Published in final edited form as:

Regen Med. 2011 January ; 6(1): 53–66. doi:10.2217/rme.10.91.

Efficient generation and cryopreservation of cardiomyocytes derived from human embryonic stem cells

Chunhui Xu^{1,†}, Shailaja Police¹, Mohammad Hassanipour¹, Yan Li¹, Yinhong Chen¹, Catherine Priest¹, Chris O'Sullivan¹, Michael A Laflamme², Wei-Zhong Zhu², Benjamin Van Biber², Livia Hegerova², Jiwei Yang¹, Karen Delavan-Boorsma¹, Anthony Davies¹, Jane Lebkowski¹, and Joseph D Gold¹

¹ Geron Corporation, 230 Constitution Drive, Menlo Park, CA 94025, USA

² Department of Pathology, Center for Stem Cell & Regenerative Medicine, University of Washington, Seattle, WA 98195, USA

Abstract

Aim—Human embryonic stem cells (hESCs) represent a novel cell source to treat diseases such as heart failure and for use in drug screening. In this study, we aim to promote efficient generation of cardiomyocytes from hESCs by combining the current optimal techniques of controlled growth of undifferentiated cells and specific induction for cardiac differentiation. We also aim to examine whether these methods are scalable and whether the differentiated cells can be cryopreserved.

Methods & results—hESCs were maintained without conditioned medium or feeders and were sequentially treated with activin A and bone morphogenetic protein-4 in a serum-free medium. This led to differentiation into cell populations containing high percentages of cardiomyocytes. The differentiated cells expressed appropriate cardiomyocyte markers and maintained contractility in culture, and the majority of the cells displayed working chamber (atrial and ventricular) type electrophysiological properties. In addition, the cell growth and differentiation process was adaptable to large culture formats. Moreover, the cardiomyocytes survived following cryopreservation, and viable cardiac grafts were detected after transplantation of cryopreserved cells into rat hearts following myocardial infarctions.

Conclusion—These results demonstrate that cardiomyocytes of high quality can be efficiently generated and cryopreserved using hESCs maintained in serum-free medium, a step forward towards the application of these cells to human clinical use or drug discovery.

Keywords

cardiomyocytes; cell transplantation; cryopreservation; differentiation; human embryonic stem cells

[†]Author for correspondence: Tel.: +1 650 473 7795, Fax: +1 650 473 7750, cxu@geron.com.

For reprint orders, please contact: reprints@futuremedicine.com

Ethical conduct of research

All animal studies were approved by the Geron Animal Care and Use Committee and were in accordance with federal guidelines.

Financial & competing interests disclosure

C Xu, S Police, M Hassanipour, Y Li, Y Chen, C Priest, C O'Sullivan, J Yang, K Delavan-Boorsma, A Davies, J Lebkowski and JD Gold are employees and shareholders of Geron Corporation. MA Laflamme is a collaborator of Geron Corporation. MA Laflamme was supported by NIH grants R01-HL064387 and K08-HL80431. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Human embryonic stem cells (hESCs) [1,2] derived from the inner cell mass of the pre-implantation embryo are a promising cell source for regenerative medicine. They are capable of extensive proliferation in the undifferentiated state (self-renewal) and, as pluripotent cells, may be induced to generate differentiated cell types such as functional cardiomyocytes [3–9]. Animal model studies indicate that hESC-derived cardiomyocytes have the ability to survive *in vivo* and to improve cardiac function [3,9–11]; if these observations can be extended to humans, the hESC-derived cardiomyocytes may help prevent progression of heart diseases and/or restore contractile function in damaged hearts.

Human embryonic stem cell-derived cardiomyocytes may also serve as cellular models for drug testing. Several noncardiovascular drugs were found to cause unanticipated cardiotoxicity and were withdrawn from the market in the late 1990s (reviewed in [12,13]), heightening concerns over cardiotoxicity within the pharmacological industry. Because of limited supplies of human cardiomyocytes, assessment of cardiotoxicity is traditionally carried out in models that use genetically modified cells or nonhuman cardiac cells (reviewed in [12,13]). hESCs represent a more appropriate and reliable cell resource for cardiotoxicity testing. They have the ability to differentiate in large quantities to cardiomyocytes with relevant physiological phenotypes, which may translate to reproducible and accurate evaluations of targets in a more cost-effective manner.

For the treatment of heart disease using hESC-derived cardiomyocytes, several important issues need to be addressed. First, the efficiency of cardiomyocyte differentiation from hESCs needs to be significantly improved. Second, regulatory-compliant materials are required for the production of the cells. Third, a scalable method without complicated processes such as cell sorting or co-culture is preferred. A clinically significant infarct can lead to the loss of more than 1 billion cardiomyocytes from the left ventricle, so a large number of transplantable cells per patient will be required. Fourth, a process of cryopreserving cardiomyocytes needs to be established. For drug discovery application, efficient and scalable methods for differentiation and cryopreservation of high-quality cells are also required.

In recent years, the stem cell field has progressed significantly in addressing some of the aforementioned issues. For example, new methods to induce cardiomyocyte differentiation using growth factors or small molecules have been developed [10,14–19], replacing the conventional methods using cell aggregates (embryoid bodies) [6,7] or co-culture of hESCs with endoderm-like cells derived from mouse carcinoma cells [8] in serum-containing media. For the growth of undifferentiated cells, many factors controlling self-renewal of undifferentiated hESCs and defined growth conditions for expansion of undifferentiated hESCs have been discovered (reviewed in [20]). However, a systematic evaluation of differentiation of hESCs maintained using optimal growth conditions has not been carried out. In addition, whether these methods are scalable and whether the differentiated cells can be cryopreserved have not been determined.

In this study, we have modified our previously used method [10] and show that hESCs maintained without conditioned medium from mouse embryonic fibroblasts (MEF-CM) retain their pluripotency and differentiate into cardiomyocytes upon sequential treatment with activin A and bone morphogenetic protein (BMP)-4 in a defined serum-free medium. Importantly, we show that this procedure is scalable and achieves high yields of viable cardiomyocytes that can be cryopreserved and thawed, and that successfully survive *in vitro* as well as after transplantation in infarcted hearts.

Materials & methods

Growth factor-directed cardiomyocyte differentiation

H7 hESCs [1] were maintained in MEF-CM [21] or transferred into a serum-free medium containing growth factors (serum-free medium/GFs) (see Supplementary material online at www.futuremedicine.com/doi/suppl/10.2217/rme.10.91) from MEF-CM or X-VIVO™10 medium [22]. To induce cardiomyocyte differentiation, confluent cultures of hESCs were incubated with collagenase IV (200 units/ml) at 37°C for 5–10 min, harvested as small clusters and cultured in Matrigel®-coated plates at 1×10^5 cells/cm² in undifferentiated culture medium (serum-free medium/GFs or MEF-CM). Cell number was estimated by dissociation of parallel cultures with 0.05–0.25% trypsin/0.5 mmol/l ethylenediaminetetraacetic acid. After growth for 6 or 7 days in the same medium in which the undifferentiated cultures had been maintained (stage 1), the cells were treated with 100 ng/ml activin A (R&D Systems Inc.) for 1 day in Roswell Park Memorial Institute (RPMI) medium (Invitrogen) supplemented with B27 supplement (Invitrogen) at a final concentration of 2% (RPMI/B27), which was designated as stage 2A. The day on which the cells were first treated with activin A was designated as differentiation day 0. At day 1, the activin A-containing medium was removed, and the cells were treated with 10 ng/ml BMP-4 (R&D Systems Inc.) in RPMI/B27 medium for 4 days without medium exchange. This BMP-4 treatment stage was designated stage 2B. Following stage 2B, the cells were cultured in RPMI/B27 medium for an additional 15–25 days with medium changes every 2–3 days (stage 3) (Figure 1a).

Electrophysiology

Human embryonic stem cells were induced to differentiate by the growth factor-directed differentiation method. At differentiation day 14, cells were dispersed using blendzyme 4 (Roche), replated onto gelatin-coated glass-bottom Petri dishes and cultured for an additional 6–10 days. The spontaneously generated action potentials (APs) of hESC-derived cardiomyocytes maintained at 35–36°C were then recorded using a HEKA EPC-10 amplifier operated in current clamp mode. After obtaining seal, electrical access to the cells was obtained via the β -escin perforated-patch technique [23,24], which improved success relative to the conventional ruptured patch approach, as it greatly minimized both seal disruption and current rundown and resulted in substantially reduced perturbation of the cytoplasmic milieu. Patch pipettes with a resistance of 2–4 M Ω were used; cells with a series resistance greater than 10 M Ω were discarded. The capacitance of the examined cells was 17.5 ± 7.6 pF (range: 5.8–32.8 pF), in comparison to the approximate 150 pF typically reported for adult human ventricular myocytes [25]. Bath medium contained (in mmol/l) 140 NaCl, 5.4 KCl, 1.8 CaCl₂, 1.0 MgCl₂, 0.33 NaH₂PO₄, 5 dextrose and 10 (4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid), adjusted to pH 7.40 with NaOH. The pipette solution contained (in mmol/l) 135 KCl, 5 Na₂-creatine phosphate, 5 MgATP and 10 (4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid), adjusted to pH 7.20 with KOH. Data were digitized at 10 kHz and filtered at 2.9 kHz. AP parameters were analyzed using Patchmaster (HEKA) and IgorPro software. hESC-derived cardiomyocytes were classified as either nodal or working chamber type based on criteria detailed in our recent publication [26]. In brief, we found the most reliable parameter when making this classification to be the slope of the AP upstroke ('phase 0'). Nodal cells were defined by their slow (maximum rate of action potential upstroke $[dV/dt_{\max}] < 15$ V/s), biphasic AP upstroke. Cells with these properties consistently demonstrated other AP properties expected of nodal cells, including a faster mean spontaneous firing rate, a narrower mean AP amplitude and a more depolarized mean maximum diastolic potential than their working-type counterparts.

Cryopreservation of hESC-derived cardiomyocytes

Differentiated cultures containing beating cardiomyocytes were dissociated with 0.25% trypsin/0.5 mmol/l ethylenediaminetetraacetic acid (Invitrogen) and neutralized with defined trypsin inhibitor (Cascade Biologics). The cells were washed and resuspended by slowly adding cryopreservation solution CryoStor™ CS-10 (BioLife Solutions Inc.). The final cell concentrations were approximately $5\text{--}10 \times 10^6$ cells in 0.25 ml/vial (small scale) or $4\text{--}8 \times 10^7$ cells in 1.5 ml/vial (large scale). Cells were frozen using a controlled rate freezer at $-1^\circ\text{C}/\text{min}$ before the temperature reached -40°C and $-5^\circ\text{C}/\text{min}$ from -40°C to -80°C . Vials were then transferred to a liquid nitrogen tank after reaching -80°C . To thaw the cells, the vials were incubated in a water bath at 37°C until no ice crystals were visible. The cell suspension was transferred to a 15 or 50 ml tube and then slowly diluted with RPMI/B27. After centrifugation at 450 g for 5 min, the supernatant was removed and cells were resuspended in RPMI/B27. The cells were subjected to flow cytometric analysis, cultured on Matrigel (BD Biosciences)-coated plates in RPMI/B27 or prepared for transplantation as described below. Comparison of cell recoveries from samples containing cardiomyocytes of different purities (indicated as mean $\pm$ standard deviation of the percentage of cardiac troponin I [cTnI], α -actinin or Nkx2.5) was carried out by Tukey's multiple comparison test using Prism software.

Preparation of cells for transplantation

Differentiated cultures were freshly harvested or cryopreserved and prepared for transplantation. Cultures were subjected to a transient heat shock at 43°C for 30 min 1 day prior to cell harvesting, and the medium was replaced with RPMI/B27 supplemented with 100 ng/ml IGF-I and 0.2 $\mu\text{mol/l}$ cyclosporine A to enhance graft survival, as described previously [10]. The cultures were washed with phosphate-buffered saline and dissociated with 0.25% trypsin/0.5 mmol/l ethylenediaminetetraacetic acid and neutralized with defined trypsin inhibitor (Invitrogen). The cells were washed and used for transplantation on the same day or cryopreserved as described earlier. To thaw the cells, the vials were incubated in a water bath at 37°C until no ice crystals were visible, and the cells were washed with RPMI/B27.

Rat model of myocardial infarction & cell transplantation

Female athymic rats (RH-Fox 1^{nu} 8–10 weeks of age, Harlan) were subjected to 1 h of myocardial ischemia–reperfusion injury [10]. Animals received an intramyocardial transplant of freshly isolated cells ($n = 6$) or cryopreserved cells ($n = 13$) 1 week after the procedure. Briefly, freshly isolated or cryopreserved cells immediately post-thawing were resuspended in medium containing a pro-survival cocktail [10] and loaded into a 100 μl Hamilton™ syringe attached to a 26-gauge needle. For transplantation, the 26-gauge needle was replaced with a 31-gauge needle and the cell suspension ($1\text{--}1.4 \times 10^7$ cells in 70 μl /animal) was divided across three injection sites: one injection in the infarct area and two injections in the myocardial zones that border the infarct. Animals were perfused with cardioplegic solution (NaCl 4 g/l, KCl 1 g/l, glucose 2 g/l, pH 7.4) 4.48 g/l, NaHCO_3 1–4 weeks after the transplantation followed by 4% paraformaldehyde. All hearts were harvested and stored in 30% sucrose overnight, embedded in a 1:1 mixture of 20% sucrose and optimal cutting temperature compound (Sakura Finetek USA, Inc.) and frozen in 2-methylbutane on dry ice prior to sectioning for histological and immunohistochemical analyses (see Supplementary material).

Other methods

Other methods, including undifferentiated hESC culture, flow cytometric analysis, real-time reverse transcription-PCR, karyotype analysis, histology, immunocytochemical analysis and *in vitro* pharmacological analysis are described in the Supplementary material.

Results

hESCs respond to specific growth factor treatment & differentiate efficiently into cardiomyocytes

We have previously reported that activin A and BMP-4 induce cardiomyocyte differentiation from hESCs maintained in MEF-CM [10]. In this study, we first performed additional characterization of the cells derived via this method. As depicted in Figure 1a, the differentiation was performed in three different stages. Undifferentiated hESC cultures maintained in adherent cultures for 6–7 days (stage 1) were induced by sequential treatment with activin A for 1 day (stage 2A) followed by BMP-4 for 4 days (stage 2B) and maintained in RPMI/B27 medium afterwards (stage 3). During stages 2A and 2B, growth factor treatment typically resulted in a cell population that had lost its undifferentiated cell morphology and led to some cell death. In stage 3, the surviving cells continued to proliferate and differentiate into multiple cell types including spontaneously contracting cells, a phenotypic characteristic associated with cardiomyocyte-lineage cells. The beating cells, appearing as both 3D cell clumps and as flat contracting regions, first arose at approximately day 9–12 (day 0 corresponds to the day of addition of activin A) and became more obvious after approximately day 15–20. By contrast, cells without growth factor treatment did not generate beating cells. In addition, inclusion of both activin A and BMP-4 was critical as removing either factor did not result in the generation of significant numbers of cardiomyocytes.

We monitored the timing of cardiomyocyte generation by quantitative immunocytochemistry. Expression of Nkx2.5, a transcription factor that is associated with precardiac mesoderm and persists in the heart during development, and α -actinin, a cardiomyocyte-associated sarcomeric protein, was determined. In cultures that were induced using sequential treatment with activin A and BMP-4, on days 6 (24 h after removing the BMP-4) and 7 of differentiation, approximately 8–10% of the total cells were positive for Nkx2.5 (Figure 1B), and by day 8, 35–40% of the cells expressed Nkx2.5. Although α -actinin was undetectable at day 6 in these conditions (A100/B10), approximately 16% of the total cells coexpressed both Nkx2.5 and α -actinin at day 8 (Figure 1B). At the final time point examined (day 23), all Nkx2.5-positive cells also expressed α -actinin, which comprised approximately 37% of the total cells (Figure 1B). By contrast, fewer than 7% of Nkx2.5-positive cells were detected in control cultures that did not receive any growth factor (Figure 1B), and Nkx2.5 and α -actinin double-positive cells were undetectable in these cultures (Figure 1B). Therefore, the growth factor-directed differentiation procedure induced formation of precardiac mesoderm as early as 6 days after the induction, and generation of cardiomyocytes by day 8, with increased percentages of cardiomyocytes observed over time.

Efficient differentiation can be achieved from hESCs without using conditioned medium

We then examined whether cardiomyocytes could be generated from undifferentiated cells maintained with serum-free medium/GFs, which would facilitate generation of large amounts of the cells. H7 cells initially maintained in MEF-CM were transferred into serum-free medium/GFs. These cells were characterized as described in the Supplementary material. Overall, the cells maintained in serum-free medium/GFs possessed characteristics of undifferentiated hESCs, including appropriate morphology, surface marker and transcription factor expression, karyotypic stability and differentiation capacity

(Supplementary Figures 1 & 2, see online at www.futuremedicine.com/doi/suppl/10.2217/rme.10.91), and therefore were suitable for testing cardiomyocyte differentiation. For differentiation analysis, confluent hESC cultures maintained in the serum-free medium/GFs were sequentially treated with activin A for 1 day followed by BMP-4 for 4 days, as described earlier. The cells showed similar morphological changes and timing of the generation of beating cells as those derived from cells maintained in MEF-CM. To quantify the efficiency of differentiation of hESCs into cardiomyocyte-lineage cells, we performed flow cytometric analysis on the cells at the end of stage 3. cTnI-, Nkx2.5- or α -actinin-positive cells, ranging from 10 to 40% of the cell population, were detected from independent cultures maintained in serum-free medium/GFs in typical experiments. These cultures showed viability of 60–88%, as determined by ethidium monoazide staining, and generated $1\text{--}5 \times 10^5$ viable cardiac cells/cm². Since 1×10^5 undifferentiated cells/cm² were seeded to set up stage 1, each input undifferentiated hESC yielded one to five viable cardiac cells. As shown in a representative experiment from three cultures, $30 \pm 5\%$ cTnI-positive cells with $86 \pm 1\%$ viability were obtained and 4 ± 2 viable cells were produced from each input undifferentiated cell (Figure 1C). Similar yields and purities of cardiomyocyte differentiation were achieved using cultures maintained in MEF-CM or the serum-free medium/GFs adapted from X-VIVO 10 medium (data not shown). The differentiation efficiency generated by this protocol was much higher than that achieved using the embryoid bodies protocol, which typically generated only approximately 1% cardiac cells [9].

We next evaluated if the differentiation process was scalable into larger culture formats. Undifferentiated cells from three independent cultures maintained in the serum-free medium/GFs were seeded into T225 flasks at 1×10^5 /cm² (23×10^6 /flask) and induced to differentiate using the direct differentiation protocol. Similar to the observations from small culture formats, beating cardiomyocytes were generated and the cultures gave rise to cell populations containing 38, 22 and 42% cardiac cells in three independent experiments (Figure 2). These cultures showed 80–88% viability and generated $31\text{--}44 \times 10^6$ viable cardiomyocytes per flask. The ratios of input undifferentiated hESCs to viable cardiomyocytes were 1:1.3 to 1:1.9, which were within the range generated from small culture formats. Therefore, the efficient cardiac differentiation process is scalable using hESCs maintained in the serum-free medium/GFs.

Differentiated cells express appropriate cardiomyocyte markers & display cardiomyocyte electrophysiological phenotypes

To further characterize the beating cells, the expression of cardiomyocyte-associated proteins was examined by immunocytochemistry. The cells showed expression of multiple markers characteristic of cardiomyocytes, including cTnI, cardiac troponin T, tropomyosin, α -actinin and sarcomeric myosin heavy chain (Figure 3a–H). Cardiomyocytes identified by the expression of cTnI or sarcomeric myosin heavy chain were positive for N-cadherin, an adherens junction protein [27], and connexin 43, a gap junction protein (Figure 3D, F & H) [28], which is consistent with the observation that cells in the beating clusters contracted synchronously. The cardiac transcription factor Nkx2.5 was detected in most nuclei of tropomyosin-, cTnI-, cardiac troponin T- or α -actinin-positive cells (Figure 3a–C, e & g). Under higher magnifications, some of the cells showed striations, a characteristic of cardiomyocytes (Figure 3g & H). In addition, a subset of the cardiomyocytes (identified by α -actinin or Nkx2.5) expressed Ki-67, a marker of actively cycling cells [29], indicating that these cells might have proliferative capacity (Figure 3i–n).

To further confirm the cardiomyocyte phenotype, we examined the cells generated by the growth factor-directed differentiation method using perforated-patch current-clamp techniques. Spontaneous APs were recorded from a total of 45 randomly selected cells, of

which 41 showed characteristic cardiac-type AP waveforms and parameters. Of these 41 cardiomyocytes, 39 exhibited AP properties that could be readily classified as either nodal or working chamber (atrial and ventricular) like. The classification of cell types was based on maximum rate of AP upstroke ($dV/dt_{\max}$), AP duration measured at 50 or 90% repolarization (APD_{50}/APD_{90}), AP amplitude, and mean diastolic potential, as summarized in Table 1. Cells with the working chamber AP comprised the majority (72%) of those that could be classified. Representative current-clamp traces obtained from cells exhibiting each of these AP subtypes are shown in Figure 4. From these results, we concluded that, despite the comparative electrophysiological immaturity of the cardiomyocytes, hESCs had the ability to generate distinct nodal/pacemaker and working chamber (atrial and ventricular) type cardiomyocytes via the growth factor-directed differentiation method. This electrophysiological diversity was similar to that previously reported for hESC-derived cardiomyocytes generated using embryoid bodies or co-culture methods [4,8,30].

hESC-derived cardiomyocytes can be efficiently cryopreserved

An important question relates to whether hESC-derived cardiomyocytes generated through the growth factor-directed differentiation could be cryopreserved, which will facilitate applications of the cells for cell therapy or drug discovery. To address this, we first evaluated if viable cardiomyocytes could be recovered after cryo-preservation, thawing and replating. Multiple cell preparations containing 25–59% cardiomyocytes were cryopreserved and then thawed. Cell viability and the percentages of cardiac cells (assessed by flow cytometric analysis of cTnI-, α -actinin- or Nkx2.5-positive cells before and after thawing) were found to be similar across the preparations, as shown in Figure 5a. To examine if the cryopreservation process could be scaled up, a cell preparation containing 59% Nkx2.5-positive cells with 86% viable (ethidium monoazide negative) cells was frozen at larger scales (4×10^7 cells in 1.5 ml/vial or 8×10^7 cells in 1.5 ml/vial, compared with 1×10^7 cells in 0.25 ml/vial at smaller scales). When triplicate vials were thawed for each condition, the percentages of cardiac cells and cell viability in all conditions were comparable (Figure 5B). Cell recovery rates obtained in all conditions were also similar, reaching an average of 70–77% (Figure 5B).

To evaluate if cardiac cell purity affects cell recovery postcryopreservation, we compared results of more than 30 vials of cells containing 21–84% cardiomyocytes. We found that cell recovery ranged from 55% to more than 90% with an average of 83%. To examine the correlation between the purity and cell recovery, we examined cell recovery in three groups of cell preparations containing low (purity of $23.6 \pm 2.3\%$, $n = 9$), medium (purity of $49.0 \pm 3.7\%$, $n = 14$) and high (purity of $76.4 \pm 5.7\%$, $n = 13$) purity of cardiomyocytes. No significant difference in cell recovery was observed among these three groups ($p > 0.05$), indicating that cardiomyocytes had a similar rate of cell recovery compared with noncardiac cells in these cell preparations.

To further determine if cryopreserved cells can survive *in vitro*, we replated the thawed cells onto Matrigel-coated plates. The majority of the thawed cells attached and recovered their contractility 1–2 days post-thawing. When these cells were analyzed using perforated-patch current-clamp recording performed by Peng and colleagues, they showed the expected cardiac electrophysiological phenotype and predicted AP responses to all of the evaluated pharmacological modulators [31].

To examine whether the cryopreserved cells survived *in vivo*, we compared cell survival after transplantation of freshly isolated cells with cryopreserved cells using an acute myocardial ischemia–reperfusion athymic rat animal model [10]. Both freshly isolated and cryopreserved cells were prepared for transplantation in a pro-survival cocktail in order to enhance graft survival, as reported previously [10]. Each animal received a total of 1.0×10^7

cells distributed in the infarct area and the zones that bordered the infarct. The hearts ($n = 6$ for each group) were processed for histological analysis 1 week after cell transplantation. Examination of heart tissue sections stained with an antibody against human nuclear antigen (HNA) revealed that multiple islands of human cells formed within the infarcted areas and border zones in all hearts examined. The overall graft sizes in the hearts transplanted with cryopreserved cells were comparable to those graft sizes formed after transplantation of freshly isolated cells, as shown in representative images of HNA labeling (grafts from freshly isolated cells in Figure 6a & C and cryopreserved cells in Figure 6B & D). To identify human cardiomyocytes surviving in the graft, sections were labeled with antibodies against cardiomyocyte markers β -myosin heavy chain (β -MHC) or Nkx2.5 in combination with the antibody against HNA. In both animals that received a transplantation of fresh (Figure 6a, C & e) or cryopreserved cardiomyocytes (Figure 6B, D & F), the majority of the HNA-positive cells expressed β -MHC or Nkx2.5, confirming the cardiac phenotype. In addition, we found that approximately 15% of β -MHC-positive cells in the grafts were immunohistochemically labeled with the Ki-67 antibody, suggesting these cardiomyocytes were at the active phases of the cell cycle (data not shown). Therefore, the survival of cryopreserved cardiomyocytes in injured myocardial tissue was comparable to that of freshly prepared cardiomyocytes.

To evaluate the long-term survival of the cryo-preserved cardiomyocytes *in vivo*, we prepared cryopreserved cells ($1.3\text{--}1.4 \times 10^7$ cells/animal) in a pro-survival cocktail [10] and injected them into the infarct area and the myocardial zones that bordered the infarct in the same animal model as described above. Histological examination of the heart tissue ($n = 7$) was performed 4 weeks after the transplantation. As identified by the antibody against HNA, the cells formed grafts in all hearts examined (Figure 7). Similar to the 1-week study, these grafts were observed not only in the border zones, but also in the central infarcted areas (Figure 7a). The majority of the human cells expressed cardiomyocyte markers such as β -MHC (Figure 7a & B), Nkx2.5 (Figure 7C) and cTnI (Figure 7D). Under higher magnifications, sarcomeric striations were clearly detected in the graft (Figure 6e & F), in contrast to the observations made on 1-week-old grafts (data not shown). The grafts contained few Ki-67-positive cells, but no signs of teratoma formation were detected in any animals. This observation is consistent with the *in vitro* characterization of cell preparations by flow cytometric analysis, which showed that the majority of the noncardiac cells expressed pan-cytokeratin and that bright staining of OCT3/4, an undifferentiated hESC marker, was undetectable (data not shown).

Taken together, these data indicated that hESC-derived cardiomyocytes generated using the growth factor-directed differentiation method can be effectively cryopreserved and that transplantation of cryopreserved hESC-derived cardiomyocytes generated through large-scale culture formats formed viable grafts in injured rat hearts.

Discussion

The above experiments serve as a proof-of-concept showing that our differentiation and cryo-preservation techniques support a robust process for the production of cardiomyocytes on a scale suitable for drug development and cellular therapy. This process does not require feeder cells or conditioned medium for the maintenance of undifferentiated hESCs, and cardiomyocyte differentiation is achieved by treatment of adherent cultures with only two growth factors, activin A and BMP-4, in a defined serum-free medium. The differentiated cells express appropriate markers and display electrophysiological phenotypes expected for cardiomyocytes. Furthermore, we show, to our knowledge, for the first time, efficient cryopreservation of hESC-derived cardiomyocytes. The cryopreserved cardiomyocytes can be recovered and survive both *in vitro* and *in vivo* and have the ability to form viable grafts

in injured rat hearts similarly to that of freshly isolated cells. The cryopreserved cells are capable of surviving even in the central regions of the infarct region for as long as 4 weeks after the transplantation. Together, this differentiation and cryopreservation technology will offer significant advantages to provide reliable materials for preclinical studies to examine if the cells improve cardiac functions and if the cells are safe post-transplant. This technology will also facilitate clinical application since it not only allows sufficient time for product qualification, but also facilitates optimal timing for transplantation to when the patient is ready to receive the cells. The high-quality cells from our method will help ensure consistency and accuracy of drug testing and convenience of the availability of premanufactured cells.

In addition to regenerative medicine and drug discovery applications, efficient cardiomyocyte differentiation from hESCs should also provide a useful tool for developmental biology studies. The stem cell field has made rapid progress on controlling hESC differentiation into cardiomyocytes in recent years. Our results, together with findings from other groups, show that cardiomyocyte differentiation from hESCs can be accomplished or enhanced using specific culture conditions that involve growth factors or small molecules (for examples see [16,19,32–34]). Since different factors and/or combinations of techniques are used in these methods, it is possible that cardiogenesis can be achieved through intervention at multiple pathways or multiple steps in a common pathway. Similar to findings by other groups, our differentiation process mimics the cardiac development *in vivo* as shown by a transient upregulation of mesoderm marker, T, right after the activin A treatment (data not shown), expression of the precardiac mesoderm marker post-BMP-4 treatment and generation of cardiomyocytes at later times. The differentiation methods we have described here, and those reported by other groups, should provide a valuable model system for further investigation of the detailed mechanisms of action by which growth factors or small molecules act in promoting cardiogenesis *in vitro*.

The recent discovery of human induced pluripotent stem cells (iPSCs) [35,36] raises the possibility of using iPSCs as a cell source for regenerative medicine, drug discovery or disease modeling [37]. We found that IMR90 clone-1 human iPSCs [36] exhibited an unambiguous cardiac phenotype upon induction using our growth factor-directed differentiation protocol, as shown by contraction of the cells (see Supplementary video), expression of the cardiomyocyte-specific marker, cardiac troponin T, and their cardiac-type APs (Supplementary Figure 3). These results are consistent with data previously reported by Yamanaka's group [35]. These experiments confirm expectations that a technology established with hESCs is also likely to be applicable to iPSCs.

Conclusion

We have demonstrated highly specific and scalable cardiomyocyte differentiation of hESCs maintained in serum-free medium, have shown that the resulting differentiated cells possess the characteristics of human cardiomyocytes and have demonstrated successful engraftment of the cryopreserved cardiomyocytes after transplantation. Further modification of the process, such as the use of completely xeno-free reagents, will be optimal for clinical applications. In the near term, controlled expansion of undifferentiated hESCs and high-frequency cardiomyocyte differentiation will provide valuable tools to further explore applications of hESC-derived cardiomyocytes in regenerative medicine and drug discovery, as well as to investigate the molecular mechanisms that control cardiomyocyte differentiation from hESCs. The technology described here will allow cardiomyocyte production sufficient to support long-term graft survival studies in which observation of functional coupling between recipient and donor cells and improvement of ventricular functions can be made. In addition, large-scale production and cryopreservation of the

cardiomyocytes will allow preclinical safety studies of sufficient size to examine biodistribution of the cells after administration and monitor any potential adverse effects such as arrhythmogenicity or teratoma formation. The study we presented here has focused on developing processes for efficient generation and cryopreservation of cardiomyocytes from hESCs, which constitute a critical first step for future applications of hESCs in the treatment of cardiac disease or drug discovery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank K Spink for critical review of this manuscript. The authors would also like to thank JA Thomson (University of Wisconsin-Madison, WI, USA) for kindly providing the IMR90 clone-1 human iPSC line to MA Laflamme.

Bibliography

1. Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. *Science* 1998;282:1145–1147. [PubMed: 9804556]
2. Reubinoff BE, Pera MF, Fong CY, et al. Embryonic stem cell lines from human blastocysts: somatic differentiation *in vitro*. *Nat Biotechnol* 2000;18(4):399–404. [PubMed: 10748519]
3. Xue T, Cho HC, Akar FG, et al. Functional integration of electrically active cardiac derivatives from genetically engineered human embryonic stem cells with quiescent recipient ventricular cardiomyocytes: insights into the development of cell-based pacemakers. *Circulation* 2005;111(1):11–20. [PubMed: 15611367]
4. He JQ, Ma Y, Lee Y, et al. Human embryonic stem cells develop into multiple types of cardiac myocytes: action potential characterization. *Circ Res* 2003;93(1):32–39. [PubMed: 12791707]
5. Reppel M, Boettinger C, Hescheler J. β -adrenergic and muscarinic modulation of human embryonic stem cell-derived cardiomyocytes. *Cell Physiol Biochem* 2004;14(4–6):187–196. [PubMed: 15319521]
6. Kehat I, Kenyagin-Karsenti D, Snir M, et al. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *J Clin Invest* 2001;108(3):407–414. [PubMed: 11489934]
7. Xu C, Police S, Rao N, et al. Characterization and enrichment of cardiomyocytes derived from human embryonic stem cells. *Circ Res* 2002;91(6):501–508. [PubMed: 12242268]
8. Mummery C, Wardvan Oostwaard D, Doevendans P, et al. Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells. *Circulation* 2003;107(21):2733–2740. [PubMed: 12742992]
9. Laflamme MA, Gold J, Xu C, et al. Formation of human myocardium in the rat heart from human embryonic stem cells. *Am J Pathol* 2005;167(3):663–671. [PubMed: 16127147]
10. Laflamme MA, Chen KY, Naumova AV, et al. Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. *Nat Biotechnol* 2007;25(9):1015–1024. [PubMed: 17721512]
11. Kehat I, Khimovich L, Caspi O, et al. Electromechanical integration of cardiomyocytes derived from human embryonic stem cells. *Nat Biotechnol* 2004;22(10):1282–1289. [PubMed: 15448703]
12. Fermini B, Fossa AA. The impact of drug-induced QT interval prolongation on drug discovery and development. *Nat Rev Drug Discov* 2003;2(6):439–447. [PubMed: 12776219]
13. Meyer T, Sartipy P, Blind F, et al. New cell models and assays in cardiac safety profiling. *Expert Opin Drug Metab Toxicol* 2007;3(4):507–517. [PubMed: 17696802]
14. BurrIDGE PW, Anderson D, Priddle H, et al. Improved human embryonic stem cell embryoid body homogeneity and cardiomyocyte differentiation from a novel V-96 plate aggregation system highlights interline variability. *Stem Cells* 2007;25(4):929–938. [PubMed: 17185609]

15. Yao S, Chen S, Clark J, et al. Long-term self-renewal and directed differentiation of human embryonic stem cells in chemically defined conditions. *Proc Natl Acad Sci USA* 2006;103(18):6907–6912. [PubMed: 16632596]
16. Yang L, Soonpaa MH, Adler ED, et al. Human cardiovascular progenitor cells develop from a KDR⁺ embryonic-stem-cell-derived population. *Nature* 2008;453(7194):524–528. [PubMed: 18432194]
17. Tomescot A, Leschik J, Bellamy V, et al. Differentiation *in vivo* of cardiac committed human embryonic stem cells in postmyocardial infarcted rats. *Stem Cells* 2007;25(9):2200–2205. [PubMed: 17540853]
18. Leschik J, Stefanovic S, Brinon B, et al. Cardiac commitment of primate embryonic stem cells. *Nat Protoc* 2008;3(9):1381–1387. [PubMed: 18772864]
19. Graichen R, Xu X, Braam SR, et al. Enhanced cardiomyogenesis of human embryonic stem cells by a small molecular inhibitor of p38 MAPK. *Differentiation* 2008;76(4):357–370. [PubMed: 18021257]
20. Lin G, Xu RH. Progresses and challenges in optimization of human pluripotent stem cell culture. *Curr Stem Cell Res Ther* 2010;5(3):207–214. [PubMed: 20214560]
21. Xu C, Inokuma MS, Denham J, et al. Feeder-free growth of undifferentiated human embryonic stem cells. *Nat Biotechnol* 2001;19:971–974. [PubMed: 11581665]
22. Li Y, Powell S, Brunette E, et al. Expansion of human embryonic stem cells in defined serum-free medium devoid of animal-derived products. *Biotechnol Bioeng* 2005;91:688–698. [PubMed: 15971228]
23. Fan JS, Palade P. Perforated patch recording with β -escin. *Pflugers Arch* 1998;436(6):1021–1023. [PubMed: 9799421]
24. Fu LY, Wang F, Chen XS, et al. Perforated patch recording of L-type calcium current with β -escin in guinea pig ventricular myocytes. *Acta Pharmacol Sin* 2003;24(11):1094–1098. [PubMed: 14627491]
25. Drouin E, Charpentier F, Gauthier C, et al. Electrophysiologic characteristics of cells spanning the left ventricular wall of human heart: evidence for presence of M cells. *J Am Coll Cardiol* 1995;26(1):185–192. [PubMed: 7797750]
26. Zhu WZ, Xie Y, Moyes KW, et al. Neuregulin/ErbB signaling regulates cardiac subtype specification in differentiating human embryonic stem cells. *Circ Res* 2010;107(6):776–786. [PubMed: 20671236]
27. Volk T, Geiger B. A 135-kd membrane protein of intercellular adherens junctions. *Embo J* 1984;3(10):2249–2260. [PubMed: 6437808]
28. Manjunath CK, Goings GE, Page E. Human cardiac gap junctions: isolation, ultrastructure, and protein composition. *J Mol Cell Cardiol* 1987;19(2):131–134. [PubMed: 3573042]
29. Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. *J Cell Physiol* 2000;182(3):311–322. [PubMed: 10653597]
30. Moore JC, Fu J, Chan YC, et al. Distinct cardiogenic preferences of two human embryonic stem cell (hESC) lines are imprinted in their proteomes in the pluripotent state. *Biochem Biophys Res Commun* 2008;372(4):553–558. [PubMed: 18503758]
31. Peng S, Lacerda AE, Kirsch GE, et al. The action potential and comparative pharmacology of stem cell-derived human cardiomyocytes. *J Pharmacol Toxicol Methods* 2010;61(3):277–286. [PubMed: 20153443]
32. Yao S, Chen S, Clark J, et al. Long-term self-renewal and directed differentiation of human embryonic stem cells in chemically defined conditions. *Proc Natl Acad Sci USA* 2006;103(18):6907–6912. [PubMed: 16632596]
33. Xu XQ, Graichen R, Soo SY, et al. Chemically defined medium supporting cardiomyocyte differentiation of human embryonic stem cells. *Differentiation* 2008;76(9):958–970. [PubMed: 18557764]
34. Tran TH, Wang X, Browne C, et al. Wnt3a-induced mesoderm formation and cardiomyogenesis in human embryonic stem cells. *Stem Cells* 2009;27(8):1869–1878. [PubMed: 19544447]
35. Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007;131(5):861–872. [PubMed: 18035408]

36. Yu J, Vodyanik MA, Smuga-Otto K, et al. Induced pluripotent stem cell lines derived from human somatic cells. *Science* 2007;318(5858):1917–1920. [PubMed: 18029452]
37. Moretti A, Bellin M, Welling A, et al. Patient-specific induced pluripotent stem-cell models for long-QT syndrome. *N Engl J Med* 2010;363(15):1397–1409. [PubMed: 20660394]

Figure 1. Cardiomyocyte differentiation induced by growth factor treatment in a serum-free medium

(A) Undifferentiated hESCs maintained in either MEF-CM or serum-free medium/GFs (stage 1) were treated sequentially with activin A (stage 2A) and BMP-4 (stage 2B) in RPMI/B27, followed by removal of growth factors and maintenance in RPMI/B27 (stage 3). Cells were analyzed for cardiomyocyte differentiation by ICC or flow cytometric analysis after stage 3. **(B)** Timing of cardiomyocyte generation. Confluent undifferentiated cells were induced to differentiate using the sequential treatment with activin A and BMP-4 method (A100/B10) or maintained in RPMI/B27 medium without growth factors (control); cultures were fixed at days 6, 7, 8 or 23 and analyzed for the presence of Nkx2.5-positive cells and for cells that were positive for both Nkx2.5 and α -actinin. Data are represented as mean $\pm$ standard deviation of three independent wells. **(C)** An example of differentiation of hESCs maintained in serum-free medium/GFs in small culture formats. Undifferentiated cells were seeded into six-well plates at 1×10^5 cells/cm² and induced to differentiate. Numerous beating cells were observed in these cultures at stage 3. Cells were harvested and analyzed for the percentage of cells expressing cTnI. The cell viability, determined by ethidium monoazide staining, and cell yields are presented as the mean $\pm$ standard deviation from triplicate wells.

BMP: Bone morphogenetic protein; cTnI: Cardiac troponin I; D: Day; GF: Growth factor; hESC: Human embryonic stem cell; ICC: Immunocytochemical analysis; MEF-CM: Conditioned medium from mouse embryonic fibroblasts; Non-CM/GFs: Serum-free medium supplemented with growth factors.

Figure 2. Differentiation of human embryonic stem cells maintained in serum-free medium/GFs in large culture formats

Undifferentiated cells from independent cultures maintained in serum-free medium/GFs were seeded into T225 flasks at 1×10^5 cells/cm²; differentiation was induced with the three stage growth factor-directed differentiation method. Similar to cultures in small formats, numerous beating cells were observed at stage three. Cells were harvested and analyzed for expression of cardiomyocyte-associated proteins, such as Nkx2.5 or α -actinin (indicated by the scatter plots). Cell viability was determined by ethidium monoazide staining. Yields of total cells and viable cardiac cells per flask as well as input undifferentiated hESC:viable cardiac cell ratio in each experiment are presented.

hESC: Human embryonic stem cell.

Figure 3. Immunocytochemical analysis of cardiomyocytes derived from human embryonic stem cells using the growth factor differentiation method

At the end of differentiation stage 3, cells were dissociated, replated and cultured for an additional 6 days before immunocytochemical analysis. Nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI; blue). (A) Nkx2.5 (red), tropomyosin (green); (B) Nkx2.5 (red), cardiac troponin I (green); (C) Nkx2.5 (red), cardiac troponin T (green); (D) N-cadherin (red), sarcomeric myosin heavy chain (green); (E) Nkx2.5 (red), α -actinin (green); (F) cardiac troponin I (red), connexin 43 (green); (G) Enlarged image of (E) shows formation of striations in these cells. (H) Enlarged image of (F); (I) α -actinin (green); (J) Ki-67 (red); (K) merge of images (I & J) and DAPI; (L) Nkx2.5 (red); (M) Ki-67 (green); and (N) merge of images (L & M) and DAPI. Scale bar = 50 μ m.

Figure 4. Electrophysiological analysis of cardiomyocytes derived from human embryonic stem cells using the growth factor differentiation method

Perforated-patch current-clamp recordings from two representative cardiomyocytes show spontaneous action potentials with distinct nodal-type (upper) and working chamber-type (lower) waveforms and characteristics.

Figure 5. Cryopreservation of cardiomyocytes derived from human embryonic stem cells

Cultures containing contracting cardiomyocytes were dissociated and cryopreserved using a controlled rate freezer. Cells before and after thawing were analyzed for cell viability, percentages of cardiac cells and cell recovery rates. **(A)** An example of flow cytometry analysis of samples before and after thawing. **(B)** Large-scale cryopreservation of human embryonic stem cell-derived cardiomyocytes. Differentiated cultures (containing 59% Nkx2.5⁺ cells; 86% viability) derived from human embryonic stem cells were cryopreserved at 1×10^7 cells in 0.25 ml/vial, 4×10^7 cells in 1.5 ml/vial and 8×10^7 cells in 1.5 ml/vial. Triplicate vials were thawed and analyzed for percentages of cardiac cells, viability and recovery. Data are presented as mean $\pm$ standard deviation from triplicate vials. EMA: Ethidium monoazide.

Figure 6. Immunohistochemical analysis of human cell grafts in the myocardium of infarcted rat hearts in the 1-week study

Freshly isolated cardiomyocytes or cryopreserved cardiomyocytes were resuspended in medium containing a prosurvival cocktail [10] and injected into the myocardium of rats that had received an ischemia–reperfusion injury. Hearts were harvested and processed for histological and immunohistochemical analysis 1 week after transplantation. As indicated by positive labeling for human nuclear antigen (HNA; brown diaminobenzidine reaction product in (A–D) and red immunofluorescence in (E & F)), human cell grafts were identified in both the group of animals that received freshly isolated cells (A,C & E) and in the group that received cryopreserved cells (B,D & F). Lower magnification images illustrate that graft sizes in the group that received freshly isolated cells (A) were comparable to those in the group that received cryopreserved cells (B). Double labeling of HNA with β -myosin heavy chain (red chromogen in (A–D)) indicated that the majority of the HNA-positive cells were cardiomyocytes, which was also confirmed by HNA/Nkx2.5 double labeling in (E & F) (red for HNA and green for Nkx2.5; cells labeled by both antibodies appear yellow). Scale bar = 500 μ m for images in (A & B); scale bar = 50 μ m for images in (C–F).

Figure 7. Immunohistochemical analysis of human cell grafts in the myocardium of infarcted rat hearts in the 4-week study

Cryopreserved cardiomyocytes were resuspended in medium containing a prosurvival cocktail [10] and injected into the myocardium of rats that had received an ischemia–reperfusion injury. Hearts were harvested and processed for histological and immunohistochemical analysis 4 weeks after transplantation. As indicated by positive labeling for human nuclear antigen (HNA; brown diaminobenzidine reaction product in **(A & B)** and red immunofluorescence in **(C–F)**), human cell grafts were identified in all animals as shown in a representative lower magnification image **(A)**. Double labeling of HNA with β -myosin heavy chain (red chromogen in **(A & B)**) indicated that the majority of the HNA-positive cells were cardiomyocytes, which was also confirmed by HNA/Nkx2.5 double labeling in **(C)** (red for HNA and green for Nkx2.5; cells labeled by both antibodies appear yellow) and HNA/cardiac troponin I double labeling in **(D–F)** (red for HNA and green for cardiac troponin I). Higher magnifications of the image in **(D)** revealed the formation of muscle striations in **(E & F)**. Scale bar = 500 μ m for images in **(A)**; scale bar = 50 μ m for images in **(B–F)**.

Table 1

Electrophysiological analysis of cardiomyocytes derived from human embryonic stem cells using the growth factor differentiation method.

	Nodal type	Working type
N	11	28
Rate (bpm)	110.6 ± 9.2 [*]	86.9 ± 6.9
dV/dt _{max} (V/s)	6.5 ± 0.9 ^{**}	48.0 ± 6.7
APD ₅₀ (ms)	115.7 ± 8.7 [*]	160.3 ± 15.7
APD ₉₀ (ms)	170.0 ± 11.6 [*]	212.9 ± 17.2
APA (mV)	72.8 ± 3.2 ^{**}	99.6 ± 2.7
MDP (mV)	-45.6 ± 1.2 ^{**}	-58.7 ± 1.6

Data are mean ± standard error.

^{*}
p < 0.05 and

^{**}
p < 0.01 versus working type. Statistical significance was evaluated by student's unpaired t-test assuming unequal variances.

APA: Action potential amplitude; APD₅₀/APD₉₀: Action potential duration measured at 50 or 90% repolarization;

dV/dt_{max}: Maximum rate of action potential upstroke; MDP: Mean diastolic potential; N: Cell number.