

Adult stem cells, scaffolds for *in vivo* and *in vitro* myocardial tissue engineering

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Summary

The main goal in the last few years in cardiac research has been to isolate cardiac potential stem cells from adult myocardium and to demonstrate their differentiation potential. We have previously demonstrated that c-Kit positive cardiac stem cells are able to organize themselves into a tissue-like cell mass. In this 3D mass, they can produce a high concentration of natural extracellular matrix, can create vessels, a capsule and, with the help of an Open-pore Poly(lactic Acid) scaffold, many cells can organize an elementary myocardium.

Drawing from this background, we decided to design and use poly-lactic scaffolds and the model of the athymic *Nude-Foxn1tm* mouse to evaluate the extent of the myogenic vs endothelial differentiation *in vivo*, and to evaluate the presence or the absence of a foreign body reaction.

Key words

Heart remodelling, tissue engineering, cardiac progenitor cells

Introduction

Cardiac stem or progenitor cells are partially differentiated cells which can be isolated from the adult heart by tissue digestion (Beltrami et al., 2003; Di Felice et al., 2009; Messina et al., 2004). Convincing evidence has been provided about the presence of multipotent progenitor cells within the heart and almost all organs, thus suggesting their implication in tissue homeostasis and their possible role as potential targets for innovative treatments. The injection of mesenchymal or cardiac stem cells *in vivo* into a damaged organ is currently under investigation (Blin et al., 2010; Noort et al., 2010). Alternatively, the engraftment of engineered tissue substitutes produced by seeding stem cells on biodegradable, biocompatible scaffolds has been proposed as a more suitable solution to repair injured tissues (Coutu et al., 2009).

Natural (fibrin, chitosan, collagen, hyaluronic acid) and synthetic (poly-lactic and poly-lactic-co-glycolic acid, poly-caprolacton) polymers have been proposed as components of scaffolds for tissue engineering. The optimal combination of accurately designed scaffolds and stem cells could represent an extraordinary chance to treat several degenerative diseases. Cardiac stem cells, in a three-dimensional environment, express and assemble phenotype-specific proteins in a medium supplemented with 5% horse serum, without adding any particular differentiation-stimulating compound (Di

Felice et al., 2009). Starting from our previous work, in the present study we decided to improve the physical properties of poly-lactic scaffolds, and to understand the steps leading cardiac progenitor cells to differentiation both *in vitro* and *in vivo*.

Materials and Methods

Cell isolation and purification

We isolated c-Kit positive cells by their proliferative potential, as previously described (Di Felice et al., 2009).

Cell seeding in 3D cultures

Cells were seeded as previously described (Di Felice et al., 2009) into the Open-pore Polylactic Acid (OPLA) scaffold (BD Biosciences) and into the customized Poly(d,l)Lactic Acid [P(d,l)LA] composite scaffolds: L150609N, L150609T, L150609U (synthesized with a porosity agent less than 224 μm), L070709A (synthesized with a porosity agent less than 150 μm). 7 days after seeding, 3D-cultures were injected into athymic Nude-*Foxn1^{nu}* mice.

Flow cytometry analysis

Cells were detached from flasks, counted, and placed into FACS tubes (200,000 cells/sample). Cells were washed in PBS once and fixed with 4% paraformaldehyde in PBS and analyzed as previously described (Di Felice et al., 2009).

Scaffold microfabrication

The porous, three-dimensional P(d,l)LA scaffolds were prepared using a solvent cast particulate leaching technique. The polymer was first dissolved in (70:30 v/v) dichloromethane/dimethylformamide solvent and then poured into a glass Petri dish containing a sufficient amount of NaCl salt. The suspension was then dried under hood overnight and washed into water for three days in order to eliminate the residual solvent and the salt. The sponges obtained were frozen at -20°C and then lyophilized for 2 days.

Scaffold treatment before cell seeding

Before cell seeding, scaffolds were sterilized with 96% ethanol for 1 h. Thereafter, scaffolds were equilibrated with M-199 medium supplemented with 20% FBS, and air dried.

Microinjection of scaffolds in the dorsal subcutaneous region of athymic Nude-*Foxn1^{nu}* mice

Three-dimensional cultures with 100,000 cells/scaffold for each type of scaffold have been injected in the dorsal subcutaneous region of athymic Nude-*Foxn1^{nu}* mice

(Harlan laboratories), one scaffold per mouse. Scaffolds maintained in culture for 21 days and subcutaneous scaffolds from athymic Nude-*Foxn1^{nu}* mice were fixed in 4% formalin for 24h and dehydrated with ethanol 30, 50, 70, 95, 100% v/v. After dehydration, tissue pieces were put into xylol for 1h and embedded into paraffin. Paraffin-embedded tissue samples were cut into 5 μm sections and stained with haematoxylin/eosin.

Results

Cardiac stem cells isolation and characterization

We isolated immature cells from mature female rats using a differential adhesion method as previously described (Di Felice et al., 2009). We improved our protocol, and two weeks after tissue digestion 96.7 ± 2.3 % (mean \pm standard deviation) of the isolated cells were positive for the stem cell marker c-Kit (Figure 1). These cells were identified as cardiac stem cells (Di Felice et al., 2009).

Microinjection of cell-embedded P(d,l)LA scaffolds in athymic Nude-*Foxn1^{nu}* mice

To investigate the survival and morpho-functional potential of the cardiac progenitor cells *in vivo*, three-dimensional cell embedded P(d,l)LA scaffolds were injected in the dorsal subcutaneous region of athymic Nude-*Foxn1^{nu}* mice. Immature cardiac stem cells embedded in Collagen I, cultured in P(d,l)LA scaffolds and left in culture for 21 days with 20% FBS as controls, showed a heavy myogenic differentiation, as shown by ematoxylin/eosin staining (Figure 2A,B). In these samples myofibrils and vessels were visible (Figure 2A, B). Cells differentiated also in the customized P(d,l)LA scaffolds.

Cardiac stem cells injected in the subcutaneous region inside the scaffolds behaved differently from the *in vitro* counterpart. Around the five scaffolds there was a capsule and inside the scaffolds there were a few activated macrophages. OPLA scaffold induced a heavier and more defined capsule (Figure 2C, E). Customized P(d,l)LA scaffolds induced a thinner capsule (Figure 2D). Inside the mass, cardiac progenitor cells created vessels which formed anastomosis with the host ones (Figure 2F). Where nutrients did not arrived, there were areas of necrosis. Air bubbles inside the scaffold derived from the scaffold embedding procedure before injection. Residues of the scaffold structure were not identified.

Discussion

The complete myocardial differentiation of cardiac stem cells is difficult to achieve, both *in vitro* and *in vivo*. Here we demonstrated that also maintaining cells in a three-dimensional culture inside a poly-lactic scaffold for 21 days, c-Kit positive cardiac stem cells may differentiate into pre-cardiomyocytes (large cells enriched in myofibrillary proteins).

The main difficulty in using stem cells *in vivo* is to induce and drive the desired differentiation. As already described, a few cardiac progenitor cells, embedded in

matrigel and inserted in the dorsal subcutaneous region of SCID mice may differentiate into cardiomyocytes (Messina et al., 2004). We have decided to use this *in vivo* model to better understand if the c-Kit positive cardiac stem cells can differentiate, if they can create an organized myocardial tissue, if they can create vessels and above all if these cells cultured inside a P(d,l)LA scaffold within collagen may induce a foreign body reaction.

Our preliminary results demonstrate that, even if in culture for the same period of time almost all c-Kit positive cardiac stem cells differentiate into pre-cardiomyocytes, *in vivo* we do not obtain the same extent of differentiation. By analysing the hematoxylin/eosin stained paraffin sections, we can identify some pre-cardiomyocytes, many neo-formed vessels, sometimes a capsule and a few macrophages. The presence of a capsule, vessels and macrophages may be considered as a foreign body reaction (Anderson et al., 2008).

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Figures

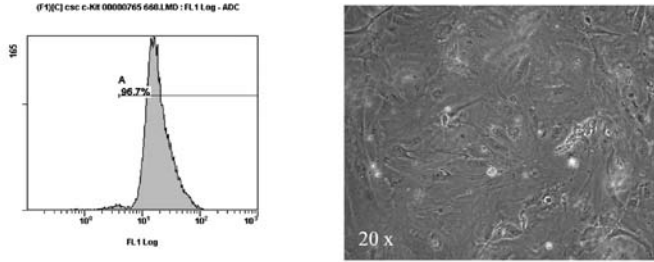


Figure 1 – Phenotyping of isolated cardiac stem cells. Percentage of cells expressing c-Kit by flow cytometry after a single differential adhesion selection, two weeks from tissue digestion. On the right an image of CSCs in culture.

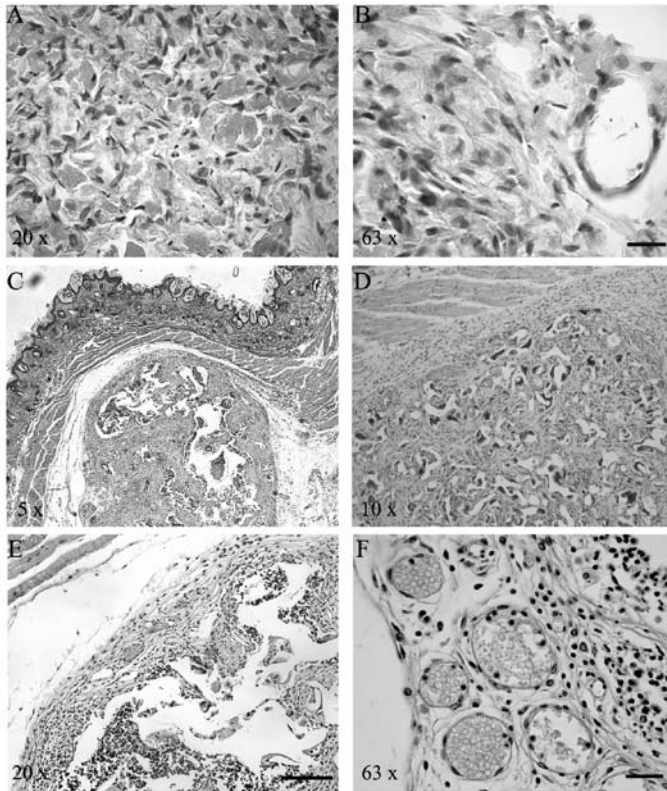


Figure 2 – Hematoxylin/eosin stained paraffin sections of *in vitro* and *in vivo* three-dimensional cultures of cardiac stem cells into P(d,l)LA scaffolds. Almost all cardiac stem cells differentiated *in vitro* after 21 days in culture, without any differentiation agent (A-B). The same three-dimensional culture, injected in the dorsal subcutaneous region of athymic *Nude-Foxn1tm* mice, did not show the same differentiation as the *in vitro* counterpart, after 21 days (C-F). *In vivo* implanted cells created a capsule (C-E), many vessels (F) and a few pre-cardiomyocytes (C). The presence of the capsule and macrophages may represent signs of a foreign body reaction.