nature portfolio

Peer Review File

Optimization of cell fate determination for cultivated muscle differentiation

Corresponding Author: Dr Joshua Flack

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This manuscript characterizes the heterogeneity during the differentiation of muscle satellite cells and distinguishes the 'reserve state' from the differentiation state through single nucleus mRNA sequencing (snRNA-seq). Since reserve cells exit the cell cycle but do not commit to myogenic differentiation, it is necessary to reduce the reserve cell state for effectively producing cultured meat. Additionally, the authors optimized serum-free cultured muscle differentiation medium to increase the proportion of cells adopting the differentiating cell fate and enhancing myotube formation. This manuscript is highly innovative and is expected to have a positive impact on the development of cultured meat. However, there are some concerns and suggestions for improving the investigation.

1. Major concerns
A. The authors defined the optimal medium formulation for muscle satellite cells differentiation, but in most of figures except Fig. 6, desmin was used as the marker of muscle differentiation. Although desmin has been reported to be major intermediate filament protein, it seems that desmin is not enough to represent the entire muscle protein since skeletal muscle is mainly composed of actin and myosin. Therefore, the title and topic of the manuscript need to be changed from optimal medium for 'differentiation of muscle cells' to 'intermediate filaments formation'. Otherwise, new data relevant to myosin and actin need to be added in most of figures, or additional evidence that desmin is a key marker of muscle cell differentiation should be added.
B. The authors stated in both the introduction and discussion that cells exiting the cell cycle make a dynamic fate decision to adopt a differentiating or reserve cell fate. However, in Supplementary Figures 6A-B, the cell cycle-inhibiting compounds (abemaciclib, narazaciclib, palbociclib) except MEKi did not promote desmin formation. Additionally, cell cycle exit is likely a characteristic of the cells that have already completed the process of becoming reserve/differentiation cells. Therefore, it is necessary to add evidence to support the claim that cell cycle exit is an important factor for differentiation, along with additional explanations of the mechanism studied in Fig. 4C-E.
C. In line 176-178, the authors stated that a synergistic effect was observed when all treatments were combined in SFDM v2. However, in Fig. 4A and Supplementary Fig. 6D, the fluorescence of desmin and myotube formation of MEKi appear to be similarly increased compared to SFDM v2. Additionally, the expression of desmin protein of MEKi seems to be significantly higher than that of SFDM v2 in Fig. 3D. Therefore, there is concern that MEKi might be more effective in desmin formation than SFDM v2. Additional data needs to be provided to demonstrate that SFDM v2 is more effective in muscle satellite cell differentiation than MEKi.

2. Minor concerns
A. In Fig. 2B and Supplementary Fig. 3B, additional explanation is needed to clarify why the quiescence marker is also expressed in the proliferating nuclei cluster.
B. In Fig. 2E, the fluorescence of Ki-67 appears similar between 0 h and 24 h, which does not match the proliferating graph in Fig. 2F. Further explanation is needed for this content.
C. In Fig. 5F, the authors need to provide the house keeping protein in the Western blot data.
D. The experimental result of ERKi is only presented in Fig. 3A. It is necessary to describe the reason why the author omitted it thereafter.
E. It is recommended to present the full names of major markers such as MYOG, MYOD, CCND1, NOTCH, etc.
F. Line 141 requires modification to improve the English expression.
G. Abstract should be restated by including background, aim, methods, results and indications.

Reviewer #2

(Remarks to the Author)
Review

In the study entitled “Optimisation of cell fate determination for cultured muscle 1 differentiation”, Melzener et al., demonstrate the improvement of primary bovine muscle cell culture fusion after single-cell RNAseq.
As far as I know, their idea is novel.
However, the presentation of results is often confusing and some of the conclusions related to muscle cell biology are not convincing.
While writing the manuscript special attention must be given as some of the sentences are not readable and unclear.
The presentation of some of the figures is unclear and should be revised for clarity and consistency.
The manuscript is scattered with specific terms from the meat industry, those should be clarified. An example: “animal component-free bioartificial muscle constructs” – this does not make any sense in a biological context.

Specific comments:
The optimization study has been developed for bovine cells and was tested only in bovine cells. This must be indicated in the abstract and the conclusions can be applied only for the cells that were studied here. It is interesting to check how general are the conclusions made from this study, are indicated supplements improve differentiation in muscle cells from other organisms?

Abstract
Line 24: “muscle protein accumulation” is written very generally, whereas only a few muscle proteins were tested, it must be revised.
Sentence 23-26 is not readable.

Introduction
Line 43: cell cycle arrest.
Line 46: add a reference.
Lines 50-54: are incorrect, it highly depends on the cell/species cell passage number, accumulation of cell culture, and the matrix (plays a prominent role). We get close to 100% fusion index.
“Reserve cells” is not a common name, describe first as quiescent cells.

Results
Figure 1: Panel 1 shows cultures over 216 hours, but panels E-F are 96 hours – this is confusing. Panels A-B are the background to panels E-F. The term Desmin index is unclear, similarly, desmin area/nuclei should be rephrased/ explained. The number of nuclei should be plotted. The image for 120 hours does not correspond to the bar.
Figure 2: the boundary between differentiating and reserve seems arbitrary and does not exactly match PAX7 and MYOG. The presentation would be more convincing if all 7 prominent muscle transcription factors were indicated (instead of 2) and more sarcomere proteins, which should be expressed later during the differentiation program (https://www.nature.com/articles/s41419-023-05725-z). The cluster of differentiating cells is unclear: muscle cell differentiation is induced by starvation, and cells stop proliferation. Please explain why in your condition cells continue to proliferate. This would be clearer if you show the cell number in Fig. 1. The genes examples in Fig. 2D are not always clear: FTL, FTH1, and RPS2 show higher expression in reserve but in all states at 75% of the cells, is this difference sufficient to mark them as biomarkers? Why show RARA, RXRA, and all genes right to them?
Did you find novel regulators? This is expected from omic studies.
The images in 3E are too small to appreciate the staining, besides for desmin. The merge image is unclear. 3F N=3 – 3 what? The cell status “committed” is unclear as all cells are muscle progenitors. The definition of cell status based on + and – two markers is not convincing. There are multiple biomarkers to describe muscle cell development, why not use them?
Figure 3: the names in panel A and panel B are unclear, better indicate the inhibitor name in panel B. Panels add C-D add cell number, this is crucial to explain the effect of the inhibitors. Desmin in panels C and D are inconsistent, please explain. Panel D – which time during differentiation? Can you explain why Notchi and RXRa are additive to MEKi? Overall, this experiment shows that the combination of the inhibitor speed-up differentiation. At 72 hours the differences between conditions are very small, and it is questionable if the difference between 80% - 85% is a biological phenomenon or a technical variation (due to segmentation limitation). In part of the conditions the triplicates show similar values, is it possible?
Figure 4: Panel A same comment as for 2E. C- the term low or high seeding density is unclear, please define it in confluence or cell nr per mm^2. It is unclear why cells proliferate in differentiation conditions (from LD at 0 to high SD in 48 hours). The experiments with the “second wave” are not sufficient to conclude.
Figure 5: A, please indicate if it is the myosin-heavy chain. The staining is unclear as MyHC is the most used marker for differentiating/fusing muscle cells. Please explain. Images in 5E are unclear.

Discussion
I’m not in the manufacturing meat production, is it allowed to add such molecules to the production process? Would it affect the ‘meat’ quality? What about potential risks for human consumption? Should you check metabolites in these cell cultures?

Reviewer #3

(Remarks to the Author)
In this manuscript “Optimisation of cell fate determination for cultured muscle 2 differentiation” the author's research, a

principle proof was provided for the generation of highly differentiated cultured muscles, which have excellent similarities with traditional muscles. This is a very interesting study, and it is recommended to receive it after minor revisions.
1 There is no conclusive description in the abstract section.
2 The purpose of the article is also unclear in the abstract.
3 Some headings in the methods section are not bolded.
4 P needs to be italicized.
5 Figure 5G the result of WESTERN BLOT needs to be reanalyzed.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The revised manuscript is acceptable.

Reviewer #2

(Remarks to the Author)
The authors have refuted all my objections well. The presentation of the revised version is sufficiently improved.
My only comment concerns the assumption that the molecular details of myogenesis are similar across organisms. While the key transcriptional fractions involved in differentiation are conserved, the post-translational modifications may be different. There have been many recent studies that attenuate the effect of transcription compared to protein steady-state in muscle cell biology. Working with human and mouse muscle cell cultures, the differences are quite obvious and well known in the field.
Nevertheless, the bovine study is relevant and could open opertiunities to investigate differences between species.

Reviewer #3

(Remarks to the Author)
Thank you for your point-by-point feedback. The quality of the article has significantly improved. I suggest acceptance with minor revisions. Here are some minor concerns.
1: Provide a brief overview of the experimental design at the beginning of the results section. This helps readers understand how the results were obtained.
2: Ensure that terms like "quiescent" and "reserve cells" are clearly defined early on, so readers unfamiliar with the field can follow the argument.
3. Consider summarizing the specific challenges faced in cultivated meat production more explicitly, which could help set up the relevance of your study.

Optimisation of cell fate determination for cultivated muscle differentiation

Manuscript #: COMMSBIO-24-0032

Response to reviewers - version 1.0

General response to editor and reviewers

Many thanks to the editor for inviting us to submit a revised version of our manuscript, and to the reviewers for their time spent reading and commenting on it. Overall, the comments were extremely helpful and we feel like the revised version of the manuscript is significantly improved in terms of content and clarity. Hopefully the reviewers agree!

Please find in this document detailed responses to each of the comments. In nearly all cases we have made a change to the figures and/or text of the manuscript, which are explained below and highlighted in red typeface on the resubmitted manuscript.

Thanks for your patience in waiting for our resubmitted version, which was delayed due to maternity leave.

Regards,

Dr. Joshua E. Flack

Assistant Professor
TU Delft
J.E.Flack@tudelft.nl

Response to Reviewer 1 comments

This manuscript characterizes the heterogeneity during the differentiation of muscle satellite cells and distinguishes the 'reserve state' from the differentiation state through single nucleus mRNA sequencing (snRNA-seq). Since reserve cells exit the cell cycle but do not commit to myogenic differentiation, it is necessary to reduce the reserve cell state for effectively producing cultured meat. Additionally, the authors optimized serum-free cultured muscle differentiation medium to increase the proportion of cells adopting the differentiating cell fate and enhancing myotube formation. This manuscript is highly innovative and is expected to have a positive impact on the development of cultured meat. However, there are some concerns and suggestions for improving the investigation.

Many thanks to the reviewer for the time spent reading our manuscript, we are very glad to hear that they found it to be interesting and innovative! Hopefully the reviewer agrees that our revised manuscript is much improved on the initial submission, and their concerns have been addressed.

Major concerns
The authors defined the optimal medium formulation for muscle satellite cells differentiation, but in most of figures except Fig. 6, desmin was used as the marker of muscle differentiation. Although desmin has been reported to be major intermediate filament protein, it seems that desmin is not enough to represent the entire muscle protein since skeletal muscle is mainly composed of actin and myosin. Therefore, the title and topic of the manuscript need to be changed from optimal medium for 'differentiation of muscle cells' to 'intermediate filaments formation'. Otherwise, new data relevant to myosin and actin need to be added in most of figures, or additional evidence that desmin is a key marker of muscle cell differentiation should be added.

It is certainly true that desmin does not constitute a high proportion of the proteome of fully differentiated skeletal muscle, and our use of the term 'desmin index' was quite confusing. However, desmin has been a very well known, established and widely used marker of myogenic differentiation for several decades[1,2], which is why we chose to use it as a marker of myofusion in the screening and mechanistic portions of our study, as we have done previously when we developed the SFDM v1 formulation[3]. It is highly expressed, easily detectable during early differentiation (including in an automated fashion, which is important for the screening experiments we performed in Fig. 3) and colocalises extremely well with fused myotubes.

Actin and (especially) myosin are harder to reliably detect and quantify in 2d cultures, because once the contractile apparatus begins to form, contraction leads to detachment of myotubes from the 2d surface (as we observed in Figure 1, and also previously

reported elsewhere). This is something we have tested quite a bit in the lab, and we suspect that in our case, there are additional limitations in terms of the antibodies for bovine myosin, which are not very sensitive, making early detection more difficult than for human or mouse samples. When demonstrating the maturity of differentiation in our 3d tissue constructs (Fig. 5; where myotube detachment is not an issue), we did look for actin and myosin, and we think those results clearly demonstrate significant actomyosin accumulation. It is also clear in the transcriptomics data that genes involved in or clearly demonstrating myogenic differentiation (such as myosin heavy chains) are strongly upregulated (e.g. Fig. 2d).

We have now made a number of changes to the revised version of the manuscript to make it more clear why we use desmin staining as our standard readout of fusion, and to show why it is a robust readout for myogenic differentiation:

- We have included a new Supplementary Fig. 1, in which we overlay desmin staining onto brightfield images, showing that desmin expression co-localises extremely well with myogenic fusion.
- We have added a note and some additional references regarding the use of desmin as a marker of muscle differentiation to the text (lines 72 to 73).
- We have performed an additional experiment to assess the expression of other well known myogenic proteins (including actin and obscurin) in SFDM v1 and v2 in 2d culture, which is now included as new Supplementary Figs. 6g, h.
- We have removed the use of the term ‘desmin index’ from the text and figures, and instead use ‘fusion index’. We have retained the use of ‘desmin area’ as a term, but hopefully this is no longer confusing for the reader.

Overall, we feel that these changes can give the reader more confidence in the status of myogenic differentiation we demonstrate and the use of desmin as our main marker of myofusion in 2d. We have therefore not changed the title of the manuscript, which we feel is accurate.

The authors stated in both the introduction and discussion that cells exiting the cell cycle make a dynamic fate decision to adopt a differentiating or reserve cell fate. However, in Supplementary Figures 6A-B, the cell cycle-inhibiting compounds (abemaciclib, narazaciclib, palbociclib) except MEKi did not promote desmin formation. Additionally, cell cycle exit is likely a characteristic of the cells that have already completed the process of becoming reserve/differentiation cells. Therefore, it is necessary to add evidence to support the claim that cell cycle exit is an important factor for differentiation, along with additional explanations of the mechanism studied in Fig. 4C-E.

Thanks for this interesting comment!

Permanent cell cycle exit definitely occurs during myogenic differentiation, since cytokinesis is no longer possible in fused cells. It is also known that this exit occurs early in differentiation[4], and expression of MyoD is incompatible with cell cycling[5,6]. However, we do agree with the reviewer that the precise moment of cell cycle exit *relative* to the differentiation/reserve cell fate decision is an unresolved and very interesting question. To some extent, it is impossible to answer that question unambiguously without significant further experiments, which we think are beyond the scope of this investigation. The wording of our mechanistic explanations were certainly unclear in a few places.

We have now made the following changes in the revised version:

- We have added some additional references to the sentence in the introduction discussing cell cycle exit and induction of myogenic transcription factors (line 46).
- In Fig. 2b, we have now added UMAPs for some additional genes, including *MYOD1*. This shows that expression of *MYOD1* and cell cycle genes (such as *CCND1*) are mutually exclusive, supporting the hypothesis that cell cycle exit occurs *prior* to the induction of myogenesis. This is also now mentioned in the results section that pertains to Fig. 2 (lines 129-131).
- In a new Supplementary Fig. 4d, we expand on that idea and provide additional bioinformatic analysis showing that expression of cell cycle genes (shown as a combined S phase score and G2M phase score) is mutually exclusive with that of the myogenic master regulators *MYOD1* and *MYOG*.
- We have changed the text of the results sections for Figs. 3 & 4 in various places (e.g. lines 169-170, 198-199, 206-207 etc) and the discussion (lines 276-279) to clarify what we think the mechanism for improved differentiation looks like, the effect that the small molecules have, and what we still can't fully conclude from this study.
- We have also modified the discussion slightly (lines 286-290) to point out that whether a differentiating/reserve cell fate decision is made prior to cell cycle exit is not a fully resolved question, and made some concrete experimental suggestions for future experiments that might be interesting here.

In line 176-178, the authors stated that a synergistic effect was observed when all treatments were combined in SFDM v2. However, in Fig. 4A and Supplementary Fig. 6D, the fluorescence of desmin and myotube formation of MEKi appear to be similarly increased compared to SFDM v2. Additionally, the expression of desmin protein of MEKi seems to be significantly higher than that of SFDM v2 in Fig. 3D. Therefore, there is concern that MEKi might be more effective in

desmin formation than SFDM v2. Additional data needs to be provided to demonstrate that SFDM v2 is more effective in muscle satellite cell differentiation than MEKi.

Thanks for this comment. We certainly agree that MEKi is the most potent of the small molecule inducers, and the difference between MEKi and SFDM v2 is not always huge, particularly if you look at individual images. Overall, however, we feel that the quantified data (i.e. Figs. 4b, e) very strongly supports a combinatorial effect for the different molecules. This data is based on multiple images quantified from different experimental replicates.

In the revised version of the manuscript, we have now performed additional statistical analysis between the individual compounds and SFDM v2, which was previously lacking in some figures (e.g. Figs 3c, 4d, 4e), and which demonstrates clear statistically significant differences between MEKi and SFDM v2 in the vast majority of experiments throughout the paper.

We do agree that the desmin blot for MEKi in Fig. 3d is a slightly unexpected result. We suspect that this might be an effect of timing (e.g. differentiation is sped up significantly in SFDM v2, and is thus already past the peak of desmin expression). However, this is clearly an outlier compared to other muscle proteins where the induction of expression occurs more slowly, and where SFDM v2 clearly shows increased expression compared to MEKi (e.g. myoglobin).

Minor concerns
In Fig. 2B and Supplementary Fig. 3B, additional explanation is needed to clarify why the quiescence marker is also expressed in the proliferating nuclei cluster.

Pax7 is a known marker of non-differentiated satellite cells (both proliferating and quiescent), it is not a quiescence marker *per se*, which is why expression is still observed in the proliferating cluster in the transcriptomics data. Our previous description of Pax7 as “a known marker of quiescence” in the text was definitely confusing in this respect; we have now reworded this section (line 108).

For Supplementary Fig. 4b (previously 3b), the ‘quiescence score’ is calculated from a large number of cell-cycle related genes, whose expression varies throughout the cycle, which explains why a small part of the proliferating cluster has a higher quiescence score. (Based on Fig. S4a, we suspect these are cells that have just completed mitosis, as they do not score highly for either S or G2M phase.)

We did try, but were not successful in identifying a marker *uniquely* expressed by quiescent cells that we could incorporate into an immunofluorescent staining panel. This explains why we used the combination of Pax7 expression and the absence of Ki-67 as our definition of quiescent cells for the quantification of IF data.

In Fig. 2E, the fluorescence of Ki-67 appears similar between 0 h and 24 h, which does not match the proliferating graph in Fig. 2F. Further explanation is needed for this content.

Significant proliferation occurs between 0 and 24h, which can be observed in the Hoechst channel (and is also evident in the nuclei counts we now present in Fig. 1d). Whilst the Ki-67 fluorescence appears similar in the image, as a proportion of total nuclei this is significantly decreased, which is reflected in the quantified data (Fig. 2f). We have amended the text of the relevant results section slightly (lines 137-139) to better explain that it is the *proportion* of proliferating cells that is reduced.

In Fig. 5F, the authors need to provide the house keeping protein in the Western blot data.

Good spot by the reviewer, this was definitely an omission. We have now added a tubulin panel to the Fig. 5f blot.

The experimental result of ERKi is only presented in Fig. 3A. It is necessary to describe the reason why the author omitted it thereafter.

MEK is directly upstream of ERK in the same kinase cascade, and thus there is no additive effect of combining MEK and ERK inhibition. A brief explanation of this is now included in the relevant results section (lines 162-163).

It is recommended to present the full names of major markers such as MYOG, MYOD, CCND1, NOTCH, etc.

We appreciate this comment from the reviewer. We did try out including the full names of genes in both text and the figures, but they became extremely difficult to read due to the number of genes we discuss!

We also checked the *Communications Biology* guidelines on gene nomenclature, which state that:

Authors should use approved nomenclature for gene symbols, and use symbols rather than italicized full names (for example Ttn, not titin). Please consult the appropriate nomenclature databases for correct gene names and symbols.

We therefore decided to stick to just using gene symbols for now, although we will of course discuss with the editorial team should the manuscript be accepted for publication.

Line 141 requires modification to improve the English expression.

This sentence has now been adjusted (now lines 148 to 151), hopefully the reviewer agrees it reads more clearly.

Abstract should be restated by including background, aim, methods, results and indications.

The abstract has been heavily reworked in our revised version. The abstract format for *Communications Biology* does not have separate subheadings, but hopefully the reviewer agrees that these items are now addressed in the revised version.

Please see also the response to Reviewers 2 and 3, who both had comments about the abstract.

Response to Reviewer 2 comments

In the study entitled "Optimisation of cell fate determination for cultured muscle 1 differentiation", Melzener et al., demonstrate the improvement of primary bovine muscle cell culture fusion after single-cell RNAseq.
As far as I know, their idea is novel.
However, the presentation of results is often confusing and some of the conclusions related to muscle cell biology are not convincing.
While writing the manuscript special attention must be given as some of the sentences are not readable and unclear.
The presentation of some of the figures is unclear and should be revised for clarity and consistency.
The manuscript is scattered with specific terms from the meat industry, those should be clarified. An example: "animal component-free bioartificial muscle constructs" – this does not make any sense in a biological context.

Many thanks to the reviewer for the time spent reading our manuscript and for the detailed feedback. We hope the revised manuscript addresses their concerns, and that the results are presented more clearly!

The optimization study has been developed for bovine cells and was tested only in bovine cells. This must be indicated in the abstract and the conclusions can be applied only for the cells that were studied here. It is interesting to check how general are the conclusions made from this study, are indicated supplements improve differentiation in muscle cells from other organisms?

This is a very interesting point, but unfortunately not straightforward to test. Because our experiments are performed in fully serum-free, defined medium conditions, we would need to first develop serum-free proliferation and differentiation medium for other species (such as porcine), which we consider beyond the scope of this study. We have now made several adjustments to the manuscript to make the limitations of our study more clear:

- We have amended the wording of the abstract (lines 19, 26), introduction (line 61) and discussion (lines 314-315) to make it clear we only performed experiments in bovine cells in this manuscript.
- We have added more specific descriptions of previous experiments in chicken cells[7] to the discussion (lines 318-319).

Due to the highly conserved nature of skeletal muscle differentiation pathways[8], we suspect that the supplements would indeed work well in cells from other species.

Hopefully in future work we can address this question, something we also now mention more explicitly in the discussion (lines 316-318).

Abstract
Line 24: "muscle protein accumulation" is written very generally, whereas only a few muscle proteins were tested, it must be revised.

Along with other changes to the abstract, this sentence has been revised to more specifically mention the actomyosin increase we observed in the 3d experiments (Fig. 5).

Sentence 23-26 is not readable.

This sentence has been adjusted as part of the reworking of the abstract, hopefully the reviewer agrees it now reads more clearly.

Introduction
Line 43: cell cycle arrest.

This is actually a very interesting point to discuss! 'Arrest' implies a reversible stop to the cell cycle, whilst 'exit' suggests something more permanent. Since our data points to a reversible exit from the cell cycle, at least for the reserve cell population, we agree that cell cycle arrest might be better terminology to use here.

Line 46: add a reference.

Good suggestion, we have now added a reference to a review from Jiang et al.[9]

Lines 50-54: are incorrect, it highly depends on the cell/species cell passage number, accumulation of cell culture, and the matrix (plays a prominent role). We get close to 100% fusion index.

Yes, very fair points, we have adjusted the text of the Introduction to soften this claim, and to specifically include these dependencies mentioned by the reviewer (lines 55-56). We do feel, however, that this issue may be underestimated in other culture systems as well; many myogenic immunofluorescence images found in the literature (or available online) clearly show a large proportion of non-fused nuclei, and this point has been raised before in the literature[10].

“Reserve cells” is not a common name, describe first as quiescent cells.
Yes, good point, we have adjusted this first mention of Reserve cells in the Introduction as suggested (line 57). This is also clarified in the revised version of the Abstract.

Results
Figure 1: Panel 1 shows cultures over 216 hours, but panels E-F are 96 hours – this is confusing. Panels A-B are the background to panels E-F.

Sorry for the confusion here. The first, longer timecourse experiments showed us that the non-differentiated cells **do** retain the capacity to differentiate later, which was an important initial observation that drew us into this study. When we came to study the differentiation process using single nuclei sequencing (which is what is shown in E-F), we decided to focus on a smaller number of timepoints in order to have more nuclei transcriptomes (and hence better resolution) for each of those timepoints. Performing a high resolution snRNA-seq across the full 216 hours would have been very interesting but was prohibitively expensive. We have now adjusted the text of the results section (line 84) to hopefully make clear that we chose to focus on the *initial* formation of the different populations for the transcriptomics experiments.

The term Desmin index is unclear, similarly, desmin area/nuclei should be rephrased/ explained.

Agreed, see also some comments from Reviewer 1 related to the use and analysis of desmin as a marker of myogenic fusion. Because the data in Fig. 1b is generated by automated analysis of nuclei within desmin-stained areas, we felt it was more scientifically accurate to use the term ‘desmin index’. However, we know desmin correlates extremely well with myofusion (see new Supplementary Fig. 1), and so it is indeed much less confusing to simply use the term fusion index. This is now adjusted in the text and figure(s).

We also agree that the normalized desmin area/nuclei plot was not very clear, and on reflection we consider it unnecessary. Based on this (and the following comment from Reviewer 2) we have now replaced that plot with the nuclei counts as panel D in the revised version of Fig. 1.

The number of nuclei should be plotted.

As mentioned above, the nuclei counts for the experiments in Figure 1 are now plotted as the new Fig. 1d. We have also added nuclei counts for the experiments in Fig. 3c (as new Supplementary Fig. 5c).

The image for 120 hours does not correspond to the bar.

Myotube detachment is a stochastic phenomenon that occurs at different times in different wells, even for otherwise identical experimental replicates. This can be observed in the greater spread in the data for the 120 h timepoint in Figs. 1b-d. We have now selected a different image for the 120 h timepoint for Fig. 1a that better corresponds to the average of the data, and hopefully gives a better visual illustration of myotube detachment kinetics! However, it is important to note that the data is the average of 9 images collected from each of multiple wells (n = 10, in this instance).

Figure 2: the boundary between differentiating and reserve seems arbitrary and does not exactly match PAX7 and MYOG. The presentation would be more convincing if all 7 prominent muscle transcription factors were indicated (instead of 2) and more sarcomere proteins, which should be expressed later during the differentiation program (https://www.nature.com/articles/s41419-023-05725-z).

The boundary between the differentiating and reserve cells is assigned mathematically (using the FindClusters() function in Seurat[11]) and takes into account all differentially expressed genes between populations, not just *PAX7* and *MYOG*. We definitely agree, however, that showing more genes would make the clustering more clear to the reader. We have thus adjusted Fig. 2b in the revised version of the manuscript to show some additional genes relating to myogenic processes (including *MYF5*, *MYOD1* and *MYH3*).

The cluster of differentiating cells is unclear: muscle cell differentiation is induced by starvation, and cells stop proliferation. Please explain why in your condition cells continue to proliferate. This would be clearer if you show the cell number in Fig. 1.

Indeed, that is the traditional understanding of myogenic differentiation in vitro, but as our data shows, this is not always the case. We feel this is a notable finding from our experiments. As mentioned earlier, we have now included the nuclei numbers as Fig. 1d of the revised manuscript, showing that cell proliferation continues after the switch to differentiation medium. This might be partly due to our serum-free medium formulation, although we also observed the same phenomenon during serum starvation-induced differentiation (Messmer *et al*., 2022[3], Fig. 1d). We have reworked the Results (e.g. lines 78, 80) and Discussion section (lines 259-261) to increase clarity on this point.

The genes examples in Fig. 2D are not always clear: FTL, FTH1, and RPS2 show higher expression in reserve but in all states at 75% of the cells, is this difference sufficient to mark them as biomarkers?

Why show RARA, RXRA, and all genes right to them?

Did you find novel regulators? This is expected from omic studies.

These are all great questions. We were trying to make a few different points with the genes we chose to display, which ended up not being clear. We have now replaced Fig. 2d with a new version, showing fewer genes and with additional labels indicating the different groups of genes we are showing. This hopefully makes it clear why (for example), *RARA* and *RXRA* are relevant to show the reader here. Many of these genes are indeed novel, or at least less well understood regulators of myogenic differentiation, which is now mentioned more specifically in the results section (lines 118-120).

With respect to the expression of *FTL*, *FTH1* and *RPS2*, these are very highly expressed genes with a lot of transcripts, so it is not surprising that even in the cell populations where they are less expressed, transcripts are identified in a large proportion of nuclei. It would be interesting to perform immunofluorescent staining at the protein or perhaps the RNA level (which might be technically more straightforward), to see if these genes could be used as unique biomarkers for the detection of reserve cells, something we now suggest specifically in the discussion (lines 268-270). However, we felt that given our immunofluorescence panel already took significant time to develop in bovine cells, this was beyond the scope of this study.

The images in 3E are too small to appreciate the staining, besides for desmin. The merge image is unclear.

Presumably the reviewer is referring to Fig. 2e here? We have now zoomed and cropped the images to provide a higher magnification view in the revised version of the manuscript.

3F N=3 – 3 what?

Assuming the reviewer is referring to Fig. 2f here, for each condition 3 separate wells were imaged, with each data point representing the analysis of a single well (itself the

average of 9 separate images collected from within that well). This is now explained more clearly in the methods section (lines 377 to 379).

The cell status "committed" is unclear as all cells are muscle progenitors.

Good point, we have now changed the legend and figure legend text for Figs. 2f and 4b to 'committed myoblast' to indicate that we mean commitment to full myogenic differentiation, not commitment to the muscle lineage. We have also adjusted the title of Fig. 2. This also has the effect of making the data labelling more consistent with the nomenclature used in the model in Fig. 4f.

The definition of cell status based on + and – two markers is not convincing. There are multiple biomarkers to describe muscle cell development, why not use them?

In bovine cells, it is not easy to find working antibodies for all markers that are commonly used in human or mouse cells. We already performed a significant amount of antibody screening to identify the panel used here. In addition, the antibodies available are often from mouse or rabbit, making it hard to create large panels to simultaneously image multiple markers. In this respect, RNA staining may be a useful methodology for multiplexed detection of marker expression in the future.

We fully agree that our panel probably provides an oversimplified view of the different cell states, and also that our categorisation of cells into just 3 distinct states might miss some of the finer details and the transitions between states. Nevertheless, we feel that the current panel does neatly capture and quantify the broad difference in muscle biology that occurs with the MEKi, NOTCHi and RXRa compounds (and their combination), as shown in the quantification in Fig. 4b.

In the new version of Fig. 2b, we now show additional canonical myogenic marker genes, so at least from a transcriptomic perspective, hopefully the reviewer agrees our classification of the different populations is more clear.

Figure 3: the names in panel A and panel B are unclear, better indicate the inhibitor name in panel B.

Great suggestion, we have now added the inhibitor names to Fig. 3b to make it clear in the first instance which molecules we are using!

Panels add C-D add cell number, this is crucial to explain the effect of the inhibitors.

Also a great suggestion, the nuclei counts for the Fig. 3c experiments are now included as Supplementary Fig. 5c. We have added an additional sentence in the Results (lines 169-170) to describe this new data and hint at the mechanism, which is further expanded upon in the results pertaining to Fig. 4.

Desmin in panels C and D are inconsistent, please explain.

The samples for immunofluorescence and protein analysis were taken from separate experiments, and in general the IF is fairly poor at quantifying protein levels, only distribution, so it is not too surprising that we see differences between these readouts. We do agree that the desmin level in Fig. 3d is slightly unexpected, but we suspect that this might be an effect of timing; please see our response to Reviewer 1 on this same topic.

Panel D – which time during differentiation?

72 hours, this is now indicated clearly in the legend.

Can you explain why Notchi and RXRa are additive to MEKi?

Unlike MEK and ERK, which are in the same pathway, NOTCH and RXR are components of separate signaling pathways, so it is not too surprising that there is a combinatorial effect on muscle differentiation. We have adjusted the results section (lines 162-163) in the results section to hopefully make this more clear. We have also slightly adjusted the relevant section of the discussion (lines 273-275).

Overall, this experiment shows that the combination of the inhibitor speed-up differentiation. At 72 hours the differences between conditions are very small, and it is questionable if the difference between 80% - 85% is a biological phenomenon or a technical variation (due to segmentation limitation). In part of the conditions the triplicates show similar values, is it possible?

We fully agree that in this particular experiment, the difference between the conditions at 72 h is smaller than at 48 h. This is why for Fig. 4, we chose to focus on the 48 h timepoint, to maximise the difference between conditions and give us better sensitivity.

The segmentation of the desmin staining is robust across a broad range of values, however, and looking at both the images and the statistical analysis, there is also a significant difference between conditions at 72 h (and especially between SFDM v1 (74.9%) and v2 (90.6%)).

We have added a sentence in the text of the relevant results section (lines 171-172) to make it clear that the formulation speeds up differentiation (which is of course also desirable from a cultured meat process perspective!).

Figure 4: Panel A same comment as for 2E.

As for Fig. 2e, we have now zoomed and recropped the images. Hopefully the reviewer agrees this higher magnification is more clear!

C- the term low or high seeding density is unclear, please define it in confluence or cell nr per mm^2.

Great point, this was previously only defined in the results text. We have now added a mention to the respective figure legend(s) as well.

It is unclear why cells proliferate in differentiation conditions (from LD at 0 to high SD in 48 hours).

As discussed in response to an earlier comment by this reviewer, this was one of the notable findings of our snRNA-seq analysis, alongside the presence of the reserve cells, and it also holds true here. Indeed, at lower seeding density, it seems like cells are *more* likely to continue proliferating, probably due to reduced pro-myogenic juxtacrine signalling. We have now adjusted the manuscript in various places (e.g. Discussion, lines 260-261) to explain the continued proliferation during the differentiation phase more clearly.

The experiments with the "second wave" are not sufficient to conclude.

We do agree with the reviewer that these experiments (Fig. 4e, S7d) are not the strongest in the manuscript. Nevertheless, we think that they help support the other experiments in Figure 4, providing a further hint that the pro-myogenic compounds have an effect on reserve cells, and also serves as a link back to the original 'second wave'

observation in Fig. 1 that prompted us to begin studying the non-differentiating cells in the first place. We have therefore retained this experiment in the manuscript, but revised the text of the relevant results and discussion sections to highlight how these results support the other data but are not alone sufficient for mechanistic conclusions (e.g. lines 215-218).

Figure 5: A, please indicate if it is the myosin-heavy chain. The staining is unclear as MyHC is the most used marker for differentiating/fusing muscle cells. Please explain.

Yes, good point. We are indeed using an antibody targeting myosin heavy chain. This is now clarified in the figure, legend and text.

Images in 5E are unclear.

We have now zoomed and cropped the images to provide a higher magnification, as for the other immunofluorescence images.

Discussion
I'm not in the manufacturing meat production, is it allowed to add such molecules to the production process? Would it affect the 'meat' quality? What about potential risks for human consumption? Should you check metabolites in these cell cultures?

We previously included a small section on the safety aspects of these small molecule differentiation inducers in the discussion, which has now been adjusted and expanded (lines 331-334). In general, the safety outlook is more favourable if the molecules are not detectable in the final product. It would therefore be very interesting to develop methods to directly measure levels of small molecules (such as MEK inhibitors) in the culture medium, although the levels are so low that this will require extremely precise analytical chemistry, which is beyond the scope of this initial proof-of-principle study.

It would certainly be interesting to compare levels of muscle metabolites after differentiation in different inducers, although again we consider this outside the scope of this study. We have now included it as a concrete suggestion for future work (lines 310-311).

Response to Reviewer 3 comments

In this manuscript "Optimisation of cell fate determination for cultured muscle 2 differentiation" the author's research, a principle proof was provided for the generation of highly differentiated cultured muscles, which have excellent similarities with traditional muscles. This is a very interesting study, and it is recommended to receive it after minor revisions.

Many thanks to the reviewer for the time spent reading our manuscript, we are very glad to hear that they found the data interesting!

1 There is no conclusive description in the abstract section.
2 The purpose of the article is also unclear in the abstract.

The abstract has now been substantially reworked in our revised manuscript, hopefully the reviewer agrees that the aim and conclusions of the manuscript are now more clearly laid out.

Please see also our response to Reviewers 1 and 2, who both had comments on the abstract.

3 Some headings in the methods section are not bolded.

Presumably this comment relates to the subheadings for the snRNA-seq analysis section? On this point we will defer to the preferred formatting of *Communications Biology* for Methods subheadings, should the article be accepted.

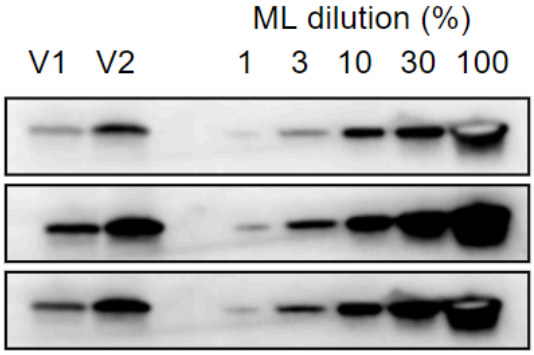
4 P needs to be italicized.

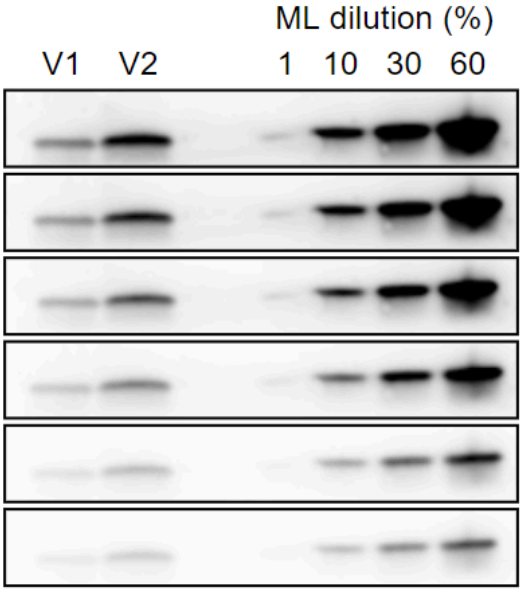
This is now adjusted throughout the manuscript.

5 Figure 5G the result of WESTERN BLOT needs to be reanalyzed.

Thanks for this comment. This was not an easy blot to get right, because we wanted to capture a broad range of Myoglobin expression levels in one exposure. We repeated this analysis, including rerunning the same samples and repeating the experiment, but did not get a version we felt was better. (Some examples of other blots and exposures we obtained are shown here.) We feel like the version we are currently showing in the

manuscript is not perfect, but does the job for the reader in terms of allowing them to compare the myoglobin expression in our samples with a set of standards.





References

1. Rangdaeng, S. & Truong, L. D. Comparative immunohistochemical staining for desmin and muscle-specific actin. A study of 576 cases. *Am. J. Clin. Pathol.* **96**, 32–45 (1991).
2. Capetanaki, Y., Milner, D. J. & Weitzer, G. Desmin in muscle formation and maintenance: knockouts and consequences. *Cell Struct. Funct.* **22**, 103–116 (1997).
3. Messmer, T. *et al.* A serum-free media formulation for cultured meat production supports bovine satellite cell differentiation in the absence of serum starvation. *Nat Food* **3**, 74–85 (2022).
4. Walsh, K. & Perlman, H. Cell cycle exit upon myogenic differentiation. *Curr. Opin. Genet. Dev.* **7**, 597–602 (1997).
5. Sorrentino, V., Pepperkok, R., Davis, R. L., Ansorge, W. & Philipson, L. Cell proliferation inhibited by MyoD1 independently of myogenic differentiation. *Nature* **345**, 813–815 (1990).
6. Crescenzi, M., Fleming, T. P., Lassar, A. B., Weintraub, H. & Aaronson, S. A. MyoD induces growth arrest independent of differentiation in normal and transformed cells. *Proc. Natl. Acad. Sci. U. S. A.* **87**, 8442–8446 (1990).
7. Eigler, T. *et al.* ERK1/2 inhibition promotes robust myotube growth via CaMKII activation resulting in myoblast-to-myotube fusion. *Dev. Cell* **56**, 3349–3363.e6 (2021).
8. Brennan, T. J., Chakraborty, T. & Olson, E. N. Mutagenesis of the myogenin basic region identifies an ancient protein motif critical for activation of myogenesis. *Proc. Natl. Acad. Sci. U. S. A.* **88**, 5675–5679 (1991).
9. Jiang, Y., Torun, T., Maffioletti, S. M., Serio, A. & Tedesco, F. S. Bioengineering human skeletal muscle models: Recent advances, current challenges and future perspectives. *Exp. Cell Res.* **416**, 113133 (2022).
10. Yoshida, N., Yoshida, S., Koishi, K., Masuda, K. & Nabeshima, Y. Cell heterogeneity upon myogenic differentiation: down-regulation of MyoD and Myf-5 generates ‘reserve cells’. *J. Cell Sci.* **111 (Pt 6)**, 769–779 (1998).

11. Waltman, L. & van Eck, N. J. A smart local moving algorithm for large-scale modularity-based community detection. *Eur. Phys. J. B* **86**, 471 (2013).

Optimization of cell fate determination for cultivated muscle differentiation

Manuscript #: COMMSBIO-24-0032

Response to reviewers - version 2.0

General response to editor and reviewers

Many thanks for the kind comments on the revised version of our manuscript, we are very glad the reviewers also found the new version to be much improved.

Our finalised version includes a few minor changes corresponding to the last remarks from the reviewers.

Regards,

Dr. Joshua E. Flack

Assistant Professor
TU Delft
J.E.Flack@tudelft.nl

Response to Reviewer 1 comments

The revised manuscript is acceptable.

Great!

Response to Reviewer 2 comments

The authors have refuted all my objections well. The presentation of the revised version is sufficiently improved.

Likewise, we are very pleased to hear this comment from the reviewer.

My only comment concerns the assumption that the molecular details of myogenesis are similar across organisms. While the key transcriptional fractions involved in differentiation are conserved, the post-translational modifications may be different. There have been many recent studies that attenuate the effect of transcription compared to protein steady-state in muscle cell biology. Working with human and mouse muscle cell cultures, the differences are quite obvious and well known in the field.
Nevertheless, the bovine study is relevant and could open opertiunities to investigate differences between species.

We have slightly modified the text of the discussion section to highlight that inter-species differences are certainly still possible.

Response to Reviewer 3 comments

Thank you for your point-by-point feedback. The quality of the article has significantly improved. I suggest acceptance with minor revisions. Here are some minor concerns.
1: Provide a brief overview of the experimental design at the beginning of the results section. This helps readers understand how the results were obtained.

We have modified the early part of the results section for Figure 1, hopefully outlining a bit more clearly how the standard 2D differentiation assay that we use a lot in the article works.

2: Ensure that terms like "quiescent" and "reserve cells" are clearly defined early on, so readers unfamiliar with the field can follow the argument.

We have slightly modified the text of the relevant section to make it more clear what quiescence means in this context.

3. Consider summarizing the specific challenges faced in cultivated meat production more explicitly, which could help set up the relevance of your study.

This sentence in the introduction is now expanded with specific mention of three main challenges, including mimicry of traditional meat (which is the main focus of this article).