Modification of a Purification and Expansion Method for Human Embryonic Stem Cell-Derived Cardiomyocytes

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Key Words

Cardiomyocytes · Expansion · Human embryonic stem cells · Purification

Abstract

Objective: This study aimed to develop a simple and efficient purification method for human embryonic stem cell (hESC)-derived cardiomyocytes (CMs) using a low-glucose culture system. In addition, we investigated whether intercellular adhesion between single hESC-CMs plays a critical role in enhancing proliferation of purified hESC-CMs. **Method:** hESCs were cultured in suspension to form human embryoid bodies (hEBs) from which ~15% contracting clusters were derived after 15–20 days in culture. To purify CMs from contracting hEBs, we first manually isolated contracting clumps that were re-cultured on gelatin-coated plates with media containing a low concentration of glucose. The purified hESC-CMs were cultured at different densities to examine whether cell-cell contact enhances proliferation of hESC-CMs. **Results:** Purified CMs demonstrated spontaneous contraction and strongly expressed the CM-specific markers cardiac troponin T and slow myosin heavy chain. We investigated the purification efficiency by examining the expression levels of cardiac-related genes and the expression of MitoTracker Red

dye. In addition, purified hESC-CMs in low-glucose culture demonstrated a 1.5-fold increase in their proliferative capacity compared to those cultured as single hESC-CMs. **Conclusion:** A low level of glucose is efficient in purifying hESC-CMs and intercellular adhesion between individual hESC-CMs plays a critical role in enhancing hESC-CM proliferation.

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Introduction

Pluripotent human embryonic stem cells (hESC) are capable of differentiating into almost all cell types in the body [1]. For this reason, cellular therapy using hESC derivatives is a potential intervention for treating various types of degenerative conditions [2]. In particular, a growing number of studies has indicated that ischemic heart disease can benefit from hESC treatment. These studies have focused on the generation of functional cardiomyocytes (CMs) capable of inducing functional re-

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covery in animal models of myocardial infarction [3–5]. As the human heart lacks regenerative capacity, CMs derived from hESC (hESC-CMs) are an attractive therapeutic approach for myocardial repair [6].

In order to develop successful hESC-based cell therapies, a number of key criteria has to be fulfilled. To ensure the safety of such treatments, it is an absolute necessity that the final hESC-derived products have to be purified from the residual undifferentiated hESC and non-target cells. Transplantation of unpurified cell populations cannot only lead to tumor development by residual undifferentiated hESC [7, 8], non-target cells can also induce adverse effects such as arrhythmia in the case of myocardial transplantation [9]. In addition, it is widely recognized that transplantation of a mixed cell population reduces overall therapeutic efficacy. Efforts have been made to remove unwanted byproducts via the use of flow cytometry and genetic modification, but it remains unclear whether such purification strategies are suitable for clinical use [10–12]. Another important criterion for developing hESC-based therapy is the ability to generate a sufficient number of target cells. It has been estimated that $\sim 10^9$ cells are required to treat myocardial infarction in humans, thus strategies to improve differentiation and expansion of hESC-CMs remains a major challenge in the clinical application of hESC-CMs in humans [13].

In this study, we have attempted to address challenges associated with purification and expansion of functional hESC-CMs. Cardiac differentiation of hESC yielded a heterogeneous population of cells from which functional CMs were purified using a medium containing a low level of glucose. Purified hESC-CMs demonstrated enhanced proliferative capacity when they were in contact with neighboring cells, indicating that intercellular adhesion promotes proliferation of contracting hESC-CMs. Our data demonstrated that purification and expansion of hESC-CMs could be achieved without the use of complicated devices or labor-intensive steps; thus, clinical application is more suitable.

Materials and Methods

hESC Culture and Differentiation into Contracting Embryoid Bodies

Undifferentiated hESCs (H9-hESC lines, Wicell, Madison, Wisc., USA) were grown on mitomycin C (Sigma-Aldrich, St. Louis, Mo., USA)-inactivated mouse embryonic fibroblast cells in DMEM/F12 (50:50%; Gibco BRL, Gaithersburg, Md., USA) supplemented with 20% (v/v) serum replacement (Gibco) and components including 1 mM L-glutamine (Gibco), 1% nonessential amino acids (Gibco), 100 mM β -mercaptoethanol (Gibco) and

4 ng/ml basic fibroblast growth factor (Invitrogen, Grand Island, N.Y., USA). The medium was changed daily, and hESCs were transferred to new feeder cells every 5–7 days with dissecting pipettes [1]. For initial induction of hESC-CMs, hESCs were detached from the feeder cells with dispase (Gibco), transferred to ultralow attachment plates [14, 15] and suspended in basic fibroblast growth factor-free hESC culture medium to form embryoid bodies (EBs) for 2 days (fig. 1a) [16, 17]. To initiate CM differentiation, EBs were cultured in high-glucose DMEM supplemented with 20% FBS for further 13 days [3]. The contracting EBs began to appear around 15–17 days after EB formation (fig. 1b).

Isolation and Purification of CMs from Contracting EBs

To separate the contracting region within contracting EBs in suspension culture condition, we first attached the contracting EBs onto 0.1% gelatin-coated plates and cultured them in DMEM supplemented with 10% FBS (Gibco). After 7 days, the attached contracting EBs were separated between the contracting region with cluster formation and the non-contracting region (fig. 1c, black arrows). Next, to obtain contracting clusters from the mixed cells of the non-contracting region, we isolated contracting clusters by the microdissection method and dissociation by a subsequent treatment with 0.25% trypsin-EDTA (Gibco) for 5 min. Following replating on 0.1% gelatin-coated plates, the digested single cells from isolated contracting clusters were cultured in DMEM containing 2% FBS (EB2 medium) for 7 days [18] (fig. 1d). To purify hESC-CMs under low-glucose conditions, isolated single cells were cultured in low-glucose DMEM (Gibco, 1 g/l) containing 2% FBS without any growth factors for 2 weeks (fig. 1e).

Quantitative RT-PCR

Total RNA was isolated using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Two micrograms of RNA were used for cDNA synthesis using the SuperScript II reverse transcriptase kit according to the manufacturer's protocol (Invitrogen). Quantitative RT-PCR was performed with the SYBR Green PCR master mix using the ABI 7300 qRT-PCR system (Applied Biosystems, Carlsbad, Calif., USA). *GAPDH* was used as an internal PCR control. The primer sequences are listed in table 1. The level of gene expression was calculated using the comparative $\Delta\Delta C_t$ method.

Confirmation of Low-Glucose Medium Purification by a Mitochondrial-Based Method

To confirm the purification of contracting hESC-CMs from non-target cells, the low-glucose medium method was evaluated using a mitochondrial-based method. The cells were labeled with MitoTracker Red dye (Invitrogen) [19, 20] and then cultured in medium with low- and high-glucose DMEM (Gibco) for 2 weeks. Cultured cells were stained with anti-cardiac troponin T (cTnT) antibody (Abcam, Cambridge, UK), a CM-specific marker. Stained cells were then analyzed with a confocal microscopic imaging system (Nikon, Chiyoda-ku, Japan) and by fluorescence-activated cell sorting (FACS; BD Biosciences, San Jose, Calif., USA).

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde for 20 min and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS; Sigma Aldrich Inc.) for 5 min. After treatment with 5% normal goat serum for 30 min, the cells were incubated with primary

Fig. 1. Differentiation, isolation and purification of hESC-CMs. **a** Schematic representation of the experimental procedure. **b** hEBs containing beating clusters. **c** The contracting clusters after attachment on gelatin-coated plates (black arrows). **d** The beating clusters were isolated and dissociated before replating. **e** Purification of hESC-CMs using low-glucose culture condition. **f** Measurement of beating rate of hESC-CMs in the low- and high-glucose culture condition.

Table 1. List of primer sequences used

Gene	Accession No.	Forward primer	Reverse primer
<i>GAPDH</i>	NM002046.3	5'GAGTCAACGGATTGTCGT	5'TTGATTTTGGAGGGATCTCG
<i>NKX2.5</i>	NM001166175.1	5'GGAATTGAATGCGGTTTCAGA	5'CGTGCGCAAGAACAACG
<i>MEF2c</i>	NM001131005.2	5'CCTGCAAATATGGCCCTAGAA	5'CGGGATTGTTCAACAGTCCTA
<i>Myh6</i>	NM002471.3	5'CGCATGAAGAAGAACATGGA	5'CGCAGCAGGTTCTTTTGTCT
<i>Myh7</i>	NM000257.2	5'CAGTGCAGGAGTGCAGGAA	5'TCCAGCTCATTCTCCAGCTC
<i>MLC2v</i>	NM182493.2	5'TGTTTGACCCTGAGGCAA	5'CCCCCTCCTAGTCCTTCTCTT
<i>cTnT</i>	NM001001420.1	5'CCATTGACCACCTGAATGAA	5'GCCCCGTGACTTTAGCCTT

antibodies that recognize lineage-specific markers for 12 h at 4°C, such as the cardiac-lineage markers cTnT (Abcam) and slow myosin heavy chain (sMHC; Millipore, Billerica, Mass., USA). The cells were washed three times with PBS and then incubated with rhodamine- or FITC-conjugated secondary antibodies (Molecular Probes Inc., Eugene, Oreg., USA) for 1 h. After three washes with PBS, the stained slides were mounted with a glycerol-based mounting solution containing 2.5% polyvinyl alcohol and 1,4-diazabicyclo(2.2.2)octane and DAPI (4,6-diamidino-2-phenylindole; Sigma). All images were analyzed with an LSM 510 META confocal microscope (Carl Zeiss Inc., Oberkochen, Germany).

Statistical Analysis

Quantitative data are expressed as means $\pm$ SD. Statistical analyses were performed with Student's paired t test or one-way analysis of variance using SPSS software (SPSS Inc., Chicago, Ill., USA). A value of $p < 0.05$ was considered statistically significant.

Results

Stepwise Purification of hESC-CMs Using a Low-Glucose Culture Medium

First, to generate CMs, undifferentiated hESC colonies were cultured in suspension in DMEM supplemented with 20% FBS in an ultralow attachment culture dish for 15 days. Ten to 15% of EBs were observed to contain spontaneously contracting areas (fig. 1b; online suppl. live image 1; for all online suppl. material, see www.karger.com/doi/10.1159/000346390). Because the contracting EBs were composed of CMs as well as other cell lineages [21], they were plated onto gelatin-coated plates to separate the contracting areas from the contracting EBs. Seven days after plating the contracting EBs, non-contracting cells migrated from the attached contracting EBs, but the contracting areas formed clusters in the center while continuously contracting (fig. 1c, black arrows; online suppl. live image 2). The contracting clusters were then isolated from the surrounding non contracting cells by microdissection and treated with 0.25% trypsin-EDTA to produce single cells. When the isolated single cells were cultured on gelatin-coated plates, approximately half of the cells exhibited spontaneous contraction, indicating that the beating clusters also had contained cells of other lineages (fig. 1d; online suppl. live image 3). To characterize the heterogeneous cell populations after isolation, we performed immunocytochemistry with CM-specific markers. Expression of the CM-specific markers cTnT and sMHC was observed in the contracting cells (data not shown). To further purify the hESC-CMs from the heterogeneous populations, we altered the culture medium composition by reducing the concentrations of FBS (from

20 to 2%) and glucose (from 4.5 to 1 g/l). When the culture was maintained in the new medium for 2 weeks, the non-CMs were eliminated while the CMs remained viable (fig. 1e; online suppl. live image 4). Interestingly, the reduction in glucose concentration was indifferent to the contractile activity as the beating rate in the low-glucose culture condition (76.6 ± 4.6 beats per minute [b.p.m.]) was comparable to that observed in the high-glucose culture condition (78.1 ± 5.3 b.p.m.; fig. 1f). These beating rates correlate well with the normal resting human heart rate of 60–100 b.p.m.

Purification Using a Low-Glucose Culture Medium Highly Enriched Population of Contracting hESC-CMs

To evaluate purification efficacy of the low-glucose culture condition in enriching hESC-CMs after differentiation, we performed quantitative PCR to compare the expression levels of the genes encoding cardiac-related transcription factors [NKX2.5 (NK2 homeobox 5) and MEF2c (myocyte-specific enhancer factor 2c)] (fig. 2a) and sarcomeric-related structural proteins [TnT, Myh6 (MHC 6; α -MHC), Myh7 (MHC 7; β -MHC) and MLC2v (myosin light chain 2v)] (fig. 2b) before and after purification. Comparative analysis revealed that the expression levels of all genes significantly increased after purification, with Myh7 and MLC2v showing the most dramatic increase (fig. 2b), which indicates that the use of the low-glucose medium efficiently enriched the population of hESC-CMs compared to the non-purified culture.

Next, using a mitochondrial tracking method, we examined whether low-glucose culture conditions enriched hESC-CMs by eliminating undesirable non-target cells. MitoTracker Red specifically accumulates in both the perinuclear and the intermyofibrillar mitochondria, thus it can be used to distinguish different CM populations as they contain a high abundance of mitochondria [19, 20]. FACS analysis of cells cultured under the high-glucose culture condition revealed two main cell populations: one with high fluorescence intensity (fig. 3a, black arrow) and the other with low fluorescence intensity (fig. 3a, white arrow). In line with this observation, immunostaining revealed that high-glucose culture contained two types of cell populations in terms of cTnT and MitoTracker Red double-positive CMs and non-CMs that expressed no cTnT and stained low levels of MitoTracker Red (fig. 3c, white arrowheads). In contrast, the low-glucose culture condition contained a homogeneous population of cells (fig. 3b, black arrow) and the staining of MitoTracker Red was strongly correlated with the expression of cTnT

Fig. 2. Comparison of gene expression between isolation and purification stages using quantitative RT-PCR. Gene expression patterns of cardiac-related transcription factors (**a**) and structural genes encoding sarcomeric-related proteins (**b**). The error bars represent means $\pm$ SD of experimental values performed in triplicate.

(fig. 3d). To quantify this finding, we counted the number of cTnT-expressing cells and compared it with the total number of DAPI-stained nuclei. Under the low-glucose culture condition, over 75% of the cells expressed cTnT, as compared to less than 50% in high-glucose condition (fig. 3e), and most purified CMs remained spontaneously contracted (fig. 3f, online suppl. live image 5). Low-glu-

cose-mediated purification yielded a heterogeneous population of cells in terms contractile ability (fig. 4a; online suppl. live image 4). The contracting hESC-CMs expressed a high level of cTnT and sMHC (fig. 4b) and maintained its contraction for a prolonged period of time in culture. Surprisingly, the non-contracting and rather flattened cells also expressed the CM-specific marker

Fig. 3. Effect of low-glucose culture condition for purification of hESC-CMs. **a, b** FACS analysis of MitoTracker Red accumulation and cTnT expression. **c, d** Immunocytochemical analysis of MitoTracker Red accumulation and cTnT expression. **e** cTnT expression in high- and low-glucose culture conditions. **f** Contraction of purified hESC-CMs with low-glucose culture condition. The error bars represent means $\pm$ SD of experimental values performed in five replicates (* $p < 0.05$)

Fig. 4. Characterization of purified hESC-CMs. **a** A heterogeneous population of purified hESC-CMs. **b** Expression of the CM-specific markers cTnT and sMHC after purification with a low-glucose culture medium. **c** Both the contracting and non-contracting cells (black arrows) highly express the CM-specific marker sMHC. **d** Quantification of marker expression. The error bars represent means $\pm$ SD of experimental values performed in five replicates (* $p < 0.05$). DIC = Differential interference contrast.

sMHC (fig. 4c, black arrows), indicating that the purification strategy using the low-glucose culture condition is highly effective in enriching hESC-CMs (fig. 4d). Taken together, these results suggest that a low level of glucose favors the survival of CMs over other types of cells, thus the use of a low-glucose medium after differentiation presents a simple purification method to enrich hESC-CMs.

Proliferative Potential of Purified hESC-CMs through Cell-Cell Contacts

To assess whether purified hESC-CMs are a suitable therapeutic agent for recovering impaired contraction of damaged hearts, we first analyzed its ability to maintain contraction over a prolonged period of time. When single hESC-CMs were plated on a gelatin-coated dish, we observed that some cells had undergone colony formation with neighboring cells (fig. 5a, white arrows; online suppl. live image 6) while others had remained as single hESC-CMs (fig. 5a, black arrows; online suppl. live image 6). Interestingly, single hESC-CMs rapidly lost the ability to maintain contractile activity as only ~40% single hESC-CMs exhibited spontaneous contraction over 2 weeks in culture (fig. 5b). In contrast, colonies of hESC-CMs demonstrated a better maintenance of contractile activity as ~80% colonized hESC-CMs appeared to be spontaneously beating over the same period of time (fig. 5b). Gap junction formation between single hESC-CMs in the colonies was evident by the expression of the gap junction marker connexin 43 (fig. 5d, black arrows), a phenomenon not observed with individual single hESC-CMs (fig. 5c). This finding indicates that cell-cell contacts may play a pivotal role in prolonging contractile activity of hESC-CMs. To examine whether these contacts are also imperative for proliferation of hESC-CMs, we performed immunocytochemistry with Ki-67 antibody 2 weeks after seeding single hESC-CMs. The proliferative capacity of CMs is very limited, but it has been shown to play an important role in recovering cardiac function [22]. Ki-67 is specifically expressed in cells undergoing active phases of the cell cycle (G1, S, G2 and during mitosis) but not in resting cells (G0) [23]. We detected only 10% of the CM-specific marker sMHC expressing single hESC-CMs stained positive for Ki-67, indicating that the majority of single hESC-CMs were not actively proliferating (fig. 5e, black arrows, 5f). However, we observed that cell-cell contacts significantly increased proliferation of hESC-CMs as ~40% single hESC-CMs in contact with the neighboring cells expressed Ki-67 (fig. 5e, white arrows, 5f). To provide evidence supporting that cell-cell contacts increase hESC-CM proliferation, we seeded single hESC-CMs at a

low (1×10^4 cells/cm²) and high (5×10^4 cells/cm²) cell density to compare the expansion ratio. The number of hESC-CMs cultured at high density increased by ~75% over 6 weeks of culture, whereas the low-density culture increased by less than 15% (fig. 5g), indicating that cell-cell contacts increased hESC-CMs proliferation.

Discussion

An increasing number of translational studies have conferred clinical benefits of hESC-CMs in animal models of myocardial infarction [6, 24]. However, a vast majority of the studies transplanted a mixed cell population containing hESC-CMs, a procedure not suitable for human clinical application due to the risk of developing adverse effects by often unknown cell types [25]. The development of unwanted cell types is inevitable following a directed differentiation of hESC into certain cell types, thus rigorous yet economical and clinically scalable purification strategies are a prerequisite for hESC-based therapies [26]. Previous attempts to purify hESC-CMs have relied on genetic manipulation, Percoll density gradient and the use of flow cytometry, however these strategies were deemed either clinically unsuitable or insufficient [10–12, 27]. In this study, we report that culturing hESC-CMs under a low-glucose culture condition presents a simple and efficient purification strategy for hESC-CMs by eliminating other contaminating cell types. Furthermore, culturing of CMs at a high density could induce proliferation of hESC-CMs.

The generation of hESC-CMs is generally initiated through EB formation [21]. The differentiation efficiency is generally low as only a small proportion of the EBs contain clusters of spontaneously contracting CMs, even in the presence of mesoderm-inducing factors [4, 27]. For this reason, even after the manual isolation of the contracting hESC-CMs, other non-contracting cells are often present (fig. 1d; online suppl. live image 3). We have demonstrated that, after generating hESC-CMs, switching to a low-level glucose medium highly purified hESC-CMs by eliminating contaminating cell types (fig. 4). The purified culture expressed high levels of cardiac-related genes and was homogeneous in terms of cells with a high mitochondrial content (fig. 2, 3). The fact that the use of a low concentration of glucose was indifferent to the survival and functionality of hESC-CMs for 2 weeks indicates that the low-glucose culture condition appears to selectively induce death of the contaminating cells.

Fig. 5. **e** The expression pattern of Ki-67 in single (black arrows) and colonized hESC-CMs (white arrows). **f** Quantification of Ki-67-expressing hESC-CMs as a measure of proliferation capacity. **g** Measurement of the expansion ratio of purified hESC-CMs in the low- and high-density culture conditions. The error bars represent means $\pm$ SD of experimental values performed in triplicate (* $p < 0.05$).

Glucose is a primary source of energy for almost all types of cells, therefore its abundant supply in the culture medium is crucial to maintain the survival and functionality of cells [28]. In the presence of oxygen, aerobic cellular respiration proceeds in which glucose is used as a substrate to yield 36 adenosine triphosphate molecules through glycolysis, the Krebs' cycle and the electron transport system [29]. It has been well documented that glucose deficiency is an important environmental stress that signals the beginning of apoptosis through mechanisms involving multiple signal pathways [30, 31]. Indeed, a low level of glucose has been shown to have a detrimental effect on the maintenance of pancreatic β -cells [32]. Here, it was reported that prolonged culture in low-glucose culture medium induced c-myc and facilitated onset of apoptosis, a phenomenon also reported in independent studies using other systems [33, 34]. In previous studies, hESC-CMs mainly coexisted with endodermal

lineage cells and their pivotal roles in inducing CM maturation were discernible [35, 36]. Based on these observations, we hypothesize that the majority of contaminating cells were endodermal lineage cells and the low-glucose culture condition may have eliminated their presence through the previously reported pathway [32]. Surprisingly, we observed that the viability and functionality of hESC-CMs were unaffected under the low-glucose culture condition (fig. 3). CMs may have exhibited a higher tolerance to the low-glucose level as they are particularly sensitive to a high concentration of glucose [37]. Indeed, studies have demonstrated that hyperglycemia induces CM apoptosis, rendering patients suffering from diabetes mellitus at high risk of developing diabetic cardiomyopathy [38]. The concept of the low-glucose medium-mediated purification of hESC-CMs coincides with an already filed patent (US20090275132) [39] although the procedure described in this study substantially differs in terms

of the low-glucose DMEM product used, the timing of the use of the low-glucose medium and the technical differences in isolating contracting hESC-CMs. In other studies, a low-glucose culture condition was also employed for in vitro maintenance of cardiac cells [40, 41], supporting evidence that CMs indeed withstand the scarce presence of glucose in the culture medium.

Our findings have demonstrated that the low-glucose culture medium favors the survival of hESC-CMs over other cell types, presenting a simple and scalable method for the purification of hESC-CMs. The ease with which hESC-CMs can be purified makes the low-glucose culture condition an attractive and a cost-effective strategy to purify hESC-CMs for future application in regenerative medicine. In addition, we have provided evidence that culture at high cell density improves proliferation of purified hESC-CMs, possibly via cell-cell contacts with neighboring cells (fig. 5e, f). This observation is in agreement with cell-cell contact-induced proliferation of endothelial cells through PI3K signaling [42]. It remains unclear whether the same signaling pathway is involved in enhancing proliferation of hESC-CMs via cell-cell contact, thus further studies are required to fully elucidate possible mechanisms. Nevertheless, the fact that hESC-CMs could proliferate is a promising observation for the devel-

opment of hESC-CM-based cell therapy, as a large number of hESC-CMs is apparently required for possible treatment of myocardial infarction [13]. It is noteworthy that the proliferative capacity was more prominent with non-contracting hESC-CMs as division of contracting hESC-CMs was not noticeably increased in high-density culture condition (fig. 5g). In conclusion, we provided evidence that hESC-CMs are tolerant to the low level of glucose and with this culture condition effectively purified hESC-CMs by eliminating the contaminating cell types. In our opinion, the simple approach for purifying hESC-CMs could facilitate the development of hESC-CM-based cell therapy for treating myocardial infarction.

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Conflict of Interest

The authors declare no potential conflicts of interest.

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