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Generation of cardiac spheres from primate pluripotent stem cells in a small molecule-based 3D system

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ABSTRACT

Pluripotent stem cell (PSC) usage in heart regenerative medicine requires producing enriched cardiomyocytes (CMs) with mature phenotypes in a defined medium. However, current methods are typically performed in 2D environments that produce immature CMs. Here we report a simple, growth factor-free 3D culture system to rapidly and efficiently generate 85.07 ± 1.8% of spontaneously contractile cardiac spheres (scCDSs) using 3D-cultured human and monkey PSC-spheres. Along with small molecule-based 3D induction, this protocol produces CDSs of up to 95.7% CMs at a yield of up to 237 CMs for every input pluripotent cell, is effective for human and monkey PSCs, and maintains 81.03 ± 12.43% of CDSs in spontaneous contractibility for over three months. These CDSs displayed CM ultrastructure, calcium transient, appropriate pharmacological responses and CM gene expression profiles specific for maturity. Furthermore, 3D-derived CMs displayed more mature phenotypes than those from a parallel 2D-culture. The system is compatible to large-scaly produce CMs for disease study, cell therapy and pharmaceutics screening.

1. Introduction

Heart failure usually results from a deficiency of specialized cardiac muscle cells known as cardiomyocytes (CMs) [1]. A robust therapy to regenerate lost myocardium using CMs provides one possibility to recover the failure. Human pluripotent stem cells (hPSCs) have indisputable CM generating abilities and have been extensively investigated for repair of the injured heart [2–5]. However, cardiac repair will require orders of magnitude more cells, because a billion CMs are lost after a typical infarct [1].

Although differentiations of hPSCs into beating CMs are well-established by monolayer differentiation [6–8], co-culture with a visceral endoderm like cell line [9], or embryonic bodies (EBs) formation following monolayer differentiation [10–13], these protocols low-efficiently produced CMs on 2D tissue culture-treated polystyrene surfaces by use of many expensive growth factors, such as Activin A, BMP4, FGF-2, vascular endothelial growth factor (VEGF) and dickkopf homolog 1 (Dkk-1) [14]. Recently, 3D aggregates of CMs (cardiospheres) from 2D differentiation cultures of hPSCs in the media including activin A and BMP4 were used to purify CMs by rotary orbital suspension culture [15]. However, these 2D differentiation systems and growth factor usages restrict the scalable productions of CMs. To solve these questions, an attractive approach is to develop a 3D differentiation system using a growth factor-free defined medium [16].

In addition, the use of *de novo* human CMs offers the pharmaceutical industry an invaluable tool for preclinical screening of candidate drugs to treat cardiomyopathy, arrhythmia and heart failure, as well as therapeutics to combat secondary cardiac toxicities [17]. Unfortunately, the CMs produced to date from hPSCs are largely immature and most analogous to fetal stages of development [17]. The lack of maturity of hPSC-derived CMs may reduce the suitability of drug testing. Thus, the usage of hPSCs in heart pharmaceutics screening requires producing enriched CMs with mature phenotypes in a defined medium. Given that cell–cell interactions are important for CM differentiation, we guessed that 3D suspension microenvironment may promote CM differentiation

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and maturation. Therefore, developing a simple growth factor-free culture system in 3D condition to efficiently produce mature CMs will be rather advantageous to realize the full promise of hPSCs for heart biomedical research, drug development and clinical applications. Here we report a simple, defined, growth factor-free and efficient 3D suspension culture system to rapid generation of cardiac spheres (CDSs). Along with small molecule-based 3D induction, this protocol produces spontaneously contractile CDSs of up to 95.7% CMs. These 3D-cultured CMs display more matured ultrastructure and gene expression profiles than those from a parallel 2D-culture.

2. Methods and materials

2.1. Homogenous suspension culture of primate PSCs spheres

Three types of primate stem cells for this study were used: Human embryonic stem cell (hESC) BG02 and H9, cynomolgus monkey embryonic stem cell (cESC) 3–12, and cynomogus monkey induced puripotent stem cell (ciPSC) J1. ciPSC J1 was reprogrammed from embryonic day 60 skin fibroblasts by Yamanaka factors as previous reported protocol [18]. Conventional maintenance of these PSCs was via culturing PSCs on MEFs in pluripotent stem cell media (PSC-M), which is composed of DMEM/F12 (1:1), 20% Knockout Serum Replacement (KOSR; Life Technologies Co, USA), and 1 mM glutamine (Invitrogen), 1% NAA (nonessential amino acids, Invitrogen), 0.1 mM β-mercaptoethanol (Sigma), 5 ng/ml recombinant bFGF (R&D, USA). Before suspension culture, PSCs were cultured on matrigel (BD Biosciences, USA) with mTeSR1 (Cat No. 05850, STEM CELL Technologies, Canada) over 3 days. Typical PSCs colonies were digested for 5–10 min with 1 mg/ml dispase (STEM CELL Technologies, Canada). The dispase treatment were stopped by removing dispase and rinsing with Ca^{2+}- and Mg^{2+}-free PBS twice once stem cell colonies began to roll up. These colonies were gently washed down by PSC-M. Maintenance of intact colonies was a key to get homogenous clusters of PSCs via mechanical dissociation. These digested colonies were gently collected by 5 ml pipette and cut into 50 μm clusters when passed through the strainer with 50 μm opening mesh in diameter (CellTrics® 50 μm, Patrec, Germany) by pipette pressure. These cut clusters were collected, re-suspended in 3 ml/well pre-warmed mTeSR1 surplus with 10 μM Y-27632 (Sigma, USA) and 0.27%–0.35% methylcellulose (R&D, USA), and cultured on ultra-low attachment plate (35 cm, Corning, USA) for propagation.

2.2. Daily maintenance and passage of PSC-spheres

A large number of homogeneous compacted spheres were formed after one night incubation of PSCs. To evaluate homogeneity of these spheres, we gently shook the dish, re-allocated these spheres into the well center, and took photos on three different spots. The media were daily changed. PSCs developing into 250 μm spheres required five days for human Bg02 and H9, and four days for cESC 3–12 and ciPSC J1. Mean 250 μm spheres were ready for passaging. Mechanical dissociation with the mesh strainers as described above was used to encourage cell propagation when passaging. PSCs were routinely passaged to 1:6 to 1:8 ratios every 5 days for human PSCs and 4 days for monkey PSCs.

2.3. Karyotype assay and teratoma formation

All the four 3D-cultured PSCs lines over 10 passages were tested karyotype by G-binding assays. The spheres were collected and replated on matrigel-coated culture dishes for another two days in mTeSR media. These cells were treated with 1 μg/ml colcemid (Wako, Japan) at the final concentration for 2 h, and then with 0.075M hypotonic potassium chloride solution for 10 min. These samples were sent to Kunming Institute of Zoology, CAS for karyotype assay. 50 M-phase cells were observed.

For teratoma formation and analysis, hPSC-spheres were injected into Hind leg muscles of 4–5-week-old SCID mice after cultured for 15 passages in 3D condition. Tumors generally developed within 6 weeks and animals were killed. All animal studies were conducted according to the guideline and following approval by the Yunnan Key Lab of Primate Biomedical Research IACUC.

2.4. In vitro differentiation of PSC-spheres into cardiac spheres in the 3D suspension culture

PSC-spheres on D5 for Bg02 and H9 as well as D4 for monkey PSC 3–12 and J1 in suspension were used to induce CM differentiation, respectively. Directed differentiation from PSCs spheres into mature CDSs was performed by a three-step induction protocol as described in Fig. 1A. First, one well of PSC-spheres were divided into 3–4 new wells for inducing into mesoderm progenitors (MPs) by replacement of mTeSR1 with MP differentiation media, which were composed of CM differentiation basic medium (CDBM) [IMDM basal medium/bFGF-free PSC-M (1:2)] surplus with 5 μM CHIR99021 (StemRD, Canada) and 5 μM BIO (StemRD, Canada). Second, 48hs later, the medium was replaced with CDBM media supplemented with 10 μM XAV939, 10 μM IWP-2 and 5 μg/ml Vitamin C (all bought from StemRD, Canada) to induce MPs into CDSs. The beating CDSs were first observed on Day 4 post-differentiation (pdD4) for cES 3–12 and ciPSC, and on pdD6-7 for BG02 and H9, respectively. The medium was changed every two days. Finally, from pdD12, the medium was changed into the maturation media, which was composed of IMDM/bFGF-free PSC-M (4:1) surplus with 5 μg/ml Vitamin C.

2.5. Monolayer differentiation of cardiomyocyte differentiation

Mean 250 μm sized hPSC-spheres were transferred onto 10 μg/ml laminin-coated tissue culture dishes to induce CM differentiation following the same protocol as CDS differentiation at pdD0. The schematic representation of CMs induction and maturation from PSC-spheres in the attachment plates was summarized in Fig. 6A. The efficiency of beating clusters were counted at pdD18 and compared with the efficiency of 3D cardiac differentiation.

2.6. Immunochemistry and flow cytometry assay

The PSC spheres and CDSs were fixed in 4% PFA for 20 min, and then immersed in 10% sucrose for 1 h, respectively. The fixed spheres were transferred onto plastic model (Tissue-Tek, Sakura, USA) and embedded in OCT. 10 μm thick slides were cryosectioned. These slides were washed three times with PBS, and incubated in blocking buffer (0.1% Tween 20 and 10% normal donkey serum (Invitrogen) in PBS) for 30 min at room temperature. The cells were incubated with primary antibody overnight at 4 °C. The following day, the cells were washed with PBS and incubated with Alexa 488 or rhodamine-conjugated secondary antibodies (Invitrogen: goat-anti-rabbit, goat-anti-mouse, donkey-anti-goat, donkey-anti-chicken, 600×) in PBS for 1 h at RT. Nuclei were visualized with DAPI staining (Sigma–Aldrich). Primary antibodies were listed as follows: pluripotent marker assay kit (SCR001, Milipore, USA) including SSEA-1, SSEA-4, Tra-1-60, and Tra-1-81 (1:500, respectively); neural markers Nestin (1:400; R&D, USA); cardiac markers NKX2.5 (1:500; Cell Signaling Technologies, USA), cTnT (1: 500; Cell Signaling Technologies, USA), α-actinin (1:400; Santa Cruz Biotechnology, USA), HCN4 (1:200; Santa Cruz Biotechnology, USA),