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The influence of physiological matrix conditions on permanent culture of induced pluripotent stem cell-derived cardiomyocytes

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ABSTRACT

Cardiomyocytes (CMs) from induced pluripotent stem (iPS) cells mark an important achievement in the development of *in vitro* pharmacological, toxicological and developmental assays and in the establishment of protocols for cardiac cell replacement therapy. Using CMs generated from murine embryonic stem cells and iPS cells we found increased cell–matrix interaction and more matured embryoid body (EB) structures in iPS cell-derived EBs. However, neither suspension-culture in form of purified cardiac clusters nor adherence-culture on traditional cell culture plastic allowed for extended culture of CMs. CMs grown for five weeks on polystyrene exhibit signs of massive mechanical stress as indicated by α-smooth muscle actin expression and loss of sarcomere integrity. Hydrogels from polyacrylamide allow adapting of the matrix stiffness to that of cardiac tissue. We were able to eliminate the bottleneck of low cell adhesion using 2,5-Dioxopyrrolidin-1-yl-6-acrylamidohexanoate as a crosslinker to immobilize matrix proteins on the gels surface. Finally we present an easy method to generate polyacrylamide gels with a physiological Young's modulus of 55 kPa and defined surface ligand, facilitating the culture of murine and human iPS-CMs, removing excess mechanical stresses and reducing the risk of tissue culture artifacts exerted by stiff substrates.

1. Introduction

Induction of pluripotency from somatic cells by defined factors facilitates the generation of iPS cells from every donor, irrespective of its age or sex [1]. Initial studies have reported on the induction of pluripotency in primary cultures of murine skin fibroblasts [2,3]. The resulting stem cells are capable to differentiate into virtually any cell type of the adult organism including functional iPS-derived cardiomyocytes (iPS-CMs). These CMs display physiological response to adrenergic and muscarinergic stimulation, express marker genes indicative of cardiac commitment, show action potentials with pacemaker-like, atrial-like or ventricular-like shape and confer force of contraction to physiological substrates [4–6].

The possibility to generate iPS-CMs not only from mice but also from any human donor, including aged and diseased persons, has gained attention of many scientists in the field of reconstructive medicine [7], toxicology and pharmacology [8]. iPS-CMs are produced from an unlimited source of pluripotent stem cells and the physiology of the cells is supposed to match the properties of the donors primary cardiomyocytes as well (discussed in Bellin et al., 2012) [9]. As an example *in vitro* model systems of QT interval prolongation (indicating increased delay between Q-wave and T-wave in the electrocardiogram) have been established with iPS cells from diseased human donors (for a review see Friedrichs et al., 2013) [10]. These models are of great interest to perform drug

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screenings on standardized setups using not only animal-derived but human cells as well.

To utilize iPS-CMs as a standardized model system in order to perform pharmacological assays and elaborate methods for reconstructive applications, a refinement of culture conditions is mandatory. Long-term culture of purified iPS-CMs is becoming necessary to evaluate effects of drugs and toxic agents *in vitro* over extended time spans and to maintain iPS-CMs without quality-loss for reconstructive purposes.

With respect to reconstructive medical applications research on iPS-CM-based therapeutic strategies is still mainly focused on animal models because most technologies are not matured yet to allow for clinical application. Murine model systems are widely used because they allow the exploration of reconstructive strategies in a mammalian model with reasonable effort.

Various culture techniques can be applied to generate long term cultures of CMs. The simplest way is a culture on traditional cell-culture-optimized polymer surfaces with neglected elasticity. We have applied such culture conditions and found them unsuitable for extended culture of iPS-CMs. Studies of the workgroup of Discher [11] have demonstrated that primary CMs of the Japanese quail react to the stiffness of a polyacrylamide (PAA) matrix, being unable to organize their sarcomeres on very soft tissues and failing to contract on very stiff substrates within only 48 h. An optimal stiffness of the culture matrix was identified at an elasticity of 11–17 kPa for these cells. The elasticity of the matrix appears therefore as a major parameter to enable culture of primary CMs and it is likely that this finding can be extrapolated to iPS-CMs.

The present study was designed to identify conditions that allow for the prolonged culture of cardiomyocytes derived from pluripotent stem cells and at high plating densities. Experiments were conducted to clarify if murine iPS-CMs can be cultured as spheroids in suspension. For comparison purified CMs were cultured on traditional cell culture surfaces. Finally a PAA matrix with optimized properties and physiological elasticity was utilized to support long-term persistence of stem cell-derived CMs to overcome common restrictions of low cell attachment and inefficient immobilization of bulky matrix proteins on PAA surfaces.

2. Materials and methods

2.1. Stem cell culture and differentiation

In order to generate purified CMs from pluripotent stem cells the established murine embryonic stem (ES) cell line αPIG44 was used [12]. For comparison a murine iPS cell line (UTF2-iPS cells) derived from adult fibroblasts and selected for pluripotency by UTF1-based selection was chosen [13]. UTF2-iPS cells were transfected with a plasmid expressing a resistance protein against the antibiotic puromycin (puromycin-acetyltransferase) under control of the cardiomyocyte-specific alpha myosin heavy chain promoter [12]. Transgenic clonal cell lines were screened for cardiac-specific expression of the puromycin-acetyltransferase (data not shown) and finally clone TαP4 was selected for further experiments.

Murine ES cells and iPS cells were maintained on a layer of mitotically inactivated feeder fibroblasts (MEFs). MEFs were obtained from e14.5 embryos of outbred mice, passaged 3 times and mitotically inactivated by incubation with Mitomycin C (10 μg/ml, Serva) for 2–4 h. For a cell culture plate with 60 mm diameter, at least 8×10^5 inactive fibroblasts were plated.

The culture of ES and iPS cells was performed at 37 °C and 5% CO_2. Cells were cultured using ES medium, composed of Iscove's modified Dulbecco's Medium (IMDM) with Glutamax (Invitrogen) supplemented with 1% Penicillin/Streptomycin 100× solution (Invitrogen) and 1% non-essential amino acids ("MEM 100× solution" (Invitrogen)) as well as 17% foetal bovine serum (Invitrogen) and 1000 U/ml of LIF (ESGRO, Chemicon). The culture was divided every 2–3 days.

To differentiate ES or iPS cells to CMs, a protocol for the production of EBs in suspension was used [14].

Briefly, to induce the EBs formation, ES or iPS cells were dissociated (0.05% trypsin/EDTA) to single cells. 2.5×10^6 cells were resuspended in 12 ml of ES medium with addition of 3 mg/ml L-Ascorbic-Acid-Phosphate-Magnesium-Salt-n-Hydrate (Wako Chemicals GmbH, Neuss, Germany) but without LIF. Cells were incubated in a bacteriological dish (non-adhesive plastic ware) at 37 °C and 5% CO_2 with continuous agitation on a rocking table inside a cell culture incubator at 50 revolutions per minutes. After two days, EBs were collected in a 15 ml Falcon tube and counted. EBs were distributed at 250 per plate (10 cm) in 12 ml ES Medium without LIF and further cultured under continuous agitation. EBs were monitored until the first spontaneous contractions were observed under an inverted microscope. Once beating EBs were found, usually on day 7th or 8th, medium containing 8 μg/ml Puromycin was used until day 15 of differentiation in order to purify CMs.

EBs containing purified CMs were then dissociated to single cells if not indicated otherwise. Cells were collected in a 15 ml centrifuge tube, left for 5 min 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 0.5 ml of trypsin/EDTA (0.05%) were added to the pellet and transferred using a 1000 μl "blue" pipette tip to a 3 cm petri dish containing 2.5 ml of trypsin/EDTA (0.05%). Cell clusters were then incubated (37 °C and 5% CO_2) for 15 min and subsequently monitored under an inverted microscope. If necessary 1 ml of fresh trypsin/EDTA (0.05%) was added and the dish was transferred again to the incubator for another 5 min. Afterwards cell suspension was briefly and carefully aspirated repeatedly with a pipette (upper limit 10 times) and dissociation was then stopped by the addition of serum containing medium.

Cells were used for downstream applications. The culture of CMs on cell culture dishes refers to tissue culture treated plastics made from polystyrene.

2.2. Human iPS cell-derived CMs

Human iPS-CMs were contributed by ReproCELL Inc. (distributed by Pelo Biotech, Planegg, Germany) as frozen single cells. Cells were cultured in ReproCardio culture medium 2. Medium was changed every alternative day.

2.3. Immunocytochemistry

For immunocytochemical staining cells on plastic surfaces were fixed by quick immersion in ice cold methanol. Fixation was performed for at least 20 min at −20 °C. Methanol was allowed to evaporate and cells were rehydrated. Blocking was performed with 5% bovine serum albumin in PBS for 1 h. Primary detection was done overnight at 4 °C with antibodies against sarcomeric actinin (1:500, clone EA53, Sigma–Aldrich), smooth muscle actin (1:500, clone 1A4, Sigma–Aldrich), and integrin-α9 (1:100, Santa Cruz Biotechnology (H-198), rabbit, polyclonal). Following rigorous washing secondary detection was performed at room temperature for 90 min with anti-mouse-IgG1-AlexaFluor488, anti-mouse-IgG2B-AlexaFluor647, and anti-rabbit-AlexaFluor647 (Molecular Probes, distributes by Invitrogen Life Technologies, Karlsruhe, Germany). Nuclei were counterstained by Hoechst33342 dye or Sytox Green. Cells on hydrogels were fixated with 4% paraformaldehyde instead and permeabilized by treatment with 0.2% Triton ×100 for 15 min at room temperature prior to antibody staining. Samples were analysed on a Zeiss Axiovert 200 inverted microscope equipped with apotome.

2.4. Scanning electron microscopy (SEM)

Murine cardiac clusters and CMs were cultured on Thermanox™ coverslips (Nunc, Thermo Scientific), made from polystyrene, the same material as in culture dishes. The samples were prepared for SEM as previously described [15]. Briefly, the samples were fixed in 2% glutaraldehyde and 2% osmium tetroxide in cacodylate puffer, and treated with 1% of tannic acid and 1% uranyl acetate in distillated water. After dehydration in increasing alcohol, the samples were dried in hexamethyldisilazane (HMDS, Sigma–Aldrich, Taufkirchen, Germany). Finally, the samples were coated with carbon and studied by SEM. After coating some samples were examined in the secondary electron (SE) mode and backscattered electron (BSE) mode. The other samples were prepared according to modified block-face SEM [16]. Briefly, the samples were embedded in epoxy resin, cross-sectioned with ultra-microtome, coated with carbon and studied by SEM in BSE mode. SEM and block-face SEM studies were performed using a field emission scanning electron microscope FESEM XL30 (Phillips, Eindhoven, Netherlands).

2.5. Preparation of PAA hydrogels

PAA hydrogels were prepared from a solution containing 0.374 ml of 15% acrylamide (w/v), 0.150 ml of 0.3% N,N′-Methylenebisacrylamide (w/v), 0.010 ml of 1.5 M NaH_2PO_4 and 0.405 ml of distilled water. The solution was briefly vortexed and degassed for 5 min. 2,5-Dioxopyrrolidin-1-yl 6-acrylamidohexanoate (12.5 mg) was added to the solution, briefly sonicated (in a water bath sonicator) and warmed to 40 °C for 3 min until it was dissolved. (Methods of Sulfo-SANPAH- and NHS-acrylate-based protein immobilization are provided on request.)

The solution was aliquoted in samples of 0.250 ml. Each aliquot was polymerized by addition of 0.015 ml of 200 mM potassium persulfate in distilled water (previously deaerated) and 0.015 ml of TEMED (diluted 1 to 10 in distilled water). Polymerization was performed in closed poly-Tetrafluoroethylene (Teflon) casts for 2 min. (See supplemental Fig. 1 for construction details of the Teflon mold.) Polymerized PAA Gels had 7 mm diameter and 1 mm thickness. After polymerization, PAA gels were washed twice with 200 mM Hepes pH 8.5 and 0.070 ml of 15ug/ml Fibronectin in 200 mM Hepes pH 8.5 was added to each gel. Activated PAA gels were left at 4 °C overnight.

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