THE EMBO JOURNAL

An Engineered Multicellular Stem Cell Niche for the 3D Derivation of Human Myogenic Progenitors from iPSCs

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Editor: Daniel Klimmeck

Transaction Report:

(Note: Please note that the manuscript was previously reviewed at another journal and the reports were taken into account in the decision making process at The EMBO Jornal. Since the original reviews are not subject to EMBO's transparent review process policy, the reports and author response cannot be published. With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision **4th Mar 2022**

Dear Florian, dear Jerome,

Thank you again for the submission of your amended manuscript (EMBOJ-2022-110655) to The EMBO Journal. We have carefully assessed your manuscript and the point-by-point response provided to the referee concerns that were raised during review at a different journal. In addition, and as mentioned before, we decided to involve two arbitrating experts to evaluate the revised version of your work, with respect to technical robustness, conceptual advance and overall suitability of your work for publication in The EMBO Journal.

As you will see from their comments enclosed below, while advisor #1 remains overall more critical, advisor #2 is broadly in favour of the work stating the interest and value of your results and s/he is supportive of publication at The EMBO Journal.

Please note that we editorially decided that, while per se well taken, advisor #1's request for additional in vivo characterization of the methods' applications is in our view beyond the scope of the current study, given the focus of the current work as a methods resource article.

We are thus pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal, pending minor revision of the following remaining issues, which need to be adjusted in a re-submitted version.

• We are piloting Structured Methods a new format for the Materials and Methods of articles published at EMBO Press. Adhering to this format is optional for research articles. However, considering the strong methodological aspect of your study, we would strongly encourage you to use it. Specifically, the Material and Methods section should include a Reagents and Tools Table (listing key reagents, experimental models, software and relevant equipment and including their sources and relevant identifiers) followed by a Methods and Protocols section in which we encourage the authors to describe their methods using a step-by-step protocol format with bullet points. More information on how to adhere to this format as well as downloadable templates (.doc or .xls) for the Reagents and Tools Table can be found in the author guidelines of our sister journal Molecular Systems Biology https://www.embopress.org/page/journal/17444292/authorguide#methodguide. An example of a paper with Structured Methods can be found here: https://www.embopress.org/doi/full/10.15252/embj.2018100300. We encourage you to be even more explicit in adding details on the experimental procedures, as this should be valuable in ensuring reproducible application if the approach.

• Re-evaluate the comparison of the current approach with earlier iPSC-derived models using in vitro differentiation assays (adv#1, pt.1).

• Re-assess integration of current method derived myogenic cells into the stem compartment by additional tissue co-stainings (adv#1, pt.2).

• Introduce caveats regarding the identity of Pax7neg cells in your culture where appropriate (adv#1, pt.3).

Based on i.p. the positive view of advisor #2 together with our own assessment, we decided to proceed with publication of your work at The EMBO Journal pending the above points related to the advisor #1's input could be conclusively addressed in a time frame of two weeks.

Once we have received the revised version, we should then be able to swiftly proceed with formal acceptance and expedited production of the manuscript.

Please submit a revised version of the manuscript using the link enclosed below, addressing the advisor's comments.

As you might have seen on our web page, every paper at the EMBO Journal now includes a 'Synopsis', displayed on the html and freely accessible to all readers. The synopsis includes a 'model' figure as well as 2-5 one-short-sentence bullet points that summarize the article. I would appreciate if you could provide this figure and the bullet points.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal, I look forward to hearing from you and receiving your final revised version of the manuscript.

Kind regards,

Daniel

Daniel Klimmeck PhD

Senior Editor
The EMBO Journal.

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Formatting changes required for the revised version of the manuscript:

>> Introduce ORCID IDs for all corresponding authors (F.B.) via our online manuscript system. Please see below for additional information.

>> Please add maximally five keywords to your manuscript.

>> The reference format needs to be corrected to EMBO Journal style and 10 author names before et al. .

>> Please introduce a 'Data availability section', detailing the sc-RNAseq data set deposition and related html links. Make the data set publicly available.

>> Move the Material and Methods part after the Discussion section.

>> Appendix File: the appendix file should be saved as a PDF with a ToC on its first page. Please change the nomenclature to 'Appendix Figure S1, S2...' and adjust references in the main text and legends.

>> Please broadly improve resolution of the figures.

>> Provide main figures and EV figures as individual, high-resolution .tiff files.

>> Please rename the current 'Conflicts of Interest' section to 'Disclosure and Competing Interests Statement'.

>> Please consider additional changes and comments from our production team as indicated by the .doc file enclosed and leave changes in track mode.

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Thank you very much in advance.

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Please check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
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See also figure legend guidelines: https://www.embopress.org/page/journal/14602075/authorguide#figureformat

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- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines
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We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (2nd Jun 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

Link Not Available

EMBOJ-2022-110655

Arbitrating advisor # 1 comments:

I have carefully read the manuscript, the reviewers' comments, and the authors' responses.

Some comments of the previous reviewers were addressed convincingly, but not all of them. In fact, some crucial experiments are still missing. In my opinion, the paper describes an interesting approach to generate myogenic progenitor cells that might be useful for therapeutic applications (regardless of the extensively discussed GMP-limitations). The technology itself is not brand new or a quantum leap but rather represents a combination of already existing protocols (co-culture plus CHIR/FGF2). Compared to already available approaches the new technique has the distinctive advantage of being much faster than previous protocols and seems to generate higher yields of myogenic cells. It is difficult to judge whether the quality of newly generated myogenic cells is equal, better or worse compared to existing protocols, since the authors essentially did not do any direct comparisons. They only compared to human skeletal myoblasts in transplantation experiments, which is a poor substitute for muscle stem cells.

Insofar, I share several of the sentiments issued by the previous reviewers. Along this line. I am not so sure, whether the rather difficult protocol, employing three different cell types (TCE), will be readily picked up by community, notwithstanding its speed and somewhat improved efficiency. I do not see any novel mechanistic insights in the paper and some claims still seem exaggerated.

I think it is fair to say that the authors have developed a technique that generates myogenic cells, which are suitable for transplantation. Essential problems, such as population of the muscle stem cell niche by TCE-derived myogenic cells and long-

term self-renewal were not addressed, which is a major shortcoming. The paper may be acceptable for publication in my view, if some critical issues are addressed.

1.) Comparison of TCE-derived myogenic cells to other hiPSC-derived muscle (stem) cells. So far, the authors only compared to human skeletal myoblasts, which perform notoriously poor in transplantation experiments. To obtain a fair assessment of the quality, it is not adequate to use a badly performing cell population, but a population that does a good job. Comparison to existing protocols was only done in respect to the generation of Pax7+ cells but not of the performance of newly generated Pax7+ cells in terms of transplantation and differentiation

2.) The authors tried to address the fate of TCE-derived myogenic cells after transplantation into mdx mice. In addition to fusion to myofibers, they claim to have observed a contribution to the muscle stem cell compartment. I do not find the results convincing, which rely on separate (!) double staining with either laminA/C (nuclear membrane of human cells) and dystrophin to laminA/C and laminin to identify the basal lamina. This approach is problematic. Lamin A/C labels all human-cell derived nuclei, not only muscle stem cells. Separate staining for laminin and dystrophin does not make much sense, since a cell above dystrophin may be either inside or outside the basal lamina. Cells below the basal lamina might be within or outside the myofiber. Only a combinatorial staining will tell whether transplanted cells have acquired a muscle stem cell position. The authors may also consider to isolate individual fibers and do the staining, which would probably be much more convincing. A specific human muscle stem cell marker would also be much better and/or a co-staining of lamin A/C with a muscle stem cell marker.

3.) The identify of non-differentiating cells TCE-derived myogenic cells in vitro is a mystery to me. According to the authors 43% of the initial Pax7-positive cells end up in MHC+ myotubes, 39% express Pax7, which means that 18% remain. What are these cells? Myoblasts that downregulated Pax7? Do the Pax7-negative cells proliferate or can they be separated into a proliferating and non-proliferating cell population? I do not think that the failure of Pax7+ cells to proliferate qualifies them as "reserve cells". If the authors think so, they should transplant such reserve cells and analyze whether they indeed demonstrate functional features of reserve/stem cells in vivo.

4.) Serial transplantations are clearly required to assess the long-term potential of TCE-derived myogenic cells to populate the muscle stem cell niche. I also think that the authors need to extend the time window to analyze the long-term fate of transplanted cells.

5.) Functional improvements were only tested at one time point, 17 days after transplantation, and compared to human skeletal myoblasts. Why did the authors omit a sham-transplanted control? Why only eccentric contraction force? In my opinion, the use of eccentric contraction creates an additional bias. Eccentric contraction damages the muscles, particularly in mdx mice, as rightly pointed out by the authors. One would like to see a force-frequency curve, assessment of recovery from fatigue, etc. Force recordings for obtained specific isometric twitch force and tetanic force should be shown after normalization to body weight.

Some minor points:
The authors suggest that they have generated a "stem cell niche", which supports generation of muscle stem cells, which is not really accurate. In fact, they used a 3D co-culture model combined with CHIR/FGF2 treatment, in which the added cells had a much lower impact than the chemical induction. Of course, some cues from growth-arrested fibroblasts and embryonic endothelial cells may support myogenic differentiation in addition to the CHIR/FGF2 treatment, but I would hardly call such an installment a stem cell niche. A more modest wording seems adequate.
The authors still state that they achieved 99% pure Pax7-positive with the TCE-technique. Technically the statement is not wrong but does it make sense to mention such a number AFTER FASCS purification? It is possible to achieve 99% purity even with the worst protocol, if the FACS purification works efficiently.
It would indeed be nice to show some disease-modelling in the paper, as suggested by my fellow reviewers but this is not a game-changer for me. Disease modelling might be done with any patient-derived myogenic cell that generates myotubes.

Arbitrating advisor # 2 comments:

I have looked over the manuscript, reviewers comments and responses.

Here are a few comments that can hopefully guide your decision making.

* I thought the comments from the four reviewers were consistent: They all wanted a lot more clarification and experimentation, specifically related to function, variation, utility and mechanism. While I'm not embedded in the cell therapy/engineering-type field unlike the reviewers, my initial reaction was that they were asking for work, way beyond what has been published in the past.

* However the general points were reasonable.

* I am very impressed with the amount of work performed by the authors to address the reviewers points. This is a robust and compelling response with substantial amount of new data to address all of the comments. The new data shows that this method is superior to two other published methods in terms of speed and function. These are two critical attributes to any cell based translational work. Whether that is cell therapy or a component of drug discovery, I don't believe it is the authors responsibility to answer which one in the present study.
* The authors have provided significant data on cell function, phenotyping and molecular characterization.
* The authors address the issue of variability by performing key experiments in different conditions and providing transparent analysis.
* The authors have gone to great lengths to understand some of the mechanistic responses between different protocols. I thought this request from the reviewer was unjustified.

* In conclusion, the authors provide an alternative approach to those published to date. This approach is superior in speed and function than two other published approaches. The authors provide in- depth characterization of the cells and discuss the potential downstream applications-with an appropriate level of circumspection. In my opinion, this work warrants publication.

Response to arbitrating advisors

Arbitrating advisor # 1 comments:

I have carefully read the manuscript, the reviewers' comments, and the authors' responses.

Some comments of the previous reviewers were addressed convincingly, but not all of them. In fact, some crucial experiments are still missing. In my opinion, the paper describes an interesting approach to generate myogenic progenitor cells that might be useful for therapeutic applications (regardless of the extensively discussed GMP-limitations). The technology itself is not brand new or a quantum leap but rather represents a combination of already existing protocols (co-culture plus CHIR/FGF2). Compared to already available approaches the new technique has the distinctive advantage of being much faster than previous protocols and seems to generate higher yields of myogenic cells. It is difficult to judge whether the quality of newly generated myogenic cells is equal, better or worse compared to existing protocols, since the authors essentially did not do any direct comparisons. They only compared to human skeletal myoblasts in transplantation experiments, which is a poor substitute for muscle stem cells.

We thank the arbitrator for highlighting that speed and efficiency are distinct advantages of our method.

We would like to emphasize that it is technically very difficult to compare cells generated using our method side-by-side with published transgene-free myogenic hiPSC differentiation protocols. Most published methods are designed to generate differentiating myogenic cells that fuse into myotubes in the end-stage of the protocol in 2D, while our approach is tailored to produce uncommitted Pax7 positive embryonic-like myogenic progenitors (eMPs) in 3D. Moreover, in a secondary screen we identified cell surface markers allowing for enrichment of 99% of the Pax7 positive eMP population from chemically induced three-component embryoids (iTCEs) (**Fig 3A-I and Appendix Table S6**). However, no enrichment strategies for Pax7 positive cells that would allow us to benchmark them to our method in downstream applications are available in published protocols. We observed that when two popular myogenic hiPSC differentiation protocols, Choi et al., 2016, and Shelton et al., 2014, were completed, they contained only between 7.8% and 10.8% Pax7+ cells at day 30 and 50, respectively (**Appendix Fig S4E-H, and Appendix Table S1**) when compared to a yield of 40-50% using our two-week protocol. Thus, we hope that the arbitrator agrees that transplantation of the mixed cultures obtained from published myogenic hiPSC protocols (containing ~90% Pax7 negative cells including differentiated myotubes) and benchmarking them to our pure Pax7 positive population would not be a very meaningful comparison. For these reasons, we decided to compare eMPs *in-vivo* to human myoblasts, the only cell type in the myogenic lineage that has been tested so far in human patients.

To still obtain a measure of the quality of eMPs compared to cells generated using other protocols, we quantified the number of Pax7 positive eMPs and their fusion index in proliferation (PM) and differentiation (DM) media and compared it to cells generated using the Choi and Shelton methods after two weeks (D13), and at the endpoint of the differentiation protocol at 30 (D30)

respectively 50 days (D50). This experiment revealed that the number of Pax7 positive eMPs in 2D culture was higher for eMPs in both PM and DM when compared to the other methods at all time-points (**Appendix Fig S7A**). Moreover, eMPs in DM fused to the same degree as cells at day 30 of the Choi et. al. protocol, while they produced more differentiated myotubes than Shelton et al. at day 50 (**Appendix Fig S7B**). Thus, next to speed and efficiency, our method also generates equal or more myogenic cells than published methods. At the same time, it allows for efficient segregation of proliferation and differentiation, which enables a wide range of possible downstream applications.

Insofar, I share several of the sentiments issued by the previous reviewers. Along this line. I am not so sure, whether the rather difficult protocol, employing three different cell types (TCE), will be readily picked up by community, notwithstanding its speed and somewhat improved efficiency. I do not see any novel mechanistic insights in the paper and some claims still seem exaggerated.

We respectfully disagree with the arbitrator. Our method requires only two widely available immortalized cell lines (embryonic endothelial cells and fibroblasts) that are used together with hiPSCs to form iTCEs. The only non-standard infrastructure that is required for our method is a horizontal shaker platform in an incubator. Compared to the complicated published multi-step protocols that take several weeks (if not months) to complete, our method takes only two weeks. Lastly, our method allows to produce unprecedented quantities of pure Pax7 positive cells in suspension culture. The latter is an important advantage for scale-up and makes handling of the cells very easy (no passaging required and a low amount of plasticware needed). Finally, we have been approached at multiple instances by groups interested in a straight-forward method to produce Pax7 positive cells and they successfully implemented our protocol. For all these reasons, we are convinced that publication of our protocol will be well received by the field.

I think it is fair to say that the authors have developed a technique that generates myogenic cells, which are suitable for transplantation. Essential problems, such as population of the muscle stem cell niche by TCE-derived myogenic cells and long-term self-renewal were not addressed, which is a major shortcoming. The paper may be acceptable for publication in my view, if some critical issues are addressed.

Comparison of TCE-derived myogenic cells to other hiPSC-derived muscle (stem) cells. So far, the authors only compared to human skeletal myoblasts, which perform notoriously poor in transplantation experiments. To obtain a fair assessment of the quality, it is not adequate to use a badly performing cell population, but a population that does a good job. Comparison to existing protocols was only done in respect to the generation of Pax7+ cells but not of the performance of newly generated Pax7+ cells in terms of transplantation and differentiation

As outlined above, due to the absence of established flow cytometry isolation protocols for enrichment of Pax7 positive cells from published protocols, we concluded that human myoblasts are the most suitable control in a transplantation application of our method.

The authors tried to address the fate of TCE-derived myogenic cells after transplantation into mdx mice. In addition to fusion to myofibers, they claim to have observed a contribution to the muscle stem cell compartment. I do not find the results convincing, which rely on separate (!) double staining with either laminA/C (nuclear membrane of human cells) and dystrophin to laminA/C and laminin to identify the basal lamina. This approach is problematic. Lamin A/C labels all human-cell derived nuclei, not only muscle stem cells. Separate staining for laminin and dystrophin does not make much sense, since a cell above dystrophin may be either inside or outside the basal lamina. Cells below the basal lamina might be within or outside the myofiber. Only a combinatorial staining will tell whether transplanted cells have acquired a muscle stem cell position. The authors may also consider to isolate individual fibers and do the staining, which would probably be much more convincing. A specific human muscle stem cell marker would also be much better and/or a co-staining of lamin A/C with a muscle stem cell marker.

We thank the arbitrator for these suggestions. Importantly, we demonstrate using bioluminescence and a multiple injury paradigm that transplanted luciferase eMPs can efficiently reactivate (**Fig 4E and F**). This experiment shows that some eMPs retain their stem cell character and can participate in repeated rounds of regeneration.

Based on the arbitrator's concerns regarding the localization of transplanted eMPs in the satellite cell position, we performed a Lamin A/C-Dystrophin-Laminin co-staining (**Appendix Fig S8B**). This experiment revealed that eMPs can indeed engraft in the satellite cell position in between the basal lamina and the muscle fiber plasma membrane. In addition, we now also show that transplanted Lamin A/C positive eMPs stain positive for the human muscle stem cell marker CD56 (**Appendix Fig S8A**).

The identify of non-differentiating cells TCE-derived myogenic cells in vitro is a mystery to me. According to the authors 43% of the initial Pax7-positive cells end up in MHC+ myotubes, 39% express Pax7, which means that 18% remain. What are these cells? Myoblasts that downregulated Pax7? Do the Pax7-negative cells proliferate or can they be separated into a proliferating and non-proliferating cell population? I do not think that the failure of Pax7+ cells to proliferate qualifies them as "reserve cells". If the authors think so, they should transplant such reserve cells and analyze whether they indeed demonstrate functional features of reserve/stem cells in vivo.

We thank the arbitrator for pointing this out. Myonuclear acquisition in human cells *in-vitro* occurs for up to 10 days (Cheng et al. 2014, Am J Physiol Cell Physiol.). Thus, we consider it likely that these cells are myocytes in the process of fusion. It has also been shown that a fraction of human primary myoblast form reserve cells (unfused cells after differentiation) that are Pax7 negative and express MyoD and Myf5 (Laumonier et al. 2017, Scientific Reports). Since both scenarios are possible, we removed any reference to "reserve cells" from the manuscript text.

C 4.) Serial transplantations are clearly required to assess the long-term potential of TCE-derived myogenic cells to populate the muscle stem cell niche. I also think that the authors need to extend the time window to analyze the long-term fate of transplanted cells.

Using bioluminescence, we demonstrate that transplanted eMPs have stem cell character and can reactivate after repeated injury (**Fig 4E and F**). Serial transplantation is yet another possible readout to assess the stem cell character of the cells. Apart from the partial redundancy with our bioluminescence readout, we feel that this goes beyond the scope of our present manuscript whose central message is merely to describe a novel method for production of Pax7 positive myogenic progenitors from hiPSCs. We'd like to emphasize that we only included the transplantation experiments to demonstrate an example of the many possible downstream applications of our method. Related methods papers often do not even include such examples, and if they do, they are rarely extensive enough to contain diverse engraftment readouts including force generation and bioluminescence after repeated injury as for our study. Thus, we hope that the arbitrator agrees that performing serial transplantation experiments would go beyond the scope of a proof-of-concept study and is not achievable within the shortened timelines of an arbitration process.

Functional improvements were only tested at one time point, 17 days after transplantation, and compared to human skeletal myoblasts. Why did the authors omit a sham-transplanted control? Why only eccentric contraction force? In my opinion, the use of eccentric contraction creates an additional bias. Eccentric contraction damages the muscles, particularly in mdx mice, as rightly pointed out by the authors. One would like to see a force-frequency curve, assessment of recovery from fatigue, etc. Force recordings for obtained specific isometric twitch force and tetanic force should be shown after normalization to body weight.

As mentioned above, our *in-vivo* experiments are illustrative of one of the possible downstream applications for eMPs and not central to the message of the paper. For these reasons, we chose to focus only on the most important aspects. Eccentric contraction is considered to be the main trigger for muscle damage in Duchenne muscular dystrophy (Blaauw, Agatea et al., 2010). Therefore, it represents an ideal paradigm to test for strengthening of myofibers after dystrophin re-expression. Since a sham control was not directly relevant to our research question and only two hindlegs are available for transplantation in a given mouse, we decided to follow the 3R ethical guidelines regarding reduction of animal experiments and did not include such an experiment.

Some minor points:

The authors suggest that they have generated a "stem cell niche", which supports generation of muscle stem cells, which is not really accurate. In fact, they used a 3D co-culture model combined with CHIR/FGF2 treatment, in which the added cells had a much lower impact than the chemical induction. Of course, some cues from growth-arrested fibroblasts and embryonic endothelial cells may support myogenic differentiation in addition to the CHIR/FGF2 treatment, but I would hardly call such an installment a stem cell niche. A more modest wording seems adequate.

The components of a stem cell niche are defined as accessory cells providing cell-cell contacts, extracellular matrix, and diffusible factors (e.g., secreted proteins, metabolites, microvesicles

etc…). Our study demonstrates that the supportive embryonic cell types dramatically increase the yield of Pax7 positive cells from hiPSC embryoids (**Fig. 1Q**) by secreting growth factors (e.g., Igf1) and by presenting cell-cell receptors (e.g., Notch; **Fig. 2J**). In addition, we show that TCEs contain extracellular matrix components such as Laminin (**Fig. 1N**). Thus, while not claiming we "reconstructed" a *bona fide* stem cell niche, we factually "engineered" one (as the title of our paper states).

The authors still state that they achieved 99% pure Pax7-positive with the TCE-technique. Technically the statement is not wrong but does it make sense to mention such a number AFTER FASCS purification? It is possible to achieve 99% purity even with the worst protocol, if the FACS purification works efficiently.

We thank the arbitrator for pointing this out. **Fig. 3A-I** shows a screen for different cell surface markers for their ability to enrich the Pax7 positive cell population from eMPs. Using the combination of CD56 and Integrin α9 we were able to isolate an eMP population that is 99% Pax7 positive, while other markers showed a much lower efficiency (ranging from 7%-88%). To accommodate the arbitrator's concern, we removed the statements regarding purity and now mention that we identified cell surface markers allowing for "enrichment of an >99% Pax7 positive eMP population from iTCEs".

It would indeed be nice to show some disease-modelling in the paper, as suggested by my fellow reviewers but this is not a game-changer for me. Disease modelling might be done with any patient-derived myogenic cell that generates myotubes.

We agree with the arbitrator. Our method opens the door to a wide range of possible applications including cell therapy, screening, modeling of disease (e.g., muscular dystrophy), and embryonic development. To illustrate an example, we chose transplantation (likely the most challenging of all options) to compare the engraftment of eMPs to human myoblasts, the only cell type in the myogenic lineage that has already been in human clinical trials for muscular dystrophy. Many of the mutations causing muscular dystrophy have been identified, and prior proof of concept demonstrated that hiPSC-derived myogenic cells can indeed be used to model disease features. Thus, although beyond the scope of our present study, we consider disease modeling an important future application of our protocol.

Arbitrating advisor # 2 comments:

I have looked over the manuscript, reviewers comments and responses.

Here are a few comments that can hopefully guide your decision making.

* I thought the comments from the four reviewers were consistent: They all wanted a lot more clarification and experimentation, specifically related to function, variation, utility and mechanism. While I'm not embedded in the cell therapy/engineering-type field unlike the reviewers, my initial reaction was that they were asking for work, way beyond what has been published in the past.

* However the general points were reasonable.

* I am very impressed with the amount of work performed by the authors to address the reviewers points. This is a robust and compelling response with substantial amount of new data to address all of the comments. The new data shows that this method is superior to two other published methods in terms of speed and function. These are two critical attributes to any cell based translational work. Whether that is cell therapy or a component of drug discovery, I don't believe it is the authors responsibility to answer which one in the present study.

* The authors have provided significant data on cell function, phenotyping and molecular characterization.

* The authors address the issue of variability by performing key experiments in different conditions and providing transparent analysis.

* The authors have gone to great lengths to understand some of the mechanistic responses between different protocols. I thought this request from the reviewer was unjustified.

* In conclusion, the authors provide an alternative approach to those published to date. This approach is superior in speed and function than two other published approaches. The authors provide in- depth characterization of the cells and discuss the potential downstream applications- with an appropriate level of circumspection. In my opinion, this work warrants publication.

We thank the arbitrator for his positive evaluation and for pointing out that our method is superior to other protocols regarding speed and function. We'd also like to highlight that our method is suspension-based, which we believe to be yet another advantage compared to published 2D based methods for transgene-free myogenic hiPSC differentiation since it allows for scale-up in bioreactor settings. We also appreciate that the arbitrator acknowledges that we went at great length to provide mechanistic insights and that we provided significant phenotyping data. With cell-therapy, we included an example of a potential downstream application of our method, which goes beyond the typical standard of published myogenic hiPSC differentiation protocols.

1st Revision - Editorial Decision **11th May 2022**

Dear Florian, dear Jerome,

Thank you for submitting the revised version of your manuscript. We have now evaluated your amended study and concluded that the remaining concerns of arbitrator #1 have been sufficiently addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing in this case the arbitrating advisors' comments and your response letter) to be published as an online supplement to each paper.

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Should you be planning a Press Release on your article, please get in contact with embojournal@wiley.com as early as possible, in order to coordinate publication and release dates.

On a different note, I would like to alert you that EMBO Press is currently developing a new format for a video-synopsis of work published with us, which essentially is a short, author-generated film explaining the core findings in hand drawings, and, as we believe, can be very useful to increase visibility of the work. This has proven to offer a nice opportunity for exposure i.p. for the first author(s) of the study. Please see the following link for representative examples and their integration into the article web page:
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Please let me know, should you be interested to engage in commissioning a similar video synopsis for your work. According operation instructions are available and intuitive.

If you have any questions, please do not hesitate to call or email the Editorial Office.

Thank you for this contribution to The EMBO Journal and congratulations on a successful publication! Please consider us again in the future for your most exciting work.

with
Best regards,

Daniel

EMBO Press Author Checklist

Corresponding Author Name: C. Florian Bentzinger & Jerome N. Feige	
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Manuscript Number: EMBOJ-2022-110655	

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Reporting Checklist for Life Science Articles (updated January 2022)
This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.
Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures

1. Data
The data shown in figures should satisfy the following conditions:
- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions
Each figure caption should contain the following information, for each panel where they are relevant:
- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	Materials and methods

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and methods

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Materials and methods

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Materials and methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	Materials and methods
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	We regularly test all cell lines for mycoplasma. This information is included in the methods

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Materials and methods
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Materials and methods

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Some core facility directors and employees are included as co-authors and some are mentioned in the acknowledgements.

Design

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If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Materials and methods and figure legends
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Yes	Materials and methods
Include a statement about **blinding** even if no blinding was done.	Yes	Materials and methods
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	No data was excluded.
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and methods and figure legends

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Materials and methods and figure legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Materials and methods and figure legends

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Materials and methods
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

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Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
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Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	All transcriptomics datasets have been deposited on GEO. This information is included in the data availability section.
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Yes	Materials and methods