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Tesi di Dottorato:

Stem Cells as a therapy for myocardial infarction in animal models

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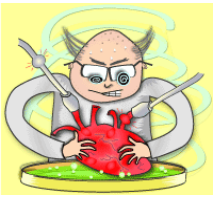
"I heard that scientists are growing human organs in lab dishes."

"Is this true?"



ABSTRACT

Advances in stem cell biology have challenged the notion that infarcted myocardium is irreparable. The pluripotent ability of stem cells to differentiate into specialized cell lines began to garner intense interest within cardiology when it was shown in animal models that intramyocardial injection of bone marrow stem cells (*MSCs*), or the mobilization of bone marrow stem cells with spontaneous homing to myocardium, could improve cardiac function and survival after induced myocardial infarction (MI) [1, 2]. Furthermore, the existence of stem cells in myocardium has been identified in animal heart [3, 4], and intense research is under way in an attempt to clarify their potential clinical application for patients with myocardial infarction. To date, in order to identify the best one, different kinds of stem cells have been studied; these have been derived from embryo or adult tissues (i.e. bone marrow, heart, peripheral blood etc.). Currently, three different biologic therapies for cardiovascular diseases are under investigation: cell therapy, gene therapy and the more recent *"tissue-engineering"* therapy .

During my Ph.D. course, first I focalised my study on the isolation and characterization of Cardiac Stem Cells (*CSCs*) in wild-type and transgenic mice and for this purpose I attended, for more than one year, the *Cardiovascular Research Institute* of the New York Medical College, in Valhalla (NY, USA) under the direction of Doctor Piero Anversa. During this period I learnt different Immunohistochemical and Biomolecular techniques, useful for investigating the regenerative potential of stem cells.

Then, during the next two years, I studied the new approach of cardiac regenerative medicine based on *"tissue-engineering"* in order to investigate a new strategy to regenerate the infarcted myocardium. Tissue-engineering is a promising approach that makes possible the creation of new functional tissue to replace lost or failing tissue. This new discipline combines isolated functioning cells and biodegradable 3-dimensional (3D) polymeric scaffolds. The scaffold temporarily provides the biomechanical support for the cells until they produce their own extracellular matrix. Because tissue-engineering constructs contain living cells, they may have the potential for growth and cellular self-repair and remodeling. In the present study, I examined whether the tissue-engineering strategy within hyaluron-based scaffolds would result in the formation of alternative cardiac tissue that could replace the scar and improve cardiac function after MI in syngeneic heterotopic rat hearts. Rat hearts were explanted, subjected to left coronary descending artery occlusion, and then grafted into the abdomen (aorta-aorta anastomosis) of receiving syngeneic rat. After 2 weeks, a pouch of 3

mm^2 was made in the thickness of the ventricular wall at the level of the post-infarction scar. The hyaluronic scaffold, previously engineered for 3 weeks with rat MSCs, was introduced into the pouch and the myocardial edges sutured with few stitches. Two weeks later we evaluated the cardiac function by M-Mode echocardiography and the myocardial morphology by microscope analysis.

We chose bone marrow-derived mensenchymal stem cells (MSCs) because they have shown great signaling and regenerative properties when delivered to heart tissue following a myocardial infarction (MI). However, while the object of cell transplantation is to improve ventricular function, cardiac cell transplantation has had limited success because of poor graft viability and low cell retention, that's why we decided to combine MSCs with a biopolimeric scaffold.

At the end of the experiments we observed that the hyaluronan fibres had not been substantially degraded 2 weeks after heart-transplantation. Most MSCs had migrated to the surrounding infarcted area where they were especially found close to small-sized vessels. Scar tissue was moderated in the engrafted region and the thickness of the corresponding ventricular wall was comparable to that of the non-infarcted remote area. Also, the left ventricular shortening fraction, evaluated by M-Mode echocardiography, was found a little bit increased when compared to that measured just before construct transplantation. Therefore, this study suggests that post-infarction myocardial remodelling can be favourably affected by the grafting of MSCs delivered through a hyaluron-based scaffold

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LIST OF ABBREVIATION

CCT = Cardiac Cell Therapy

MI = Myocardial Infarction

ICM = Inner Cell Mass

ES = Embrionic Stem cells

HSCs = Hematopoietic Stem Cells

SP cells = Side Population cells

CSCs = Cardiac Stem Cells

MSCs = Mesenchymal Stem Cells or Mesenchymal Stromal Cells

BM-MSCs or BMSCs = Bone Marrow derived Mesenchymal Stem or Stromal Cells

MAPC = Multipotent Adult Progenitor

TCSC = Tissue Committed Stem Cells

CFU = Colony Forming Units

FACS = Fluorescent-Activated Cell Sorting

SDF-1 = Stromal cell-Derived Factor-1

GFP = Green Fluorescent Protein

VEGF = Vascular Endothelial Growth Factor

HGF = Hepatocyte Growth Factor

AM = AdrenoMedullin

IGF-1 = Insulin Growth Factor-1

MCP-1 = Monocyte Chemeattractant Protein-1

MR and MRI = Magnetic Resonance and Magnetic Resonce Imaging

ECM = Extra Cellular Matrix

PLA = PolyLactic Acid

PGA = PolyGlicolic Acid

PCL = PolyCaproLactone

GAGs = GlycosAminoGlycans

PLGA = PolyLactic-co-Glycolic Acid

PIPAAm = poly(N-isopropylacrylamide)

PVDF = Poly(Vinylidene difluoride)

HA = Hyaluronic Acid

ICAM-1 = IntraCellular Adhesion Molecule-1

HASs = Hyaluronic Acid Synthases

HYALs = Hyaluronidases

HABPs = Hyaluronic Acid Binding Proteins

RHAMM = Receptor for Hyaluronan Mediated Mobility expressed protein

LYVE-1 = Lymphatic Vessel Endothelial Hyaluronan Receptor-1

COX-2 = Cycloxygenase-2

HYAFF® 11 = Hyaluronan biopolymer produced by Fidia Advanced Biopolymer

BSA = Bovine Serum Albumin

FBS = Fetal Bovine Serum

PBS = Phosphate Buffered Salin

DAPI = 4',6-Diamidino-2-Phenylindole

SEM = Scanning Electron Microscopy

TEM = Transmission Electron Microscopy

CFDA-SE = CarboxyFluorescein DiAcetate Succinimydil Ester

MTT = (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

LM = Light Microscopy

BrdU = 5- Bromo- 2' DeoxyUridine

LAD = Left Artery Discendent

FS = Fractional Shortening

LVDd or LVDs = Left Ventricular Diameter in diastole or systole

H&E = Hematoxylin and Eosin

cTnI = cardiac Troponin I

MP = Phagocyte Marker

CM = cardiomyocytes

ODC =Ornithine decarboxylase

α-MHC = α-Myosin Haevy Chain

INTRODUCTION

1. Heart failure

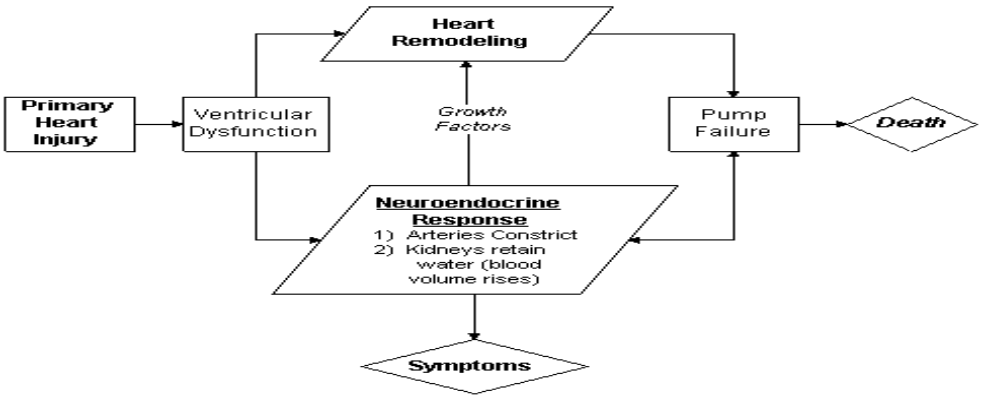
Heart failure is a major cardiovascular health problem worldwide. Despite advances in medical therapy and coronary revascularization strategies, ischemic heart disease remains the leading cause of congestive heart failure and cardiovascular mortality in industrialized countries. For example in USA about 5.2 million patients have heart failure, and the lifetime risk of developing chronic heart failure for both men and women is 1 in 5 [1] . Coronary artery disease is the most common cause of heart failure, followed by idiopathic dilated cardiomyopathy and valvular heart disease [2, 3]. Obstruction of coronary artery leads to myocardial infarction (heart attack) with associated necrosis of the myocardium, followed by infiltration of inflammatory cells. Then a scar forms, leading to the loss of cardiac function, ventricular remodelling and progressive dysfunction, and, finally, congestive heart failure [4-6].

Current therapy of heart failure is limited to the treatment of already established disease and is predominantly pharmacological, aiming primarily to inhibit the neurohormonal axis that results in excessive cardiac activation through angiotensin- or norepinephrine-dependent pathways. For patients with end-stage heart failure treatment options are extremely limited, with fewer than 3,000 being offered cardiac transplants annually due to the severely limited supply of donor organs [7, 8], and implantable left ventricular assist devices being expensive, not proven for long-term use, and associated with significant complications [9-11]. Therefore, there is a need to develop more effective, less invasive therapeutic strategies for heart failure. Because the self-renewal capacity of adult cardiomyocytes is limited, the development of strategies to regenerate damaged myocardium and improve heart function represents a major challenge. Analysis of cardiac myocyte growth during early mammalian development indicates that cardiac myocyte DNA synthesis occurs primarily in utero, with proliferating cells decreasing from 33% at midgestation to 2% at birth [12]. While ventricular karyokinesis and cytokinesis are coupled during fetal growth, resulting in increases in mononucleated cardiac myocytes, karyokinesis occurs in the absence of cytokinesis for a transient period during the postnatal period, resulting in binucleation of ventricular myocytes without an overall increase in cell number. A similar dissociation between karyokinesis and cytokinesis characterizes the primary adult mammalian cardiac response to ischemia, resulting in myocyte hypertrophy and increase in nuclear ploidy rather than myocyte hyperplasia [13, 14]. Throughout life a mixture of young and old cells is present in the normal myocardium. Although most myocytes seem to be terminally differentiated, there is a fraction of younger myocytes (15–20%) that retains the capacity to replicate [15].

Moreover, recent observations suggest that some human ventricular cardiomyocytes also have the capacity to proliferate and regenerate in response to ischemic injury [16, 17]. The dividing myocytes can be identified on the basis of immunohistochemical staining of proliferating nuclear structures such as Ki67 and cell surface expression of specific surface markers, including c-kit (CD117). Whether these cells are derived from a resident pool of cardiomyocyte stem cells or from a renewable source of circulating bone marrow derived stem cells that home to the damaged myocardium remains to be determined. More importantly, the signals required for homing, in situ expansion, and differentiation of these cells are at present unknown. Gaining and understanding of these issues would open the possibility of manipulating the biology of endogenous cardiomyocytes to augment the healing process after myocardial ischemia [18].

Over the past several years a number of studies have suggested that stem cells can be used to generate cardiomyocytes and endothelial cells *ex vivo* and *in vivo* [19-23] and so Cardiac Cell Therapy (*CCT*) should represent a new approach to replacing damaged myocardium.

The cell therapy for heart failure could has the potential to restore cardiac function by inducing neovascularization, and regenerating and protecting cardiomyocytes [4]. Replacement and regeneration of functional cardiac muscle after an ischemic insult to the heart could be achieved by either stimulating proliferation of endogenous mature cardiomyocytes or resident cardiac stem cells or by implanting exogenous donor-derived or allogeneic cardiomyocytes. The newly formed cardiomyocytes must integrate precisely into the existing myocardial wall to augment contractile function of the residual myocardium in a synchronized manner and avoid alterations in the electrical conduction and syncytial contraction of the heart, potentially resulting in life-threatening consequences. In addition, whatever the source of the cells used, it is likely that concurrent myocardial revascularization must also occur to ensure viability of the repaired region and prevent further scar tissue formation [18].



2. Cardiac regenerative medicine: the Stem Cells Therapy

It's widely agreed that regenerative capacity of human myocardium is grossly inadequate to compensate for the severe loss of heart muscle presented by catastrophic myocardial infarction or other myocardial diseases.

The ideal therapy for heart failure would have the following activities; it would minimize loss of cardiomyocytes by reducing cell death, promote return of stunned and hibernating myocardium to normal function, stimulate revascularization of the ischemic region by enhancing angiogenesis, and regenerate viable cardiomyocytes to replace those lost to the initial ischemia thereby preserving contractile function and reducing the opportunity for scarring [4]. Recent advances in biotechnology and in the understanding of tissue regeneration have allowed development of novel therapeutics with the potential to approximate this ideal: the stem cell therapy.

Cardiac stem cell therapy involves delivering a variety of cells into hearts following myocardial infarction or chronic cardiomyopathy [Fig.1]. Suitable sources of cells for cardiac transplant will depend on the types of diseases to be treated. For acute myocardial infarction, a cell that reduces myocardial necrosis and augments vascular blood flow will be desirable. For heart failure, cells that replace or promote myogenesis, reverse apoptopic mechanisms and reactivate dormant cell processes will be useful.

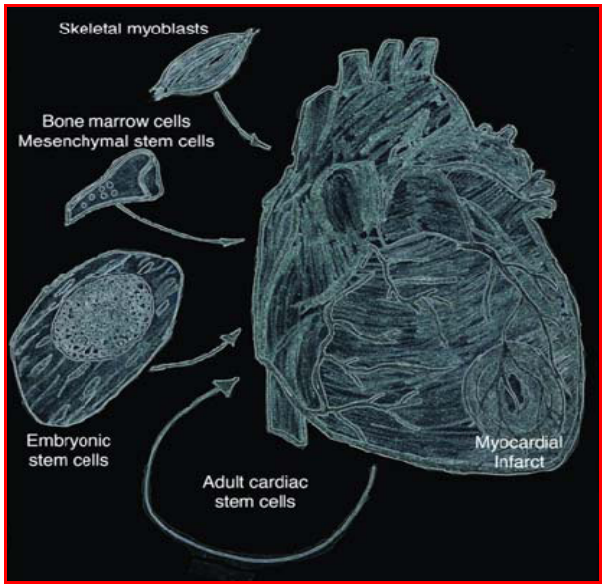


Figure 1. Stem cells as applied for the treatment of myocardial infarcts. The main purpose of stem cell therapies for the treatment of myocardial infarcts is the prevention and or regeneration of dying muscle. A variety of cell types have been used for such a treatment. These cells include skeletal myoblasts, bone marrow-MSCs, cardiac resident stem cells, and embryonic stem cells including their differentiated progeny (Figure from Christoforou N and Gearhant JD, 2007).

Stem cells might play a role in cardiac repairing becoming a specialized cell. One important type of cell that can be developed is the cardiomyocyte, the heart muscle cell that contracts to eject the blood out of the heart's main pumping chamber (the ventricle). Two other cell types are important to a properly functioning heart are the vascular endothelial cell, which forms the inner lining of new blood vessels, and the smooth muscle cell, which forms the wall of blood vessels. The heart has a large demand for blood flow, and these specialized cells are important for developing a new network of arteries to bring nutrients and oxygen to the cardiomyocytes after a heart has been damaged. The potential capability of both embryonic and adult stem cells to develop into these cells types in the damaged heart is now being explored as part of a strategy to restore heart function to people who have had heart attacks or have congestive heart failure.

3. Stem Cells

In the last few years, great emphasis has been placed on the isolation, characterization and potential therapeutic uses of stem cells. Stem cells are defined as cells capable of both self-renewal and commitment to differentiation into one or more mature cell types. However different types of stem cells can be distinguished on the basis of their developmental potential.

The real totipotent stem cells are the fertilized oocyte (the zygote) and its descendants of the first two divisions. These cells are indeed able to form the embryo and the trophoblasts of the placenta. After about 4 days, these totipotent cells begin to specialize, forming a hollow ball of cells, the blastocyst, and a cluster of cells called the inner cell mass (ICM) from which the embryo develops. The ICM cells are considered to be pluripotent, namely able to differentiate into almost all cells that arise from the three germ layers, but they are unable to give rise to the placenta and supporting tissues. ICM cells can be maintained in culture and the cell lines derived, known as embryonic stem (ES) cells, are also considered to be pluripotential [Fig.2]. In the adult, most tissues have multipotential stem cells, defined a cells capable of producing a limited range of differentiated cell lineages appropriate to their location. Also adult stem cells are heterogeneous with respect to developmental potential. For example, small intestinal stem cells can produce all four indigenous lineages (Paneth, goblet, absorptive columnar, and enteroendocrine), while central nervous system (CNS) stem cells have tri-lineage potential, giving rise to neurons, oligodendrocytes, and astrocytes. At the bottom of the stem cell hierarchy are unipotential stem cells, capable of generating one

specific cell type. Examples are epidermal stem cells in the basal layer that produce only keratinized squames and certain adult hepatocytes that have long-term repopulating ability [24].

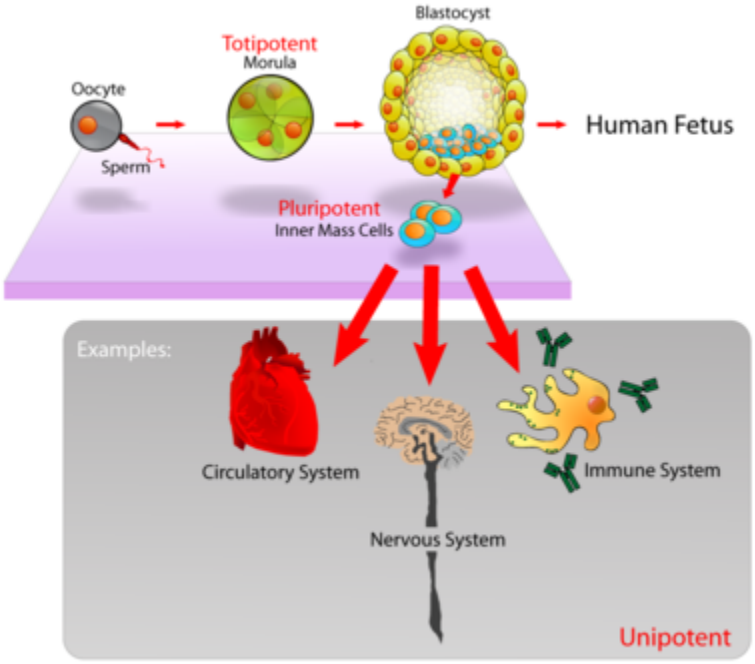


Figure 2. Pluripotent, embryonic stem cells originate as inner mass cells within a blastocyst. The stem cells can become any tissue in the body, excluding a placenta. Only the morula's cells are totipotent, able to become all tissues and a placenta.

Tissue-specific stem cells appear to be present in most organs of the body, and share some common properties:

(1) Stem cells are a self-maintaining population.

This is achieved if, on average, each stem cell division gives rise to one replacing stem cell and one transit-amplifying cell (asymmetrical division). Equally well, stem cell numbers would remain constant if only symmetrical divisions occurred, provided that each time a stem cell gave rise to two daughter transit amplifying cells, another stem cell gave rise to two daughter stem cells.

(2) Stem cells are a small percentage of the total cellularity.

In the mouse small intestine, there are perhaps 4–5 stem cells in a ring near the bottom of the crypt [25] out of a total crypt population of about 250 cells. Likewise, in skeletal muscle, satellite cells comprise about 5% of all nuclei. In the bone marrow, the multipotential hematopoietic stem cell (HSC) is even more rare, with a frequency of perhaps 1 in 104-105 bone marrow cells.

(3) Stem cells are undifferentiated.

In most tissues, stem cells do not have the specialized functions of the progeny that they originate.

(4) Stem cells are slowly cycling but highly clonogenic.

In theory, it would seem prudent to restrict stem cell division because DNA synthesis can be error-prone. Thus, in many tissues we see that stem cells divide less frequently than transit-amplifying cells. In the intestine, stem cells cycle less frequently than transit-amplifying cells, located more luminally [26] and in human epidermis, integrin-bright cells have a lower level of proliferation as compared to other basal cells. In hair follicles, the hair shaft and its surrounding sheaths are produced by the hair matrix, which is itself replenished by the bulge stem cells. The bulge cells divide less frequently, but are more clonogenic than the transit-amplifying cells of the hair matrix [27] thus showing an extensive proliferative ability.

In many tissues and organs, the identity of the stem cells has remained either elusive or at least equivocal [Fig.3]. However, in the bone marrow the identification of cells with the properties of self-renewal and multi-lineage differentiation potentialities well advanced. Indeed, such cells were functionally defined in the mouse back in 1961 by Till and McCulloch [28] as cells that, upon transplantation, were able to form multilineage hematopoietic colonies in the spleen of lethally irradiated animals [colony forming units – spleen (CFU-S)].

In human bone marrow, the sialomucin CD34 is a hematopoietic cell surface antigen that has been extensively exploited for the selection of long-term repopulating cells with multi-lineage potential, although not all HSCs express this. Nowadays, murine HSCs are empirically recognized on the basis of their immunoprofile and known as KLS cells (selected using several markers; Kit $^{+}$/Lin^{-}/Sca-1^{+}). An alternative method for enriching HSCs exploits the fact that some cells have evolved a cellular protection mechanism against toxic metabolites and xenobiotics. This mechanism involves the activity of efflux pumps that belong to the ATP-binding cassette (ABC) superfamily of membrane transporters, and such cells are able to efflux a combination of Hoechst 33342 and Rhodamine 123, thus appearing at the bottom left corner of a dual parameter FACS analysis – hence called the side population (SP) [29]. SP cells have been found in many other tissues, and the association between SP phenotype and stemness seems to be true in most of these tissues.

In the central nervous system, neural stem cells and probably their transit-amplifying descendants express both the intermediate filament nestin and a 39 kD RNA-binding protein known as Musashi1 [30,31]. Musashi was first identified in Drosophila and thought to be responsible for the asymmetric divisions of sensory organ precursor cells [32]; it may also be a marker for intestinal crypt stem cells.

A tissue that has been traditionally considered completely post-mitotic is the heart. However, in the last few years a number of studies suggested the presence of a cardiac stem cell population capable of (re)generating the cardiac tissue throughout life, raising the possibility to speculate about new potentially therapeutic strategies for cardiac repair. Initial evidence, though in part controversial, has been presented that bone marrow cells injected into damaged myocardium or mobilized into the circulation may transdifferentiate into heart cell types [33-36]. Additional, exciting approaches for cardiac repair have been raised by the remarkable discovery that the heart contains a reservoir of stem and progenitor cells.

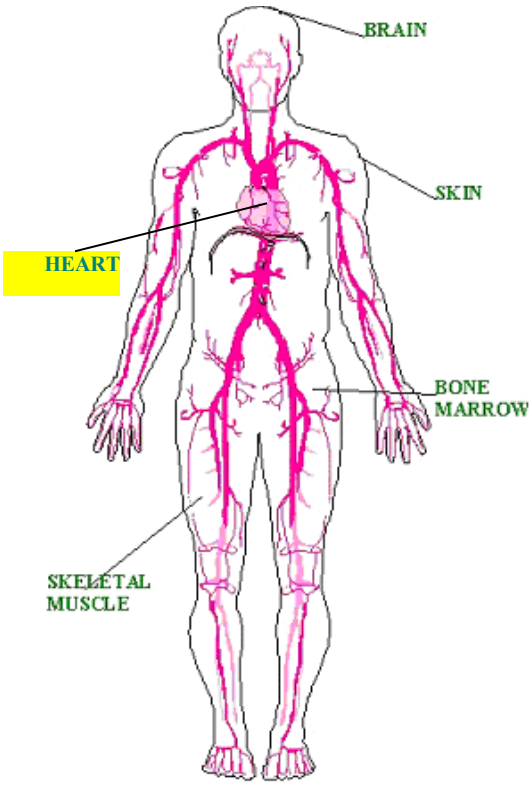


Figure 3. Adult stem cells locations

Comparing embryonic and adult stem cells, it's clear that both of them have advantages and disadvantages regarding potential use for cell-based regenerative therapies. Of course, adult and embryonic stem cells differ in the number and type of differentiated cells types they can become. Embryonic stem cells can become all cell types of the body because they are pluripotent. Adult stem cells are generally limited to differentiating into different cell types of their tissue of origin. However, different scientific evidences suggest that adult stem cell plasticity may exist, increasing the number of cell types a given adult stem cell can become. A great potential advantage of using stem cells from an adult is that the patient's own cells could be expanded in culture and then reintroduced into the patient. The use of the patient's own adult stem cells would mean that the cells would not be rejected by the immune system. This represents a significant advantage as immune rejection is a difficult problem that can only be circumvented with immunosuppressive drugs. Moreover, the use of adult stem cells overcomes the ethical problem of embryo "destruction"; that's why the interest of researchers about stem cells derived from adult tissues is increasing.

Actually, the most stunning aspect of current progress towards cardiac regeneration is the wide variety of cell types that have been considered as candidates for therapeutic delivery in humans [Fig. 4]. This myriad of cell types reflects the unmet medical need for treating heart disease, and hence the large amount of experimental effort being put into devising cell-based therapies. It also points to the lack of mechanistic understanding at many levels. The ideal cell type has not yet emerged, and few clinical studies are still comparing different adult stem-cell types such as skeletal myoblasts, endogenous cardiac stem cells, bone-marrow-derived cells (i.e. haematopoietic stem cells, endothelial progenitor cells, mesenchymal stem cells etc.) but also embryonic stem cells.

Some of these tested stem cells have been shown to improve cardiac function through various mechanisms, including the formation of new myocytes, endothelial cells and vascular smooth muscle cells, as well as trough paracrine effects.

Different experimental data have shown that, for cardiac regenerative medicine, cardiac stem cells (CSCs) and bone marrow- derived mesenchymal stem cells (BMSCs) have shown the greatest regenerative potential.

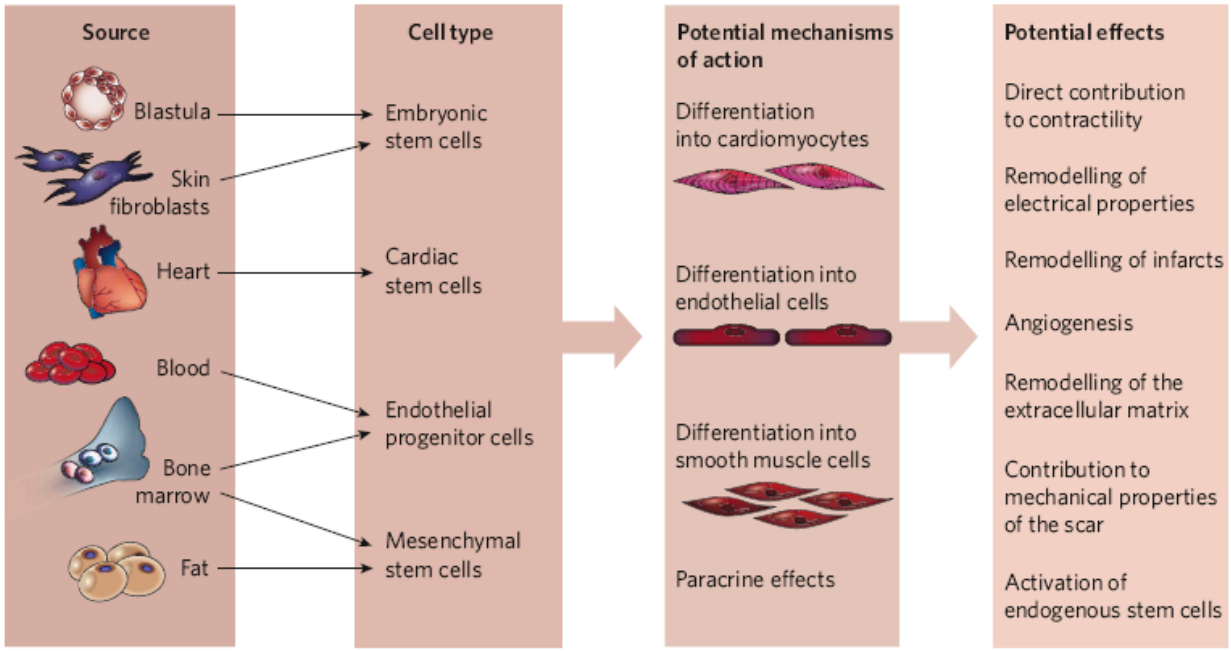


Figure 4. Different cell types and mechanisms proposed for cardiac stem cell therapy (Figure from Vincent F. M. Segers and Richard T. Lee, Nature 2008)

3.1 Cardiac stem cells

The adult heart was said for many years to be a postmitotic organ [12, 37, 38]. Of course, it was known that the endothelial, smooth muscle, and fibroblast cells of the heart do proliferate. But the cells that make up the meat of the heart—the myocardium—were thought to be terminally differentiated and therefore had lost their proliferative capacity [12, 37-39]. A major implication of this belief is that the myocytes comprising the adult myocardium are present at birth and are not replenished during an individual's life span. This traditional viewpoint was supported by several lines of evidence. First, studies examining mitosis in the heart showed that the number of myocytes undergoing proliferation was either very low or nonexistent in the adult myocardium. When comparing the heart to other tissues that have obvious high levels of cellular regeneration (e.g., the bone marrow, liver, etc.), it was reasonable to conclude that the myocardium was postmitotic. Second, it has been well established that the myocardium responds to increased load, due to physiological and pathological stress, principally by cellular hypertrophy (increase in cell size) without obvious cellular hyperplasia (increase in cell number). Third, in response to severe injury of the myocardium, as occurs in an infarct, the myocardium is unable to restore functional cardiac muscle within the damaged area [12, 39, 40].

Throughout the years, there were several reports suggesting that cells within the myocardium undergo cell division [41, 42], thus challenging the prevailing concept that the adult myocardium is postmitotic. However, the idea that the adult heart contains at least some newly regenerated myocytes has only been taken seriously during the past five years [43-45]. In 2003, in fact, several independent groups reported the discovery of resident progenitor cells in heart tissue, which would have the capacity to regenerate sections of the healthy or injured myocardium [46-48].

In one such study, a Lin^{-} $c\text{-}Kit^{+}$ cell was isolated from the adult rat heart, and it was reported to be self-renewing, clonogenic, and multipotent, being able to give rise to cardiomyocytes, smooth muscle cells, and endothelial cells [49]. The cells were injected into ischemic hearts and were reported to reconstitute up to 70% of the myocardium, forming both new vessels and cardiomyocytes. More importantly, the authors reported a significant increase in cardiac function of the animals that had received these cells. In a similar study, the same cell type was delivered to the coronary arteries of rats that had had a myocardial infarct, via a catheter positioned into the aortic root [50]. The cells were reported to induce myocardial regeneration through the decrease of the infarct size by 29% and also to improve the function of the injured heart.

At about the same time, a different type of adult cardiac stem cell was reported by Oh *et al* [51]. The cells, isolated from adult mouse hearts, were $Sca\text{-}1^{+}$ and initiated in vitro cardiac gene expression after treatment with the DNA demethylating agent 5-azacytidine. A mouse model of ischemia/ reperfusion injury was used to assay the in vivo capacity of the $Sca\text{-}1^{+}$ cardiac stem cells to regenerate the infarcted myocardium. Transplanted cells initiated production of cardiac functional proteins and were identified in the mouse hearts 2 weeks after injection. Fusion between the donor cells and the host cardiomyocytes was evident in about 50% of the identified cells, whereas the other 50% had differentiated into cardiomyocytes.

Martin *et al* [52] reported on the identification of a resident cardiac population of adult stem cells with the unique expression of the Abcg2 transporter protein (Side Population Cells-SPCs). The $Abcg2^{+}$ cells were reported to differentiate into alpha actinin-positive cells when cocultured with adult cardiomyocytes; however, when cultured individually in methylcellulose, they gave rise to hematopoietic colonies. Finally, gene expression analysis revealed a unique transcriptional signature similar to that of endothelial and hematopoietic progenitor cells. Cell-based cardiac therapies using the $Abcg2^{+}$ cells are underway.

Islet-1, a LIM homeodomain transcription factor, is uniquely expressed in the adult heart by the fourth identified cardiac stem cell population. This marker is expressed by cardiac progenitor cells

in the secondary cardiac field, a structure present during early development, which contributes to most cells in the heart [53]. During development, proliferating progenitor cells in the outflow tract, the right ventricle, and the atrium express isl1, without which they cannot contribute to the heart. Isl1$^+$ cells were identified in the postnatal rat, mouse, and human myocardium [54].

A technique of conditional genetic marking was used to identify the isl1$^+$ population in a temporal/spatial manner. This allowed the authors to selectively isolate the isl1$^+$ cells at a particular developmental stage. When cocultured with isolated cardiac mesenchymal cells, the isl1$^+$ cells maintained isl1 expression and proliferated in culture without differentiating. The isolated cells could be induced to differentiate in culture into cardiomyocytes after exposure to 4-OH-TM or after coculture with neonatal cardiac myocytes. No cell-based cardiac therapy experiments have yet to be reported involving the isl1+ cardiac stem cells.

The four adult cardiac stem cell populations reported isolated are Lin$^-$/c-Kit$^+$, Sca-1$^+$, Abcg2$^+$, and isl1$^+$ cells. When these cell types were examined for markers expressed by the rest of the stem cell populations, it was found that the c- Kit$^+$ cells did not express Sca-1, the Sca-1$^+$ cells did not express c-Kit, and the isl1$^+$ cells did not express c-Kit or Sca-1. The Abcg2$^+$ cells were reported to be approximately 50% Sca-1$^+$ and only about 2% c-Kit$^+$ (no data were given for isl1).

Therefore, cardiac stem and progenitor cell types characterized so far exhibit significant differences in their immunophenotypic, developmental and biological properties. Thus, the heart probably contains various types of stem and progenitor cells. This is in agreement with the emerging consensus that more than one stem cell may be present in a particular tissue [55], and with recent evidence that heart endothelial, cardiac and muscle cells may arise from a hierarchy of multipotent/bipotent stem progenitor cells [56-58, Fig.5]. The developmental origin(s) of stem and progenitor cells of the adult heart has been studied only to a very limited extent [59]. Mouse chimera experiments suggest that most (at least 95%) cardiomyocytes are derived from a relatively small embryonic founder population, ruling out any major contribution to the adult cardiomyocyte population from “immigrant” non-cardiac stem cells, at least under physiological conditions [60]. On the other hand, several findings, including the presence of cardiac chimerism in patients receiving allogeneic bone marrow transplantation, suggest that immigrant bone marrow-derived cells could behave, under pathological conditions leading to heart damage, as precursors of cardiac stem cells [61]. Thus, the important issue of extra-cardiac sources of CSCs, following cardiac damage, still needs to be addressed. If extra-cardiac cells can be induced, not only to migrate to the

heart, but also to reconstitute a pool of extensively replicating CSCs, exciting therapeutic possibilities may arise.

Moreover, if these $Lin^{-}/c\text{-}Kit^{+}$, $Sca\text{-}1^{+}$, $Abcg2^{+}$, and $isl1^{+}$ described progenitor/stem cell populations reside in the myocardium, it would be informative to examine why under normal circumstances they do not regenerate the myocardium and how can we stimulate them to do so.

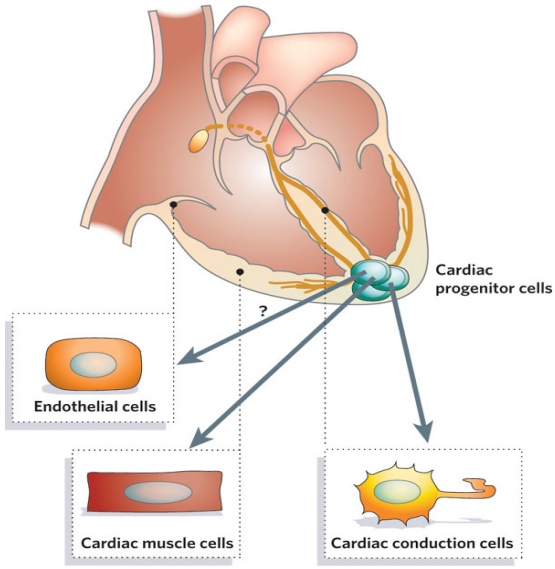


Figure 5. Stem and progenitor cells are present in heart tissue.

3.2 Bone marrow mesenchymal Stem Cells

About 40 years ago Friedenstein described stromal cells in the bone marrow that were spindle shaped and proliferate to form colonies [62]. These cells attach to plastic and are able to differentiate under defined *in vitro* conditions into multiple cell types present in many different tissues, e.g. osteoblasts, chondroblasts, adipocytes and, more recently, cardiomyocytes and vascular endothelial cells [21, 63, Fig.6]. Later on these cells, obtained from postnatal bone marrow, were called mesenchymal stem cells (MSCs) or stromal stem cells [64, 65]. The term mesenchymal stem cells (MSC) was popularized by Caplan [66], in reference to work by Friedenstein and Owen [67], describing a plastic-adherent fibroblastic cell isolated by Percoll density centrifugation, reactive with monoclonal antibodies SH2 and SH3. The adjective ‘mesenchymal’ is fraught with some ambiguity since ‘mesenchyme’ describes tissue of mesodermal origin, the middle embryological germ layer, giving rise to the musculoskeletal, blood, vascular and urinogenital systems, and to

connective tissue (including dermis). Thus, developmentally speaking, the term ‘mesenchymal’ should include both blood and connective tissue cells. In practice however, only the latter cells are usually described as being derived from MSC and considered distinct from haematopoietic stem cells (HSC), which are responsible for the development, maintenance, and regeneration of blood forming tissues [68]. It is quite possible that MSC and HSC have a common precursor in the elusive ‘‘haemangioblasts’’ [69], in the cells identified by the group of Verfaillie originally termed ‘‘mesodermal progenitor’’ [70], later ‘‘multipotent adult progenitor’’ (MAPC) [71] cells, or in ‘‘pluripotent stem cells” [72] , or ‘tissue committed stem cells’ (TCSC) [73]. However, this is contentious and the physiological relevance of these cells remains to be demonstrated.

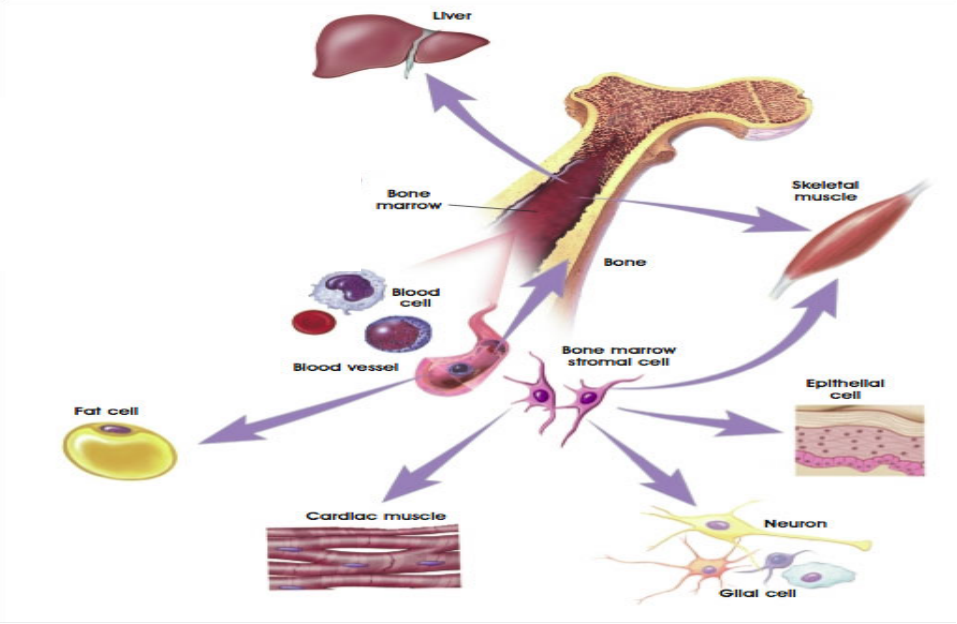


Figure 6. Bone marrow stem stell are multipotent stem cells.

Recently the presence of somewhat similar cells has been demonstrated in many other tissues too. In fact, different studies have shown that MSCs reside not only in bone marrow but also in other tissues such as adipose tissue [74] , synovium [75] , periosteum [76] , muscle [77] , dental pulp [78], periodontal ligament [79] , placenta [80] and umbilical cord blood [81]. A recent study suggests that MSC reside in virtually all postnatal organs and tissues, and may be localized to vessel walls [82]. Bone marrow-derived MSC, which are most investigated, are a rare subpopulation of bone marrow cells (approximately 0.001-0.01%) [21].

So far the nomenclature is not consistent. Designations for cells with nonhaematopoietic multipotency have included ‘‘colony-forming-unit-fibroblasts’’, ‘‘stromal (stem) cells’’, ‘‘bone marrow (stromal) cells’’, ‘‘skeletal stem cells’’, ‘‘mesodermal progenitor cells’’, ‘‘non-haematopoietic stem cells’’, ‘‘(bone marrow) stem cells’’, ‘‘mesenchymal progenitor cells’’ and others [71, 83]. There is also an understandable tendency to designate such cells as ‘‘pre-(lineage-under-investigation)’’ cells (e.g. pre-osteoblast etc.). It has also been suggested that MSC are simply pericytes [84]. Some of the inconsistencies surrounding the identification of MSC arise from the fact that specific markers have not yet been agreed on. In the absence of a universal antigenic indication (analogous to $CD34^+$ for HSC) and an universal assay (analogous to the repopulation assays for HSC) MSC are often identified simply by testing a cultures' differentiation potential into colony forming units (CFU) indicative of proliferative capacity and into several lineages of mesenchymal tissue as defined above [21]. Also, the ability to adhere to tissue culture plastic and a fibroblastlike morphology are taken as characteristic markers for MSC [66]. Recently, different surface markers have been associated with MSC including D7fib [85], Stro1 [86], CD45 and glycophorin A [21, 70, 87], BMPR1a [83, 88].

Further complications arise when different sources, extraction and cultivation methods are used. Even when narrowing sources to bone marrow, the site of extraction is reported to influence cell behaviour: e.g. MSC from alveolar bone show less chondrogenic and adipogenic potential compared to iliac bone [89] . Isolation is usually conducted by density centrifugation (sometimes enhanced by gradient solutions) to obtain the mononuclear fraction of marrow cells and by using the widely reported ability of MSC to adhere to tissue culture plastic [90]. Newer methods employ magnetic beads [86] or FACS sorting [91] in conjunction with antibodies to the proposed MSC markers above. Additionally, widely differing standards regarding serum composition, culture conditions, and growth factor application in MSC cultivation exist. Differing conditions can lead to enrichment of different subsets of MSC with differing clonogenic potential. All these potential deviation points in current methods are summarised in Table 1.

Table 1

Extraction sites (Prinz et al., 1999)
- Bone marrow (live donors—partial samples only): hip, sternum, broken bones (rare)
- Bone marrow (cadaver donors): all sites (rare)
- Other tissue: teeth, fat, muscle, cartilage, synovial fluid, skin
- Developmental tissue: foetal, umbilical, placenta

Dissociation method
- Trypsination
- Scraping
- Suspension culture

Marker combinations
- $CD10^+$, $CD13^+$, $CD34^+$, $CD56^+$, $CD90^+$, MHC-1^+ (Young et al., 1999)
- $CD10^-$, $CD13^+$, $CD31^-$, $CD34^-$, $CD44^+$, $CD45^-$, $CD90^+$, $CD105^+$, $CD133^-$ $Wnt2^+$,$Wnt4^+$, $Wnt5a^+$, $Wnt11^+$, $Wnt16^+$, $Fz2^+$, $Fz3^+$, $Fz4^+$, $Fz5^+$, $Fz6^+$ (Etheridge et al., 2004)
- $VCAM^+$, STRO-1^+, $CD73^+$, $CD105^+$ (Tuli et al., 2003)
- $GlyA^-$, $CD45^-$ (Reyes et al., 2001)
- D7-FIB^+, $CD13^+$; $CD45^-$, GPA^-, $LNGFR^+$ (Jones et al., 2002)
- $SH2^+$; $SH3^+$; $CD14^-$; $CD29^+$, $CD34^-$, $CD44^+$; $CD45^-$, CD71+, $CD90^+$, $CD106^+$, $CD120a^+$, $CD124^+$ (Pittenger et al., 1999)

Identification
- Adherence to plastic
- Magnetic bead
- FACS cell sorting

Other factors
- Cell line or ex vivo
- Donor age
- Donor sex
- Donor disease status
- Point of first analysis
- Seeding density
- Feeder cells used
- Culture conditions (temperature, motion, etc.)
- Differentiation agent
- Medium composition
- First medium change
- Frequency of medium change

Listed are some variables in MSC description where differing standards reportedly or likely result in influencing cell behaviour or otherwise lead to variant data

Since MSC can be rather easily isolated from the bone marrow and can also be expanded *in vitro* they have become a prime target for researchers of tissue regeneration. These cells have now been extensively used for transplantation experiments in animals and also for some therapeutic trials in humans. However, much new research is needed to learn enough on the molecular mechanisms of MSC differentiation to evaluate their full capacity for tissue regeneration. Mesenchymal stem cells have been studied in great detail and scientists have advanced knowledge about how to grow these cells in culture. Unlike most other human adult stem cells, mesenchymal stem cells can be obtained in quantities appropriate for clinical applications, making them good candidates for use in tissue repair. Techniques for isolation and amplification of mesenchymal stem cells in culture have been established and the cells can be maintained and propagated in culture for long periods of time, without loosing their capacity to form all the above cell types.

3.2.1 Bone Marrow-MSC and cardiac repairing

In vitro studies have demonstrated that bone marrow stem cells (BMSC) can differentiate not only into adipocytes and osteocytes, but also into cardiomyocytes and vascular endothelial cells *in vivo* and *in vitro* [21, 63, 92-94]. Conversely, it has also been reported that the differentiation of BMSC into cardiomyocytes occurs rarely, if at all [95-97]. There is no consensus on the plasticity of BMSC, including their differentiation into functional cardiomyocytes; however, experimental studies and clinical trials have demonstrated that the implantation of bone marrow-derived cells can improve regional perfusion and cardiac function of the injured heart, by rebuilding the damaged myocardium and cardiac vessels [98-100]. Thus, autologous BMSC are still one of the most studied cell sources for myocardial repair.

Different researchers tested the in vitro capacity of mouse bone marrow–derived MSCs to differentiate into cardiomyocytes in cultures containing either BMSC alone, treated with 5-azacytidine or a cocktail of growth factors and in co-culture with cardiomyocytes [101, 102]. The in vitro differentiation of BMSC into cardiomyocytes was first reported in 1999 by Makino et al. [92]. The authors induced the cells to differentiate by treating them with 5-azacytidine: a global DNA demethylating agent that acts as a cytosine analog capable of altering expression of certain genes that may regulate differentiation. The morphology of about 30% of the treated cells changed within a week of treatment, and by the second week, the cells were spontaneously contracting and expressing cardiac specific proteins. Bittira et al [103] also reported the isolation of rat bone

marrow–derived MSCs. LacZ labeled cells received either 5-azacytidine treatment or no treatment and were subsequently injected into the cryoinjured myocardium of isogenic rats. The authors reported that 4 to 8 weeks postinjection, the treated cells appeared myotube-like while expressing the cardiac marker troponin I-C. The data from both studies suggest that cell treatment with a DNA demethylating agent is necessary for the differentiation of MSCs into cardiomyocytes.

The effect of bone marrow–derived MSCs on cardiac function after myocardial infarction was also examined. A study reported by Shake *et al* [104] focused on the implantation of autologous MSCs in a swine myocardial infarct model. Labelled cells were administered 2 weeks postmyocardial infarction in the infarcted area through direct injection. The authors reported a significant attenuation in the degree of contractile dysfunction in the transplanted animals with reduced wall thinning in the infarcted region of the myocardium. In the second study, bone marrow MSCs were injected into the tail vein of rats that had had a myocardial infarct (MI) [105]. The MSCs were reported to be recruited to the injured heart through the expression of the stromal cell-derived factor-1 (SDF-1), enhance angiogenesis, and improve cardiac function. The same authors also reported in another study that lacZ labelled rat bone marrow–derived MSCs were injected into the tail vein. The cells were able to home to the injured area of the heart and were found at high concentrations in the peri-infarct region of the myocardium [106].

Toma *et al* [107] reported the injection of lacZ labelled human bone marrow– derived MSCs into the left ventricle of mice. One week postinjection, only a limited number of cells had survived; however, these cells were reported to express cardiac-specific markers similarly to the host myocardium. Hattan *et al* [108] used a transgene that allowed expression of GFP under a ventricular-specific promoter. After MSC differentiation, GFP^+ cells were sorted and transplanted into the adult mouse myocardium with a reported long-term survival. In a most recent report, MSCs cultured in the presence of cardiogenic growth factors were injected into the myocardium of dogs that had had a myocardial infarct (coronary artery ligation) 8 weeks prior to the injections. The authors report significant functional recovery of the transplanted hearts [109]. Bone marrow–derived MSCs are reported to have the ability to home to the areas of the heart that have sustained an injury as a result of a myocardial infarct. They have also been shown to express cardiac markers in the myocardium independent of 5-azacytidine treatment. One of their advantages is that they are an autologous cell source obviating the need for immunosuppression therapy [110].

However, the time needed for MSCs to proliferate in culture to a sufficient cell number needed for the transplantation is far longer than the short amount of time the patient has postinfarction for

the injection of these cells as shown by Bittira *et al* [103]. The capacity of these cells to completely regenerate the infarcted myocardium has not been proven yet; so far it's clear that the presence of the cells in the myocardium decreases the potential infarct size but the mechanism is still unclear and poorly understood.

More recently, it has been shown that MSC should exert their effect on cardiac regeneration not only by differentiation into specific cell types, but also through paracrine actions. In vitro studies have demonstrated that MSC can secrete a variety of angiogenic, antiapoptotic and mitogenic factors such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), adrenomedullin (AM) and insulin-like growth factor-1 (IGF-1) [111, 112]. Interestingly, administration of conditioned medium obtained from MSC culture exerted cytoprotective effects on the myocardium in an animal model of myocardial infarction [113]. Ohnishi *et al* [114] demonstrated that cultured cardiomyocytes were injured in response to monocyte chemoattractant protein-1 (MCP-1), which plays an important role in myocarditis, whereas this effect was significantly attenuated by conditioned medium derived from MSC culture. These results suggest a cardioprotective effect of MSC acting in a paracrine manner, demonstrating the importance of secreted factors in cardiac repair.

4. Cell delivery in cardiac stem cell therapy

Functional benefits of cell therapy for cardiovascular applications may arise from induction of angiogenesis, cardiomyogenesis or mechanical interstitial support. The former two modes of benefit may result from site-specific transdifferentiation of administered cells or by secretion of paracrine factors that may stimulate endogenous repair mechanisms. On this basis, improvements in regional myocardial perfusion, systolic function, diastolic function and adverse ventricular remodelling would be predicted. The additional mechanical interstitial support provided by cell administration may itself impact beneficially on the ventricular remodelling process. Clearly these potential benefits need to be weighed against the potential toxicity from cell therapy, such as exacerbation of atherosclerosis, arrhythmogenesis, inappropriate calcification and local or ectopic tumor formation.

The optimal cell preparation for each of the variety of potential cardiovascular applications remains to be determined. It cannot be assumed that one cell preparation will be equally efficacious for all clinical applications, and different cell preparations may have varying toxicity profiles .

Indeed, it is unclear if administration of a highly selected cell population is preferable to a heterogeneous unselected or combination cell product [115].

Other unresolved issues include determination of the optimal number of cells to be delivered, timing of cell administration, importance of growth factor preconditioning of cellular products prior to administration, how to follow transplanted cells once they are in the body, effects of *ex vivo* cell expansion and prolonged cell culture prior to administration, and the use of allogeneic rather than autologous cell populations [Table 2].

Table 2
Comparison of allogeneic and autologous cell preparations (from R de Silva and RJ Lederman, 2004)

	Autologous	Allogeneic
Advantages	• No immune rejection of cells • Minimal risk of anaphylaxis, transfusion reaction and alloimmunization • Minimal risk from transmissible infectious agents • Rapid access to large numbers of cells, e.g. post-mobilization leukapheresis product	• "Off the shelf '' cell product, immediate access useful for acute interventions • Immediate access to large numbers of cells • Immediate access to genetically modified cells • BM aspirate, cytokine mobilization ± leukapheresis or skeletal muscle biopsy not required • Cells from young donors may overcome issues of age-related decline in regenerative capacity
Disadvantages	• BM aspirate, cytokine mobilization ± leukapheresis or skeletal muscle biopsy required • Time-consuming *ex vivo* expansion may be required (e.g. skeletal myoblasts, stromal cells), cell administration for acute conditions delayed • Potential age-related loss of regenerative capacity • Extra expense of patient-specific cell processing	• Anaphylaxis, acute transfusion reaction, alloimmunization • Potential immune rejection • Risk of transmissible infectious agents • Decline in regenerative capacity and accumulation of cytogenetic abnormalities as a consequence of prolonged *ex vivo* expansion and culture

About the sources of cell for cardiac transplant, cardiac and bone marrow stem cells are described by different researchers as the cells with the best potentiality in cardiac regeneration. Very little data is available to guide cell dosing. Scientific data suggests that there is a dose-dependent improvement in function but it is still unclear which is the best dose to warranty a real functional cardiac improvement.

Determining optimal delivery methods raise issues not only of dose, but also of timing. Moreover, assessing the fate of injected cells is critical to understanding mechanisms of action.

For therapeutic purposes, cells should be delivered to the target tissue of interest in sufficient number to confer functional benefit with minimal toxicity to the recipient.

For cardiovascular applications, modes of cell delivery may be broadly categorized as systemic or local [Table 3]. Systemic delivery may consist of intravenous infusion of cells or cytokine mobilization of cells into the circulation. The homing of systemically administered cells to the target tissue of interest requires appropriate chemokine signalling from the target tissue. The intensity of these homing signals appears to decline with time following an acute injury and may determine the time window during which systemic cell administration may confer functional benefit. Studies in rodents have demonstrated the ability of human progenitor cells from G-CSF-mobilized leukapheresis products [99] and rat allogeneic mesenchymal stromal cells [116] to home to regions of acute myocardial infarction following intravenous administration. In the former study, improvements in ejection fraction and attenuation of adverse cardiac remodelling were noted on the basis of echocardiography. Other animal studies have demonstrated that cytokine mobilization may confer hemodynamic benefits in a murine model of acute coronary artery ligation [117]. However, scientific and clinical data suggest that systemic cell delivery may not be optimal in the setting of acute or chronic coronary syndromes, although further studies are required.

Table 3
Route of cell delivery (from R de Silva and RJ Lederman, 2004)

Systemic	Local
• Intravenous infusion + cell homing	• Cardiac: percutaneous catheter **based**
• Cytokine mobilization + cell homing	• Selective intracoronary infusion
	• Transcoronary sinus retrograde infusion
	• Endomyocardial needle injection
	• Transcoronary vein
	• intramyocardial injection
	• Intrapericardial
	• Cardiac: surgical
	• **Open** chest transepicardial intramyocardial injection
	• Lower limb
	• **Intra**-arterial infusion
	• Direct intramuscular injection

Local delivery is theoretically more attractive than systemic in that larger numbers of cells may potentially be administered to specific regions of interest within the target organ. Local delivery systems should be biocompatible with the cell preparation to be administered, such that there is minimal loss of cell number and cell viability as a consequence of passage through the delivery system. Clinically, local cell delivery can be achieved by direct injection at the time of open heart surgery or by percutaneous catheter-guided intracoronary infusion or intramyocardial injection.

For example, surgical delivery is excellent for performing intramyocardial cell injection targeted to infarct borders. Using this method the cells are directly transplanted close to the damaged area in order to facilitate cell grafting and the regenerative process. Others local delivery methods are represented by catheter delivery. Percutaneous catheter-based approaches are available for delivery of cells to the myocardium via intracoronary infusion, endomyocardial injection, transcoronary vein intramyocardial injection, transcoronary sinus retrograde infusion and intrapericardial injection. Intracoronary infusions can be performed down the infarct-related artery [100] and endomyocardial injections can be localized to peri-infarct regions using an electromechanical mapping system [118,119]. Targeted cell delivery may also be achieved using real-time magnetic resonance imaging (MRI) [120, 121] techniques and cells labelled with particles [122], which appear as signal voids on the magnetic resonance (MR) image. Using this sophisticated technology, cell delivery can be targeted in real time precisely to infarct borders.

Anyway, there are several major unresolved issues in cell delivery. The optimal delivery route has not been established. There are few quantitative data addressing cell distribution and cell retention as a function of the mode of cell delivery. Biodistribution studies using technetium-99m labeled BM-derived mesenchymal stromal cells in recently infarcted rats suggest that, following intravenous infusion, the vast majority of infused cells are entrapped in the lungs with little distribution to the heart [123]. The number of cells in the heart was increased by infusion of cells directly into the left ventricular cavity [123]. Retention of cells following direct intramyocardial injection was not assessed in this study. Other pre-clinical data suggest that at best only 30–40% of particulate material is retained within the myocardium following a successful endomyocardial injection [124]. Clearly, significant improvements in cell retention and its quantification are required. Furthermore, the biocompatibility of interventional devices with therapeutic cell preparations needs further assessment. Variability in cell number, viability, migratory, proliferative and differentiation capacity as a function of cell handling and interaction with the delivery system may be important determinants of the efficacy of cardiac stem cell therapy.

5. *Tissue-engineering*: a new approach for cell delivery in cardiac regenerative medicine

The poor survival of grafted cells has been a concern of researchers. Given the poor vascular supply after a heart attack and an active inflammatory process, grafted cells survive with difficulty. As mentioned above, the efficacy of cell engraftment by injection of a cell suspension is generally very low as more than 90% of the cells injected is lost and does not engraft [125]. It is necessary to provide cells an environment that is suitable to proliferation and differentiation for the induction of tissue regeneration.

Thus, much effort is now conveyed to the development of alternative cell delivery system. One of these new strategies is the *tissue-engineering strategy,* in which biomatrices are used to generate three-dimensional cell constructs able to provide a physiological support to the cells, allowing to successfully engrafting new cells into the myocardium.

Tissue-engineering combines cells, engineering and materials methods, and suitable biochemical and physio-chemical factors to improve or replace biological functions. While most definitions of tissue engineering cover a broad range of applications, in practice the term is closely associated with applications that repair or replace portions of or whole tissues (i.e., bone, cartilage, blood vessels, heart etc...).

Often, the tissues involved require certain mechanical and structural properties for proper function. The term has also been applied to efforts to perform specific biochemical functions using cells within an artificially-created support system.

A commonly applied definition of tissue engineering, as stated by Langer and Vacanti, is "an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ" [126]. Tissue engineering has also been defined as "understanding the principles of tissue growth, and applying this to produce functional replacement tissue for clinical use" [127].

Scientific advances in biomaterials, stem cells, growth and differentiation factors, and biomimetic environments have created unique opportunities to fabricate tissues in the laboratory from combinations of engineered extracellular matrices ("scaffolds"), cells, and biologically active molecules. Tissue-engineering utilizes living cells as engineering materials, the scaffold for cell proliferation and differentiation, and, eventually, growth factors which has the potential to accelerate tissue regeneration.

The utilized cells are often categorized by their source in:

- **Autologous** cells are obtained from the same individual to which they will be reimplanted. Autologous cells have the fewest problems with rejection and pathogen transmission, however in some cases might not be available. Moreover since this category of cells needs to be harvested from the patient, there are also some concerns related to the necessity of performing such surgical operations that might lead to donor site infection or chronic pain. Autologous cells also must be cultured from samples before they can be used: this takes time, so autologous solutions may not be very quick. Recently there has been a trend towards the use of mesenchymal stem cells from bone marrow and fat. A large number of cells can be easily and quickly isolated from fat and bone marrow.

- **Allogenic** cells come from the body of a donor of the same species. While there are some ethical constraints to the use of human cells for *in vitro* studies, the employment of dermal fibroblasts from human foreskin has been demonstrated to be immunologically safe and thus a viable choice for tissue engineering of skin.

- **Xenogenic** cells are those isolated from individuals of another species. In particular animal cells have been used quite extensively in experiments aimed at the construction of cardiovascular implants.

- **Syngeneic' or isogenic** cells are isolated from genetically identical organisms, such as twins, clones, or highly inbred research animal models.

- **Stem cells** are undifferentiated cells with the ability to divide in culture and give rise to different forms of specialized cells. According to their source stem cells are divided into "adult" and "embryonic" stem cells, the first class being multipotent and the latter mostly pluripotent some cells are totipotent, in the earliest stages of the embryo. While there is still a large ethical debate related with the use of embryonic stem cells, it is thought that stem cells may be useful for the repair of diseased or damaged tissues, or may be used to grow new organs.

Cells are often implanted or 'seeded' into an artificial structure capable of supporting three-dimensional tissue formation. These structures, typically called scaffolds, are often critical, both *ex*

vivo as well as *in vivo,* to recapitulating the *in vivo* milieu and allowing cells to influence their own microenvironments. Scaffolds usually serve at least one of the following purposes:

- Allow cell attachment and migration
- Deliver and retain cells and biochemical factors
- Enable diffusion of vital cell nutrients and expressed products
- Exert certain mechanical and biological influences to modify the behavior of the cell phase
- Protect trasplanted cells from the immunological attack.

It is necessary for tissue regeneration to increase the number of cells constituting the tissue as well as reconstruct a structure of extracellular matrix (ECM) to support the proliferation and differentiation of cells for regeneration induction. In addition, it is necessary to allow cells of high potential to proliferate as well as to maintain their biological function.

To achieve the goal of tissue reconstruction, scaffolds must meet some specific requirements. The materials should be well deliquesced with contiguous tissues after transplantation without or with a very low inflammatory reaction. They must have a high porosity and an adequate pore size, necessary to facilitate cell seeding and diffusion throughout the whole structure of both cells and nutrients. Biodegradability is often an essential factor since scaffolds should preferably be absorbed by the surrounding tissues without the necessity of a surgical removal. The rate at which degradation occurs has to coincide as much as possible with the rate of tissue formation: this means that while cells are fabricating their own natural matrix structure around themselves, the scaffold is able to provide structural integrity within the body and eventually it will break down leaving the neotissue, newly formed tissue which will take over the mechanical load.

Many different materials (natural and synthetic, biodegradable and permanent) have been investigated. Most of these materials have been known in the medical field before the advent of tissue-engineering as a research topic, being already employed as bioresorbable sutures. Examples of these materials are collagen or some linear aliphatic polyester.

New biomaterials have been engineered to have ideal properties and functional customization: synthetic manufacture, biocompatibility, non-immunogenicity, transparency, nano-scale fibers, low concentration, resorption rates, etc.

A commonly used synthetic material is polylactic acid (PLA). This is a polyester which degrades within the human body to form lactic acid, a naturally occurring chemical which is easily removed

from the body. Similar materials are polyglycolic acid (PGA) and polycaprolactone (PCL): their degradation mechanism is similar to that of PLA, but they exhibit respectively a faster and a slower rate of degradation compared to PLA. Scaffolds may also be constructed from natural materials: in particular different derivatives of the extracellular matrix have been studied to evaluate their ability to support cell growth. Proteic materials, such as collagen or fibrin, and polysaccharidic materials, like chitosan or glycosaminoglycans (GAGs), have all proved suitable in terms of cell compatibility, but some issues with potential immunogenicity still remains. Among GAGs hyaluronic acid, possibly in combination with cross linking agents (e.g. glutaraldehyde, water soluble carbodiimide, etc...), is one of the possible choices as scaffold material. Functionalized groups of scaffolds may be useful in the delivery of small molecules (drugs) to specific tissues.

5.1 Materials as scaffolds for cells transplantation in cardiac tissue-engineering

One of the first materials used for tissue-engineering of the heart was based on hydrolytically degradable biocompatible polymers composed of polyactic acid (PLA), polyglycolic acid (PGA) and their copolymer polylactic-*co*-glycolic acid (PLGA) [128]. Subsequently, researchers realized that the mechanical properties of the material used had to be adapted to the elastic properties of the heart tissue. Therefore, most research is focusing on the use of scaffold made of different synthetic and/or natural polymers. Table 4 gives an overview on different materials used in the past few years for the regeneration of the heart [125]. It has to be pointed out that only a few materials were tested in humans, while many animal studies were carried out in rats and dogs.

Table 4
Materials used in the past three years for cardiac tissue engineering

Natural
Gelatin scaffolds
Acellular porcine scaffolds
Porous alginate scaffolds
Alginate–gelatin–PEGa scaffolds
Collagen scaffolds
Fibrin glue
Synthetic
PLA–PGA
Poly-L-lactide-gelatin–PGA
Electrically conducting membrane layers composed of PGA, gelatin, alginate and/or collagen
Polyvinyl alcohol
PGA-co-polyhydrobutyrates
e-Caprolactone-co-L-lactide
Polyurethanes
TMC-co-e-caprolactone-co-D-L-lactide

(Table from (Zammaretti and Jaconi, 2004).

Several groups are currently working with scaffold materials composed of natural polymers such as collagen [125, 129, 130], this latter being the major constituent of the cardiac extracellular matrice (ECM). Promising results in the development of collagen-based grafts or 'patches' containing beating cardiomyocytes were obtained in Canada [129] and Germany [130]. These studies comprised the application of cardiomyocyte-seeded collagen strings that were cyclically stretched, thus providing patches with improved morphology and contractile function. Zimmermann *et al* [125] demonstrated that these collagen patches could survive and beat for up to eight weeks after engraftment on the heart of immunosuppressed rats.

Similar approaches and results were obtained using alginate-based scaffolds by Cohen *et al*. in Israel [131, 132]. Alginate, a negatively charged polysaccharide from seaweed which forms hydrogels in the presence of calcium, offers the advantage of detecting ECM formation by cardiac cells to follow not only the proliferation and migration of the cells, but also the kinetics of ECM formation. After implantation into the infarcted rat myocardium, the alginate-biografts were shown to stimulate intense neovascularization and to attenuate left ventricular dilatation and failure, compared with control rat hearts [132].

Composites of natural and synthetic polymers were also developed; for example, sponges based on caprolactone-*co*-L-lactide reinforced with knitted poly-L-lactide fabric (PCLA), gelatin or PGA. Using rat aortic smooth muscle cells, an increased colonization of the right ventricular outflow tract was obtained using gelatin or PCLA, but not with PGA-reinforced grafts [133]. Another interesting synthetic material is based on 1,3-trimethylene carbonate and D,L-lactide copolymers [134, 135], which have the ability to be reabsorbed over a ten month period and to sustain the cyclic loading of the heart muscle under physiological conditions. However, as yet no animal studies have been carried out with this amorphous material. The most fascinating approach to the regeneration of heart has been proposed by Shimizu and co-workers [136], who used materials to create electrically communicating three-dimensional cardiac tissue layers [Fig. 7, panel c]. In this case, cells were adhered on tissue-culture plates previously coated with poly(*N*-isopropylacrylamide) (PIPAAm), a temperature-sensitive polymer. At 37°C PIPAAm is hydrophobic, enabling cell adhesion and access to the binding sites offered on this modified surface; at a lower temperature such as 32°C, the surface becomes hydrophilic and inappropriate for cell adhesion due to the rapid hydration and swelling of PIPAAm. Using poly(vinylidene difluoride) (PVDF) membranes, which are hydrophobic, the detaching cell layers can be collected and handled, providing up to four conducting layers of synchronously beating cardiomyocytes. When these patches were implanted on rats with

induced myocardial infarction, an improved myocardial contractility was observed, concomitant with the appearance of a vascular network within a few days after implantation [137]. Figure 7 summarizes three of the major approaches to cardiac engineering described above, based on the use of collagen, hydrogel or multiple layers.

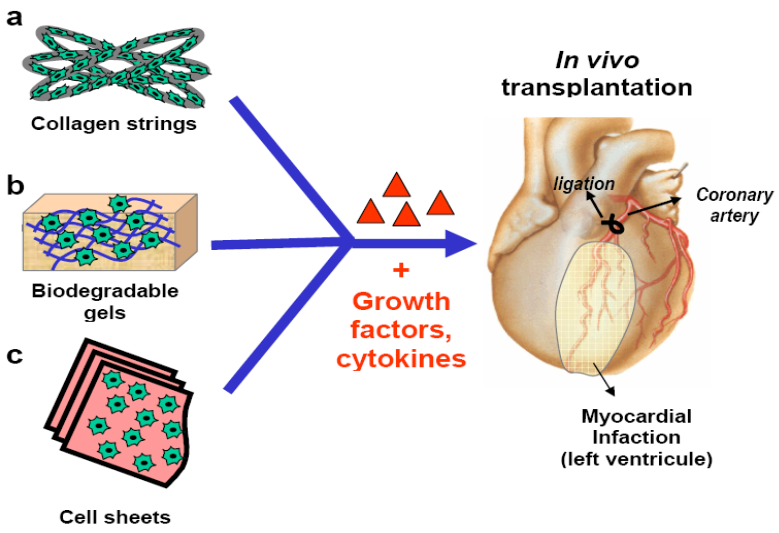


Figure 7. Scheme of the present major strategies of cardiac tissue engineering using (a) collagen strings, (b) biodegradable gels or (c) cardiac cell sheets. The incorporation of growth factors and/or cytokines (triangles) may have a crucial role to support cell differentiation, engraftment and survival, both within the scaffolds and in vivo, thus improving the overall cardiac function. (Figure from (Zammaretti and Jaconi, 2004)

More recently researchers are using another natural component of ECM as support for cardiac stem cell transplantation: hyaluronic acid (HA). HA is a naturally occurring polysaccharide, and is one of the basic components of the ECM of connective tissue. HA is biocompatible and biodegradable and exhibits an unusual and distinctive set of physicochemical and biological properties, enabling it to regulate and control several physiological functions of tissue [138]. It plays a major role in tissue growth and remodelling, interacting specifically with endogenous receptors such as CD44 and intracellular adhesion molecule-1 (ICAM-1) to guide and control cellular migration, growth and adhesion [139]. It also regulates macromolecular traffic within the interstitial space in joints contributing to lubrication and to the strength in compression of soft tissue [140].

Twenty years after the initial discovery of HA, Meyer's laboratory determined the exact chemical structure of HA, a non sulfated, highmolecular-weight glycosaminoglycan composed of

repeating polymeric disaccharides d-glucuronic acid and *N*-acetyl-dglucosamine linked by a glucuronidic β(1→3) bond [141]. The disaccharide units are then linearly polymerized by hexosaminidic β(1→4) linkages (Fig.8). Hyaluronan can be 25,000 disaccharide repeats in length and reach a molecular weight of 4000 kDa. HA is synthesized by membrane-bound synthases (HASs) at the inner surface of the plasma membrane, and the chains are extruded through pore-like structures into the extracellular space [142].

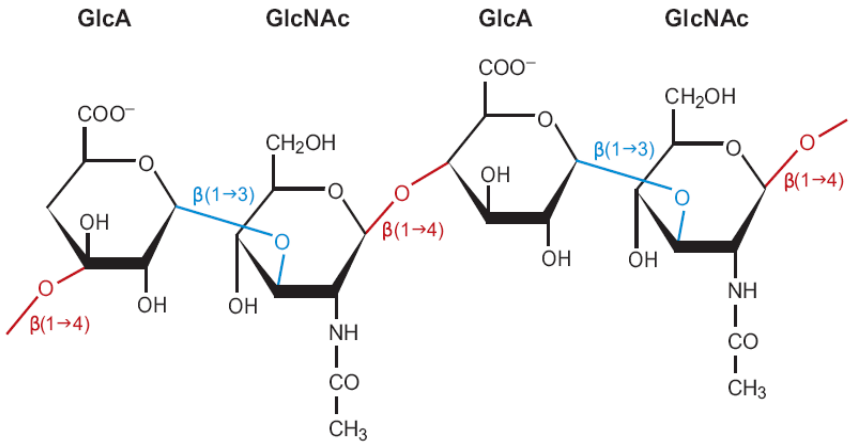


Figure 8. Structure of hyaluronan. Hyaluronan iscomposed of repeating units of d-glucuronic acid- β(1→3)-*N*-acetyld-glucosamine. The disaccharide units are linked by a β(1→4) bond (*red*). Two disaccharide units are shown. GlcA, d-glucuronic acid; GlcNAc, *N*-acetyld- glucosamine

HA is degradeted by hyaluronidases. Hyaluronidases (HYALs) hydrolyze the hexosaminidic β(1→4) linkages between *N*-acetyld- glucosamine and d-glucuronic acid residues in HA. These enzymes also hydrolyze β(1→4) glycosidic linkages between *N*-acetyl galactosamine or *N*-acetylgalactosamine sulfate and glucuronic acid in chondroitin, chondroitin 4- and 6-sulfates, and dermatan. Therefore, HA is easly degradated *in vivo*.

HA covalently binds to a variety of proteins (HABPs), also referred to as hyaladherins [143]. These include receptors such as CD44, RHAMM (receptor for hyaluronan mediated motility expressed protein), and LYVE-1 (lymphatic vessel endothelial hyaluronan receptor-1). Some hyaladherins are associated with cell membranes, whereas others are found in the extracellular matrix. CD44 is the major cell surface HA binding proteins. Most cells—including stromal cells such as fibroblasts and smooth muscle cells, epithelial cells, and immune cells such as neutrophils, macrophages, and lymphocytes—all express CD44 [144]. HA-CD44 interactions, as mentioned

above, may play an important role in growth, development, cell migration, cell adhesion but also in inflammation process.

As showed by Camenish TD *et al*. HA is involved in heart development during embryogenesis. In fact, the targeted deletion of HAS2 gene results in embryonic death at approximately E9.5, with extensive abnormalities of the developing cardiovascular system [145]. HA is important also for cardiomyocytes, endothelial [145] and mesenchymal stem cells migration [146]. Moreover, HA has been implicated in angiogenesis. It appears to exert its biological effects through binding interactions with at least two cell surface receptors: CD44 and receptor for HA-mediated motility (RHAMM) [147]. Again, the engagement of CD44 with HA induces cyclooxygenase-2 (COX-2), generates VEGF, and thus leads to an increase in endothelial cells proliferation [148].

Owing to its distinctive behaviour, versatility and biological properties, HA has been investigated by many researchers with a view to its potential use in biomaterials for heart tissue regeneration. However, due to its poor mechanical properties, the use of unmodified HA has been limited to viscosurgery applications. To overcome these limitations, several approaches have been taken to improve mechanical properties by structural modification of the HA molecule. These have generally involved altering the ratio of hydrophobic to hydrophilic substituents on the polymer backbone, or the introduction of intermolecular crosslinking. For example, HA has been chemically modified by a coupling reaction – such as esterification via its carboxyl groups – with various alcohols, both therapeutically active and inactive. This has led to a wide range of derivatives with enhanced processability and mechanical properties in both the dry and the wet state [149].

6. Strategies, challegenges and further directions

The most obvious question to be answered by preclinical studies is which type of cell is the best candidate for cardiac therapy [150]. Different cardiac pathologies — for example, acute myocardial infarction and chronic ischemic cardiomyopathy — might require different types of stem cell or progenitor cell.

Moreover, a crucial issue in designing more rational cell-based therapy approaches for cardiac disease is understanding the mechanisms by which each of the stem-cell or progenitor-cell types used can affect myocardial performance [151, 152].

Another issue is determining the optimal route of delivery; retention of cells immediately after delivery is highly dependent on the delivery strategy. Survival in the inflammatory environment of

infarcted myocardium is a challenge common to all types of transplanted cell, as typically 90% of the cells die within a week [151]. A sufficiently large cell graft with appropriate structural and functional properties will be needed.

Finally, long-term electromechanical stability and appropriate structural and functional electromechanical integration [153] with host tissue will be essential for cardiac regeneration.

Therefore, each steps in cardiac stem cell therapy [Fig.9] is important and requires careful consideration and better understanding in order to improve the final result: cardiac regeneration.

All these step can be summarized in:

- Stem cell isolation
- Stem cell delivery method
- Survival and proliferation factor
- Electromechanical integration
- Stability and safety

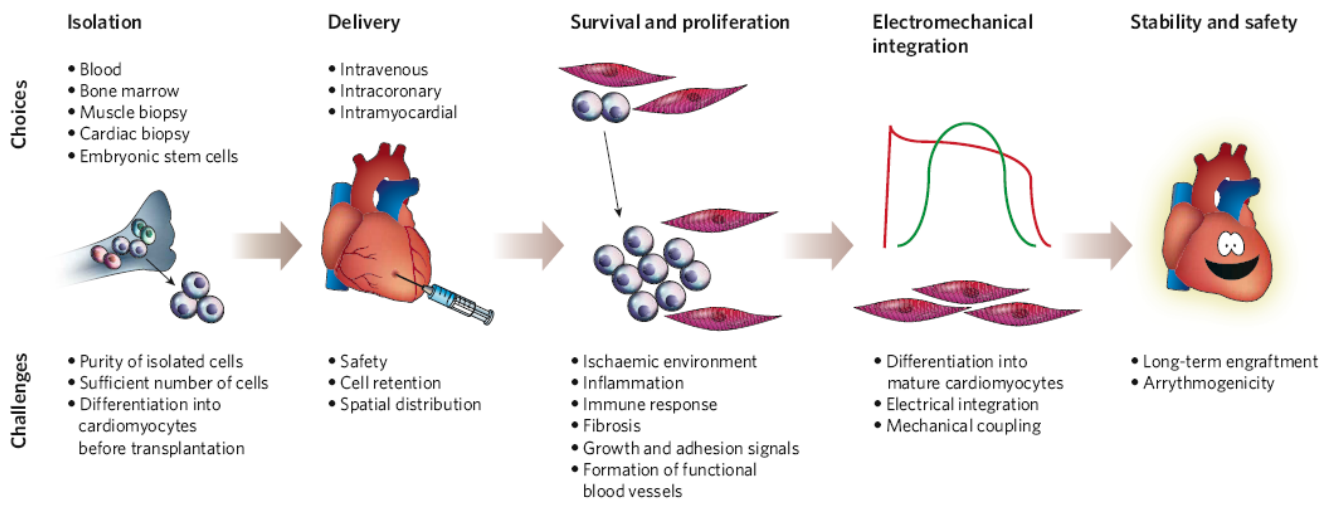


Figure 9. True cardiac regeneration with stem cell therapy will require careful consideration at each step, from isolation of the cells to their stable and safe long term integration (Figure from Vincent F. M. Segers and Richard T. Lee, Nature 2008)

Moreover, understanding cardiomyocyte development and turnover — both in normal development and after injury — will be essential for guiding the development of stem-cell-based therapies [154].

Defining the factors present in the hostile microenvironment of injured myocardium that limit the survival and functional integration of transplanted cells is also crucial. The realization that regenerative mechanisms do exist in mammalian myocardium brings into sharp focus the problem of defining the barriers that could be preventing regeneration, including the ischemia, inflammation and fibrosis that characterize various stages of infarcted myocardium [Fig.10]. This hostile microenvironment might prevent the activation of resident CSCs and thus also reduce the success of exogenous cell therapies. Some components of the inflammatory response might be essential for promoting angiogenesis and progenitor-cell recruitment, but excessive inflammation might also prevent the recruitment and survival of progenitor cells. Similarly, some degree of fibrosis is required to prevent myocardial rupture after a myocardial infarction, but dense fibrosis presents a formidable physical barrier to regenerating cells. It is likely that no single factor defines the hostile microenvironment of injured myocardium [154].

Of course, a major effort by basic researchers is required to better understand all these factors affecting cardiac stem cell therapy before application in clinical trials.

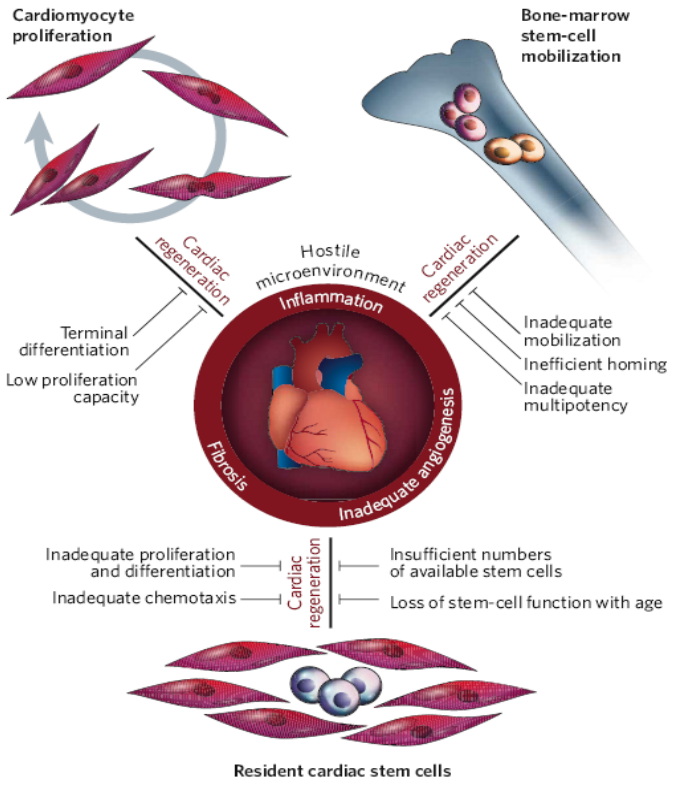


Figure 10. Mechanism of, and potential barrier to, cardiac regeneration (Figure from Vincent F. M. Segers and Richard T. Lee, Nature 2008)

EXPERIMENTAL SECTION

AIM OF THE STUDY

Stem cells offer the opportunity of transplanting a live source for self-regeneration. The isolation of stem and progenitors cells is now being developed for many different clinical applications including heart failure. Traditional clinical therapies such as pharmacological medications, organ transplants and other methods have proved far from effective to full recovery of such patients.

Stem cell-based therapy, focusing on repairing damaged heart tissue, is a promising option for treatment of ischemic diseases. Several cell types have been shown to increase the functional recovery of the heart after ischemia, but unfortunately scientists are still far to identify the best cell and/or the best cell delivery method.

This study was designed to investigate the relevance of tissue engineering strategies for the regeneration of the heart following myocardial infarction in animal model.

At this purpose we tested cell adhesion, proliferation and vitality of rat bone-marrow mesenchymal stem cells seeded onto a hyaluronan-biopolymer, HYAFF®11, developed by Fidia Advanced Biopolymer (FAB - s.r.l., Abano Terme, Italy). Subsequently, we transplanted the scaffold into heterotopic infarcted rat hearts, in order to evaluate the effects of this new strategy for cardiac cell delivery.

Moreover, in order to investigate the fate of endogenous resident cardiac stem cells in animal hearts, I attended for more than one year, the *Cardiovascular Research Institute* at New York Medical College, (Valhalla, NY, USA) directed by Doctor Piero Anversa.

There I characterized the cardiac phenotype of α-MHC-ODC-transgenic mice using both immunohistochemical and biomolecular techniques (see *APPENDIX*).

MATERIALS&METHODS

Isolation and culture of rat bone marrow mesenchymal stem cells

Rat mesenchymal stem cells (rBMSCs) were isolated from rat bone marrow according to *Javazon et al 2001*. Bone marrow plugs were extracted from the femurs of 2-months old Lewis syngeneic rats (Harlan, Italy) by inserting a 21-gauge needle into the shaft of the bone and flushing the bone marrow cavity with 30 mL of 20% FBS α-modified Eagle's Medium (BioWhittaker, Walkersville, MD, USA) without deoxyribonucleosides and added of 100 U/mL penicillin/streptomycin (BioWhittaker, Walkersville, MD, USA) and 0,1 μg/mL L-glutamine (BioWhittaker, Walkersville, MD, USA). Marrow plug suspension was dispersed by passing it through 70 μm filter (Falcon, Franklin Lakes, NJ, USA), plated in T-75 culture flask (Sarstedt, Nümbrecht, Germany) and incubated at 37°C, in a 5% CO_2 humidified atmosphere. The following day, cultered cells were washed twice with PBS in order to remove non-adherent cells then 10% FBS α-MEM was added.

The mesenchymal population was isolated on the basis of its ability to adhere to the culture plate. Culture medium was changed every 3-4 days. At 90% confluence, the cells were trypsinized by incubation with 0.25% trypsin-EDTA (Sigma, St. Louis, MO, USA), centrifuged at 1000 rpm for 10 minutes and resuspended in 10 mL complete medium, counted manually in duplicate using a Bürker's chamber, and plated as P1 on 58-cm^2 plates at densities of 2000 cells cm^2. Complete medium was replaced every 3–4 days over the 18 to 24-day period of culture.

First-passage rBMSCs were used in all experiments.

Immunological characterization of rBMSCs

For immunostaining BMSCs were seeded onto glass coverslips and fixed in 4% paraformaldehyde at room temperature for 15 minutes and then washed twice with Ca^{2+} and Mg^{2+} free PBS. Non-specific antibody-binding sites were blocked with 1% BSA/0.1% Tween20/PBS (blocking buffer) for 45 min at room temperature following which, the primary antibodies anti CD90 (Pharmigen, San Diego, CA, USA) 10 μg/mL in blocking buffer, anti CD34, CD45 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) 1:100 in blocking buffer were added and incubated overnight at 4° C or 1h at room temperature. Cells were washed 5 min three times in PBS and incubated 1 hour in FITC-conjugated or Cy3-conjugated anti-mouse IgG secondary antibody

(Sigma, St. Louis, MO, USA) at room temperature. The cells were washed 5 min three times in PBS and cells' nuclei labelled with, 4',6-Diamidino-2-phenylindole (DAPI; Sigma, St. Louis, MO, USA) 15 min at room temperature. After immunofluorescent staining cells were mounted with Vectashield mounting medium (Vector Laboratories, Burlingam, CA, USA) on standard glass slides.

The images were taken using the IX50 Olympus Fluorescence microscope (Olympus, Tokyo, Japan). No significant fluorescent signal was detectable with any of the secondary antibodies alone.

Flow cytometry analysis

Flow cytometry analysis was performed using an Epics Elite (Beckman Coulter, Fullerton, CA, USA) flow cytometry equipped with a 15-mW argon ion laser tuned to 488 nm. Green and red fluorescences were collected at 525 nm and 605 nm, respectively.

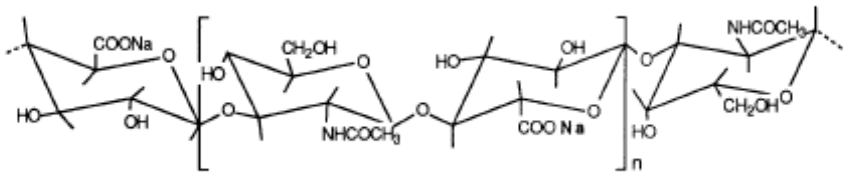
Cells were detached with a solution containing 500 mg/mL trypsin and 200 mg/mL EDTA (BioWhittaker, Walkersville, MD, USA), washed in PBS, and centrifuged at 1000 rpm for 10 minutes. The pellets were resuspended in 3 % paraformaldehyde for 15 minutes at room temperature and then washed in PBS. Non specific antigens were blocked by incubating the cells at room temperature for 1 hour in a solution containing 1% BSA and 0.1% FBS in PBS. Aliquots (2 to 3 x 10^5 cells) were incubated with mouse anti CD90 (Pharmigen, San Diego, CA, USA) or rabbit anti CD59 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibodies for 45 minutes at room temperature. The cells were washed by centrifugation in PBS, and the pellet was resuspended in antimouse FITC-conjugated (Sigma, St Luis, MO, USA) or antirabbit rhodamine-conjugated (Sigma, St Luis, MO, USA) secondary antibodies for 20 minutes at room temperature in the dark. Primary antibodies were used 1:100 for CD90 and 2 µg/mL for CD59 diluited in blocking buffer.

The cells were then washed in PBS and resuspended in 1 mL PBS for fluorescent- activated cell sorting (FACS) analysis. Non specific fluorescence was determined using equal aliquots of the cell preparation that were incubated with antimouse FITC-conjugated or antirabbit rhodamine-conjugated antibodies.

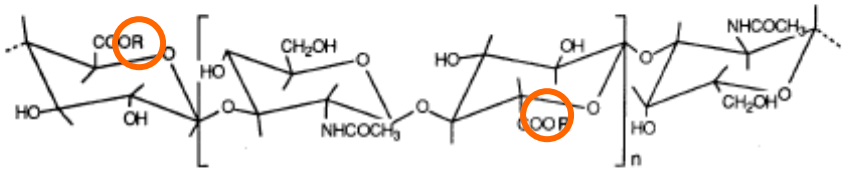
Biomaterial

The biomaterial used in this study was a hyaluronic acid - based polymer, i.e. HYAFF®11, developed by Fidia Advanced Biopolymer – FAB - s.r.l., Abano Terme, Italy. HYAFF®11 is derived through the total esterification with benzyl alcohol of the carboxyl groups of glucuronic acid along the polymeric backbone of sodium hyaluronate.

The experiments have been performed on a non-woven structured HYAFF®11 construct. HYAFF®11 is biocompatible and resorbable and integrates with damaged tissues (*Campoccia et al, 1998*).



Hyaluronan



HYAFF®

Fig. 1. Structure of HA and HYAFF® polymers. R represents one of the possible substituent ester groups.

HYAFF®11 morphological analysis

Samples from non-woven structured HYAFF®11 were initially examined under an inverted light microscope and then processed for scanning (SEM) and transmission (TEM) electron microscopy. For SEM the samples were directly mounted on aluminium stubs and then coated with a thin layer of gold before examination. For TEM, both glutaraldehyde fixed and unfixed 2x2 mm^3 samples

were embedded in Epoxy resin. Before examination, thin section were stained with lead citrate and uranyl acetate.

Culturing rBMSCs on hyaluronan-scaffold

Cells at the first passage were trypsinized at 90% of confluence, resuspended in 0.5 ml 10% α-MEM medium and counted manually in duplicate using a Bürker's chamber. Patches of 0.3-0.5 cm^2 of non-woven structured HYAFF®11 were pre-treated with complete culture medium for 1 to 3 hours before seeding. Then a suspension of 50 µl of cells was seeded at the concentration of $2x10^6$ cell/cm^2 on the upper surface by gently dropping on the medium-wetted scaffolds. The scaffolds were left at 37°C, in a 5% CO_2 humidified atmosphere for 4 hours to allow the cells to attach. Afterwards, 0.5 ml medium was added and further cell culturing was carried out in 24-well plates (Sarstedt, Nümbrecht, Germany) at 37°C, in a 5% CO_2 humidified atmosphere.

Interaction between rMSCs and HYAFF®11 was investigated on days 1, 3, 7, 14, and 21 using appropriate techniques. Cell culture media was changed twice a week.

At least three scaffolds were analysed for each experimental condition and at each time-point.

rBMSCs adhesion on HYAFF®11

The trypan blue exclusion staining method was used to quantify rBMSC adhesion onto the biopolymer. rBMSCs were seeded on the scaffold as described above.

After 24 h, the number of cells bound to the fibres was calculated by subtracting from the known number of total seeded cells the amount of cells which adhered to the well bottom and those still present in the supernatant, determined by Bürker hemocytometry chamber counting:

[n° HYAFF®11 adherent cells = n° seeded cells – (n° well-adherent cells + n° suspended cells)].

rBMSC viability and proliferation into HYAFF®11

rBMSC viability onto HYAFF®11 was analysed at 1, 7, and 14 days by using the vital dye carboxyfluorescein diacetate, succinimidyl ester (Vybrant™ CFDA-SE Cell Tracer Kit, Molecular Probes, Eugene, Oregon, USA) following the manufacturer instructions. CFDA-SE is a non-fluorescent dye that passively diffuses into the cytoplasm of cells. Once inside the cell, it is cleaved

by intracellular esterases, becoming fluorescent. This highly amine-reactive product then forms dye-protein adducts that are retained by the cells throughout their development. Moreover CFSE is inherited equally by daughter cells after division, resulting in the sequential halving of mean fluorescence with each generation.

rBMSC proliferation was determined by the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO, USA) assay at day 4, 7, 14 as previously reported (*Lisignoli et al, 2001*). After removing the supernatant, the scaffold was washed with PBS, transferred into a new well containing 1 ml of MTT solution (1 mg/ml) and incubated for 3 h at 37°C in a 5% CO_2 humidified atmosphere. The scaffold was then transferred into an eppendorf tube containing 1 mL of solubilization solution (0.01 M HCl in isopropanol) and vortexed for 5 min. This provoked the release from the scaffold of MTT which was actively reduced by viable cells acquiring a yellow colouring. Each sample was centrifuged at 15,000 x g for 5 min and the supernatant was read at 595 nm using a multiwell spectrophotometer (Victor 2 microplate reader, Perkin Elmer, Wellesley, MA).

Immunofluorescence detection of CD90 expression

HYAFF®11-rBMSC cultures were fixed at day 21 at room temperature for 24 hours with 10% formalin and then embedded in paraffin. After deparafinization and re-hydration, samples were blocked with 1% BSA dissolved in 0.2% tween-20/PBS and then rinsed three times with PBS. The primary antibody monoclonal mouse anti-rat CD90 (Pharmingen, San Diego, CA) was diluted 1:100 in blocking buffer for 1 h at room temperature and washed three times in PBS. The secondary Cy3-conjugated polyclonal anti-mouse antibody (Sigma, St Luis, MO, USA) was diluted 1:2000 in blocking buffer, incubated 45 min at room temperature and then washed three times in PBS. Nuclei were stained with DAPI (Sigma, St. Louis, MO, USA) diluted 1:1000 for 15 min and washed three times in PBS. Cells were mounted with Vectashield mounting medium (Vector Laboratories, Burlingam, CA, USA). The samples were observed with an IX50-Olympus inverted microscopy (Olympus, Tokyo, Japan).

Light, transmission and scanning electron microscopy analysis

On day 1, 3, 7, 14 and 21, HYAFF®11-rBMSC cultures were processed for light and electron

microscopy.

For light microscopy (LM), the samples were fixed in formalin and embedded in paraffin; the samples were stained with hematoxylin and eosin.

For TEM, the samples were fixed in 2.5% buffered glutaraldehyde, post-fixed with 1% osmium tetroxide and embedded in Epoxy resin; semithin sections were stained with toluidine blue and thin sections with uranyl citrate and lead citrate; the thin section were observed in a Philips 400T transmission electron microscope.

For SEM, following fixation the samples were dehydrated and critical point dried. The samples were mounted on aluminium stubs and then coated with a thin layer of gold using a sputtering device.

The samples were observed in a Philips 505 scanning electron microscope at 15kV.

Confocal microscopy analysis of CD44 expression

4 μm thick dewaxed histological sections were incubated overnight with anti-CD44 monoclonal antibody (Chemicon, Temecula, CA, USA) 1:100 in blocking-buffer. After washing in PBS, immunolabelling was carried out by incubating sections for 1 hour with a goat anti-mouse IgG Alexa-Fluor-488-conjugate (Molecular Probes, Eugene, Oregon) 1:200 in PBS. The sections were finally mounted with a fluorescent mounting medium and analyzed by a LSM 510 confocal laser microscopy system (Zeiss, Jena, Germany) which incorporates two lasers (Argon and HeNe).

rBMSC BrdU-labelling and immunofluorescence detection of BrdU

In order to detect the cells after biopolymer construct transplantation within the heart , cultured cells were labelled with the thymidine analogue 5-bromo-2' deoxyuridine (BrdU; Pharmigen, San Diego, CA, USA). The cells were seeded on hyaluron-based scaffold dishes (HYAFF-11®) of 0.3-0.5 cm^2 area at the density of $2x10^6$ cells/cm^2 for 3 weeks and incubated with BrdU 10 μM in complete culture medium for 72 h.

HYAFF®11 –labelled rBMSC cultures were fixed at day 21 at room temperature for 24 hs with 10% formalin and then embedded in paraffin. After deparafinization and rehydration, samples were blocked with 1% BSA dissolved in 0.2% tween-20/PBS and then rinsed three times with PBS. DNA

was denatured by incubating sections in 2N HCl for 30 minutes at 37 °C, and then the acid was neutralized by immersing sections in 0.1M borate buffer for 2x5 min. The primary ready-to-use monoclonal mouse antibody anti-BrdU (BioGenex, San Ramon, CA, USA) was incubated over night at 4°C and the following day washed three times in PBS. Unspecific bindings were blocked by incubating the samples with 1%Triton-X100/1M glycine/5% goat serum 1 h at room temperature. The secondary Fitch-conjugated polyclonal anti-mouse antibody (Sigma, St Luis, MO, USA) was diluted 1:2000 in blocking buffer, incubated 45 min at room temperature and then washed three times in PBS. Nuclei were stained with DAPI (Sigma, St Luis, MO, USA) diluted 1:1000 for 15 min and washed three times in PBS. Cover glasses were mounted with Vectashield mounting medium (Vector Laboratories, Burlingam, CA, USA) and observed with an IX50-Olympus inverted microscope (Olympus, Tokyo, Japan).

Heterotopic heart transplant surgery

The heterotopic heart transplantation [Fig.2] between syngeneic male LEWIS rats (210 to 230 g weight) was performed according to the method of *Lee et al 1970*. In brief, donor rats were anesthetized with Enfluran via inhalation. Cardiac arrest was achieved by injecting cold (4°C) Bretschneider cardioplegic solution (Custodiol, Dr. F. Ko¨hler Chemie, Alsbach-Ha¨hnlein, Germany), first in a retrograde manner into the vena cava inferior, then by perfusion of the coronary arteries via the aorta. After ligation of vessels, the heart-lungs block was carefully excised, left lung excised and infarction induced by left descending coronary artery (LAD) ligation on the bench. A 6-0 polypropylene suture was passed through the epicardium layer beneath the LAD 1 to mm from its origin, tying the ligature permanently occluding the LAD (Ye *et al.*, 1997). The occlusion was confirmed by the appearance of a hypokinetic whitish area.

Syngeneic Lewis recipients rats were also anesthetized with enfluran via inhalation. The allograft was transplanted by end-to-side anastomosis of the aorta to the abdominal aorta of the recipient. Arterial blood from the recipient's abdominal aorta enters the donor left heart via the donor left lung and is ejected into the donor ascending aorta by the transplanted left ventricle. Using this model, the donor left ventricle performs work comparable to that of the native heart. This model is easily reproducible and can be applied to various studies, including long-term assessments of cardiac remodelling, long-term efficacy of cardiac preservation, and post transplantation ventricular performance under more physiologic conditions.

Two weeks after surgery, abdomen of receiving rats was opened and a pouch of 3 mm^2 was made in the thickness of the ventricular wall at the level of the post-infarction scar in heterotopic heart. The hyaluronic scaffold, previously engineered for 3 weeks with rBMSCs, was introduced into the pouch and the myocardial edges sutured with few stitches. Two weeks later, after heterotopic-heart functional analysis, rats were sacrificed and heart fixed in 10% formalin for immunohistochemistry analysis.

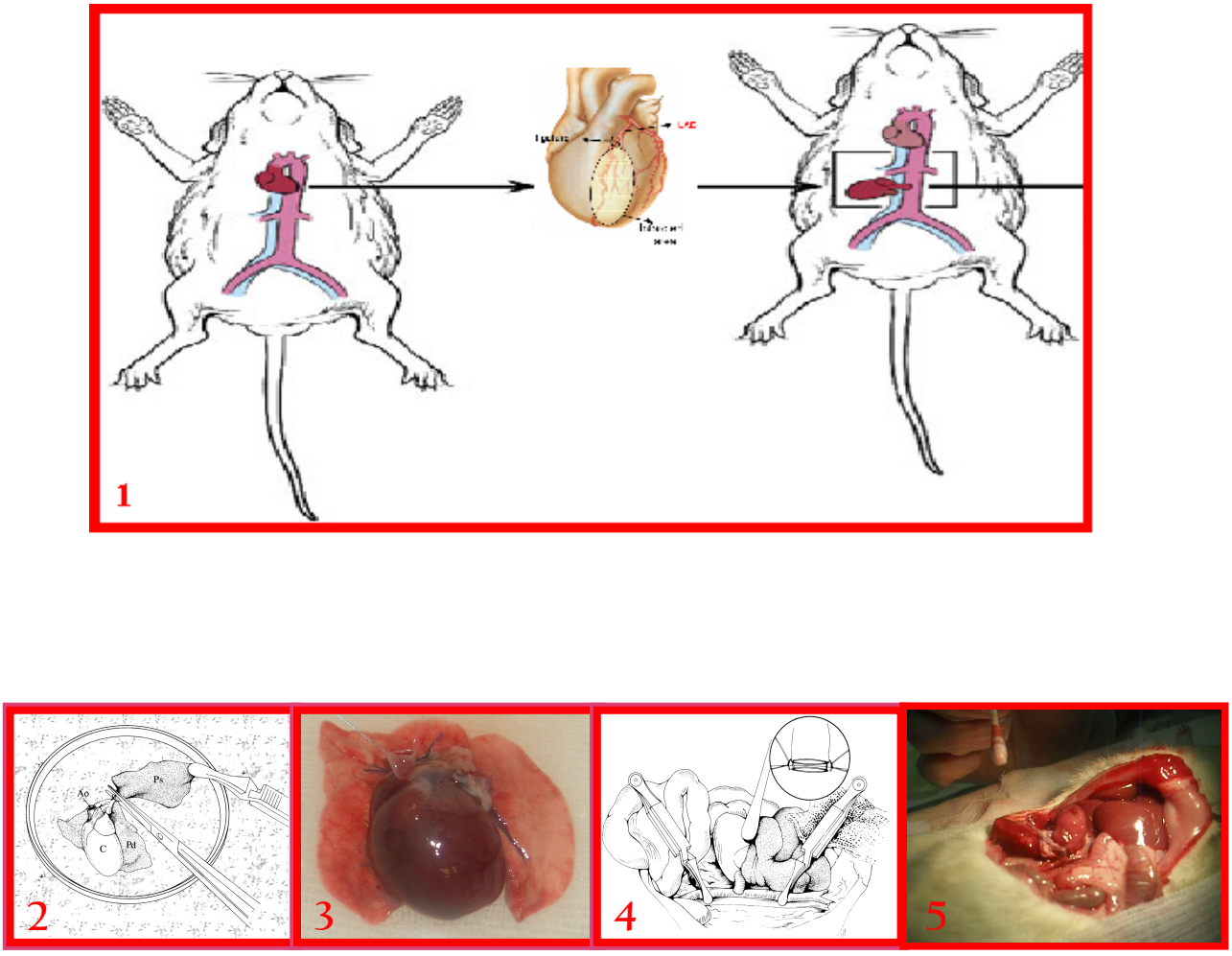


Fig. 2 *Heterotopic heart transplant surgery (1).* After ligation of vessels, the heart-lungs block was carefully excised, left lung excised (2) and infarction induced by left descending coronary artery (LAD) ligation on the bench (3). The allograft was transplanted by end-to-side anastomosis of the aorta to the abdominal aorta of the recipient (4, 5).

Experimental protocol

All the animal experiments were performed under the guidelines on animal care and welfare determined by the Italian Bioethics Committee. A total of 12 male syngeneic Lewis rats were included in this experiment: 6 of them were donors and 6 were recipients. As a control, normal rats with no treatment at all were used for the study. In the receiving rat heart, scaffold implantation was performed after two weeks from infarcted heart implantation. All rats were kept for 2 weeks after HYAFF®11 -rBMSC implantation before sacrifice; heterotopic heart function was evaluated by echocardiography immediately after scaffolds transplantation and two weeks later.

Fig. 3 summarizes the experimental animal protocol

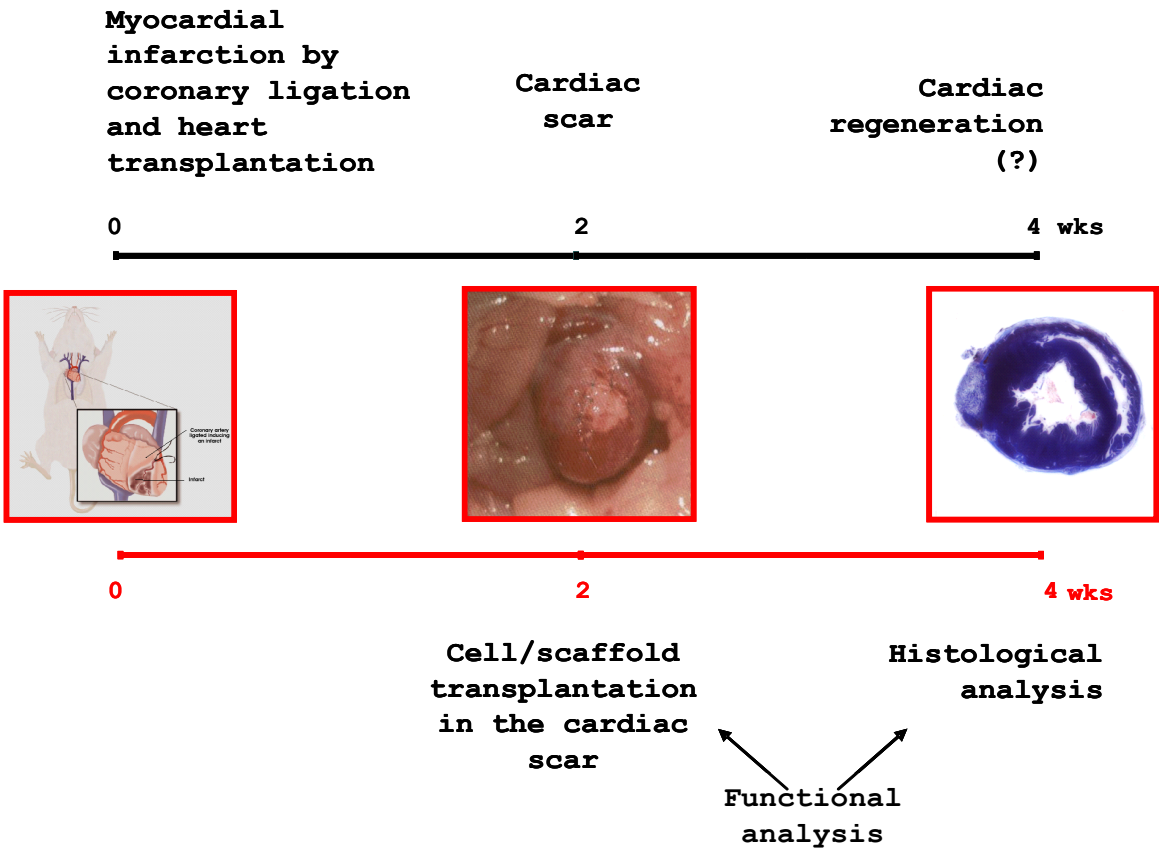


Fig. 3 *Experimental animal protocol*. Time 0: heterotopic infarcted heart implantation; after 2 weeks: functional analysis and scaffold transplantation; after 4 weeks: functional analysis and rat sacrifice for histological analysis.

M-Mode Echocardiography analysis

M-Mode echocardiography of heterotopic hearts was performed 2 weeks after heterotopic-heart transplantation and 2 weeks after scaffold heterotopic-heart implantation just before rats sacrifice. After the abdomen was shaved, the rats were placed in dorsal decubitus position. Isoflurane (1-2%) was continuously supplied via a mask. In vivo heart function was evaluated by echocardiography using a fully digitized Vingmed System Five (GE Vingmed Ultrasound, Horten, Norway) with a 10-MHz linear array transducer. The transducer was positioned on the left anterior side of the abdomen. At first, longitudinal images of the heart were obtained, including the left ventricle, the left atrium, the mitral valves and the aorta, followed by the cross-sectional images from the plane of the base to the left ventricular apical region. Two-dimensional (2D) images of the left ventricle were obtained both in long and short axes. Short axis recordings and M-mode tracings were performed at the level of the papillary muscles. All of two-dimensional images, M-mode tracings and Doppler curves were recorded on videotape for later analysis.

Fractional shortening (FS), in percent, was calculated according to the following formula:
LV FS (%) = (LVDd – LVDs)/LVDd x 100
where LVDd is left ventricular diameter in diastole, and LVDs is left ventricular diameter in systole according to the M-mode tracing from the cross-sectional view.
All measurements were averaged for 3 consecutive cardiac cycles and were analyzed by an observer who was blinded to the treatment status of the animals.

Immunohistochemistry procedure and microscope analysis

At the experimental end-time point, heterotopic heart were rapidly excised, blocked with an injection of potassium chloride (2 ml KCl 15%) in order to stop the heart in diastole. Cardiac cavities were rinsed with PBS to remove blood and thrombus, then the hearts were fixed with 10% formalin for 24 hours. Afterwards, the hearts were cut into 3 pieces apex, middle portion and base, before being dehydrated in alcohol at increasing concentration and embedded in paraffin**.** Blocks were cut into 6-µm sections. Serial sections were stained with haematoxylin/eosin (H&E) for morphological analysis or used for immunofluorescence analysis.

The standard deparaffinization protocol was used, tissue sections were serially rehydrated in 100%, 95%, 70% ethanol and distilled water. Endogenous peroxidase activity was blocked by 3% H_2O_2 10 min at room temperature and the antigens were retrieved in a microwave for 10 minutes at

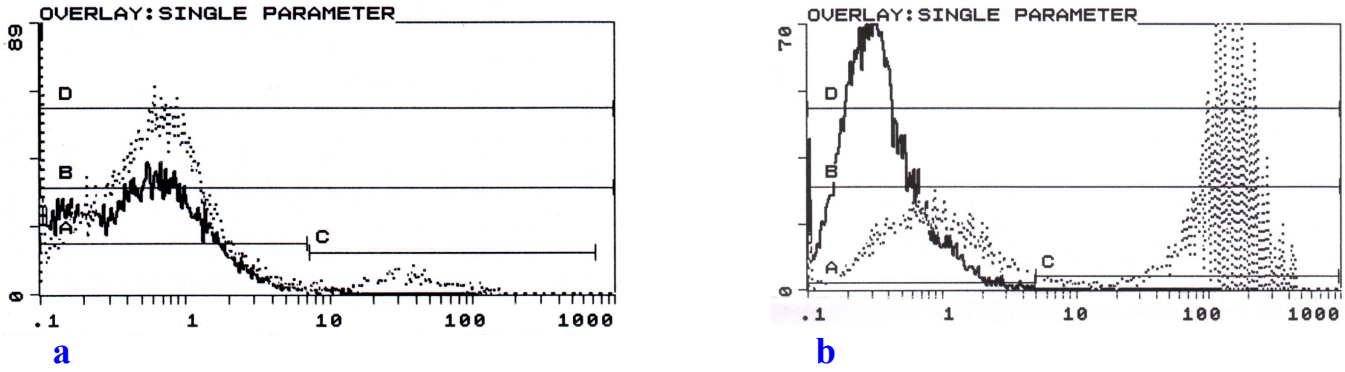
full power in pH=6.0, 10mM TP citrate solution. Non-specific bindings were blocked with 10% serum (goat serum for anti-mouse secondary antibody and sheep serum for anti-rabbit secondary antibody) for 45 minutes. Then the slides were incubated with the following prime antibodies: mouse-monoclonal ready-to-use anti-BrdU (BioGenex, San Ramon, CA, USA) overnight at 4 °C, rabbit polyclonal anti-Cardiac Troponin-I 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) 1 hour at room temperature. Afterwards, the slides were incubated for 45 minutes at room temperature with one of the two following secondary antibodies Fitch- or Cy3 anti mouse IgG (Sigma, St Luis, MO, USA) 1:2000 in blocking buffer. Afterwards, slides were washed 10 min in PBS and then mounted with Vectashield-with DAPI mounting medium (Vector Laboratories, Burlingam, CA, USA).

For light microscopy the BrdU visualization was performed using NovoLink™ Polymer Detection System (Novocastra, New Castle, UK) following the manufacturer instructions.

RESULTS

Rat-Bone marrow Mesenchymal Stem Cells characterization

In the first part of the study, we analyzed by immunofluorescence and FACS analysis the immunophenotype of extracted rat bone marrow mesenchymal stem cells (rBMSCs). The results show that after 10-12 days of culture expansion on polystyrene surface adherent rBMSCs did not express the hematopoietic markers CD34 and CD45, nor a specific mononuclear phagocyte marker (MP). On the contrary, a very low positivity to CD59, an Sca-1 homologue, was observed, whereas 53% of total rBMSC were positive to CD90, a superficial antigen expressed by rBMSCs. CD90 and CD59 superficial antigens were first determined by immunofluorescence and then quantified by FACS analysis. CD34, CD45 and MP marker were determined only by immunofluorescence because rBMSCs resulted negative to these antigens. After 11 days from seeding, adherent bone marrow cells were mostly positive to CD90, dimly positive to CD59, and completely negative to the other tested hematopoietic cell markers [Fig. 4].



CD90 +++
CD59+ (dimly)
CD34-
CD45-
MP-

Figure 4. Cytofluorimetric analysis of rBMSC for CD90 expression. rBMSCs were labelled with primary antimouse CD90 antibody and FITC-conjugated secondary antibody and then analized by FACS. a) after 8 days of culturing b) after 11 days of culturing .

The positivity to CD90 antigen was confirmed by fluorescent immunocytochemistry analysis [Fig. 5].

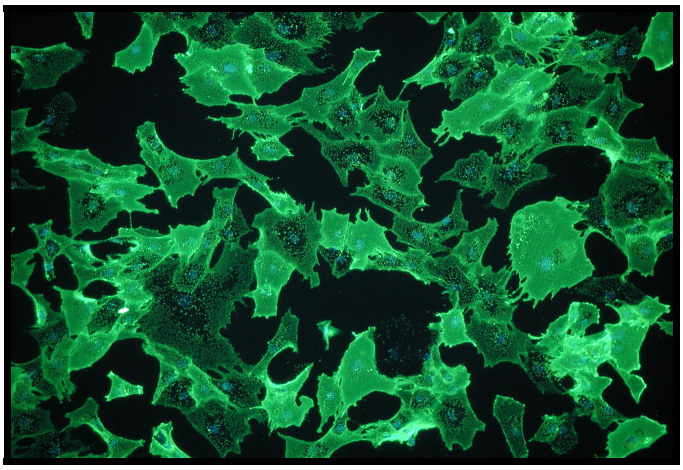


Figure 5. Immunofluorescence analisys of rBMSCs for CD90 antigen.
Merged images : rBMSCs labelled with primary mouse monoclonal anti CD90 antibody and FITC-conjugated secondary antibody (green); nuclei are stained with DAPI (blue).

Interactions between rBMSCs and a hyaluronan biopolymeric scaffold

Secondary, we investigated the interactions between a microfibrous three-dimensional scaffold based on hyaluronic acid and rBMSCs using different techniques. The hyaluronan based scaffold was chosen because its molecule is constitutively present in the extracellular matrix of all soft tissues and can be selectively modified in its chemistry to obtain a derived compound potentially suited for tissue repair. In this study we used the non-woven structured HYAFF®11 produced by Fidia Biopolymers (Abano Terme, Italy). Analysis were performed on cell-untreated or cell-treated scaffolds using light microscopy (LM), scanning (SEM) and transmission (TEM) electron microscopy or immunohistochemistry techniques.

HYAFF®11 structural analysis

Samples from non-woven structured HYAFF®11 were initially examined under an inverted light microscope (LM) and then processed for scanning (SEM) and transmission (TEM) electron microscopy.

At LM the HYAFF®11 scaffold was composed by randomly distributed layers of translucid fibres in the micron dimensional range [Fig. 6a]. The scaffold overall morphology was better appreciated under SEM investigation [Fig. 6b]; here the scaffold fibres had a solid appearance with

interconnected voids among them such that a clear gross porous structure was recognizable; the fibre diameter ranged between 12 to 16 µm with an average of 15 µm diameter. At higher magnification the fibre surface was slightly irregular. At TEM the fibres were moderately electrondense and at higher magnification were composed by a tight mesh of nanofibrils which bulged in correspondence with the free edge of the fibre surface [Fig. 6c,d].

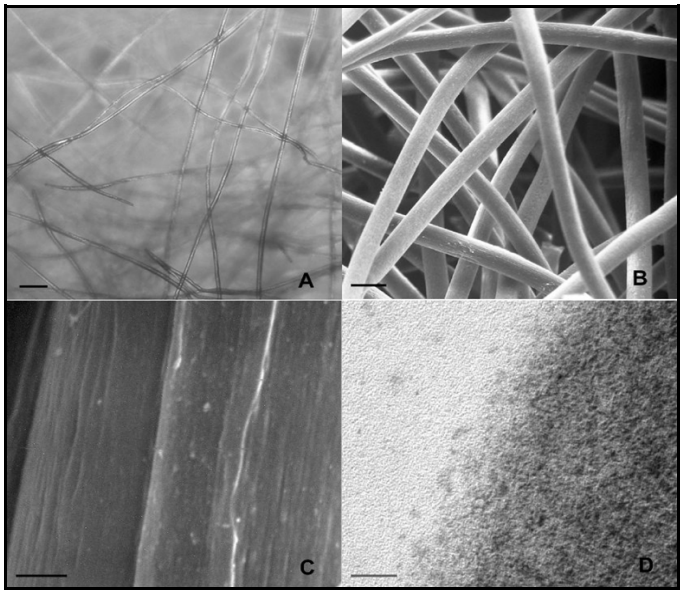


Figure 6. HYAFF®-11 morphological analysis. Inverted LM (a) showing layers of translucid fibres which SEM reveals having a micron dimensional range (b); at higher magnification, SEM (c) shows that the fibre surface is rough; TEM (d) of an individual fibre composed by a tight mesh of nanofibrils bulging at the free border edge. Scale bars: a) 100 µm; b) 25 µm; c) 1 µm; d) 20nm.

Analysis of cell adhesion and proliferation in HYAFF®11

rBMSCs were seeded at the concentration of 2 x 10^6 cell/cm^2 on the upper surface of the hyaluronan scaffold, cultured up to 21 days and then analyzed using appropriate techniques. As detected by the trypan blue exclusion assay, the rBMSC adhesion on the scaffold was high.

After 24 hours of rBMSC seeding, the number of cells entrapped in the scaffold was similar to the number of cells which had been previously seeded, i.e., 96 ± 0.4% of the overall number of the initially seeded cells. As viewed with SEM, rBMSCs adhered on the HYAFF® 11 surface efficiently forming extremely thin cell sheets since early days of culture; at day 1, the cells covered an area of about 12 ± 2% of the entire seeded scaffold; cell adhesion on the scaffold steadily increased and after 21 days the rBMSCs covered an area of 40 ± 10% [Fig.s 7a,b].

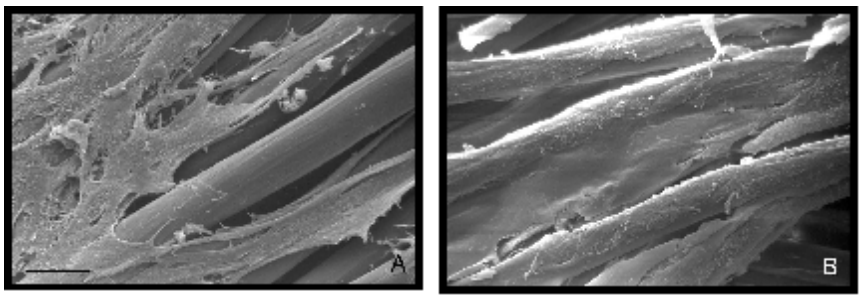


Figure 7. Surface analysis of cell adhesion on the scaffold. At day 1 (a), rMSCs cover small areas of the seeded scaffold as thin cell layers, whereas after 21 days of culture (b) rMSCs spread over the surface forming large confluent cell layers. Scale bars: a, b = 20 μm.

During their growth in the scaffold, rBMSCs remained viable and progressively proliferated during incubation times. To test cell viability and proliferation we used respectively the fluorescein-derived dye CFDA-SE (carboxyfluorescein-diacetate succinimidyl ester) method and the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test. CFDA provides a simple and sensitive tool for the analysis of the division history of individual cells, MTT is a simple colorimetric method to assay cell proliferation (*see Meterial&Methods section for details*).

Results are shown in Figure 8.

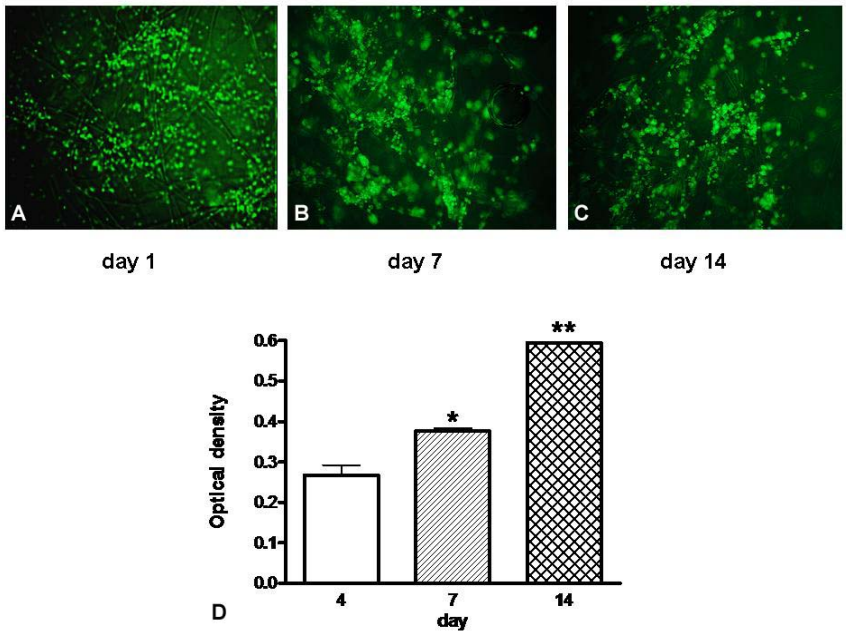


Figure 8. rBMSC viability and proliferation into HYAFF® -11 non-woven scaffold. Micrographs (a-c; magnification 10x) show representative CFDA staining of rBMSCs at different times of culture onto Hyaff-11®. Proliferation of rMSCs up to 14 days (d) was estimated by the MTT assay as described in Materials and Methods. Values are expressed as mean ± S.E.M. (n = 3). One-way ANOVA was performed as statistical analysis ($p < 0.01$), followed by Dunnett's comparison test. *$p < 0.05$, **$p < 0.01$ vs. day 4.

LM showed that rBMSCs were able to migrate throughout the entire thickness of the scaffold. After 3 days of culture cells start permeating the inner regions of the scaffold even though rBMSCs appeared more homogeneously distributed in the scaffold thickness only starting from day 7 of culture [Fig. 9a, b]. This finding mimics one of the pivotal "in vivo" properties of hyaluronate that is facilitating cell migration.

At day 7 of culture, TEM showed the rBMSCs tightly adhered to the fibres through well-spaced focal contacts, which are, as it is known, specifically involved in the interaction of MSCs with extracellular matrix (ECM); nuclear chromatin was finely dispersed and the cytoplasm rich in dilated cisternae of rough endoplasmic reticulum. After 15 days, a filamentous fibronexus was found in the free narrow space located between cell membrane and biomaterial surface; at this time, rBMSCs still had a synthetic phenotype and no significant degenerative changes, e.g. vacuoles, secondary lysosomes, lipid inclusions, were observed. After 21 days, autologous extracellular matrix, including collagen fibres and proteoglycan particles were found between rBMSCs and HYAFF®11 fibres. A corresponding finding was seen at SEM where filamentous extracellular material was observed in close contact with rBMSC plasma membrane.

Representative results of the rBMSC interaction with HYAFF®11 are illustrated in Figure 10.

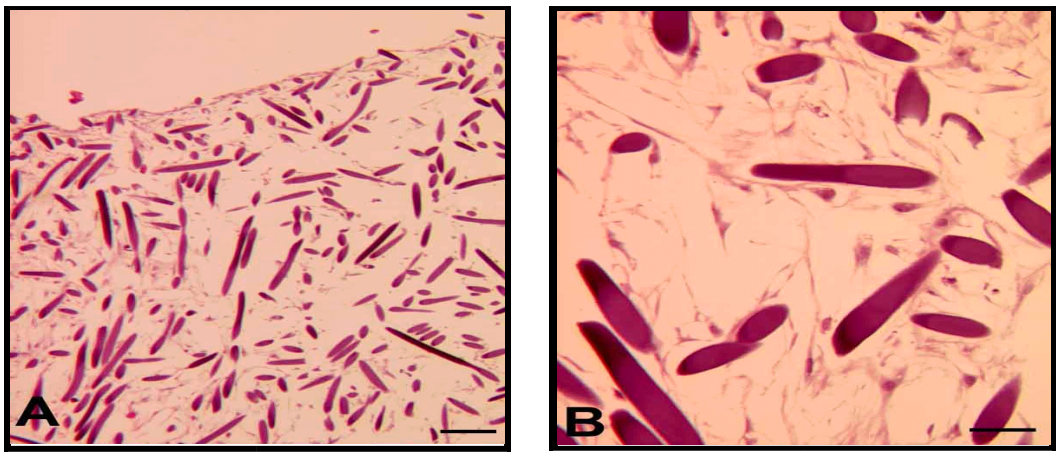


Figure 9. rBMSC migration through HYAFF®-11 fibres. LM showing rBMSCs migration in the inner portions of the scaffold at day 3 (a). At higher magnification (b) cells are spindle and viable. Scale bars: a) 200 µm; b) 50 µm.

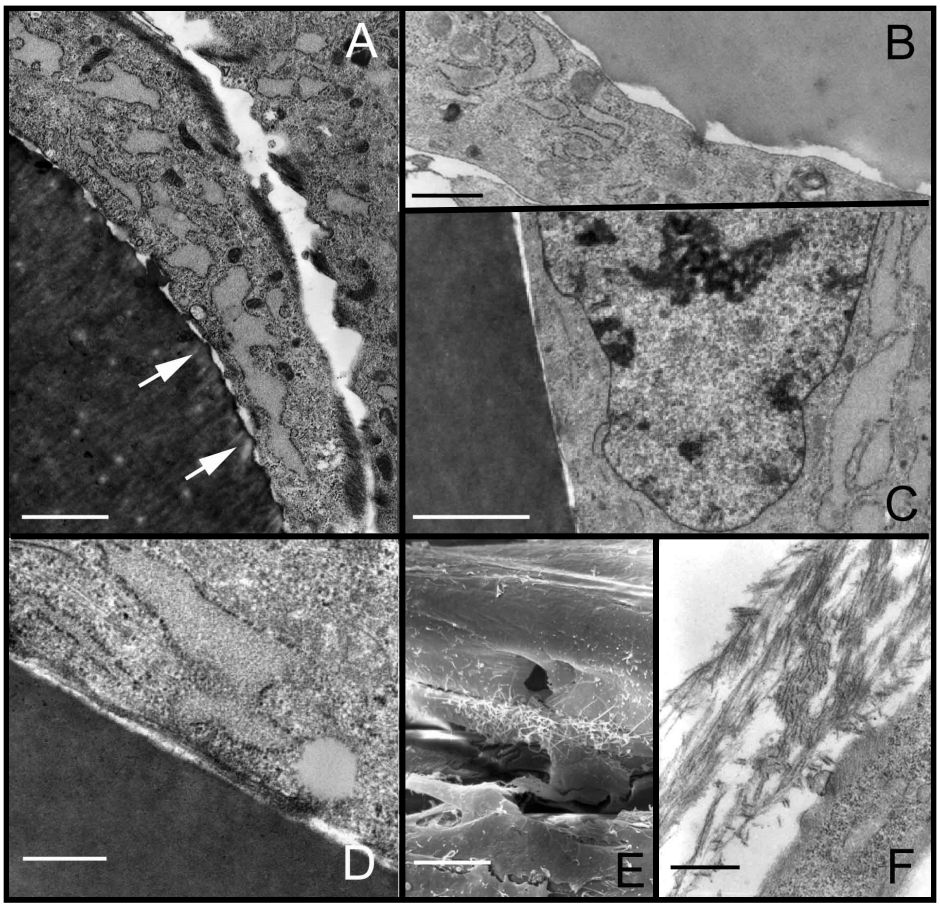


Figure 10. rBMSC interaction with HYAFF® -11 fibres. TEM of 7 day culture (a, b) showing a rBMSC wrapping a scaffold fibre; the arrows indicate regularly-spaced focal contacts. rBMSCs have nuclei with finely dispersed chromatin and numerous cisternae of rough endoplasmic reticulum in the cytoplasm (c). After 14 days (d), a distinct fibronexus is seen while connecting the contractile cell cytoskeleton with the outer portion of a scaffold fibre. Autologous fibrillary extracellular matrix is seen after 21 days of culture (e, f). Scale bars: a) 1 μm; b) and c) 2 μm; d) 0.5 μm; e) 10 μm; f) 1 μm.

Confocal and immunofluorescence analysis of CD44 and CD90 expression

At day 3, confocal images of rBMSCs cultured on non-woven structured HYAFF®11 showed a polarized cell membrane expression of CD44 molecule, the native receptor for hyaluronic acid [Fig. 11a,c]. The CD44 molecule was preferentially expressed in correspondence with the material-cell contact.

After 21 days, the abundance of CD90, a marker of MSC stemness, was not negatively influenced by cell-scaffold interaction showing a homogeneous membranous pattern of expression [Fig. 11d,e], suggesting that rBMSC prolonged interaction with the substrate did not modify the multipotency of the investigated cell model.

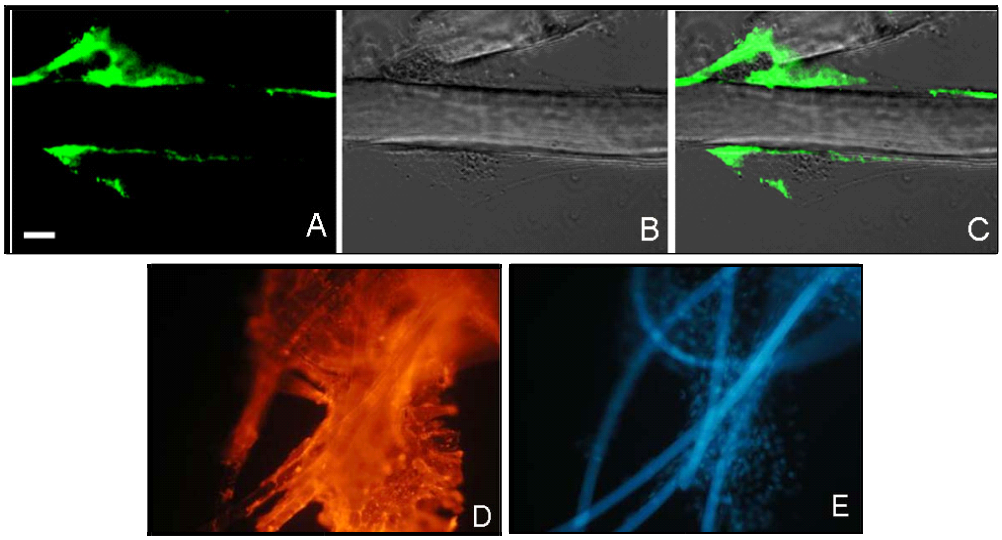


Figure 11. Confocal and immunofluorescence microscopy of CD44 and CD90 expression of rBMSCs grown in the scaffold. Confocal images of immunohistochemical analysis of CD44 antigen (a-c) in rMSCs at day 3. a) CD44 expression (green); b) phase contrast; c) merged image. CD44 expression is polarized in correspondence with the scaffold contact. Scale bars: a, b, c = 10 µm. Immunofluorescence detection of CD90 at day 21 (d, e - magnification 20x). d) CD90-related Cy3 fluorescence (red) was extended to a large portion of the sample; e) nuclear staining with DAPI (blue) shows the high number of MSCs which are present in the sample. In the background the hyaluronan fibres are weakly stained with DAPI

In vivo application of HYAFF®11 engineered with rBMSCs

The *in vitro* studies showed that the microfibrous nature, the gross porous structure and the three-dimensional architecture of HYAFF®11 facilitates adhesion, migration, and proliferation of rBMSCs as well as the synthesis and delivery of autologous extracellular matrix components under static culture condition without any chemical induction.

The high retention rate and viability of the seeded cells, as well as the fine mechanisms of cell interaction with the substrate, suggested that such scaffold could be potentially useful in cardiac tissue repairing.

At this purpose we tested the HYAFF®11 engineered with rBMSC in a heterotopic infarcted rat heart model.

Experimental animal model

rBMSCs at first passage were cultured for 21 days on HYAFF®11 before transplantation into the infarcted region of a heterotopic rat heart. For the heterotopic heart animal model we used

syngeneic Lewis two-months old rats to avoid allograft rejection; half of them were donors, half recipients. In brief, hearts were excised from the donor, left descending coronary ligation was performed on the bench and the infarcted heart implanted into the abdomen of recipient rats (*see Materials&Methods section for more details*). We chose this model because the donor left ventricle performs work comparable to that of the native heart and because this procedure is easily reproducible.

After infarcted heterotopic heart implantation we waited 2 weeks, therefore we measured cardiac function by M-Mode echocardiography and we transplanted HYAFF®11-rBMSCs construct into the heart infarcted area. Again, two weeks later we measured heterotopic cardiac function, afterwards we sacrificed recipient rats and we fixed the heterotopic heart for histological analysis.

The experimental protocol is summarized in Figure 3.

rBMSC labelling

In order to investigate the fate of rBMSCs delivered through HYAFF®11 into the infarcted heart, cells were labelled by incubation with bromodeoxyuridine (BrdU) 10 μM for 72 hours before construct transplantation. BrdU is a thymidine analog that is used in cell proliferation studies. BrdU in culture is incorporated into the DNA during DNA synthesis; rBMSCs incorporation of BrdU was detected using an anti-BrdU specific antibody by immunocyto- (HYAFF®11-rBMSCs construct) or immunohistochemistry (HYAFF®11-rBMSCs construct after heart transplantation) techniques.

$BrdU^+$-cells were detected by both fluorescent and light microscopy [Fig. 12].

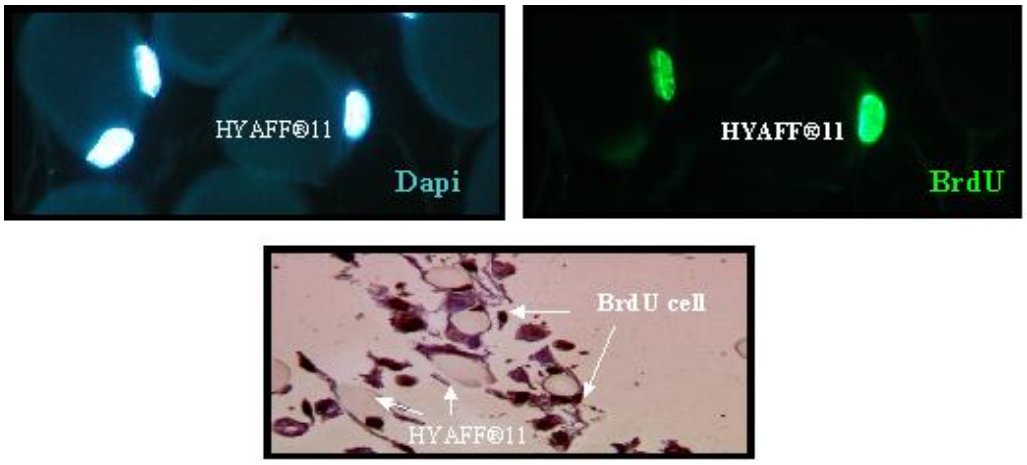


Figure 12. BrdU labelled rBMSCs. Fluorescent and light microscope images of rBMSCs seeded onto HYAFF®11 and incubated with BrdU for 72hs. A) nuclei stained with Dapi; B) nuclei labelled with antimouse anti-BrdU primary antibody and Fitch-conjugated secondary antibody; C) $Brdu^+$positive cells (dark spots) detected on HYAFF®11 by Novo Link™Polymer detection system Kit.

Histological analysis

At the end of the forth week, after cardiac functional analysis, recipient rats were sacrificed and heterotopic heart rapidly excised, fixed, embedded in paraffin and used for histological analysis. Heart sections were used for morphological studies (stained with H&E), fluorescent analysis or light microscopy study.

Heart structures observed by H&E staining had a relatively normal morphology and no malformations were found. We observed slightly more loose connective tissue containing signs of an active neo-vascularization near the infarcted area as compared to non-infarcted hearts [*pictures not shown*]. As shown in Figure 13, the hyaluronan fibres of the transplanted scaffold had not been substantially degraded 2 weeks after HYAFF®11-rBMSCs construct heart-implantation and they are surrounded by non-specific inflammatory and giant cells. We expected a non-specific inflammatory response after scaffold implantation, because it is known that following the implantation of a biomaterial, an inflammatory process, called Foreign Body Reaction (FBR), could initiate a complex series of reactions tending to prevent the ongoing tissue damage, isolate and destroy the foreign material and activate the repair process [155]. Anyway, if the inflammatory reaction is limited and not chronic it can be considered as a normal response and the material could be considered biocompatible [155].

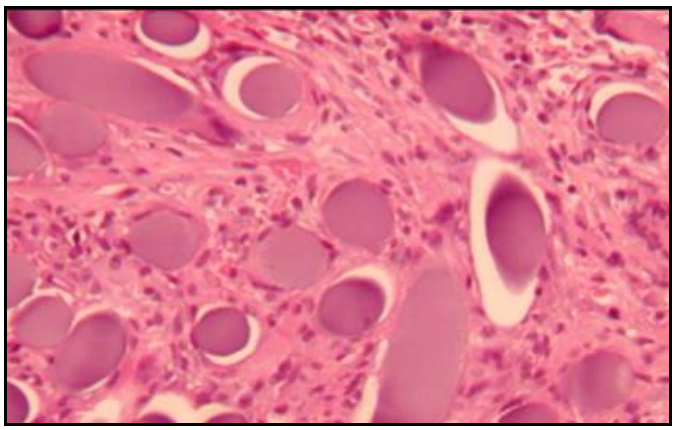
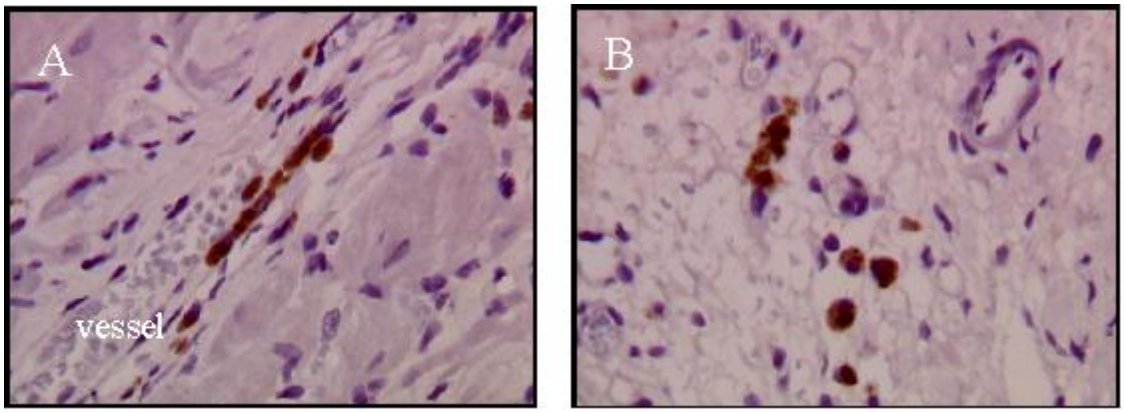


Figure 13. H&E tissue section. After 2 weeks from construct implantation Hyaff-11® fibres had not been degraded even if they start to be surrounded by non-specific inflammatory and giant-cells (10X magnification).

Analyzing H&E sections in which BrdU-labelled rBMSCs were detected using light microscopy, we observed that most of the transplanted cells had migrated to the surrounding infarcted area.

The $BrdU^+$- cells were especially found close to small-sized vessels or integrated into the wall of

bigger vessels [Fig. 14a]. Moreover, we found $BrdU^+$-cells at the cytoplasmatic level instead of the nuclear one. This finding shows that some of the delivered rBMSCs were digested by endogenous cells of the recipient rat [Fig. 14b].



Fugure 14. H&E tissue section. BrdU-labelled rBMSCs (dark spots) are migrated to the surrounding infarcted area. $BrdU^+$ cells are detected using a pre-diluited mouse monoclonal anti-BrdU antibody and the NovoLink™ Polymer Detection System. A part of delivered rBMSCs is integrated into small-sized vessels (A; 40X magnification) and a part is digested by haematic rat-recipient cells (B; 40X magnification).

In order to evaluate if some of the survived transplanted cells might contribute to heart tissue regeneration, we stained some heart sections simultaneously with BrdU and cardiac Troponin I (cTnI) antibodies. cTnI is a later cytoplasmatic marker of differentiation into cardiac phenotype. Fluorescent microscopy analysis showed that a small number of cells, mainly found close to HYAFF®11 fibres or in the periphery of the infarcted region, express both these markers [Fig. 15].

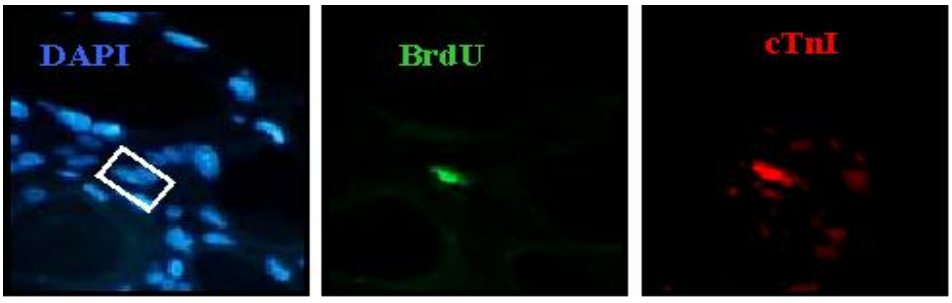


Figure 15. Immunofluorescence analysis of heart sections. Rapresentative image of rBMSCs positive for both markers BrdU and cTnI two weeks after construct implantation. A) Nuclei stained with DAPI, blue; B) Nuclaer BrdU labelling, green; C) cytoplasmatic positivity to cTnI, red

This result indicates that rBMSCs delivered into the infarcted region through a hyaluronan scaffold preserve the ability to transdifferentiate into cardiomyocytes.

Functional analysis

Functional analysis of infarcted heterotopic hearts were performed by M-Mode echocardiography 2 weeks after heart implantation, but before HYAFF®11-rBMSCs transplantation, and 2 weeks later the second surgery, before rat sacrifices (*see Material&Methods section for more details*).

Analyzing the M-mode images emerged that scar tissue was moderated in the engrafted region and the thickness of the corresponding ventricular wall was comparable to that of the non-infarcted remote area.

Posterior wall (PW) thickening, as well as fiber elongation and intra ventricular septum (IVS) thickening, are index of cardiac hypertrophy. As shown in Figure 16, IVS and left ventricular PW thickening in heterotopic hearts after HYAFF®11-rBMSCs transplantation are similar, or even slightly decreased, if compared to the same parameters measured before construct transplantation. These data suggest that the treatment of infarcted heart with MSCs delivered through a biopolymeric support might contribute to avoid the wall thickening typical of post-infarction left ventricular remodelling.

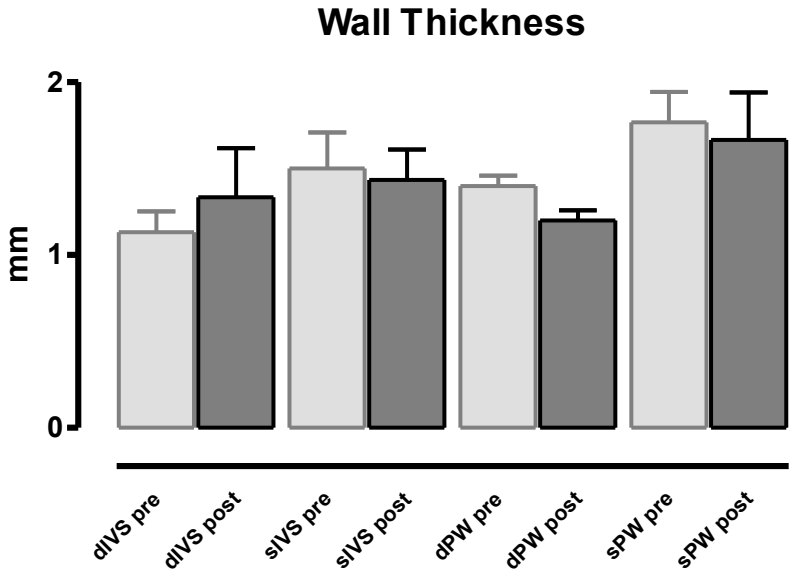
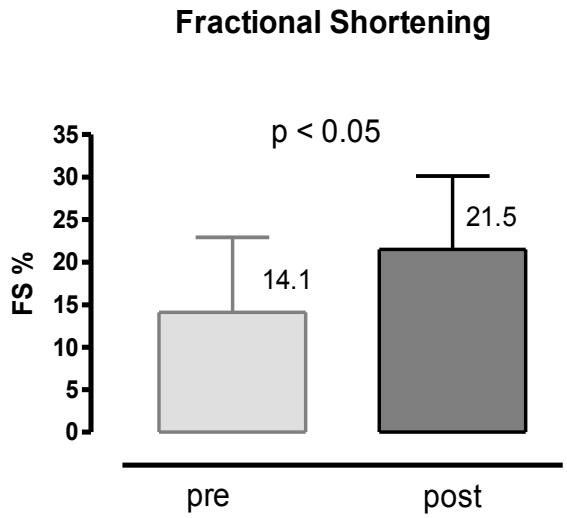


Figure 16. Heterotopic Heart Wall Thickness before and after HYAFF®11-rBMSCs construct heart-implantation. d IVS = diastolic Intra Ventricular Septum; s IVS = systolic Intra Ventricular Septum; pre = before implantation; post = after implantation

Again, the left ventricular shortening fraction, evaluated by M-Mode echocardiography, was found increased when compared to that measured just before construct transplantation [Fig. 17]. The shortening fraction is a slightly different way of measuring left ventricle performance. Instead of measuring and ratioing blood volumes, the shortening fraction measures and ratios the change in the diameter of the left ventricle between the contracted and relaxed states. An increase in the shortening fraction indicates improvement of cardiac function.



	PRE	POST
LVDd (mm)	4.60 ± 0.70	4.43 ± 0.55
LVDs (mm)	3.93 ± 0.73	3.43 ± 0.40
FS %	14.10 ±15.27	21.50 ± 14.90

Figure 17. **Fractional Shortening evaluatred by M-Mode echocardiography**. FS in percent, was calculated according to the following formula: LV SF% = (LVDd – LVDs)/LVDd x 100 ; LVDd = Left Ventricular Diameter in diastole; LVDs = Left Ventricular Diameter in systole; Pre = before scaffold transplantation; Post = after scaffold transplantation; FS % = Fractional Shortening x100. One-tailed Student's T test , paired data was performed as statistical analysis ($p < 0.05$)

Moreover, there is no evidence of an increase in LV volumes and dimensions in treated heterotopic rat hearts.

Thus, taken together, all these results indicate that inserting a cell engineered-scaffold into the infarcted region could favourably affect the heart disfunction consequent to MI.

DISCUSSION

In this thesis, it has been studied a new strategy for cell delivery for the regeneration of the heart in a rat model of myocardial infarction: the tissue-engineering therapy.

At this purpose we used HYAFF®11 as hyaluronan biopolymeric scaffold. It is a biomaterial derived from the esterification of native hyaluronan, which is an abundant non-sulfated glycosaminoglycan component of synovial fluid and extracellular matrices ubiquitous in the body. In appropriate experimental settings HYAFF®11 scaffolds have demonstrated to have properties which are interesting for the field of tissue engineering. In fact, HYAFF®11 is biocompatible [156], biodegradable [157, 158] and it promotes the adhesion and growth of several cytotypes, including keratynocytes [159], endothelial cells [157, 160] and chondrocytes [161,162].

The possibility of combining such biological properties of HYAFF®11 with those offered by multipotent stem cells has been investigated by others with the aim at improving cartilage repair [158, 162, 163].

In the present study, first we studied the basic abilities of HYAFF®11 on facilitating the adhesion, migration and proliferation of rBMSCs, and then we evaluated the *in vivo* effects of the construct HYAFF®11-rBMSCs implanted into the infarcted area of heterotopic rat hearts.

The plain morphological analysis of the non-woven HYAFF®11 scaffold confirms its microfibrous nature and gross porous structure [Fig. 6 a,b]. The presence of a rough surface [Fig. 6d] dramatically expands the sites of possible chemical interactions between cells and substrate; this characteristic could contribute to early effective cell adhesion on HYAFF®11 surface as documented by both SEM and TEM analysis [Fig.7 a,b]. Coherently with this view, the rBMSC retention in the substrate was high at day 1 and the number of rBMSCs entrapped within HYAFF®11 was found to be comparable to the number of the originally seeded cells by using the trypan blue exclusion stain method.

No significant cell loss and/or degeneration was found up to 21 days of cell culture on the substrate as detected by CFDA assay [Fig.8] and LM. Moreover, MTT analysis demonstrated that rBMSCs proliferated steadily during culture conditions [Fig.8] and SEM revealed a subconfluent surface monolayer at the end of culture condition. As a logical consequence, the interaction between rBMSCs and HYAFF®11 was functional and this was also supported by the development of fibronexus, a distinct ultrastructural structure which documents anchorage of cell contractile cytoskeleton to extracellular matrix components [164].

The HYAFF®11 three-dimensional architecture could be essential in facilitating the migration of the seeded rBMSCs within its entire thickness. Accordingly, starting 3 days after the initial cell seeding, we found cells permeating the innermost region of the scaffold [Fig. 9 a,b].

It is generally accepted that cells attach and organize better around fibres with diameters smaller than the diameter of cells [165]. However, the present study demonstrates that even the microfibres of HYAFF®11 provides adequate anchorage for cell adhesion. SEM analysis clearly evidenced that rBMSCs firmly adhered on the substrate as thin sheets thus recovering their phenotypic shape rapidly and, more importantly, TEM revealed that the adhesion was achieved through regularly spaced adhesion structures, namely focal contacts, which are specifically involved in the interaction of MSCs with extracellular matrix (ECM) [Fig.10]. Focal contacts represent the ultrastructural mirror of a complex, highly regulated process, where integrins in the plasma membrane are clustered with other molecules pushing cells toward many adhesion-mediated signalling pathways. Schematically, many fundamental cellular functions including motility, proliferation, differentiation, and apoptosis are regulated through cell – ECM interactions [166].

Also, an additional finding supporting a functional interaction is the confocal microscopy demonstration that the CD44 molecule, the native receptor for hyaluronan acid, was preferentially expressed in correspondence with the material-cell contact [Fig.11 a,b,c].

An additional point of interest is that the rBMSC growth on the HYAFF®11 was accompanied by ultrastructural documentation of expanded protein synthesis and late autologous extracellular matrix production. TEM analysis of early cultures showed that rBMSCs had euchromatinic nuclei and numerous rough endoplasmic reticulum cisternae in the cytoplasm; at day 21 SEM revealed distinct meshworks of extracellular fibrils which TEM demonstrated to be fibrillar collagen admixed with small proteoglycan particles [Fig.10].

In vivo hyaluronan surrounds migrating and proliferating cells and, expecially in the embryonic tissues, facilitates cell differentiation according to their orchestrated destiny. In the present study this ability has not been investigated; however human MSC chondrogenesis in a similar polymer scaffold was well induced by 20 ng/ml of TGFß1 [167] and consequently a cardiac or endothelial differentiation potential can be expected also in our model whether appropriate growth factors are utilized. Herein was found that the CD90 surface antigen, a stemness molecule which belongs to the basic phenotype of mesenchymal stem cell, was retained up to the end of the culture condition [Fig. 11 d,e], thus suggesting that rBMSC prolonged interaction with the substrate did not modify the multipotency of the investigated cell model.

The high retention rate and viability of the seeded cells, as well as the fine mechanisms of cell interaction with the substrate, suggested that such scaffold could be potentially useful in cardiac tissue repairing, thus we tested the HYAFF®11 engineered with rBMSC in a heterotopic infarcted rat heart model. At this purpose we transplanted the HYAFF®11-rBMSCs in the infarcted region of a heterotopic rat heart and after two weeks we performed functional and histological analysis.

Our results showed that after two weeks the HYAFF®11 fibres have not been degradated but they are surrounded by inflammatory cells [Fig. 13]. Hyaluronan-scaffold activates innate alloimmune responses acting as a foreign body (Foreign Body Reaction, FBR). Anyway, if the immuno-response is limited and not excessive, it might contribute to scar digestion and consequently tissue regeneration. Improving our understanding of the nature of the immuno-reaction in tissue-engineering strategy may lead to improve therapeutics in heart tissue repearing.

By light and fluorescent microscopy analysis we found that transplanted rBMSCs are still present in the heart tissue but they are not close to HYAFF®11 fibres as we expected according to the *in vitro* studies. In fact, most of the trasplanted cells were especially found close to the vessels [Fig.14] in the periphery of scar tissue area or migrated in the viable myocardium. Only a very few cells were found close to HYAFF®11 fibres; some of them were differentiating into cardiomyocytes, as showed by the expression of cardiac markers such as cTnI [Fig. 15]. We are not able to explain this phenomenon but we can hypothesized that cell migrate in the sourranding area because of the inflammatory process.

Finally, analyzing functional analysis emerged that there was no IVS or PW thicknening in the treated infarcted hearts [Fig. 16] and even the shortening fraction parameter was slightly increased [Fig. 17] two weeks after HYAFF®11-rBMSCs transplantation. These results indicate that trasplanting a hyaluronan biopolymer engineered with MSCs into the infarcted area could attenuate the cardiac disfunction and LV remodelling subsequent to myocardial infarction.

CONCLUSION

The HYAFF®11 non-woven scaffold has many characteristics which makes it attractive for tissue engineering.

In the present study we have demonstrated that HYAFF®11 facilitates adhesion, migration, and proliferation of rBMSCs as well as the synthesis and delivery of autologous extracellular matrix components under static culture condition without any chemical induction. The high retention rate and viability of the seeded cells, as well as the fine mechanisms of cell interaction with the substrate, suggest that such scaffold could be potentially used for post-infarction cardiac regeneration.

In fact, the *in vivo* results indicate that the treatment of infarcted rat hearts with MSCs delivered through a biopolymeric support might contribute to attenuate the progression of pathologic LV remodelling and that post-infarction myocardial function could be favourably affected by the grafting of MSCs delivered through a hyaluron-based scaffold.

APPENDIX

I spent the first year of my PhD course attending the laboratories of the *Cardiovascular Research Institute* at New York Medical College in Valhalla (New York, USA), directed by Doctor Piero Anversa.

Piero Anversa, professor of medicine and director of the *Cardiovascular Research Institute*, is a well-known cardiovascular researcher who has made landmark discoveries in the pathophysiological mechanisms of heart disease. Dr. Anversa's work on heart failure is reshaping our understanding of myocardial growth and death by providing evidence that the heart has its own adult stem cells capable of regenerating heart muscle tissue following a coronary event [17]. Under the guide of this brilliant scientist I started learning about stem cells and I started working into this interesting field.

During that period I studied proliferation and cell death of cardiomyocytes (CMs) and resident cardiac stem cells (CSCs) in a transgenic animal model. I characterized the phenotype of cardiac cells of transgenic mice overexpressing, at cardiac level, ornithinedecarboxylase (ODC), the first and potential rate-limiting enzyme of polyamines biosynthesis. Polyamines (putrescine, spermidine and spermine) are aliphatic polycations essential for the maintenance of cell growth, survival and for macromolecular biosynthesis. They exert them role by interacting with nucleic acids, proteins and membranes through ionic interaction. Polyamines are involved in many cellular functions including gene expression, transcription, signal transduction, cell growth, cell cycle regulation, proliferation, cell attachment, cell migration and cell signalling. Consistent with their metabolic and biological features, polyamines can be potential targets for multiple intervention also in stem cell manipulation.

The overexpression of ODC in the heart tissue, as shown by Shantz LM *et al* [168], leads to an increase in intracellular polyamines level and to a slight cardiac hypertrophy. It is known that agents responsible of cardiac hypertrophy activate ODC enzyme, thus polyamines biosynthesis, at cardiac level. In fact, increased polyamine concentrations were found in the ventricular myocardium of animal subjected to protocols known to induce cardiac hypertrophy, including aortic stenosis, intense muscle exercise or treatment with β-adrenergic agonist (ODC inducers).

The aim of my work was, first, to evaluate the cardiac hypertrophy level in ODC-trangenic mice and secondarily to try of clarifying if it could exist a relationship between polyamines-induced cardiac hypertrophy and cardiomyocytes hyperplasia and death and/or resident CSCs proliferation and differentiation.

For developing this research I used different techniques that were very useful also for my subsequently studies in tissue-engineering therapy illustrated in the first part of my thesis; some of them are described below.

TECHNIQUES

Mouse heart perfusion: Cardiomyocytes and Small Cell isolation

Two-months old ODC-transgenic and wild type mice were injected with 200 uL of heparin solution 20 minutes before sacrifice. Immediately after they were anesthetized with $CHCl_3$ (200-300uL/100g Body Weight); chest was opened and heart incannulated from the aorta. Heart was perfused 1 minute in Langerdoff System with Basic Buffer (Minimal eagle's essential medium, 0.7 g HEPES, 0.3 g Glutamine, 1.25 g Taurine, 20 units Insuline, 5 mL Pen-Strep, pH 7.2-7.3). Afterwards, Basic Buffer was removed and heart was perfused 10 minutes with Collagen Solution (50 mg Collagenase TypeII in 50 mL of BASIC BUFFER, 25 nM of $CaCl_2$). Then it was put in a dish with Incubation Buffer (100 ml Basic Buffer, 0.5 g BSA, 60 uL $CaCl_2$ 0.5 M, pH 7.2-7.3), sminced with forceps and centrifuged 1 minute at 100g. Surnatant containing the Small Cells fraction was collected in a Falcon and the pellet, cardiomyocytes fraction, was washed with IB and centrifuge again 1 minute at 100g.

Cardiomyocytes culture

Two dishes 30 mm for each digested heart were coated with laminin 50 uL/mL PBS and used for cardiomyocytes colture. Cardiomyocytes were resuspended in IB seeded in laminin-coated dishes and incubated at 37°C, in a 5% CO_2 humidified atmosphere until they were attached to the bottom. Then medium was changed and cardiomyocytes kept in colture. For immunohistochemical analysis or cardiomyocytes measurement 1/3 of resuspended cardiomyocytes was put in 1 mL of 10% formaline .

Small Cell isolation and selection of ckit$^+$ (CD117) cells

The surnatant from cardiomyocytes isolation was filtered with 40 um filter and centrifuged at 1800rpm for 10 minutes. The pellet was resuspended in 80 uL PBS/BSA/EDTA for MACS® cell sorting (Miltenyi Biotec) and 20 uL of CD117-micro Beads for each sample were added. Then samples were mixed at 4°C for 15-20 minutes. Afterwards, cell suspension containing $CD117^+$ cell magnetically labelled with CD117 microbeads was loaded on a coloumn which was placed in the magnetic field of MACS® Separator. The magnetically labelled cells were retained on the coloumn while the unlabelled cells run throughout. After removal of the coloumn from the magnetic field, the magnetically retained $CD117^+$ cell were eluated as the positevely selected cell fraction.

Mice BrdU injection

In order to evaluate CSCs or cardiomyocytes proliferation, all mice used for the experiments were injected with 200 uL of BrdU solution (50 mg/10 mL PBS) twice a day for one week before sacrifice.

Immunohistochemical analysis

ODC-transgenic and wild type mouse hearts were arrested in diastole by injection of potassium chloride (2 ml KCl 15%) and fixed in formaline 10% for 24 hours. Afterwards, the hearts were cut into 3 pieces apex, middle portion and base, before being dehydrated in alcohol at increasing concentration and embedded in paraffin**.** Blocks were cut into 6-μm sections. Serial sections were stained with haematoxylin/eosin (H&E) for morphological analysis or used for immunofluorescence analysis. The standard deparaffinization protocol was used, tissue sections were serially rehydrated in 100%, 95%, 70% ethanol and distilled water. Antigens were retrieved in a microwave for 10 minutes at full power in pH=6.0, 10 mM TP citrate solution. Non-specific bindings were blocked with 10% donkey serum for 45 minutes. Then the slides were incubated with the following primary antibodies: mouse-monoclonal anti-BrdU (from BD bioscience-kit) overnight at 4°C, mouse monoclonal anti-CD117 (Dako Cytomation) diluted 1:20 in PBS 2 hours at 37°C, rabbit polyclonal anti-Ki67 (Pharmigen) 1:300 in PBS Over Night at 4°C. Afterwards, the slides were incubated for 45 minutes at room temperature with one of the two following secondary antibodies Fitch- or Tritc-anti-mouse or anti-rabbit IgG (Sigma, St Luis, MO, USA) 1:2000 in blocking buffer. Then slides were washed 10 minutes in PBS and then mounted with Vectashield-with DAPI mounting medium (Vector Laboratories, Burlingam, CA, USA).

For apotosis detection was used the apoptosis kit from BD-Bioscience (Clonech-ApoAlert DNA Fragmentation Assay Kit) following the manufacturer instruction.

The results are rapresentative of the measurement of 20 sections for each animal heart; 6 mice for each group were used. The results are expressed in percent of the total number of cardiomyocytes for heart section.

The total number of CM *per* section was calculated with the following formula:

CM/mm^2 * Section area

Functional and anatomical measurements

Before mice sacrifice anatomical and functional heart parameters were measured. The functional parameters were taken using the Millar's procedure [169]. Mice were anaesthetized with chloral hydrate 400 mg/kg body weight, intraperitoneally, and the right carotid artery was cannulated with a microtip pressure transducer (model SPR-671; Millar) for the measurements of LV pressures, and LV +dP/dt and LV- dP/dt in the closed-chest preparation.

Cardiomyocytes density and otal number of cardiomyocytes

For the cardiomyocytes density measurement pictures of the H&E heart sections were taken and sections area were measured using the IMAGE PRO program. Then, ten pictures for each sample with at least 3 nuclei for the measurements of nuclear length were taken.

Cardiomyocytes density (CMs Density) was calculated with following formula:

CMs DENSITY in mm^3 = myocytes in mm^2/nuclear length *1000

The total number of cardiomyocytes was calculated with the following formula:

CMs Density*Heart Volume

(Heart Volume = Heart weight/1,06)

RESULTS and DISCUSSION

The experiments described in the present study use a transgenic approach to examine the effects of overexpression of ODC in the heart. At this purpose I used ODC-transgenic mice kindly provided by Dr. Anthony Pegg's laboratory from Hershey Medical Center (Hershey, PA, USA).

Directed overexpression of ODC to the heart is accomplished through the use of the well-characterized α-myosin heavy-chain (α-MHC) promoter. The use of this promoter allows expression of ODC in both the atria and ventricles, but not in other organs, of transgenic animals throughout adult life. The measures of ODC expression and cardiac polyamines level were performed by Shantz LM *et al* [168].

In order to evaluate if the overexpression of the ODC protein into the heart tissue and the subsequently increase in polyamines level lead to cardiac hypertrophy and/or heart failure anatomical and left ventricular functional analysis were performed.

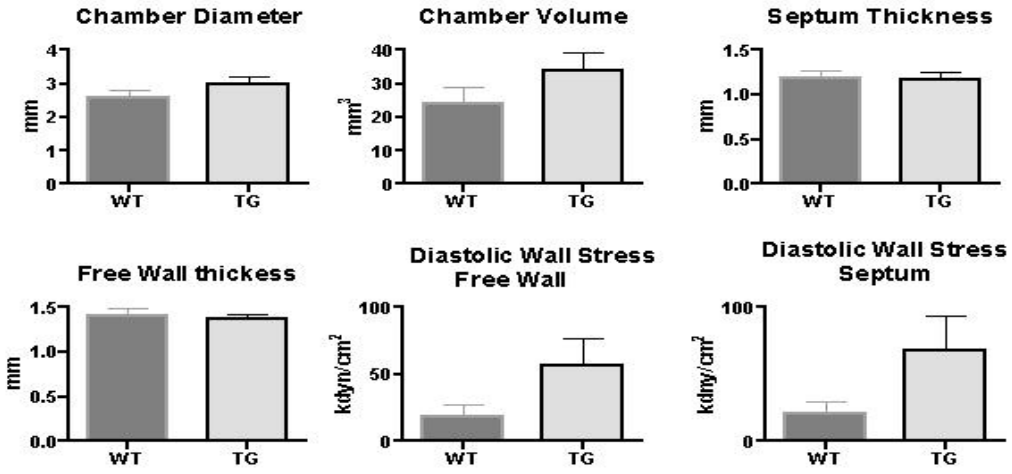


Figure 18. Anatomical analysis. In the graphs are compared the anatomical heart measurement of wild type and ODC transgenic mice. All data are representative of 6 animals for each group.

As shown in Figure 18, the anatomical results indicate that while free wall (FW) and septum thickness are similar comparing wild type to transgenic mice, the chamber diameter and volume of ODC-overexpressing mice are 16% and 41% higher than the same parameters of wild type respectively. Moreover, the diastolic wall stress both of FW and septum of transgenic mice are

three times higher then wild type. An increase in these latter parameters indicates a developed mild cardiac hypertrophy in the ODC-transgenic mice. For evaluating if cardiac hypertrophy is associated to heart failure, hemodynamic parameters were measured before animal death.

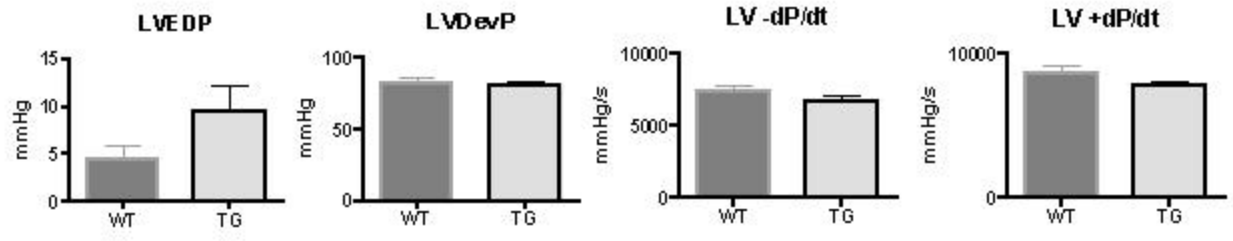


Figure 19. Hemodynamic measurement by Millar's procedure. LVEDP = Left Ventricular End Diastolic Pressure; LVDevP = Left Ventricular Developed Pressure; LV -dP/dT = rate of pressure decay; LV +d*P*/d*t* = rate of pressure rise. All data are representative of 9 animals for each group.

The results indicate that in comparison with wild type mice, the transgenic group exhibited indices of a slight cardiac insufficiency. In fact in ODC mice left ventricular (LV) end-diastolic pressure (LVEDP) was 47% higher, and developed pressure (LVDevP), LV + dP/dt and LV - dP/dt were 6%, 11% and 10% respectively lower than wild type parameters [Fig. 19].

These observations indicate that overexpression of ODC in the heart can be itself sufficient to cause an obvious hypertrophic phenotype that could lead to heart failure.

It is widely agreed that cardiac hypertrophy occurs to preserve normal cardiac function in response to different stimulus as hemodynamic overload, adrenergic stimulus or whatever could lead to heart failure. For many years, the lateral expansion of pre-existing myocytes has been considered to be the only cellular process available for the augmentation of wall width, which tends to normalize the higher peak systolic wall stress. More recently, hyperplasia of muscle cells has been suggested to represent an additional important component of the response of the myocardium in humans [170,171]. Myocyte proliferation has been proposed to characterize the phase of transition from compensated physiological hypertrophy to cardiac dysfunction and overt failure [170].

Thus, considering the notion that myocyte cellular hyperplasia could be a significant component of the growth mechanism of the diseased heart, and considering that polyamines are involved in the

regulation of cell proliferation, I tried to clarify if, in the ODC-overexpressing mice hypertrophy, this mechanism could be involved.

At this purpose I performed immunohistochemical analysis evaluating proliferation markers such as Ki67 and BrdU [Fig. 20]. Ki67 is a protein strictly associated with cell proliferation. The fact that the Ki67 protein is present during all active phases of the cell cycle (G1, S, G2, and mitosis), but is absent from resting cells (G0), makes it an excellent marker for determining the so-called growth fraction of a given cell population. BrdU (bromodeoxyuridine) is a synthetic nucleoside which is an analogue of thymidine. It can be incorporated into the newly synthesized DNA of replicanting cells (during the S phase of the cell cycle), substituting for thymidine during DNA replication. Antibodies specific for BrdU can then be used to detect the incorporated chemical, thus indicating cell that were actively replicating their DNA. At this purpose, I injected all mice used in the experiments with a BrdU solution twice a day for one week.

The immunohistochemical analysis indicates that CMs proliferation is very low both in transgenic and wild type mice and that there is no significant difference between the two compared animal groups [Fig.20].

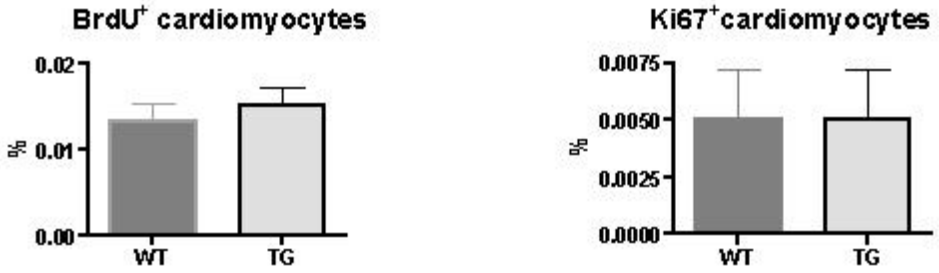


Figure 20. Fluorescent immunohistochemical analysis. Heart sections were stained for proliferation markers such as BrdU and Ki67 using appropriate primary and secondary antibody . The results are rapresentative of the measurement of 20 sections for each animal heart; 6 mice for each group were used. Results are expressed in percent of the total number of cardiomyocytes of the heart section calculated with the following formula: CM/mm^2 * Section area.

Considering that polyamines could regulate programmed cell death inhibiting or favouring cell apoptosis in consequence to different stimuli, I hypothesized that it would be a difference in CMs cell death between wild type and transgenic mice. For evaluating CMs apoptosis I performed a Tunel.

The results show that the cardiomyocyte programmed cell death is very low in both the

experimental group and the difference between wild type and ODC-overexpressing mice is not so high to justify a major number of CMs in the transgenic group [Fig.21].

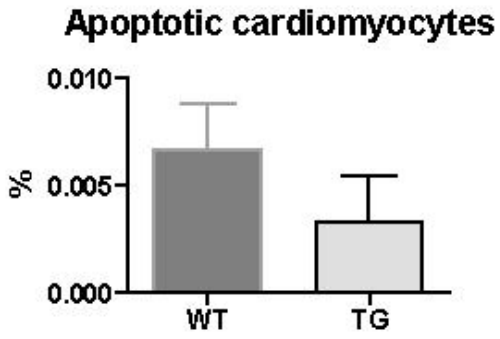


Figure 21. Apoptosis immunohistochemical analysis. Heart sections were used to perfome a Tunel test using the Clonech-ApoAlert DNA Fragmentation Assay Kit visualized by fluorescent microscopy. The results are rapresentative of the measurement of 20 sections for each animal heart; 6 mice for each group were used. The results are expressed in percent of the total number of cardiomyocytes of the heart section calculated with the following formula: CM/mm^2 * Section area.

Thus, all the results collected until now indicate that the cardiac hypertrophy manifested in the ODC-transgenic mice is not a consequence of a cardiomyocytes hyperplasia as I hypothesized, but it is much probably due to an increase in cardiomyocytes size (CMs hypertrophy).

To confirm this new hypothesis I measured cardiomyocytes density and the total number of cardiomyocytes both in wild type and transgenic mice hearts. As shown in the graphs below [Fig. 22], both cardiomyocytes density and total number of cardiomyocytes are lower in ODC-overexpressing mice than in wild type. Thus, these results confirm that in our animal model the manifested cardiac hypertrophy is not dependent by an increase of cardiomyocytes number but by an increase in their cell size. Thus, ODC overexpression at cardiac level is responsible of cardiomyocytes hypertrophy but not of cardiomyocytes hyperplasia.

Considering that all the mice used in these experiments are young mice (two-months old mice) and considering that the transgenic type manifests cardiac hypertrophy and heart failure, we could conclude that the overexpression of ODC protein within the heart tissue might lead to a precocious heart aging. Of course more experiments are necessary to confirm this hypothesis and to understand the right mechanism by which a high level of polyamines induces this cardio- pathological phenotype.

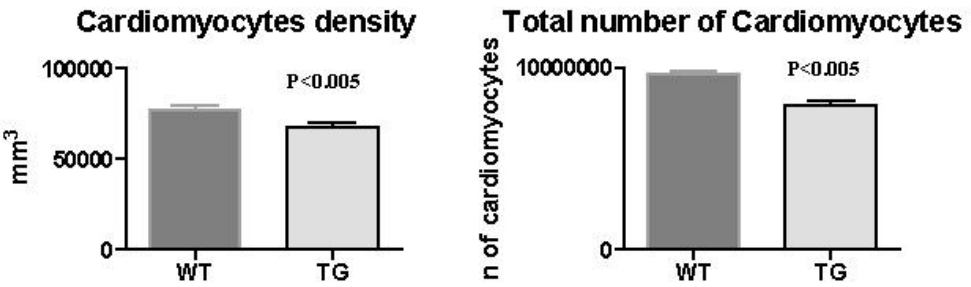


Figure 22. Cardiomyocytes density and total number of cardiomyocytes. Cardiomyocytes density was calculated using the following formula: CMs DENSITY in mm^3 = myocytes in mm^2/nuclear length *1000; the total number of cardiomyocytes was calculated with the following formula: CMs Density*Heart Volume. All the data are representative of 6 animals for each group. Two-tailed Student's T test , unpaired data was performed as statistical analysis ($p < 0.05$)

The recently discovery of resident cardiac stem cells (CSCs) introduced a new concept according to which the myocardial aging could be a stem cell problem [172]. Aging could affect the growth and differentiation potential of CSCs interfering not only with their ability to sustain physiological cell turnover but also with their capacity to adapt to an incoming heart failure.

In accordance to this notion and considering that polyamines regulate cell cycle and cell proliferation, I investigate if the ODC overexpression and the consequent increased in polyamines level within the heart could affect the proliferation (cell number) of resident cardiac stem cell identified as $ckit^+$ cells.

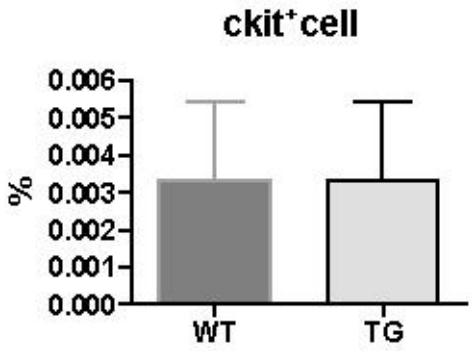


Figure 23. Fluorescent immunohistochemical analysis. Heart sections were stained for ckit using an anti CD117 primary antibody and an appropriate FITC conjugate secondary antibody. The results are rapresentative of the measurement of 20 sections for each animal heart; 6 mice for each group were used. Results are expressed in percent of the total number of cardiomyocytes of the heart section calculated with the following formula: CM/mm^2 * Section area.

The immunohistochemical analysis, reported in the Figure 23, shows that there is no evidence of a difference in the number of resident ckit$^+$ cardiac stem cells comparing ODC-transgenic mice to the wild type. Thus, this indicates that the high level of polyamines doesn't effect the proliferation of the ckit population of resident cardiac stem cells. Anyway, this result doesn't exclude that high level of polyamines could affect ckit-CSCs differentiation or the proliferation and/or differentiation of the other populations of resident CSCs, such as Sca-1$^+$, Abcg2$^+$, and isl1$^+$ cardiac stem cells.

CONCLUSION

In the present study I characterized the cardiac phenotype of young ODC-transgenic mice overexpressing ODC at cardiac level. By anatomical and functional analysis emerged that the transgenic mice manifested a mild cardiac hypertrophy correlated to a slight cardiac insufficiency [Fig. 18, 19]. I tried to clarify if the high level of polyamines, consequent to the increased ODC protein expression within the heart, was correlated to this pathological phenotype considering the important role of these polycations in regulating cell proliferation and cell cycle. The results indicate that the manifested transgenic-mice hypertrophy was due to a simply increase in cell size and not to a cardiomyocytes proliferation [Fig. 20, 21, 22]. This phenomenon could be justified as a precocious aging process of the myocardial tissue induced by polyamines. Because of the new concept according to which myocardial aging is a stem cell problem, I evaluated if the number of ckit$^+$-cardiac stem cell was affected by the increased in polyamines concentration. According to the result [Fig.23], it can be excluded that ODC-overexpression at cardiac level influences the ckit-CSCs number and proliferation, but it cannot be excluded that an increase in polyamines level could affect the proliferation or differentiation of Sca-1$^+$, Abcg2$^+$, and isl1$^+$ cardiac stem cells population.

Thus, more experiments have to be performed to elucidate the relationship that could exist between polyamines and cardiac hypertrophy.

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