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Development of a novel two-dimensional directed differentiation system for generation of cardiomyocytes from human pluripotent stem cells

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ABSTRACT

Background: Human pluripotent stem cells (hPSCs) hold great promise for treating ischemic heart disease. However, current protocols for differentiating hPSCs either result in low yields or require expensive cytokines.

Methods: Here we developed a novel two dimensional (2D) stepwise differentiation system that generates a high yield of cardiomyocytes (CMs) from hPSCs without using special cytokines. Initially, undifferentiated hPSCs were transferred onto Matrigel-coated plates without forming embryoid bodies (EBs) for a few days and were cultured in bFGF-depleted human embryonic stem cells (hESCs) medium. When linear cell aggregation appeared in the margins of the hPSC colonies, the medium was changed to DMEM supplemented with 10% fetal bovine serum (FBS). Thereafter when cell clusters became visible, the medium was changed to DMEM with 20% FBS.

Results and conclusions: At about two weeks of culture, contracting clusters began to appear and the number of contracting clusters continuously increased, reaching approximately 70% of all clusters. These clusters were dissociated by two-step enzyme treatment to monolayered CMs, of which ~90% showed CM phenotypes confirmed by an α-myosin heavy chain reporter system. Electrophysiologic studies demonstrated that the hPSC-derived CMs showed three major CM action potential types with 61 to 78% having a ventricular-CM phenotype. This differentiation system showed a clear spatiotemporal role of the surrounding endodermal cells for differentiation of mesodermal cell clusters into CMs. In conclusion, this system provides a novel platform to generate CMs from hPSCs at high yield without using cytokines and to study the development of hPSCs into CMs.

1. Introduction

Heart failure is the leading cause of death worldwide. Current therapies including surgical and pharmacological interventions are only capable of delaying the progression of this devastating disease [1]. Since human CMs have very little or no regenerative capacity, the damage to CMs resulting from various heart diseases is irreversible and frequently leads to heart failure [2]. Recent discovery of pluripotent stem cells (PSCs) such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) has allowed the use of PSCs for cell therapy [3]. hESCs proliferate unlimitedly and differentiate into cells of all three germ layers [4]. hESC-derived CMs (hESC-CMs) hold similar structural and functional properties to native CMs and showed a potential to integrate into the recipient heart when transplanted in vivo [5–8]. Thus, hESCs can be an appropriate source for cardiac cell-based therapy. However, the use of hESCs for therapy has been limited due to ethical controversy. Recent discovery of hiPSCs opened a new avenue for using similar PSCs while avoiding ethical controversy and immunological mismatches associated with hESCs. hiPSCs have been generated from various human somatic cells by introducing pluripotency factors through viral and non-viral delivery techniques, and possess almost identical cell biologic characteristics to hESCs [9–13]. Several independent groups have successfully generated CMs from human PSCs [14–19]. These differentiated CMs displayed many of the properties of primary CMs including the appearance of spontaneous contraction, expression of CM-specific genes and proteins, and electrophysiological characteristics including the expected responses to known pharmacological agents [20–22]. The most common method to induce CM differentiation is EB-mediated differentiation. EBs are round multi-cellular three dimensional aggregates which are formed via suspension culture

Abbreviations: hiPSCs, human induced pluripotent stem cells; CMs, cardiomyocytes; hESCs, human embryonic stem cells; 2D, two dimensional; hPSCs, human pluripotent stem cells; EB, embryoid body; AP, action potential; APD 90, AP duration from the peak to 90% repolarization.

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and consist of cells representing all three germ layers [23,24]. Typically, a part of the aggregated EBs mature into spontaneously contracting CM-like cells during culture [16,18]. While the results of EB-mediated CM differentiation have been fairly successful, the method has several limitations. Conventional methods to form EBs yield significant variations in the size and cell number of EBs, and human EBs (hEBs) have different differentiation capabilities dependent on the size [25]. hEBs do not induce specific distribution patterns of three germ layers, and their descendent lineages are heterogeneous, resulting in variations in cardiomyogenic differentiation [23,24]. Moreover, EBs are often fused together to form large aggregates causing extensive cell death [23]. Finally, the process for forming EBs is labor-intensive and therefore is considered an obstacle for mass production for therapeutic use.

Another approach for inducing CM differentiation is to co-culture PSCs with a mouse visceral endoderm-like cell line, END-2 [6]. It has been demonstrated that direct cell–cell interactions or secreted factors from the END-2 cells are capable of stimulating CM differentiation in vitro [26]. Although this system is reliable for generating CMs, it results in a relatively heterogeneous and undefined mixture of other cell types, which may require another step for isolating a pure population of CMs [14]. Recently, a high-density monolayer model has been introduced for CM differentiation [15,17]. In this protocol, PSCs are cultured in a feeder-free system for cell expansion and undergo cardiomyogenic differentiation by the addition of serum-free medium supplemented with several developmental-stage-specific cytokines such as BMP4, Activin A and VEGF [15,17]. However, this system requires expensive cytokines and yielded ~30% CMs. Common problems for the above methods are the difficulties in enrichment of CMs, as these systems result in heterogeneous cell mixtures. To purify CMs, Percoll density gradient or antibody-based cell isolation for KDR have been developed; however, the yield was variable and KDR^+ cell sorting resulted in cardiac progenitor cells but not pure CMs [3,15,27].

In the present study, we developed a novel 2D, directed differentiation system which can generate a high yield of differentiated CMs without using cytokines. The identity of differentiated CMs was verified by immunocytochemistry, gene expression studies, electrophysiology, and a CM-specific viral reporter system. Of note, we discovered the significant contribution of endodermal lineage cells to the differentiation and maturation of CMs as seen in developmental cardiomyogenesis. This differentiation system can serve as an important platform to investigate cardiomyogenesis.

2. Materials and methods

The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology.

2.1. hPSC culture

hiPSC lines were generated by the lentivirally-mediated four transcription factors (OCT4, SOX2, c-MYC and KLF4) as previously described [28]. In this study, we used a hiPSC line derived from human dermal fibroblasts (BJ1) and a hESC line H1 [4,28]. Briefly, undifferentiated hPSCs were grown on mitotically inactivated STO cells (ATCC, Manassas, VA) in DMEM/F12 (50:50; Gibco BRL, Gaithersberg, MD) supplemented with 20% (vol/vol) serum replacement (Gibco) and basic hESC medium, including 1 mM L-glutamine (Gibco), 1% nonessential amino acids (Gibco), 100 mM mercaptoethanol (Gibco), and 4 ng/mL basic fibroblast growth factor (bFGF; Sigma Aldrich Inc., St. Louis, MO) [29]. The medium was changed every 24 h, and hPSCs were transferred to new feeder cells every 5 to 7 days using dissecting pipettes.

2.2. Differentiation of hPSCs into CMs using a 2D system

For hPSC-CMs differentiation by the 2D system, cultured hPSCs were plated at 3×10^5 cells per well on Matrigel (BD Biosciences, Bedford, MA)-coated 6-well plates (Nunc, Roskilde, Denmark) without feeder cells as previously described [30]. Attached hPSCs were expanded in hESC media for 4 days (stage 1), and then cultured in hESC media without bFGF for three days (stage 2). Next, the aggregated cells were transferred into DMEM containing 10% FBS (HyClone, Logan, UT) and cultured for 7 days to form clusters (stage 3). Finally, FBS was increased to 20% and the clusters were more clearly demarked, with some clusters showing contraction within 7 days (stage 4). All culture medium was changed every 24 h.

2.3. Isolation of CMs using two step enzyme treatments

To isolate purified CMs from contracting clusters at stage 4, we used a two-step enzyme treatment. First, peripheral cells surrounding contracting clusters were removed by 0.05% trypsin-EDTA treatment (Invitrogen, Grand Island, NY). The remaining contracting clusters were detached by 0.25% trypsin-EDTA (Fig. 1C). The isolated contracting clusters were replated onto 0.1% gelatin-coated plates and cultured with DMEM containing 2% FBS [18]. Under these conditions, the contracting clusters formed a monolayer-like structure (Fig. 1E, left panel). We further digested these compact clusters by incubation with 0.25% trypsin-EDTA and made true monolayered cell sheets by culturing the cells on 0.1% gelatin (Sigma)-coated plates. Single cells and small clusters composed of less than 10 cells continued to beat even after digestion (supplemental online Movie 4).

2.4. Real-time RT-PCR (qRT-PCR)

To analyze gene expression patterns at each stage (Fig. 1A, cell populations indicated by white arrows), we performed real-time RT-PCR (qRT-PCR) as described previously [31]. Total RNA was isolated using TRIzol Reagent (Molecular Research Center, OH) according to the manufacturer's instructions. Two microgram of RNA was used for cDNA synthesis with SuperScript II reverse transcriptase according to the manufacturer's instructions (Invitrogen). Real time PCR was performed with SYBER Green PCR master mix using 7500 Fast RT-PCR System. GAPDH cDNA was used as an internal control. Primer sequences are listed in Supplemental online Table 1.

2.5. Construction of α-MHC viral reporter system

We utilized lentiviral vectors (courtesy of Dr. Miyoshi) as backbones to construct a human α-myosin heavy chain (MHC) viral reporter system.

(http://www.brc.riken.go.jp/lab/cfm/Subteam_for_Manipulation_of_Cell_Fate/Plasmid_List.html). CSII-EF-Venus was incubated with AgeI to delete its EF-1 promoter. The digested vector was self-ligated with T4 DNA ligase. CSII-ΔEF-Venus was double-digested by BamHI and XbaI, then ligated with the fragment of IRES2-BSD containing the same double-digestion sites from CSII-CMV-MCS-IRES2-BSD. To construct CSII-ΔEF-GFP-IRES2-BSD, eGFP was amplified by PCR using the following primers:

5′ ATATATGCGGCCGCAAGGATCCCCGGGTACCGGTCGCCACC,
5′ ATATATAGATCTTTACTTGTACAGCTCGTCCATG,

with pEGFP-N1 as a template, and were double-digested by NotI and BglII. The eGFP fragment was pasted into the sites of NotI and BamHI in CSII-ΔEF-IRES2-BSD. The fragment of human α-MHC promoter (3068 bp) was amplified by PCR using the following primers

5′ ATATATGCGGCCGCCCAGACAGAGCTCCCTCAAACCA,
5′ ATATATGGATCCTCCTCAAAGCTCCAGTTCCTTTAT,

with human genomic DNA as a template. These synthetic DNAs were double-digested by NotI and BamHI and ligated with NotI/BamHI-digested CSII-ΔEF-GFP-IRES2-BSD for the construction of α-MHC-GFP-IRES2-BSD. Lentiviral particles were produced in 293T cells using an α-MHC reporter with packaging plasmids pMDL-gp-RRE and pCMV-VSV-G-RSV-Rev. The sequences of the constructs were verified by DNA sequencing.

2.6. Immunocytochemistry

Immunocytochemistry was performed as described previously [32]. Briefly, cells were fixed with 4% paraformaldehyde (PFA) for 20 min and permeabilized with 0.1% Triton X-100 in phosphate buffered saline (PBS, Sigma) for 5 min. After treatment with 5% normal goat serum for 30 min, the cells were incubated for 12 h at 4 °C with antibodies against the following proteins: a pluripotency marker OCT4 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), mesodermal lineage markers Brachyury (Abcam Inc., Cambridge, MA) and HAND1 (Santa Cruz Biotechnology), cardiac-lineage markers NKX2-5, cardiac troponin T (cTnT), α-MHC (Abcam), connexin 43, GATA4 (BD Bioscience), MEF2C (Abgent Inc., San Diego, CA), β-MHC (Millipore, Billerica, MA), MLC2a, and MLC2v (Synaptic Systems, Goettingen, Germany), and endodermal lineage markers FOXA2 (Abcam) and AFP (Abcam). Cells were washed three times with PBS and then incubated with rhodamine or FITC-conjugated secondary antibodies (Molecular Probes Inc., Eugene, OR) for 1 h. DAPI counterstaining was performed. All images were analyzed with an LSM 510 META confocal microscope (Carl Zeiss Inc., Oberkochen, Germany).

2.7. Electrophysiologic study

The intracellular AP was recorded as described previously with slight modification [5]. The beating cells isolated from contracting clusters by two step enzyme treatment at stage 4 were transferred and cultured on 0.1% gelatin-coated glass bottom microwell dishes for 5 to 7 days. For intracellular recording, the 35-mm dishes were mounted on an inverted microscope (Olympus IX71, Japan) and heated by a heating/cooling bath temperature controller (DTC-200, Dagan Corporation, Minneapolis, MN). The contracting clusters were perfused with Tyrode's solution containing (mmol/L) 140 NaCl, 5.4 KC1, 1 $MgCl_2$, 10 HEPES, 10 glucose, 1.8 $CaCl_2$, pH 7.4 with NAOH 37 °C. Glass microelectrodes were fabricated from borosilicate glass (PG52151-4, World Precision Instruments, Inc., Sarasota,