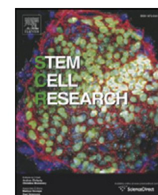
Stem Cell Research 20 (2017) 21–29

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr



Differentiation and characterization of rhesus monkey atrial and ventricular cardiomyocytes from induced pluripotent stem cells

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ARTICLE INFO

Article history:
Received 6 February 2016
Received in revised form 2 February 2017
Accepted 8 February 2017
Available online 11 February 2017

Keywords:
Rhesus monkey
Induced pluripotent stem cells
Atrial cardiomyocytes
Ventricular cardiomyocytes

ABSTRACT

The combination of non-human primate animals and their induced pluripotent stem cell derived cardiomyocytes (iPSC-CMs) provides not only transplantation models for cell-based therapy of heart diseases, but also opportunities for heart-related drug research on both cellular and animal levels. However, the subtypes and electrophysiology properties of non-human primate iPSC-CMs hadn't been detailed characterized. In this study, we generated rhesus monkey induced pluripotent stem cells (riPSCs), and efficiently differentiated them into ventricular or atrial cardiomyocytes by modulating retinoic acid (RA) pathways. Our results revealed that the electrophysiological characteristics and response to canonical drugs of riPSC-CMs were similar with those of human pluripotent stem cell derived CMs. Therefore, rhesus monkeys and their iPSC-CMs provide a powerful and practicable system for heart related biomedical research.

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1. Introduction

Human pluripotent stem cells (HPSCs) present a promising therapeutic approach for a number of conditions, owing to their indisputable and varied ability to differentiate into many cell types (Takahashi et al., 2007; Xu et al., 2015; Qiu et al., 2015). Human cardiomyocytes derived from pluripotent stem cells (hPSC-CMs), provide a promising cell resource in future cell-based therapies for heart infarction treatments (Caspi et al., 2007; Chong et al., 2014; Laflamme et al., 2007; Matsa et al., 2014; Shiba et al., 2012; van Laake et al., 2007). Although hPSC-CMs have been successfully transplanted into non-human primate (monkey) models for heart repair (Chong et al., 2014), issues such as differences in cardio-physiology between species, and immuno-rejection, continue to hinder the long-term observation and accurate evaluation of cell transplantation therapies. The use of monkey iPSC-CMs in cell-based heart repair research with a monkey model, especially the same individual, would therefore avoid these issues.

Because monkeys share a high degree of genetic, anatomic, physiological, and cardiological similarity with humans (Oishi et al., 2014; Han et al., 2007; Hosseinkhani et al., 2007a,b; Khaitovich et al., 2005), they represent an ideal *in vivo* model for drug testing. Furthermore, monkey iPSC-CMs would provide a good cellular model for *in vitro* drug testing. The combination of monkey iPSC-CMs and primate models for drug discovery could provide a two dimensional strategy for assessing heart-related drugs.

More research efforts are underway for the cardiac differentiation of hPSCs, which can be efficiently induced into CMs using growth factors or small molecules that regulate Wnt signaling (Lian et al., 2012; Mummery et al., 2012; Yang et al., 2008). Moreover, hPSCs can be directionally differentiated into high homogeneous atrial and ventricular CMs by manipulating the retinoic acid pathway after mesodermal cell formation (Gassanov et al., 2008; Xavier-Neto et al., 1999; Zhang et al., 2011). Conversely, research has shown that even though monkey CMs can differentiate from monkey ESCs, which were cultured on mouse embryonic fibroblast (MEF) feeder cells, using an embryoid body (EB) based approach, the differentiation efficiency and purity cannot satisfy the amount of cells needed for transplantation studies and large-scale drug discovery (Hosseinkhani et al., 2007a,b; Chen et al., 2008; Schwanke et al., 2006).

Here we report that rhesus iPSCs (riPSCs) were generated successfully from rhesus monkey dermal fibroblasts using episomal vectors to deliver human genes (Okita et al., 2011). To support efficient growth of these cells, we developed a feeder-free culture system which supported riPSCs long-term self-renewal. These cells were further differentiated into atrial and ventricular CMs using the small molecules, CHIR99021 (glycogen synthase kinase 3 inhibitor), IWR-1 (Wnt pathway inhibitor), and RA/BMS493 (RA pathway inhibitor). The riPSC-CMs possessed human cardiomyocyte characteristics, including cardiac-specific gene expression, electrophysiology and chronotropic drug response. These characteristics make riPSC-CMs an ideal cell type for studies of human heart regenerative therapy and drug discovery.

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http://dx.doi.org/10.1016/j.scr.2017.02.002
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2. Materials and methods

2.1. Generation of rhesus induced pluripotent stem cells (riPSCs)

Ear skin biopsies (0.5 cm^2) were taken from rhesus monkeys, which were housed in individual cages at the Institute of Psychology, and diced into small pieces. Samples were digested in 0.25% Trypsin-EDTA (Invitrogen) for 30 min at 37 °C. Rhesus ear fibroblasts were routinely maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 20% fetal bovine serum (FBS, Hyclone) in T25 flasks (NUNC). The reprogramming plasmids, pCXLE-hSK, pCXLE-hUL and pCXLE-hOCT4-shp53 (Addgene), were amplified in *Escherichia coli* (Transgene) and purified using the Plasmid Mini kit (OMEGA) prior to quantification using a NanoDrop 2000 (Thermo Scientific). Total plasmids (~1.25 μg for each vector) were electroporated into 5×10^5 cells *via* AMAXA progress U-023 using NHDF Nucleofector kit (LONZA, VPD-1001). The cells were seeded into six-well plates (NUNC) coated with 0.1% gelatin in DMEM supplemented with 10% FBS. Cells were passaged at a ratio of 1:6 on MEFs (3×10^5 cells per well) in hESC medium containing 80% DMEM-F/12 (Invitrogen), 20% Knock-out Serum Replacement (Invitrogen), 1 mM L-Glutamine (Invitrogen), 1% MEM non-essential amino acids (Invitrogen), 0.1% 2-Mercaptoethanol (Invitrogen), supplemented with 20 ng/ml basic fibroblast growth factor (bFGF, PeproTech) after 6–7 days. Obvious clones appeared after 3–4 weeks. Clones were picked and further cultured on a feeder layer in hESC medium supplemented with 20 ng/ml bFGF.

2.2. riPSCs culture

riPSCs were incubated at 37 °C, in 5% CO2. In brief, riPSCs were cultured with MEF feeders on 0.1% gelatin in hESC medium supplemented with 20 ng/ml bFGF. Cells were then maintained in the monkey stem cell medium (MM) system containing hESC medium supplemented with 0.5 μM CHIR99021 (Selleck), 2 μM IWR-1 (Selleck), 100 ng/ml bFGF, and 10 ng/ml Activin A (R&D) on recombinant human vitronectin (VTN). Clones were passaged every five days following exposure to 1 mg/ml collagenase IV (Invitrogen) for 9–15 min, and were washed twice with DMEM-F/12 prior to plating the cells as clusters.

2.3. riPSCs survival and proliferation assay

riPSCs were dissociated into a single cell suspension and 3×10^4 cells were plated per well (12-well plate). Cells were maintained in different media, including hESC medium (hESC), hESC medium conditioned by MEF feeder (CM), hESC medium supplemented with CHIR99021 and IWR-1 (hESC + C + I), hESC medium supplemented with Activin A and bFGF (hESC + A + b), E8 medium (E8), and E8 supplemented with CHIR99021 and IWR-1 (E8 + C + I), 10 μM Y27632 (Selleck) was added to each media on day 1 and all media were changed daily. Cells were digested by 0.25% trypsin-EDTA for 1–3 min, neutralized by FBS and counted every 24 h. Experiments were carried out in triplicate for each media type on each day.

2.4. In vitro differentiation

For embryoid body (EB) formation, riPSCs were dissociated into pieces using 1 mg/ml collagenase IV. Cells were then cultured on 10 cm low-attachment culture dishes in differentiation medium, consisting of 80% DMEM (Invitrogen) supplemented with 20% FBS. After 7 days of floating culture, cell cluster were seeded into 24-well plates coated with 0.1% gelatin. EBs were collected for quantitative polymerase chain reaction (Q-PCR) and immunofluorescence on day 14. For ectoderm differentiation, riPSCs were dissociated into single cells and cultured to 90% confluence. By adding 2 μM Su5402 (Sigma) and 2 μM dorsomorphin (Sigma) for 4 days, ectoderm cells (Sox2 and Tuj1 double positive) were yielded after 7 days. EBs were treated with 100 ng/ml Activin A for 3 days and then 1 μM RA for 2 days, endoderm cells could be detected at day 14. For mesoderm differentiation, riPSCs were cultured in monolayers to 90% confluence prior to addition of CHIR99021 for one day, resulting in mesodermal lineage cells (brachyury positive).

2.5. Quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted by Trizol reagent (Invitrogen), and then reverse transcribed using the RT Reagent kit (Takara). Quantitative RT-PCR was performed using a SYBR Green PCR kit (Qiagen) with the primers listed in Table S1.

2.6. Teratoma formation

Approximately 10^7 riPSC colonies were harvested using collagenase IV and were re-suspended in 200 μl Matrigel matrix. This mixture was then hypodermically injected into non-obese diabetic/severe combined immunodeficiency (NOD-SCID) mice, Matrigel as a negative control. After 6–8 weeks, teratomas were fixed in 4% paraformaldehyde (PFA, Amresco), imbedded in paraffin and stained with hematoxylin-eosin (HE) after slicing. Photographics images were obtained using a Leica SCN400 at a magnification of 20×. NOD-SCID mice were raised in a specific pathogen free (SPF) laboratory animal room at the Institute of Biophysics, University of Chinese Academy of Sciences.

2.7. Karyotype analysis

riPSC colonies on VTN were cultured in MM for about 4–5 days. G-banded karyotypes analysis tested at the Peking University Center of Medical Genetics.

2.8. Cardiomyocytes differentiation

riPSCs were dispersed into small clusters containing 10–20 cells by 0.48 mM EDTA (Invitrogen), plated at approximately $2.5–3 \times 10^5$ cells per well (24-well dishes) on VTN and cultured for 2 days. All riPSCs were treated with 1 μM CHIR99021 on day 1 and 5 μM IWR-1 on days 3–5. The cells were differentiated in RPMI1640 medium supplemented with S12 without insulin. S12 containing only 12 components was developed in our lab, which was a albumin-free and chemical defined

Fig. 1. riPSCs were generated from fibroblasts. (A) Time schedule of riPSCs generation (B) Morphology of rhesus fibroblasts (Ba), and riPSCs (Bb) riPSCs were positive for alkaline phosphatase (Bc). (C) Karyotype analysis displayed the normal karyotypes of rhesus cells (42, XY, P15 + 31). (D) RT-PCR analysis of riPSCs revealed that they were positive for endogenous pluripotent stem genes, *OCT3/4*, *SOX2* and *NANOG* (end), but negative for exogenous pluripotent stem genes, *OCT3/4* and *SOX2* (pla). (E) Cell growth curves of riPSC in different media for 5 days, indicated that MM supported riPSC initial survival and proliferation, which was similar to CM. There were seven media used in our work, including hES medium (hESC), hES medium conditioned by MEF feeders (CM), hES medium supplemented with 0.5 μM CHIR99021 and 2 μM IWR-1 (hESC + C + I), hES medium supplemented with 10 ng/ml Activin A and 100 ng/ml bFGF (hESC + A + b), E8 medium (E8), E8 supplemented with 0.5 μM CHIR99021 and 2 μM IWR-1 (E8 + C + I), and E8 supplemented with 10 ng/ml Activin A and 100 ng/ml bFGF (E8 + A + b). (F) Flow cytometric analyses showed that MM maintained riPSCs self-renewal better than the other media, as demonstrated by the percentage of OCT3/4 and SOX2 positive cells. riPSCs (P15 + 2) were cultured in four media, including, hESC + C + I, hESC + A + b, E8, and E8 + C + I, riPSCs (P15 + 7) in CM, and riPSCs (P15 + 30) in MM. (G) riPSCs were cultured on feeders (Ga) and in MM (Gc) for more than 30 passages, and which were subsequently analyzed by immunofluorescence of Tra-1-81(Gb,Gd). (H) These riPSCs were also positive for Oct3/4, Sox2, Nanog and SSEA4 in MM.

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