Cardiomyocyte differentiation of human pluripotent stem cells in suspension culture

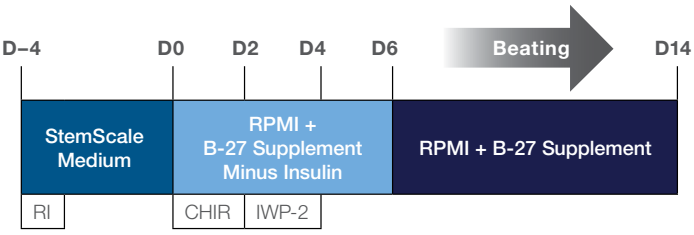
Introduction

Cardiovascular disease (CVD) is the leading cause of death around the world [1]. In the US alone, about 30 million adults are living with heart disease [2]. Since 1998, patient-specific pluripotent stem cells (PSCs) have been seen as a potential tool to regrow muscle tissue in damaged hearts. Transplantation of human PSC-derived cardiomyocytes into animal models has convincingly demonstrated the concept while pointing to some challenges [3]. In addition, *in vitro* production of functional cardiomyocytes is starting to be used for large-scale drug screening with more human-relevant results. Putting both of these developments into practice will require methods for reproducible and safe scale-up of PSC growth and cardiac differentiation.

To facilitate the expansion of authentic and capable human PSCs, we developed Gibco™ StemScale™ PSC Suspension Medium. Here we describe how researchers can differentiate PSCs grown in this medium to obtain abundant functional cardiomyocytes.

Overview

The method used here to differentiate PSCs is derived from a published method to induce cardiomyocytes by manipulating Wnt/β-catenin signaling [4]. Figure 1 summarizes the protocol, which we adapted from a monolayer system to a suspension culture using spheroids grown in StemScale medium and maintained in rotation throughout differentiation. Briefly, mesoderm is induced by placing PSC spheroids into Gibco™ RPMI 1640 Medium with Gibco™ B-27™ Supplement Minus Insulin and exposing the PSC spheroids to the Wnt/β-catenin activator CHIR99021 for 48 hours. The spheroids are then washed in the same medium and exposed to IWP-2 for the next 48 hours to inhibit PORCN-mediated palmitoylation of Wnt and block its activity. Insulin is returned to the system after 6 days of differentiation by switching to complete B-27 Supplement.



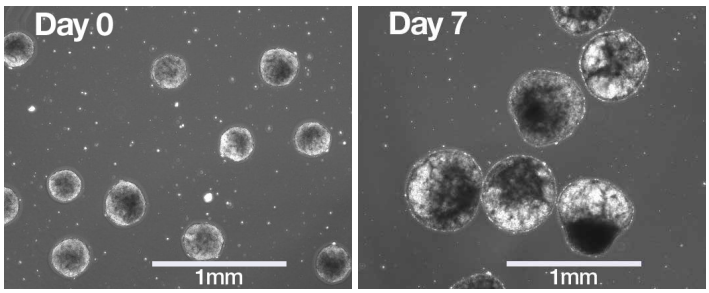


Figure 1. Timeline for induction of cardiomyocyte differentiation. Schematic summary of cardiomyocyte induction in suspension from human PSCs grown in StemScale medium. Day –4 (D–4) represents the day at which PSCs are passaged as single cells prior to induction; the duration of this growth phase should be optimized for starting spheroid size. Day 0 is the time at which PSC spheroids are switched into RPMI medium with B-27 Supplement Minus Insulin and dosed with CHIR99021 (CHIR). The medium is changed completely at day 0, day 2, day 4, and day 6, and thereafter refreshed by half fluid changes every second day. The day 0 image shows typical spheroids of appropriate size to begin induction. The day 7 image shows beating spheroids that are enlarged and round, and enclose fluid-filled spaces.

The first week of differentiation produces rounded spheroids with large fluid-filled cavities (Figure 1). Spontaneous contractions of spheroids can be observed as early as day 7. The onset of contraction may vary from experiment to experiment; in monolayer cultures, earlier contraction has been correlated with a greater proportion of cardiomyocytes [6]. FACS quantification of cardiac troponin T2 (TNNT2) expression in dissociated spheroids has shown that the fraction of cardiomyocytes can approach 100%, although there is substantial variation between experiments (Figure 2A). By 2 weeks of differentiation, abundant cardiomyocytes co-labeled for TNNT2 and α-sarcomeric actinin can be observed in whole-mount spheroids (Figure 2B). At this time point, organization of cardiomyocytes towards myofibrillar structures is not evident. Further maturation of cardiomyocytes may be promoted by the addition of small molecules or a switch to specially designed media [6,7].

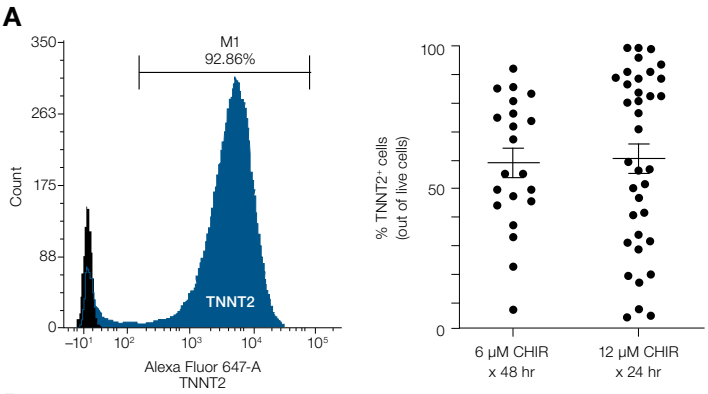


Figure 2. Immunofluorescent detection of cardiomyocyte markers in spheroids. (A) Flow cytometric quantification of TNNT2-positive cardiomyocytes as a percentage of live cells in dissociated spheroids. Each point represents a biological replicate (data combined from 7 independent batches for 48 hr treatment with 6 µM CHIR and 12 independent batches for 24 hr treatment with 12 µM CHIR). **(B)** Spheroid stained in whole mount for the cardiac markers α-sarcomeric actinin (green) and cardiac troponin T (red). Nuclei are counterstained with DAPI (blue). The right panel is a maximum intensity projection for each channel, corresponding to the marked area in the left panel.

Critical factors for success

Two factors are critical to optimize for each PSC line to be used: the starting size of the PSC spheroids prior to induction, and the concentration of CHIR99021 used to begin induction.

The size of PSC spheroids in StemScale medium depends on several factors, including seeding density, time in culture, vessel size, volume of medium, rotation speed, and radius of rotation. Guidance for choosing these values is provided in the StemScale PSC Suspension Medium user guide [5]. This guidance was followed to determine the optimal seeding density, volume of medium, and rotation speed for each vessel type used here; this left time in culture as the main variable affecting PSC spheroid size. As seen in Figure 3, the initial spheroid size plays a large role in the success of cardiomyocyte induction. We compared PSC spheroids grown in suspension for 2, 3, or 4 days prior to initiating differentiation. In this interval, the average spheroid size increases with time in culture. RT-PCR at 2 weeks of differentiation showed a substantial impact of time in PSC culture on cardiomyocyte differentiation. Expression of each of three cardiomyocyte markers—myosin heavy chain 6 (*MYH6*), homeobox transcription factor *NKX2-5*, and *TNNT2*—responded strongly to initial spheroid size. All three markers were ultimately expressed more than 100-fold higher when induction was started at day 4 than when it was started at day 3, and at least 600-fold higher than when induction began at day 2 (Figure 3B). The optimal starting spheroid size may vary by cell line; external researchers found that the most successful cardiac inductions began with spheroid diameters of 280 ± 50 µm (see Acknowledgment).

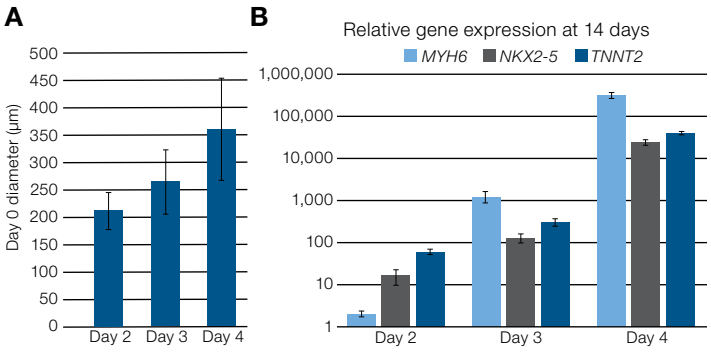


Figure 3. Effects of starting spheroid size on cardiomyocyte differentiation. (A) Average spheroid diameter for the WTC11 PSC line grown in StemScale medium for 2, 3, or 4 days. **(B)** RT-PCR quantification of expression of three cardiomyocyte marker genes, relative to undifferentiated PSCs, shown in log scale. Expression was assayed after 14 days of differentiation starting from PSCs grown in StemScale medium for 2, 3, or 4 days.

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The second critical factor is the GSK-3α/β inhibitor CHIR99021, which we have observed to induce cardiac differentiation only within a narrow range of concentrations. A **support protocol** is provided to help users identify a concentration of this small molecule that efficiently induces cardiomyocytes in their cell line of interest. Interestingly, differentiation can also be initiated by a higher dose of CHIR99021 given for only 24 hours (Figure 2A). In either case, Wnt activity is shut down 48 hours after the start of induction with the inhibitor IWP-2. To help completely reverse Wnt activation, it is advised to wash the spheroids and flask once in a medium without any small molecules, prior to adding medium with IWP-2.

Conclusions

Human PSCs grown in StemScale medium can be efficiently differentiated to mesodermal fates such as cardiomyocytes. Careful optimization of the protocol for each candidate cell line can produce cardiomyocytes as a high proportion of the live cells in the culture. Suspension culture is advantageous for producing PSCs and cardiomyocytes in bulk, as cell growth is not limited by confluency.

Acknowledgment

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References

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