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Establishing optimal culture conditions for electrophysiological characterization of human pluripotent stem cell-derived cardiomyocytes.

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Abstract

Functional analysis of human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) often includes patch clamp electrophysiology and acquiring action potential (AP) profiles. Protocols to induce hPSC differentiation towards the cardiac lineage vary and it is not clear the extent to which the protocol used affects the recorded APs. Here we investigated the impact of factors such as differentiation time, addition of compounds that influence cell fate and cell seeding density on hPSC-CM AP characteristics. The study showed that: a) AP was affected most noticeably by the cell density used after the dissociation of cardiac structures; b) day 21 was the time point of differentiation that was most favourable for AP analysis; c) The use of XAV939 in combination with the monolayer differentiation protocol improved cardiac morphology and the yield of cells of consistent quality that could be used for analysis. Our results help establish optimal culture and differentiation conditions for AP analysis frequently used in studies that utilize hPSC-CMs as a cardiac disease model.
Introduction

Improving the electrophysiological phenotype of human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CMs) such that they mature to resemble cells of adult rather than fetal heart is one of the major challenges of hiPSC-CM research. The acceptability of hiPSC-CMs for disease modeling and gaining insight into the pathophysiology of cardiac disease would be greatly improved if this issue was solved. As mentioned in Chapter 2 [1], various strategies have been used to enhance maturity of the hiPSC-CM phenotype. Aside from altering the transcriptome of hiPSC-CMs [2], co-culturing them with other cell types [3] or adding specific combinations of hormones and growth factors [4], changes in hPSC-CM culture conditions may also promote maturation of the electrophysiological output of the cells. Since hiPSC-CMs are cultured and differentiated in a variety of formats [5] including monolayer cultures, aggregates (or embryoid bodies/EBs) and multicellular co-culture, which format yields cardiomyocytes with the best functional phenotype has remained unexplored. Furthermore, although the long term culture of hiPSC-CMs has been shown to benefit cardiomyocyte maturity [6-8], total differentiation times above thirty days are considered impractical for research use. That aside, the question of whether there is a best time point to record the electrophysiological properties of hiPSC-CMs between the moment they start beating (~day 9) until the threshold of day 30 has not yet been answered. Apart from their electrophysiological immaturity it has been shown that cardiomyocytes from different hiPSC (or embryonic hESC-) also have significant variability in baseline electrophysiological characteristics [1,9], that could be attributed to the distinct genetic background of the individuals from which they were derived. Factors such as sex, ethnic background or age might also impact the electrophysiological profile of generated hiPSC-CMs. Compounds added at the initial step of hPSC-CM differentiation could also be relevant. For instance, the growth factors that trigger mesoderm formation in the initial step of cardiac differentiation are crucial for the quality of the resulting cardiomyocytes. BMP4 and Activin A are of particular significance and require cross-titration to determine their optimal concentrations for differentiation of each hPSC line. Other compounds such as Wnt inhibitors also improve cardiac differentiation when added at the early stages of mesoderm induction [10]. In many protocols a tankyrase inhibitor, XAV939 is added at day 3 of differentiation to inhibit Wnt/β-catenin signaling and enhance cardiac specification. However its role in determining cardiomyocyte function at the end of differentiation has not yet been determined.

In this chapter we present studies that address the issues above, by recording and analysing the AP characteristics of multiple hiPSC-CMs and hESC-CMs prepara-
tions: 1) at various time points of differentiation; 2) with and without addition of XAV939 3) using the monolayer or aggregate differentiation approaches 4) seeding them in a low or high density after their dissociation. Not all conditions yield cells of the same quality of cardiomyocytes in terms of their ability to undergo patch clamping and the consistency with which they do so.
**Results**

**hPSC lines selected for analysis and experimental scheme**

The following lines were selected for inclusion in this study: 1) hESC reporter cells designated ΔN3 [11] in which the promoter of the NK2 homeobox 5 targeted by GFP; 2) An hiPSC reporter line designated hiPSC-NKX2-5 with the same targeted locus (Berg et al unpublished data); 3) An hiPSC line designated DF6 [12]; 4) An hiPSC line designated Ctrl04 [13]. All lines were derived from healthy individuals. Table 1 provides a description of the lines used and their characteristics.

Cardiac differentiation was induced using a monolayer [12] or embryonic body [14] approach with defined medium (BPEL) and cytokines to direct the cells towards the cardiac mesoderm lineage (Fig.1). After dissociation the action potential profile of the cardiomyocytes from all lines was acquired at various experimental conditions.

<table>
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<th>Name of the line</th>
<th>Type</th>
<th>Reprogramming method</th>
<th>Cell type reprogrammed</th>
<th>Origin</th>
<th>Line characteristics</th>
<th>Study in which line was used</th>
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<td>-</td>
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<td>NKKX2.5 targeted with eGFP</td>
<td>Elliott et al [10]</td>
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<td>Berg et al [in prep]</td>
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<td>hiPSC</td>
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<td>Fetal fibroblasts</td>
<td>Female</td>
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<tr>
<td>Ctrl04</td>
<td>hiPSC</td>
<td>Sendai vector</td>
<td>Dermal fibroblasts</td>
<td>Male/European</td>
<td></td>
<td>Kosmidis et al [12]</td>
</tr>
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</table>

**Action potential characteristics of cardiomyocytes at various stages of differentiation.**

To determine the optimal time point during differentiation for dissociation of cell aggregates to single cardiomyocytes that resulted in the best AP profile, we compared the AP parameters of cardiomyocytes from the ΔN3 hESC line dissociated at day 13, day 21 or day 31 of differentiation. Forced cell aggregation by centrifugation to “spinEBs” was used as the basis for cardiomyocyte generation. No significant differences were observed in the maximal AP upstroke velocity ($V_{max}$), maximal diastolic potential (MDP), AP amplitude (APA), AP duration at 50% (APD$_{50}$) and 90% (APD$_{90}$) of repolarization (Fig.2), between any of these three conditions. We did however observe that cardiomyocytes from 13 day old
EBs were more difficult to be patched and could not be readily paced at 1 Hz. This is reflected on the lower cell number of cells that could be measured from this condition (n=11) as opposed to the other two n=16.

Figure 1. Experimental scheme.

hPSCs were differentiated as a cardiac monolayer or aggregates (called spinEBs) in defined conditions. After dissociation at various time points the resulting single cells were left to recover an additional 7-10 days in coverslips before the recordings of action potentials took place.

Figure 2. AP properties measured in ΔN3 hESC-CMs at 13, 21 and 31 days post differentiation.

APs were recorded in single cells paced at the stimulation frequency of 1 Hz. Data were collected from 3 distinct differentiations.
The impact of XAV939 Wnt inhibitor on the morphology and action potential profile of hPSC-CMs

XAV939 is not strictly necessary as a medium supplement for cardiomyocyte generation but is often added to increase cardiomyocyte yield. In order to evaluate the effect of XAV939 on the AP profile hPSC-CMs, we compared AP recordings of cells derived with and without XAV939. The ΔN3 hESC line differentiated as spinEBs was used for this comparison. We did not observe any noticeable differences in AP parameters between the two conditions (Fig.3A); however the XAV939 treated EBs appeared morphologically intact and expressed GFP throughout their whole surface in contrast to the poor morphology and GFP expression of non-treated EBs (Fig.3B). Furthermore, the dissociation of XAV939 treated EBs to single cardiomyocytes was more efficient and yielded higher cell numbers (data not shown).

Figure 3.

A. AP properties measured in XAV939 treated and untreated ΔN3 hESC-CMs dissociated at day 21 using the spinEB protocol. AP were recorded in single cells paced at the stimulation frequency of 1 Hz. Data were collected from 3 distinct differentiations. B. Morphology of XAV939 treated and untreated ΔN3 hESC-CMs. Left panel: Images of 21 day old EBs treated with XAV939 (Brightfield and GFP channel images). Right panel: Images of 21 day old EBs that were not treated with XAV939 at day 3 of differentiation.
The influence of the differentiation protocol on the action potential phenotype of hPSC-CMs. Monolayer vs spinEB derived cardiomyocytes

Cells from the ΔN3 hESC line were differentiated using either the spinEB or monolayer differentiation protocols to identify possible variances in AP characteristics of resulting cardiomyocytes. Although a small decrease in AP durations was noted in cardiomyocytes from the monolayer protocol, it was not statistically significant (Fig. 4). The other AP properties were similar between the two conditions. It is worth noting that we were able to dissociate cultures following monolayer differentiation using milder conditions (less mechanical stress and lower concentration of Tryple Select). Also the quality of generated cardiomyocytes was more consistent when the monolayer protocol was implemented; 3 out of 3 monolayer differentiations yielded cells that could be patched as opposed to 3 out of 5 of the spinEB differentiations.

![Figure 4.](image)

**Figure 4.** AP properties measured in ΔN3 hESC-CMs dissociated at day 21 using the spinEB and monolayer protocols.

AP were recorded in single cells paced at the stimulation frequency of 1 Hz. Data were collected from 3 distinct differentiations.

Action potential profile of cardiomyocytes from various hESC and hiPSC lines

The variability in AP values between hPSC-CMs from different lines under baseline conditions was assessed by recording the APs of four different lines in parallel. The cardiomyocytes resulting from the Ctrl04 hiPSC line had a considerably better AP phenotype when compared with those from the other three lines. More specifically, the $V_{\text{max}}$ and APA values of the Ctrl04 derived cardiomyocytes were significantly higher than those of the other cardiomyocytes (Fig. 5). APD was also longer and MDP was more negative in Ctrl04 derived cardiomyocytes compared to the other two hiPSC lines DF6 and NKX2-5. Furthermore, an apparent dif-
difficulty in generating cardiomyocytes that could be analysed was observed with DF6 and the NKX2-5 hiPSC lines. Only two out of six differentiations performed using the DF6 line yielded cardiomyocytes that could be patched while two out of four were successful using the NKX2-5 line. EBs from these two lines were difficult to dissociate and increased cell death after dissociation was observed.

Figure 5. AP properties measured in NKX2-5 hiPSC-CMs, DF6 hiPSC-CMs, ΔN3 hESC-CMs and Ctrl04 hiPSC-CMs dissociated at day 21.

AP were recorded in single cells paced at the stimulation frequency of 1 Hz. Data were collected from 3 distinct differentiations for DN3 and Ctrl04 lines while from 2 distinct differentiations for NKX2-5 and DF6 lines. Statistical significance was calculated using a one-way ANOVA with Tukey’s multiple comparison test, * P<0.05 (between groups).

The impact of cell density in the action potential recordings.

In our standard protocol after EB or monolayer dissociation, the resulting single cardiomyocytes (along with any non-cardiomyocytes) were plated at a cell density of 1.2-1.5 x 10⁵ cells per 10 cm² coverslip. To see whether a lower cell density would influence electrophysiological output, we plated cardiomyocytes at a density of 20,000 cells per coverslip (designated low cell density condition) and compared the AP parameters acquired with those of the standard 1 x 10⁵ cells per coverslip (designated high density condition). The Ctrl04 line was used in this set of experiments, since it yielded cells with the best AP phenotype of this study. A significant difference in V_max and MDP values was observed in favour of the high cell density condition, while APA and APD values did not differ (Fig.6A).
Figure 6.

A. AP properties measured in Ctrl04 hiPSC-CMs dissociated at day 21 and plated in a high or low cell density for analysis. High density is designated as 150,000 cells per coverslip. Low density is designated as 20,000 cells per coverslip. AP were recorded in single cells paced at the stimulation frequency of 1 Hz. Data were collected from 3 distinct differentiations. B. Brightfield images of a single cardiomyocyte patched in a low cell density format (left image) and in a high cell density format (right image). Cell is completely isolated in the left image while in close proximity with other cells in the right image.

Discussion

Patching single cardiomyocyte cells that could be paced at the frequency of 1 Hz was the selection used in all experiments of this study. Recordings at 2 Hz and 3 Hz were also acquired but for simplicity (results between conditions did not differ at higher frequencies) they are not shown. An alternative approach, patching “clumps” or clusters of cells, was not undertaken. The term “clusters of cells” does not define the number of coupled cells present per cluster that could influence the recorded AP. The presence of other cell types in a cluster might also impact the AP phenotype in a way that cannot be evaluated. Measuring single cardiomyocytes is the most reliable approach for acquiring results.

The MDP is of key importance in establishing the quality of an AP in a particular cell. Four categories of cardiomyocytes could be defined in our study based on their MDP values. Cells with MDP above -45 mV do not spark APs that are worth analysing. Cells with a MDP in the range of -45 mV to -55 mV have poor
AP phenotypes and are in such an immature electrophysiological state, that is impossible to utilize them in any comparisons or disease modelling. Cells with a MDP in the range of -55 mV to -65 mV have much better AP phenotypes that can be analysed whilst finally cells with a MDP below -65 mV that will spark a proper AP that has good characteristics, although still retaining a somewhat immature phenotype. It is this final set of cells that is recommended for disease modelling and other studies.

Our comparative data indicate that although XAV939 did not influence AP phenotype, its addition was of value because the resulting cardiomyocytes had improved (ie more mature) morphology: fewer triangular or circular, more elongated / oblong cells. Our results indicated the best time point for dissociating the spinEBs or monolayers for AP analysis is day 21 of differentiation. Prolonging the culture for an additional 10 days did not have a beneficial impact on AP phenotype. On the other hand, cardiomyocytes resulting from cultures dissociated on day 13, could not be easily patched or paced at 1 Hz. It was also interesting to observe that in cultures after day 30, cells were hard to dissociate into single cardiomyocytes using our standard (mild) dissociation conditions. The dissociation itself is crucial to preparing cells for AP analysis and its importance is often neglected. The milder the dissociation conditions, the higher the yield of good quality cardiomyocytes that can be patched. Successful dissociation of spinEBs or monolayer cultures older than 30 days remains challenging. In this respect, it is worth noting that monolayer cultures at day 21 were easier to dissociate and were more consistent in quality of generated cardiomyocytes, than those of spinEBs on the same day. The use of monolayer cultures is therefore more favourable for AP analysis.

The density used to seed cardiomyocytes after their dissociation is also of great importance. Plating a high number of cells results in a pattern in which cardiomyocytes are adjacent providing them support and improving their AP phenotype. Our results showed that the $V_{\text{max}}$ and MDP, the two parameters directly linked to electrical cardiac maturity, were considerably better in high density plated cells. Determining the identity of these non-cardiomyocyte cells and standardizing their ratio towards the cardiomyocytes seeded on the coverslip, should be examined and optimized in future experiments.

It is evident from our results that there is a considerable variation between lines in terms of their baseline AP properties. The cardiomyocytes generated from the DF6 hiPSC, ΔN3 hESC and NKX2-5 hiPSC lines had a rather poor AP pheno-
type with low $V_{\text{max}}$ and MDP values, in contrast with those from the Ctrl04 line in which the same values were closer to the mean of baseline measurements found in other studies [1]. This variation and differences also involve other factors such as the differentiation efficiency and capacity to generate cells that could be analysed under patch clamp. These aspects should be taken into consideration when starting the functional analysis of a new line. Based on our results for instance, the DF6 and NKX2-5 lines had a very poor yield of decent quality cardiomyocytes and are therefore not recommended for further use in other studies.

Additional starting points for achieving increased cardiac maturity in hPSC derived cardiomyocytes could be: a) the use of more potent Wnt inhibitors; b) compounds that influence the activity of the $I_{\text{K1}}$ current (the driving force behind the MDP value of the AP); c) growth factors that increase the emergence of cardiac progenitors during the early stages of cardiac differentiation; and of course other approaches that mimic key aspects of cardiac development [1]. Future experiments that would provide insight and help moderate the functional immaturity of hPSC-CM, are of great necessity to improve the robustness of hPSC-CMs as a model system.

**Materials and Methods**

**hiPSC culture and cardiac differentiation**

All hPSC lines were maintained in mTeSR1 medium (Stem Cell Technologies) and mechanically passaged on Matrigel- (BD Biosciences) coated tissue culture plates according to the suppliers protocol. Differentiation was induced in either a monolayer culture format [12] or in a spinEB culture format [14] using timed addition of growth factors BMP4, Activin A, CHIR-99021 (a small molecular activator of Wnt signaling) and Xav939 (inhibitor of Wnt signaling) in BPEL medium. For electrophysiological recordings and immunofluorescence measurements the cultures were dissociated to single cells with 5x-10x (depending on the line and condition) Tryple Select (Gibco Life Technologies) and seeded on Matrigel coated glass coverslips. Electrophysiological properties were measured 7-10 days after dissociation from at least 2-3 independent differentiations.

**Action potential measurements**

APs were measured at 37°C with the perforated patch clamp technique using an Axopatch 200B amplifier and pClamp10.2 software (Axon Instruments). Analysis of the APs was performed with custom-made software. APs were filtered and
digitized at 5 and 40 kHz, respectively, and the potentials were corrected for the calculated liquid junction potential of 15 mV.

Bath contained (in mM): 140 NaCl, 5.4 KCl, 1.8 CaCl₂, 1.0 MgCl₂, 5.5 glucose, 5.0 HEPES; pH 7.4 (NaOH). The pipette solution contained (in mM): 125 K-gluconate, 20 KCl, 5 NaCl, 0.22 amphotericin-B, 10 HEPES; pH 7.2 (KOH). APs were elicited at the stimulation frequency of 1 Hz, 2 Hz and 3 Hz by 3 ms, 1.2x threshold current pulses through the patch pipette. Maximal diastolic potential (MDP), maximal upstroke velocity (V_max), AP amplitude (APA), and AP duration (APD) at 50% and 90% repolarization (APD_{50} and APD_{90}, respectively) were analysed. Data from 10 consecutive APs were averaged.

**Statistical analysis**

Results are expressed as mean ± s.e.m. Comparisons of AP parameters were performed with the unpaired t-test or One-Way ANOVA when applicable (when comparing more than two conditions). P<0.05 was considered statistically significant.

**Acknowledgement**

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References


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