Int. J. Dev. Biol. 65: 537-543 (2021)
https://doi.org/10.1387/ijdb.210115fs

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Human C2a and C6a iPSC lines and H9 ESC line have less efficient cardiomyogenesis than H1 ESC line: Beating enhances cardiac differentiation

ANA SEPAC[1], ZELJKO J. BOSNJAK[2], SVEN SEIWERTH[1,3], SUNCANA SIKIRIC[1], TIHANA REGOVIC DZOMBETA[1,4], ANA KULIC[5], JELENA MARUNICA KARSAJ[6] and FILIP SEDLIC*[,7]

[1]Department of Pathology, University of Zagreb School of Medicine, Zagreb, Croatia, [2]Department of Medicine, Medical College of Wisconsin, Milwaukee, WI, USA, [3]Department of Pathology and Cytology, University Hospital Center Zagreb, Zagreb, Croatia, [4]Department of Pathology, University Hospital Center Sestre milosrdnice, Zagreb, Croatia, [5]Department of Oncology, Clinical Unit of Pathophysiology and Experimental Oncology, University Hospital Center Zagreb, Zagreb, Croatia, [6]University Hospital Center Sestre milosrdnice, Zagreb, Croatia and [7]Department of Pathophysiology, University of Zagreb School of Medicine, Zagreb, Croatia

ABSTRACT Background: Human induced pluripotent stem cells (hiPSCs) need to be thoroughly characterized to exploit their potential advantages in various aspects of biomedicine. The aim of this study was to compare the efficiency of cardiomyogenesis of two hiPSCs and two human embryonic stem cell (hESC) lines by genetic living cardiomyocyte labeling. We also analyzed the influence of spontaneous beating on cardiac differentiation.

Methods: H1 and H9 hESC lines and C2a and C6a hiPSC lines were induced into *in vitro* directed cardiac differentiation. Cardiomyogenesis was evaluated by the analysis of cell cluster beating, cardiac protein expression by immunocytochemistry, ability of cells to generate calcium transients, and cardiomyocyte quantification by the myosin light chain 2v-enhanced green fluorescent protein gene construct delivered with a lentiviral vector.

Results: Differentiation of all cell lines yielded spontaneously beating cell clusters, indicating the presence of functional cardiomyocytes. After the cell dissociation, H1-hESC-derived cardiomyocytes exhibited spontaneous calcium transients, corresponding to autonomous electrical activity and displayed ability to transmit them between the cells. Differentiated hESC and hiPSC cells exhibited striated sarcomeres and expressed cardiac proteins sarcomeric α-actinin and cardiac troponin T. Cardiomyocytes were the most abundant in differentiated H1 hESC line (20% more than in other tested lines). In all stem cell lines, cardiomyocyte enrichment was greater in beating than in non-beating cell clusters, irrespective of cardiomyogenesis efficiency.

Conclusion: Although C2a and C6a hiPSC and H9 hESC lines exhibited efficient cardiomyogenesis, H1 hESC line yielded the greatest cardiomyocyte enrichment of all tested lines. Beating of cell clusters promotes cardiomyogenesis in tested hESCs and hiPSCs.

KEY WORDS: *cardiomyogenesis, hiPSC, hESC, beating, calcium transients*

Introduction

Various disorders in cardiomyocyte structure and function may lead to cardiac injury and failure (Svaguša *et al.*, 2020). However, a shortage of human myocardium available for research hampers understanding of precise disease mechanisms. Human induced pluripotent stem cells (hiPSCs) derived from patients, and their differentiation to cardiomyocytes, offers a powerful tool for the study of cardiac pathophysiology (Yoshida and Yamanaka, 2017). Stem cell derived-cardiomyocytes exhibit many similarities with adult

Abbreviations used in this paper: EGFP, enhanced green fluorescent protein; hESC, human embryonic stem cell; hiPSC, human induced pluripotent stem cell; MLC-2v, myosin light chain-2v.

***Address correspondence to:** Filip Sedlic. Department of Pathophysiology, University of Zagreb School of Medicine, Kispaticeva 12, 10 000 Zagreb, Croatia. Tel: +385-1-236-7293. E-mail: filip.sedlic@mef.hr - https://orcid.org/0000-0002-3733-5985

Submitted: 4 August, 2021; *Accepted:* 13 December, 2021; *Published online:* 13 December, 2021.

ISSN: Online 1696-3547, Print 0214-6282

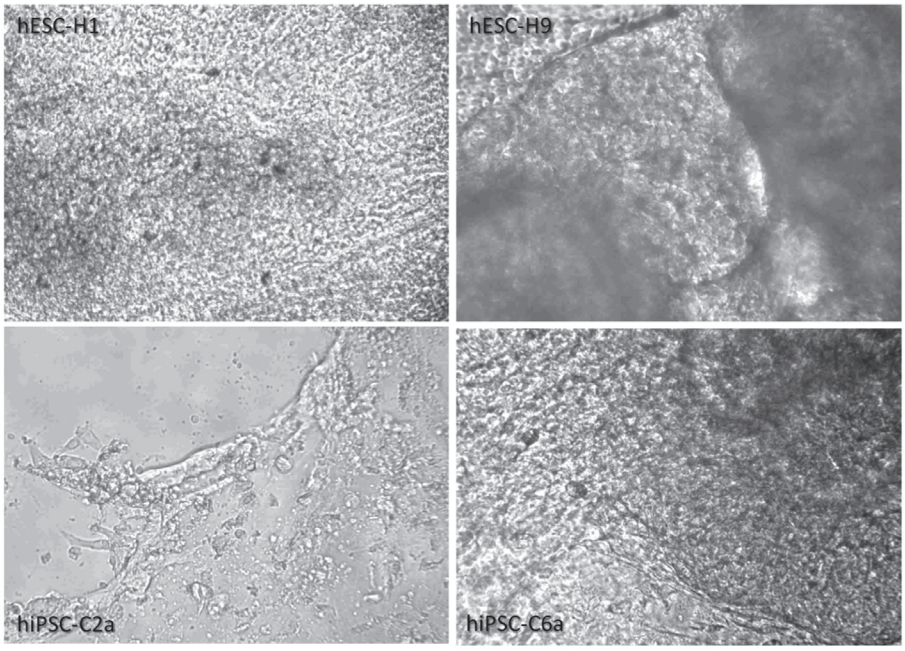

Fig. 1. Cardiac differentiation yields beating cell clusters. *Representative phase-contrast images (x400), 40 days after directed differentiation of hESC and hiPSC lines. All hESC and hiPSC lines yield spontaneously and rhythmically beating cell clusters, indicating presence of cardiomyocytes. Cardiomyogenesis of hESC and hiPSC lines formed interconnected and multilayered clusters of cells.*

cardiomyocytes, and can therefore be used for modeling various cardiac diseases. Human stem cell-derived cardiomyocytes are especially useful in studying ischemia-reperfusion injury, because these cells can be preconditioned (Sepac *et al.*, 2010), similarly to adult rat cardiomyocytes (Sedlic *et al.*, 2017), exhibiting delay in mitochondrial permeability transition pore opening (Canfield *et al.*, 2012, Sepac *et al.*, 2010), involvement of reactive oxygen species as preconditioning signaling molecules (Sepac *et al.*, 2010), and other characteristic elements of endogenous cardioprotective machinery (Canfield *et al.*, 2012). Human embryonic stem cells (hESCs) are considered the gold standard for pluripotency (Puri and Nagy, 2012, Thomson *et al.*, 1998). Many studies indicate that hiPSCs exhibit similar pluripotency and differentiation capacity to hESCs (Marei *et al.*, 2017). While some studies show similar cardiomyogenic potential between hESCs and hiPSC (Gherghiceanu *et al.*, 2011, Jin *et al.*, 2018), others do show differences (Lundy *et al.*, 2013, Sepac *et al.*, 2012) and a lower potential of hiPSC in cardiomyogenesis (Toivonen *et al.*, 2013, Zhang *et al.*, 2009). The advantage of hiPSCs is that they are not hampered by the majority of ethical issues, or transplant rejection problems if derived from the same donor, and can be derived from cells of patients with genetic disease to generate cellular disease models. On the other hand, there are studies that show considerable differences in gene expression between hiPSCs and hESCs (Doi *et al.*, 2009). hiPSCs are also considered to be problematic due to epigenetic memory (transcriptional memory of the cell of origin), incomplete differentiation and genetic instability (Noguchi

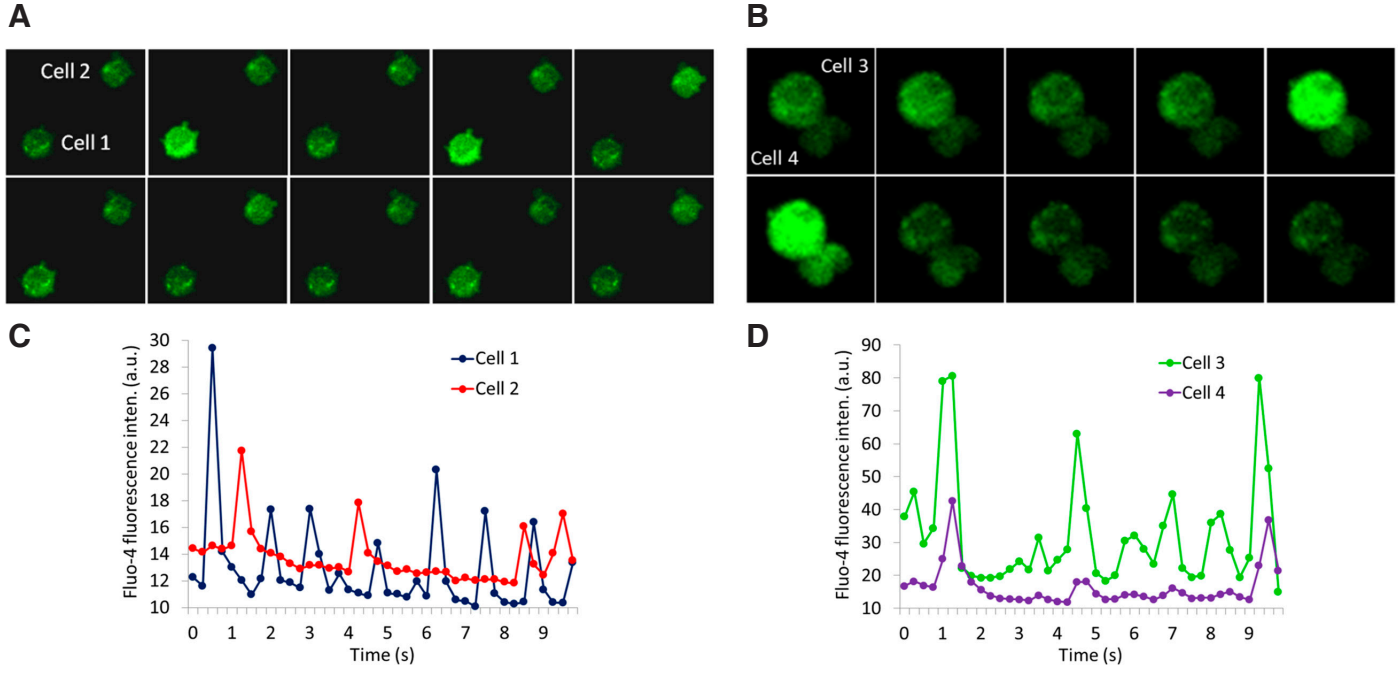

Fig. 2. Spontaneous calcium transients in hESC-H1-cardiomyocytes. (A) *and* **(B)** *Following the dissociation, some cells spontaneously generated rhythmical calcium transients of different frequencies.* **(C)** *and* **(D)** *Cell 3 generates calcium transients and transmits them to Cell 4. Images in (A) and (C) correspond to the first 10 data points in sequences shown in (B) and (D), respectively.*

et al., 2018). The rising need for the utilization of hiPSC-derived cardiomyocytes in biomedicine urges investigators to characterize mechanisms for their differentiation and find improved cardiomyogenesis protocols.

We designed this study to compare efficiency of cardiomyogenesis in two hESC and two hiPSC lines by genetic labeling of living cells, and to explore whether spontaneous contractions affect cardiomyocte enrichment during directed cardiac differentiation.

Results

Directed differentiation of hESCs and hiPSCs yields cell clusters with beating cardiomyocytes

One to two weeks after directed differentiation of hESC-H1, hESC-H9, hiPSC-C2a and hiPSC-C6a cells, areas with beating cell clusters and areas without obvious cell contractions can be observed in culture dishes (Fig. 1). Corresponding videos are provided as supplementary material (Suppl. videos 1-4). Spontaneous and rhythmical contractions suggested presence of cardiac cells. The most extensive areas with beating cells were observed in differentiated hESC-H1 line.

hESC-H1 cardiomyocytes spontaneously generate calcium transients

To verify existence of functional cardiac cells capable of generating calcium transients, which are essential for electromechanical coupling, differentiated hESC-H1 cardiomyocytes were dissociated and the cytosolic calcium dynamics was analyzed. We observed existence of individual cells capable of generating calcium transients that are important elements of cardiomyocyte action potentials (cells 1 and 2 in Fig. 2 A,B). Different calcium oscillation frequency of cells 1 and 2 indicates a degree of heterogeneity among differentiated cells. In Fig. 2 C,D, the spreading of calcium from cell 3 to cell 4 confirms the ability of cells to transmit calcium signals.

Differentiated hESCs and hiPSCs exhibit cardiac-specific markers and striated sarcomeres

Immunocytochemical staining for cardiac proteins sarcomeric α-actinin and cardiac troponin T verified abundant presence of cardiomyocytes in all hESC and hiPSC lines (Fig. 3). Moreover, in all tested lines, sarcomeric α-actinin displayed striated pattern of expression, indicating presence of striated sarcomeres, a hallmark of highly organized and relatively mature sarcomeres. However, striations appeared more extensive in hESC lines, especially in hESC-H1 cells. Z-stack image analysis of differentiated hESC-H1 cells shows abundance of striated sarcommeres, extending in all three dimensions as continuous structures and beyond borders of individual cells (Fig. 4A and supplemental video 5). This suggests that sarcomeres form trans-cellular continuum and contractions units, acting synergistically for efficient force generation of the entire syncytium, as in adult hearts. Sometimes, rod-shaped cardiomyocytes resembling adult cardiomyocytes can be found, suggesting a high degree of maturity of such cells (Fig. 4C).

Cardiomyocyte differentiation is less efficient in hiPSC lines than in hESC-H1 cells

A 40 days after induction of cardiomyogenesis in all hESC and hiPSC lines, cells were isolated from beating and non-beating areas within the same dish, and treated with a lentiviral vector delivering MLC-2v-EGFP genetic construct, as shown in Fig. 5. Biological replicates were used for statistical analyses. Living cells expressing EGFP were identified as cardiomyocytes, since EGFP expression was under the transcriptional control of a cardiac-

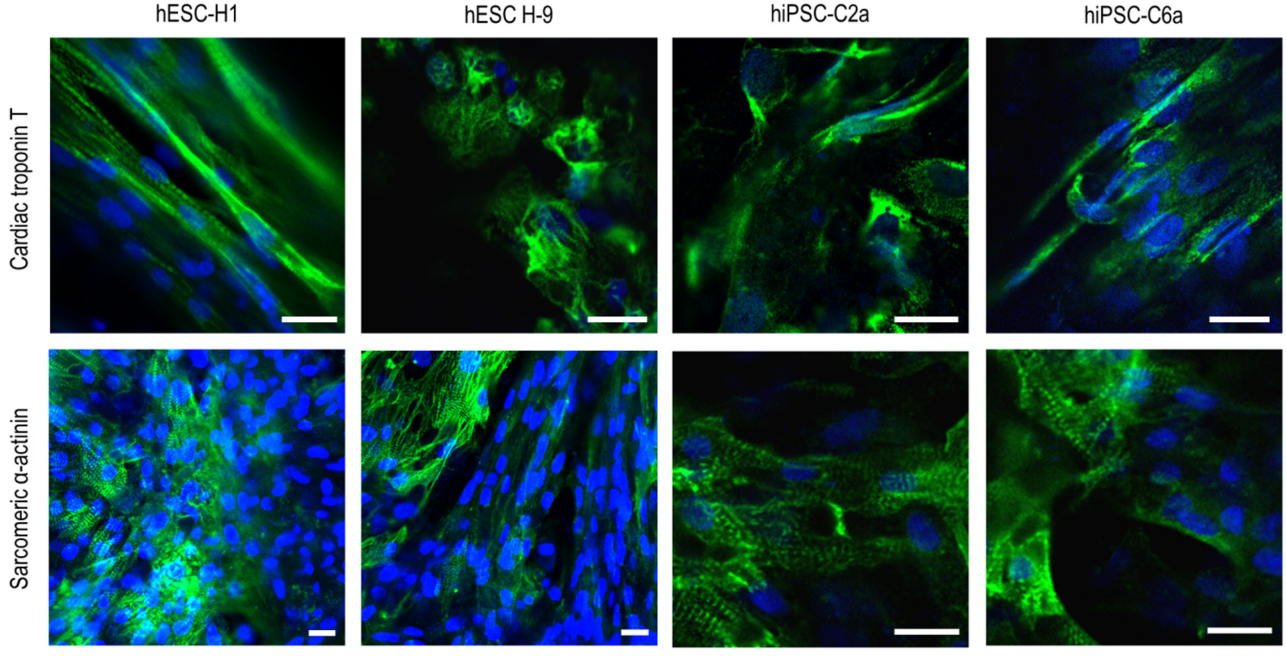

Fig. 3. Immunocytochemical staining of differentiated cells. *Representative images of cardiac-specific markers, sarcomeric α-actinin and cardiac troponin T, which are expressed in all hESC and hiPSC lines at day 40. Striated pattern of sarcomeric α-actinin expression indicates presence of highly organized sarcomeres in all differentiated hESCs and hiPSCs. Scale bar is 20 μm long.*

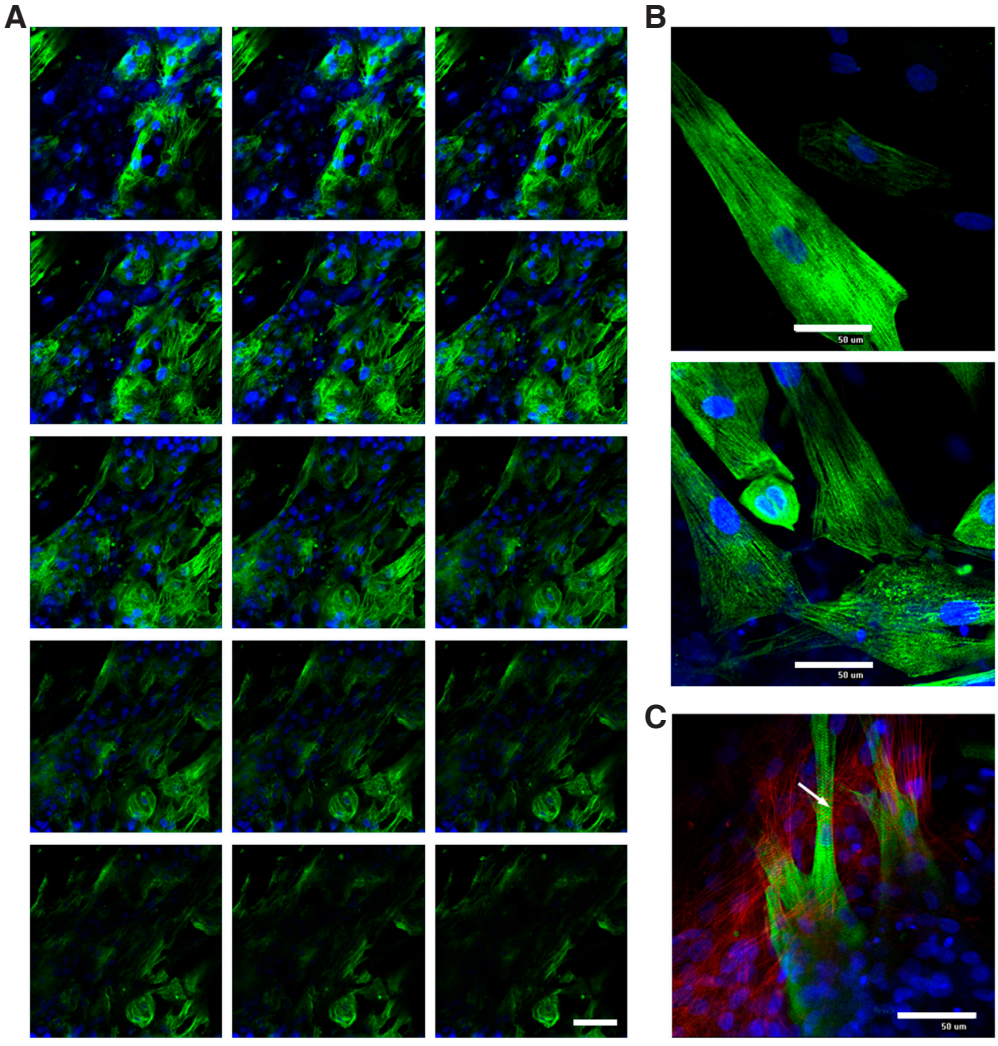

Fig. 4. Highly organized sarcomeres in hESC-H1 cells. (A) *Z-stack images of differentiated hESC-H1 cells stained for sarcomeric α-actinin. An extensive networking of sarcomeric structures in three dimensions exhibits continuum beyond borders of single cells. Scale bar is 20 μm long. Very large cells (sarcomeric α-actinin)* **(B)** *and rod-shaped cells (titin; arrow), resembling mature adult cardiomyocytes* **(C)** *can occasionally be observed.*

specific promoter MLC-2v (Fig. 5A). hESC-H1 cells exhibited greatest cardiomyocyte enrichment (>80% in beating and >70% in non-beating areas) compared to all other tested stem cell lines, which is approximately 20% more than in other cell lines. However, efficient cardiomyogenesis of >60% in beating areas and >50% in non-beating areas was also observed in both hiPSC lines and hESC-H9 cells, indicating that both hiPSC lines can be efficiently differentiated *in vitro* into cardiomyocytes. Approximately 10% more cardiomyocytes were found in beating than in non-beating cell clusters in all cell lines.

Discussion

Here we showed that hESC-H1, hESC-H9, hiPSC-C2a and hiPSC-C6a cell lines readily form cardiomyocytes after directed differentiation. This was observed by occurrence of spontaneously and rhythmically beating cell clusters, expression of cardiac-specific markers (sarcomeric α-actinin and cardiac troponin T), expression of cardiac specific reporter gene in living cells (MLC-2v-EGFP construct) and presence of striated sarcomeres. The spontaneous beating of hESC-H1 cells was paralleled by rhythmical generation and transmission of calcium transients. Moreover, hESC-H1 line produced the most extensive beating areas, the most extensive areas containing cells with striated sarcomeres and the greatest enrichment of cardiomyocytes. However, both hiPSC lines also displayed high degree of cardiac differentiation. A greater proportion of cardiomyocytes in spontaneously beating than in non-beating cell clusters was observed consistently in hESC and hiPSC lines.

Quantification of the differentiated cardiomyocyte yield is critical for evaluating cardiomyogenic potential of hiPSC lines. To achieve the accurate quantification of cardiomyocyte differentiation, we applied highly efficient directed differentiation protocol (Sepac *et al.*, 2010, Sepac *et al.*, 2012) and genetic labeling of living cells that allows unequivocal identification of cardiomyocytes. Directed cardiac differentiation mimics natural signaling in heart development, and increases cardiomyocyte yield when applied *in vitro* (Kattman *et al.*, 2011). Genetic labeling of cardiomyocytes in our study included lentiviral vector delivery of MLC 2v-EGFP gene construct. This construct allows the expression of the fluorescent reporter gene EGFP in cells that contain MLC-2v transcription factor, which is specific for cardiac cells. Our results show considerable cardiomyogenesis in hESC and hiPSC lines, consistent with our previous studies (Canfield *et al.*, 2012, Sepac *et al.*, 2010, Sepac *et al.*, 2012) and studies by others (Lewandowski *et al.*, 2018). We demonstrated previously that during the directed differentiation, hESC-H1, hESC-H9, hiPSC-C2a and hiPSC-C6a lines exhibit comparable changes in the expression of various markers specific to different stages of cardiomyogenesis (Sepac *et al.*, 2012). In that study, we showed that following the complete decline in pluripotency factor OCT4 expression, transient expression of mesodermal marker Brachyury occurred, after which a substantial increase in the expression of early and late cardiomyogenic markers was detected including: MESP1, NKX2.5, ISL1, GATA4, MEF2C, TNNT2, TBX20 and MYL7, as well as cardiac ion channels: HCN4, SCN5A, CACNA1C, KCNH2, KCNJ2 and KCND3. In accordance with our current findings that H1 hESC line more readily differentiates toward cardiomyocytes are studies by Zhang *et al.,* (Zhang *et al.*, 2009) and Toivonen *et al.,* (Toivonen *et al.*, 2013), showing that hESC lines produce more areas with beating cells than hiPSC lines. Contrary to our results, Zhang *et al.,* showed that

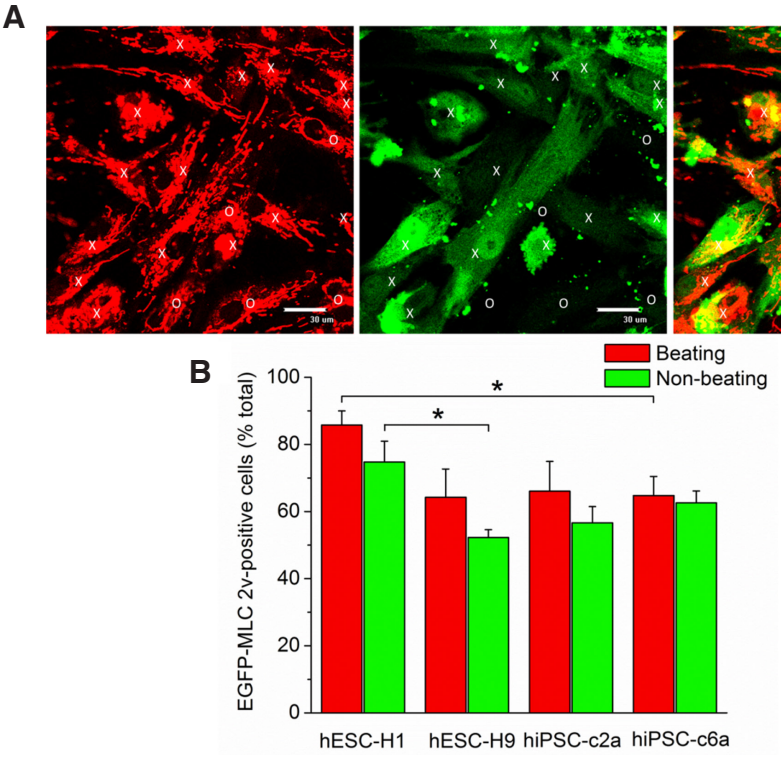

Fig. 5. Comparison of cardiomyogenesis in hESCs and hiPSCs. (A) *Following the dissociation at day 40, bodies of all living cells were visualized by staining mitochondria with TMRE (red), a potentiometric dye attracted by mitochondrial membrane potential. Cardiomyocytes (X mark) were identified and counted as cells expressing MLC-2v-driven EGFP (green), while non-cardiac cells had no EGFP expression (O mark).* **(B)** *Summary of cardiomyocyte quantification from beating and non-beating areas of three independent culture dishes (biological replicates) of individual hESC and hiPSC lines. Cardiomyocytes were the most abundant in beating areas of differentiated hESC-H1 cells. In all lines, there was a tendency for greater cardiomyocyte enrichment in beating than non-beating cell clusters. Data are means ~ SD. *P < 0.05.*

hESC-H9 line was superior in cardiomyogenesis to hESC-H1 line (Zhang *et al.*, 2009). It is possible that such discrepancies occur due to differences in differentiation methodology (embryoid body vs. directed differentiation). However, numerous studies found no differences in cardiomyogenesis among ESC and iPSC lines (Gherghiceanu *et al.*, 2011, Jin *et al.*, 2018). An underlying cause of possible lower cardiomyogenic potential of hiPSC in our study may be their greater propensity toward spontaneous random differentiation because of the epigenetic memory (Noguchi *et al.*, 2018). Yamamoto *et al.,* found lower expression of CHD7 in three hiPSC than in hESC lines, which was associated with reduced differentiation capacity of hiPSC (Yamamoto *et al.*, 2018). A recent study demonstrated that human pluripotent stem cell acquire mutations during cell culture induced by oxidative stress (Kuijk *et al.*, 2020). However, mutation rate can be substantially reduced when oxygen is kept low (Thompson *et al.*, 2020). Thus, mutations in somatic cell from which hiPSC lines were generated, as well as mutations in hESC and hiPSC lines arising during cell culture, may also have contributed to observed differences in cardiomyogenic potential of tested pluripotent cell lines.

We found greater proportion of cardiomyocytes in beating than in non-beating cell clusters in all cell lines, suggesting that spontaneous beating provides additional cardiomyogenic signals during directed differentiation. Our finding is supported by several studies showing that electrical stimulation increases cardiac differentiation of hESCs (Serena *et al.*, 2009) and hiPSCs (Hernández *et al.*, 2016), and that cyclic mechanical stretching also promotes cardiomyogenesis (Shradhanjali *et al.*, 2017). Mechanical stretching alter expression of more than 1,500 genes in neonatal cardiomyocytes, which included pathways for cell growth and proliferation (Rysä *et al.*, 2018). On the other hand, electrical currents upregulate GATA4, cTnT and α-MHC of human cardiosphere-derived cells (Nazari *et al.*, 2020). Unlike previous studies that tested external electrical or mechanical signals on cardiac differentiation, here we tested spontaneous beating that occurs in normal heart development. In our study, cardiomyogenesis was compared among the cell clusters within the same culture dish, and the only difference was the presence or absence of electromechanical signals arising from spontaneous beating.

We also observed the presence of pacemaker cells that are responsible for spontaneous and rhythmical beating of cell clusters (Lee *et al.*, 2017). Upon dissociation, these cells continue to exhibit spontaneous electrical activity that we observed as spontaneous and rhythmical calcium oscillations, which are key elements of electromechanical activity in cardiomyocytes (Verkerk *et al.*, 2013). hiPSC-derived cardiac pacemaker cells are heterogeneous population characterized by different firing rates (Yechikov *et al.*, 2016). We also showed that differentiated cells have the capacity to transmit calcium signals to adjacent cells, which is a basis for electrical coupling among stem cell-derived cardiomyocytes (Si-Tayeb *et al.*, 2010b).

In conclusion, we found that despite efficient cardiomyogenesis in both hiPSC lines (>60% in beating and >50% in non-beating cell clusters), cardiomyocytes are more enriched following directed differentiation of H1 hESC line (>80% in beating and >70% in non-beating cell clusters). Beating of cell clusters promotes cardiomyogenesis in all hESC and hiPSC lines and increases it by an average of 10%.

Materials and Methods

hESC and hiPSC lines

hESC-H1 and hESC-H9 lines were purchased from the National Stem Cell Bank (WiCell, Madison, WI), while hiPSC-C6a and hiPSC-C2a were a kind gift from Dr. Stephen Duncan, MUSC. hiPSC cells were derived from human fibroblasts as previously published (Si-Tayeb *et al.*, 2010a), by introduction of OCT3/4, SOX2, NANOG and LIN28. Upon generation, both

iPSC lines were thoroughly characterized, which included karyotype and DNA fingerprint analysis, pluripotency gene expression profiling, alkaline phosphatase activity and ability to differentiate into all three germ layers (Si-Tayeb *et al.*, 2010a). Genotyping showed a complete match between both hiPSC lines and the somatic cells from which they were generated. We did not perform additional genotyping of hiPSC or hESC lines after the cells were passaged. Pluripotent cells were cultured in hypoxic conditions (4% O_2, 5% CO_2) on mouse embryonic fibroblasts, as we previously published (Sepac *et al.*, 2012). The experiments were conducted on cell passage 29-36 and on cardiomyocytes, 40 days after the induction of differentiation.

Directed cardiomyocyte differentiation

Directed cardiac differentiation was conducted as we previously described (Sepac *et al.*, 2010). Briefly, a five-day treatment with activin-A (50 ng/ml; R&D Systems) and bone morphogenetic protein-4 (10 ng/ml; R&D Systems) was used to induce cardiac differentiation. After that treatment, cells were placed in normoxic conditions in RPMI/B27/insulin media (Thermo Fisher Scientific).

Calcium transients

Calcium transients were recorded, following cell dissociation with trypsin. Fluo-4 AM (2 μM; Thermo Fisher Scientific) was used as a calcium-sensitive indicator and images were acquired with the laser-scanning confocal microscope (Eclipse TE2000-U; Nikon), as we previously described (Si-Tayeb *et al.*, 2010b). Images were acquired every 250 μs, and data were analyzed with ImageJ software (NIH).

Immunocytochemistry

Cell staining was performed according to our previously published protocol (Sepac *et al.*, 2012). Briefly, we used following primary antibodies: anti-cardiac troponin T (1:100; AMPA6687, Thermo Fisher Scientific), anti-sarcomeric αactinin (1:100; A37732, Merck) and anti-titin (1:200; 9D10, Developmental Studies, Hybridoma Bank). Secondary antibody was labeled with Alexa Fluor 488 (1:1000; Thermo Fisher Scientific). Confocal microscope and Image J software were used to for image acquisition and analysis, respectively.

Cardiomyocyte genetic labeling and counting

Genetic labeling with human myosin light chain-2v (MLC-2v)–driven enhanced green fluorescent protein (EGFP) was used for identification of cardiomyocytes, as we previously described (Sepac *et al.*, 2010). For each of the hESC or hiPSC cell lines, three different cell batches were independently induced into differentiation in separate culture dishes, and were independently transduced after completion of cardiomyogenesis, making each of them a biological replicate. Briefly, following the cardiac differentiation for 40 days, single cells from beating and non-beating areas of the culture dish were dissociated by enzymatic digestion of extracellular matrix with a 0.05% trypsin- EDTA (Invitrogen) applied for 4 min. After dissociation, cells were plated onto Matrigel-coated coverslips and recovered for 24 hours, which was followed by 24-hour long cell incubation with a lentiviral vector pHR(+)c.MLC-2v.EGFP.R(-)W(+), carrying MLC-2v-EGFP construct at the multiplicity of infection of $2.2x10^4$. Cells were then incubated with TMRE for 20 min (100 nM; Thermo Fisher Scientific), which allowed us to visualize mitochondria and thereby cell bodies, as we previously published (Sedlic *et al.*, 2020). The cell counting was performed using a confocal microscope. Transduction efficiency was determined by using the similar vector which contained ubiquitin instead of MLC-2v promoter (pHR(+)c.Ub.EGFP.R(-)W(+)). In all four lines, transduction efficiency was approximately 97% (Supplemental Fig. 1). Hence, the transduction efficiency was high, and similar in all tested lines. The production and tittering of lentiviral vectors was performed as described previously (Park *et al.*, 2008).

Statistical Analyses

SigmaStat software (Systat Software, Inc.) was used for statistical analyses. Comparisons were performed using one-way ANOVA with Tukey *post hoc* tests for multiple group analysis and *t* test for two-group analysis. Differences at $P < 0.05$ were considered significant.

References

CANFIELD, S.G., SEPAC, A., SEDLIC, F., MURAVYEVA, M.Y., BAI, X. and BOSNJAK, Z.J. (2012). Marked hyperglycemia attenuates anesthetic preconditioning in human-induced pluripotent stem cell-derived cardiomyocytes. *Anesthesiology* 117: 735-744.

DOI, A., PARK, I.H., WEN, B., MURAKAMI, P., ARYEE, M.J., IRIZARRY, R., HERB, B., LADD-ACOSTA, C., RHO, J., LOEWER, S. *et al.,* (2009). Differential methylation of tissue- and cancer-specific CpG island shores distinguishes human induced pluripotent stem cells, embryonic stem cells and fibroblasts. *Nat Genet* 41: 1350-1353.

GHERGHICEANU, M., BARAD, L., NOVAK, A., REITER, I., ITSKOVITZ-ELDOR, J., BINAH, O. and POPESCU, L.M. (2011). Cardiomyocytes derived from human embryonic and induced pluripotent stem cells: comparative ultrastructure. *J Cell Mol Med* 15: 2539-2551.

HERNÁNDEZ, D., MILLARD, R., SIVAKUMARAN, P., WONG, R.C., CROMBIE, D.E., HEWITT, A.W., LIANG, H., HUNG, S.S., PÉBAY, A., SHEPHERD, R.K. *et al.,* (2016). Electrical Stimulation Promotes Cardiac Differentiation of Human Induced Pluripotent Stem Cells. *Stem Cells Int* 2016: 1718041.

JIN, Y., LIU, Y., LI, Z., SANTOSTEFANO, K., SHI, J., ZHANG, X., WU, D., CHENG, Z., WU, W., TERADA, N. *et al.,* (2018). Enhanced differentiation of human pluripotent stem cells into cardiomyocytes by bacteria-mediated transcription factors delivery. *PLoS One* 13: e0194895.

KATTMAN, S.J., WITTY, A.D., GAGLIARDI, M., DUBOIS, N.C., NIAPOUR, M., HOTTA, A., ELLIS, J. and KELLER, G. (2011). Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. *Cell Stem Cell* 8: 228-240.

KUIJK, E., JAGER, M., VAN DER ROEST, B., LOCATI, M.D., VAN HOECK, A., KORZELIUS, J., JANSSEN, R., BESSELINK, N., BOYMANS, S., VAN BOXTEL, R. *et al.,* (2020). The mutational impact of culturing human pluripotent and adult stem cells. *Nat Commun* 11: 2493.

LEE, J.H., PROTZE, S.I., LAKSMAN, Z., BACKX, P.H. and KELLER, G.M. (2017). Human Pluripotent Stem Cell-Derived Atrial and Ventricular Cardiomyocytes Develop from Distinct Mesoderm Populations. *Cell Stem Cell* 21: 179-194.e4.

LEWANDOWSKI, J., ROZWADOWSKA, N., KOLANOWSKI, T.J., MALCHER, A., ZIMNA, A., RUGOWSKA, A., FIEDOROWICZ, K., ŁABĘDŹ, W., KUBASZEWSKI, Ł., CHOJNACKA, K. *et al.,* (2018). The impact of *in vitro* cell culture duration on the maturation of human cardiomyocytes derived from induced pluripotent stem cells of myogenic origin. *Cell Transplant* 27: 1047-1067.

LUNDY, S.D., ZHU, W.Z., REGNIER, M. and LAFLAMME, M.A. (2013). Structural and functional maturation of cardiomyocytes derived from human pluripotent stem cells. *Stem Cells Dev* 22: 1991-2002.

MAREI, H.E., ALTHANI, A., LASHEN, S., CENCIARELLI, C. and HASAN, A. (2017). Genetically unmatched human iPSC and ESC exhibit equivalent gene expression and neuronal differentiation potential. *Sci Rep* 7: 17504.

NAZARI, H., KEHTARI, M., RAD, I., ASHTARI, B. and JOGHATAEI, M.T. (2020). Electrical stimulation induces differentiation of human cardiosphere-derived cells (hCDCs) to committed cardiomyocyte. *Mol Cell Biochem* 470: 29-39.

NOGUCHI, H., MIYAGI-SHIOHIRA, C. and NAKASHIMA, Y. (2018). Induced Tissue-Specific Stem Cells and Epigenetic Memory in Induced Pluripotent Stem Cells. *Int J Mol Sci* 19: 930.

PARK, F., SWEENEY, W.E., JIA, G., ROMAN, R.J. and AVNER, E.D. (2008). 20-HETE mediates proliferation of renal epithelial cells in polycystic kidney disease. *J Am Soc Nephrol* 19: 1929-1939.

PURI, M.C. and NAGY, A. (2012). Concise review: Embryonic stem cells versus induced pluripotent stem cells: the game is on. *Stem Cells* 30: 10-14.

RYSÄ, J., TOKOLA, H. and RUSKOAHO, H. (2018). Mechanical stretch induced transcriptomic profiles in cardiac myocytes. *Sci Rep* 8: 4733.

SEDLIC, F., MURAVYEVA, M.Y., SEPAC, A., SEDLIC, M., WILLIAMS, A.M., YANG, M., BAI, X. and BOSNJAK, Z.J. (2017). Targeted Modification of Mitochondrial ROS Production Converts High Glucose-Induced Cytotoxicity to Cytoprotection: Effects on Anesthetic Preconditioning. *J Cell Physiol* 232: 216-224.

SEDLIC, F., SEIWERTH, F., SEPAC, A., SIKIRIC, S., CINDRIC, M., MILAVIC, M.,

BATELJA VULETIC, L., JAKOPOVIC, M. and SEIWERTH, S. (2020). Mitochondrial ROS Induce Partial Dedifferentiation of Human Mesothelioma via Upregulation of NANOG. *Antioxidants (Basel)* 9: 606.

SEPAC, A., SEDLIC, F., SI-TAYEB, K., LOUGH, J., DUNCAN, S.A., BIENENGRAEBER, M., PARK, F., KIM, J. and BOSNJAK, Z.J. (2010). Isoflurane preconditioning elicits competent endogenous mechanisms of protection from oxidative stress in cardiomyocytes derived from human embryonic stem cells. *Anesthesiology* 113: 906-916.

SEPAC, A., SI-TAYEB, K., SEDLIC, F., BARRETT, S., CANFIELD, S., DUNCAN, S.A., BOSNJAK, Z.J. and LOUGH, J.W. (2012). Comparison of cardiomyogenic potential among human ESC and iPSC lines. *Cell Transplant* 21: 2523-2530.

SERENA, E., FIGALLO, E., TANDON, N., CANNIZZARO, C., GERECHT, S., ELVASSORE, N. and VUNJAK-NOVAKOVIC, G. (2009). Electrical stimulation of human embryonic stem cells: cardiac differentiation and the generation of reactive oxygen species. *Exp Cell Res* 315: 3611-3619.

SHRADHANJALI, A., RIEHL, B.D., LEE, J.S., HA, L. and LIM, J.Y. (2017). Enhanced cardiomyogenic induction of mouse pluripotent cells by cyclic mechanical stretch. *Biochem Biophys Res Commun* 488: 590-595.

SI-TAYEB, K., NOTO, F.K., NAGAOKA, M., LI, J., BATTLE, M.A., DURIS, C., NORTH, P.E., DALTON, S. and DUNCAN, S.A. (2010a). Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. *Hepatology* 51: 297-305.

SI-TAYEB, K., NOTO, F.K., SEPAC, A., SEDLIC, F., BOSNJAK, Z.J., LOUGH, J.W. and DUNCAN, S.A. (2010b). Generation of human induced pluripotent stem cells by simple transient transfection of plasmid DNA encoding reprogramming factors. *BMC Dev Biol* 10: 81.

SVAGUŠA, T., MARTINIĆ, M., MARTINIĆ, M., KOVAČEVIĆ, L., ŠEPAC, A., MILIČIĆ, D., BULUM, J., STARČEVIĆ, B., SIROTKOVIĆ-SKERLEV, M., SEIWERTH, F. *et al.,* (2020). Mitochondrial unfolded protein response, mitophagy and other mitochondrial quality control mechanisms in heart disease and aged heart. *Croat Med J* 61: 126-138.

THOMPSON, O., VON MEYENN, F., HEWITT, Z., ALEXANDER, J., WOOD, A., WEIGHTMAN, R., GREGORY, S., KRUEGER, F., ANDREWS, S., BARBARIC, I. *et al.,* (2020). Low rates of mutation in clinical grade human pluripotent stem cells under different culture conditions. *Nat Commun* 11: 1528.

THOMSON, J.A., ITSKOVITZ-ELDOR, J., SHAPIRO, S.S., WAKNITZ, M.A., SWIERGIEL, J.J., MARSHALL, V.S. and JONES, J.M. (1998). Embryonic stem cell lines derived from human blastocysts. *Science* 282: 1145-1147.

TOIVONEN, S., OJALA, M., HYYSALO, A., ILMARINEN, T., RAJALA, K., PEKKANEN-MATTILA, M., ÄÄNISMAA, R., LUNDIN, K., PALGI, J., WELTNER, J. *et al.,* (2013). Comparative analysis of targeted differentiation of human induced pluripotent stem cells (hiPSCs) and human embryonic stem cells reveals variability associated with incomplete transgene silencing in retrovirally derived hiPSC lines. *Stem Cells Transl Med* 2: 83-93.

VERKERK, A.O., VAN BORREN, M.M. and WILDERS, R. (2013). Calcium transient and sodium-calcium exchange current in human versus rabbit sinoatrial node pacemaker cells. *ScientificWorldJournal* 2013: 507872.

YAMAMOTO, T., TAKENAKA, C., YODA, Y., OSHIMA, Y., KAGAWA, K., MIYAJIMA, H., SASAKI, T. and KAWAMATA, S. (2018). Differentiation potential of Pluripotent Stem Cells correlates to the level of CHD7. *Sci Rep* 8: 241.

YECHIKOV, S., COPACIU, R., GLUCK, J.M., DENG, W., CHIAMVIMONVAT, N., CHAN, J.W. and LIEU, D.K. (2016). Same-Single-Cell Analysis of Pacemaker-Specific Markers in Human Induced Pluripotent Stem Cell-Derived Cardiomyocyte Subtypes Classified by Electrophysiology. *Stem Cells* 34: 2670-2680.

YOSHIDA, Y. and YAMANAKA, S. (2017). Induced Pluripotent Stem Cells 10 Years Later: For Cardiac Applications. *Circ Res* 120: 1958-1968.

ZHANG, J., WILSON, G.F., SOERENS, A.G., KOONCE, C.H., YU, J., PALECEK, S.P., THOMSON, J.A. and KAMP, T.J. (2009). Functional cardiomyocytes derived from human induced pluripotent stem cells. *Circ Res* 104: e30-41.

Further Related Reading, published previously in the *Int. J. Dev. Biol.*

Construction and characterization of a sox9b transgenic reporter line
Jessica S. Plavicki, Tracie R. Baker, Felipe R. Burns, Kong M. Xiong, Alex J. Gooding, Peter Hofsteen, Richard E. Peterson and Warren Heideman
Int. J. Dev. Biol. (2014) 58: 693-699
https://doi.org/10.1387/ijdb.140288jp

Structure and function of gap junction proteins: role of gap junction proteins in embryonic heart development
Bhavesh K. Ahir and Margaret K. Pratten
Int. J. Dev. Biol. (2014) 58: 649-662
https://doi.org/10.1387/ijdb.140188dp

Sarcosin (Krp1) in skeletal muscle differentiation: gene expression profiling and knockdown experiments
Leonie du Puy, Abdelaziz Beqqali, Helena T.A. van Tol, Jantine Monshouwer-Kloots, Robert Passier, Henk P. Haagsman and Bernard A.J. Roelen
Int. J. Dev. Biol. (2012) 56: 301-309
https://doi.org/10.1387/ijdb.113327lp

Cytoskeletal heart-enriched actin-associated protein (CHAP) is expressed in striated and smooth muscle cells in chick and mouse during embryonic and adult stages
Willemijn van Eldik, Abdelaziz Beqqali, Jantine Monshouwer-Kloots, Christine Mummery and Robert Passier
Int. J. Dev. Biol. (2011) 55: 649-655
https://doi.org/10.1387/ijdb.103207wv

The enigmatic role of the ankyrin repeat domain 1 gene in heart development and disease
Alexander T. Mikhailov and Mario Torrado
Int. J. Dev. Biol. (2008) 52: 811-821
https://doi.org/10.1387/ijdb.082655am

Embryonic development of the proepicardium and coronary vessels
Anna Ratajska, Elzbieta Czarnowska and Bogdan Ciszek
Int. J. Dev. Biol. (2008) 52: 229-236
https://doi.org/10.1387/ijdb.072340ar