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

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\textbf{A R T I C L E I N F O}

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\textbf{A B S T R A C T}

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-\textalpha, 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.

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1. Introduction

Heart failure (including myocardial infarction and Ischemic heart disease) is one of the most deadly diseases \cite{1}. In the defected heart, the heart muscle cells (cardiomyocytes) undergo necrosis and apoptosis which result in scar formation and lead to heart failure \cite{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 \cite{4}. In fact, the lack of suitable cell sources is a major hurdle to be overcome before clinical application of novel cardiac regenerative therapies \cite{4,5}.

Cumulative evidence has proven the safety, feasibility, and efficacy of stem cell therapy in order to cardiomyocyte replacement in heart failure treatments \cite{6,7}. Recent reports have mentioned the capability of mesenchymal stem cells \cite{8-10}, cardiac progenitor cells \cite{11,12}, embryonic stem cells (ESCs) \cite{13,14} and induced pluripotent stem (iPS) cells \cite{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 \cite{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 \cite{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 \cite{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 \cite{23}. However, the recently applied protocols to differentiate iPS cells into cardiomyocytes have low efficiency and a direct and robust
differenctiation 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 miRNAs 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 this protocol 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 (pLentilil-mir-1-GFP) and pLentilil-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 using 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 pLentillil-mir-1-GFP or pLentillil-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 1 h 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 antimouse IgG (SCB) in 0.2% PBS/BSA. The cells were rinsed twice with PBS containing 0.1% BSA for 1 h at 37 °C. Nuclei were stained with DAPI (1 μg/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
differentiation, we evaluated the morphological changes of the EBs and iPS cells during differentiation and the cardiomyocyte markers, both transcriptionally (by qPCR) and translationally (by immuno-cytochemistry). Prior to these analyses, the transduction efficiency was also assessed.

3.1. Quantitative evaluation of mir-1 expression

The transcription of mir-1 was significantly elevated on day 28 in comparison to un-transduced self-differentiated iPS cells (Supplementary Fig. 2). As an internal control, Small nucleolar RNA (SNORD) was evaluated.

3.2. Morphological evaluation of embryoid bodies (EBs)

Since pLentiIII-mir-1-GFP plasmid contained the GFP-gene marker, the miRNA transcription was long with the expression of GFP; the latter was assessed by fluorescent microscopy on days 3 and 6 in three groups (control group (un-transduced EBs), mir-1 transduced group and Azacitidine treated/mir-1 transduced (AZA & mir-1) group) to verify the probable morphological alterations. As shown in Fig. 1, application of both Azacitidine and mir-1 increased the shininess of the EBs. In addition, iPS cells in form of EBs spread out more in day 6 than day 3. Therefore, morphological analysis represented that the mentioned treatments led to observable alterations.

3.3. Transcriptional evaluation of cardiomyocyte markers

It is well known that GATA4, Troponin-T and MHC-α are cardiomyocyte markers. Therefore, in order to investigate the cardiomyocyte differentiation, their transcriptional alterations were quantitatively evaluated in two groups: AZA&mir-1 transduced cells and self-differentiated ones (control untransduced group, 28 day cultured iPS cells). Beta-actin was assessed as internal control to normalize the other data. As presented in Fig. 2, all of these markers were upregulated significantly in the AZA&mir-1 group.

3.4. Translational evaluation of cardiomyocyte markers

3.4.1. Fetal liver kinase 1 (Flk-1)

Fluorescent microscopy evaluation of the adherent iPS cells after treatment by PE-conjugated anti-Flk-1 on days 7 and 14 are shown in Fig. 3. The nuclei were stained with DAPI. As seen, more Flk-1 was expressed in day 14. Also application of both AZA and mir-1 resulted in more Flk-1 expression in both time points.

3.4.2. Troponin

Similarly, fluorescent microscopy evaluation of the adherent EBs (spread to iPS cells) after treatment by PE-conjugated anti-Troponin on days 7 and 14 are shown in Fig. 4. The nuclei were stained with DAPI. Observably, the transduced iPS cells with mir1 under induction of AZA expressed much more Troponin than the other groups. Worth of note, a clear time-dependence was observed since the troponin expression was greater on day 14 than on day 7.

4. Discussion

Cardiac disorders affect the quality of people lives; so many researches have been performed to heal the heart both functionally

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**Table 1**

The sequence of the human primers.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Sequence Up: Forward, Down: Reverse</th>
<th>Annealing temp.</th>
<th>Amplicon length</th>
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<tr>
<td>Troponin-T</td>
<td>CCA GGG CAG AAG ATG CCA TTC TCT CAT CCG</td>
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<td>135</td>
</tr>
<tr>
<td>Alpha MHC</td>
<td>GCT TCA TCT AAG AGA AAG GCC ACT TGT CAC TCT CAT C</td>
<td>58</td>
<td>135</td>
</tr>
<tr>
<td>GATA4</td>
<td>CAC CAG CTC TCT CAT CAC CCG AGT CAT CTT G</td>
<td>59</td>
<td>138</td>
</tr>
<tr>
<td>Beta ACTIN</td>
<td>CTT CCT TCG GCA TG GTC TTI GCG GAT GTC CAC</td>
<td>55.5</td>
<td>85</td>
</tr>
</tbody>
</table>

**Fig. 1.** Fluorescent microscopy of transduced EBs on day 3 and day 6 after treatment, when they were spread on the plate surface. Scale bars: 20 μm.
Establishment of cardiac cellular model helps to i) improve developmental study of human cardiogenesis in vitro; ii) provide a continuous supply of these precious cells for both novel drug discovery testing for regenerative medicine, and for the study of human cardiac disease.

Utilization of iPS cells as self-stem cell therapy and stimulation of these cells into cardiomyocytes by induction factors (i.e. hormones or epigenetic factors such as miRNAs and chemical agents) significantly improves future cardiac regenerative therapies [39]. miRNAs are all expressed endogenously and are supposed to be safe for normal cells [40]. Therefore any cardiac induction protocol, based on RNA, is safe and thus, more applicable in clinical

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**Fig. 2.** Transcriptional evaluation of the cardiomyocyte markers (GATA4, Troponin-T and MHC-α) on day 28 of culture of AZA&mir-1 transduced cells versus self-differentiated ones (untransduced control group). Beta-actin was assessed as internal control and the data were normalized to day one of culture.

**Fig. 3.** Immunocytochemistry evaluation of FLK as a cardiomyocyte marker in iPSC clones after transduction with mir-1 or mir-1&AZA on days 7 and 14 of culture. Scale bars: 20 μm.
treatments than the protocols based on growth hormones. Among miRNAs, mir-1 has been investigated as cardiomyogenesis agent in many researches [41-43].

In the present work, we provided a novel protocol for iPS cells induction to cardiomyocytes using both mir-1 induction and 5’-Azacitidine treatment in a period of 28 days. iPS cells were generated and characterized as mentioned in previously published work of this team [20].

Since EBs have spherical shapes with masked inner cells, ectopically lentiviral transduction by hanging drop method during EBs formation ensured us about the exposure of most of the cells to lentiviral particles. Quantitative evaluation of mir-1 confirmed its overexpression in the transduced cells compared to control (self-differentiated cells) (Supplementary Fig. 2).

Efficiency of mir-1 transduction using a GFP-containing plasmid was evaluated by fluorescent microscopy. As illustrated in Fig. 1, the EBs were more spread in day 6 compared to day 3. Interestingly supplementation of AZA led to shinier colonies that could be due to co-transmission of GFP-containing plasmids with AZA during the passive diffusion process across the iPSC membrane [44] which increased the transduction efficiency.

For the evaluation of cardiomyogenic differentiation, we assessed cardiac markers at the transcriptional and translational levels. Troponin is a sarcomeric molecular regulator, directly regulating the contractile event in concert with intracellular calcium signals [45,46]. In addition, Troponin is the regulatory complex of the myofibrillar thin filament that plays a critical role in regulating excitation-contraction coupling in the heart [47]. Cardiac troponin is exclusively expressed at high concentrations in the cardiac muscle [48,49].

The zinc finger transcription factor GATA4 is among the best-studied cardiac transcriptional factors [50]. GATA4 binds and participates in establishing active chromatin regions that changes markedly between fetal and adult heart. Cardiac stress restored GATA4 occupancy to a subset of fetal sites [51].

Changes in the relative abundance of MHC-β and MHC-α (the fast isoform of cardiac myosin heavy chain) correlate with the contractile velocity of cardiac muscle [52,53].

Quantitative investigation of GATA4, Troponin-T and MHC-α as cardiac transcription markers showed significant transcriptional upregulation in differentiated iPS cells (on day 28 of culture) compared to undifferentiated ones (Fig. 2).

The Flk-1 as the cell marker associated with the earliest commitment stages in heart development, has been proposed by several investigators [54–57].

Investigation of these two cardiac markers, Flk-1 and Troponin, indicated their upregulation in protein levels (Fig. 4) and confirmed the previous findings in transcript patterns. For both markers, increasing in the period of culture-time led to higher expression. Moreover, AZA and mir-1 synergistically induced cardiomyogenesis in iPS cells. This finding was in concurrence with transcriptional results.

Accumulating evidences revealed by quantitative evaluation of transcription and translation of cardiac markers supports a new direct protocol for cardiac differentiation of iPS cells. From a clinical point of view, this raises a possibility that administration of miRNA mimic or miRNA inhibitor therapies could increase allocation of iPS cells into the cardiac lineage. Although microRNAs are emerging as promising therapeutic agent for cardiac programming, development of a safe delivery system directed at the target tissues is the key obstacle for their successful clinical application [58]. Other barriers have to be overcome prior to their clinical application are...
optimize programming efficiency, epigenetic barriers to cell programming, requisite growth factors and environmental cues [59]. Taking all the results into account, this study could provide further progress in the patient’s own cells for more effective therapies; making this cellular model appealing as a useful target for cell-based therapeutic strategies and personalized drug screening.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.biologics.2017.09.003.

References


