Differentiation of Murine Induced Pluripotent Stem Cells into Cardiomyocytes

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Abstract

Background: The recent breakthrough in the generation of induced Pluripotent Stem (iPS) cells, which are almost indistinguishable from Embryonic Stem (ES) cells, facilitates the generation of human patient-specific disease-specific stem cell lines. The ability to generate a reproducible murine iPS-cardiomyocyte differentiation system may bring unique value to the emerging field of personalized medicine: For the establishment of patient-and disease-specific disease models, for drug screening and for the establishment of future autologous myocardial cell-replacement therapies.

Aim of Work: The aim of this study was to characterize the cardiomyocyte differentiation potential of murine iPS cells.

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 80% of spontaneously contracting EBs at day 13 of differentiation. Analysis on structural level demonstrated that iPS cell-derived cardiomyocytes show typical features of ES cell-derived cardiomyocytes. Immunofluorescent staining confirmed expression of cardiomyocyte-typical protein; sarcomeric α-actinin.

Conclusions: iPS cells differentiate into functional cardiomyocytes. In contrast to ES cells, iPS cells allow derivation of autologous myocardial cell-replacement therapies. These results hold great promise for the development of in vitro models of cardiac genetic disorders, for drug discovery and testing, and for the emerging field of cardiovascular regenerative medicine.

Key Words: Stem cells – Pluripotency – Differentiation – Embryoid bodies – Cardiomyocytes.

Introduction

EMBRYONIC Stem (ES) cells are widely used as an in vitro model to study developmental processes. Pluripotency is maintained by culturing murine ES cells on a layer of embryonic fibroblasts (“feeder cells”) with Leukemia Inhibitory Factor (LIF). The addition of LIF is necessary to activate STAT3 signaling, which is critical for stem cell pluripotency [1,2]. The network that regulates pluripotency is complex and involves genes of the pluripotency core circuits, including Oct4, Nanog [3,4] and Utf1 [5,6], as well as several markers that are associated with the pluripotency phenotype.

Differentiation of murine ES cells is usually initiated by dissociation of the cells, aggregation of single ES cells in spherical clusters in suspension (spheroids) and LIF withdrawal. Spheroids that form by agitation of a cell suspension or by clustering of ES cells in hanging drops are known as Embryoid Bodies (EBs). EBs resemble features of early embryonic development during in vitro culture. The endoderm, mesoderm and ectoderm are formed, and a variety of different cell types are developing, including cardiomyocytes [7], smooth muscle cells [8], chondrocytes [9] and cells of the nervous system, such as glial cells [10].

In 2006, Takahashi and Yamanaka [11] were able to reprogram mouse fibroblasts by ectopic retroviral expression of 4 transcription factors (Oct4, Sox2, c-Myc, and Klf4), yielding cells with characteristics similar to those of mouse ESCs. The resulting mouse induced Pluripotent Stem (iPS) cells were demonstrated to be able to differentiate into cell derivatives of all 3 germ layers, including functional cardiomyocytes [12,13]. In the present study, we aimed to maintain murine induced pluripotent stem cell cultures, embryoid bodies formation and their differentiation into cardiac cells.

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).

**Experiment:**

Here we demonstrate an approach to generate functional cardiomyocytes from murine iPS cells by EB generation using Ascorbic acid to stimulate cardiogenesis. It relies on treatment of undifferentiated murine iPS with ascorbic acid in the absence of LIF in IMDM 20%.

**Maintenance of murine iPS cells:**

To maintain their undifferentiated state, the undifferentiated murine iPS colonies were cultured on monolayer of irradiated, mitotically inactivated 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, 0.2mmol/L L-glutamine, 0.1 mmol/L P-mercaptoethanol, and 0.1mmol/L non-essential amino acid stock with the addition of leukemia inhibitory factor (LIF; 500U/ml). On reaching confluency (every 2 days), colonies were dissociated using 0.05% trypsin/EDTA for 5min at 37ºC and 5% CO₂. 1 X 10⁶ cells were plated in 10mm bacteriological dish (non-adhesive plastic ware). The differentiation medium was based on IMDM and supplemented with 20% fetal calf serum, 0.2mmol/L L-glutamine, 0.1mmol/L P-mercaptoethanol, and 0.1mmol/L nonessential amino acid stock without LIF with the addition of Ascorbic Acid (500 µg/ml) at 37ºC and 5% CO₂. Cells were incubated in suspension on a horizontal shaker inside a cell culture incubator.

**2- EB splitting:**

At day 2 of differentiation, EBs were collected in a 15ml Falcon tube and counted. EBs were 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- Dissociation of EBs into single cells:**

On day 13, EBs were collected in a 15ml centrifuge tube, left for 5min to slowly collect by gravity at the bottom of the tube, supernatant was then carefully aspirated. Afterwards, cells were washed twice with calcium-and magnesium-free PBS, supernatant was removed and 0.25% Trypsin/EDTA was added to the pellet and cell clusters were then incubated (37ºC and 5% CO₂) for 20min 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 passed through a 40- µm sieve to achieve single-cell suspensions. Single cells were plated on fibronectin-coated cell culture dishes and cultured for up to 3 days at 37ºC and 5% CO₂ in IMDM 5%. The medium was changed daily.

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**Fig. (1):** Timeline of murine iPS cell differentiation into cardiomyocytes.
4- Characterization of murine iPS Cell-derived cardiomyocytes:

**Immunostaining:**

On day 16 of differentiation, cells were fixed with 4% ice-chilled methanol for 20min at room temperature. Blocking of non-specific binding was accomplished with Roti-Immunoblock reagent (1:100, Carl Roth, Karlsruhe, Germany) for 60-120 min at room temperature. Primary detection was done overnight at 4°C with antibodies against mouse monoclonal IgG1 anti-sarcomeric α-actinin (clone EA53, 1:800, Sigma-Aldrich; Germany; Prod# A7811). Following rigorous washing, the preparations were subsequently incubated with appropriate secondary antibodies for 2 hours at room temperature in the dark. Secondary detection was performed with goat anti-mouse-IgG-Alexa-Fluor 647 (Polyclonal, 1:1000, Prod# A-21236). After washing in the dark, the coverslips were sealed to glass slides with ProLong Gold Antifade Reagent with DAPI (Life Technologies) and analyzed on a Zeiss Axiovert 200 inverted microscope equipped with apotome. For analysis, the Zeiss AxioVision 4.5 software package (Zeiss, Göttingen, Germany) was applied.

**Results**

1- **Murine iPS cell culture:**

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

**In vitro cardiomyocyte differentiation of murine iPS cells:**

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 until 80% on day 13 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.

On day 13, EBs were dissociated into single cells which were plated on fibronectin-coated cell culture dishes. Plated beating single cells are shown in Fig. (2D).

**Structural characterization of the murine iPS cell-derived cardiomyocytes:**

Immunofluorescent staining confirmed expression of cardiomyocyte-typical protein, sarcomeric α-actinin. Staining of single beating cells of murine iPS cell-derived cardiomyocytes on day 16 post differentiation revealed a clearly cross-striated pattern of cardiac myofilamental protein sarcomeric α-actinin Fig. (2E). These results show that the differentiated cells developed well-organized sarcomeric structures and express typical sarcomeric markers.

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Fig. (2): Cardiomyocyte differentiation of murine iPS cells:
A- Undifferentiated murine iPS colonies are propagated on an MEF feeder layer.
B- To induce differentiation, the murine iPS cells are cultivated in suspension where they form EBs.
C- Spontaneously beating EBs were observed at day 7.
D- Spontaneously beating plated single cells from dissociated beating EBs.
E- Immunostaining of murine iPS-cell derived cardiomyocytes show well organized cross striation pattern of sarcomeric α-actinin, nuclei stained with DAPI (scale bar 50µm).
Discussion

The heart is the first organ to function in the embryo, and this function is essential for survival during fetal life. The beating heart is the most readily detectable sign of life. Heart development is a finely tuned complicated process. It has been well-studied, and the pathways that coordinately regulate cardiac differentiation and morphogenesis are well-defined [14].

A series of recent articles [15-21] based on the groundbreaking study of Takahashi and Yamanaka [6] demonstrated that the reprogramming of somatic cells to a pluripotent state can be achieved by simple retroviral overexpression of specific transcription factors and that the resulting iPS cells are almost indistinguishable from ES cells [7,15,18]. The latest development showing production of human iPS cells, even in the absence of the proto-oncogene c-Myc [13,21] and without the need for drug selection [13,22], opens up new opportunities for the establishment of patient-specific stem cell lines.

Although pluripotency has been demonstrated by the generation of teratomas and by the live birth of chimeric mice after blastocyst injection [15,17,20], very limited data are available concerning the in vitro differentiation potential of iPS cells into specific cell lineages [11,16]. It was therefore the aim of the present study to investigate the cardiac differentiation potential of a murine iPS cell clone (TiB7-4) and to characterize the iPS cell-derived cardiomyocytes on structural level.

To maintain pluripotency, murine embryonic stem cells were cultured on fetal fibroblasts commonly referred to as feeder cells. Alternative culture protocols describe the maintenance of pluripotent cells under feeder-free conditions. Under both culture conditions, the application of exogenous LIF is mandatory to maintain the pluripotent state, maintain the cells in a state of self-renewal and prevent the onset of differentiation. EB formation is a commonly used model to study the differentiation of ES cells in vitro. Differentiation is initiated by the aggregation of pluripotent cells in suspension and withdrawal of LIF. The developing EBs show some characteristics of early embryonic development, including specification of the three germ layers and axis formation [23].

Maturing CMs can be identified by the expression of cardiac structural proteins such as α-actinin, α-MHC or the cTnT. By initiating the complex myocardial cross-regulatory network, these factors are believed to be involved in morphogenetic events leading to the formation of the heart [24].

On the basis of a well-established standard differentiation protocol for ES cells, iPS cell clone (TiB7-4) showed a maximum of 80% spontaneously beating EBs on day 13 of differentiation. Immunofluorescence analyses on day 16 of differentiation showed that the iPS cells expressed cardiomyocyte-specific proteins and had well-organized cross-striations that were indistinguishable from those seen in ES cell-derived cardiomyocytes. Our results showing the ability to achieve cardiomyocyte differentiation of murine iPS cells are in agreement with 2 recent studies showing similar cardiomyocyte differentiation potential in the mouse iPS cell system [12,13].

Conclusion:

In conclusion, our study shows that iPS cells are capable of generating cardiomyocytes that are functionally comparable to ES cell-derived cardiomyocytes. Our results establish a valuable basis for creating animal models for the development of iPS cell-based cellular cardiomyoplasty. Although there are many unresolved questions and many hurdles to overcome, the ability to generate a reproducible murine iPS-cardiomyocyte differentiation system may bring unique value to the emerging field of personalized medicine: For the establishment of patient-and disease-specific disease models, for drug screening and target validation, and for the establishment of future autologous myocardial cell-replacement therapies.

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References


تمايز الخلايا الجزيئية الخاصة بالفئران المحفزة لتكون متعددة القدرات إلى خلايا عضلة القلب

الخلايا الجزيئية المتعددة (iPS)، والتي لا يمكن تمييزها تقريباً من الخلايا الجذعية الجنينية (ES) في الفئران، تم تمييزها باستخدام طريقة جذعية خاصة بالمرض المحدد. إن تميز خلايا iPS الخاصية بالفئران إلى خلايا عضلة القلب قد تجلب قيمة كبيرة من ناحية في مجال الطب الشعبي من حيث إنشاء نماذج لأمراض خاصة بمريض محدد، وبالتالي يمكن للبحث تأثير الأدوية وتكوين العلاج بإنتاج خلايا عضلة القلب بالخلايا الذاتية في المستقبل.

الهدف من الدراسة: كان الهدف من هذه الدراسة هو تحديد خصائص خلايا عضلة القلب المولدة من الخلايا الجزيئية المحفزة لتكون متعددة الخلايا الخاصة بالفئران.

طرق البحث والنتائج: باستخدام بروتوكون التماثيل الخاص بالخلايا الجنينية عن طريق الأجسام المضيفة (EB), تم تمييز خلايا iPS الخاصية بالفئران إلى خلايا عضلة القلب. أدى التمثيل الابتكاري إلى متوسط 80% من الأجسام المضيفة للخلايا المتميزة في اليوم 12 من التمثيل. أظهر التحليل على المستوى الهيكلي أن خلايا عضلة القلب المشتقة من الخلايا الجنينية, 

α-actinin أظهرت التعبير عن علاجم محددة للقلب. 

الاستنتاجات: خلايا iPS قادرة على التماثيل إلى خلايا عضلة القلب الوراثية وعلى التقييم من الخلايا الجذعية الجنينية, خلايا iPS تسمح بإنشاء الخلايا الجذعية الوراثية ذاتية. تقدم هذه الدراسة وعالية كبيرة لتمثيل نماذج من الإضطرابات الوراثية للقلب في المختبر، والإكتشاف الأنثوي والدليل، وبالتالي استخدام في مجال الطب التجريبي للقلب والأوعية الدموية.