Differentiation of the human PAX7-positive myogenic precursors/satellite cell lineage in vitro

Ziad Al Tanoury, Jyoti Rao, Olivier Tassy, Bénédicte Gobert, Svetlana Gapon, Jean-Marie Garnier, Erica Wagner, Aurore Hick, Arielle Hall, Emanuela Gussoni and Olivier Pourquié
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Original submission

First decision letter

MS ID#: DEVELOP/2019/187344

MS TITLE: Differentiation of the human PAX7-positive myogenic precursors/satellite cell lineage in vitro

AUTHORS: Ziad Al Tanoury, Olivier Pourquie, Jyoti Rao, olivier tassy, benedicte Gobert, Svetlana Gapon, Jean-Marie Garnier, qiuyi wang, Erica Wagner, Aurore Hick, margarete diaz-cuadros, arielle Hall, and Emanuela Gussoni

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.

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

This manuscript by Al Tanoury et al examines the differentiation of human iPS cells along the myogenic lineage to generate populations of muscle stem cells and progenitors. The authors build upon previous work from this laboratory that defined culture conditions to generate mouse muscle stem/progenitors from pluripotent stem cells. Here, they use similar conditions with the human iPS cells that were engineered to express fluorescent reporter proteins under control of the Pax7 and MyoG loci. Importantly, they show that they are able to general pure populations of Pax7+ muscle stem/progenitors (some quiescent, some proliferating) that are capable to differentiating into multinucleated myotubes and to self-renew as Pax7+ stem cells in vitro. Furthermore, they show that the Pax7+ cells are capable of contributing to regenerating myofibers in vivo after transplantation into injured, immunodeficient mice.

This is a careful and thorough study of this differentiation program which the authors correlate with developmental myogenesis. The characterization of the myogenic potential is rigorous, and the single cell analysis is as expected – cells transition from Pax7+/MyoG-, through an intermediate Pax7+/MyoG+ phase, and into a Pax7-/MyoG+ phase. The in vivo myogenic potential, while quite limited (the authors did not use irradiation to reduce the competition with endogenous muscle stem cells), is comparable to that of human fetal myogenic cells.

Comments for the author

I have just a few questions and comments.

- The authors write that, after 3 weeks of culturing in differentiation medium, ~20-25% of the mononucleated cells expressed the reporter from the Pax7 locus, and 12-15% expressed the reporter from the MyoG locus. Is there any information about the phenotype of the large percentage of mononucleated cells that are apparently Pax7-/MyoG-?
- From the transcriptomic analysis, the authors report that genes encoding sarcomeric proteins are expressed in the Pax7Venus+ cells, and their suggestion is that perhaps there are more differentiated cells in the population due to the stability of the Venus protein. Is there any data on differentiation-specific protein expression in this population, or could it be that the genes are transcribed but not translated (which has been shown for muscle stem cells)?
- I'm confused by the interpretation of Fig 3e. The authors write that many cells express the "quiescence marker" p57, which is upregulated in clusters 3 and 4. They indicate that this is consistent with these cells being "post-mitotic cells committed to myogenic differentiation". However the quiescent muscle stem cells would be expected to be highly undifferentiated, and likely residing in clusters 1 and 2. By "quiescence", do the authors mean just non-cycling?
- Some of the points on Notch signaling are confusing. The authors state that DLL is expressed by differentiating, MyoG+ cells, referring to Fig. 4a. It's not clear to me that the data in that figure strongly support that statement. Second, the issue of Notch signaling in muscle stem progenitors is complex. On the one hand, there is a long literature on Notch signaling inhibition myogenic differentiation, consistent with the data shown in Figs. 4b-g. However, there are also data supporting the role of Notch signaling in promoting the quiescence of muscle stem cells. This is not specifically examined here, but may be playing a role in the work the authors refer to that examines the generation of myogenic cells from ES/iPS cells in vitro (Choi et al; Salveraj et al).

Minor points

- What are the arrowheads in Figs. 1e and 1f highlighting?
- The authors refer to cells not incorporating BrdU is studies related to Fig. 3f. I think that they mean to write EdU.

Reviewer 2

Advance summary and potential significance to field

This manuscript provides highly anticipated single cell transcriptome resolution for the field of in vitro human iPSC differentiation into the myogenic lineage. The authors use CRISPR knock-in reporter systems to specifically provide information on myogenic precursor cells expressing Pax7 and myogenin. This allows to distinguish between potential muscle stem cell and committed myocyte populations in human development as well as providing valuable alternative candidates to current cell markers used to enrichment myogenic precursors from in vitro differentiated iPSCs.

Comments for the author

The main concern with the manuscript lies in the heterogeneity of the Pax7 Venus reporter expressing population. The authors conclude that their population is highly and purely myogenic and suggest in the introduction that previously published techniques give rise to neural crest cells, which also express Pax7.
Upon closer inspection of the transcriptome data in Table S1, it is discernible that the Pax7-Venus cells also include neural crest and neural progenitor cell populations. Genes that are enriched in the Pax7 population substantiate this concern with examples such as ASCL1, ITGA4, BMP4, SNAI1, SNAI2 TNFRSF19, VIM in the dataset. We therefore recommend that:
1. The introduction be revised to disclose that some neural crest cell presence is observed in the differentiation process used for this dataset, unless proven otherwise with experiments suggested under the following point.
2. The conclusion be revised such that claims about the myogenic purity of the differentiated cells be removed unless substantiated by the following:
a. Single cell data presented in Fig2b to include transcript count plot examples of neural crest and neuronal lineage markers to verify myogenic purity of the analysed cell population, e.g. B3GAT1 (HNK1/CD57), TUBB3 (TUJ1).
b. Flow cytometry data to show limited CD57+ or A2B5+ cells within the Pax7-Venus population at both the scRNA-Seq (D30) and microarray (D21) timepoints of differentiation;
c. Immunohistochemistry of differentiated cells at both the scRNA-Seq (D30) and the microarray (D21) timepoints to confirm lack of TUJ1(TUBB3) and HNK1(CD57) staining. In case either of these markers turn out to be present then co-staining with a Pax7 antibody or Venus reporter overlay will be required to verify that none of these alternative lineage cells express Pax7.

Some additional concerns are listed below:
1) Use of primary foetal donor tissue derived CD56+/CD82+ cells is featured in the manuscript, but the source of these cells remains elusive. The authors should report the ethics board approval document reference and assessment organisation for obtaining the donor samples and detail the isolation methods as well as cell sorting strategies (including antibody information) for these cells. Currently there is no mention of these cells in the methods section other than the number of the double-positive cells injected into mouse TA muscles. Were the cells cultured after sorting to expand them and did they thus potentially lose their muscle stem cell potential during culture or were cells freshly isolated from donor tissue immediately transplanted into mouse muscle?
2) Further to the above-mentioned, the methods section states that 2x105 cells were transplanted into TA muscles, while the figure legend for Fig5 states that 105 cells were injected. Can this please be clarified and if different numbers of primary foetal and iPSC-derived Venus reporter cells were transplanted then the implications of this discussed in the results section.
3) The Pax7+/LaminA/C+ cell shown in Fig5k-n only displays faint LaminA/C signal on one side of the Pax7+ nucleus in comparison to other LaminA/C+ nuclei in the same field of view. The whole nucleus should be encompassed by positive signal as the protein is present throughout the nuclear membrane.
There are also areas in the same single channel panel for LaminA/C (Fig5l) with non-specific sarcolemmal signal. An alternative – or at least one additional – example of a Pax7+/LaminA/C+ cell should therefore be shown. If this cell was the only example the authors observed in their transplantation experiments then the data presented is not an improvement on Hicks et al. (2018), especially comparing the numbers of human protein expressing fibers. It may therefore be beneficial to transplant CD57-/CD56+/ERBB3+/NGFR+ cells rather than Venus+ cells without any

negative selection because of the heterogeneous population of 57-/56+/E+/N+ showing higher engraftment potential.
4) The authors claim that the Pax7 cells observed in the differentiating iPSC in vitro culture system are located adjacent to fast myosin heavy chain expressing fibers. The MF20 antibody stains for pan-myosins and the fast-MHC antibody reported (MY-32) is a mouse IgG1 and can therefore not be used to co-stain with Pax7 (The DSHB antibody is also IgG1), unless the Pax7 reporter system is used. Upon quick inspection of the Chal et al (2015 and 2018) publications we were unable to find such co-staining images other than Fig3l in the 2015 Nat Biotechnol paper, where it is likely that the MF20 antibody was used rather than the MY32 clone considering co-staining with a Pax7 antibody is reported. Can the authors please verify that fast-MHC/Pax7 co-staining has been published with alternative antobodies, referring to the specific image in their referenced previous publications? We recommend that the authors do not confuse the field about human iPSC-derived Pax7+ progenitors having a propensity to associate with fast twitch fibers unless they present immunofluorescence data to directly substantiate the claim. Therefore please present the data in this manuscript if not previously published, or revise the introduction such that distinction between Pax7 cells associating with fast or slow fibers is not made.
5) Single channel DAPI image panels should be included for the data presented in the following figures: Fig1c, Fig1j, Fig5f.
6) Immunohistochemistry methods section needs some further details with either clone numbers or product codes included for those antibodies that currently only have vendor names reported.
7) Data availability statements and transcriptome data uploading into repositories need to be reported for the datasets included herein.

First revision

Author response to reviewers' comments

Reviewer 1 Advance summary and potential significance to field
This manuscript by Al Tanoury et al examines the differentiation of human iPS cells along the myogenic lineage to generate populations of muscle stem cells and progenitors. The authors build upon previous work from this laboratory that defined culture conditions to generate mouse muscle stem/progenitors from pluripotent stem cells. Here, they use similar conditions with the human iPS cells that were engineered to express fluorescent reporter proteins under control of the Pax7 and MyoG loci. Importantly, they show that they are able to general pure populations of Pax7+ muscle stem/progenitors (some quiescent, some proliferating) that are capable to differentiating into multinucleated myotubes and to self-renew as Pax7+ stem cells in vitro. Furthermore, they show that the Pax7+ cells are capable of contributing to regenerating myofibers in vivo after transplantation into injured, immunodeficient mice.

This is a careful and thorough study of this differentiation program which the authors correlate with developmental myogenesis. The characterization of the myogenic potential is rigorous, and the single cell analysis is as expected – cells transition from Pax7+/MyoG-, through an intermediate Pax7+/MyoG+ phase, and into a Pax7-/MyoG+ phase. The in vivo myogenic potential, while quite limited (the authors did not use irradiation to reduce the competition with endogenous muscle stem cells), is comparable to that of human fetal myogenic cells.

Reviewer 1 Comments for the author
I have just a few questions and comments.

-The authors write that, after 3 weeks of culturing in differentiation medium, ~20-25% of the mononucleated cells expressed the reporter from the Pax7 locus, and 12-15% expressed the reporter from the MyoG locus. Is there any information about the phenotype of the large percentage of mononucleated cells that are apparently Pax7-/MyoG-?
We have recently published a single cell analysis of the first four days of the differentiation protocol which shows that our protocol generates an almost pure (>90%) population of presomitic mesoderm cells (Diaz-Cuadros et al, Nature 2020). We are currently characterizing the phenotype

of the entire mononucleated population during the myogenic differentiation process by single cell RNA sequencing (scRNAseq). We see that the vast majority of non-myogenic cells is composed of fibroblastic cell types such as muscle connective tissue or dermis precursors. This is not unexpected as these lineages are also derived from the paraxial mesoderm. This data is however still preliminary and will be included in a comprehensive scRNAseq study of the cultures differentiating in vitro.

-From the transcriptomic analysis, the authors report that genes encoding sarcomeric proteins are expressed in the Pax7Venus+ cells, and their suggestion is that perhaps there are more differentiated cells in the population due to the stability of the Venus protein. Is there any data on differentiation-specific protein expression in this population, or could it be that the genes are transcribed but not translated (which has been shown for muscle stem cells)?
Our scRNAseq data show the expression of mRNAs coding for several sarcomeric proteins including ACTA1, TTN, TNNC2, ML1, MYH8 in the purified PAX7-Venus cells (Supplementary Figure 3). The cells expressing these mRNAs form a small distinct cluster (cluster 4) and do not express the PAX7 mRNA. Our developmental trajectory analysis (Figure 2d, PAGA graph) suggest that these cells represent the most mature stage of the PAX7-Venus cells. Together, our data support the idea that PAX7+MYOG- cells give rise to PAX7+MYOG+ cells, which then give rise to PAX7-MYOG+ cells, which express sarcomeric proteins and differentiate into myocytes. We believe these PAX7- cells are purified together with cells expressing PAX7 due to the stability of the YFP protein which remains in cells for some time after downregulation of PAX7 mRNA. In our microarray data of purified PAX7+ cells, cells of the four clusters are mixed and thus one can detect expression of both PAX7 and sarcomeric proteins mRNAs in the same samples. We have performed many immunostainings of the differentiated cultures with antibodies against sarcomeric proteins such as ACTA2, TTN or MYHC but have never seen co-expression with the PAX7 protein. Thus, so far, our evidence suggests that PAX7 RNA and protein expression are mutually exclusive with expression of sarcomeric proteins.

-I'm confused by the interpretation of Fig 3e. The authors write that many cells express the "quiescence marker" p57, which is upregulated in clusters 3 and 4. They indicate that this is consistent with these cells being "post-mitotic cells committed to myogenic differentiation". However, the quiescent muscle stem cells would be expected to be highly undifferentiated, and likely residing in clusters 1 and 2. By "quiescence", do the authors mean just non-cycling?
We agree that the wording was confusing as p57 mostly controls cell cycle exit, therefore it should have been defined as 'post-mitotic' marker instead of "quiescence". P57(CDKN1C) has been shown to control cell cycle exit during muscle differentiation at the MYOG activation step (Zhang …Elledge et al, Genes and Dev, 1999). Clusters 3 and 4, which express the strongest levels of p57, are composed of cells expressing MYOG but not Ki67 or PCNA (Figure 3h, supplementary Figure 3), consistent with the idea that these cells are becoming post-mitotic myocytes. The lower levels of p57 expression in clusters 1 and 2 (where Notch activation is high as shown by expression of HES1/HEY1, Figure 4a) are in line with the role of Notch signaling in repressing p57 in muscle progenitor cells in mouse (Zalc et al, Development, 2014).
In the initial version of the text, we referred to the cluster 2 of PAX7+MYOG- cells as quiescent because its cells are mostly in G0/G1 phase as shown by scRNAseq and FACS analysis (Figure 3). These cells form a distinct cluster from the PAX7+MYOG- cycling cells (cluster 1, Figure 2a). Moreover, like human adult satellite cells (Barruet et al, elife 2020), they do not express the proliferation markers Ki67 or PCNA (Supplementary Figure 3). However, our data also indicate that virtually all PAX7+MYOG- cells are labeled following a 40h incubation with EdU. In addition, regressing the cell cycle genes from the scRNAseq analysis show that cluster1 and cluster2 merge into a single cluster, suggesting that the two clusters identify cells of similar identity but in different phases of the cell cycle. This argues that cluster2 cells are not quiescent but cycling cells exhibiting a strikingly long G1 phase.
While our data initially suggested the existence of a small fraction of EdU negative PAX7+MYOG- cells, which could correspond to quiescent cells, our reanalysis of the scRNAseq data using the most recent version of Scanpy (New Figure 2 and 3) does not support the existence of this population any longer. We have rewritten the text of the revised version to make these points clearer and do not refer to the PAX7+ cells as quiescent anymore.

-Some of the points on Notch signaling are confusing. The authors state that DLL is expressed by differentiating, MyoG+ cells, referring to Fig. 4a. It's not clear to me that the data in that figure strongly support that statement.
In Figure 4a, light blue cells are mostly found in cluster3 and 4 (MYOG+) whereas clusters1 and 2 (MYOG-) mostly contain dark blue cells. This suggest that DLL1 mRNA is activated concomitantly with MYOG mRNA.
Second, the issue of Notch signaling in muscle stem progenitors is complex. On the one hand, there is a long literature on Notch signaling inhibition myogenic differentiation, consistent with the data shown in Figs. 4b-g. However, there are also data supporting the role of Notch signaling in promoting the quiescence of muscle stem cells. This is not specifically examined here, but may be playing a role in the work the authors refer to that examines the generation of myogenic cells from ES/iPS cells in vitro (Choi et al; Salveraj et al).

We believe that our data is consistent with the well-described role of Notch signaling in inhibiting myogenic differentiation reported during mouse and chicken embryo development. The most plausible explanation for the phenotype observed is that Notch inhibition caused by DAPT treatment in the myogenic cultures leads to premature differentiation of PAX7+ cells thus exhausting this population (as observed in vivo in the mouse mutants of the Notch pathway). As discussed above, our revised analysis of the scRNAseq data does not support the existence of a quiescent PAX7+ population in our cultures anymore and thus it is unlikely that we interfere with the role described for Notch signaling in maintaining adult satellite cell quiescence. The two papers mentioned (Choi et al and Salveraj et al, only report an effect of DAPT on the maturation of myofibers but do not examine the status of PAX7+ cells in the cultures. We have revised the text to better discuss these points.

Minor points
-What are the arrowheads in Figs. 1e and 1f highlighting?
The arrowheads were to exemplify the double positive cells but they are not really useful, so we have removed them.

-The authors refer to cells not incorporating BrdU is studies related to Fig. 3f. I think that they mean to write EdU.
We have corrected this in the revised text.

Reviewer 2 Advance summary and potential significance to field
This manuscript provides highly anticipated single cell transcriptome resolution for the field of in vitro human iPSC differentiation into the myogenic lineage. The authors use CRISPR knock-in reporter systems to specifically provide information on myogenic precursor cells expressing Pax7 and myogenin. This allows to distinguish between potential muscle stem cell and committed myocyte populations in human development, as well as providing valuable alternative candidates to current cell markers used to enrichment myogenic precursors from in vitro differentiated iPSCs.

Reviewer 2 Comments for the author
The main concern with the manuscript lies in the heterogeneity of the Pax7 Venus reporter expressing population. The authors conclude that their population is highly and purely myogenic and suggest in the introduction that previously published techniques give rise to neural crest cells, which also express Pax7. Upon closer inspection of the transcriptome data in Table S1, it is discernible that the Pax7-Venus cells also include neural crest and neural progenitor cell populations. Genes that are enriched in the Pax7 population substantiate this concern with examples such as ASCL1, ITGA4, BMP4, SNAI1, SNAI2, TNFRSF19, VIM in the dataset.
We respectfully disagree with the conclusions of this reviewer on this point. Table S1 lists the genes enriched in the purified PAX7 cells compared to ES cells which are identified using Affymetrix microarrays. Out of the 7 genes mentioned to be specific for the neural crest by the reviewer, 5 of them are also well-known to be expressed in the paraxial mesoderm. My laboratory and others have reported the expression of SNAI1 and SNAI2 in the paraxial mesoderm (Dale et al, Dev Cell 2006). BMP4 has long been known to be expressed in the paraxial mesoderm (see for instance Amthor et al, Development 1999). TNFRSF19 is also expressed in the paraxial mesoderm (Dequeant et al, Science 2006), and Vimentin as well (Olson and Capetanaki, 1989 Oncogene). When ITGA4 is searched in combination with neural crest on Pubmed, it gives 0 results. While this does not mean that it may not be expressed by neural crest cells, it suggests that it is not an

unequivocal marker for this lineage. Only one gene ASCL1 (MASH1) can be considered more neural specific. However, this gene is found in position 388 in the list and it shows a high FDR being close to 0.1. Thus, we believe that there are significant chances that it might correspond to a false positive in the microarray dataset. Importantly, as shown below, ASCL1 is not detected in the scRNAseq dataset of purified PAX7 cells (see plot below), further supporting this idea.

Finally, while the reviewer criticism is based on the microarray analysis, our scRNAseq analysis of the purified PAX7-Venus positive population is extremely clear and only reveals four myogenic clusters and no Neural Crest cluster.
Therefore, based on these arguments, we feel that the assertion from this reviewer that the PAX7-Venus population is contaminated by Neural Crest cells is unfounded and the experiments requested below are not justified.
We therefore recommend that:
1.The introduction be revised to disclose that some neural crest cell presence is observed in the differentiation process used for this dataset, unless proven otherwise with experiments suggested under the following point.
2.The conclusion be revised such that claims about the myogenic purity of the differentiated cells be removed unless substantiated by the following:
a.Single cell data presented in Fig2b to include transcript count plot examples of neural crest and neuronal lineage markers to verify myogenic purity of the analysed cell population, e.g.B3GAT1 (HNK1/CD57), TUBB3 (TUJ1).
b.Flow cytometry data to show limited CD57+ or A2B5+ cells within the Pax7-Venus population at both the scRNA-Seq (D30) and microarray (D21) timepoints of differentiation;
c.Immunohistochemistry of differentiated cells at both the scRNA-Seq (D30) and the microarray (D21) timepoints to confirm lack of TUJ1(TUBB3) and HNK1(CD57) staining. In case either of these markers turn out to be present then co-staining with a Pax7 antibody or Venus reporter overlay will be required to verify that none of these alternative lineage cells express Pax7.

Some additional concerns are listed below:
1)Use of primary foetal donor tissue derived CD56+/CD82+ cells is featured in the manuscript, but the source of these cells remains elusive. The authors should report the ethics board approval document reference and assessment organisation for obtaining the donor samples and detail the isolation methods as well as cell sorting strategies (including antibody information) for these cells. Currently there is no mention of these cells in the methods section other than the number of the double-positive cells injected into mouse TA muscles. Were the cells cultured after sorting to expand them and did they thus potentially lose their muscle stem cell potential during culture or were cells freshly isolated from donor tissue immediately transplanted into mouse muscle?
To address this concern, we have added the section below to the revised manuscript.
Human fetal muscle cell isolation. Human de-identified, discarded fetal tissue was collected under a protocol approved by the Committee of Clinical Investigation at Boston Children's Hospital (IRB-P00020286). Primary tissue was dissociated into mononuclear cells, then cells were frozen and stored at -140˚C as previously described (Pakula and Gussoni, Methods Mol Biol. 2019). For purification of myogenic cells, frozen cells were thawed and plated overnight in DMEM-high glucose (4.5g) media supplemented with 20% FBS and antibiotics. Cells were purified by FACS as described in (Pakula and Gussoni, Methods Mol Biol. 2019) using APC anti-human CD56 antibody, Clone HCD56 (BioLegend, catalog nu mber:318310) and PE anti-human CD82 antibody, Clone ASL-24 (BioLegend, catalog number: 342103). Antibodies were added at a concentration of 5µl antibody per million cells, as recommended by the manufacturer. Double positive (CD56+CD82+ sorted cells were plated overnight in DMEM high glucose media before injection in animals. For injections, sorted human fetal cells were trypsinized and resuspended in physiological grade saline at a concentration of (100,000 cells/15 µl).

2)Further to the above-mentioned, the methods section states that 2x105 cells were transplanted into TA muscles, while the figure legend for Fig5 states that 105 cells were injected. Can this please be clarified and if different numbers of primary foetal and iPSC-derived Venus reporter cells were transplanted then the implications of this discussed in the results section.
We apologize for the oversight. We have now corrected the method section in the revised version of the MS as shown below.

15 µl of cell preparation containing 105 cells were injected into TA muscles of 3- to 4-month-old mice. Injections were done under general anesthesia. Grafted TA muscles were collected 6-8 weeks after transplantation and processed for cryosection and immunofluorescence analyses.

3)The Pax7+/LaminA/C+ cell shown in Fig5k-n only displays faint LaminA/C signal on one side of the Pax7+ nucleus in comparison to other LaminA/C+ nuclei in the same field of view. The whole nucleus should be encompassed by positive signal as the protein is present throughout the nuclear membrane. There are also areas in the same single channel panel for LaminA/C (Fig5l) with non-specific sarcolemmal signal. An alternative – or at least one additional – example of a Pax7+/LaminA/C+ cell should therefore be shown. If this cell was the only example the authors observed in their transplantation experiments then the data presented is not an improvement on Hicks et al. (2018), especially comparing the numbers of human protein expressing fibers. It may therefore be beneficial to transplant CD57-/CD56+/ERBB3+/NGFR+ cells rather than Venus+ cells without any negative selection because of the heterogeneous population of 57-/56+/E+/N+ showing higher engraftment potential.
As requested by the reviewer, we now show an alternative example of a human LaminAC positive cell co-stained with PAX7 and located under the laminin-positive basal lamina, as expected for a satellite cell.

4)The authors claim that the Pax7 cells observed in the differentiating iPSC in vitro culture system are located adjacent to fast myosin heavy chain expressing fibers. The MF20 antibody stains for pan-myosins and the fast-MHC antibody reported (MY-32) is a mouse IgG1 and can therefore not be used to co-stain with Pax7 (The DSHB antibody is also IgG1), unless the Pax7 reporter system is used. Upon quick inspection of the Chal et al (2015 and 2018) publications we were unable to find such co-staining images other than Fig3l in the 2015 Nat Biotechnol paper, where it is likely that the MF20 antibody was used rather than the MY32 clone considering co-staining with a Pax7 antibody is reported. Can the authors please verify that fast-MHC/Pax7 co-staining has been published with alternative antobodies, referring to the specific image in their referenced previous publications? We recommend that the authors do not confuse the field about human iPSC-derived Pax7+ progenitors having a propensity to associate with fast twitch fibers unless they present immunofluorescence data to directly substantiate the claim. Therefore, please present the data in this manuscript if not previously published, or revise the introduction such that distinction between Pax7 cells associating with fast or slow fibers is not made.
In figure 5b-c, we show a co-staining with the mouse anti-Fast-MyHC antibody clone MY32 (Sigma) and a chicken anti-GFP. This information was not in the methods and this omission has been corrected in the revised MS. Elsewhere in this study, the mouse anti-alpha-actinin (ACTA2) antibody was used. The MF20 antibody (the name of the anti-MyHC antibody from DSHB) was not used in this study. Based on this data, we can conclude that human iPS cells can differentiate in fast skeletal myofibers. However, since we have not examined slow myosin expression in these experiments we prefer to refrain from speculating on whether a specific fiber type is privileged in the cultures. We have amended the revised text accordingly.

5)Single channel DAPI image panels should be included for the data presented in the following figures: Fig1c, Fig1j, Fig5f.
As the labeling presented is reasonably scarce in this picture and the non-labeled DAPI-positive cells are clearly visible, we are concerned that adding single channel image would force us to decrease the overall size of the other pictures, whose details will be more difficult to appreciate.

6)Immunohistochemistry methods section needs some further details with either clone numbers or product codes included for those antibodies that currently only have vendor names reported.
These details have now been added to the Material and Methods section in the revised version.

7)Data availability statements and transcriptome data uploading into repositories need to be reported for the datasets included herein.

We agree and have now deposited the data in GEO and it can be found under the accession number GSE149057 for the microarray data. The scRNAseq data is being processed for submission and will be also deposited to GEO prior to publication.

Second decision letter

MS ID#: DEVELOP/2019/187344

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AUTHORS: Ziad Al Tanoury, Olivier Pourquie, Jyoti Rao, olivier tassy, benedicte Gobert, Svetlana Gapon, Jean-Marie Garnier, qiuyi wang, Erica Wagner, Aurore Hick, margarete diaz-cuadros, arielle Hall, and Emanuela Gussoni

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.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

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.

Reviewer 1

Advance summary and potential significance to field

This study advances the field of muscle stem cell biology by characterizing the myogenic progenitors that are generated from iPS cells. The findings reported here may ultimately be of value in advancing stem cell therapeutics for various muscle disorders.

Comments for the author

The authors have addressed my several concerns in detail. My only remaining suggestion is that they include in the Results section that there is a significant percentage of cells (and specify that percentage) that do not express either Pax7 or MyoG, and that this population is composed of fibroblastic cell types. I understand that the analysis of these cells at the scRNAseq level will be included in a future publication, but it seems only appropriate to give reader a sense of the purity here and the nature of the non-myogenic cells.

Reviewer 2

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This manuscript provides highly anticipated single cell transcriptome resolution for the field of in vitro human iPSC differentiation into the myogenic lineage. The authors use CRISPR knock-in reporter systems to specifically provide information on myogenic precursor cells expressing Pax7 and myogenin. This allows to distinguish between potential muscle stem cell and committed myocyte populations in human development as well as providing valuable alternative candidates to current cell markers used to enrichment myogenic precursors from in vitro differentiated iPSCs.

Comments for the author

We thank the authors for having addressed most of the concerns raised in the first round of revisions. However, we are disappointed that our main concern, regarding spontaneous

differentiation into alternative lineages, has not been sufficiently addressed. We address the authors' response to our previous comments below, and suggest that addressing the two issues detailed below will significantly improve the impact of the final manuscript.

It is unlikely that the authors are seeing paraxial mesoderm cells in their transcriptome analysis samples where evidence for alternative lineage marker expression was previously highlighted by us in our main concern. The state that the experiment was performed with cells at day 21 of their differentiation protocol when cells have differentiated past the paraxial mesoderm stage. Furthermore, the previously highlighted markers (ASCL1, BMP4, SNAI1, SNAI2, TNFRSF19, VIM) are unlikely to be expressed by any residual paraxial mesoderm cells also because characteristic paraxial mesoderm markers, such as TBX6, MSGN1 RSPO3, CDX2 are not differentially expressed in the dataset in question, suggesting that there is no significant population of those cells remaining in the sample used for transcriptome analysis.
Regarding ITGA4, this gene expresses integrin-alpha4, which is frequently referred to as CD49D in publications. CD49D plays a role in neural crest cell migration (McKeown et al., 2013 Dev Biol) and it has previously been used as a marker to isolate neural crest cells, e.g. by Fattahi et al. (2016 Nature) as well as Hindley et al. (2016 Sci Rep).
In response to comments from Reviewer1 regarding the same concern, given the Pax7 and Myogenin Venus reporter systems only accounting for a proportion of the cells in the total population, the authors refer to their own publication from earlier this year (Diaz-Cuadros et al., 2020 Nature). In that same publication in Extended Data Fig.2 they list mouse tail bud neural crest enriched genes that are also listed in the microarray data comparison in question here (PKNOX2 and ID3).
We would like to highlight that Hicks et al. (2017 Nat Cell Biol), using the same differentiation technique also generated CD57+ in culture (Fig.2a Method2). The transcriptome data reported here supports that observation. The authors also suggest in this manuscript that the Pax7+ in Wu et al. (2018 Cell Rep) was of neural crest origin. This publication could be a good opportunity for the authors of this manuscript to clarify whether they are also observing spontaneous differentiation towards neural crest and lead by example, providing proof for their highly myogenic nature of their Pax7+ population by providing appropriate population characterisation experiments.
In conclusion, we stand by our request for the authors to either rephrase their claims for pure myogenic differentiation using their protocol, or verify that they are not seeing spontaneous neural crest or neuronal cell differentiation in their differentiating populations, not only by single-cell transcriptome plots as they already did for ASCL1, but also by immunofluorescence for TUJ1 and flow cytometry for CD57 and A2B5 at both time-points used for the microarray (Day 21) and the single-cell RNA-Seq (Day 30). This is important to allow the muscle field to move forward with the most efficient technique for generating myogenic cells and to avoid frequent dismissal of previously published techniques by researchers coming up with new techniques, claiming to yield no spontaneous differentiation into alternative lineages with insufficient evidence to support such claims.

We also thank the authors for having provided further information in their immunofluorescence methods section. We did notice that the product code reported for the mouse laminin antibody from Sigma (L9393) is in fact also a rabbit antibody and considering the only LaminA/C antibody in the same section was also raised in rabbit, we would like the authors to add the missing LaminA/C or laminin antibody details for the staining in Fig.5k-o. In light of additional details having been added to this section we also noticed that the reported dystrophin antibody used for the human cell engraftment experiments is not actually human-specific. The Leica DYS1 antibody binds to mouse and human dystrophin and mdx-5Cv mouse muscles have revertant dystrophin-expressing myofibers. Considering that only a limited few dystrophin-positive fibers in Fig.5i-j also contain centrally located human laminB2-positive nuclei, we fear that human myofiber numbers may have been over-represented by counting revertant mouse dystrophin-positive fibers. For this reason, we would like to ask that the authors check the dystrophin antibody used for the IF panels in Fig.5i-j and if indeed DYS1 was used for those images then we recommend replacing those panels with IF images for human-specific spectrin or a human-specific dystrophin antibody (e.g. MANDYS104) co-staining with either human laminA/C or human LaminB2.

Second revision

Author response to reviewers' comments

Reviewer 1 Advance summary and potential significance to field
This study advances the field of muscle stem cell biology by characterizing the myogenic progenitors that are generated from iPS cells. The findings reported here may ultimately be of value in advancing stem cell therapeutics for various muscle disorders.

Reviewer 1 Comments for the author
The authors have addressed my several concerns in detail. My only remaining suggestion is that they include in the Results section that there is a significant percentage of cells (and specify that percentage) that do not express either Pax7 or MyoG, and that this population is composed of fibroblastic cell types. I understand that the analysis of these cells at the scRNAseq level will be included in a future publication, but it seems only appropriate to give reader a sense of the purity here and the nature of the non-myogenic cells.

We thank the reviewer for his/her positive comments and as suggested, we have added the sentences below in the revised text.
Thus at 3-4 weeks of differentiation, the proportion of myogenic cells (PAX7 and MYOG-positive) in the mononucleated fraction of the culture is around 30%. The remaining fraction appears to be mostly composed of fibroblastic populations.

Reviewer 2 Advance summary and potential significance to field
This manuscript provides highly anticipated single cell transcriptome resolution for the field of in vitro human iPSC differentiation into the myogenic lineage. The authors use CRISPR knock-in reporter systems to specifically provide information on myogenic precursor cells expressing Pax7 and myogenin. This allows to distinguish between potential muscle stem cell and committed myocyte populations in human development, as well as providing valuable alternative candidates to current cell markers used to enrichment myogenic precursors from in vitro differentiated iPSCs.

Reviewer 2 Comments for the author
We thank the authors for having addressed most of the concerns raised in the first round of revisions. However, we are disappointed that our main concern, regarding spontaneous differentiation into alternative lineages, has not been sufficiently addressed. We address the authors' response to our previous comments below, and suggest that addressing the two issues detailed below will significantly improve the impact of the final manuscript.

It is unlikely that the authors are seeing paraxial mesoderm cells in their transcriptome analysis samples where evidence for alternative lineage marker expression was previously highlighted by us in our main concern. The state that the experiment was performed with cells at day 21 of their differentiation protocol, when cells have differentiated past the paraxial mesoderm stage. Furthermore, the previously highlighted markers (ASCL1, BMP4, SNAI1, SNAI2, TNFRSF19, VIM) are unlikely to be expressed by any residual paraxial mesoderm cells also because characteristic paraxial mesoderm markers, such as TBX6, MSGN1, RSPO3, CDX2 are not differentially expressed in the dataset in question, suggesting that there is no significant population of those cells remaining in the sample used for transcriptome analysis. Regarding ITGA4, this gene expresses integrin-alpha4, which is frequently referred to as CD49D in publications. CD49D plays a role in neural crest cell migration (McKeown et al., 2013 Dev Biol) and it has previously been used as a marker to isolate neural crest cells, e.g. by Fattahi et al. (2016 Nature) as well as Hindley et al. (2016 Sci Rep).
In response to comments from Reviewer1 regarding the same concern, given the Pax7 and Myogenin Venus reporter systems only accounting for a proportion of the cells in the total population, the authors refer to their own publication from earlier this year (Diaz-Cuadros et al., 2020 Nature). In that same publication in Extended Data Fig.2 they list mouse tail bud neural crest enriched genes that are also listed in the microarray data comparison in question here (PKNOX2 and ID3). We would like to highlight that Hicks et al. (2017 Nat Cell Biol), using the same differentiation technique, also generated CD57+ in culture (Fig.2a Method2). The transcriptome data reported here supports that

observation. The authors also suggest in this manuscript that the Pax7+ in Wu et al. (2018 Cell Rep) was of neural crest origin. This publication could be a good opportunity for the authors of this manuscript to clarify whether they are also observing spontaneous differentiation towards neural crest and lead by example, providing proof for their highly myogenic nature of their Pax7+ population by providing appropriate population characterisation experiments.
In conclusion, we stand by our request for the authors to either rephrase their claims for pure myogenic differentiation using their protocol, or verify that they are not seeing spontaneous neural crest or neuronal cell differentiation in their differentiating populations, not only by single-cell transcriptome plots as they already did for ASCL1, but also by immunofluorescence for TUJ1 and flow cytometry for CD57 and A2B5 at both time-points used for the microarray (Day 21) and the single-cell RNA-Seq (Day 30). This is important to allow the muscle field to move forward with the most efficient technique for generating myogenic cells and to avoid frequent dismissal of previously published techniques by researchers coming up with new techniques, claiming to yield no spontaneous differentiation into alternative lineages with insufficient evidence to support such claims.

We are afraid that there might be a misinterpretation in the reading of our data and conclusions. In this paper, we exclusively focus on cell populations sorted based on PAX7-YFP expression (or MYOG-YFP expression). In the report, we unambiguously show using single cell RNA sequencing of these sorted cells that these cells constitute a homogenous group of myogenic precursors. This fraction does not include any neural derivatives.
What we are stating is that "We also demonstrate using scRNAseq, that our differentiation conditions can yield a pure myogenic human PAX7+ cells population free of neural contaminants (Kim et al., 2017)". We do not make any claim about the unsorted mononucleated population, which contains a small fraction of neural derivatives, which could be of neural crest origin as well as fibroblastic cells as discussed above. These cells do not express PAX7 and hence are not part of our analysis. We are currently characterizing the kinetics of the entire mononucleated population differentiating in our myogenic conditions which we intend to publish as a separate study.

We also thank the authors for having provided further information in their immunofluorescence methods section. We did notice that the product code reported for the mouse laminin antibody from Sigma (L9393) is in fact also a rabbit antibody and considering the only LaminA/C antibody in the same section was also raised in rabbit, we would like the authors to add the missing LaminA/C or laminin antibody details for the staining in Fig.5k-o.

We thank the reviewer for this comment. In Figure 5k-o, the anti-laminin antibody used is an unconjugated chicken polyclonal antibody purchased from LSbio (cat LS-C96142), together with anti lamin A/C (human specific, raised in rabbit) and the Pax 7 antibody (mouse monoclonal from DHSB). The new information on the chicken anti-laminin antibody has been included in the figure legend and material and methods.

In light of additional details having been added to this section we also noticed that the reported dystrophin antibody used for the human cell engraftment experiments is not actually human-specific. The Leica DYS1 antibody binds to mouse and human dystrophin and mdx-5Cv mouse muscles have revertant dystrophin-expressing myofibers. Considering that only a limited few dystrophin-positive fibers in Fig.5i-j also contain centrally located human laminB2-positive nuclei, we fear that human myofiber numbers may have been over-represented by counting revertant mouse dystrophin-positive fibers. For this reason, we would like to ask that the authors check the dystrophin antibody used for the IF panels in Fig.5i-j and if indeed DYS1 was used for those images then we recommend replacing those panels with IF images for human-specific spectrin or a human-specific dystrophin antibody (e.g. MANDYS104) co-staining with either human laminA/C or human LaminB2.

We thank the referee for these thoughtful comments. The original sentence mistakenly suggested that the anti-DMD antibody (DYS1) is human-specific, whereas it can recognize both mouse and human as correctly stated by this reviewer. With respect to misidentifying the DMD-expressing fibers as revertants in the grafted NOD; Rag1-/-; Dmdmdx-5Cv we would like to point out that we use this particular mouse strain because it has been described as having a very limited number of revertants (Danko et al, 2012) and thus the probabibility of having a human nucleus in such a

revertant is extremely low. We have now changed this in the revised text and clarified the legend of Figure 5h-i.

Third decision letter

MS ID#: DEVELOP/2019/187344

MS TITLE: Differentiation of the human PAX7-positive myogenic precursors/satellite cell lineage in vitro

AUTHORS: Ziad Al Tanoury, Olivier Pourquie, Jyoti Rao, olivier tassy, benedicte Gobert, Svetlana Gapon, Jean-Marie Garnier, Erica Wagner, Aurore Hick, arielle Hall, and Emanuela Gussoni
ARTICLE TYPE: Techniques and Resources Report

I have now received the referee's report on the above manuscript, and have reached a decision. The referee's 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.

The overall evaluation is positive and we would like to publish a revised manuscript in Development. Please address the one remaining reviewer concern as soon as possible so we can move forward with your manuscript. As it does not require any further experimentation, I believe it should be straight forward to deal with this issue.

Reviewer 2

Advance summary and potential significance to field

We thank the authors for having provided further information for the engraftment experiments and we have no further concerns regarding that dataset.

Comments for the author

However, the one major concern we have had throughout this revision process, regarding population purity, has still not been addressed. We are basing our interpretation of the presented data on the methods and results sections, where the authors state that cells for the microarray dataset were enriched by sorting for the Pax7-Venus reporter positive cells. This dataset showed non-myogenic marker expression in the Pax7-Venus positive population. We therefore asked for no further experiments, in case these genes were artefacts of bulk transcriptome analysis and either these genes or characteristic markers for neural crest and neuronal cells (B3GAT1 and TUBB3, respectively) expressed in the scRNA-Seq dataset, to easily rule out the presence of non-myogenic Pax7-expressing cells in the Venus-enriched population.

We appreciate that these putative non-myogenic cells, if present, would presumably cluster out as a standalone population, but some of the previously highlighted alternative lineage marker genes from the microarray – BMP4, SNAI1, VIM – have also been shown to be expressed in myogenic cells and may be enough to force the potential non-myogenic cells to cluster with the myogenic populations in scRNA-Seq data. Therefore, we request that the authors at least provide data to illustrate the lack of non-myogenic marker expression – B3GAT1, TUBB3 as well as SNAI2, TNFRSF19 and ITGA4 – in the scRNA-Seq data, like they already did for ASCL1. If these markers are not expressed in the sorted Pax7-Venus cells by scRNA-Seq then there will be no need for further experiments.

It is of utmost importance for us to emphasise that this is in no way a criticism of the authors' dataset overall. Quite the contrary, we believe this manuscript presents a very coherent and nicely laid out story showing enrichment of a highly myogenic population. We are making these

suggestions to improve the manuscript and especially to avoid any potential misinterpretations and future offhand comments by other experts in the muscle field in their publications in the same way as the dataset by Wu et al (2018) has been speculated to contain contaminants by the authors in the first results section of this manuscript. We reiterate that this is a question that can quickly and easily be addressed by presenting further figures from an existing dataset and hope that the authors appreciate our genuine concern for not leaving their conclusions open for interpretation.