Disease modeling of hypertrophic cardiomyopathy from patients carrying sarcomeric mutations

Background and introduction

Familial hypertrophic cardiomyopathy (HCM) is an inherited heart disease that affects approximately 1 in 500 people worldwide. Over the past 20 years, researchers have identified more than 500 different mutations across 20 or more genes that may play a role in disease development. Patients with HCM exhibit abnormal thickening of the left ventricular myocardium and are at heightened risk for clinical complications such as arrhythmia, progressive heart failure, and sudden cardiac death.

Stem Cell Theranostics (SCT) was founded in 2011 and is focused on accelerating the discovery of novel therapeutics using patient stem cell–derived cardiac models. Through its exclusive partnership with Stanford University Cardiovascular Institute (CVI), Stem Cell Theranostics has built a library of induced pluripotent stem cells (iPSCs) derived from human donors harboring a spectrum of common inherited cardiovascular diseases, along with their family-matched controls. This library is being used for disease modeling studies, novel target identification/validation, and efficacy and toxicity screening. The foundation for this "clinical trials in a dish" platform was built on a decade of discoveries by SCT's founders, Drs. Joseph Wu, Robert Robbins, and Andrew Lee.

To enable this vision, Stem Cell Theranostics recently partnered with Thermo Fisher Scientific to establish standard workflows that would enable the routine generation of donor-specific cardiomyocytes (CMs) from a variety of genetic backgrounds and source tissues. In the work described here, skin-punch biopsies or blood samples were collected from donors harboring genetic forms of HCM, as well as from healthy controls. After expansion of fibroblasts from the skin biopsies, or $CD71^+$ erythroid progenitor cells from blood samples, iPSCs were generated using the Invitrogen™ CytoTune™-iPS 2.0 Sendai Reprogramming Kit. After expansion and characterization for pluripotent markers and karyotypic integrity, cardiomyocytes were prepared using the Gibco™ PSC Cardiomyocyte Differentiation Kit. After additional characterization for appropriate cardiomyocyte markers, cells were monitored for disease-relevant phenotypes.

Sample collection and expansion of cells for reprogramming

Somatic samples were collected from patients with familial HCM at the Stanford CVI, following Human Institutional Review Board (IRB) guidelines from Stanford University, and were transferred to Stem Cell Theranostics with appropriate consent documentation.

Fibroblast samples were obtained from a donor harboring an R663H mutation in the *MYH7* gene, which codes for the myosin heavy chain 7 protein. This mutation has previously been shown to cause disease-relevant phenotypes that are observable in iPSC-derived cardiomyocytes [1]. Peripheral blood mononuclear cell (PBMC) samples were obtained from a donor harboring an R145W mutation in the *TNNI3* gene, which codes for the troponin I protein. To our

knowledge, iPSC-derived cardiomyocytes have not been described with this mutation.

Fibroblasts were then expanded in DMEM containing 10% FBS, and PBMCs were isolated using CPT tubes (BD Bioscience). CD71+ cells were obtained from PBMCs in culture, in the presence of FLT-3, IL-6, SCF, and TPO, according to the protocol supplied with the CytoTune-iPS 2.0 kit. All somatic cell samples were tested and cleared for the presence of mycoplasmas.

Reprogramming to iPSCs

Fibroblasts and CD71+ erythroid progenitor populations were expanded in culture and transduced using the CytoTune-iPS 2.0 kit. Based on the included protocol, cells were cultured in their respective somatic cell media and transitioned to Gibco™ Essential 6™ Medium containing bFGF. After successful reprogramming, colonies were observed by day 10 and subsequently picked on days 15–20 for fibroblast reprogramming, or on days 18–25 for CD71+ cell reprogramming. Twelve colonies were picked from each line and individually plated in wells that had been treated overnight with Geltrex™ matrix in the presence of Gibco™ Essential 8™ Medium. Reprogramming efficiency ranged between 0.25% and 1%. Based on cell morphology and growth kinetics, 2 clones were further expanded for each parental sample. After retesting as negative for mycoplasmas, cells were analyzed for the presence of the expected disease-linked mutations by DNA sequencing, and characterized for pluripotency by immunostaining with mouse anti–Tra-1-60 antibody and mouse anti-SSEA4 antibody (Figure 1).

These lines were then further expanded to create iPSC master banks using the Gibco™ PSC Cryopreservation Kit. In general, this kit yielded better cell recovery after thawing compared to other freezing media. All banked cells showed a normal karyotype by G-band analysis.

Differentiation of iPSCs to cardiomyocytes

The iPSC lines were thawed and passaged onto Geltrex matrix–coated plates and cultured for 3–4 days before differentiation using the PSC Cardiomyocyte Differentiation Kit. The effect of tissue culture vessel size on differentiation efficiency was assessed by evaluating 6- and 12-well plates, and 10 cm^2 and 15 cm^2 dishes. Although the differentiation efficiency was strongly influenced by the specific cell line, we did also observe a correlation

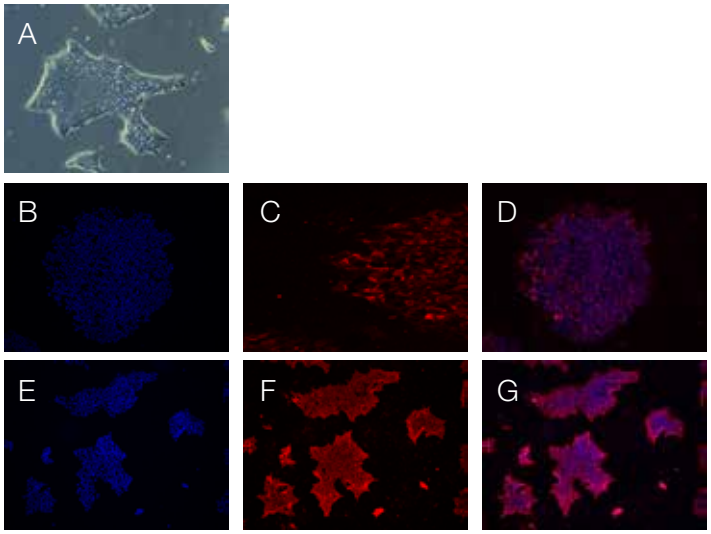

Figure 1. Immunocytochemistry with iPSCs. (A) Representative phase contrast image of a human iPSC colony. **(B–D)** Immunostaining for the extracellular marker Tra-1-60: **(B)** DAPI nuclear stain, **(C)** Tra-1-60, **(D)** the images merged. **(E–G)** Immunostaining for the extracellular marker SSEA4: **(E)** DAPI nuclear stain, **(F)** SSEA4, **(G)** the images merged.

showing that, in general, the larger the vessel, the lower the efficiency. As a result, differentiation was experimentally optimized by plating ~115,000 cells per well in 6-well plates to balance throughput and efficiency. The medium was changed as described in the protocol. Beating foci were typically observed between days 7 and 10, and the beating area progressively expanded with increasing days in

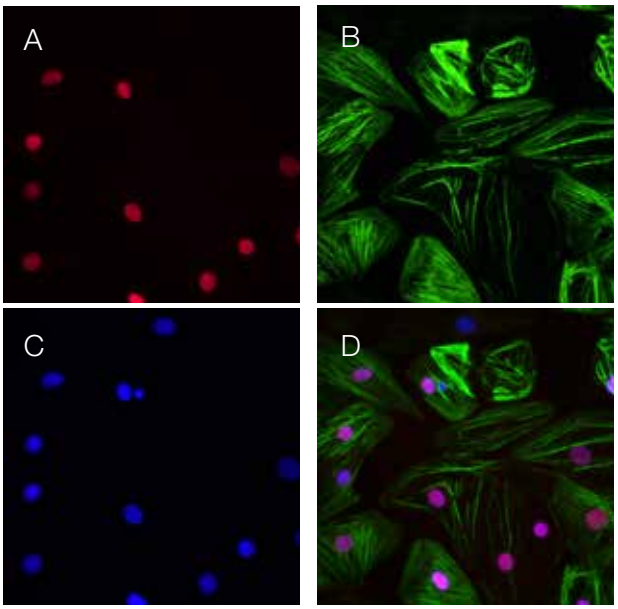

Figure 2. Immunofluorescence characterization of iPSC-derived cardiomyoctyes using the Invitrogen™ Molecular Probes™ Human Cardiomyocyte Immunocytochemistry Kit. Dissociated cardiomyocytes from day 35 after differentiation were plated and labeled with antibodies to **(A)** Nkx2-5 and **(B)** cardiac troponin T (cTnT), and stained with **(C)** Invitrogen™ Molecular Probes™ NucBlue™ nuclear stain. **(D)** Merged image of the three channels shows clear co-expression of the cardiac transcription factor Nkx2-5 and the cardiac sarcomeric protein cTnT in our control iPSC-derived cardiomyocytes.

culture. Despite the relative simplicity of the protocol, a subpopulation of undifferentiated cells was observed in differentiations from all iPSC lines studied. Although lower-yield differentiation may be acceptable for single-cell studies, functional studies that necessitate the formation of a cardiac syncytium, such as MEA or calcium transient analysis, are hampered by the presence of lower-purity preparations. To address this challenge, we incorporated a glucose deprivation selection step at the end of the differentiation protocol to enrich for cardiomyocytes [2].

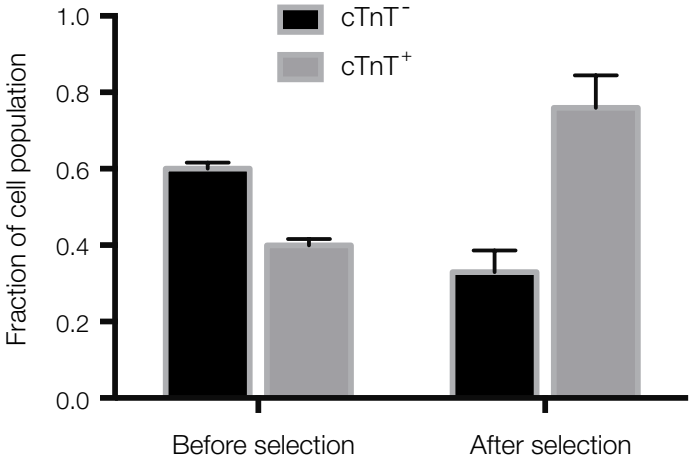

Figure 3. Glucose deprivation results in an enrichment of cTnT⁺ cardiomyocytes. Dissociated cardiomyocytes from differentiations with and without glucose deprivation were plated and stained for cTnT using the Human Cardiomyocyte Immunocytochemistry Kit. Images were acquired and analyzed for the fraction of cells that showed expression of cTnT. Glucose deprivation results in a significant increase in the fraction of cTnT⁺ cells.

Cardiomyocytes were characterized using the Invitrogen™ Molecular Probes™ Human Cardiomyocyte Immunocytochemistry Kit (Figure 3), and the cTnT marker was used to determine differentiation efficiency for all preparations. Preparations that demonstrated >80% cardiomyocyte differentiation were advanced to phenotype assessment studies.

Observation of disease-relevant phenotypes in iPSC-derived cardiomyocytes

It has previously been shown that cardiomyocytes derived from patients carrying the *MYH7* R663H mutation present with an arrhythmic beating phenotype [1]. To evaluate whether this phenotype is replicated in the HCM patient lines derived in this study, we subjected cardiomyocytes to electrophysiological assessment using the Maestro multielectrode array (MEA) platform from Axion Biosystems (Figure 4).

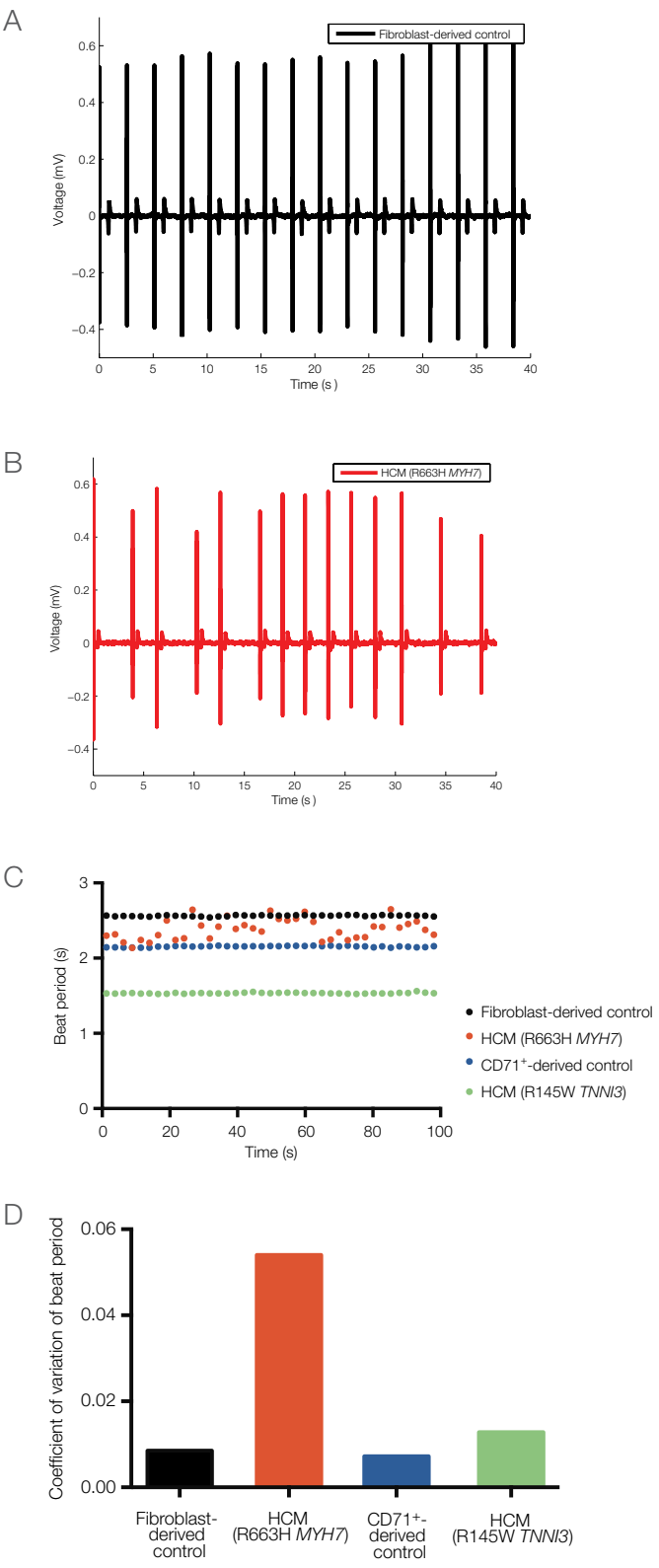

Figure 4. Electrophysiological assessment of iPSC-derived cardiomyocytes using the Maestro multielectrode array (MEA) platform (Axion Biosystems). Comparing representative electrical activity from **(A)** control and **(B)** *MYH7* R663H mutation–carrying cardiomyocytes shows arrhythmic beating in the diseased cardiomyocytes. The arrhythmic beating of the cardiomyocytes with the mutation is evident when comparing its **(C)** beat period and **(D)** coefficient of variation of beat period to those of cardiomyocytes derived from the other cell lines.

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After selection, cardiomyocytes were dissociated and replated onto fibronectin-coated MEA plates. Medium was changed after 24 hours and then every 3 days thereafter to allow for cells to recover. MEA experiments were typically conducted 5–7 days after plating. Electrical activities across the cardiac syncytium were captured using the Maestro MEA system. Functional features such as beat rate and repolarization characteristics were measured from the different cardiomyocytes derived from the control and HCM-derived lines.

Cardiomyocytes derived from the iPSC line carrying the *MYH7* R663H mutation displayed arrhythmic beating during baseline recording, whereas the two control lines and the line carrying the *TNNI3* R145W mutation displayed steady beating under baseline conditions. Coefficient of variation analysis was conducted to determine variation and statistical significance of these preliminary observations.

Future studies

In this study, we have demonstrated that the PSC Cardiomyocyte Differentiation Kit can play a central role in the establishment of standard workflows that enable the routine generation of donor-specific cardiomyocytes. We have further shown that we can replicate the published disease phenotype for the HCM *MYH7* R663H mutation. Although these results are promising, it is clear that more detailed studies are required to further evaluate and uncover disease phenotypes, such as hypertrophy, calcium handling defects, and arrhythmic behavior, in the presence of stress conditions that more accurately replicate the true disease environment.

References

1. Lan F, Lee AS, Liang P et al. (2013) Abnormal calcium handling properties underlie familial hypertrophic cardiomyopathy pathology in patient-specific induced pluripotent stem cells. *Cell Stem Cell* 12(1):101–113.
2. Tohyama S, Hattori F, Sano M et al. (2013) Distinct metabolic flow enables large-scale purification of mouse and human pluripotent stem cell-derived cardiomyocytes. *Cell Stem Cell* 12(1):127–137.

Ordering information

Product	Cat. No.
CytoTune-iPS 2.0 Sendai Reprogramming Kit	A16517
PSC Cardiomyocyte Differentiation Kit	A25042-SA
Essential 6 Medium	A1516401
Essential 8 Medium	A1517001
PSC Cryopreservation Kit	A2644601
Human Cardiomyocyte Immunocytochemistry Kit	A25973
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Mouse anti-SSEA4	41-4000
Geltrex Matrix	A1413301
DMEM	11965-092
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