Entrapment of Embryonic Stem Cells-Derived Cardiomyocytes in Macroporous Biodegradable Microspheres: Preparation and Characterization

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Key Words

Cardiomyocyte • Electrophysiology • Embryonic stem cell • Block-face scanning electron microscopy • Confocal laser scanning microscopy • Microcarrier • Biomaterials

Abstract

Embryonic Stem (ES) cells-derived cardiomyocytes can possibly be applied for cell therapy of diseases such as heart failure. Biodegradable scaffolds will significantly improve the expansion of sufficient functional ES cell-derived cardiomyocytes and may also increase the survival rate of cardiomyocytes after their transplantation. In the present study, we cultivated cardiomyocytes isolated from a transgenic α -myosin heavy chain (α -MHC) ES cell lineage expressing both puromycin resistance and enhanced green fluorescent protein (EGFP) under the control of the α -MHC promoter in macroporous gelatine microspheres using small-scale bioreactors and

proved that cardiomyocytes function after their cultivation in microspheres. The average number of cultivated cells per microsphere was optimised once the most suitable agitation conditions and the optimal timeframe of cultivation were identified. Our study shows that 72% of CultiSpher-S beads were colonised by cardiomyocytes under optimal conditions. Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) showed that colonization of the beads was not limited to the surface, but that cells also invaded the inner surfaces of the microspheres. Electrophysiological experiments demonstrated that the action potentials (APs) of α -MHC⁺ cardiomyocytes entrapped in microspheres were identical to action potentials of control cells. This attractive approach for cultivation and expansion of functional cardiomyocytes in biodegradable macroporous may offer a perspective for higher transplantation efficiencies of ES cell-derived cardiomyocytes.

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Introduction

Congestive heart failure (CHF) remains a major cause of morbidity and mortality worldwide [1]. CHF as a result of structural defects due to insufficient number of intact and functional cardiomyocytes impairs the ability of the heart to pump a sufficient amount of blood throughout the body. Embryonic stem (ES) cell-derived cardiomyocytes could be attractive candidates for a transplantation therapy of heart failure. In this context, multiple strategies were developed to obtain cardiomyocytes from ES cells for cellular transplantation therapy of the heart [2-4]. Specific investigations on the generation of cardiomyocytes with high purity and the characterisation of their gene expression signatures have been conducted so far from a transgenic ES cell line (CGR8) exhibiting puromycin resistance and EGFP under the cardiomyocyte-specific α -MHC promoter [5, 6].

CultiSpher-S (Percell Biolytica) is a macroporous protein-based, enzymatically degradable microcarrier that is manufactured from pharmaceutical grade porcine gelatin and offers a highly cross-linked gelatin matrix with high mechanical and thermal stability. The easily adhesion surface area of the microspheres, when used in cell cultures, provide enhanced cell production without harmful effects to the cells and in parallel entrapment of the cells support their viability [7]. Therefore, macroporous microcarriers may be utilised as excipient component of cellular components intended for implantation resulting in a longer survival of the implanted cells [8]. Till now, porcine gelatin microbeads microcarriers have been developed to produce autologous living dermal equivalents on large scale [9] and to evaluate a stem cell delivery system in bone tissue engineering [10].

In the present work we cultivated α -MHC⁺ cardiomyocytes within Cultispher-S microcarriers and tested their survival rate as well as their functionality. The use of biodegradable microcarriers (CultiSpher-S) as a potential system for carrying and delivery of functional cardiomyocytes is evaluated and discussed in detail.

Materials and Methods

ES cell culture

Murine CGR8 ES cells (strain 129) were cultured in the absence of feeder cells in Glasgow minimum essential medium (GMEM) (Invitrogen, UK) supplemented with 10% fetal calf serum (FCS) (Invitrogen, UK), 2 mM L-glutamine, 100 units/mL leukemia inhibitory factor (LIF) and 50 μ M β -mercaptoethanol

(β -ME) (Invitrogen, UK) in 0.2% gelatine coated flasks as previously described [11].

Generation of ES-derived α -MHC⁺ cardiomyocytes

A transgenic α -MHC embryonic stem cell lineage was generated, exhibiting puromycin resistance and expressing EGFP under the control of the α -MHC promoter as described previously [5, 6]. A puromycin-resistant, EGFP- and α -MHC-positive cardiomyocyte population was isolated with over 92% purity as described previously [5, 6]. Briefly, a suspension of 2.5×10^4 α -MHC⁺ ES cells per ml was prepared in Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen, UK) supplemented with 20 % FCS, 1 % non-essential amino acids (vol/vol) (Invitrogen, UK), 2 mM L-glutamine and 100 μ M β -ME. 20 μ l of this ES cell suspension was spotted on the inside of the upper lid of a 10 cm bacteriological dish and then covered over its bottom dish containing 5 ml PBS. On day 2, the formed multicellular aggregates (EBs) were transferred to suspension in a new dish with 20 ml IMDM supplemented with 20% FCS, 1% non-essential amino acids (vol/vol), 2 mM L-glutamine and 100 μ M β -ME. On day 8, contracting clusters were observed. At this stage, cultures were treated with puromycin (4 μ g/ml) for 7 days with a medium change on every alternative day. The α -MHC/EGFP⁺ beating cardiomyocytes were examined under a fluorescence microscope and the structures observed were documented through videos and pictures (Axiovert 10, Zeiss, Germany) (Fig. 1, left). On day 15, the beating clusters were trypsinised and used for Fluorescence Activated Cell Sorting (FACS) analysis resulting in a 92% purity of α -MHC⁺ cardiomyocytes or used for the experiments below.

Dissociation of α -MHC⁺ cardiomyocytes

The 15-days old beating α -MHC⁺ cardiomyocytes were dissociated with Collagenase B (1 mg/ml) and dissolved in Ca²⁺- and Mg²⁺-free PBS. The cell clusters were incubated at 37°C in the CO₂ incubator for 30-45 minutes. The supernatant was removed and the dissociated cardiomyocytes were re-suspended in IMDM supplemented with 20% FCS, 1% non-essential amino acids (vol/vol), 2 mM L-glutamine and 100 μ M β -ME (Fig. 1, left).

Preparation of Cultispher-S microcarriers

CultiSpher-S microcarriers (diameter: 130-380 μ m, Percell Biolytica, Astrop, Sweden) were prepared according to the manufacturer's instructions. Dry microcarriers (100 mg dry CultiSpher-S) were swollen and hydrated in 5 ml Ca²⁺- and Mg²⁺-free PBS for 2 hours at room temperature. The microcarriers were then sterilized by autoclaving at 121°C for 15 min. After removal PBS the microcarriers were washed twice in IMDM medium. The sterile CultiSpher-S were counted by Haemocytometer and divided in aliquots over 3 cm bacteriological dishes, each containing 200 microspheres.

Expansion of α -MHC⁺ cardiomyocytes in Cultispher-S in vitro

The dissociated α -MHC⁺ cardiomyocytes (5×10^5 cells/ml) were washed twice with Ca²⁺ and Mg²⁺-free PBS, and mixed in a ratio of 200 prepared CultiSpher-Ss per dish. The culture

was agitated at 50 U/min on a 3006 GFL Shaker (Gesellschaft für Labortechnik, Germany) and maintained in the CO₂ incubator at 37°C for 4 days. The entrapment of the cells was monitored before, during and after the agitation of the cells in the CultiSpher-S microcarriers by the use of a fluorescence microscope (Axiovert 10, Zeiss, Germany) (Fig. 1, right).

The 7 days old α -MHC⁺ cardiomyocytes entrapped in CultiSpher-S were transferred into 15 ml Falcon tubes and were washed with Ca²⁺ and Mg²⁺-free PBS. To release cells, CultiSpher-S microspheres carrying α -MHC⁺ cardiomyocytes were treated with collagenase B (2 mg/ml) for 2 hours until the complete dissociation of the microcarriers. After releasing of the cardiomyocytes, trypan blue treated cells were counted by Haemocytometer (Fig. 1, right).

Long term growth (see above) and estimation of the entrapment efficiency (see above) in entrapped α -MHC⁺ cardiomyocytes was determined by cultivating the cells in time intervals of up to 4 weeks. Briefly, 3 cm bacteriological dishes containing a cardiomyocyte concentration of 5×10^5 cells/ml entrapped into 200 CultiSpher-S were incubated under constant stirring conditions and the medium was refreshed every 3 to 4 days. Dishes representing three replicates were dissociated weekly and the released cells were counted.

Sharp electrode electrophysiological studies

Due to the firm structure of microcarriers, glass microelectrodes were broken when they were impaled. Thus, microcarriers with α -MHC⁺ cardiomyocytes entrapped for 10-15 days were dissociated into EB-like clusters by incubation in Collagenase B solution (1 mg/ml, 20 min.), and dissociated clusters were allowed to recover and attach overnight in DMEM supplemented with 20% FCS at 37°C in presence of 5% CO₂.

Functional characterization of α -MHC⁺ cells was then performed by recordings of action potentials (APs) with sharp electrodes (20-50 M Ω when filled with 3 M KCl) made of borosilicate glass capillaries (WPI, Sarasota, USA). Signals were amplified with a SEC-10LX amplifier (npi electronic, Tamm, Germany) and acquired with the Pulse software (HEKA, Lambrecht/Pfalz, Germany). Experiments were performed at 37°C in DMEM without FCS bubbled with 95% O₂ and 5% CO₂.

Block face scanning electron microscopy (SEM) of CultiSpher-S entrapped with cardiomyocytes

CultiSpher-S microcarriers containing entrapped α -MHC cardiomyocytes were washed with 0.25 M HEPES buffer and were fixed with 2% glutaraldehyde in sodium cacodylate buffer (v/v). The suspension was washed with sodium cacodylate buffer, pH 7.2, and was embedded in 2% low melting agarose (Sigma-Aldrich, Taufkirchen, Germany). The post fixation was performed by the treatment of the microcarriers suspension in three steps using 2% osmium tetroxide, 1% tannic acid and 1% uranyl acetate in water as previously described [12]. Dehydration and drying of the suspension was carried out in increasing series of ethanol, while the last dehydrating step was performed in acetone using the Polaron high resolution sputter coater SC 7640 (Quorum Technologies Ltd.). The CultiSpher-S microcarriers entrapped with the cells were prepared for block-face scanning electron microscopy (SEM)

Fig. 1. Outline of the protocol used for generating ES cell derived cardiomyocytes (left) and expansion of α -MHC⁺ cardiomyocytes in CultiSpher-S *in vitro* (right).

as previously published [12]. Briefly the microspheres entrapped with the cells were embedded in epoxy resin, cross-sectioned with ultra-microtome. Sectioned resin blocks were coated with carbon in SCD-030 (Balzers, Lichtenstein) and examined in a field emission scanning electron microscope (FESEM) XL30 (Philips, USA) using backscattered electron (BSE) modes with 12 kV accelerating voltage and 10 mm working distance.

Confocal laser scanning microscopy (CLSM)

Detection of α -MHC⁺ cardiomyocytes on CultiSpher-S microcarriers was done by the use of confocal laser scanning microscopy (Eclipse TE2000-U microscopy, D-Eclipse C1 CLSM, Nikon, Japan). The entrapped microspheres with α -MHC⁺ cardiomyocytes were cultured in 20% IMDM medium, and placed into a μ -dish (IBID GmbH, Munich, Germany). A Z-stack was performed with 5 μ m step size. Images were taken at room temperature, in separated channels for red and green emission. A three dimensional image was constructed afterwards by merging single Z-stack images by software EZ-CE1 (Nikon, Japan).

Fig. 2. Progressive entrapment of α -MHC⁺ cardiomyocytes in CultiSpher-S. A-C, Generation of α -MHC⁺ cardiomyocytes after treatment of 8-day old EBs with 4 μ g /ml puromycin for 7 days. D, Single cardiomyocytes after dissociation of the EBs with Collagenase B. E, CultiSpher-S microspheres without cells. F, Suspension of α -MHC⁺ cardiomyocytes and CultiSpher-S prior to agitation (day-0). G-I, Entrapped CultiSpher-S with α -MHC⁺ cardiomyocytes (day 7). Magnification A-H 10x and I 32x. Scale bar = 200 μ m.

Fig. 3. Cell entrapment curve of α -MHC⁺ cardiomyocytes in CultiSpher-S microcarriers. The average (%) of long term entrapped α -MHC⁺ cardiomyocytes was estimated by the dissociation of three replicates weekly and the count of the released cardiomyocytes. The results are expressed as the mean $\pm$ Standard Deviation (SD) of four independent experiments in triplicate.

Results

The cultivation of highly purified cardiomyocytes obtained from a transgenic α -MHC⁺ ES cell line was validated by the increase of the EGFP fluorescence during the differentiation by the hanging drop protocol. The EGFP-expressing beating clusters of α -MHC⁺ cardiomyocytes were observed at day 8 and then treated with 4 μ g/ml puromycin for further 7 days resulting in

Fig. 4. CLSM pictures of an entrapped CultiSpher-S microcarriers with α -MHC⁺ cardiomyocytes (7 days). A three dimensional presentation of merged Z-stack images. A-H, Different sections of the scanned slides. The internal and external entrapment of the microcarriers is confirmed. Magnification 20x. Scale bar = 100 μ m.

Fig. 5. Block-face SEM inverted images of the CultiSpher-S microspheres colonised with α -MHC⁺ cardiomyocytes. A-C, block-face SEM images from low to high magnification of the attached α -MHC⁺ cardiomyocytes on the CultiSpher-S's surface. B, cardiomyocyte squared in A; C, view of area squared in B, rotation 90°. D-F, block-face SEM images indicating the entrapment of cardiomyocytes in the pore channel of CultiSpher-S gelatine beads. E, view of area squared in D, rotation 90°; F, details of cardiomyocyte ultrastructure (area squared in B). G-I, block-face SEM images demonstrating the microcarrier - cardiomyocyte contact and typical features of cardiomyocytes. White arrows - myofilament bundles; white discontinuous arrows - Z disks; black arrows - microcarrier - cardiomyocyte contact.

progressive enrichment up to day 15, while non-puromycin-resistant cells were in parallel killed by the puromycin (Fig. 2A-C).

Single α -MHC⁺ cardiomyocytes dissociated with collagenase B (1 mg/ml) were visualised as shown in the Fig 2D. CultiSpher-S treated only with Ca²⁺ and Mg²⁺-free PBS are shown in Fig. 2E. Prior to the agitation on day 0, α -MHC⁺ cells and CultiSpher-S were in a mixture (Fig. 2F) and the entrapment of the α -MHC⁺ cells after 7 days was monitored (Fig. 2.G-I).

Long-term cultivation of α -MHC⁺ cardiomyocytes *in vitro* was achieved by performing experiments in which cardiomyocytes were seeded with microcarriers under constant agitation in 3 cm bacteriological dishes. Cells were seeded simultaneously in a ratio of 2.5×10^2 cells per microcarrier and the medium was replaced every day. The expansion of the α -MHC⁺ cardiomyocytes in microcarriers was performed simultaneously in three replicates for up to 4 weeks. To monitor the entrapment efficiency every 7 days, one of the triplicates was dissociated and the released cells were counted.

We monitored the *in vitro* release of cells from the CultiSpher-S to determine the percentage of entrapment of the α -MHC⁺ cardiomyocytes into CultiSpher-S microcarriers during the long-term incubation step after dissociating of the cells with Collagenase B. The biodegradable CultiSpher-S microcarriers reached an entrapment efficiency of 72% during the first week. As shown in Fig. 3, after 2 and 3 weeks of cultivation, the entrapment efficiency declined from 53 to 45%. After 4 weeks, cell entrapment efficiency declined to 39%. The decreasing entrapment efficiency of the stirred culture

Fig. 6. Electrophysiology. Action potentials of α -MHC⁺ cells dissociated from microbeads had cardiac-specific properties. Action potential duration was variable, reaching from a fast pacemaker- or atrial-like repolarisation (A) to a pronounced ventricular-like plateau phase (B). Diastolic potential was either stable (B) or there was a spontaneous diastolic depolarisation (A).

system may be attributed to the progressive biodegradable nature of the CultiSpher-S (Fig. 3).

The entrapment profiles were analysed, to investigate the optimum number of cardiomyocytes that could be entrapped per bead. The average number throughout 3 independent experiments was approximately 1400 cells per bead. This approximate number of cardiomyocytes could be taken into consideration for preliminary judgment, during the optimisation conditions of the entrapment of cardiomyocytes in CultiSpher-S microspheres.

CLSM was carried out to confirm entrapment of the 3 weeks old α -MHC⁺ cardiomyocytes, after 1 week of their co-culturing with the CultiSpher-S microcarriers. The entrapment analysis by CLSM showed massive colonisation in different sites of the microspheres. As demonstrated by the three-dimensional image of CLSM, the α -MHC⁺ cardiomyocytes are found on the

surface as well as deeper in different layers of the sphere (Fig. 4).

The entrapment behaviour of α -MHC⁺ cardiomyocytes were examined by block-face SEM, to investigate the cell adherence in pore channel and on the surface of the microcarriers' matrix. Moreover, block-face SEM was used to determine the size of the 7-days entrapped microcarrier, the inner and outer surface morphology and the cells expansion into the inner pores of the CultiSpher-S microcarriers (Fig. 5). Consistent with the block-face SEM data, attached cardiomyocytes on the surface of the CultiSphere-S were observed. Fig. 5A-C shows the same focus of the cell colonized microcarrier at different magnifications. The migration of cardiomyocytes into the pore channel of CultiSpher-S gelatine beads was good visible (Fig. 5D-F). As shown in Fig. 5G and I (black arrows) the cardiomyocyte and microcarrier contact was well defined in one week expanded cardiomyocytes into CultiSphere-S. Typical features of cardiomyocytes, myofilament bundles were observed (Fig. 5C, H, white arrows). Also a tight electron dense bands (Fig. 5H, discontinuous arrows) were identified that can be interpreted as completely preserved Z discs.

Functional characterization of the entrapped α -MHC⁺ cardiomyocytes in beads was performed by measuring their typical spontaneous APs. Intracellular recordings showed typical cardiac AP properties of the entrapped α -MHC⁺ cardiomyocytes as the α -MHC⁺ control EBs cardiomyocytes. During the diastole, there was either a spontaneous diastolic depolarisation or a stable resting membrane potential. In the latter case, APs could be stimulated via the recording electrode. AP duration and morphology were variable, reaching from a fast pacemaker-like or atrial-like repolarisation to a pronounced ventricular-like plateau phase (Fig. 6). The electrophysiological characterization of α -MHC⁺ cardiomyocytes entrapped in CultiSpher-S revealed typical morphologies of cardiac APs, which have considerably longer AP durations as compared to neuronal or skeletal muscle potentials. Reflecting the variety of cardiac AP shapes *in vivo*, which include pacemaker, atrial and ventricular potentials, APs measured in α -MHC⁺ cells were not uniform, but had different durations and shapes, as reported before for cardiomyocytes from the CGR8 line [1]. AP morphologies of entrapped α -MHC⁺ cardiomyocytes corresponded to those of α -MHC⁺ control EBs, an alteration of electrophysiological properties due to the entrapment in microcarriers was not found.

Discussion

Heart failure is characterized by the loss of functional cardiomyocytes [13, 14]. Cardiomyocytes generated from a transgenic ES cells line that were used in this study showed functional characteristics as the mature cardiomyocytes [5].

Biodegradable CultiSpher-S can be colonized with cardiomyocytes and transplanted into the heart to enhance regeneration. Notably, the cardiac cell expansion in the CultiSpher-S can be used to maintain cultures in steady state from several days up to four weeks, indicating efficient supply of nutrients to the cells and the removal of waste products that may inhibit cell growth and survival.

The macroporous microcarriers allow cardiomyocytes to attach to both the external and the internal surfaces of the matrix, increasing the culturing surface area and providing protection from mechanical stress. For a multitude of cells, the macroporous three-dimensional collagen surface has been demonstrated to contribute to increased cellular attachment, resulting in high cell density [15, 16]. A wide variety of microcarriers including Cytodex 3 [17, 18] and CultiSpher-S microcarriers laying on the polymer scaffold [10] have already been investigated using mouse embryonic stem cells with potential applications for tissue regeneration. Additionally polystyrene-based cationic trimethyl ammonium-coated microcarriers in spinner flasks have been successfully used for expansion of human fibroblasts enabling extended self-renewal while retaining full differentiation potential [19]. Stirring conditions such as agitation or spinning are important for the facilitation of the cells attachment to the microspheres. Recently, it has been reported [17] that a stirred culture system (spinner flask) with mouse ES cells and CultiSpher-S microspheres was used to scale up mouse ES cell expansion. The EGFP/ α -MHC⁺ cardiomyocytes intended for transplantation could possibly be cultivated on the biodegradable macroporous microspheres to prolong the survival rate of cardiomyocytes prior to transplantation process.

The entrapment and the adhesion of the cardiomyocytes in CultiSpher-S gelatine microspheres was critically dependent on the stirring or shaking conditions, providing an excellent cell attachment, spreading and expansion of the cells. The decreasing entrapment efficiency of the stirred culture system can be attributed to the progressive biodegradable nature of the CultiSpher-S. The microscopical examination of the microcarriers showed an efficient entrapment in case of

stirred or agitated cultivation system and less efficient in not stirring condition.

Of major importance is the fact that the attached and entrapped α -MHC⁺ cardiomyocytes to gelatine beads, exhibited similar growth kinetics and beating behaviour to those of the control α -MHC⁺ cardiomyocytes clusters. Based on these findings it can be assumed that cardiomyocytes could be easily released from gelatine beads after transplantation and could help in the integration of the cells into damaged heart areas.

The highly cross-linked matrix of the CultiSpher-S permits the entrapment of high cell numbers. We estimate that about 1400 cells can be entrapped per microcarrier in both interior and exterior surface. Microscopically investigations by block face SEM and CLSM indicate cardiomyocytes in pore channels after 14 days of co-culturing demonstrating migration and growth of cardiomyocytes even in the inner parts of the gelatine bead. We assume that the cardiomyocytes can be slowly released from the CultiSpher-S because of their biodegradable nature. Nevertheless, we treated the CultiSpher-S microspheres carrying cardiomyocytes with collagenase B for cell release [7]. If all other experimental parameters like oxygen and nutrients are provided in excess, the final cell yield in a microcarrier culture will be proportional to microcarrier concentration. Thus, it is not necessary to increase the surface area by increasing the number of the beads in excess required for the number of cells that can be supplied under optimal culture conditions (sufficient nutrients and oxygen). As we demonstrated the number of cells on each microcarrier decreases when the concentration of CultiSpher-S increases and the probability that a cell will interact with a bead is directly proportional to the bead concentration. The cells will probably only grow on the outer parts of CultiSphers when beads are in excess compared the cardiomyocytes. CultiSpher-S beads are suitable for expansion of mesenchymal stem cells and may be applied to regenerate bone and other tissues [8]. Moreover, porous gelatin and collagen spheres have been proven to be valuable in cell-based therapies for the treatment of acute and persistent wounds [10, 20-23]. A variety of animal and human cell lines have been successfully cultivated in macroporous gelatine spheres [16-18].

Identification features representative of the cardiac cytoskeleton including muscle myofilaments and Z discs were well detected in the entrapped CultiSpher-S microcarriers. The lateral boundaries of the sarcomeres showing their characteristic Z discs are the primary

conduits of the force generated by contraction [24]. Crosslinked cardiac actinin and titin are organised into repeated sarcomeres along the length of myofibril and give skeletal and cardiac muscles their striated appearance.

The electrophysiological characterization of α -MHC⁺ cardiomyocytes entrapped in gelatin CultiSpher-S revealed typical morphologies of cardiac APs, which have considerably longer AP durations as compared to neuronal or skeletal muscle potentials. Reflecting the variety of cardiac AP shapes *in vivo*, which include pacemaker, atrial and ventricular potentials, APs measured in α -MHC⁺ cells were not uniform, but had different durations and shapes, as reported before for cardiomyocytes from the CGR8 line [5]. AP morphologies of entrapped α -MHC⁺ cardiomyocytes corresponded to those of α -MHC⁺ control EBs, an alteration of electrophysiological properties due to the entrapment in microcarriers was not found.

Transplantation of cardiomyocytes into infarcted hearts is a promising new therapeutic approach, yet it is hampered by a low persistence of transplanted cells. Especially embryonic stem cell-derived cardiomyocytes have a low rate of persistence (<10%), which may be caused by apoptosis, necrosis and a wash-out of transplanted cells shortly after injection from the transplantation site into other organs or the pericardium (unpublished observation). This washout may be diminished by the injection of microspheres due to their huge diameter as compared to single cells, leading to an enhanced persistence of cells after transplantation.

In summary, α -MHC⁺ cardiomyocytes derived from ES cells attached efficiently endogenously and exogenously to CultiSpher-S microspheres in a stirred cultivation system and successfully expand *in vitro*. The cardiomyocytes within the CultiSpher-S microcarriers remain functional as demonstrated by the intact action potential and cytoskeletal features of the entrapped cardiomyocytes. This study thus offers a perspective form transplantation strategies.

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