Generation of Pure Cardiomyocytes from Murine Induced Pluripotent Stem Cells by Puromycin Purification of Cardiomyocytes

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Abstract

Background: Embryonic and induced Pluripotent Stem cells (ES/iPS cells) are promising cell sources for cardiac regenerative medicine but their clinical use is associated with tumor formation as generated cardiac cells will always have few escaping pluripotent cells that proliferate after transplantation and make problems. To realize ES/iPS cell-based cardiac cell therapy, efficient induction, purification, and transplantation methods for cardiomyocytes are required. Though marker gene transduction or fluorescent-based purification methods have been reported, fast, efficient and scalable purification methods with no genetic modification are essential for clinical purpose.

Aim of Work: In this study, we attempted to purify murine iPS cell-derived cardiomyocytes. The desired result is to eliminate all pluripotent cells and to deliver 100% pure cardiomyocytes.

Methods and Results: With the use of a standard Embryoid Body (EB)-based differentiation protocol for ES cells, murine iPS cells were differentiated into cardiomyocytes. The differentiation resulted in an average of 70% of spontaneously contracting EBs at day 9 of differentiation. As murine iPS expressed the neomycin-resistance gene under the control of the cardiac α-Myosin Heavy Chain (α-MHC) promoter, addition of puromycin to the beating EBs resulted in 100% beating EBs. Further puromycin purification of plated single cells derived from dissociated EBs results in pure beating cardiomyocyte production. It constitutes 92% of cultured cells by flow cytometry.

Conclusion: We succeeded in efficiently inducing cardiomyocytes from murine iPS cells and selecting the murine iPS cell-derived cardiomyocytes for robust, efficient and scalable purification. These findings would offer a valuable technological basis for iPS cell-based cell therapy.

Key Words: Stem cells – Pluripotency – Cardiomyocytes – Purification – Puromycin.

Introduction

STEM cells, especially Embryonic Stem (ES) cells, which are able to self-renew and differentiate into cells of all 3 germ layers, have become a potential source for cell-based therapy and tissue engineering [1]. Recent studies show that it is now possible to convert somatic cells into pluripotent stem cells that closely resemble ES cells [2-6]. These induced Pluripotent Stem (iPS) cells can be generated from the patient's own somatic cells without using embryos or oocytes, thus bypassing many ethical issues and solving the problems of immunological rejection associated with the use of allogeneic ES cell-derived progenitors [7-12].

The lineage-specific differentiation of hPSCs, including human Embryonic Stem Cells (hESCs) and human induced Pluripotent Stem Cells (hiPSCs), into functional mature cell types is an important step in unfolding their full potential. Ultimately, such in vitro-generated hPSC-derived progenies are potential building blocks for novel therapeutic approaches or cell type-specific in vitro assays in drug discovery. Some of these applications require large numbers of cells to be produced [13].

To establish ES/iPS cell-based cardiac cell therapy, efficient induction, purification and transplantation methods for cardiomyocytes are required. High differentiation efficiencies of cardiomyocytes (approximately 30-80%) have been reported in some protocols [14,15]. Nevertheless, these efficient methods still did not provide pure cardiomyocytes. Contamination of undifferentiated ES/iPS cells would cause teratoma formation after transplantation. Moreover, for application of ES/iPS cell-derived cardiomyocytes to clinical purpose, large-scale purification would be required. Thus, the establishment of cardiomyocyte purification methods has been long awaited [16].
As there are relatively few antibodies available for the isolation of cardiac progenitors, investigators have genetically engineered ES cells to enable specific selection of cells representing different stages of development within the lineage. An ES cell line has been reported in which lacZ expression is under the control of the cardiac specific promoter human cardiac α-actin [17]. This allows also a vital stain approach, in order to investigate early stage cardiomyocytes functionally. This approach has been further improvised by the establishment of stably transfected ES-cell lines, where in vivo reporter genes are under control of very early, cardiac specific promoters. ES cells have been generated to express either drug-resistance or fluorescent genes under the control of promoters that drive expression at specific stages of cardiac development. In the first of these approaches [18], expressed the neomycin-resistance gene under the control of the cardiac α-Myosin Heavy Chain (α-MHC) promoter. With G418 selection at appropriate stages of development, populations highly enriched (>99%) for cardiomyocytes were isolated. When applied to large-scale cultures, this strategy enabled the generation of large numbers of cardiomyocytes [19]. Other strategies involve expressing the Green Fluorescent Protein (GFP) from cardiac specific promoters including Nk X 2.5 [20], cardiac α-actin [21] and myosin light chain-2v [22]. Expression from myosin light chain-2v was designed to specifically select for ventricular cells from the ES cell differentiation cultures. Cells selected on this basis displayed electrophysiological properties of ventricular cardiomyocytes, indicating that the strategy was successful. Recently, a FACS-based isolation of cardiac progenitors from murine iPS cells expressing Green Fluorescent Protein (GFP) under the control of Nk X 2.5 promoter has been reported [23]. Similarly, Rust et al., [24] have used CD166 (ALCAM) specifically expressed in cardiac cells by puromycin treatment. Selection is based on activation of the cardiac α-MHC promoter.

Material and Methods

This work was performed at the Institute of Neurophysiology, University of Cologne, Germany during the period from the 15th of March 2015 to the 15th of September 2015.

Murine iPS cell line:

The murine iPS cell line used in the present study is TiB7-4; kindly provided by the institute of Neurophysiology, University of Cologne, Germany. It was generated by Meissner and Jaenisch (Whitehead Institute of Technology, MA, USA) by reprogramming of adult murine fibroblasts with defined factors (Oct4, Sox2, cMyc and Klf4). TiB7-4 murine iPS cells were transfected with an Undifferentiated Transcription Factor (UTF1)-promoter-driven G418 (“Neo”) resistance, genetically selected and enriched for stable pluripotent phenotypes. UTF1-Neo can serve as a selection marker to standardize iPS cell quality. TiB7-4 murine iPS cells were stably genetically modified by plasmid electroporation to express a resistance protein against the antibiotic puromycin (puromycin-acetyltransferase) under control of the cardiomyocyte-specific α-myosin heavy chain (α-MHC) promoter. The α-MHC promoter-driven puromycin resistance was introduced in order to purify murine iPS cell-derived cardiomyocytes after differentiation.

Experiment:

Here we demonstrate an approach to purify functional cardiomyocytes derived from murine iPS cells. It relies on the cardiac differentiation of the transgenic murine iPS cells expressing neomycin resistance using EB-based differentiation protocol followed by purification of the generated EBs as well as the EBs-derived plated single beating cells by puromycin treatment. Selection is based on activation of the cardiac α-MHC promoter.

1- Murine iPS cell culture:

Murine iPS cells were maintained on mouse embryonic fibroblasts (MEFs; 50,000 cells/cm²) feeder layer in 6-cm culture plates. Exposure to 10 µg/mL mitomycin C (Sigma-Aldrich, Taufkirchen, Germany) was used for inactivation of the MEFs. The culture medium was based on Iscove’s modified Dulbecco’s medium (Invitrogen) (IMDM) and supplemented with 17% fetal calf serum, 0.2 mmol/L L-glutamine, 0.1 mmol/L β-mercaptoethanol, and 0.1 mmol/L nonessential amino acid stock with the addition of leukemia inhibitory
factor (LIF; 500U/ml). Colonies were passaged as single cells once every 2 days using 0.05% Trypsin/EDTA for 5 min at 37°C and 5% CO₂ and plated on feeder layer in IMDM 17%.

2- Murine iPS differentiation into cardiomyocytes:
Cardiomyocyte differentiation was induced by embryoid bodies-based differentiation protocol as previously reported (the first paper, unpublished data). At day 0 of differentiation, murine iPS cells maintained on feeder layer in IMDM 17% were dissociated into single cells using trypsin/EDTA (0.05%), seeded in 10mm bacteriological dish (non-adhesive plastic ware) in IMDM and supplemented with 20% fetal calf serum, 0.2mmol/L L-glutamine, 0.1mmol/L β-mercaptoethanol, and 0.1mmol/L nonessential amino acid stock without LIF with the addition of Ascorbic Acid (500 µg/ml). Cells were incubated in suspension on a horizontal shaker inside a cell culture incubator.

On day 2 of differentiation, EBs were collected, counted, distributed at a density of 1000EBs/10mm dish in 20% IMDM without LIF with the ascorbic acid and continuous agitation. Starting from day 7 of differentiation, EBs were examined daily for spontaneously beating areas under an inverted microscope. Once starting to beat, medium was changed with IMDM 5%.

3- Purification of culture by puromycin:
Once beating, the EBs were treated with Puromycin (8 µg/ml) on day 9 and 11 of differentiation in order to purify the culture.

On day 13, EBs were collected, washed and 0.25% Trypsin/EDTA was added to the pellet for 20 min and subsequently monitored under an inverted microscope. Afterwards cell suspension was briefly and carefully aspirated repeatedly with a pipette (10 times) and dissociation was then stopped by the addition of FCS-containing medium. Dissociated Cells were passaged through a 40-µm sieve to achieve single-cell suspensions. Single cells were plated on fibronectin-coated cell culture dishes and cultured in IMDM 5%. The medium was changed daily.

Plated EB-derived beating single cells were treated with puromycin (8 µg/ml) on day 14 and 15 of differentiation. On day 16 of differentiation, the cardiomyocytes were ready for characterization.

Fig. (1): Timeline of murine iPS cell differentiation into cardiomyocytes with puromycin purification.

4- Characterization of puromycin selected murine iPS cell-derived cardiomyocytes:
• Dissociation of murine iPS cell-derived cardiomyocytes:
  I- The differentiated cells were washed with 1 mL of PBS -/–. The PBS was aspirated and 2 mL of 0.25% (wt/vol) trypsin-EDTA was added per well. The mixture was incubated in a 37°C, 5% CO₂ incubator for 5 min.
  II- 2 mL of IMDM 5% was added to stop trypsin with the contained FBS. The mixture was pipetted 5-10 times with a P1000 blue tip to singularize the cells.
  III- Cell mixture was transferred into a 15-ml conical tube, centrifuged at 1000 rpm for 5 min at RT and the supernatant was aspirated.
  IV- The cell pellet was resuspended in IMDM 5% and counted by hemocytometer. The cells are now ready for characterization.
• Flow cytometry:
  I- On day 16 post-differentiation, the cell pellet of the dissociated cells was resuspended in 1 mL of IMDM 5%, counted by hemocytometer and divided into 3 X 1.5 mL tubes at a concentration of 500,000 cells each and used as follows:
    Tube 1: Negative control.
    Tube 2: Isotypic control.
    Tube 3: Characterized murine iPS cell-derived cardiomyocytes.
  II- The cell pellet of each tube was resuspended in ice-chilled methanol and then incubated at 4°C for 20 min.
III- Then methanol was aspirated and 2mL of FlowBuffer-1 was added, centrifuged at 200g for 5min at RT. The supernatant was then aspirated. This wash was repeated two times to remove the methanol.

IV- Then every tube was treated differently.

• The cell pellet in tube 1 was resuspended in 300μL of FlowBuffer-1 ready for analysis.

• The cell pellet in tube 2 was resuspended in 100μL of FlowBuffer-2 with the appropriate dilution of isotype control, mouse IgG2a isotype-control immunoglobulin (1:100; Santa Cruz Biotechnology; Prod# Sc-3878).

• The cell pellet in tube 3 was resuspended in 100μL of FlowBuffer-2 with the appropriate dilution of the primary antibody, cardiac Tropinin-T, mouse monoclonal IgG2a anti-cardiac Tropinin-T (Clone CT3, 1:100; Santa Cruz Biotechnology; Prod# sc-20025). The mixture was in-cubated for 1 hour at RT.

V- The cells were washed with 2mL of FlowBuffer-2 and the cell pellet in tube 2 and 3 were resuspended in 100μL of FlowBuffer-2 containing 1:1000 dilution of secondary antibody, goat-anti-mouse-IgG-AlexaFluor 555 (Polyclonal, 1:1000, Life technologies, Prod# A-21424). The mixture was incubated for 30min at RT in the dark.

VI- The cell pellet in tube 2 and 3 were washed with 2mL of FlowBuffer-2 twice; the cell pellet was resuspended in 300μL of FlowBuffer-1 and transferred into flow round-bottom tubes. The flow tubes were kept on ice and the flow cytometric analysis was done using attune acoustic focusing cytometer.

Results

In vitro cardiomyocyte differentiation of murine iPS cells:

The murine iPS colonies were propagated in the undifferentiated state on top of the MEF feeder layer Fig. (2A) in the presence of LIF.

To induce cardiomyocyte differentiation, the murine iPS cells were removed from the MEF feeder and cultivated in suspension, where they formed 3-dimensional differentiating cell aggregates (EBs) Fig. (2B). After 7 days in suspension, rhythmically contracting EBs started to appear Fig. (2C). The percentage of iPS cell-derived EBs with beating areas continued to increase to 70% on day 9 of differentiation. The contracting areas had a diameter ranging from 0.2 to 1.5mm and continued to beat vigorously for several weeks in culture.

Puromycin purification:

On day 9 and 11 of differentiation, the EBs were treated by puromycin. During puromycin treatment, the non-puromycin-resistant cells died, and beating clusters of puromycin-resistant beating EBs were progressively enriched.

On day 13, EBs were dissociated into single cells which were plated on fibronectin-coated cell culture dishes. Plated beating single cells shown in Fig. (2D) were treated by puromycin on day 14 and 15 of differentiation. Puromycin-resistant 15-day-old cardiomyocytes were progressively enriched.
Characterization of puromycin-selected murine iPS cell-derived cardiomyocytes by flow cytometry:

To determine the percentage of cardiomyocytes within the purified cultures and to evaluate the overall cardiomyocyte differentiation efficiency, we performed flow cytometric analysis of the murine iPS cell-derived cardiomyocytes using cTnT as a cardiac-specific marker on day 16 post-differentiation after 2 times of puromycin treatment: CTnT was specifically expressed on the differentiated cells giving rise to a population consisting of 92% Fig. (3) contracting cTnT-positive cells with subsequent purification.

On day 16 of cardiac differentiation, the purified murine iPS cell-derived cardiomyocytes were analyzed by flow cytometry after being subjected twice to puromycin selection giving rise to 92% cTnT+ cardiomyocytes. Red indicates the differentiated cell population stained positively for cTnT, purple indicates differentiated murine iPS cells that don't stain with cTnT while blue indicate the isotope control.

![Flow cytometric analysis of puromycin selected murine iPS cell-derived cardiomyocytes.](image)

**Discussion**

Myocardial infarction and heart failure are leading causes of death worldwide. As the myocardium has a very limited regenerative capacity, endogenous cell regeneration cannot adequately compensate for heart damage caused by myocardial infarction. The concept of cell replacement therapy is an appealing approach to the treatment of these cardiac diseases. Human Pluripotent Stem Cells (hPSCs) are an attractive cell source for cell replacement therapies because they can be expanded indefinitely in culture and efficiently differentiated into a variety of cell lineages, including cardiac cells [25].

The importance of derivation of homogenous cell population is not limited to pluripotent state but also to the mature cells types like cardiomyocytes. Purification of cardiomyocytes from the differentiating cultures of pluripotent stem cells is especially important to study cardiac specific developmental, functional and transcriptional process.

In the present study, we have analysed whether selection for murine iPS cell-derived cardiomyocytes based on puromycin purification is suitable to generate high quality cell lines, such as required for future transplantation studies and therapeutic applications. The murine iPS cell line TiB7-4 used for this study was generated by reprogramming of adult murine fibroblasts with the four transcription factors Oct4, Klf4, Sox2 and c-Myc [26]. The selection was performed based on a human UTF 1 promoter/enhancer driven neomycin-resistance transgene led to virtually absolute pluripotent cultures that could be maintained for at least 40 passages.

Our results go hand in hand with other studies that showed that genetic enrichment strategies using drug selection markers expressed under the control of cardiac specific promoters have been successfully applied for purification of murine and human ES-cardiomyocytes [27-31]. These approaches permitted genetic and physiological studies of pure cardiomyocytes without the potentially confounding influence of other cells present in differentiating EBs. These cell lines represent excellent tools for identification of new agents that direct differentiation of pluripotent stem cells to cardiomyocytes and for optimization of scalable production of pure cardiomyocytes in controlled bioreactors. In addition, the ability to produce high numbers of well characterized cardiomyocytes that will facilitate progress in basic research leading to cell based therapy, disease modeling, drug screening and drug discovery.
The establishment of homogenous cell populations is of importance for a variety of purposes. Lines of ES as well as iPS and pre-differentiated cells that contain a mixture of pluripotent and pre-differentiated cells might be less suited to perform in vitro differentiations to study aspects of differentiation or to form specific derivatives for detailed analysis [32-34]. It is therefore highly desired to generate homogeneous populations of iPS cells since heterogeneous cultures will potentially obscure findings.

Conclusion:

Making use of an UTF1 promoter driven G418 cassette inserted in murine iPS cell line, we succeeded in efficiently inducing cardiomyocytes from murine iPS cells and selecting the murine iPS cell-derived cardiomyocytes for robust, efficient and scalable purification. These findings would offer a valuable technological basis for iPS cell-based cell therapy.

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