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IPS-DERIVED CARDIOMYOCYTES IN CARDIAC DAMAGE AND REGENERATION

Antoni Bayés Genís

Institut d'Investigació en Ciències de la Salut Germans Trias i Pujol

Mercè Martí Gaudes

Centre Medicina Regenerativa de Barcelona

1. Summary

The growing worldwide epidemic of heart failure is causing a great need for organs, which is presently unmet due to the scarcity of donations. Myocardial infarction (MI) occurs after heart blood supply interruption and causes an irreversible cardiac muscle loss and non-contractile collagen scar formation. Cardiac homeostasis self-regeneration is insufficient after MI. Over the past decade tissue-engineering procedures have emerged as an advanced opportunity to promote the regeneration of whole organs or locally damaged tissues. In particular, this novel therapeutic option is based on the combination of stem cells with regenerative capacity, biological matrices, biocompatible synthetic polymers and/or online registration systems. In this context, our group have relevant experience in the design, development and testing of bioprostheses filled with therapeutic or reparative cells. Furthermore, some tissue engineering prototypes have been tested on animal models with promising results, yet cardiac contractility and tissue restoration remain limited. Thus, new cardiac grafts need to be generated and tested with novel biomaterials and more committed cardiac cell precursors or progenitors. This project represents a step forward in our aim to further develop more efficient tissue-engineered cardiac grafts for cardiac regeneration and repair. In particular, we expect this may open a whole new approach for cardiac repair in those patients suffering MI, to whom current options are either very aggressive or limited, leading to increase in contractile cardiac forces and better quality of life and longer life expectancy.

In addition, development of human cardiomyocytes derived from induced pluripotent stem cells (iPS) opens new opportunities for in vitro models of cardiac diseases, screening for new drugs, and patient-specific cardiac therapy. iPS-derived cardiomyocytes may be proper cell candidates since they do self-renewal and are a highly reproducible cellular model to study pathophysiology and therapy of human diseases, i.e. inherited hypertrophic cardiomyopathy (HCM). Thus, we propose to create novel bioactive implants by combining iPS-derived cardiomyocytes with biocompatible scaffolds, both in the murine and the swine models of myocardial infarction (MI). Particularly, the main objective is the generation of iPS and iPS-derived cardiomyocytes, which could be used to:

1) analyse the value of iPS-derived cardiomyocytes for cardiac repair. In this context, we plan to electromechanically stimulate iPS-derived cardiomyocytes and test them using a fibrin construct in the murine model of MI or a cardiac bioprosthesis of decellularized biomatrices in the MI swine model.

2) explore the value of iPS-derived cardiomyocytes (iPS-CMs) in inherited HCM modelling. To that end, iPS will be derived from peripheral blood mononuclear cells (PBMCs) from inherited HCM patients, and then differentiated into functional cardiomyocytes.

2. Results

First, we successfully generated and characterised iPS derived from swine dermal fibroblasts by a non-integrative transfection vector (Sendai Virus) carrying the factors OCT4, SOX2, KLF4 and MYC. Then, p-iPS colonies were singularized and adapted to matrigel-coated 6-well culture plates. After culture purification, line passaging and cell stocking, we started the cardiodifferentiation process. In particular, the potential of two distinct cardiodifferentiation protocols was evaluated on the p-iPS. The protocols are those based on the use of BMP4, activin A, ascorbic acid and VEGF, and CHIR99021, IWR-1 and glucose restriction, respectively. Following both protocols, p-iPS were not totally differentiated into the cardiomyogenic lineage since we were not able to observe spontaneous contractile activity in any of the treated primary cultures generated.

Second, for mechanical stimulation h-iPS-CMs were dispensed into a biomimetic stretching device and incubated for 1h at 37°C in 5% CO2 and humid atmosphere to allow gelation. The construct was then subjected to mechanical stimulation for 7 days. This device, designed to reproduce the cyclic stimulation of the heart, has been manufactured with polydimethylsiloxane (PDMS) (Sylgard® 184, Dow Corning) in circular moulds (18mm). It is made of a silicone membrane integrated into the surface of a 6-well culture plate containing a side hole that allows air to enter. Thus, the membrane is deformed, and consequently stretches the cell matrix placed on its surface. This air inlet was been at a frequency of 1Hz (350ms input, 650ms output). The evaluation by confocal microscopy showed an increase in the number of cardiac T-troponin strains in mechanically stimulated constructs compared to non-stimulated

ones. Furthermore, in the analysis by electronic transmission microscopy there was a greater definition and number of sarcomeres as well as a greater number of gap unions in mechanically stimulated CM-IPSs compared to the control. In terms of gene expression, the genes ACTC1, CACNAC1, CONNEXIN43, cTNT, MLC2V, MYH6, MYH7, and RYR2 were analysed for qPCR, using human heart tissue as a control. The results showed increased values of cTNT and MHY7 under conditions of mechanical stimulation compared to controls. It should be noted that the genes CACNAC1, CX43, cTNT, MYH6, and RYR2 showed values of the same order of magnitude as the human left ventricle. With the results observed, we can conclude that the mechanical stimulation of the CM-IPSs results in an increase in the parameters of cardiac maturation and, therefore, this type of stimulus can be useful in obtaining mature cardiomyocytes for therapeutic purposes.

Mature iPS-CMs were then administered in a mouse MI model. Cardiac function was evaluated by echocardiography at baseline, post-infarction, and pre-sacrifice. Further analysis revealed no significant differences between groups regarding cardiac function parameters. Results from the morphometric analyses of heart cross-sections through the infarcted myocardium 30 days after surgery showed no differences in infarct size between experimental groups. Masson's trichrome staining analyses of cross-sections of excised hearts corroborated that the constructs were well adhered to the mouse myocardium, covering the infarcted scar. Immunohistochemistry against GFP also demonstrated a partial engraftment of the cells into the host myocardium. Moreover, 30-day post-transplantation iPS-CM-GFP+ were also stained for alpha actinin and Cx43 and detected in the scaffolds (both control and pumped) in each individual. Most of the iPS-CM-GFP+ exhibited organized Z-bands confirming their cardiac phenotype. Interestingly, in some specimens, iPS-CM-GFP+ were found within the host tissue, which was considered a sign of cell migration from the scaffold to the host myocardium.

Remarkably, the regenerative potential of porcine iPS delivered through a decellularized tissular matrix after MI was also studied in swine. Cardiac magnetic resonance imaging was performed at 1.5 T in all animals using a 4-channel phased array surface coil. Cardiac functional parameters and necrotic mass were measured at baseline, 2 days post-MI, and before sacrifice. Our analyses of parameters for cardiac function over time showed no differences in cardiac function or necrotic mass, as

assessed by late gadolinium enhancement between groups. Histopathological examination of the heart, pericardial scaffold, APG, lung, mediastinal lymph node, pancreas, liver, kidney, and spleen, ruled out at 90 days of follow-up, confirmed the absence of undesired teratoma formation. Moreover, no evidence of p-iPSCs could be found in any of the p-iPSC-enriched constructs, neither at the infarct zone, nor within the border and remote locations. Using real time PCR, GFP was undetectable in the heart, lung, lymph node, liver, spleen, pancreas, or kidney, after 30 and 90 days of follow-up. Infarct size was similar for all treatment groups, irrespective of p-iPSC co-delivery. The arm that combined scaffold with AGTP (transposition of a cardiac adipose flap from the same recipient directly over infarcted myocardium), used on the presumption of a beneficial additive effect, showed the opposite, with a trend towards larger infarct size than the scaffold group. Moreover, the AGTP-scaffold arm also showed significantly higher vascularization, irrespective of p-iPSC delivery, compared to the other treatment groups both in infarct and border zones.

Third, in regard to the generation of iPS-CMs derived from hypertrophic cardiomyopathy family members, PBMCs were obtained from all members of a family affected by hypertrophic cardiomyopathy. PBMCs were then reprogrammed to iPS by virus reprogramming with Sendai virus carrying the factors necessary for their reprogramming i.e. OCT4, SOX2, KLF4 and MYC. To confirm the generation of bona fide iPS, the colonies obtained were stained with specific stem cell markers, such as SSEA-4, TRA-1-60 and NANOG. In addition, pluripotency was evaluated by the formation of embryonic bodies in vitro. Subsequently, iPS cardiodifferentiation was performed using a previously described protocol based on the addition of a variety of small molecules (B-27, insulin, CHIR-99021 and IWR-1 endo).

Regarding morphological and functional analysis of hypertrophic cardiomyopathy iPS-CMs, 90% of differentiated cells were positive for specific cardiac markers such as troponin T and α-actinin, as determined by flow cytometry. In immunocytochemistry experiments we confirmed protein levels for cardiac troponin T and tropomyosin I, as well as the typical sarcomeric organization in the iPSC-CMs analysed. Representative action potentials typical for ventricular and atrial phenotype could be also measured in our cell cultures using the patch clamp technique. Collectively, these results indicated that differentiation of iPS towards a mature cardiomyogenic lineage was successfully achieved. Importantly, the arrhythmias of the disease were reproduced in the form of

delayed depolarization in the cardiac action potentials observed. Thus, the behaviour and phenotype of CMs in the context of hypertrophic cardiomyopathy were recreated in vitro to potentially study intrinsic genotype and mechanisms of action, and for testing new medications.

Finally, although it was not planned in the original proposal, we have advanced further by taking advantage of generated iPC-CMs. Particularly, we have assessed potential genetic mutations in some of the family members. As a result, three distinct gene mutations have been found in all the family. Subsequently, we focused on one of these mutations which was corrected by using gene editing or CRISPR technology. Then we confirmed the normalization of the action potential register in the genetically edited iPC-CMs.

3. Scientific relevance

For decades, the mammalian adult heart was thought to be terminally differentiated. Later our team, following the fate of the Y chromosome in sex-mismatch cardiac transplants, pioneered cardiac chimerism, which involves mobilization of recipient extra-cardiac cells (e.g. from bone marrow) into the myocardium. Foetal-maternal cell cross-talk or foetal microchimerism was also demonstrated in the hearts of women with male offspring. These findings, which were verified by others, changed the prevailing dogma and prompted the rise of regenerative medicine for cardiovascular diseases. In this context, cardiac tissue engineering (TE) is an innovative therapeutic strategy for the regeneration of post-infarcted myocardial tissue. These therapeutic approaches are based on the administration of combinations of different types of stem cells (with great regenerative capacity), extracellular matrices of biological origin or synthetic biocompatible polymers, molecules or multifunctional factors (growth, differentiation, etc.) and, in some cases, electronic non-invasive online tracking and stimulatory or pre-conditioning/maturation systems.

In this project, our tested bioprostheses or biocompatible constructs show certain benefits (in terms of revascularisation) and safety when placed in post-infarcted animal models (basically in pigs, given the similarity of their cardiovascular system). However, it is necessary to further optimize the degree of survival, differentiation and electrical

coupling of implanted cells. For that purpose, we have accomplished the pre-conditioning or maturation of our regenerative cells using mechanically-stimulated human cardiomyocytes from iPS (iPS-CMs) prior to *in vivo* implantation. This approach induces a more proper or effective cell maturation within our tested TE constructs, making them even more suitable for cardiac regeneration and repair. For instance, stimulated constructs comprising iPS-CMs from human origin show increased cardiac T troponin strains and sarcomeric organization as well as greater number of gap junctions compared to those non-stimulated or control constructs. Of note, with the results observed, we can conclude that mechanical stimulation of well-designed TE constructs results in an increase in the level of cardiac maturation exhibited by regenerative cells. Therefore, this type of stimulus can be useful in obtaining mature cardiomyocytes for the design and development of more efficient therapeutic procedures or approaches against MI.

On the other hand, iPS have created new expectations in the field of regenerative medicine. iPS are somatic cells reprogrammed for pluripotency avoiding the ethical and immunological concerns present in embryonic stem cells. The development of iPS-derived cardiomyocytes (iPS-CMs) opens new opportunities for *in vitro* models of cardiac diseases, screening for new drugs and patient-specific tissue engineering-based therapies. iPS-CMs may be proper cell candidates because they do self-renewal and are a highly reproducible cellular model to study pathophysiology and post-infarcted myocardium treatment. In this project, we have developed several new cardiac TE grafts by combining iPS or iPS-CMs with biocompatible scaffolds both in the murine and swine myocardial infarction models for the assessment of *in vivo* benefits.

Alternatively, we used iPS to obtain differentiated CMs reproducing inherited hypertrophic cardiomyopathy (HCM)-associated mutations *in vitro*. Also, we have been able to correct one of the reported HCM-associated mutations by using gene editing or CRISPR technology. As a result, corrected iPS-CMs normalize their characteristic action potential registers and performance. Collectively, this is of an extreme complexity since, by using this approach, whole organisms can be reduced or simplified in a single crop plate to analyse their related mechanisms. Importantly, we have reached our objective since the derivation of iPS-CMs in a reproducible manner from peripheral blood-monocytes either from healthy or affected members of an HCM family has permitted us to comparatively compare all the generated iPS-CM cultures.

Undoubtedly, our model may be valuable *in vitro* to study HCM-specific mechanisms or find more efficient drugs or treatments against this type of cardiomyopathy, which is a tremendous alteration that significantly limits normal heart function.

4. References directly generated

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