Biologicals xxx (2017) 1–7

Contents lists available at ScienceDirect

Biologicals

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

A novel protocol to provide a suitable cardiac model from induced pluripotent stem cells

Mahsa Rasekhi [a], Masoud Soleimani [b, *], Behnaz Bakhshandeh [c], Majid Sadeghizadeh [d]

[a] National Institute for Genetic Engineering and Biotechnology, Tehran, Iran
[b] Department of Hematology, School of Medical Sciences, Tarbiat Modares University, Tehran, Iran
[c] Department of Biotechnology, College of Science, University of Tehran, Tehran, Iran
[d] Department of Genetics, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran

ARTICLE INFO

Article history:
Received 21 May 2017
Received in revised form
6 September 2017
Accepted 11 September 2017
Available online xxx

Keywords:
Cardiomyogenesis
microRNA-1
5′-Azacitidine
iPS cells
Differentiation protocol

ABSTRACT

Cumulative evidence has proven the safety, feasibility and efficacy of stem cell therapy for cardiomyocyte replacement in heart failure treatment. In contrast to embryonic stem cells, induced pluripotent stem cells (iPS cells) provide a route to the production of patient-specific stem cell lines with no ethical concerns. Recent studies have revealed that myogenic transcription factors activated the expression of conserved microRNAs (miRNAs), such as mir-1, that 'fine-tuned' the output of the transcriptional networks. To introduce an efficient and applicable protocol for establishment of autologous cardiac cellular models, herein we introduced a novel protocol for induction of iPS cells into cardiomyocytes using both microRNA-1 transduction and 5′-Azacitidine treatment. Quantitative evaluation of transcription and translation of cardiac markers such as MHC-α, GATA4, FLK and troponin, demonstrated that this new direct protocol led to cardiac differentiation of iPS cells. From a clinical point of view, these results raise the possibility that administration of miRNA mimic or miRNA inhibitor therapies could increase allocation of iPS cells into the cardiac lineage. Taking all the results into account, our novel protocol provides further progress in the application of patient's own cells for more effective therapies. Moreover, such cellular models could be used in personalized drug screening.

1. Introduction

Heart failure (including myocardial infarction and Ischemic heart disease) is one of the most deadly diseases [1]. In the defected heart, the heart muscle cells (cardiomyocytes) undergo necrosis and apoptosis which result in scar formation and lead to heart failure [2,3]. Replacement of damaged area may prevent heart failure but engineering of bioartificial cardiac muscle is hampered by the fact that adult cardiomyocytes have almost no potential for proliferation [4]. In fact, the lack of suitable cell sources is a major hurdles to be overcome before clinical application of novel cardiac regenerative therapies [4,5].

Cumulative evidence has proven the safety, feasibility, and efficacy of stem cell therapy in order to cardiomyocyte replacement in heart failure treatments [6,7]. Recent reports have mentioned the capability of mesenchymal stem cells [8–10], cardiac progenitor cells [11,12], embryonic stem cells (ESCs) [13,14] and induced pluripotent stem (iPS) cells [15–17] for cardiomyocyte differentiation. Most of these cells are not accessible in the proper amount for tissue repair and immunological rejection of mismatched cellular grafts is a considerable obstacle [18].

In contrast to ESCs, not only iPS cells are not ethically controversial but also they provide a route to the production of patient-specific (autologous) stem cell lines as a solution for immunological reactions. The gene expression profile, DNA methylation status and chromatin configuration in iPS cells are similar to those of ES cells [19,20]. The human iPS cells also had all the essential properties typical of human ES cells, and differentiated into derivatives of all three primary germ layers, including beating cardiomyocytes [21,22].

Terzic and colleagues generated iPS cells by the use of three factors (Oct3/4, Sox2, Klf4 without c-MYC oncogene) and also established their potential to generate cardiac myocytes [23]. However, the recently applied protocols to differentiate iPS cells into cardiomyocytes have low efficiency and a direct and robust

* Corresponding author. Hematology Dept., Faculty of Medical Science, Tarbiat Modares University, P.O.Box: 14115-111, Tehran, Iran.
E-mail address: soleim_m@modares.ac.ir (M. Soleimani).

https://doi.org/10.1016/j.biologicals.2017.09.003
1045-1056/

differentiation method for iPS cells has not been established yet [22,24,25]. Therefore, more efficient and applicable protocols for the establishment of autologous cardiac cellular models are recommended [26,27].

Recently progress in molecular research has uncovered various regulatory agents such as microRNAs (miRNAs). miRNAs are small noncoding gene products that play an important regulatory role in determining cell differentiation post-transcriptionally [28]. Recent studies have revealed that myogenic transcription factors activate the expression of a set of conserved miRNAs [28,29]. In addition, miRNA expression profiling studies demonstrated the involvement of miRNAs in cardiomyopathies [30,31].

miR-1 is highly enriched in the heart and acts as an important regulator for heart development and myocyte differentiation. Studies in both Drosophila [32,33] and mice [34] demonstrated the importance of miR-1 during cardiogenesis. miR-1 promotes myoblast differentiation as a consequence of its repressive influence on histone deacetylase 4 as a transcriptional repressor of myogenesis [35]. Another study suggested the positive role of miR-1 in myocardial differentiation during embryoid body (EB)-based culture of mouse and human ES cells [36]. Moreover, mice lacking miR-1-2 suffered from cardiac arrhythmia and congenital malformation [37]. So we focused on mir-1 as a potent factor for cardiac differentiation.

Azacitidine acted epigenetically though hypomethylation and histone acetylation mediated hyper expression of cardiogenesis-associated genes, involving activation of ERK signaling. Abbey et al. showed that Azacitidine is a potent cardiac inducer when applied during the initial phase of mouse P19 embryonic carcinoma cells differentiation [38].

Herein we introduced and evaluated a novel protocol for iPS cells induction for cardiomyocyte differentiation using both mir-1 induction and 5′-Azacitidine treatment. The efficiency of thisprotocol was evaluated by cardiomyocyte markers both transcriptionally and translationally.

2. Materials and methods

2.1. Cell culture

The iPS cells (a gift from Stem Cells Technology Research Center (Tehran, Iran), supplementary Doc. 1) [20] were cultured on previously mitomycin-inactivated SNL cells, in a 60 mm culture dish coated with gelatin 0.1%. They were maintained in DMEM F12 (Gibco) supplemented with 15% ES (Gibco) (v/v), 1% non-essential amino acids (Sigma), 4 ng/ml bFGF (peprotech) and 1 mM L-glutamine (Gibco) with every 5 day passaging. In order to investigate any spontaneous differentiation, iPS cells dissociated by collagenase IV (Gibco) 1 mg/ml and diluted in iPS medium lacking bFGF. 25 μL drops (each containing approximately 150 cells) were placed on the inside of a petri dish lid. Two days later the embryoid bodies (EBs) were plated in 6-well non-adherent petridishes and cultured for 2 more days in suspension medium. On fifth day, the EBs in DMEM F12 supplemented with 5% FBs were transferred to 0.1% gelatin coated plates.

2.2. Lentivirus production and titration

To produce lentivirus particles the miRNA harboring plasmid (pLentiIII-miR-1-GFP) and pLentiIII-GFP backbone (abm) were cotransfected to the Hek293 cells with psPax as packaging plasmid and PMD2G as an envelope plasmid using calcium phosphate transfection method (Supplementary Fig. 1). After 16 h, culture medium was replaced with fresh DMEM and 10% FBS. Then the supernatant (containing viral particles) was collected 2 times with 24 h intervals. The viral supernatants of 24, 48 and 72 h cultures were then filtered through 0.45 μm membranes and mixed over night with 5% PEG-8000 and 0.15 M NaCl by mild shaking. The next day, the viruses were concentrated with ultracentrifuge in 25000 g for 1 h and suspended in EB medium. The viral titration was performed using serial dilutions of HEK transfected cells and subsequent FACS analysis for GFP expression evaluation.

2.3. Cardiomyocyte differentiation

Hanging drop method was used for iPS cells transduction except that in each 25 μL drop, the concentrated virus particles in EB medium for pLentiIII-miR-1-GFP or pLentiIII-GFP backbone (abm) with the MOI of 30, were added to the iPS cells which led to transduction during EBs formation. For cardiomyocyte differentiation, EBs were cultured in 6-well plated using DMEM supplemented with 15% FBS-ES qualified. After 70% confluency of the cultured cells, the medium was exchanged with DMEM, FBS-ES qualified, non-essential amino acids, L-glutamin and 5′-Azacytidine (10^{-6} M). The concentration of FBS-ES qualified was decreased to 5% gradually during 28 days. After 28 days of induction, the differentiation evaluation was performed.

2.4. Real time PCR

Total RNA of iPS cells was extracted using Trizol reagent (Invitrogen). cDNAs were generated using Vivantis reverse transcriptase. The specific primers were designed for cardiac marker genes; GATA4, Troponin-T and MHC-α (Table 1). Real Time PCR was performed on RotorGene6000 (Corbett, Australia) followed by melting curve analysis to confirm PCR specificity. The threshold cycle average was used for data analysis by Rotor-gene Q software (Corbett). Relative expression was quantified using the comparative ΔΔCt method. Target genes were normalized against for cardiomyocyte genes and to SNORD for miRNAs and calibrated to undifferentiated iPS cells.

2.5. Immunocytochemistry

The cells were rinsed twice with PBS and fixed with 4% paraformaldehyde (in PBS) for 20 min at 4 °C and 10 more minutes at room temperature. Since both cardiomyocyte markers (FLK and troponin) are intracellular, after washing the cells 2 times with PBS they were permeabilized for 10 min in PBS with 0.1% triton x-100. Goat serum 5% was used as blocking solution for 1 h at room temperature. Cells were incubated overnight at 4 °C with appropriate dilution of first antibodies (FLK and troponin; both from Santacruz bio) in 0.2% PBS/BSA. After rinsing with PBS the cells were incubated with second antibody PE conjugated goat anti-mouse IgG (SCB) in PBS containing 0.1% BSA for 1 h at 37 °C. Nuclei were stained with DAPI (1 μ/ml) for 1 min at room temperature.

2.6. Statistics

The student t-test was used for comparison of two groups. P value < 0.05 was considered as significantly different in all cases. All experiments were done in triplicate, unless otherwise stated and data were shown as mean ± standard deviation (SD).

3. Results

In order to establish an easy and robust method for differentiation of iPS cells into cardiomyocytes we used 5′-azacytidine treatment and miRNA induction. To confirm the cardiomyocyte

Please cite this article in press as: Rasekhi M, et al., A novel protocol to provide a suitable cardiac model from induced pluripotent stem cells, Biologicals (2017), https://doi.org/10.1016/j.biologicals.2017.09.003