contrast, claims regarding which cell types and what percentage cells are Hoxa11+ are central to the authors' study and main conclusions, and need to be supported by the requested quantifications. A minor comment: some typos still persist, e.g.: p. 9 l. 212, typo: similar to; and p. 15 l. 378, typo: Like what was… Overall, I am still highly supportive of this study as it represents an important addition to the field of cellular heterogeneity of myogenic progenitor cells. I sincerely hope that the authors can address my last remaining concern, which precludes me from fully endorsing the manuscript for publication in its current state.