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Peer Review File

Advanced physiological maturation of human iPSC-derived human cardiomyocytes using an algorithm-directed optimization of defined media components

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This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In the present study, Callaghan et al. presented a new formula for hPSC-CM maturation media and demonstrated that it promotes more mature hPSC-CM functional and morphological properties. More specifically, cells appear to have increased size and improved striations, improved calcium handling, electrophysiological and contractile properties, and better aerobic metabolism. The authors have done a significant amount of revision work, and their culture medium appears to convincingly promote a more mature hPSC-CM phenotype.

However, there are three main residual concerns regarding their manuscript. (1) The transcriptomic data on treated cells is rather controversial at times, especially as it relates to sarcomeric genes. (2) the authors do not provide any evidence using hPSCs from a disease model to prove the applicability of their approach. (3) The main criticism of this work involves the comparisons of hPSC-CMs in C16 vs other previously published maturation media. Although I do understand that repeating all experiments and analyses of cells grown in the Feyen et al. media protocol (which admittedly has had a more consistent effect on maturation properties) is quite burdensome, this would significantly strengthen the novelty and significance of this work. I think the authors should at least perform morphometric/structural analyses and calcium transient recordings that are critical for the functional maturation of hPSC-CMs comparing C16 with the Feyen medium. Moreover, and given the discrepancy in the expression of sarcomeric proteins, it will be useful if the authors could compare contractile properties of hPSC-CMs in Feyen vs C-16 media.

Minor comments:

Individual values should be added in each graph in Fig. 3n-u to assist the readers and reviewers interpret the number of experiments performed.

Reviewer #2

(Remarks to the Author)
The manuscript has substantially improved since its last version submitted to NBT. There are, however, still some points to address:
1. This revised statement is still too negative and general on the current state-of-the-art: “However, despite growing acceptance, current iPSC-CMs remain phenotypically immature in all aspects of CM-specific physiology…” There are multiple studies showing conditions for positive force-frequency for example, well-organized sarcomeres, t-tubules, action potentials of adult cardiomyocyte magnitude, etc. Perhaps defining “mature” would help: postnatal is what most studies achieve with some physiological parameters “adult-like” although none typical of aged cells. Please modify.
2. Regarding the comments on the low and variable n in the electrophysiology data: this hardly seems to have increased. Just a different method of statistical analysis has been used: the only text revision is “Data were analyzed by non-parametric

Mann-Whitney U-test."
Otherwise, the work will be a useful addition to the literature in this area although the question of whether the algorithm is relevant for the maturation of other cell types remains open. You could conclude that the algorithm has demonstrated its value for CM maturation and therefore its value is complete.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed most of my concerns. Although I remain somewhat skeptical about the comparisons of their approach to the Feyen et al. protocol and the novelty of their work, I do think that their methodology can likely have applications for the in vitro maturation of hPSC-CMs.

Reviewer #2

(Remarks to the Author)
The authors have made some useful additions to the manuscript which brings some improvement.
Regarding points of reviewer 1:

1. There are still some overstatements: eg "and are not satisfactorily predictive of patient responses to pharmaceuticals (ref 35). There are multiple reports such as the CiPA study, showing for arrhythmias, monolayer hiPSC-CMs are predictive of drug responses. In addition Saleem et al (doi: 10.1093/toxsci/kfaa058) showed in Engineered Heart Tissues that contraction responses to drugs are >80% predictive of responses in humans cf ±70% in routinely-used primary rabbit cardiomyocytes. There are multiple other studies of this type. This would seem (more than) satisfactory especially as here neither an extensive screen of test compounds nor multiple diseased hiPSC-CMs have been examined.

Minor points: phenotypically immature IN most aspects : IN has been deleted in error
Please use the conventional hiPSC and hiPSC-CMs for human iPSCs, throughout, as also reviewer 2. iPSC would normally refer to mouse iPSCs.

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Title: **Advanced physiological maturation of iPSC-derived human cardiomyocytes using an algorithm-directed optimization of defined media components**

RESPONSE TO REVIEWERS

Thank you to the editor and reviewers for their careful and constructive review of our manuscript. We believe that, having fully considered all comments and with the addition of new experiments, we have strengthened the manuscript to make it suitable for publication. We have included directly below a point-by-point response to the reviewer comments, including a summary of all of our changes to the manuscript in accepting the reviewers' suggestions.

Reviewer comments are provided in blue below, with our responses in black and changes to the text in the manuscript in red.

REVIEWER #1:

Remarks to the Author:

The manuscript has substantially improved since its last version submitted to NBT. There are, however, still some points to address:

1. This revised statement is still too negative and general on the current state-of-the-art: "However, despite growing acceptance, current iPSC-CMs remain phenotypically immature in all aspects of CM-specific physiology…" There are multiple studies showing conditions for positive force-frequency for example, well-organized sarcomeres, t-tubules, action potentials of adult cardiomyocyte magnitude, etc. Perhaps defining "mature" would help: postnatal is what most studies achieve with some physiological parameters "adult-like" although none typical of aged cells. Please modify.

We have included additional context summarizing important advancements in iPSC-CM maturity by parameter in the introductory paragraph to highlight both the range and magnitude of change of morphological and functional metrics of interest, while still highlighting the gap between maturing PSC-CMs and adult CMs *in situ* (Line 23):

> Both disease phenotypes and drug responses are emergent composites of interactions across multiple aspects of physiology [8,9], necessitating higher-fidelity *in vitro* models of myocardium. Important advances have been made in evoking physiological maturation in both iPSC-CM monolayers and organoids in translational and preclinical applications [10–12], including transcriptomic or proteomic profiles [13–30], morphometrics [13,15,27,29–31,16,18–22,25,26], metabolic profile [13,14,28–31,15,16,20–23,26,27], Ca^{2+} handling [16,19,29–31,20–27], electrophysiology [15,16,20,23,24,26,27,29–31], and

> disease modeling [13,16,24,26,27,29,30,32,33]. However, despite the advancements of many physiological parameters of current iPSC-CMs to neonatal levels or beyond, they ~~are~~ remain phenotypically immature ~~in all~~ most aspects of CM-specific physiology; they do not recapitulate the contractile force, morphology, electrophysiology, Ca^{2+} handling characteristics, or metabolic profile associated with adult myocardium [34], and are not satisfactorily predictive of patient responses to pharmaceuticals [35].

2. Regarding the comments on the low and variable n in the electrophysiology data: this hardly seems to have increased. Just a different method of statistical analysis has been used: the only text revision is "Data were analyzed by non-parametric Mann-Whitney U-test."

We completed new experiments in which I_{K1} measurements were made with larger sample sizes (**Figure 2g**). In keeping with Reviewer 2's suggestion of comparing additional metrics against the high-efficacy maturation medium of Feyen *et al*, we have additionally included this treatment in the experiment. These independent studies confirm our original findings (Line 133):

> Measurement of I_{K1} density slope between -115 and -95 mV further demonstrated significant differences between C16-treated cells to those treated with RPMI + B27 or the high-performing medium of Feyen *et al.* [29] (**Figure 2g**).

3. Otherwise, the work will be a useful addition to the literature in this area although the question of whether the algorithm is relevant for the maturation of other cell types remains open. You could conclude that the algorithm has demonstrated its value for CM maturation and therefore its value is complete.

We thank the reviewer for their appreciation of the value of our work and for their constructive feedback to improve it.

REVIEWER #2:

Remarks to the Author:

In the present study, Callaghan et al. presented a new formula for hPSC-CM maturation media and demonstrated that it promotes more mature hPSC-CM functional and morphological properties. More specifically, cells appear to have increased size and improved striations, improved calcium handling, electrophysiological and contractile properties, and better aerobic metabolism. The authors have done a significant amount of revision work, and their culture medium appears to convincingly promote a more mature hPSC-CM phenotype.

However, there are three main residual concerns regarding their manuscript:

(1) The transcriptomic data on treated cells is rather controversial at times, especially as it relates to sarcomeric genes.

We agree that some of transcriptomic data, including certain sarcomeric genes, do not completely mirror the more immediately relevant proteomic or functional metrics. We discussed this on Line 275:

> Furthermore, the transcriptional downregulation of key traditional markers of iPSC-CM maturity such as *MYH7* and *TNNI3* in the C16 treatment was not reflected in the expression of their protein products, suggesting that transcriptional assessment without

corresponding protein or functional verification may not be sufficient in determining the maturation status of advanced iPSC-CM cultures.

We speculate that given the electrophysiological basis for non-spontaneous contraction in iPSC-CMs treated with C16 for 3 weeks, it is possible that decreased cyclic mechanical strain contributed to lower turnover and therefore gene expression, despite the stability of corresponding protein expression. We have added a short passage to this effect in the discussion (Line 278):

> Additionally, given the electrophysiological basis for non-spontaneous contraction in the C16-treated iPSC-CMs, it is possible that decreased cyclic mechanical strain contributed to lower turnover and therefore gene expression, despite the stability of corresponding protein expression.

(2) the authors do not provide any evidence using hPSCs from a disease model to prove the applicability of their approach.

We include in the Results (Line 100) and Supplementary Figure 1 a demonstration of differential function of a disease model based on CRISPR knockout of *MYBPC3*, which shows modified contractile kinetics that vary based on maturation state, demonstrating the importance of iPSC-CM maturity in modeling cardiomyopathy.

> Optical contractile traces, obtained using particle image velocimetry, demonstrated… pathologically slowed and biphasic contraction in a MYBPC3-KO CRISPR-generated PGPC17 mutant iPSC line (**Figure S1g**).

(3) The main criticism of this work involves the comparisons of hPSC-CMs in C16 vs other previously published maturation media. Although I do understand that repeating all experiments and analyses of cells grown in the Feyen et al. media protocol (which admittedly has had a more consistent effect on maturation properties) is quite burdensome, this would significantly strengthen the novelty and significance of this work. I think the authors should at least perform morphometric/structural analyses and calcium transient recordings that are critical for the functional maturation of hPSC-CMs comparing C16 with the Feyen medium. Moreover, and given the discrepancy in the expression of sarcomeric proteins, it will be useful if the authors could compare contractile properties of hPSC-CMs in Feyen vs C-16 media.

To complement our existing global proteomic data that comprehensively compare the Feyen medium to C16 and other control media (Fig. 5), we performed new experiments to compare morphometric and structural parameters including the Feyen medium (Figure 1f/h, Figure S1f) (Line 90):

> Compared to treatment with both STEMCELL and a leading high-efficacy maturation formulation by Feyen et al. [29], iPSC-CM monolayers treated with C16 medium exhibited increased spontaneous alignment (circular variance $p < 0.0001$), cell elongation (eccentricity $p = 0.0019$), and intracellular organization (α-actinin:connexin-43 (Cx43) colocalization coefficient $p < 0.0001$; Figure 1f), while confocal microscopy demonstrated that C16 medium enhanced myofibrillar density, α-actinin striation, and Cx43 junctional expression (Figure 1g). C16-treated cells demonstrated clear sarcomeric striations under phase-contrast in C16-treated monolayers (Figure S1d-e) with increased sarcomere length ($p = 0.0003$; Figure 1h), as well as increased hypertrophy (projected area $p <$

0.0001; perimeter $p < 0.0001$) and anisotropy (form factor $p < 0.0001$), but not α-actinin integrated density ($p = 0.91$) (Figure S1f).

In expanding our electrophysiological experiments per the suggestions of Reviewer 1, we have now included iPSC-CMs treated in parallel with the Feyen medium (Figure 2g). As the reviewer is aware, the medium of Feyen *et al.* is highly effective at arresting cell proliferation and in advancing multiple metrics of maturity. We were unfortunately unable to replicate the extent of the absolute magnitude of I_{K1} current reported in the Feyen *et al*. paper (c.a. -7.5 pA/pF at -115 mV), despite very favourable cell hypertrophy, morphological changes, contractility, and metabolic performance. This discrepancy could be due to differences in cell lines, replating technique, or electrophysiological technique. Regardless, we were able to demonstrate outsized performance of the Feyen medium relative to maintenance media and of C16 relative to both, which is included as Figure 2g as voltage-dependent slope of current density (Line 130):

> Measurement of IK1 density slope between -115 and -95 mV further demonstrated significant differences between C16-treated cells to those treated with RPMI + B27 or the high-performing medium of Feyen et al. [29] (Figure 2g).

Minor comments:

Individual values should be added in each graph in Fig. 3n-u to assist the readers and reviewers interpret the number of experiments performed.

We have added the individual values as suggested.