HIGHLIGHT DIVISION HEART

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Towards an hiPSC-CMs derived model of the heart

Cardiovascular diseases (CVDs) remain the foremost cause of death worldwide, based on WHO statistics. Due to the scarcity of human-derived material and the limitations of maintaining these samples due to the non-proliferative nature of cardiomyocytes, research to elucidate the mechanism underlying pathological conditions as well as to develop new therapeutics for cardiac diseases often relies on non-human models. Although these models have been a useful source of information to understand the aetiology and pathophysiology of cardiac diseases, differences between humans and these models in terms of the genetic background, cardiac morphology [1], action potential [2] and contraction capabilities [3] have limited the translational power of the non-human models.

Advancements in new techniques like culturing human-induced pluripotent stem cells (hiPSCs) have overcome these limitations, as these cells are patient-derived materials which use non-invasive techniques for sample collection to generate hiPSCs. This enables the generation of patient-specific cell lines to establish models with high disease specificity [4], [5], [6]. Thanks to the ability of hiPSCs to differentiate into the three germ layers, these cells are an infinite source of different cell populations.

hiPSCs have already proved to be an extremely valuable tool to study human patho-physiology at cellular level, also in the cardiac field [2], [6].

The first example of cardiomyocytes differentiated from hiPSCs (hiPSC-CMs) dates back to 2011 and utilised BMP4 and FGF2 to generate beating embryoid bodies [7][8]. A few years later, hiPSC-CMs cultured in monolayer form were prepared via temporal modulation of canonical Wnt signalling [9], which has become the leading way to generate hiPSC-CMs in a monolayer fashion. Multiple protocols, which differ in terms of complexity, type of inhibitors or exposure time, have been developed since the first examples. These protocols generally require around 12 days or more to establish hiPSC-CMs, and they can be followed by a phase in which the CM population is purified, which also facilitates the maturation of the cells. Overall, the procedure from hiPSCs to fully differentiated hiPSC-CMs can take up to 20-25 days.

Since hiPSC-CM differentiation and culture are expensive and lengthy processes, it seems reasonable to maximise data collection by exploiting non-invasive techniques that do not require cell collection or treatment. Acquisition of video

images of the culture using a camera and a bright-field microscope is an example of non-invasive data collection to evaluate the beating of cardiomyocytes without interfering with the experiment or the need for special equipment.

Together with other advanced cell-culture techniques such as spheroids, organoids, on-a-chip, 3D engineered tissue and bio-printed scaffolds, hiPSC-CMs aim to generate highly physiological in vitro models to mimic the human environment as closely as possible. In this context, it is not only the source of the cells which is important, but also the way in which the culture is maintained and the microenvironment in which this is done. The extracellular matrix (ECM) defines the microenvironment in vivo and is also a key factor for in vitro modelling. Indeed, ECM plays a role in the migration, differentiation and maturation of cardiac cells[10] and it has been shown that, in vivo, ECM goes through a series of chemical modifications after birth, which affect the electrophysiological properties of cardiac muscle [11], [12]. The gold standard for the culturing and differentiation of hiPSCs into cardiac lineage is the use of Matrigel, a basement membrane extracted from murine tumor [13] as the ECM [12]. Few studies have investigated the effect of physico-chemical properties of Matrigel on cell behaviour, in particular the stiffness of the matrix. In this regard, the literature has reported contradictory results, with certain studies reporting that intermediate to moderately high stiffness (around 50 kPa) promotes differentiation and contraction in CMs [14], [15], [16], while others have suggested that low (around 34 kPa) [17] or even super-low stiffness (around 0.6 kPa) [18] is ideal for the culture. Apart from the debate regarding the preferred stiffness of the Matrigel, it seems obvious that identifying the **optimal microenvironment** for culturing and differentiating hiPSC-CMs should always be considered.

Due to their nature as a patient-derived in vitro model, hiPSC-CM cultures find their most useful application in disease modelling and drug development, especially in the field of personalised medicine. In this research area, parallel testing of different conditions, or new or previously developed drugs, is a common procedure, which requires the use of multiple technical replicates per experiment. Generally, hiPSC-CMs are produced in 6- or 12-well plates [19] or culture dishes [9], but these platforms are not considered to be easily scalable, while 48- and 96-well plates constitute the right compromise between high-throughput need and manual handling feasibility. Scaling down the hiPSC-CMs culture from traditional flasks or 6-well plates to higher-throughput platforms makes the model a better tool for medium-scale testing, which fits in with the purpose of disease modelling and personalised medicine.

Here we present some data regarding the maximisation of data collection, finding the optimal microenvironment for hiPSC-CM differentiation and scaling down the process to proceed in the direction of generating a reliable hiPSC-derived in-vitro model of the heart.

MAXIMISING DATA COLLECTION

Acquisition of data throughout non-invasive or end-point assays is always a valuable option, especially in research that employs expensive high-maintenance models. An example of this in the context of in-vitro models is differentiated hiPSCs.

In Prof. Leon de Windt’s group, we establish hiPSC-CMs by inducing the canonical Wnt signalling through GSK3 inhibition for mesodermal germ-line specification, followed by Wnt inhibitors to restrict the differentiation to cardiac lineages and a chemically driven metabolic selection to

purify the culture [20]. The entire protocol takes 25 days in total and requires different mediums and active compounds.

Possible approaches to collecting data without intervening in the culture are medium collection to investigate the release of biomarkers, metabolites to monitor physiological parameters, and image-based techniques which do not require labelling or incubation with fluorescent substances, such as bright field or phase-contrast microscopy. The latter is particularly suitable for hiPSC-CMs, thanks to the spontaneous contraction of these types of cells, which makes it possible to record video images of the beating cells for later analysis of the images, in order to extract important parameters regarding contractility.

There are various examples of the use of specific platforms developed to quantify the beating with expensive high-speed cameras [21], the use of Matlab codes [22] as well as specific software to analyse the videos [23].

Here we report a simple and cheap method to acquire video images of beating CMs (Figure 1.A) by using a standard camera and a bright-field microscope, and then to analyse the images by using a macro developed by Grune et al. [24] for use in the opensource imageJ program to extract information about beat time [sec], frequency [1/sec] and amplitude of the contractions [a.u.], as well as to allow certain parameters like the diastole and systole portions of the beating to be derived (Figure 1.B).

The analysis of the frames is used to generate a graph with the beating profile (Figure 1.C), with green and red indicating the local minimum and maximum for each individual peak, representing a beat. From this plot, various parameters are extracted and calculated (Figure 1.D). This non-invasive analysis can be used as a standard quality control (QC) to monitor and evaluate the success of the differentiation as well as the perpendicular or parallel assay to be used in the experiments.

OPTIMAL MICROENVIRONMENT

Identifying the optimal microenvironment for the culture and differentiation of hiPSC-CMs is crucial to establish reliable and physiologically relevant in-vitro models.

We tested the effect of storing the Matrigel on the differentiation outcome, in terms of the expression of cardiac markers and the contractile capability of hiPSC-CMs. We also decided to evaluate the expression of inflammation, to exclude the possibility that storing the Matrigel could be detrimental to the culture. We noticed a massive increase in GATA4 and NKX2-5 [25], [26], both capable of activating genes driving cardiac lineage differentiation, as well as cardiac troponin expression, which indicates the presence of functional CMs (Figure 2.a-c). Storing the Matrigel does not seem to affect the culture, as the inflammatory markers were considerably decreased, as in the case of ILB1, or were comparable, as the differences in IL6 and TNF-alpha were not statistically significant Figure 2.d-f). Video images of beating CMs were also acquired, and parameters were extracted using the MYOCETER macro for ImageJ (Figure 2.g-k). One observation is the number of beating CMs that could be extracted from the two conditions tested, and in fact, the same number of video images of beating hiPSC-CMs was acquired. The graphs depicted in Figure 2.g-i show the discrepancy in terms of numbers of datapoints per condition, with each data (dot) representing beating cells. Next to this, hiPSC-CMs cultured on stored Matrigel had shorter* beat times and higher frequency, characteristics generally linked to increased maturation [27]. The video

presented in Figure 2.j-k (by scanning the QR code) shows that in the stored version there is a bed of beating hiPSC-CMs with synchronised contractions.

Overall, this indicates that storing the Matrigel has a beneficial effect, and it is a promising parameter to tone to improve hiPSC-CMs cultures.

SCALING DOWN THE CULTURE TO INCREASE THROUGHPUT

Improving the throughput of in-vitro models is always preferable and in line with the tendency to develop complex models suitable for drug development. In any case, it is not a straightforward process, and always requires some optimisation. Here we report some preliminary results regarding the culture and differentiation of hiPSC-CMs in 48-well plates versus the traditional T25. The aim was to reliably replicate the model established in T25 in a platform with higher throughput/capable of hosting multiple technical replicates. Unlike IRX4, which showed lower expression in the 48-well plate compared to T25, the markers TNNT2, GATA4 and NKX2-5, investigated with qPCR to evaluate the quality of the differentiation were comparable between platforms (Figure 3.a-d). The same can be said for the contractile capability of hiPSC-CMs, which was also comparable (Figure 3.e-g). A qualitative difference in favour of the 48-well plate condition is the formation of larger patches of hiPSC-CMs compared to T25, based on the video images acquired (Figure 3.h,i). These preliminary results indicate the suitability of generating hiPSC-CMs on a high-throughput-friendly platform.

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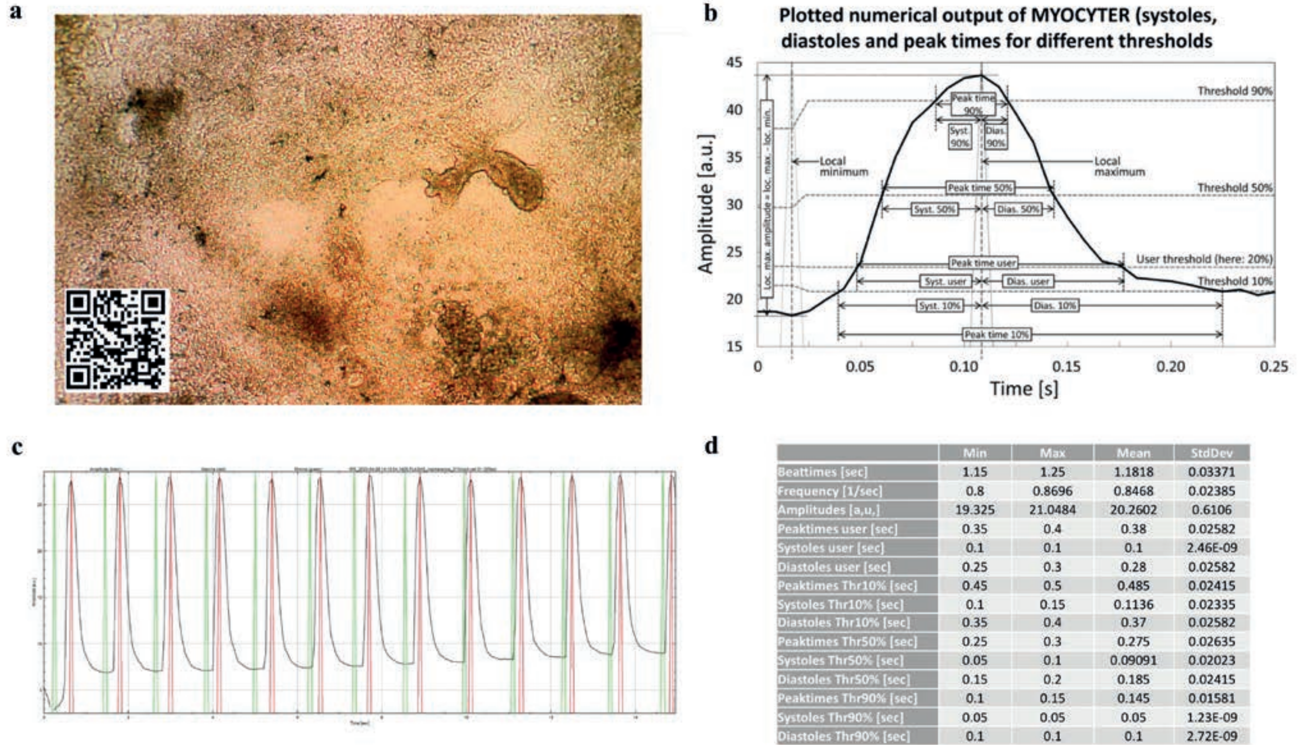

	Min	Max	Mean	StdDev
Beattimes [sec]	1.15	1.25	1.1818	0.03371
Frequency [1/sec]	0.8	0.8696	0.8468	0.02385
Amplitudes [a,u,]	19.325	21.0484	20.2602	0.6106
Peaktimes user [sec]	0.35	0.4	0.38	0.02582
Systoles user [sec]	0.1	0.1	0.1	2.46E-09
Diastoles user [sec]	0.25	0.3	0.28	0.02582
Peaktimes Thr10% [sec]	0.45	0.5	0.485	0.02415
Systoles Thr10% [sec]	0.1	0.15	0.1136	0.02335
Diastoles Thr10% [sec]	0.35	0.4	0.37	0.02582
Peaktimes Thr50% [sec]	0.25	0.3	0.275	0.02635
Systoles Thr50% [sec]	0.05	0.1	0.09091	0.02023
Diastoles Thr50% [sec]	0.15	0.2	0.185	0.02415
Peaktimes Thr90% [sec]	0.1	0.15	0.145	0.01581
Systoles Thr90% [sec]	0.05	0.05	0.05	1.23E-09
Diastoles Thr90% [sec]	0.1	0.1	0.1	2.72E-09

FIGURE 1 Maximising data collection: (a) Video of beating hiPSC-CM acquired with a standard camera and a bright-field microscope. (b) Data extraction from the peak profile. (c) Plot of beating profile of the hiPSC-CM shown in a, with green and red indicating the local minimum and maximum of each peak. (d) Example of data extracted from a video of beating CMs, generated with the MYOCYTER macro for ImageJ.

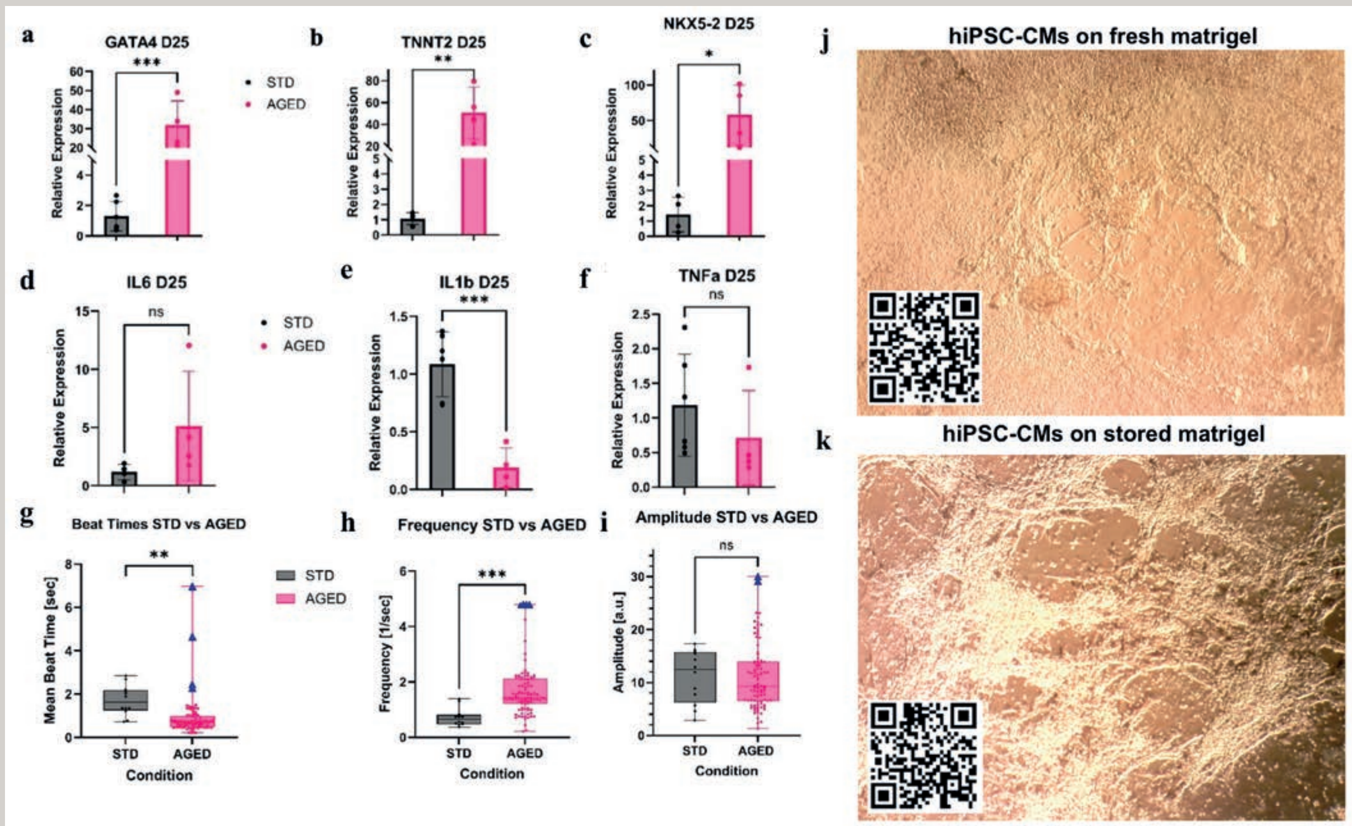

FIGURE 2 hiPSC-CMs culture on fresh and stored Matrigel. RNA expression (quantified with qPCR) of cardiac markers: (a) GATA4, (b) TNNT2 (c) NKX5-2; inflammatory markers: (d) IL6, (e) ILb1 and (f) TNF-alpha (values normalised against the standard condition of hiPSC-CMs on fresh Matrigel); Contractile properties: (g) beat time [sec] (h) frequency [1/sec] (i) amplitude of the contraction. Images extracted from the video of beating hiPSC-CMs cultured in (j) fresh (STD) (k) stored (AGED) Matrigel (scan the QR code to watch the video). In graphs (a)-(i), data in grey show the fresh Matrigel condition, while those in pink show the stored version. In all graphs, data are expressed as mean ± standard deviation. Data were analysed with unpaired student's t-test: *** $p < 0.001$, ** $p < 0.01$ * $p < 0.05$. In graphs (g) to (i), data points highlighted with a blue triangle were identified as outliers based on the ROUT method.

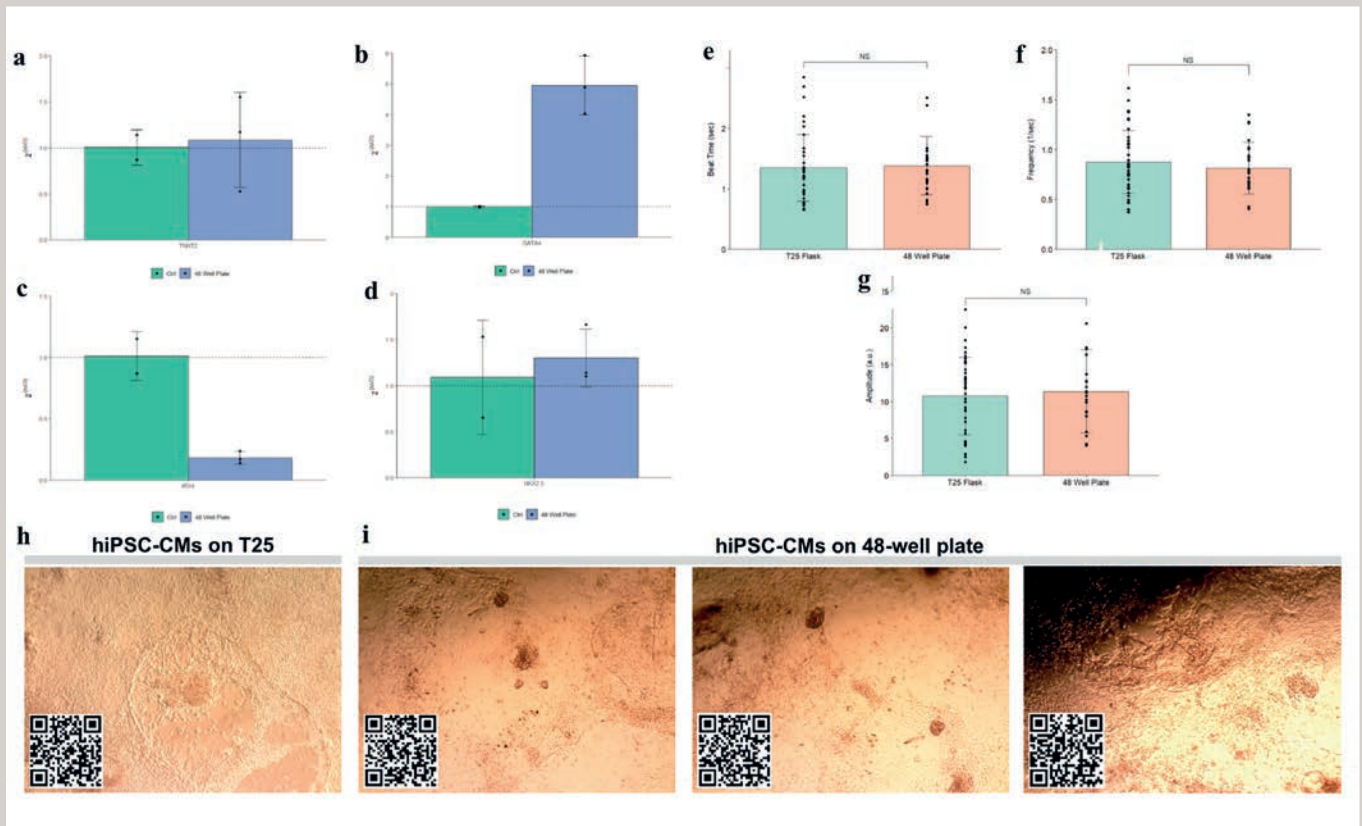

FIGURE 3 hiPSC-CMs culture on T25 and 48-well plates: RNA expression (quantified with qPCR) of cardiac markers: (a) TNNT2 (b) GATA4, (c) IRX4 (d) NKX5-2, with values normalised against the standard condition of hiPSC-CMs in T25. Contractile properties: (e) beat time[sec] (f) frequency [1/sec] (g) amplitude of the contractions. Images extracted from the video of beating hiPSC-CMs cultured on (h) T25 (i) 48-well plates (scan the QR code to watch the videos). In graphs (a)-(d), T25 and 48-well plates are shown in green and blue, respectively, while in graphs (e)-(g) they are shown in light green and salmon, respectively. Data shown are mean ± standard deviation. Data in graphs (e)-(g) were analysed with unpaired student's t-test.

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