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Comparison of Cardiomyogenic Potential among Human ESC and iPSC Lines

Ana Sepac[1], **Karim Si-Tayeb**[2], **Filip Sedlic**[1], **Sara Barrett**[1], **Scott Canfield**[1], **Stephen A. Duncan**[2], **Zeljko J. Bosnjak**[1], and **John Lough**[2]

[1]Department of Anesthesiology, Medical College of Wisconsin, Milwaukee, WI53226 USA

[2]Department of Cell Biology, Neurobiology and Anatomy, Medical College of Wisconsin, Milwaukee, WI 53226 USA

Abstract

We recently reported that following induction of clumps of pluripotent H1 human embryonic stem cells (hESCs) with activin-A and Bmp4 in defined medium for five days, widespread differentiation of rhythmically contracting cardiomyocytes occurs within 3–4 weeks. In this study the same approach was used to assess whether human induced pluripotent cells (hiPSCs), which may theoretically provide an unlimited source of patient-matched cells for transplantation therapy, can similarly undergo cardiomyocyte differentiation. Differentiation of four pluripotent cell-lines – H1 and H9 hESCs, and C2a and C6a hiPSCs – was compared in parallel by monitoring rhythmic contraction, morphologic differentiation, and expression of cardiomyogenic genes. Based on expression of the cardiomyogenic lineage markers MESP1, ISL1 and NKX2-5, all four cell-lines were induced into the cardiomyogenic lineage. However, in contrast to the widespread appearance of striations and rhythmic contractility seen in H9 and especially in H1 hESCs, both hiPSC lines exhibited poor terminal differentiation. These findings suggest that refined modes of generating hiPSCs, as well as of inducing cardiomyogenesis in them, may be required to fulfill their potential as agents of cardiac regeneration.

INTRODUCTION

Two distinct characteristics define pluripotent stem cells: self-renewal and ability to differentiate into derivatives of all three embryonic germ layers. Thus, virtually any cell type in the body can be derived from pluripotent stem cells. Therefore, pluripotent stem cells have immense potential for various applications. For example, pluripotent cells may serve as an *in vitro* model for cell differentiation, which may be particularly useful for elucidating the earliest steps of cardiomyocyte differentiation since this process occurs during the earliest stages of embryonic development when tissue quantities are sparse. Also, derivatives of pluripotent cells, which are capable of differentiation into rhythmically contractile cardiomyocytes(3,11,16)and can be modified to increase avidity for engraftment into sites of infarction (17), may provide a robust source of human cells for transplantation therapy.

Until recently, lines of pluripotent cells derived from human embryos – human embryonic stem cells (hESCs) – have been the most widely investigated. However, the potential for therapeutic use of hESCs is hampered by ethical issues, immune rejection of allogeneic grafts, and oncogenic risk. The first two of these issues may have been recently resolved by the ability to induce pluripotent stem cells (hiPSCs) from human dermal fibroblasts, via

Correspondence to: John W. Lough, PhD, Professor, Department of Cell Biology, Neurobiology and Anatomy, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226-0509, Phone: (414)-955-8459, FAX: (414) 955-6517, jlough@mcw.edu.

transduction of pluripotency factors encoded in retroviral vectors (13,15). Similar to hESCs, hiPSCs exhibit indefinite self-renewal and the ability to differentiate into derivatives of all embryonic germ layers. Hence, hiPSCs provide the possibility of performing cell replacement therapy with patient-matched cells. In addition, disease-matched hiPSC-lines will enable studies to determine the influence of genetic background on the differentiation of specific cell types, which has already been accomplished in the instance of a congenital heart defect that causes hypertrophic cardiomyopathy (1).

Despite these advances, the issue of oncogenic risk remains, which is conversely related to the ability to efficiently induce pluripotent cells to specific differentiated endpoints. Current methods to induce cardiomyocytes when initiated in embryoid bodies (EBs) are only 50–70% efficient (2). Hence, the twofold challenge of efficiently inducing cardiomyocytes and demonstrating that hiPSCs possess the cardiomyogenic potential of hESCs remains. Although it has been reported that mouse (4,5,7,10) and human (16,18) iPSCs can emulate ESCs in this regard, these studies utilized the embryoid body (EB) differentiation model wherein multiple cell types spontaneously appear and in which the extent of cardiomyocyte differentiation is highly variable. Using a non-EB directed-differentiation format we recently demonstrated that H1 hESCs, when exposed to factors which induce heart development in the embryo, can differentiate into rhythmically contracting cardiomyocytes within two weeks (11). Using the same approach we have compared the extent of cardiomyogenic differentiation in H1 hESCs with H9 hESCs and with two lines of lentivirus-generated hiPSCs. Although biochemical evidence of cardiomyogenesis was similar in all four cell-lines, terminal differentiation into cardiomyocytes exhibiting well-defined sarcomeres and rhythmic contractions was most pronounced in H1 and H9 hESCs.

MATERIALS AND METHODS

hESCs & hiPSCs

Human ESC lines H1 and H9 (14) were purchased from the National Stem Cell Bank (NSCB; WiCell, Madison WI). Induced pluripotent stem cell lines C2a and C6a were induced from human foreskin fibroblasts (ATC #CRL2097) as previously described (12) by transduction with lentivirus-encoded Oct4, Sox2, Nanog and Lin28. Pluripotent cells were maintained under hypoxic conditions (4% O_2, 5% CO_2) on mouse embryonic fibroblasts (MEFs) inactivated with mitomycin C (#M0503; Sigma-Aldrich, St Louis, MO), in medium consisting of DMEM/F12 (#11330-032, Invitrogen, Carlsbad, CA) supplemented with 20% knock-out serum replacement(#10828-028; Invitrogen), 1% non-essential amino acids (11140-050, Invitrogen), 1% penicillin-streptomycin (#15140-148, Invitrogen), L-glutamine (#25030-081, Invitrogen), 100 μM β-mercaptoethanol (#M-6250, Sigma-Aldrich) and 4 ng/mL human recombinant basic fibroblast growth factor (#PHG0026, AA 10-155, Invitrogen). Colonies of hESCs and hiPSCs were passaged every five days by manual microdissection with a 27-gaugeneedle to create ~500 μm^2 clumps, followed by a 1 → 4 split. The experiments described in this paper employed H1 and H9 hESCs at passage 36, and C2a and C6a hiPSCs at passage 29.

Induction of Cardiomyocyte Differentiation

To differentiate pluripotent cells into cardiomyocytes, we used “directed differentiation” previously described (11). Briefly, colonies were mechanically dissected into small clumps that were plated onto wells of a 6-well dish (without MEFs) that had been pre-coated with Reduced Growth Factor Matrigel (354230; BD-Biosciences, San Jose, CA)at a final concentration of 0.05 mg/ml in DMEM/F12; counting of cells dissociated from clumps estimated that each well contained 250,000 cells. For the next seven days pluripotency was maintained under hypoxic conditions using hESC medium that had been conditioned by

MEFs during the previous 24-hours period (i.e. MEF-conditioned medium). To induce differentiation, medium was exchanged for RPMI Medium 1640 (#22400, Invitrogen) fortified with B-27 Serum-Free Supplement (#17504-044, Invitrogen, which contains a proprietary amount of insulin), supplemented with activin-A (50 ng/ml; #338-AC, R&D Systems, Minneapolis, MN) and BMP4 (10 ng/ml; 314-BP-010 R&D Systems)and the cultures were placed in normoxic conditions; this time point is referred to as “day 0”. After five days’ induction with activin-A and BMP, these growth factors were removed and differentiation was continued in RPMI/B27/insulinalone. Cultures were monitored daily for the onset of spontaneous contractions.

Immunostaining

Cardiomyocytes derived from hESCs and hiPSCs were characterized by immunostaining 40 days after inducing differentiation. Cells were fixed in1% paraformaldehyde for 1 hr and permeabilized with 0.5% Triton X-100 for 30 min. Cultures were rinsed 3x with PBS, blocked with 3% bovine serum albumin/PBS, followed by 3x rinses with PBS. Primary antibodies prepared in 1% BSA/PBS were applied overnight at 4 C. The following primary antibodies were used: anti-α-actinin (1:100; A37732,Sigma), anti-troponin T (1:100; AMPA6687, Thermo Scientific, Rockford, IL), and anti-titin (1:200; 9D10, Developmental Studies Hybridoma Bank, Iowa City, IA). Primary antibodies were rinsed 3x with PBS, followed by application of an appropriate secondary antibody labeled with Alexa Fluor 488 (1:1000; A21202 Invitrogen). Secondary antibodies diluted in 1% BSA/PBS were applied for 2 hr on a rotator. Cells were rinsed 3x with PBS and nuclei were stained with TOPRO-3 (1:1,000; T3605, Invitrogen) for 10 min. Images were acquired using a laser-scanning confocal microscope (Eclipse TE2000-U; Nikon, Tokyo, Japan) and analyzed using MetaMorph 6.2 software (Universal Imaging, West Chester, PA).

Real-Time PCR

Expression of genes expressed during cardiomyogenic differentiation was assessed by real-time quantitative PCR (qPCR). Beginning at the time of induction (day 0), cells were harvested on alternate days during the first 20 days, followed by harvest of a terminal time point on day 40. RNA purification and qPCR were performed as previously described(11). Briefly, cells were lysed, vigorously pipetted and stored at -80 C until purification. Immediately after thawing, total RNA was purified using the RNeasy Mini Kit (#74104, Qiagen, Valencia, CA), with Qiashredder (#79654, Qiagen), per the manufacturer’s instructions. Following quantification at A_{260}, 1.0 μg total RNA from each sample was reverse-transcribed (#C-03 RT^2 First Strand Kit, SABiosciences, Frederick, MD). qPCR was performed by adding 10 μl of each RT product to 90 μl master mix (#PA-011 RT^2 SYBR Green/Fluorescein qPCR Master Mix, SABiosciences) in wells of 96-well plates containing custom pre-arrayed primer pairs (Custom RT^2 Profiler PCR Array, SABiosciences). PCR reactions were performed using an iCycler (BioRad, Hercules, CA).

RESULTS

Differentiation of Beating Cardiomyocytes is more Extensive in hESCs than in hiPSCs

We simultaneously compared the ability of two widely used hESC lines (H1 and H9) and two hiPSC (C2a and C6a) lines to differentiate into beating cardiomyocytes, using identical conditions of induction and cell culture. As described in Supplemental Figure 4 of Si-Tayeb et al. (2010), pluripotent C2a and C6a hhiPSCs exhibit morphologic, gene expression, and karyotype characteristics that are indistinguishable from pluripotent H9 hESCs. Moreover, C2a and C6a hiPSCs form teratomas containing cells from all three germ layers. And, these hiPSCs can reproducibly and efficiently differentiate into hepatocytes in stepwise, directed

fashion by sequential treatment with growth factors that induce liver development in the embryo (12).

In this study, all four lines were induced to differentiate in defined medium (RPMI/B27) supplemented with cardiomyogenic growth factors BMP4 and activin-A at time zero (day 0) for five days, after which the cells were treated with RPMI/B27 only, up to day 40. The experimental scheme is depicted in Figure 1. Although rhythmic beating was observed by day 10 in both hESC lines, contractions were not observed in the hiPSC lines until the fourth week. In all lines, contractions were spontaneous and rhythmic, always occurring in cell clusters rather than in individual cells, which is characteristic of cardiomyocytes derived from pluripotent cells (3,9). Figure 2 shows characteristic morphologies of cultures derived from each cell-line at day 40. Corresponding videos showing the extent of rhythmic contractions in culture dishes on day 40 are presented as Video Clips 1–4, which can be viewed by accessing the following link: http://www.mcw.edu/cellbiology/johnloughphd.htm. In some instances (H1 hESCs), contractions were sustained for a year or longer (not shown). Contractility was most extensive in H1 hESC cultures, wherein multilayered networks of contracting cells formed that were interconnected, nearly spanning the entire dish (Video Clip 1); in accord with our previous experience using H1 cells, this indicated that the contractile tissue was comprised of contiguous myocytes that were electrophysiologically synchronized (11). H9 hESCs also exhibited contractile three-dimensional areas (Video Clip 2); however, these were neither as networked nor as extensive as in cultured H1 hESCs. By contrast, cultures containing hiPSClinesC2a and C6aexhibited beating in only a few clumps of cells that were not interconnected (Video Clips 3 & 4); with time, contractile areas in hiPSC cultures did not expand or become multilayered, in contrast to H1 and H9hESCs.

Sarcomere Differentiation is more Extensive in hESCs than in hiPSCs

Immunostaining was performed 40 days after inducing differentiation to monitor the presence of cardiac-specific proteins and their assembly into sarcomeres. All panels of Figure 3 show immunostaining patterns in rhythmically beating are as of the dish. This revealed that cardiac troponin-T (cTnT) and sarcomeric α-actinin were organized into striated patterns in H1 and H9 hESCs (Fig. 3a-f). However, striations in both hiPSC lines (Fig. 3h,k) were less conspicuous, and not as widespread as in the hESC cultures, consistent with the abundance of beating clusters shown in the Video Clips (http://www.mcw.edu/cellbiology/johnloughphd.htm). It is also noteworthy that the titin immunostaining pattern was remarkably extensive only for H1 hESCs (Fig. 3m,n), which displayed three-dimensional organization of sarcomeric structures. These characteristics were less evident in H9 hESCs, and were almost completely absent in contractile areas of C2a and C6a hiPSCs (not shown).

qPCR Monitoring of Marker Gene Expression

Although percentages of immunostained cells could not be enumerated due to extensive cellular multilayering, expression of selected mRNAs was monitored by qPCR at multiple points during the differentiation period (Fig. 4). In all four lines, induction of differentiation at day 0 was followed by diminishing levels of the pluripotency marker *OCT4*, concomitant with transient expression of the mesendoderm marker *brachury* (*T, Tbx1*); this pattern was anticipated for these markers. The earliest phases of cardiomyogenic induction are characterized by expression of *MESP1*, *NKX2.5* and *ISL1*; expression of these markers was similar in all lines, except that expression *of ISL1* was low in both hiPSC lines but remarkably high in H9 hESCs. As cardiomyocytes begin to terminally differentiate, they express mRNAS including *GATA4*, *MEF2C*, *TNNT2*, *TBX20*, and *MYL7*. Although these markers increased as expected during the second week of the 40 day culture period,

expression of *TNNT2*, *TBX20* and *MYL7* was remarkably low in both hiPSC lines. Expression of mRNAs encoding various ion channel genes (*HCN4*, *SCN5A*, *CACNA1C*, *KCNH2*, *KCNJ2*, *KCND3*) was also monitored, revealing that with the exception of HCN4 which peaked in all lines at day 6, all began to undergo steady, subtle increases late in the second week; however, no cell line-specific differences were noted. Finally, expression of mRNAs indicative of differentiation into ectodermal (*PAX6*) and endodermal (*SOX17*) lineages revealed that whereas all four lines exhibited strong *SOX17* expression (300x-800x) during the first week, induction of ectoderm was minimal, with the exception of increased *PAX6* levels in c2a hiPSCs on days 4–12, and especially on day 40.

DISCUSSION

We simultaneously compared the cardiomyogenic potential of two human iPSC lines with two well-characterized human ESC lines, under controlled conditions using a directed differentiation protocol. We show that cells from all four pluripotent stem cell lines can differentiate toward a cardiomyocyte fate. However, H1 hESCs exhibited the most cardiomyogenic competence, forming extensive areas of rhythmically contractile cells(Video Clips 1–4) that contained organized sarcomeres(Fig. 3). Accordingly, in terms of biochemical differentiation, qPCR revealed that H1 cells were the most robust in terms of cardiomyogenic gene expression (Fig. 4).

Several groups have reported that human and mouse iPSCs possess cardiomyogenic competence (4,5,7,10,16,18). Relative to our findings, those of Zhang et al.(16) are most noteworthy because these investigators compared differentiation of H1 and H9 hESCs with two different lines of lentivirus-induced human iPSCs, observing that sarcomere organization and expression of cardiomyogenic genes were indistinguishable in hiPSCs and hESCs, although the ability to contract was highest in H9 hESCs. Despite similarities in approach it is difficult to compare their findings with those described here since Zhang et al. (16) used an embryoid body (EB) rather than a directed differentiation format, and because their gene expression analysis was based on a single endpoint determination. Regarding the relative competence of H1 and H9 hESCs, differences in cardiomyogenic potency have previously been noted, attributed to epigenetic marks (6,8); other reports have indicated that cardiomyocytes are most successfully differentiated from H1 (this paper and (11)), H7 (3), orH9 (16) hESCs. These differences are possibly related to differences in spontaneous differentiation that we noted during expansion and passaging of the pluripotent cells used in this study. Specifically, spontaneous differentiation in H1 hESCs was minimal during more than 50 passages, in contrast with H9 hESCs which exhibited differentiation after only seven passages. Both hiPSC lines displayed even greater propensity to spontaneously differentiate, requiring the establishment of fresh colonies after every fourth passage. Although epigenetic factors may have influenced these results, differences in cell culture format and induction conditions, for example directed differentiation vs. the EB format, likely affected differentiated outcomes. Hence, while the results described here indicate that both of the hESC and hiPSC lines can enter the cardiomyogenic pathway while only the hESC lines exhibited substantial terminal differentiation, such a conclusion must remain tentative until universal conditions are established that are capable of efficiently, reproducibly and rapidly inducing pluripotent cells to terminally differentiated cardiomyogenic endpoints.

Regarding the relative inability of C2a and C6a hiPSCs to differentiate into functional cardiomyocytes, the possibility that differentiation is suppressed due to the integration of pluripotency genes in lentivirus-induced hiPSCs has been previously noted (16). However, the decline of Oct4 expression in C2a and C6a cells was essentially equivalent to that observed in H1 and H9 hESCs (Fig. 4). Moreover, evidence that C2a hiPSCs are differentiation-competent was recently demonstrated by the equivalent ability of C2a

hiPSCs and H9 hESCs to undergo growth factor-directed differentiation into mature hepatocytes, with efficiency exceeding 80% (12). Although the relative inability of hiPSCs to undergo terminal myocyte differentiation is unexplained, it is noted that the highly efficient differentiation of hepatocytes was performed in monolayer culture format wherein all cells are exposed to equivalent growth factor levels, in contradistinction to the induction of pluripotent cells organized in three-dimensional clumps as employed here. It is therefore speculated that the relative inability of hiPSCs to terminally differentiate may indicate that while initial cardiomyogenic signals in clumped cells were sufficient to overcome the presence of integrated pluripotency genes, signals regulating terminal differentiation were somehow compromised. It is nonetheless encouraging cells in C2a and C6a cultures expressed cardiomyogenic RNAs, suggesting that these cells occupy a position in the cardiomyogenic pathway from which improved signaling may support terminal differentiation. In this regard we are currently inducing pluripotent cells maintained in monolayer culture, with the goal of establishing conditions that mimic the high levels of efficiency that have been reproducibly achieved during hepatocyte differentiation (12).

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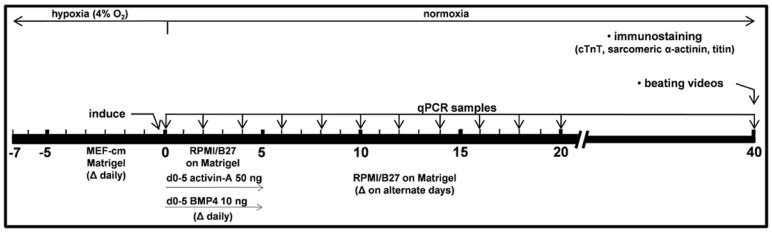

Figure 1.
Experimental scheme for inducing the cardiomyogenic lineage in clumps of pluripotent hESCs and hiPSCs.

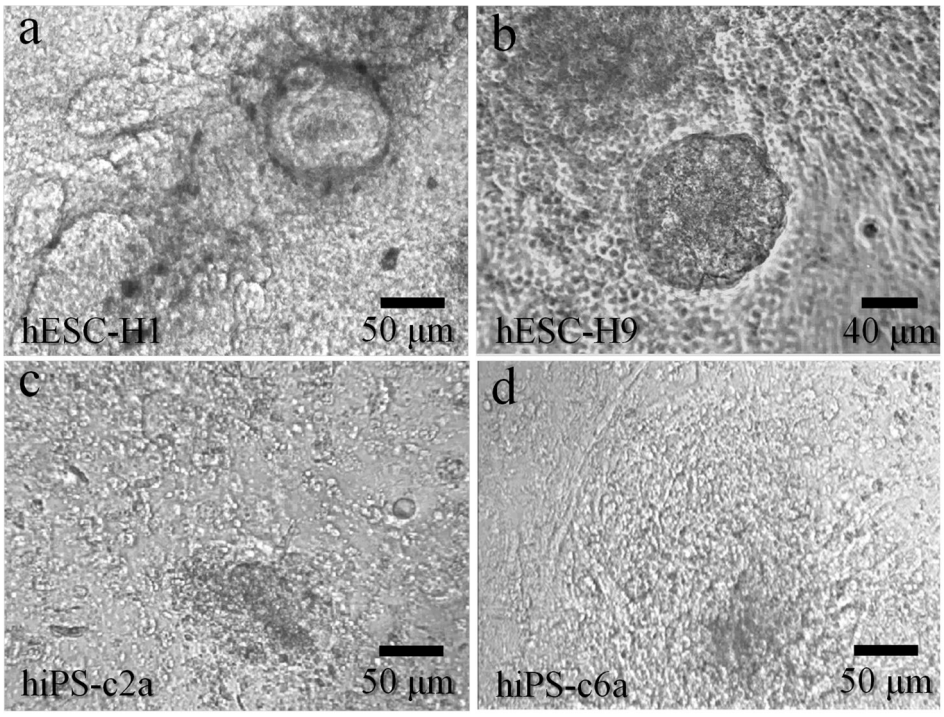

Figure 2. Phase contrast images of differentiated hESCs and hiPSCs after 40 days in culture

Representative light microscope images of hESC-and hiPSC-derived cells at high magnification (400x) on the day 40 of differentiation. Note thatH1 and H9 hESCs formed multilayered confluent cluster of cells that exhibited spontaneous rhythmic contractions (viewed in Video Clips 1 & 2). hiPSC lines (c2a and c6a) also form multilayered and interconnected cell clusters that also spontaneously and rhythmically contracted(Video Clips 3 &4); however, the hiPSC cells for medless confluent clusters than hESCs.

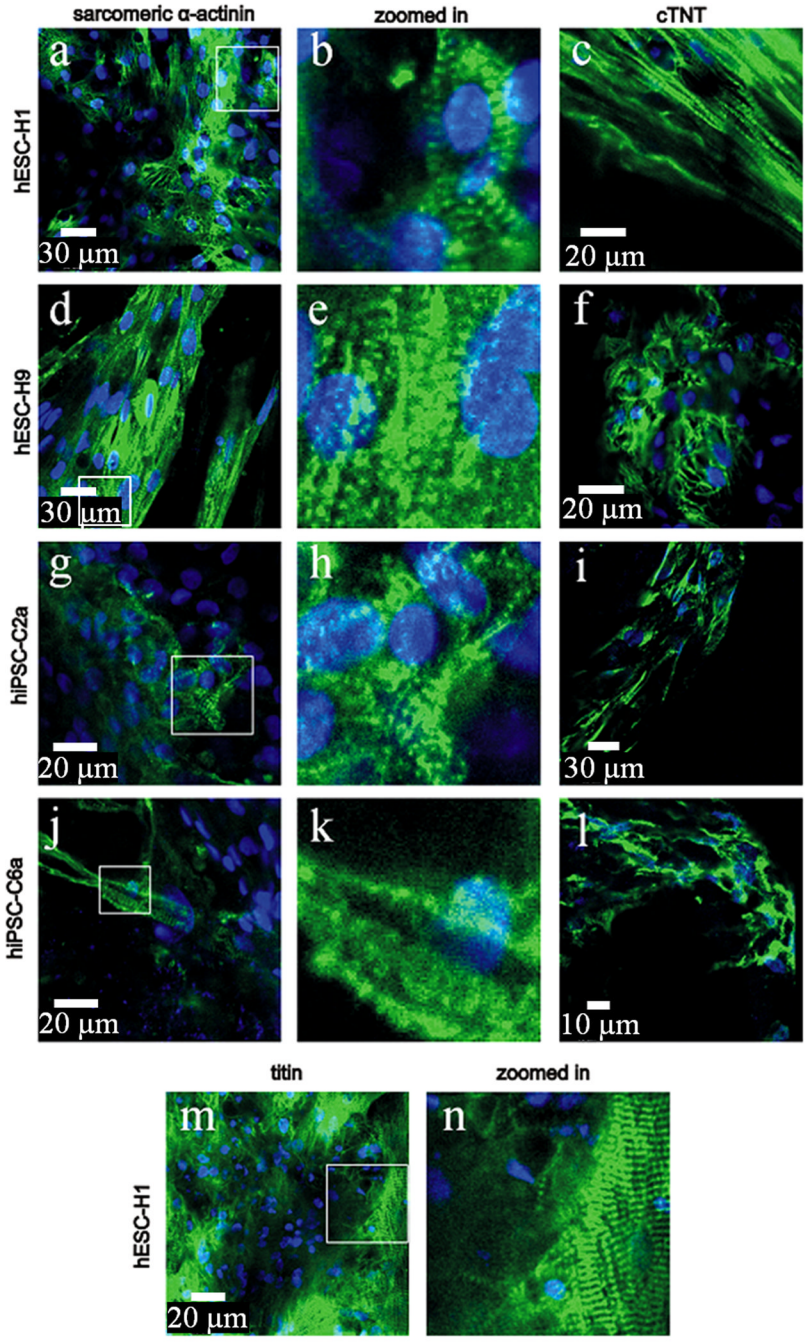

Figure 3. Immunostained cardiomyocytes derived from hESCs and hiPSCs

(a–l), representative images of immunostained cardiac troponin T (TnT) and sarcomeric α-actinin in all four cell-lines at day 40. Striated staining patterns indicative of sarcomeres are seen in all hESC and hiPSC lines; however striations are most extensive in H1 cells, wherein titin immunostaining **(m–n)** reveals a level or sarcomeric networking that was not observed in the other cell-lines.

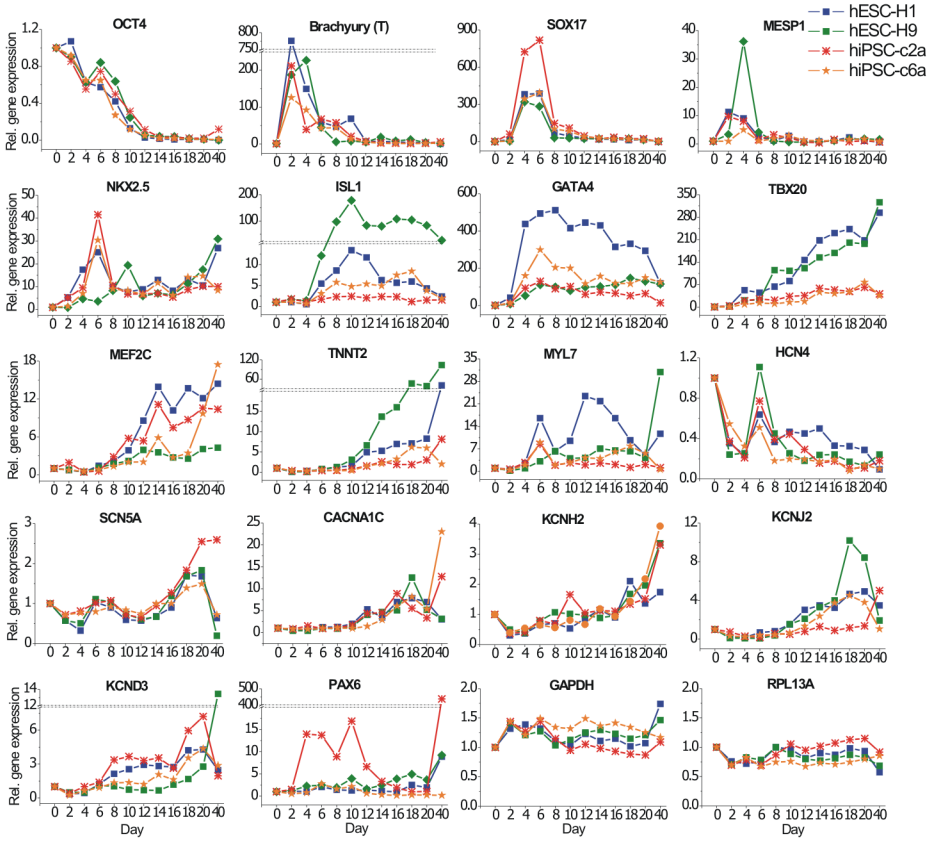

Figure 4. Gene expression during cardiomyogenesis in hESC and hiPSC lines

Expression of the indicated genes was monitored during the 40 day differentiation period by qPCR. Each data point is normalized to values at day 0 (induction of differentiation). GAPDH and RPL13A served as loading controls. Each point represents the mean of duplicate samples; vertical lines indicate the range.