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Cardiac differentiation potential of human induced pluripotent stem cells in a 3D self-assembling peptide scaffold

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ABSTRACT

In the past decade, various strategies for cardiac reparative medicine involving stem cells from multiple sources have been investigated. However, the intra-cardiac implantation of cells with contractile ability may seriously disrupt the cardiac syncytium and de-synchronize cardiac rhythm. For this reason, bioactive cardiac implants, consisting of stem cells embedded in biomaterials that act like band aids, have been exploited to repair the cardiac wall after myocardial infarction. For such bioactive implants to function properly after transplantation, the choice of biomaterial is equally important as the selection of the stem cell source. While adult stem cells have shown promising results, they have various disadvantages including low proliferative potential *in vitro*, which make their successful usage in human transplants difficult.

As a first step towards the development of a bioactive cardiac patch, we investigate here the cardiac differentiation properties of human induced pluripotent stem cells (hiPSCs) when cultured with and without ascorbic acid (AA) and when embedded in RAD16-I, a biomaterial commonly used to develop cardiac implants. In adherent cultures and in the absence of RAD16-I, AA promotes the cardiac differentiation of hiPSCs by enhancing the expression of specific cardiac genes and proteins and by increasing the number of contracting clusters. In turn, embedding in peptide hydrogel based on RAD16-I interferes with the normal cardiac differentiation progression. Embedded hiPSCs up-regulate genes associated with early cardiogenesis by up to 105 times independently of the presence of AA. However, neither connexin 43 nor troponin I proteins, which are related with mature cardiomyocytes, were detected and no contraction was noted in the constructs. Future experiments will need to focus on characterizing the mature cardiac phenotype of these cells when implanted into infarcted myocardia and assess their regenerative potential *in vivo*.

1. Introduction

In every year since 1900 with the exception of 1918 cardiovascular disease accounted for more deaths than any other major cause of death in the United States (Lloyd-Jones et al., 2010). More than 50% of the cardiovascular diseases present as coronary events, including sudden cardiac death and nonfatal myocardial infarction (Alagona and Ahmad, 2015). Permanent myocardial cell death, scar formation and left ventricle dilation belong to the secondary damages incurred during myocardial infarction ultimately leading to post infarction heart failure. Despite the advancements in clinical medicine over the past century current treatment regimens fail to prevent disease progression only leaving heart transplantation as a means to save the patient.

Owing to the shortage of organ donors and the limited ability of adult cardiomyocytes to proliferate after injury, many efforts focus on improving, preserving and restoring function after myocardial infarction exploiting tissue engineering principles. Mesh made of various materials (i.e. polypropylene, knitted polyester) is used to cover or encapsulate the dilating ventricle and to slow further expansion of the cardiac wall. In addition, the injection of multiple sources of stem cells, mostly of adult origin, has been exploited with the hope for true tissue repair and restoration of cardiac function (Vunjak-Novakovic et al., 2010). However, the engraftment of such cells is currently inefficient due to poor cell retention in the injury site caused by the mechanical forces the

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myocardium exerts with every pump.

We and others have previously hypothesized that a combination of a restraining biomaterial with an appropriate cell source may proof superior in restoring cardiac function. Specifically, we and other members of the RECATABI (REgeneration of CArdiac Tissue Assisted by Bioactive Implants) Consortium created a bioactive cardiac implant consisting of partially biodegradable elastomeric membranes made of polycaprolactone methacryloyloxyethyl ester filled with a self-assembling peptide hydrogel that carried adult progenitor cells with cardiac potential (Soler-Botija et al., 2014). While providing structural support for both the ventricular wall and the transplanted cells as they differentiate, these material-cell constructs created three-dimensional tissue-like structures mimicking the natural organization of the tissue and improved cardiac function *in vivo*.

While the elastomeric membrane provided a comparable resistivity to myocardium (Chachques et al., 2013; Salazar et al., 2004), the function of the RAD16-I hydrogel within this construct was to improve cell distribution, to facilitate uniform colonization of the membrane pores and to support the differentiation of the progenitor cells into the cardiac lineage (Soler-Botija et al., 2014; Thonhoff et al., 2008; Castells-Sala et al., 2012; Cui et al., 2010; Hsieh et al., 2006; Tokunaga et al., 2010). Cell delivery scaffolds based on peptide hydrogels, such as RAD16-I, are neutral, easy to tailor with bioactive motifs, biodegradable, porous, permeable and flexible. In addition to promoting cardiac differentiation (Soler-Botija et al., 2014; Prat-Vidal et al., 2014), RAD16-I (Zhang et al., 1995; Yokoi et al., 2005) has been shown to promote osteogenic (Garreta et al., 2006; Horii et al., 2007) and chondrogenic (Quintana et al., 2009; Quintana et al., 2009; Johnstone et al., 2013; Kopesky et al., 2010) differentiation of multiple cell sources. In addition, these synthetic self-assembling peptides may be modified to deliver drugs and other bioactive molecules (Puig-Sanvicens and Semino, 2013).

Beside the biomaterial, the choice of cell type for such an implant is equally critical. Multiple cell sources have so far been exploited as starting material for the *in vitro* and *in vivo* generation of functional cardiomyocytes, including allogeneic and autologous sources (Bunnell et al., 2008). The latter category of cells includes skeletal myoblasts (Taylor et al., 1998; Menasché et al., 2008; Dib et al., 2005), resident cardiac stem cells (Beltrami et al., 2003; Oh et al., 2003), bone-marrow-derived hematopoietic (Orlic et al., 2001) and mesenchymal stem cells as well as adipose-tissue-derived stem cells with mesenchymal potential (Wang et al., 2009; Valina et al., 2007), such as the subcutaneous adipose tissue-derived progenitor cells (subATDPCs) that were used in RECATABI's bioactive cardiac implant (Soler-Botija et al., 2014). While eliminating the potential for allogeneic rejection, the listed cell sources are afflicted with other challenges: the existence of adult cardiac progenitor cells in the heart is still debated (Beltrami et al., 2001), bone-marrow-derived cell types do not appear to transdifferentiate into cardiac muscle and the limited expansion of subATDPs in culture may jeopardize the harvest of clinically relevant cell numbers for human implants.

In contrast, allogeneic embryonic stem cells have the clearest track record in differentiating cells of the cardiac lineage (Wobus et al., 1991; Mummery et al., 2002; Riegler et al., 2015). While these are potentially immunogenic, the related induced pluripotent stem cells (iPSCs) have become increasingly attractive in the last few years. These cells are converted from an autologous somatic cell through the process of reprogramming, creating immuno-compatible pluripotent cells that are highly proliferative and capable of tri-lineage differentiation (Takahashi et al., 2007). Indeed, iPSCs have successfully been differentiated into cardiomyocytes *in vitro* (Zwi-Dantsis et al., 2012). Towards the application in a cardiac implant, the objective of this study was therefore to investigate the potential of human iPSCs for cardiac differentiation *in vitro* and when embedded in the RAD16-I hydrogel.

2. Materials and methods

2.1. Cell culture

Human iPSCs (RIV9, Chatterjee et al., 2011) were cultured in Matrigel pre-coated plates with mTeSR (Stem Cell Technologies). Confluent cells were expanded and passaged (1:3 to 1:6) with accutase. Medium was changed every two days.

2.2. Cardiac differentiation of hiPSCs

In order to induce differentiation, mTeSR was replaced with cardiac induction medium composed of DMEM, 15% lot-tested fetal bovine serum (PAA), 1% non-essential amino acids, 0.5% penicillin-streptomycin and 0.1 mM β-mercaptoethanol either with or without ascorbic acid (50 μg/ml) when cells had reached confluence.

For hydrogel seeding, hiPSCs were treated with ROCK inhibitor Y-27632 for one hour at 37 °C and 5% CO_2, prior to trypsin treatment and then mixed with RAD16-I (PuraMatrix™ Peptide Hydrogel, BD Bioscience, 354250) to a final concentration of 4×10^6 cells/ml in 0.15% RAD16-I, 10% sucrose. Cell/matrix suspension was loaded onto pre-wetted cell culture inserts (Millipore, PICM01250 or PICM03050) and inserts were fully topped off with medium 20 min after initial seeding. Half of the medium was replenished every day.

2.3. Cell viability assay

Viability of cells was tested with a LIVE/DEAD assay. After washing in PBS three times, hiPSCs were incubated with 2 μM calcein and 1 μM ethidium homodimer-1 (both Invitrogen) in 1XPBS for 15 min in the dark. Dyes were removed with three PBS washes and cells observed under a fluorescent microscope (Nikon Eclipse TI).

2.4. Immunocytochemistry (ICC)

Adherent cultures of hiPSCs were washed with 1XPBS and fixed with 4% paraformaldehyde at 4 °C for 30 min and permeabilized with 0.1% Triton X-100 for 15 min at room temperature (RT). Unspecific binding was blocked with 10% FBS, 0.5% BSA in 1XPBS for one hour at RT. Cells were then incubated with respective primary antibodies in blocking buffer at 4 °C overnight. Primary antibodies were rabbit anti-human Nanog (Millipore, AB9220), goat anti-human Brachyury (R&D system, AF2085), rabbit anti-human Gata4 (Santa Cruz, sc-9053), rabbit anti-human Nkx2.5 (Santa Cruz, sc-14033), mouse anti-human MHC (Abcam, ab15), rabbit anti-human Connexin 43 (Santa Cruz, sc-9059), rabbit anti-human Troponin I (Santa Cruz, sc-15368), rabbit anti-human Tbx5 (Santa Cruz, sc-48782), goat anti-human FOXA2 (Santa Cruz, sc-6554). Appropriate Alexa Fluor conjugated secondary antibodies (Invitrogen) were added with 20 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) diluted in blocking buffer for 2 h at RT and photographed in PBS.

Cells from constructs were harvested by trypsinization and cytospun (Shandon Cytospin 3) onto slides at 500 rpm for 4 min. Cells were then treated the same as the plastic-adherent cultures. For staining of complete constructs, hydrogel cultures were washed with 1XPBS and fixed as above. Consecutively, hiPSCs-constructs were incubated with 0.1% H_2O_2 in methanol for 45 min. Non-specific binding was blocked with blocking buffer (20% FBS,