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CARDIAC STEM CELLS AND BIOMATERIALS: INDUCTION OF MYOGENIC DIFFERENTIATION AND IMPLANTION OF BIOSYNTHETIC AND NATURAL MATRICES IN THE ADULT HEART

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Introduction

1.The anatomy of heart

The heart is a muscular pump that serves two functions: (1) collect blood from the tissues of the body and pump it in the lungs and (2) collect blood from the lungs and pump it in all tissues of the body. The human heart is located in the thorax,protected posterior to the sternum and costal cartilages, rests on the superior surface of the diaphragm, assuming an oblique position in the thorax, with two-thirds of the left of midline. It occupies a space between the pleural cavities called the middle mediastinum, defined as the space inside the pericardium, covering around the heart. This serous membrane has a inner and outer layers, with a lubricating fluid in between. The fluid allows the inner visceral pericardium to “glide” against the outer parietal pericardium.

The internal anatomy of the heart reveals four chambers composed of cardiac muscle or myocardium. The two upper chambers (or atria) function mainly as collecting chambers; the two lower chambers (ventricles) are much stronger with the function to pump blood in system circulation. The role of the right atrium and ventricle is to collect blood from the body and pump it to the lungs. The role of the left atrium and ventricle is to collect blood from the lungs and pump it throughout the body. There is a one-way flow of blood through the heart; this flow is maintained by a set of four valves. The atrioventricular valves (tricuspid and bicuspid) allow blood to flow only from atria to ventricles. The semilunar valves (pulmonary and semilunar) allow blood to flow from the ventricles out of the heart and through the great arteries [1]

The division of the heart into four chambers produces boundaries that are visible externally as grooves (sulci). The interatrial groove is a shallow groove separating the two atria. The lateral limits are defined by the borders of the atria. The atrioventricular (coronary) groove (or sulcus) separates the atria from the ventricles. This groove, containing the main trunks of the coronary arteries, is oblique. It descends to the right on the sternocostal surface, separating the right atrium (and its auricle or appendage) from the oblique right margin of the right ventricle and its infundibulum. Its upper left part is obliterated where it is crossed by the pulmonary trunk and, behind this, the aorta, from which the coronary arteries originate. Continuing to the left, the groove curves around the obtuse margin and descends to the right, separating the atrial base from the diaphragmatic surface of the ventricles.

Internally, the ventricles are separated by the septum. The mural margins of the septum correspond to the anterior and inferior (diaphragmatic) interventricular grooves. The anterior groove, seen on the sternocostal cardiac surface, is near and almost parallel to the left ventricular obtuse margin. On the diaphragmatic surface, the groove is closer to the midpoint of the ventricular mass. The

interventricular grooves extend from the atrioventricular groove to the apical notch on the acute margin, which is a little to the right of the true cardiac apex.

A cross section cut through the heart reveals three layers: a superficial visceral pericardium or epicardium; (2) a middle myocardium; and (3) a deep lining called the “endocardium”. The endocardium is a sheet of epithelium called endothelium that rests on a thin layer of connective tissuebasement membrane. It lines the heart chambers and makes up the valves of the heart.

The myocardium is the tissue of the heart wall and the layer that actually contracts. The myocardium consists of cardiac muscles willing in a spiral arrangement of myocardium that squeezes blood through the heart in the proper directions (inferiorly through the atria and superiorly through the ventricles). Unlike all other types of muscle cells, cardiac muscle cells: (1) branch, (2) join at complex junctions called intercalated discs so that they form cellular networks, and (3) each contain single, centrally located nuclei. A cardiac muscle cell is not called fiber because the term is used for a long row of joined cardiac muscle cells [2].

1.1 *Cardiac microstructure*

In cardiac muscle, as in skeletal muscle, the contractile proteins are organized structurally into sarcomeres which are aligned in register across the fibres, producing fine cross-striations that are visible in the light microscope. They both contain the same contractile proteins (although many are cardiac isoforms), which are assembled in a similar way, and the molecular basis for contraction, but not its regulation, is the same. The release of calcium into the sarcoplasm triggers contraction, which corresponds to cardiac systole, the pumping phase of the heart cycle. Reuptake of calcium produces relaxation, which corresponds to cardiac diastole, the filling phase of the cycle.

The myocardium, the muscular component of the heart. Each cell has one or two large nuclei, occupying the central part of the cell, whereas skeletal muscle has multiple, peripherally placed nuclei. The cells are branched at their ends, and the branches of adjacent cells are so tightly associated that the light microscopic appearance is of a network of branching and anastomosing fibres. Cells are bound together by elaborate junctional complexes, the intercalated discs .

Fibrocollagenous connective tissue is between cardiac muscle fibres, equivalent to the endomysium of skeletal muscle. Numerous capillaries and some nerve fibres are found within this layer. Connective tissue, equivalent to the perimysium of skeletal muscle, separates the larger bundles of muscle fibres, developed near the condensations of dense fibrous connective tissue that form the ‘skeleton’ of the heart.

Longitudinal section about cardiac muscle cells show that the myofibrils are separate before they pass around the nucleus, leaving a zone that is occupied by organelles, including sarcoplasmic reticulum, golgi complex, mitochondria, lipid droplets, and glycogen.

The cross-striations of cardiac muscle are less conspicuous than those of skeletal muscle. This is because the contractile apparatus of cardiac muscle lies within a mitochondria-rich sarcoplasm but the proportion of the cell volume occupied by mitochondria (approximately 35%) is even greater in cardiac muscle skeletal muscle. The high demand for oxygen is also reflected in high levels of myoglobin and an exceptionally rich network of capillaries around the fibres.

The force of contraction is transferred through the ends of the cardiac muscle cells via the junctional strength provided by the intercalated discs. As in skeletal muscle, the force is transmitted laterally to the sarcolemma through the extracellular matrix via vinculin-containing elements, bridge between the z-discs of peripheral myofibrils and the plasma membrane.

The sarcolemma of ventricular cardiac muscle cells invaginates to form t-tubules with a wider lumen than those of skeletal muscle; atrial muscle cells have few or no t-tubules. Unlike skeletal muscle, most t-tubules penetrate the sarcoplasm at the level of the z-discs . The t-tubules are interconnected at intervals by longitudinal branches to form a complex network. They probably serve a similar function in skeletal and cardiac muscle, i.e. To carry the wave of depolarization into the core of the cells. The actin-binding proteins, spectrin and dystrophin, are important components of the cardiac muscle cell cytoskeleton, which associate independently with the sarcolemma to provide mechanical support.

Sarcoplasmic reticulum which bears junctional processes has been termed junctional sarcoplasmic reticulum, to distinguish it from the free sarcoplasmic reticulum, which forms a longitudinal network. Reticulum junctional sarcoplasmic makes contact with both the t-tubules and the sarcolemma (of which the t-tubules are an extension). Sarcoplasmic reticulum forms small globular extensions (corbular sarcoplasmic reticulum) in the vicinity of the z-discs, but not in immediate relation to t-tubules or the sarcolemma.

1.2 *Intercalated discs*

Intercalated discs are unique in cardiac muscle. In the light microscope they are seen as transverse lines crossing tracts of the cardiac cells, they have step irregularly within or between adjacent tracts. At the ultrastructural level these structures, which are complex junctions between the cardiac muscle cells, are seen to have transverse and lateral portions. The transverse portions occur wherever myofibrils abut the end of the cell, and each takes the place of the last z-disc. At this point, the actin filaments of the terminal sarcomere insert into a dense subsarcolemmal matrix

which anchors them, together with other cytoplasmic elements such as intermediate filaments, to the plasma membrane. Prominent desmosomes, often with a dense line in the intercellular space, occur at intervals along each transverse portion. This junctional region is homologous with, and probably similar in composition to, the structure found on the cytoplasmic face of the myotendinous junction, and is a type of fascia adherens junction. It provides the connection between cells for transmission of contractile force from one cell to the next.

The lateral portions of the intercalated disc runs parallel to myofilaments, long axis of the cell, for a distance which corresponds to one or two sarcomeres, it turning again to form another transverse portion. The lateral portions contain gap junctions, which are responsible for the electrical coupling between adjacent cells. Conductance channels within these junctions enable the electrical impulse to propagate from one cell to the next, spreading excitation and contraction rapidly along the branching tracts of interconnected cells. In this way the activity of the individual cells of the heart is coordinated so that they function as if they were a syncytium [2].

2. Heart development

The earliest progenitors of the heart are located in cardiogenic fields in the splanchnic layer of anterior lateral of mesoderm plate. The first morphological sign of heart development begins when two bilateral troughs of of myocardium called endocardial tubes are brought to the ventral midline during closure of the ventral foregut. The borders of cardiogenic fields form myocardial troughs that are brought into apposition to fusing at the ventral midline, forming the future outer curvature. After fusion of the endocardial tubes that the arms of the w-shaped myocardium fuse just beneath the foregut form the dorsal midline seam of myocardium that will become the inner curvature and dorsal mesocardium, thus creating the primary cardiac tube. In this process, the myocardium secretes thick acellular matrix called cardiac jelly that forms a layer separating the myocardium and the endocardium [3]. Because the heart begins to beat while the myocardium is still a tube, the functional development of the myocardium is a critical step. This involves development of appropriate channels to allow electrical currents to be propagated through the myocardium himself.

Signals from to the endoderm anterior (cranial) induce a heart region forming region, overlying the splanchnic mesoderm and they inducing the transcription factor NKX2.5. The signals require secretion of BMPS 2 and 4 secreted by the endoderm and lateral plate mesoderm. Concomitantly, the activity of WNT protein, secreted by the neural tube, must be blocked because they normally inhibit heart development. Inhibitors (crescent and cerberus) of the WNT proteins are produced by

endoderm cells immediately adjacent to heart forming mesoderm in the anterior half of the embryo. The combination of bone morphogenetic protein (BMP) activity and WNT inhibition by crescent and cerberus causes expression of NKX2.5, the master gene for heart development. Bmp expression also upregulates expression of FGF8 that is important for the expression of cardiac specific proteins. NKX2.5 contains a homedomain and a homologue of the gene tinman that regulates heart development in drosophila. Tbx5 is another transcription factor that contains a dna-binding motif known as T-box. The phase of cardiac looping is dependent upon several factors, including the laterality pathway and expression of the transcription factor PIZX2 in the lateral plate mesoderm on the left side. PIZX2 may play a role in the deposition and function of extracellular matrix molecules that control looping. In addition, NKX2.5 upregulates the expression of HAND1 and HAND2, transcription factors expressed in the primitive heart tube. Downstream effectors of these genes participate in looping cardiac. Hand1 and Hand2, regulated by NKX2.5, also contribute to expansion and differentiation of the ventricles [4]. Most of cells that will differentiate in the developing myocardium are derived from a population of progenitor cells located in the pharyngeal mesoderm, termed the secondary heart field (SHF). Cells belonging to the pre-cardiac mesoderm differentiate into early cardiac progenitors which can in turn to differentiate in primitive cardiomyocytes, smooth muscle cells and endothelial cells. Known markers such as pre-cardiac cells of the primary and the secondary heart field are NKX2.5 and GATA-4. The expression of these two transcription factors is noted common in the LIM HOMEOBOX transcription factor positive (Isl-1+) of cardiac progenitor cells (CSC) in the developing pharyngeal mesoderm as well as fetal and post-natal mice hearts. This small number of isl-1+ cells has been identified as a residue of migrating SHF cells and considered as resident progenitor cells in the myocardium in post-natal age [5,6].

3. The stem cell

Stem cells have generated more public and professional interest than other topic in biology. Stem cells attract the attention of many because understanding their unique capacity may provide a path toward therapy for a variety of degenerative diseases. Although studies about stem cell biology are several, there is still a conflicting options on the nature of stem cells. This confusion can be partly attributed to the sometimes idiosyncratic terms and definitions used to describe stem cells [7].

The first work carried out in the early 60s by Till and McCullough [8] on hematopoietic stem cells of mice have laid the bases for all subsequent strategies stem cell research. This work, in fact, pointed to the conceptual paradigms that still makes use of the scientific community to design research and clinical applications.

Stem cells are defined functionally as cells that have the power to self-renew as well as the capacity to generate differentiated cells[9]. Particularly, stem cells can produce daughter cells identical to their mother (self-renewal) as well as generate progeny with more restricted potential (differentiated cells). This simplistic and broad definition may be satisfactory for embryonic or fetal stem cells that don't endure for the lifetime of an organism [10].

In recent years, several models have been proposed, which clarify the behavior of the stem cell :

1) the stem state model

2) the phase space model

3) the chiaroscuro stem cell model[11]

In the stem state model the cell enters a state stem of the reversible type that could bring a result variation of the concept of stem cell, implying that stemness would be a state rather than cell entity : from cellular entity to a true state [12]. In the phase space model is developed starting from the analysis that in stem cell biology, " a principle, still unclear, where it isn't possible to measure the differentiation capabilities and the proliferative ability of a cell, since the evaluation of these properties would result in the irreversible alteration of the other [13].

An effect of this alternative model, which input its principles on continuous variables, is that the outcome of the division of stem cell must be described by a probability function, rather than a discrete probability distribution. Therefore, the cell that is generated by the division of a stem cell may have more skills and attitudes stem than the mother cell (de-differentiation). Morover, in this model, the concept of self-renewal is replaced by the concept of renewal of the population, while the plasticity of stem cell isn't only possible but it is a fundamental trait of stem cell .

In the model chiaroscuro there isn't a classification in "progenitor/stem cell", but rather a reversible continuum. This may depend on the variability of chromatin and various genes expressed during the

cell cycle. Following this pattern, the imput of a primitive cell in the cell cycle will match to a variation of the expression of adhesion molecules, cell surface antigens, cytokine receptors, and a set of variations that cause the possible modulation of complex functions, such as the ability to form progenitors, the homing and engraftment in tissues [14]. Then have been analyzed the ability of these immature precursor cells that can potentially differentiate in a multilineage, where stem cell plasticity, however, is believed to be a "not proven properties that stem cell tissue possesses, able increase its power in response to physiological demands or insults. According the stem cells differentiation capabilities, are classified as:

- Totipotent (able to generate all the cells and tissues that organize the embryo including extraembryonic membranes such as the placenta and umbilical). Generically embryonic cells retain this property up to 4-5 days from the composition of the zygote ;
- Pluripotent (originators of the cells of the three germ layers) ;
- Multipotent (generate cells that are derived from the same germ layer) ;
- Tissue-specific (retain the ability to originate own cells of the tissue in which they reside)

Therefore, the difference between totipotency and pluripotency is the ability to form an entire organism *in vivo*, while germline competence is the main difference between toti/pluripotent cells and multipotent cells. Consequently, if we adhere to this definition, embryonic stem cells (ESC) are pluripotent, while fetal and ASCs can be considered, with the exception of spermatogonial stem cells multipotent [15]. The ESCs could be a possible source of cells with infinite capacity, self-healing, which can in turn give rise to all the somatic cells and germ cells of an organism but are still significant obstacles to ethical and biological agents in the use of stem cells embryonic that led to the search for other possible sources of undifferentiated cells defined by their abilities to self renew and differentiate into mature cells. Stem cells found in fully developed tissues are defined as Adult Stem Cells (ASC). The function of ASCs is the maintenance of adult tissue specificity by homeostatic cell replacement and tissue regeneration [16]. ASCs are presumed quiescent within adult tissues, but divide infrequently to generate a stem cell clone and a transiently-amplifying cell. The transiently-amplifying cells will undergo a limited number of cell divisions before terminal differentiation into mature functional tissue cells. The existence of ASCs has been reported in multiple organs; these include: brain, heart, skin, intestine, testis, muscle and blood, among others [17]. Effective functioning of the body’s tissues and organs depends upon innate regenerative processes that maintain proper cell numbers (homeostasis) and replace damaged cells after injury (repair). In many though not all tissues, regenerative potential is determined by the presence and functionality of a dedicated population of stem and progenitor cells, which respond to exogenous cues to produce replacement cells when needed [18].

4. Adult stem cell and stem cell therapy

In order that the ideal customized stem cell therapy to be efficacious have to be realized four terms. First, stem cells used in these therapies have to be genetically stable and epigenetically controlled to ensure the safety of stem cells used in any future therapies. This is indispensable to ensure that patients undergoing stem cell therapy are not exposed to greater risks of tumorigenesis and other mutagenic illnesses. Second, stem cells should be capable to elude the innate immune response of patients, perhaps through the secretion of immunosuppressive molecules that impede immune responses or by showing host cellular recognition markers. The attendance of transplanted stem cells is essential for the strategy of an effective therapy. Moreover the capacity of transplanted stem cells to avoid immune detection and inflammatory responses will prevent undesired symptoms such as graft-versus-host-disease in patients. Third, stem cells employed in these treatments should be location specific. These stem cells should have specific cell surface markers, where the homing will consent them to locate and migrate to specific localities [19]. Over the last years proofs on the probable presence of stem cell plasticity have been showed [16]. Numerous mechanisms, responsible for this property, have been suggested, among others, cell fusion [20], the persistence of usually multipotent cells in adult tissues, and the aptitude of ASCs to de-differentiate, obtaining higher degrees of stemness [12, 13]. Considerate these latter two premises, scientists have analyzed whether adult mammalian tissues could host multipotent cells whose differentiation potential was not delimited to the germ layer of their origin. Molecular signals for pluripotency, genetic reprogramming of ASCs, and widely multipotent [11]. Recent studies in the literature indicates the possibility of obtaining stem cells from sources of different origin, accordingly it has been possible to study their different capacity to give rise to different cell types using as a starting point a multicellular organism. So the study of Adult Stem Cells (ASC) originates from different types of tissue such as the bone marrow, the dental pulp, neural progenitor cells, the blood that flows from the umbilical cord, the amniotic fluid cells, etc. Nevertheless, the ability to undergo a differentiation process of an adult cell is only partial, and generally can only form only types of cell similar to the progeny of origin of the tissue where it originated (multipotent stem cells). A long time of ASCs have been widely used becoming a viable alternative in the context of uses in the therapy with the use of cells, several preclinical studies have pointed out that the use of undifferentiated precursor cells has shown encouraging results for as regards the type of bone marrow transplant autologous or increase in cardiac function after myocardial infarction and chronic coronary artery disease [21]. In animal models of the Thomas Moon group and in the case of spinal cord injury, the use of ASCs have provided a substantial improvement of the capacities of sensorimotor type [22]. Yet, in most

cases, the positive outcomes have subjected a serious risks, such as showed in other laboratories, which is given by the protocols able to provide positive results but are not may be included completely in order to be provide a result only one capable of being used in clinical practice. In addition, the functional recovery could be attributed, in some situations, to events of cell fusion or following release of paracrine factors that may intervene in the signaling between adjacent cells to support so the start of differentiation processes [23]or inhibition of apoptosis [24]. In some cases, highlighted by the work of Thomas of 2011 has been documented that the possible functional recovery could also be due to phenomena which affect the survival and differentiation of transplanted cells [22]. Several clinical studies have employed the ASCs in various different types of pathological processes such as the treatment of heart disease[25], in diabetes [26], lesions of the spinal cord [27], in disorders affecting the nervous system [28], in diseases of the liver and intestinal [29], lesions of the retina and neuromuscular dystrophies [30]. In this studies, mentioned above, the cells whose origin is unipotent and tissue-specific, originate from different sources depending on the condition treated, they were noticed evident, but, reduced improvements of health conditions that once again, raising many doubts on positive results. The results obtained could have been attributed to both the differential properties of the injected cells, in addition to the effects of anti-inflammatory certainly not secondary, that to paracrine action are able to reform a specific network that takes active part in the healing process [31]. In contrast, human ESCs derived from the inner cell mass (ICM) capable of developing in the embryonic stage starting from the blastocyst stage, which are able to expand indefinitely *in vitro* cultures, where they show in an indefinite manner the undifferentiated state. It was highlighted by most research groups their ability, in specific culture conditions to differentiate in cell types representative of the three germ layers: ectoderm, mesoderm, and endoderm. However, a characteristic emerged in various works, during and subsequent to the use of ESCs is their ability to give rise to teratomas when they are injected into immunocompromised mice, the ability to create masses abnormal cell [32]. The difference between ASCs and ESCs is due the first cell represent a source with further development capacity but especially as regard their use in cell therapy where their differentiation potential is certainly much higher. As early as 1988, with the work carried out by Thomson, has gradually increased the interest addressed to the embryonic cell lines due largely to their number that registered has seen a rapid increase, and more than 800 different ESCs human lines are now available at present [33]. Contrary to their differentiation potential, the use of human ESC research and cell therapy has been controversial because of various implications such as of a political, religious and ethical issues regarding the use of human embryos. In fact, much of the public opinion always deployed against research on ESCs largely due the use of preimplantation embryos should have the same right to life

as any person, regardless of whether we are talking about crops *in vitro* or during gestation. But now the discussion about the origin and the ethical use of human embryos was mostly passed in permissive countries such as China and Belgium, where embryos created in clinical practice of *in vitro* fertilization, are used for the study and the development of human ESCs, thanks to the informed consent of the parents [34]. The most important problem concerning the use hESC-based therapy is related to its clinical safety, more specifically, the intrinsic potential of pluripotent cells to originate teratomas or teratocarcinomas when injected into the patient [35]. But as explained above, several recent studies have pointed out an unexpected development potential of ASCs tissue-specific. In fact, most work on the activity of multipotent stem cells in adult tissues have raised many hopes, these could represent an alternative source and more acceptable from the standpoint of regeneration. Even here there remain many questions about the nature and status of stem cells, both *in vivo* and *in vitro*, and their ability of proliferation and differentiation. ASCs represent one of the first successes of the experimental therapy with human stem cells. There are several reasons why ASCs collect a greater number of consents within the framework of cellular therapies compared to immortal cell lines or embryonic pluripotent cells. The therapies with ASCs are often of autologous origin with a risk for a possible immune rejection that is reduced to a minimum. The most of the studies carried out, the use of ASC imply that have undergone a number of probable genetic mutations minimum with a reduced chance of chromosomal aberration. ASCs were seen also in large tumors when tested *in vivo*. Finally, in the last years,the use of ASCs in therapy isn't considered controversial since they can be easily extracted from the tissues of the patient and don't require the destruction of embryos, these advantages of ASCs have led to their wider use in research and in various clinical settings [19]. For example, the epidermis is a tissue capable to rapid and persistent self-renewal. Indeed when it is exposed to UV radiation and other chemicals, start a mechanism of self-renewal. The latter may take place thanks to the presence of the cellular fraction in active development but unipotent. The epidermis have a single inner layer (basal) of dividing cells, which cyclically come out from the replicative cycle, terminally differentiate and migrate towards the outside, on the skin surface [36]. In response to injury, the stem cells of edematous surrounding regions are activated, and these leave these areas, migrate and contribute to repopulation of the epidermis. In addition, even the cells derived from bone marrow contribute to the regeneration epidermic and differentiate in keratinocytes [37]. The bone marrow serves as a reservoir for different types of stem cells, including hematopoietic stem cells (HSC), endothelial progenitor cells (EPC) and mesenchymal stem/stromal cells (MSC)[38]. MSCs are one of the first attempts of adult multipotent cells to use in stem cell therapies. These stem cells have the ability to differentiate into various types of tissue such as bone, cartilage, and adipose tissues. While the bone

marrow is the most common source of MSCs for clinical use, also can be found in various areas including adipose tissue and synovial fluid, skeletal muscle, peripheral blood, umbilical cord, etc. [39]. The mesenchymal stem cells indicated a heterogeneous population of cells that showing differences in proliferative capacity, cell markers, and morphological aspects. For example, bone marrow-derived MSC (M-MSC) have a lower proliferative capacity, compared to the mesenchymal stem cells of adipose tissue (A-MSC) and from umbilical cord blood (U-MSC), which have the highest proliferative capacity [40]. MSC also have different differentiation potentials. For example, the bone marrow MSCs have a higher potential chondrogenic while the adipose tissue MSC have a low potential chondrogenic [41]. Moreover, both the bone marrow MSC and adipose cells form phenotypes adipogenic unlike cord blood that show a lesser ability to form adipocytes [42]. MSC also express different cell markers. For example, A-MSCs express CD34, CD49d, CD54, while M-U-MSC and MSC show higher levels of CD106 [43]. M-MSC and A-MSC also have higher levels of CD90 and CD105 expression compared to U-MSC [40]. The morphology of the mesenchymal stem cells may also differ significantly, in the literature are indicated various morphological characteristic such as, spindle shaped, round, fibroblastoid cells, flattened cells, and blanket cells [44]. Further studies to understand the differences in various subpopulations of mesenchymal stem cells could lead to a better understanding about epigenetic differences that regulate stem cells and their fate differentiation, homing to specific recognition sites, the rates of proliferation and senescence. M-MSC are currently being used in clinical trials and therapies. In fact, one of the main reasons to the success of therapy with the use of M-MSC is due to their intrinsic immunomodulatory properties that allow to inhibit and evade the danger of a possible immune rejection when transplanted [45]. M-MSC are able to inhibit the maturation and function of various immune populations such as dendritic cells, natural killer cells, B and T lymphocytes [46]. Furthermore, the M-MSCs have a low immunogenicity due to the reduced surface expression of MHC proteins II and the lack of stimulatory proteins such as CD80 and CD86 [47]. Another important reason of the initial success of therapies based on M-MSC is these cells have low oncogenic potential and therefore a low risk of forming tumors [47]. Moreover, the presence of ASCs within specific niches is important to the possibility of maintaining a sufficient communication and active within the environment which ensures a high reactivity in the activities of cell turnover and repair following to damage. In many tissues, the balance between protection and interaction factors appears to be crucial to maintain ASCs in a specialized microenvironment, or niche, which provides mutual influence from the point of view of time and space to support and coordinate the activities in which they are used [18].

5. Pathology of acute myocardial infarction: The traditional view

The tradition vision of myocardial infarction is characterized an initial phase in which the cardiomyocytes undergo apoptosis starting from the innermost layers of the endocardium. When the blood supply doesn't occur in the first 6 hours, the result that follows is necrotic site around to the occluded artery. The cardiac tissue loss starts a articulated process which has the purpose of repairing the damaged tissue and preserve the structural integrity. Different chemio-attracted factors arrive in the infarcted area and they work in the stage of tissue repair. Inflammatory cells in the lesion area quickly remove necrotic and apoptotic cardiomyocytes, release active mediators that lead to synthesis of new blood vessels that begin to sprinkle the infarcted area [49].This initial inflammatory phase is usually followed by a phase in which the fibroblasts come to border area and slowly invade the infarct region, give rise to the scar tissue. Instead, after the infarct the cardiomyocytes that remain functionals, to compensate for the loss of contractile tissue, become hypertrofic. In humans, the healing process lasts six to eight weeks, with progressive changes in ventricular size. However, the left ventricle remodeling and a large part of the heart tissue was believed to be irreversible, since the heart was been considered a postmitotic organ without any capacity for self- renewal. However, recent evidence on cardiomyocytes overtun this conception [50], in particular the discovery of stem cells tissue-specific resident in the adult heart in active phase of cellular cycle [51]. Indeed more papers indicate a possible clinical approach [52], rejecting the classic view classic where the adult heart is a postmitotic organ, suggesting an alternative therapeutic about myocardial infarct that supporte the heart regeneration and cell turnover .

6. Tissue regeneration

Stem and progenitor cells have been identified in most organs including, skeletal muscle, liver, lung, skin, kidney, blood, bones, reproductive tissues, intestines and heart. The function of these cells seems to be tied to a cellular homeostasis, in the phases of cellular turnover of or due to damage and replace so insure normal and correct functions of the organ. Some progenitor cells also can renewing the tissue after minor injury, or even after a major damage or removal. Relevant examples are given by regeneration in the blood and in the liver. Indeed, hematopoietic stem cells transplanted have a high ability to repopulate the hematopoietic system of an irradiated mice, and

the liver is able to renew its mass within two weeks after the removal of two-thirds of the whole liver tissue[53,54]. In the other hand, not all organs have the same degree of regeneration. The heart and the central nervous system are particularly resistant to regeneration after injury, for example, the brain, has a reduced propensity to regenerate in the case of neurodegenerative disease or stroke. For hundreds of years, biologists have been fascinated and inspired by the spectacular regenerative capabilities of different species. Some animal models of invertebrates such as Hydra and planaria have an unlimited capacity of regeneration, able to recreate a full head and a tail from a small piece of tissue [55]. Lizards, frogs and fish have been employed as models for vertebrate regeneration; yet salamander research in the work of Spallanzani in 1768 who was the first that indicate the ability of salamanders to regenerate an entire limb amputated [56].

6.1 *Understanding heart regeneration in animal models: from adult to neonatal regeneration*

The study of the natural regenerative capacity of the heart tissue of amphibians has been examined in frogs, newts and axolotl, carried out as early as 1960 by leading Soviet scientists. The first data obtained, have shown that amphibians survive after an extensive and severe damage to the ventricle, and further the removal of approximately one quarter of the ventricular portion. This resistance likely due to a lower degree of dependence of the blood circulation compared to mammals [57]. The scientific popularity of the zebrafish model has encouraged the research of its regenerative capacity. Zebrafish seven fins that can regenerate also after an amputation of the tissue until 95%. The caudal fin is often used in regeneration experiments, and completes regeneration in 10-14 days. The process of fin regeneration is epimorphic, involving the formation and proliferation of an undifferentiated multipotent cells [58]. The simple structure of the fin, its external accessibility and the fact that his amputation doesn’t cause significant changes in lifestyle and the main reasons for its success as a organ model. Many groups have focused in understanding the events underlying the regeneration of zebrafish fin, with the aim of exploiting the tools and techniques developed by embryologists to apply them to problems and fundamental questions which regulate in particular the activation of regenerative properties of cardiac tissue in mammals and specifically in humans.

6.2 *The Amphibian Heart*

Unlike the mammalian heart, the heart of zebrafish form a reduced scar tissue and that compensate with a high regenerative response. Different important work suggests that after amputation of 20 %

of the apex of the ventricle, after the formation of fibrin clots, follows an intense regenerative activity that recreates the lost heart muscle in a period of 30 to 60 days [59] thus confirming the idea that implies a migration of cardiomyocytes through the heart [60]. One of the cellular pathways involved in cycle cell reactivation is definitely Notch that seems crucial for the reactivation of these cellular phases which remains inactive until activation of important pathways for the formation of the heart [59]. Scientific networks have questioned the possible origin of newly synthesized cardiomyocytes in animal models of zebrafish, currently there are many papers in the literature that indicate that the new muscle doesn't come from differentiation processes of stem cells. Others work group suggest that there is a reactivation of the cell cycle in adult cardiomyocytes undergoing dedifferentiation and subsequent proliferation, such as demonstrated by several laboratories [61]. During genetic testing before mentioned, cardiomyocytes that dedifferentiate and that proliferate, they are characterized by re-expression of progenitor cardiac marker such as GATA4, by disassembly of the sarcomeres, and by formation of a mesenchymal-like phenotype infiltrate [60]. When the cardiac function is rehabilitated in zebrafish, it was seen that cardiomyocytes return to a state of quiescence after the migration in their site of origin. Not surprise that regenerative response involves a considerable amount of cardiomyocytes in active proliferative phase. Other groups show a basic level, when the zebrafish cardiomyocytes show a higher degree of activity of the cell cycle compared with their mammalian counterparts. In fact, one study showed that about 3% of cardiomyocytes, in the myocardium of adult hearts undamaged of Zebrafish, are able to absorb thymidine analogue bromodeoxyuridine (BrdU) during a seven-day pulse-labeling experiment.Two weeks after the amputation of cardiac apex, the fraction of BrdU-positive cardiomyocytes was increased tenfold, and this parameter remains to 20% even after 30 days from amputation [62]. Initial experiments have suggested that undifferentiated progenitor cells were the main source regenerative in zebrafish cardiomyocyte, although lately other experimental data with genetic fate-mapping experiments have shown that cardiac regeneration starts from pre-existing cardiomyocytes. The research on regenerative properties has been consistent over the last few years in zebrafish, but didn't clarify whether the processes as seen in amphibians are also possible in mammalian cardiomyocytes. In fact, in experiments carried out by Porrello and colleagues in 2011[63], it has been observed a very similar regenerative response to that observed in Zebrafish in the hearts of neonatal mice. The rats subjected to 10% -15% to amputation of ventricle were able to elicit a regenerative response in the first days of life (up to 7 days). Also here, the synthesis of new cardiomyocytes from cardiomyocytes pre-existing, such as shown by genetic approaches lineage-tracing with genes Cre/lox, is involved in a complete cardiac activity with recovery after 30 days. The authors have reported many features in common with the zebrafish model, the presence of de-

differentiation with the characteristic disassembled sarcomeres and the absence of hypertrophic scar tissue, the last characteristics typical of mammalian heart postinjure. Lately the group Porrello et al. has showed a model of ischemic myocardium in postnatal mice, demonstrating a robust regenerative response with the active proliferation of cardiomyocytes differentiated capable of supporting functional recovery in 21 days. Inside the animal model before mentioned, it was possible to analyze such as the functional inhibition of miR-15 has able to reactivate the cell cycle in cardiomyocytes supporting thereby the full recovery of ventricular systolic function after myocardial infarction[64]. Even the use of genetic manipulation techniques allow relevant similarities between zebrafish and neonatal mice models but also important differences. It is important to note that the zebrafish model provides a heart with a small percentage of adult cardiomyocytes compared to mouse studies where it is assessed with a heart actively growing with proliferative cardiomyocytes within a tissue infarction partially still immature and in the development phase. Despite the differences between evolutionary state and differentiation of heart in the two species is definitely remarkable the efficiency of the response also in neonatal rat cardiomyocytes in manner similar that seen in zebrafish. These observations suggest that pathways that lead to proliferation of adult cardiomyocytes are much conserved, hypothesizing so the beginning of studies on proliferative activity of heart tissue[62].

7. Cardiac regeneration in mammalian heart

Acute myocardial infarction (AMI) is caused by the abrupt closure of a coronary artery due primarily to thrombus formation. The most effective therapy for AMI is represented by timely revascularization of infarcted area, with thrombolytic agents, with coronary intervention, or with the use of by-pass. With the advent of reperfusion therapies, the institution of intensive care units, the introduction of effective drugs such as beta-blockers and ACE-inhibitors, the occurrence of complications in patients with AMI has been reduced, life-expectancy improved. Despite these advances, AMI produces a high morbidity and mortality, especially in those patients who missed the opportunity for timely reperfusion. In patients with large infarcts, ventricular remodeling often leads to congestive heart failure (CHF). Recently, the use of stem cell in clinical practice has been indicated such as possible regenerative or reparative therapy for AMI. This clinical strategy is based on the hypothesis that multipotent stem cell types, that injected into the heart, will be able to repopulate the necrotic tissue and differentiate in new cardiomyocytes, promoting the function

recovery contractile. Stem cell therapy has also been tested in models of chronic myocardial infarction (CMI) and chronic ischemic heart disease (CIHD)[65]. Few are so far the improvements in human cardiac regeneration, though of great clinical impact especially in the prospects of improvement of quality life of affected people. Indeed, it is generally accepted that mature mammalian cardiomyocytes have a low capacity for cell division [66]. Studies in rodents have suggested that hypertrophy in cardiomyocytes lead to an increase in the size of the cells, as opposed of hyperplasia that brings an increase in the number of cells[67]. The paradigm that the heart is a postmitotic organ incapable of regenerating their parenchymal cells was established in 1970, and this dogma has profoundly influenced basic research and clinical research in cardiology probably in the last 30 years [68]. The heart failure is often the result of cardiomyocytes loss after the onset of events, such as myocardial infarction. The majority of patients hospitalized with heart failure have a similar prognosis to patients with common cancers [69]. With the difference that the organ transplantation or implantation, depending on the case, imply of assist devices mechanical ventricular, but currently further efforts by the clinical world have turned to find alternative to current therapies that don't resolve the central problem of decreased pumping ability of heart due to myocytes reduction. Thus, it was born an relevant interest in recent years from many networks, on the study of regenerative properties of heart. Until recently, the prospect of cardiac regeneration was considered by many in large part such as a concept of pure science fiction. The long-standing paradigm that stated that the mammalian heart is a terminally differentiated organ, it incapable to to initiate the cellular turnover. However, over the past decade, relevant studies have showed that cardiac myocytes of mammals retain of division degree[70], but highlight the presence within the bone marrow [71] and in particular in adult heart of cardiac progenitor cells [72]. A significant issue related to ability of heart to support the cardiac pathologies could be due to ability to start the infarction reconstruction in postnatal stage that represent an adult stage of the organ development with reduced proliferation capacity in phase postmitotica, limiting the response of heart with increase in workload. When myocardial hypertrophy exhausted its effects, can accumulate diseases such as ventricular dysfunction. Over the past three decades, the study of molecular cardiology and of the signaling pathways that regulate the activation and the inhibition of gene transcription, was been essential to understanding the hypertrophic response in cardiomyocytes in early stages of physiological development, in aging and in pathological states of heart [68].

The mechanism that rules the ability of heart to renew its parenchymal cells has been rejected by many, and in fact the myocardial repair is viewed with suspicion but also with some trepidation. A new point of view promotes a biological perspective extremely interesting, indicated first by the group of Prof. Anversa, which in sick adult heart is demonstrated to degree of cellular regeneration

that occurs both in humans but also in animals after acute infarct [70, 73], after pressure prolonged overload [74], and in decompensated heart failure in aging [75]. Despite the intense scientific production and several data published over the last 20 years [76] and the new networks on cardiac regeneration, nevertheless, many researchers has rejected this alternative view on cardiac biology, safeguarding the old conception considered unshakable and immovable. But the conviction in some research groups that nothing could be done to generate new myocardium was so strong that only scientific results published in Nature in 2001 where new myocardial cells from bone marrow (BMC) were synthesized in necrotic tissue after myocardial infarction [71] call into question the view ancient point [77]. The controversy on myocardial regeneration by undifferentiated cells with exogenous or endogenous origin, play on relevant comments about the history of the heart as a postmitotic organ are employed to understanding of shift paradigm required for implementation of new field of regenerative cardiology. Numerous studies on human heart begins already in the first half of the 800 where the common thought was that the hypertrophy of the myocardium was the natural consequence of hyperplasia and hypertrophy in damaged cardiomyocytes. Scientific data where the myocytes weren't able to divide it was inspired by the complexity of determining mitotic figures within myocardial cells. they have gained the support of many in the early '20s thanks to experiments autoradiographic of incorporation of thymidine in hearts animals of during the post-natal growth and after the occurrence of conditions of work overload [78]. In fact the results obtained doesn't show appreciable synthesis of deoxyribonucleic acid in nuclei of cardiac cells and thus it has been introduced in the scientific community that the dogma that heart survives and exerts its function, until the death of organism, with almost the same number of cells that are present at birth and that the increase in cardiac muscle mass in heart disease was the result of mechanisms of hypertrophy cellular [79]. In additiont he number of myocytes synthesized that established only few months after birth [79], and that cells themselves are considered able to contract 70 times for minute for a lifetime, could be a risked consideration that lead to consider the cardiomyocytes such as immortal cells both from the functional point of view that structural especially in centenarian subjects with an age greater than the average. This hypothesis contradicts basic concept that indicate cellular aging and cell death by apoptosis going beyond the simple logic which assign a slow but constant cellular turnover therefore capable of supporting normal tissue function during the life of the organism [80]. The observations carried out for decades have been challenged by experiments where was possible to identify male cells in female hearts transplanted. In these experiments hearts of women in male guest has a significant number of myocytes chromosome-positive Y and the synthesis of new coronary vessels. Although there are differences between the groups about degree of cardiac chimerism [81], these findings have raised the possibility that these

cells could populate, both heart male and female, differentiating in myocytes and vascular structures. These early data were consistent with the thesis according to which stem-like cells can migrate to area of damaged heart and it give rise to progeny cardiac[80]. A new type of method to be used for replacement of traditional transplantation has been shown by research into stem cells, which have enriched the last decade our understanding of the biology of development and cell differentiation, providing a type of instrument alternative to organ transplant to correct degenerative diseases and tissue loss. The stem cell treatment seems to be interesting therapy for cardiac tissue damage[82]. Several mechanisms work together to contribute to improving cardiac regenerative capacity sometimes observed in mice, which is often associated to increased vascularity and proliferation cellular. Substantial studies indicate that activation of responses pro-rigenerative is realy possible in the hearts of mammals, but the mechanisms involved remain currently poorly understood [62]. Few answers to the many questions born of the understanding of the steps involved in cardiac regeneration were been definitively resolved. But the aspect that at moment seems to be certain is that regenerative percentage in human myocardium is quite insufficient to counterbalance the serious loss of heart muscle that occurs following an acute myocardial infarction or other myocardial diseases. The implementation of reduced regenerative mechanisms that exist in adult cardiac tissue of mammals, highlights the problem of physiological barriers, that may prevent the synthesis of new cardiomyocytes, such as they could be the onset of inflammatory and fibrotic phenomenon that characterize the various phases of the infarcted myocardium including ischemic phenomena. 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. Perhaps the most surprising aspect of the current progress towards cardiac regeneration is the wide variety of cell types that have been considered as candidates for tissue reconstruction become necrotic. The different cell types constituting the intricate structure of the heart involved in myocardial damage and in reducing the performance of the organ reflect the need to find a medical solution for the treatment of heart disease, and therefore giusticano the large amount of experimental efforts in development of cellular therapies targeted. It's important to underline that different are experiments carried out to study the different cell types able to support the reconstruction of lost or damaged tissue. Different cell types have been proposed and they studied with important projects for recovery of damaged heart: skeletal myoblasts [83], fibroblasts [84], SMC [85], fetal myocytes [86], ESCs [87], cells derived from bone marrow (BMC) [88], and cells, induced pluripotent stem cells (iPS) [89]. Fibroblasts, SMC, and fetal myocytes

form a portion of newly synthesized tissue with different criticality, it is showed which the decrease of stiffness in parenchymal tissue resynthesized, however, a positive effect on the ventricular remodeling is functional, creating a positive effect on the contraction. Also totipotent ESCs seem grow up in myocardium root but quickly die due to lack of new blood vessel formation besides possible problems of immune rejection they are observed [90]. Important complications they are showed in some studies where it has been shown that the ES cells give rise to teratomas and teratocarcinomas [91]. Similarly, also the iPS cells were recently identified a potential carcinogen on their development ability, moreover these types of cells weren't considered for possible clinical uses .The advent of new technologies about (IPSs) the synthesis of pluripotent stem cells it offers a possible solution the problem to immune rejection. This technology involves reprogramming of adult human fibroblasts terminally differentiated into pluripotent stem cells where it is showed a expression of four pluripotency factors: Oct4, Sox2 c-MYC, and KLF4. The reprogrammed cells show many characteristic features of mature ESCs, morphology, cell surface antigens, gene expression, telomerase activity, proliferation degree, and ability differentiate in cells of the three embryonic germ layers [92]. Since, the fibroblasts adults can be obtained directly by the patient, it is assumed that patient-specific IPSC cells, derived from the CM could be transplanted without immune rejection. But another barrier to iPSs-based therapy is the reduced time window to get cells patient-specific. Also taking advantage of the protocols currently accessible, iPSCs cannot be used in exigent circumstances, such as in context of myocardial acute infarction or heart failure progressive. It's also important to reminder that iPSCs were created using viral vectors, which increase the risk of genomic integration and an eventual potential oncogenic risks. A first attempt to replace infarcted myocardium with patch of skeletal muscle has been performed over the years 30. Later in the 80, layers of skeletal muscle tissue were placed on surface epicardial ischemic, stimulating the contraction with a pacemaker [93]. The way indicated above has formed new approach called cellular cardiomyoplasty and consists of direct injection of skeletal myoblasts in ischemic area. The cells autologous origin, inoculated in damage area, create a clear benefit in this type of cardiac repair. Moreover, the skeletal myoblasts, it is indicated, to be more resistants to ischemia compared to myocardial cells, in turn capable of support their chance of survival in necrotic myocardium. The use of skeletal muscle cells has been viewed by many as a viable alternative to transfer in clinical environment, thanks to data obtained in some experiments on infarcted patients subjected to this type of treatment [94]. However, the lack of integration of skeletal myoblasts within the myocardium creates more of a reason of apprehension about the final consolidation of their use in therapeutic practice. Indeed, the analysis of the interface graft-host in the area of inoculum, doesn't showed prove of mechanical or electrical coupling between cardiac

and skeletal muscle [95]. Specific markers of cardiac weave as connexin 43 and N-cadherin were detected in skeletal muscle cells injected, to which is added the increased synthesis of layer of scar tissue created between cardiomyocytes and skeletal muscle which is opposed to the grafting process. As has already occurred in some animal models by the group of Reinecke et al [95], the lack of synchronous contraction could be connected of responsible factors for arrhythmic events seen in patients treated with inoculum of skeletal myoblasts [94]. A further limitation to the use of skeletal myoblasts is represented by the initial phase of amplification *in vitro*, insufficient to meet the clinical requirements, this need represents a limit for clinical application in this procedure. This problem is particularly significant since it has been shown that success of use of skeletal myoblasts is directly correlated with number of donor cells. The need for a large amount of cells to be implanted is dictated by the fast cell death which undergo a large number of skeletal myoblasts with another important consideration which indicates the fast differentiation of myoblasts with synthesis of myotubes which limits any possible proliferation of inoculated cells [96]. Another cellular type are ESCs, prototype of stem cells. Able to meet all requirements of stem cells: clonality, self-renewal and pluripotency. To grow and divide indefinitely while maintaining the ability to develop into all three germ layers. Under appropriate culture conditions, a small fraction of human ESCs (5-15%) was seen spontaneously differentiate into cardiomyocytes with structural and functional properties characteristic of cardiac tissue [93]. ES cells can differentiate into any cell present in the adult and they have the potential to completely regenerate the myocardium. Several they showed the obstacles to therapeutic use of ESCs including the tendency to immunological rejection and the ability of ES cells to form teratomas when injected *in vivo*. This problem suggests the deepening of many paths that lead to the differentiation of ES cells, to study the alternatives to the limit that leads the teratoma formation where a possibility can be through the genetic selection of differentiated stem cells, or the *in vitro* differentiation of stem cells into cardiomyocytes and endothelial cells before injection, for example, utilization of tumor necrosis factor it was observed to promote the differentiation of ES cells into cardiomyocytes. Many groups believe that differentiated ES cells are able to survive and improve myocardial function if inoculated in the damaged myocardium but supported by different of tissue specific factors able to support cellular growth and differentiation. In some recent studies it has been studied the control of time proliferation and differentiation of ESC cells that could activated to sustain specific signaling pathways. For example, recent studies on rat and zebrafish embryos reveal the role of pathways of Wnt-catenin in cardiac development which can vary depending on stage of development [97]. Also in the field of ESCs, progress in understanding the pathways involved in gene regulation and epigenetics that leads to myocardial differentiation, have suggested potential new approaches to generation of cardiomyocytes derived

from undifferentiated cells useful for therapy. Effects regulators by some RNAs are indicated, microRNA (miR)-1 and miR-133, are specifically expressed in hearts of mice, and their deletion was targeted to phenomena of gene rearrangement with the result of creating a series of alterations in cardiac morphogenesis, in the electrical conduction in the cell cycle, and in cardiac hypertrophy. Different experiments have shown that transplantation of human ESCs in animal models of myocardial infarction gave low results until now to which are added the risks described first over, besides to problems of engraftment monitoring of the inoculum [98]. These results a complex indicate to partial creation of new tissue in the area infarcted creating cardio-specific incentives in the engraftment *in vivo* that appear not stable [93]. Non-resident stem cells, like BMSCs, were initially thought to adopt such as possible myocardial cell. Extensive research has published recently shown that therapies with nonresident BMSC are able to providing a cardioprotective and cardiogenic paracrine contribution in injured hearts rather than creating new myocardium cells. This type of cell therapy is used to facilitate cardiac repair through revascularization, producing positive physiological effects. In facts, BMSCs secrete angiogenic factors, activating several *pathways* involved in cellular proliferation. Many reviews it has been suggested that BMCs can be migrate in the heart, creating niches that might be involved in the physiological homeostasis of heart, it intervening both in the healthy organ that in regeneration of damaged tissue [99]. The specific contribution of this cell type in the processes of coronary vasculogenesis and cardiomyogenesis is currently little studied. Also some studies have allowed us to assess the chimerism degree in cardiac transplantation and in hearts of patients who received allogeneic bone marrow transplantation. In the latter case, 14-16% of myocytes and endothelial cells in transplanted hearts were found chimeric. These observations suggest the intracardiac origin of the recipient cells of donor heart and the extracardiac origin of chimeric cells in host heart after bone marrow transplantation. It have been suggested more than a hypothesis, in the first case, the host cells can be migrated from strains atrial residues to the heart of the donor and, in the second, the donor cells may have reached the myocardium due to high level of blood chimerism [100]. This particular cellular type of hematopoietic origin but with a cardiac differentation cardiac can be detected only when the peripheral blood contains a large number of hematopoietic stem cells (HSCs). Experimental results support this theory. The experimental test that HSCs were been able to induce regeneration after myocardial infarction, was obtained from the experiment when BMCS mononuclear cells and CD34-positive were administered to patients with various cardiac diseases such as acute myocardial infarction and chronic dilated cardiomyopathy, and refractory angina. The results though still under study, to which is added a certain degree of variability between studies, indicate that BMCs are able to procure therapeutic effects in subjects before indicated with a 3-4% increase in ejection fraction.

The safety in the mechanisms involved in therapy of BMC on humans remains to be confermed. Ratings on coronary blood flow suggest, however, that in the phenomena of vasculogenesis there is probably the release of active cytokines that you think would be probably decisive for the activation of one of the new growth target in the cardiac cell therapy such as the activation of cells resident stem in the adult heart (CSC) [96].

8. Cardiac stem cell

The recent identification of CSCs has shifted the attention on endogenous cell mechanisms about the reactivation of cardiac cellular development in adult organ such as a novel target of cell therapy for the failing heart. Different groups have isolated cardiac stem and progenitor cells from the adult mammalian heart. For the purpose of easier understanding have grouped in the same acronym the cardiac cells that appeared with characteristics progenitor and stems (CSCs). However, the difference between these two cell types is minimum. Many network of research on cardiac stem cell determine this cell such as a primitive cell, clonogenic, self-renewing, and multipotent, and it capable of differentiating into the three main cardiac lineages: myocytes, smooth muscle cells (SMC), and endothelial cells (EC). In contrast, a progenitor cell is an proliferating cell immature capable of differentiating into only one of the three above-mentioned lineages (myocyte or SMC or EC) [97]. The difficult recognition of cardiac stem cells and the singularity of their antigenic characteristics still remains a much argument discussed, in fact the stem characteristics can be identified in the delimitation and in the identification of functional characteristics of adult cardiac stem cells relate to inherent ability of the cell to self-renew, to clone and express different phenotypes mature (multipotency) *in vitro* and *in vivo*. The propensity the autorenewal, in fact is the ability of undifferentiated cells to divide indefinitely without errors during DNA replication. Furthermore, the daughter cells, that originate, have the same tissue-specific properties of the mother cell. In fact, the accumulation of mutations through the life, leads to aging of stem cells and affect the functionality of ASCs [102]. A typical example of accumulation of mutations occurs in the case of CSC mature in telomeric sequences of about 10 kilobase pairs, after each cell division occurs a subtraction of about 130 base pairs in length telomere, this could to have negative effects, as indicated by several research groups, on replicative capacity and further accumulation of oxidative damages in the DNA that occur during the cellular life cycle. The riduction of telomere

lengths may be reflected on the aging of CSC and the heart itself, reducing the possibility of action on cell turnover and thus on organism lifespan [103]. Similarly, clonogenicity, indicated as ability of a single cell to proliferate independently to form a colony, it is another specific feature found in adult CSCs, although still numerous are the limits of understanding of the capabilities of ex vivo expansion. Addition to two above mentioned properties, is added the multipotency, ability to generate distinct cell types from the same progenitor, in fact as mentioned above, the CSC have been shown a propensity to differentiate into the three main types of cardiac cells: cardiomyocytes, smooth muscle and endothelial cells. More than a marker antigens has been suggested in the identification about the regenerative potential stem cell and their relative interpretation in various classifications, that is still poorly defined. However, the study on replicative capacity and regenerative was analyzed in cells residing in the adult myocardium, where the selection markers directly related to stem cells are been identified such as factors cardiac transcriptions essential. The reference markers indicated in adult CSC are c-Kit (CD117) analyzed in cardiac tissue of different species such as rodents, dogs and humans, the protein MDR-1 indicated also as protein to multidrug resistance, and finally the Sca-1, antigen of generic stem cells. In murine animal models has been observed considerable increase of c-Kit positive cells after acute myocardial infarct. Even after inoculation, always in *in vivo* models of myocardial infarction, the cells c-kit seem able of regenerating the damaged myocardium, thereby improving cardiac function. In fact, c-Kit has different roles in the homeostasis of the organism, the Kit protein in CSC adult acts in development of bone, mobilization of hematopoietic progenitor cells, by decreasing the release of cytokines and chemokines that may participate in paracrine signaling with effects cardioprotective. Initial investigations suggested also which CSCs only transiently express c-kit, in rodents, and in humans, where is been found often the expression of Sca-1. A different subset of cardiac progenitors deriving from the secondary heart field, express the LIM-homeodomain transcription factor Islet1 (Isl-1), identified in rodent but also in the human fetal atrium after greater outflow blood. Nevertheless, the continued study about the cardiac phenotype of CSC demonstrates the real need to define certain markers with antigenic model to redefine the universally accepted procedures for the manipulation of stem cells. However, c-Kit has been proposed such as unique marker for adults CSC capable of differentiating in contractile cells, while cells Isl-1 positives, also if it observes a certain degree of differentiation in cardiomyocytes and smooth muscle cells, they are detectable in murine animal models in murine fetal life, such as it observed in human beings, but not in adult stage. Conversely, progenitor cells Sca-1 positive weren't considered such as suitable for synthesis new cardiomyocytes. However, recent studies, show that Sca-1 is a stable marker of adult progenitor cells whose expression increases progressively in adult CSCs, suggesting a possible

hypothesis about the expression of SCA-1 linked to more mature of cellular development [102] Oh [104] and Matsuura [105] isolated Sca-1 positive cells from hearts of adult mice, and they have demonstrated that they can differentiate, *in vitro*, in different cell lines, including cardiomyocytes. The actual role of Sca-1 positive cells isolated from myocardium is still unknown, but it is possible, that are involved in cell signaling, in myocardial regeneration, although only 9% of cellular population Sca-1, were found also c-Kit positive. Others groups have isolated several cells with "immature" potentials in the adult myocardium, which expressed c-Kit in different combinations; however, the cells c-Kit +/Sca-1+ constitute only a cellular subtype in the subpopulation of cardiac cells immature. Indeed, Anversa et al have suggested that CSCs classification and their progeny in three major subpopulations, it was based on the fact that they only express only c-Kit, only MDR-1, Sca-1 only, or a combination of these three markers and proposing a classification of the immature heart cells into four classes: CSC, progenitors, precursors, and amplifying cells. They suggest that co-localization of the expression of c-kit, Sca-1 and MDR-1 in primitive cells, but also that presence of an antigen does not exclude the others, or a combination of these (or c-Kit/Sca-1 c-Kit/Sca-1/MDR-1 or c-Kit/MDR-1 or MDR-1/Sca-1) can be expressed in individual clones [97]. Further evidence on presence of CSCs adult c-Kit positive, were obtained from rodents about 8 years ago, where gradually have been outlined identifying characteristics of CSC c-Kit, such as clonogenic cells that divide symmetrically and asymmetrically *in vitro*, able to differentiate in cardiomyocytes and vascular cells. Newly formed cardiomyocytes, showed an increase of mechanical and electrical properties in functionally competent cells, improving the performance in the damaged heart ventricle, showing also regenerative abilities in a restoring the structure of infarcted myocardium [96]. Moreover, it is very important the idea that cell populations in the heart are grouped in cluster/progenitor stem cells capable of creating functionally competent cardiomyocytes and coronary vessels, consistent with the regenerative response observed after acute myocardial infarction and chronic pain in humans. In addition, it has been noticed in the transplantation of CSCs cloned in animal models of myocardial infarction, their ability to differentiate *in vivo* in a progeny with electricals, mechanicals that include the synthesis of calcium channels transients, typical of differentiated myocytes. The work of Braezi et al. in 2007 showed that the generation of cardiomyocytes is impressive and markedly higher than the numbers of cells lost chased to myocardial infarction. Furthermore, it was observed, the synthesis of marked coronary arterioles, events considered by many as one of the major factors capable of stimulating the formation of new myocardial tissue structurally and functionally similar to the parenchyma of neonatal heart, indicating the remarkable hyperplasticity of cardiac tissue of newly synthesized [100]. This specific population of stem cells, residing in specific anatomical areas called specialized

niches, which provide a microenvironment designed to safeguard the survival and activation of replicative potential of CSC residents in adult heart tissue [106]. The niche concept was introduced in 1978 by Schofield, who defined niche as a stable microenvironment that can control the behavior of hematopoietic stem cells [107]. Currently, the niche is seen as a subset of tissue cells and extracellular substrates that can indefinitely contain one or more stem cells and verifying via via their ability to self-renewal [106]. It is believed that the architectural organization in the niches of adult heart, provides several factors able to drive the differentation in different types of stem cells such as, CSCs mature, progenitors, precursors and cells in initial stage of differentiation. The role of niches is in turn connected with the surrounding environment through the expression of gap and adherens junctions. The specifics junctions, between CSCs and niches, are foundamental to transmit and receive signals from the surrounding tissue in phisiological stages such as cell survival, proliferation, but especially the induction to differentiate. Adherens junctions appear to be involved in maintaining the undifferentiated state of CSC. However, deregulation of the expression of connexins and cadherins, may alter the configuration of junctional complexes, influencing the growth and commitment of stem cells. The niches were detected in different sites of the wall of left ventricle indicating that CSC are, preferentially but not exclusively, present in protected areas of the heart. The formation of ventricular niches may be dictated, also by the migration of CSC from their sites of origin to damaged regions. The activation of the CSCs in order to migrate may be dictated by physical forces transduced in the ventricular wall in intracellular responses that regulate the cellular behavior and fate. It is important to emphasize that the abnormal pathological loads may be associated with the start of the activation of active phase of replication, however, the peculiar topographical distribution of CSC in heart suggests that a relationship may exist between the function of CSC and the level of hemodynamic stress [96]. With the increase of work in CSCs adults have emerged a new area of cardiac research, also if it is one of the most controversial. Most of the work has focused on cell culture and transplantation, driven by clinical need for cardiac repair. In fact there are few reliable data to indicate the specific role of adults CSC in the spontaneous repair of damaged myocardium [108]. The inevitable evolution of ischemic injury is the formation necrotic myocardial with loss of mass and contractile function. In fact, the extent of spontaneous cardiac repair after infarction is minimal, the prevailing regenerative response is restricted to the non- infarcted tissue spared, [50] and the size of the scar formed negatively affects on the remodeling. Similarly, many believe that the regeneration in adult heart where it is believed that the loss of cardiomyocytes is replaced by newly synthesized tissue, could regenerate new cells but not prevent or reverse the progression [74] of heart failure. The adaptation to heart failure and the pathological loads can reflected in the lack of mechanical transduction signals from the cardiac

tissue to the niches, it can lead then to lack of regeneration. Indeed, the absence of differentation in SMC, Endothelial cells, and myocytes, in some cases, could be linked to reduction of number niches with possible formation of voids and depletion of functional adults CSCs that could severely reduce the synthesis of cells important in regeneration cardiac with the result in excessive myocyte hypertrophy and altered coronary perfusion[96]. It is believed that maturation and survival of myocytes of new synthesis that invade the infarcted area both in relation to the availability of oxygen. There are two prerequisites for the successful integration of cells in the ischemic region [80]. Coronary arterioles and capillary structures must be formed in order to provide the necrotic tissue, restablish communication with large vessels of the normally perfused myocardium. In addition, the new blood flow through the vessels of new synthesis creates a vascular supply capable of permeating the myocytes, preserving their survival, favoring their growth, differentiation and contractile function [79].

At the moment, two clinical trials are underway in the United States : a study of intracoronary injection of CSCs in coronary heart disease with congestive heart failure so as to test the ability to reform new cardiomyocytes [109]. Instead other aims, such as assess the safety and efficacy of intracoronary delivery, was been examined an other form of CSC such as the Cardiospheres-derived (CSCs) from the patients with ischemic left ventricular dysfunction and a recent myocardial infarction [110]. In these two trials, stem cells are inoculated by intracoronary injection. However, the grafting of stem cells in infarcted myocardium without any structural support, it does not allow the cells to synthesize new tissue such as it was seen in papers where was showed that stem injected cells don't integrate properly in myocardium, not receiving the stimuli to grow or differentiate from the mechanical forces that are known to drive myocardial cell physiology [111].

8.1 *Regenerative medicine and tissue engineering*

Cell therapy includes different branches of regenerative medicine, such as tissue engineering biological, where is provided the use of cell and tissue culture protocols for synthesize and increase of number specific cells to able of replace cells with functional alteration or completely lost, in various disease states. Major efforts have been directed towards two different strategies useful for the application of regenerative medicine based on the use of stem cells. The first involves the direct inoculation of a cell suspension into the bloodstream or into the damaged area of the target tissue. The second therapy, called "cell therapy", is made possible by the use of construct, similar to a fabric, formed by a three-dimensional matrix, which will be colonized by cells cultured *in vitro* and then subsequently implanted in the tissue of site recipient [112].

The concept that cell growth and survival could be recreated *in vitro* has been recognized as a possible almost one hundred years ago, when the German zoologist Wilhelm Roux, where has described a experiment with the neural crest of caught and maintained in saline solution for few days, putting the foundations of mechanics of development supporting the functional adaptation of the various parts of the body. It's relevant indicate the tissue like an aggregate of morphologically similar cells that interact together to perform specific functions within organism macrosystem . Tissue repair can be understood such as the reconstitution of the architecture of parenchyma but also of specific features which it carries on [113]. Therefore includes both regeneration processes that could ensure an alternative method of healing, where the growth of new tissue completely restores portions of damaged tissue returning them to their normal state. Systems that regenerate tissue repair and/or damaged tissue replacement, as defined above, identify complex systems that allow cell growth in natural or synthetic polymer matrices [114]. Langer and Vacanti have summarized that tissue engineering is an interdisciplinary area that applies the principles of bio-engineering towards the development of biological substitutes with the purpose to restore, maintain, or improve the perfomance of the functional organ [115]. Tissue engineering is important for the synthesis of artificial tissue three-dimensional (3D). Indeed, the correct of biological and mechanical functionality is of great importance for tissue created in artificial structures, argument, this, still under development. It is of fundamental importance, to understand that the synthesis of a functional tissue requires a high efficiency of growth of different cell types within of scaffolds 3D, often it isn't supported for low number of cells, that appear in many works it is not adequate. The main function of scaffold is to regulate and guide the cell behavior such as migration, proliferation, differentiation, differentiation phenotype, facilitating the interactions between various types of cell-matrix and cell-cell. The design of scaffold and the fabrication are major areas of biomaterial research, since biomaterial scaffold is the substrate within which cells are Instructed to form a tissue or an organ through a pathway highly adjusted [116]. A common approach in field of engineering techniques is the isolation of cell populations with a specific phenotype through small biopsy from a patient, increasing in number inside the scaffold, under controlled culture conditions. After the phase *in vitro*, the construct is placed in the desired anatomic site, with the aim of inducing the formation of new tissue where the scaffold rule the growth, but subsequently it will be followed a series of degradative events. An alternative approach is to implant the scaffolds for tissue growth directly *in vivo*, with the aim to stimulate and induce the formation of tissue in situ: the advantage of this approach is a reduced number of operations required [117], resulting in recovery in time minor for patient. They distinguish grafts depending on the origin, in:

- ✓ Allografts: when the cells, tissues or engineered tissues derived from another individual of the same species .
- ✓ Xenografts: when the cells, tissues or engineered tissues derived from another species (eg. heart valve devitalized from pig or bovine pericardium) .
- ✓ Autografts: when the cells, tissues or engineered tissues derive from the same individual

It's important the cellular kind, because tissue engineering techniques allow loading in polymeric matrixes (3D) (scaffolds) with differentiated stem cells to restore the original structure in damaged organs. For some years, encouraging results have been obtained in the reconstruction of tissues such as bone, cartilage, and in soft tissue, such as cornea and skin, while the greatest problems are represented in myocardium, between the most critical in the reconstruction *in vitro*. Currently, the type of autologous stem cells considered the best candidate for synthesis of bio-active substitutes, must be pluripotent and not induce a foreign body reaction [118].

The main protocols of three-dimensional cell culture are directly related to influence of cellular environment linked to basic aspects such as its dimensionality, stiffness, elasticity, composition and remodeling during the illness. Numerous studies have shown that the absence of extracellular matrix (ECM) and its main components such as fibronectin, collagen, laminin, aggrecan, etc. can lead to phenotypes, which altered in embryonic development and post-natal, have serious problems in their function [119]. In addition, recent discoveries such as Cdc42, important for the acquisition of the apical and basal polarity during morphogenesis or cell differentiation at the time of tubulogenesis, would not be rendered feasible without the EMC [120]and its three-dimensional characteristics able to create a specific microambiante for cell growth [18]. The attempt to induce the greatest efforts in synthesis of engineered scaffold that mimics ECM active, actively promoting tissue regeneration *in vitro*, as does the native ECM *in vivo*. Despite the incredible diversity of the structures of the ECM, due to the presence of different biomacromolecules and the system with which these are organized in space, in fact, a main feature native ECM is the nanoscopic size of its physical structures. In a typical connective tissue, structural proteins such as collagen fibers and elastic fibers have diametrical range from several tens to several hundreds of nanometers. The protein fibers clump together to form tissue structure which gives tensile strength and elasticity to tissues. Adhesive proteins, such as fibronectin and laminin use specific binding sites for cell adhesion, alike occurs exist between nanoscopic fibers and ECM. ECM in heart and in wall vascular includes fibrous proteins (collagen and elastin), adhesive glycoproteins (eg laminin, fibronectin) and proteoglycans. This organized network create in the ECM directs the development of cardiomyocytes, allowing a mechanical interaction between them and the adjacent network of capillaries and nerves, providing, also, an elastic support during the filling of the ventricular portion

[121]. The arrangement of proteins, in the cardiac ECM, is disposed such as relatively rigid fibrous rings that surround each heart valve. The collagenous connections between adjacent myocytes show the Z-lines sarcomeric across the cell membranes and it is relevant ensure the extension equal between cells contiguous while preventing slippage between of them. In fact, alteration of fibers collagen can decrease the myocardial systolic performance without to alter the contractility of myocytes [122]. A further consideration about the ECM is that cannot be considered as a passive tool, because it provides the organization microstructural to able regulate and direct the cardiac morphogenesis as well as myocardial function. For example, it has been observed already in 1988 the elevated importance of the ECM in neonatal cardiomyocytes with a serious differences of their contractile properties to depending of type of substrate on which were grown which differed in the protein composition of the ECM [123]. So it is crucial to understand such as the ECM regulate the self-assembly of sarcomeres in differentiated myocytes, providing directional cues that guide the synthesis and placement of heart muscle myofibrils [124]. The understanding the way in which ECM interacts with tissues and cells allows to define the transmission of mechanical forces existing. It's important, concentrate the interest on receptors for integrins detectable on cell surface. Integrins are transmembrane proteins, dimeric structure with two sub-units α and β, linked to ECM proteins in extracellular and cytoplasmic such as actin-linker (eg vinculin, talin, α-actinin, paxillin and zyxin) or inside the cell, promoting mechanically the coupling between the integrins and cytoskeleton. Cardiac myocytes express various integrin receptor types that bind to collagen I (α3β1), to fibronectin (α3β1, α5β1) and laminin (α1β1, α3β1, α7β1). When integrins bind ECM, a change occurs in the conformation that leads the receptors to work together with specific protein kinases such as src kinase and focal adhesion kinase (FAK), and small GTPases such as Rho, on the cytoplasmic side of the membrane, supporting the assembly and the focal adhesion, between protein complexes of the matrix. These adhesion plaques specialized mechanically link the cell with the ECM, in which it stand include several clusters of integrins, proteins associated to molecules actin-linker and multiple signal transduction. Important to reminder, the focal adhesions play a central role in mechanotransduction, the process in which cells convert mechanical signals into biochemical responses [126].

As pointed out in a review of 2012 by Karam et al. [127], the majority of cells transplanted in heart undergoes apoptosis in the early days after inoculation. Cell death in this particular situation is a phenomenon that has different and multiple factors that involves an inflammatory reaction in response to inoculation, apoptosis, ischemic conditions in response to reduced synthesis of the blood vessels and then poor vascularization and possible loss of contact between the cells and molecules of extracellular matrix (ECM). These issues have become critical in the last decade for

those that work on cardiac regeneration, through a continuous search to signals and new strategies to improve the survival of cells inoculated.

The strategies for tissue engineering are suggest in some studies where is been possible to make the combination between cell and biomaterials in the structure of scaffold, it is indicated in important results from group of Di Felice et al. [128] where they were shown encouraging results about to interesting perspectives for repair heart. Specialized branch of tissue engineering is certainly the heart that aims to find solutions in specific case as the repair or regeneration of a damaged portion of the heart [129]. The biomimetic approach carried out by the CTE is to develop the production *in vitro* of immature tissues but functional, mimicking the key factors in native myocardium as the high cell density with multiple cell types, proposing expecially the diffusive transport of oxygen through network and an excitation-contraction coupling of fibers forming the newly formed tissue [130]. The design of 3D scaffolds for artificial tissues, tends to create a microenvironment that mimics the physical properties of the extracellular matrix as natural as the signals useful to the development of cardiac cells. Many are the qualities required for the scaffold such as the ability to not induce adverse immune response, a correct degree, also called "controlled biodegradability or bio-resorbability" and allow cell adhesion and migration, and dissemination of vital cellular nutrients. The cells that are on inside must be able to proliferate and differentiate, able to promoting tissue integration and vascularization, and, subsequently, to synthesize and organize a new ECM in time useful to organize the new tissue and to replace the artificial scaffold. The scaffold keeped *in vitro* or *in vivo*, is a structure capable of providing the necessary support for the cells to proliferate and maintain their differentiated functions, defining with its architecture the final shape of the intended tissue. Inevitably, the design and synthesis of a scaffold have become the main development areas in search of suitable biomaterial, covering a major role for tissue engineering and regenerative medicine research [116]. The more difficult task in the field of cardiac tissue in creation of new cardiac muscle, over the characteristics of biomaterials, there are but many adverse factors that counteract the realization, such as disease states in myocardial infarction and ischemia, where myocardial tissue puts in place a series of complex events such as an inflammatory acute state, the synthesis of granulation tissue, and the formation of scar tissue [131]. It observes the amplified synthesis of cytokines and growth factors that are released into the damaged area and in order to recruit white blood cells, neutrophils and macrophages. Macrophages are responsible for various processes such as the removal of apoptotic cells and necrotic from the infarcted area, in the recall of fibroblasts, endothelial cells and stem/progenitor cells, besides factors that lead to the synthesis of granulation tissue. The granulation tissue is replaced by new ECM synthesis, that differs from the normal condition mainly by fibroblasts that produce an area where we observe a

reduced number of functional cells compared to formation of granulation tissue. A fundamental understanding of relationship between the structure of the biomaterial and cell function in disease states is made possible thanks to numerous jobs in synthesis of structural tissues such as skin, cartilage, bone, and bladder [132].

9. Immunological behaviour to stem-cell based to cardiac repair

The clinical use of cardiac stem cells has done emerge a number of issues regarding the immunological behavior after inoculation in host organism. Currently the problems recognized in the field of stem cell therapy for cardiac repair can be grouped into the following categories: teratoma formation, vascularization graft, cell viability in long term, integration in anatomical site and immune rejection of transplant. The immunogenicity of stem cells is often mentioned as a potential danger to the host, in fact, to date few have tried to address the issue even if current findings about inoculum of cardiac stem cells in immunosuppressed or immunodeficient animal models are still incomplete [133]. It's particularly important the state of inflammatory environment cardiac tissue damaged that you can be aggravated further to inflammatory reaction of host. The inoculum of cells from genetically unrelated organisms was been seen generate a robust immune response and, consequently, to increase the risk of transplant rejection. In stage initial, they was showed that T lymphocytes recognize foreign antigens in the form of peptides, they are presented in association with self molecules of major histocompatibility complex (MHC). Antigens of MHC class I are found on the membrane outer portion of cell nucleated able to show fragments of proteins synthesized within cell to cytotoxic T lymphocytes, or CD8+ T cells. In parallel, the MHC class II antigens are expressed only on a few specialized cell types defined antigen presenting cells such as macrophages, dendritic cells and lymphocytes B. Thus the function of the molecules MHCII is to present peptides of exogenous proteins to the T-helper lymphocytes, or CD4+ T cells . Are recognized different types of recognition such as the direct Allo-recognition where both CD8+ T cells that are stimulated by CD4+ antigen-presenting cells. The indirect allo-recognition occurs when non- self peptides are then processed and presented by MHC class II molecules of the host to be presented to CD4+ T lymphocytes. The presence of MHC molecules is the determining factor that largely determines the degree of rejection. Important considerations must been done before

inoculating that concern relevant problem such as the three phases of rejection. The three phases that distinguish the rejection are defined in: hyperacute, with a trend that occurs within minutes after injection or transplantation, activeted by self antibodies pre-existing that bind to cell antigens not self. This binding activates the complement system and a sequence that generate a different physiologic reactions of response from the organism, such as an influx of mononuclear cells from the peripheral blood, the formation of platelet thrombi , and the inevitable destruction of transplant. Acute rejection, differently,occurs after more time about 1-2 weeks after transplantation, it is characterized by a high number of monocytes/macrophages, lymphocytes and dendritic cells that infiltrate in surface of contact between host tissue and inoculum. The third phase is the "hypersensitive rejection", in this reaction there is the action mediated by cytotoxic T lymphocytes. After, the last stage is the chronic rejection that occurs after a long period of time that can vary months or years after transplantation. Charateristic important of this reaction where is showed a relevant association with deposits of immunoglobulins and complement C3 molecules of cells inoculated [134]. The role played in the response to immune by cell transplantation is given by different biological and pharmacological agents implicated, whose role is important in the success of organ transplantation. Different pre-clinical studies were started with aim of studying cardiac regeneration by the inoculation of different tissue stem cells, including iPS cells, MSC and human stem cells derived from amniotic membrane mesenchymal. In autologous transplant of iPS, mentioned above, cells have an reduced risk of a possible immunological rejection, but oncological risks are indicated such as higher than the normale, indeed it is to keep in account the presence of formations neoplastic masses unwanted [135]. An other cellular type such as MSCs II were detected the expression intermediate levels of human leukocyte antigen (HLA) class I and many are led to suppose that this particular cell is capable of withstanding a potential adverse immunological response as immunologically tolerated in the host heart.

For the cure of myocardial deseases, in the last years, were indicated for potential therapeutic use the CSCs autologous human for clinical protocols in patients with cardiomyopathy, and in particular two trials, such as Scipio and Caduceus, it were focused many attentions. In the first autologous cells c-Kit positive, differently in the second are used autologous stem cells obtained from Cardiosphere-derived to reverse ventricular dysfunction. Data obtained in both trials have provided the first evidences about feasibility of the utilization. Indeed, initial clinical results showed important and significative scientific evidence for a possible clinical utility [136]. However, a obstacle is indicated by immunogen response, intrinsic in host organisms. Allogeneic responses can lead to graft rejection largely that is mediated by cells CD4+ with pro-inflammatory phenotypes Th1 and Th2 [137]. An other cellular population that work in these phases cell type T are

lymphocyte CD4+ FoxP3+, they have important regulatory ability (Treg), adept at reducing the allo-immunity action [138]. The action of the two distinct lymphocyte populations indicates a high plasticity due to properties proinflammatory Th1/Th2 but also with tolerogenic properties Treg, thanks to subsets shown in 2001 by the work of Amarnath [139] in nude mice models. Furthermore, additional results have shown that cells hypo-immunogenics because of inflammatory conditions and not, don't exhibit costimulatory molecules CD80/CD86 that were requests the stimulation of Th1 or Th2. In contrast, the expression the costimulatory molecules PD-L1, ensure the ability to limit the possible immune response due to stimulation of Treg cells. Patients with heart failure after myocardial infarction have showed the reduction of synthesis of Treg circulating cells [140]. The number of Treg cells has been shown to increase significantly, on the contrary, in conditions that reduce the environment physiological/pathological such as the myocardial damage, which is followed the inoculation of CSC and with their assets they stimulate the synthesis of Treg cells rather than proinflammatory cells. These results are fundamental to understanding the mechanisms involved in the immune rejection and reduce the possible risk and allow the persistence of repair cells in order to facilitate the repair/regeneration rate [136]. Even the complement is important component immune that plays an important role in the development and differentiation of CSC. An *in vitro* study was showed from Astiaso et al, such as the CSC cells express receptors anaphylatoxin functionals, that stimulation with C3a C5a induces the proliferation and the synthesis of CSC in addition to an increased activity of telomerase (NFκB -dependent). Taken together these data support a role for anaphylatoxins in healing rate and in the formation of scars. Indeed, apart from the role chemotactic known that C3a and C5a have on leukocytes , the anaphylatoxins are also chemotactic factors for other cells such as mesenchymal stem cells or neural stem cells [141].

The MSC are among the most studied cells, about the influences after their inoculation in host tissue by immune system. It was observed that MSCs are able to interfere in the differentiation and function of dendritic cells (DC), which are considered to be antigen presenting cells (APC), playing a crucial role therefore regards the regulatory of responses cellular immune against self and non-self antigens. MSCs interfere in stages of endocytosis of DC, and affecting the ability of these cells to secrete interleukin-12 and to activate alloreactive cells T. Results obtained from different studies observe that the inhibition of molecules associated to the antigen presentation, such as CD40, CD83, CD80, CD86 and HLA-DR, during the differentiation of DC to following to the contact with MSC cells [142]. Additional scientific data are supported by other experiments in which MSCs inhibit the differentiation of naive CD4 T cells in T helper 1 (Th1). During T cell differentiation, the human MSC registers a significant decrease in the amount of IFN-γ (about 50 %). In contrast, when the cells MSC were present during the differentiation of Th2 cells , it was seen a significant increase

in synthesis of interleukin-4. These results indicate that MSCs can play an anti-inflammatory role, directing the profile of cytokines produced by these immune cells. MSC exert an inhibitory effect on the proliferation of B cells and on the differentiation of these cells into plasma cells. In Addition, MSCs are been indicated to inhibit the chemotactic properties of B cells, because receptors for chemokine CXCR4, CXCR5, CXCL12 and CXCR4 were downregulated by the reduction of their specific ligand. When B cells are maintained in co-culture with MSCs,they are influenced by the immunomodulatory potential of MSCs. When MCSs were in co-cultured with NK-cells (stimulated by interleukin-2), there was a decreased secretion of IFN-γ from these cells. Furthermore, activating receptors: such as NKp30 and NKp44 were seen downregulated in these innate immunity cells, also, a marked decrease in the cytolytic capacity of NK cells was observed[142].

10. Biomaterials

Biomaterial is one of the essential concepts that underlie the methods of tissue engineering, regarded in biomedical applications to interact with biological systems. The main functions of a biomaterial are numerous and how already outlined previously favoring the complex cell - material and initiate a series of steps vital for the synthesis of new portions of tissue such as growth, differentiation, vascularization at the same time, it should allow for the replacement of the its structure with the newly formed tissue with a degradation rate equal to that of formation of new tissue, to be finally removed from the body by natural metabolic pathways without producing toxic by products [129]. The synthesis of the biomaterial is considered as the combination of synthetic or natural origin [143], with plenty of progress in various areas of chemistry, physics and biology were synthesized in new biomaterials with characteristics optimized for cell development *in vitro* and *in vivo*[144]. The biomaterials can be classified according to their performance and policies, such as polymers, metals, ceramics and composites. The polymers are probably the most biomaterials used in clinical practice. They represent the most important class of biomaterials in medical applications, because they have the best properties, in fact are inert, absorbable and easy synthesis compared to the other. In fact, the polymers may embrace a wide range of biomaterials with physical and chemical properties available in many different forms, including solids, fibers, films, and gels, to able to interact in conjunction with other materials or coated on the surfaces [145]. The characteristics and mechanisms that make them suitable for replacement of tissues, are simple to realize and may be engineered in an infinite ways for their characteristic chemical in structure modular. The polymers are composed of very long molecules and flexible, formed by repetitions of simple units and from the monomer units, arranged between them due to covalent bonds. In turn, the long chains are held together by Van der Waals forces or by hydrogen bonds or, even, by covalent bonds called "crosslink"; addition, each chain may have other chains or side groups which influence the organization global. The polymers are arranged with chains partially ordered, acquiring the so-called semi-crystalline structure, characterized by regions where ordered crystals are immersed in an amorphous matrix. The basic parameter of the polymers is the degree of polymerization, which defines the average number of repeat units within a molecule, the increase in the degree of polymerization decreases the relative mobility of the chain and changes the mechanical properties of the polymer. For the study of these materials is important, also, to know their thermal characteristic, that is their behavior in function of the temperature. Polymers, natural or synthetic, are the largest class of engineered biomaterials that have probably the best chance to utilization in the reconstruction of the myocardial tissue. As well as their utilization in

cardiovascular systems, in orthopedics, in soft tissue and in dental implants because the polymers can be synthesized tailored in relation to the characteristics of the target site. However, it is reported in some cases negative aspects such as the absorption of water that could alter the polarity of the surface such as biodegradation and toxic reactions unwanted that halt their medical fitness[129]. One of the first considerations when designing a scaffold for tissue engineering is the choice of material. The three types of polymers most used with success are: (i) natural polymers, (ii) synthetic polymers, and (iii) ceramics [146]. Natural polymers are commonly derived, from protein or carbohydrate polymers, used as scaffolds for growth of different types of tissues [147]. Examples of natural polymers designed for biomedical application is the collagen.

Collagen is considered by many scientists as ideal matrix for tissue engineering, structural protein, component of the ECM capable of retaining its shape after sowing or cellular infiltration *in vitro* and *in vivo*. It provides support to the connective tissues, such as skin, tendons, bones, cartilage, blood vessels, ligaments. Its influence is important in regulation of the activity of connective tissues supporting the transduction of signals essential for the regulation for the migration, proliferation, differentiation and cell survival. Ideal characteristics that resultant *in vivo*, are became interesting for *in vitro* use in design, such as high mechanical strength, good biocompatibility, low ability to induce an immune response by the host tissue and a percentage of mechanical degradation compatible with the formation of new tissue[148].

The collagen is synthesized in a variety of formats, including porous sponges, gels and sheets [147]. The chitosan, cationic polymer, obtained from chitin, comprising copolymers of β (1→4)-glucosamine and N-acetyl-D-glucosamine. Chitin is a natural polysaccharide that is found mostly in the shell of crustacean, cuticles of insects and fungal cell walls and is the second most abundant polymerized carbon that is found in nature [149]. Presents a high adaptability of use which favors the use in the medical field especially in the administration of drugs. A other type of biomaterial are agarose and alginate, linear polysaccharides, are undergo numerous of purification reaction for avoid unwanted immune responses and of difficult health treatment, *in vivo*, after their implantation [146]. Their employment in clinical practice, has shown good attitudes in tissue engineering of the liver, nerves, heart, and cartilage. Drawbacks due by the use of alginate, are indicated mechanical weakness and lack of cell adhesion. To limit these drawbacks the composition of the alginate has been improved with unions with other materials [147].

A biomaterial with discrete consensus is the fibronectin (FN), glycoprotein present on the outside of the cell membrane, such as other proteins of ECM, composed of tandem repeats of three distinct types (I, II and III) of individually folded modules [129]. A polymer produced by Bombyx mori, the cocoons of silkworms, originates a fascinating material of protein nature called silk fibroin (SF).

The SF is synthesized with natural fibers with comforting result about the resistant, attributed to its particular chemical structure of the protein itself. The amino acid sequence of SF contains repeated sequences of glycine-alanine-glycine-alanine-glycine-serine (GAGAGS) that self-assemble in a anti-parallel β-sheet structure. These β-sheets are highly crystalline, the disposttion of the is essentially crosslink, maintained through strong intra and inter-molecular hydrogen bonds, but also by strong Van der Waals interactions between stacked β-sheets, giving the material a high mechanical functionality properties. Advantages in use of SF, expecially, in biomedical applications include the excellent mechanical perfomance, slow auto-degradation process and good adaptability in hydrophilic environment. The synthesis of β-sheet domains with hydrophobic properties prevents the penetration of water and the slowing of protease activity with resulting in slow biodegradation of silk fibroin *in vivo* [150]. The silk fibroin has aroused increasing interest in the biomedical field for its stability in the tissue microenvironment thanks to excellent levels of biocompatibility, flexibility, morphological and mechanical properties, mentioned above. Already some groups have achieved excellent results in different areas tissue engineering in pharmacological application, since the release of the drug to optical equipment. To meet the various requirements of different applications, researchers still strive to extend the capabilities of fibroin from hydrophobic material such as hydrophilic material, by filaments films, sheets, and scaffolding. Interesting developments in study of silk have improved the hydrophilic ability and hydrophobic and mechanical rigidity and depending on the requirements. However, the degradation is a serious obstacle for its applications *in vivo* and *in vitro* even if they are been proposed a series of changes in its synthesis or with the addition of enzymes to promote cell adhesion. Some studies, where is evaluated the inflammatory response *in vitro* and after inoculation of human MSC *in vivo* rat. The *in vitro* response to silk fibroin was similar to response to collagen and to the tissue culture in bi-dimensional. *In vivo*, silk showed a lower inflammatory response when compared to collagen. The inflammatory potential of the silk fibroin was evaluated, also, *in vitro* in macrophages with percentages of synthesis of tumor necrosis factor alpha (TNF-α), a cytokine indicative of an inflammatory response, at levels equal to that of the tissue culture on plastic [151]. The fields of application of fibroin-based materials are several such as bone, cartilage, tendons, and skin [152]. In recent times is demonstrated that porous scaffolds in fibroin can be used to cultures of chondrocytes 3D, where the cells able to adhere to biomaterial structure using integrin subunits α5, β1, and β3 [111]. Alternative to natural biomaterials are synthetic polymers, widely used by over twenty years in surgical sutures. However, synthetic polymers current don't possess a surface chemistry able to consent cellular development *in vivo*, even if several are their advantages in their use, indeed, synthesized to ensure a wide range of mechanical and degradative properties. Some of these synthetic polymers used in tissue engineering

are briefly described.

Poly lactic acid (PLA) is a biodegradable polyester produced by the poly-condensation of lactic acid, amonomeric precursor obtained from renewable resources. Lactic acid is a chiral molecule available in stereoisomers forms L and D. L-lactic acid is in metabolism of all animals and microorganism, is a non-toxic degradation product. The Pla has showed excellent results in terms of biocompatibility and biodegradability such as to be used with excellent results in three-dimensional scaffold for the transplantation of cell *in vivo* [153].

Poly-glycolic acid (PGA) is highly crystalline with high melting point and low solubility in organic solvents. In recent times, a research aimed has studied the improvement of properties with positive results in preparation of copolymers more hydrophilic compared to PLA.

Poly-e-caprolactone (PCL), an aliphatic polyester biocompatible and bioresorbable, used in synthesis of pharmaceutical products. It shows a low melting point of about 60°, degraded by hydrolysis of ester linkages in physiological conditions (for example in the human body), getting a lot of attention in use of scaffolds *in vivo*[154].

Poly ethylene glycol (PEG), also known as polyethylene oxide (PEO), has low immune response after implantation. Additionally, it is showed that this polymer is useful to seal cellular membranes after injury, making it useful for limiting cell death. Hydrophilic PEG hydrogels can be made through a variety of cross-linking schemes to create scaffolds with varying rates of degradation. Further, procedures chemistries, have changed in these gels to add sites for cell adhesion or (ECM), molecules that allow cells to infiltrate into these scaffolds, extending their potential applications [116].

10.1 *Methods used for scaffolds design*

Several techniques have been developed for the synthesis of polymeric matrices with synthetic and natural materials in porous structures. An overview of these different protocols, include: Electrospinning, solvent-casting, particulate-leaching, gas foaming, fiber meshes/fiber bonding, phase separation, melt molding, emulsion freeze-drying, solution casting and freeze drying [155]. Electrospinning is a technique for fabrication of nano-fibrose scaffolds. Various synthetic and natural polymers are produced with this technique, with fibers of diameters variable by nano to micrometer. They are characterized by a high surface to volume ratio and offer wide area in relation to the cellular substrate for cell attachment. In this technique, polymers are dissolved into a proper solvent or melted before being subjected to a very high voltage to overcome the surface tension and viscoelastic forces, able to forming different fibers (50 nm-30 mM) with high morphologic

similarity to the extracellular matrix of natural tissue and effective mechanical properties. These nanofibrous scaffolds can be utilized to provide a environment for cell attachment, migration, proliferation and differentiation, when compared with traditional scaffolds [156]. In general, the process of electrospinning is characterized by factors different, such as polymer molecular weight, viscosity, surface tension, conductivity, and process parameters, such as flow rate, electric potential, distance between capillary and etc. The intended use of products of Electrospinning can be used to create thin films coated with biocompatible or with surface structures that have specific architectures that allow the deposited on implantable area. One of the major materials obtained from this technique is the collagen, organized nanofibers characterized by a wide range of distribution of the pore size, high porosity, excellent mechanical strength and high surface area in volume ratios, favorable parameters for cellular activity. The Freeze-drying, is a of the fabrication technologies for polymers, based on particulate-leaching techniques, heat compression, and extrusion. However, the harsh operating conditions of these processes may limit the incorporation of bioactive proteins and cells, indeed, amounts of the chemical solvents required to production, can cause toxicity *in vivo*. This method, leading to formation of ice crystals that can sublimate from the polymer, creating a catteristica micro-architecture. Since the growth direction and the size of ice crystals are functional of temperature gradient, different structures are produced with porosity linear, radial, and/or random [157]. On the other hand, the pore size can be controlled by the freezing rate and pH in fast freezing rate that produces smaller pores.

11. Foreign body reaction to biomaterials

As indicated in the review of 2012 by Bryers et al., the device biomaterials market consist of $ 250B in 2014 where millions of humans have implanted medical devices with the objective improve the quality of life. The device failure or any complication remain high, indeed, many devices that could impact medical practice do not work at all or fail early [158]. It has been estimated that in the United States at least 20 million people have implanted a device consisting of biomaterial. The possible problems to success of a biomaterial implant is strongly associated with acute infections which can have disastrous consequences for function of device implanted. Although the risk of implant-associated infections is reduced (1-7%), these infections occur with high morbidity, to which are added to high costs for health care and prolonged use of antibiotics. The foreign body

reaction (FBR), also known as the host response vs to implanted biomaterials, defines the activity of complex series of immune modulators that are released in circulation in site implantation, including various types of cells, soluble mediators, and specific cellular interactions. The obstacles to successful implantation are mainly two; first is fibrotic encapsulation of the device (for example, the glucose sensor system) and the second the increase in release of enzymes and reactive intermediaries from activated cells capable of degrading the biomaterials[159]. The reactions from the host system vs biomaterials include specifically, interactions blood-material, the formation of provisional matrix, acute and chronic inflammatory conditions, development of granulation tissue, foreign body reaction, synthesis fibrotic tissue and development of fibrous capsule surrounding the implant. In the early stages of implant, the biomaterial is in contact with blood stream, a layer of proteins of the circle linked to the surface of the material, among these blood proteins included, such as fibrinogen (FG), fibronectin and vitronectin, opsonins such as immunoglobulin G (IgG) and C3b fragment of activated complement [158].

Following the interactions blood/material and the formation of a provisional matrix, after the onset of an inflammatory state of acute and chronic type occurs in a sequential manner. Neutrophils (polymorphonuclear leukocytes, PMN) characterize the acute inflammatory response, indeed, mast cell degranulation with histamine release and absorption of fibrinogen is noted to mediate acute inflammatory responses against implanted biomaterials [160]. In these phases, the release of interleukin-4 (IL-4) and interleukin-13 (IL-13) from mast cells, plays an important role in defining the size and the degree of subsequent development of the foreign body reaction [161]. The histamine is a fundamental regulator for the inflammatory responses and the recall of phagocytes, which in turn are in contact with implant surface supported by the action of fibrinogen. It is confirmed that receptor antagonists for histamine H1 and H2, significantly reduce the recruitment of monocytes/macrophages and neutrophils on surface of biomaterial. The acute inflammatory response against the biomaterial results in a short amount of time, in relation to the type of damage and the anatomical site of implant, usually resolves quickly, usually less than a week, depending on the extent of the damage in implantation site. Unlike the presence of mononuclear cells, including lymphocytes and plasma cells, identified the onset of chronic inflammation that is usually short-lived, limited to site of implantation. The persistence of inflammatory state acute indicate the presence of infection in which it detects the synthesis of granulation tissue with the presence of macrophages, the infiltration of fibroblasts, and neovascularization. The granulation tissue, the precursor of fibrous capsule, is composed of layers of monocytes, macrophages and foreign body giant cells that separate the implant from the microenvironment in which it was fixed [162].

The mechanisms that lead, biomaterials inert and non-toxic, to recall the accumulation of

inflammatory cells requires the activation of process such as the chemotaxis of leukocytes that they move through the towards the implant and across the endothelium. This process is supported by several types of molecular signals that acting in the implantation site, including chemoattractants for initial recruitment of inflammatory cells. In the early stages of the system is initially records the presence of small molecules such as ATP, uric acid, and bioactive lipids that are leaked from the damaged cells. Also activated platelets release several growth factors, to which is added the release various products of activated mast cells including histamine, proteoglycans, proteases and cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-16 (IL-16). Within these phases the mast cells are able to favoring the synthesis and the release of various other cytokines and chemokines, such as GM-CSF, IL-4, IL-6, IL-10, IL-13, MIP-α, and (MCP-1) macrophage inflammatory protein monocyte chemotactic protein [158]. One of aspects that stimulates the adhesion of macrophages to the biomaterial is the the interaction with integrin, the binding protein, which is assumed to provides intracellular signals that modulate the action of macrophages. Due to the action of podosomes, adhesion structures of macrophages with β2 integrins, useful for the initial adhesion of monocytes. FAK, focal adhesion kinase, of the Src family, becomes active after the interaction with integrins and it is responsible for regulating cytoskeleton dynamics of macrophages and of the assembly and disassembly of focal adhesions. A series of events are planned that finely regulate cell-cell fusion to the biomaterial where adherent macrophages merge to form foreign body giant cells. Foreign body giant cells show an antigenic phenotype similar to monocytes and macrophages. The adhesion of macrophages and foreign body giant cells to the surfaces of biomaterials creates a microenvironment between the cell membrane and the surface of the biomaterial. In a process described by Henson as frustrated phagocytosis, in which macrophages and foreign body giant cells release mediators such as reactive oxygen molecules (ROI oxygen free radicals), degradative enzymes in cell membrane and the biomaterial surface, acting as mediators of degradation [162]. Lymphocytes are an other important cellular immune population, present in implantation site during the inflammatory response The response of lymphocytes to biomaterials was not entirely studied in where many sides are still by understanding. The lymphocytes influence the behavior of macrophages to surfaces of biomaterials *in vitro*, they calling the monocytes from circulation, supporting adhesion, fusion and activation of degradative stage of macrophages. IFN-γ, IL-4 and IL-13 are other molecules secreted by lymphocyte cells that modulate the action of macrophages. For example, IFN-γ is known to activate macrophages promoting a pro-inflammatory state that is reflected in the ability to upregulation of presenting the antigen, and to favor the phagocytosis. In contrast IL-4 and IL-13 promote a response downregulatory which is indicated by an increased production of anti-inflammatory molecules such as IL-8 [163].

12. The use of biomaterial scaffold and his properties in cardiac repair

Karan et al point out a set of guidelines that cover both the macromolecular appearance that micromolecular. In fact the planning of a appropriate biomolecular support for cardiac repair must be biocompatible to the needs to respecting the size of the heart, physical properties and topography, general parameters that must be taken into account in the synthesis of the scaffold to be used on the ischemic heart tissue [127]. Also the size of the scaffold and its consistency are carefully taken into account, because for the biomaterial is indicated a porous texture and sufficiently thin. If it is less than 0.5 mm allows adequate oxygenation and nutrition within the scaffold structure-cell. Furthermore, the microstructured and nanostructured particles that make up the architecture of the scaffold must to possess small size enough to be easily implanted in the myocardium, making it possible also repeated inocula in well-defined areas with use of mini-invasive surgical techniques such as to injection catheter, with the use of echocardiography or fluoroscopy [164]. Recently, Al Kindi et al. emphasize the importance of the size scaffold for injection intra - myocardial where it has been reported an increase between 10 mm to 400 mm, the size of microspheres of alginate- poly-L-lysine-alginate was observed a four-fold increase in the retention rate of cells [165]. Topography, charge, roughness, hydrophilicity and hydrophobicity are also of great importance for the purposes of cell therapy. The hydrophobic and hydrophilic property play an important role in the kinetics of protein adsorption,regulating the native conformation of the protein and the dependent cellular functions in the new tissue. When biomaterials are exposed to biological microenvironments, ECM proteins and molecules are non- specifically adsorbed on the surface of the scaffold. An intriguing study, using in vivo models of rat, showed that the hydrophilic charge on substrate surface can influence apoptosis after the increase on surface of the scaffold with a anionic charge and hydrophilic compared to hydrophobic surfaces of the cells. Indeed, the surfaces hydrophilic and anionic have a reduced percentage of adhesion and increase the number of apoptotic cells [166]. A growing number of works show the elastic characteristics how another important parameter. Most biomaterials used for cardiac cell therapy are developed to improve the conservation of inoculated cells and their consequential survival but as regards the contractile function of the heart, the scaffold must be able to respond with a certain degree of flexibility in a manner synchronous contraction of the myocardium, transferring mechanical stimuli but also in the infarcted microenvironment, especially to the cells of the differentiation process, providing stimulus for the proper development of the contractile phenotype. The complex cell-scaffold indirectly

interacts through their integrin receptors localized on the cell surface, able to regulate the activity of the cardiac cell by automatism called mechano-transduction [167]. The rigidity may be another phenomenon strongly connected to the cell behavior and their mechanical load. Indeed, cardiac cells, smooth muscle cells and endothelial cells of blood vessels are subject to strong and continuous mechanical stress. Cellular responses to the loads depend on the load conditions that are subject to multiple factors such as magnitude, duration and frequency of the load, but also by proteins of the surrounding matrix, as well as soluble factors. Some studies have also suggested that the orientation of the neonatal rat cardiomyocytes contractility and promotes stability of contractile fibres [127]. Therefore, the recent advances in the field of tissue engineering have popularized the concept of cell stem/progenitor for using in the useful synthesis biomatrices for tissue regeneration in situ. The process described before, joined to new generation of coated biomaterials with bioactive molecules to extended release, could be able to unlock the replicative processes of differentiation in complicated tissues such as hatt cardiac. In turn, this could lead promote the recruitment of stem/progenitor cells to the damaged area, acting on the processes of cell differentiation and functional [145].

Aim

Principal purpose of regenerative medicine for the cardiac tissue is to find the best way to inoculate stem cells in a specific myocardial area damage, improving their homing, integration and survival. To achieve this goal, the field of biomaterials is important to bypass this obstacles, modulating the environment for implanted cells and enhance CSC function in the heart. Biomaterials can mimic or include naturally occurring extracellular matrix and instruct stem cell function in different ways: promoting angiogenesis, enhancing stem cell engraftment and differentiation, and accelerating electromechanical integration of transplanted cells. The aim of this thesis was to assess whether the properties of three-dimensional polymer matrices in synthetic biomaterial such as polylactic acid and in natural origin as silk fibroin, if and how influence differentiative process of stem cells cardiac c-kit +. Another point considered was been the evaluation of expression of cardiac markers and sarcomeric proteins of cells isolated, inoculated in different types of scaffold and maintained in colture for 21gg in vitro and analyzed in RT-PCR and Real-time quantitative RT–PCR analysis. Also it was analyzed the immunogenicity of the scaffold when implanted in the dorsal subcutaneous region of nude mice, nude rats and SCID mice in order a possible use in vivo in the cardiac regeneration. These experiments showed a myocardial-like differentiation, in which the CSCs acquired a muscle-like shape, with the formation of initial intercalated disks, and a striated-like myofilament organization. In results shown below highlights evidence of an higher degree of differentiation using 3D scaffold for CSCs c-Kit+ that can be induced to differentiate definitely into cardiomyocytes thanks to three-dimensional culture of the scaffold, where is possible an environment similar to a cardiac niche in vivo.

Material and Methods

1. P(d,l)LA scaffold microfabrication

Becton Dickinson (BD) three-dimensional Open-pore Poly-lactic Acid (OPLA)® scaffold. The 3D OPLA® scaffold is a synthetic polymer synthesized from D, D-L, L polylactic acid.

2. P(d,l)LA scaffold (Trento)

Poly(d,l) Lactic Acid (P(d,l)LA) scaffold were produced by salt leaching, using a porous agent between 150 and 224 µm. The polymer was dissolved in dichloromethane/dimethylformamide solvent (70:30 v/v) and then mixed with a sufficient amount of sodium chloride salt. The resultant mixture was dried under chemical hood and, in order to eliminate the residual solvent and salt, subjected to continuous washes into water for three days. Finally the sponges were frozen at -20°C and lyophilized for 2 days. The size of the porogen particles will affect the size of the scaffold pores, while the polymer to porogen ratio is directly correlated to the amount of porosity of the final structure. After the polymer solution has been cast the solvent is allowed to fully evaporate. Then the composite structure in the mold is immersed in a bath of a water for dissolving the porogen. Once the porogen has been fully dissolved a porous structure is obtained.

2.1 *Fibroin–water solution*

Bombyx mori cocoons (kindly provided by Socio Lario, Cassina Rizzardi, Como, Italy) were boiled for 1.5 h in an aqueous solution containing 1.1 g/l Na_2CO_3 (10 g silk/l solution) and then for another 1.5 h in bath of water containing 0.4 g/l Na_2CO_3. The cocoons were rinsed thoroughly with distilled warm water to extract the glue-like sericin proteins and finally air-dried. The fibroin–water solution was prepared by dissolving fibroin in an aqueous solution containing 9.3M LiBr (10% w/v; Fluka Chemical) at 65 C for 2 h, followed by dialysis (for 3 days) against distilled water with a 3500Da MWCO membrane (Slyde-A-Lyzer, Pierce) to eliminate the salt. The resulting solution was concentrated by dialysing against a PEG–water solution (25%w/v) for 5 h and filtered through a 160–250mm filter (Duran Group). The final concentration of silk fibroin in the aqueous solution was approximately 15% w/v, as determined using a NanoDropND-1000 Spectrophotometer (A280).

2.2 *Freeze-dried sponges*

3D porous fibroin scaffolds were prepared using a freezedrying technique with different parameters to obtain different pore sizes and orientations. The aqueous fibroin solution was diluted to 5%, poured into polystyrene Petri dishes, frozen at –80 C and freeze-dried (sample RP). Spongeswith an orientated structurewere obtained using the 5% fibroin solution, but the Petri dish was positioned vertically to induce a temperature gradient (sample O). All obtained samples were stabilized by

treating with a methanol:water solution (80:20) for 10min, rinsed with distilled water to eliminate the solvent and freeze-dried again.

2.3 *Electrospun nets*

The aqueous fibroin solution was frozen at –20 °C, lyophilised at –50 °C and stored in a desiccator until use. The freeze-dried fibroin was dissolved in formic acid (98–99%) at 15% w/v. A 3ml syringe with a metal needle was filled with the solution and mounted in a programmable syringe pump (Model 11 Plus, Harvard Apparatus), providing a constant flow rate of 0.002 ml/min. A positive voltage (15 or 20 kV) was applied to the solution by the needle, while a rotational mandrel, which was covered with aluminium, was connected to the ground electrode. The deposition was performed at room temperature at constant rotational speed (3250 rpm) for 8 h. The resulting non-woven nets were stabilized in methanol/ H_2O (80/20 v/v) for 10 min and washed in distilled water for 2 days to remove the residual solvent (sample F).

2.4 *Field-emission scanning electron microscopy (FE-SEM)*

All scaffolds were examined with a field-emission scanning electron microscope (FE-SEM; Supra 40, Zeiss). Samples were observed after coating with gold in a reduced argon atmosphere. SEM photos, fibre diameters of the nanofibrous membranes and pore sizes of the sponges were analysed using the image visualization software ImageJ, which was developed by National Institutes of Health (http://rsb.info.nih.gov/).

2.5 *Water content*

The water uptake of the three different sponges was measured by weighing the samples in the dry state and then in the wet state. Water was added one drop at a time onto the surface of the sample, avoiding the entrance of air into the pores. Finally, the water content percentage was calculated as follows:

$$Water\ Uptake(\%)W = \frac{W_{wet} - W_{dry}}{W_{dry}} \times 100$$

3. Cell isolation and purification

In contrast with previous studies, in which cells were isolated by antibody-mediated cell sorting, our c-Kit+ cells were isolated for their proliferative potential and not for the expression of particular surface markers. Mature (150–200 gr) female Sprague–Dawley rats were anesthetized with 2% halothane in O_2 and their hearts were excised away from the chests, still beating. Each excised heart was put (HBSS) with 50 U/ml collagenase II (Gibco; Invitrogen Corp., Carlsbad, CA), and 3 mmol/L $CaCl_2$, to avoid blood coagulation into the coronary vessels. Under a laminar flow hood, the atria were separated from the ventricles and the last ones were cut into four pieces, and these pieces were put into 20 ml of HBSS with 50 U/ml collagenase II and 1.25 mmol/L $CaCl_2$ 1 heart/tube. After 10 min of incubation at 37°C in a rotating dry incubator, the solution was collected and pieces left on the bottom of the Falcon tube. Isolated cells were removed from the collagenase solution by centrifugation and put into fresh M-199 medium (BD Biosciences, Franklyn Lakes, NJ) supplemented with 20% fetal bovine serum (FBS; Biolife Italiana S.r.l., Milano, Italy), 3mg/ml fungizone, 300 mg/nml streptomycin, and 300 U/ml penicillin. Another 20 ml HBSS with 50 U/ml collagenase II and 1.25 mmol/L $CaCl_2$ were put in the Falcon tube with the four pieces, and one 10-min incubation started again. Hence, we performed repeated cycles of 10-min incubation followed by 5-min centrifugation; five to six cycles were considered a fraction, and three fractions were obtained in 4 h. The second one, got after the second five centrifugations, was filtrated with nylon net filters (Millipore) whose diameter was 80 μm .This method of tissue digestion allowed a rapid recovery of cells, a higher yield and a shorter exposure of the isolated cells to the collagenase solution. All fractions of one heart were placed into 25 cm2 flasks (Braun Medical, Inc., Bethlem, PA, http://www.bbraunusa.com) with fresh M-199 medium supplemented with 20% FBS and cultured for were obtained from only the 2nd and 3rd fractions; a good yield was 500–600,000 cells/heart within 1 week. The quality of plastic-ware greatly affected the growth potential and the selection of these cells. In fact, in Braun culture flasks c-Kit POS cells scarcely attached, showed a different morphology compared to cardiac fibroblasts (as viewed with phase contrast light microscopy) and tended to live in suspension. In particular, when cell suspension collected from tissue digestion was placed in Braun flasks, after 4–5 days, many fibroblasts adhered to the flask and a few groups of cells preferred to live on the fibroblast layer or in suspension. Braun-flask adherent cells were identified as cardiac fibroblasts. They were negative for c-Kit, CD34, nestin, GATA-4 and positive for vimentin (Fig. 2). Cells collected from the suspension and obtained by a light trypsinization were plated onto BD or Corning flasks (BD Biosciences; Corning Incorporated, Corning, NY), where they attached firmly and grew quickly. Poly-D-lysine 5mg/ml (Sigma–

Aldrich, St. Louis, MO) is a good substrate for improving adhesion. In this manner, even without a flow cytometer, we isolated a population of cells that was 80–94% positive for c-Kit.

4. Cell seeding in 2D and 3D cultures

For bi-dimensional cultures, the plasticware was coated with 0.1mg/ml poly-D-lysine in PBS. The cells were plated in 75 cm^2 flasks, grown in presence of M-199 (BD Biosciences, Franklyn Lakes, NJ, USA) grow medium, supplemented with 20% FBS (Biolife Italiana), antibiotic-antimycotic solution 1x (Lonza), L-glutamine 1x (Gibco) for 21 days, as previously described [58].

For three-dimensional cultures, cells were cultured into the Open-pore Polylactic Acid (OPLA) scaffolds (BD Biosciences) and into customized Poly(d,l) Lactic Acid (P(d,l)LA) composite scaffolds: P(d,l)LA-N, P(d,l)LA-U (synthesized with a porosity agent less than 224 μm), Silk fibroin random porosity (RP) (synthesized with a porosity agent less than 28-85μm), Fibroin electrospin nets (F) (synthesized with Fiber diameter 332±74 nm) Scaffold with fibroin porous partially oriented (O) (synthesized with a porosity agent less than 200-400 nm) kindly provided by Biotech Laboratories (University of Trento). Before cell seeding, customized scaffolds were sterilized with 96% ethanol for 1 h. Thereafter, scaffolds were equilibrated with M-199 medium supplemented with 20% FBS, and air dried. A mix of CSCs collagen type I rat tail (BD Biosciences) diluted 1:8 in M-199 grow medium was prepared on ice. Small pieces of scaffolds were embedded with cells and collagen mix and kept for half-hour at 37°C inside inserts for 24-well plates (BD Biosciences); each insert contained $4x10^5$cells. Then scaffolds were layered with collagen I in M-199 (dilution 1:8) and kept at 37°C for 3 hours in order to allow the solidification of collagen I. Afterwards, inserts were filled with growth medium and left in incubator for 21 days changing medium every two days. After incubation, the 3D cultures were fixed with acetone:-methanol:water (2:2:1), for immunofluorescence analysis, or 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in 100mM sodium cacodylate buffer (Sigma-Aldrich) for transmission electron microscopy (TEM) to enhance visualization of the cell membrane in 3D cultures, as previously described [168].

5. Flow cytometry analysis

After detaching from flasks, cells were counted and placed into FACS tubes ($2x10^5$ cells/sample). Initially, cells were washed in PBS and then fixed in cold methanol on ice for 20 minutes. After washing, cells were blocked with incubation buffer (9 PBS : 1 M-199 + 10% FBS) at room temperature for 15 minutes. Cells were incubated with primary antibodies diluted 1:200 in incubation buffer for 45 minutes at room temperature anti-c-Kit, [KAP-TK005], (Stressgen

Bioreagents, Ann Arbor, MI, USA); anti-Ly-6A/E (Sca-1), clone E13-161.7 (Santa Cruz Biotechnology, Inc); anti-MDR1, SC-71557 (Santa Cruz Biotechnology, Inc), rinsed twice in PBS and incubated with secondary antibodies diluted 1:200 in incubation buffer for 45 minutes at room temperature in the dark. After two washes in PBS, cells were analyzed with a FACSCalibur Flow Cytometer (BD Biosciences). The unstained sample was used to calibrate the analysis.

6. Immunofluorescence on CSCs

Cells cultured on poly-D-lysin (Sigma–Aldrich) coated chamber slides were fixed firstly with 4% PFA for 15 min and after with ice-cold methanol for 30 min. Antigen retrieval with 10 mM citrate buffer (pH 6.0) with 0.05% Tween 20 for 10min. After incubation with 5% Bovine Serum Albumin (Sigma–Aldrich) for 30 min, cells were incubated with primary antibodies, diluted 1:50 in PBS, overnight at 4°C [anti-c-Kit, KAP-TK005, (Stressgen Bioreagents, Ann Arbor, MI, USA); anti-Ly-6A/E (Sca-1), clone E13-161.7 (Santa Cruz Biotechnology, Inc); anti-MDR1, SC-71557 (Santa Cruz Biotechnology, Inc)]. Primary antibody was detected with: 1:50, FITC-conjugated anti-goat secondary antibody (Sigma–Aldrich). Nuclei were stained 10 minutes with 10 μg/ml Hoechst33342 staining in PBS (Invitrogen Corp., Carlsbad, CA). Cells were observed using Leica CTR5000 fluorescent microscope.

7. Selection of positive cells Sca-1

For isolation of Sca-1 positive cells was used the Do-It-Yourself kits of EasySep (Stem Cell Technology cod. 18098). It consists of a cocktail of selection previously prepared, a mixture of magnetic nanoparticles and a magnet (RoboSep®). In this mix there the antibody is anti-Ly-6A/E (Sca-1), clone E13-161.7 (Santa Cruz Biotechnology, Inc), cell specific object of the search. The antibody and mix together, form the tetrameric Antibody Complexes (TAC), bi-specific, in fact the one hand recognizes the surface antigen of the cells (in this case, Sca-1) and the other magnetic nanoparticles, as it recognizes the residues of dextran by which they are covered. The preparation of the cocktail were taken 75μl of antibody Sca-1 (corresponding to 15μg of antibody, from the initial concentration of 200μg/ml). In test tube were added 100 μL of solution A and solution B was extensively mixed and incubated at 37°C overnight. The volume of the vial was brought to 1 ml by adding sterile PBS (phosphate buffered saline). The cells are detached from the flask by trypsinization (0.05% trypsin and 0.02% EDTA) (Trypsin -EDTA solution 10x , Sigma-Aldrich) and resuspended in the recommended medium (composed of 49 ml of PBS, 2% FBS, EDTA), so as to add 10μl of cocktails previously prepared per ml of cells. The mix composed was left at room

temperature for 15 minutes. At this point, magnetic nanoparticles were added (50µl of particles per ml of cells), mixed to the solution and left at room temperature for 10 min. The entire solution was resuspended in final volume of 2.5 ml, adding the medium recommended. Finally, the tube was placed in the magnet RoboSep® and incubated for 5 min. Subsequently for manual inversion, the cells are separated due to the link of magnetic nanoparticles conjugated with anti Sca-1. The cells remain at the bottom of the tube, isolated from the supernatant. The magnet was removed and the tube were added 2.5 ml of medium recommended and mixed, and then be put back into the magnet, left 5 minutes and invert manually. The above procedure was repeated 2 more times so you have a complete selection of Sca-1 positive cells .

8. Tumorigenicity tests: Tumorigenicity of CSC was assessed *in vitro* and *in vivo*.

In vitro

CSCs, the cell line VERO (negative control) and the cell line Hep-2 (positive control) were cultured in vitro into three different 75cm^2 flasks (BD Biosciences). Later, each cell culture (1x10^5 cells) was inoculated in six-well plates containing the solid medium (MEM plus Agar Noble, BD Bioscience) and incubated at 37°C. They were observed at inverted microscope for 3 weeks. The number of colony forming units (CFU) were counted and compared to the control (Hep-2).

In vivo

Cardiac progenitor, VERO and Hep-2 cells were inoculated in vivo by intracutaneous injection. Every cell type was injected in 10 Nude mice. Animals have been observed for 21 days and any new-formation of nodules in the injection area was check. The size and weight of the neoplasia were measured to evaluate the growth after 21 days. The test was considered valid if at least 9 mice inoculated with the positive control cells (Hep-2) produced a neoplasia.

9. Animal models

Constructs of CSC and collagen I gel were injected into 5-week old athymic Nude-*Foxn1*^{nu} (Nude) mice, C.B-17/IcrHan®Hsd-*Prkdc*^{scid} (SCID) mice and athymic Hsd:RH-*Foxn1*^{rnu} (Nude) rats (Harlan Laboratories Inc., Indianapolis, USA). All experimental protocols used in this study were approved by the Animal Care and Use Committee of “Ministero della Sanità”.

10. Three dimensional construct microinjection

Constructs were injected with a 0.5-mm trocar in the dorsal subcutaneous region of Nude mice, SCID mice, and Nude rats, one injection per animal. The animals were kept in captivity for 45 days. After that period, they were sacrificed by displacement of the first cervical vertebra, and nodules were excised, fixed in a solution of acetone:methanol:water (2:2:1 v/v) and embedded in paraffin. Finally, the embedded nodules were cut into 5-µm sections and stained with haematoxylin/eosin or Masson's trichrome staining or treated for immunohistochemistry or fluorescence in situ hybridization (FISH) analysis.

11. Transmission electron microscopy

Three-dimensional cultures were grown in M-199 medium (Life Technologies) supplemented with 20% FBS (Life Technologies) for 21 days and then fixed twice. In the first step, scaffolds were fixed with 2.5% glutaraldehyde (Electron Microscopy Sciences), in 100 mM sodium cacodylate (Life Technologies) buffer (pH 7.4) for 30 min and rinsed 3 times with 100 Mm sodium cacodylate buffer. A second step of fixation was performed with 2% OsO_4 (Electron Microscopy Sciences) in 100mM sodium cacodylate buffer. After fixation, samples were rinsed twice with 100 mM sodium cacodylate buffer (pH 7.4), and dehydrated with ethanol (25%, 50%,70%,95% and 100%). After dehydration scaffolds were embedded into EPON resin (Electron Microscopy Sciences) with passages of 1:3 of resin/70% ethanol, 3:1 resin/70% ethanol for 2 h, pure resin overnight, pure resin with 0.1 ml DMP-30 (EMS) for 3h before inclusion and for 65°C at 48 h. Semi-thin sections were prepared and stained with Methylene Blue (Sigma-Aldrich).

12. Haematoxylin/eosin staining

To assess the aspect of the constructs, paraffin sections were stained with haematoxylin and eosin (H&E). Scaffolds with cells were fixed in a solution of acetone, methanol and water (2:2:1) for 12h, washed in tap water and dehydrated with ethanol at 30%, 50%, 70%, 95% and 100% v/v. After dehydration, the tissue pieces were placed in xylol for 1 h and embedded in paraffin. The paraffin-embedded tissue samples were cut into 5 mm sections. The sections were deparaffinized with xylene for 10 min and hydrated with a decreasing ethanol gradient. They were stained with haematoxylin for 4 min (Merck KGaA, Darmstadt, Germany), blocked for 15 min in tap water, treated with eosin (Merck KGaA) for 1 min and rinsed in water. The sections were dehydrated and mounted using Canada balsam (Panreac Química S.L.U., Barcelona, Spain). Images were captured using a Leica DM5000 microscope.

13. Masson's trichrome staining

The tissue sections were placed in distilled water and subsequently stained with hematoxylin ferric (Bio-Optica, Milan, Italy) for 10 minutes. Subsequently, without carrying out any washing, on the sections was placed picric acid (Bio-Optica, Milan, Italy), and left to act for 4 minutes. Were washed quickly in distilled water and place on them the solution ponceau (Bio-Optica, Milan) for 4 minutes. Washed again in distilled water and incubated for 10 minutes with a solution of phosphomolybdic acid (Bio-Optica, Milan). Finally, the slides were drained, not washed, and stained with aniline blue (Bio-Optica, Milan, Italy) for 5 minutes. Washed in distilled water, rapidly dehydrated through ascending alcohols, passed in xylene and mounted with Canada balsam (Panreac Quimica SLU, Barcelona, Spain). The preparations were examined by optical microscope Leica DM5000.

14. Immunofluorescence on scaffold section

Scaffolds without cells were fixed in a solution of acetone, methanol and water (2:2:1) for 12h, washed in tap water and dehydrated with ethanol at 30%, 50%, 70%, 95% and 100% v/v. After dehydration, the tissue pieces were placed in xylol for 1 h and embedded in paraffin. The paraffin-embedded tissue samples were cut into 5 mm sections. The sections were deparaffinized with xylene for 10 min and hydrated with a decreasing ethanol gradient. The section were incubated with primary antibodies diluted 1:100 overnight at 4°C with IL-4 (M-19, SC-1261), IL-13 (C-19, SC-1292) and CD11b (p170, SC-6614), (Santa Cruz, CA). Bound primary antibody was detected with 1:50, FITC-conjugated goat anti-rabbit secondary antibody (Sigma–Aldrich). TOTO®-3 (T3604, Life technologies) stain is a carbocyanine dimer with far-red fluorescence similar to Alexa Fluor® 647 used as a nuclear counterstain. The sections were mounted using Fluoro Care Mountant 5 ml (Biocare medical, FP001G5) Images were captured using a ZEISS LSM710 Exciter Laser Scanning Confocal Microscope.

15. Immunohistochemistry

Immunohistochemistry was performed with a biotin-free technology using a specific probe to detect rabbit and goat antibodies followed by a horse radish peroxidase (HRP)-polymer, (MACH 1 Kit for rabbit antibodies, Biocare Medical, Concord, CA, USA) or alkaline phosphatase (AP)-polymer (Goat AP-polymer kit for goat antibodies, Biocare Medical). After deparaffination and rehydration, the tissue sections were incubated with a 3% hydrogen peroxide solution (Sigma–Aldrich) for 5 min. After incubation with the protein blocking agent Background Sniper (Biocare Medical) for 15 min, the tissue sections were incubated with the anti-CD3 antibody (rabbit polyclonal, AO452,

DAKO Denmarks A/S, Glostrup, Denmark) or IL-4 (SC-1261 Santa Cruz Biotechnology) diluted 1:100 for 1 h. The linked primary antibody was detected using MACH 1 or Goat-AP kits respectively, according to manufacturer's instructions. Biocare's Betazoid Diaminobenzidine (DAB) was used as a HRP substrate. Warp Red™ Chromogen was used as AP substrate. The sections were then washed in tap water and counterstained with haematoxylin for 1 minute. VectaMount (Vector Laboratories Inc., Burlingame, CA, USA) was used as the mounting medium.

16. Fluorescence in situ hybridization

Paraffin embedded sections were kept at 37°C O.N. and then at 60°C for 10 minutes. The sections were deparaffinised in xylol at 60°C for 10 minutes, hydrated through a graded ethanol series, and immersed for 15 minutes in citrate buffer with 1% Tween 20 for heat-induced target retrieval. Slides were cooled to RT for 5 minutes and placed in PBS with 0.1% Tween (Sigma–Aldrich). To catalyse the degradation of RNA, the slides were treated with 10 μg/μl RNase at 37°C for 1 hour. FISH was performed on sections by hybridising with a 5'-3' 6-carboxyfluorescein (FAM) labelled Custom LNA™ FISH Probe that recognises mouse centromeres but not rat centromeres (/5'-FAM/ATTCGTTGGAAACGGGA /3'-FAM/ -Exiqon A/S, Vedbaek, Denmark). The LNA probe was used at a concentration of 6.4 pmol in hybridisation buffer (50% formamide, 2x saline-sodium citrate buffer-SSC, pH 7), and the slides were denatured at 75°C for 5 minutes, followed by 1 hour hybridisation at 60°C. The sections were washed three times with 0.1x SSC at the hybridisation temperature for 5 minutes and two times with 4x SSC with 0.05% Tween 20 at 37°C, followed by a wash in PBS at RT for 5 minutes. Nuclei were counterstained with a 1:1000 dilution of TOTO-3 (Life Technologies) in PBS at RT. The sections were mounted using Vectashield (DAKO) for confocal analysis. Images were captured using a ZEISS LSM710 Exciter Laser Scanning Confocal Microscope.

17. RNA extraction and RT-PCR

CSCs were cultured into BD, P(d,l)LA scaffold or fibroin scaffolds inside inserts (2 x 10^5 cells/insert; BD Biosciences) and allowed to grow and differentiate for 21 days in M-199 medium supplemented with 20% FBS in a cell culture incubator.

Total RNA from cell grown for 21 days in bi-dimensional and three-dimensional cultures was purified by using ChargeSwitch® Total RNA Cell Kit (Life Technologies Europe BV Kwartsweg, Bleiswijk, Netherlands), whereas total amount of RNA from cardiac tissue was extracted using QuickPrep Total RNA Extraction Kit (GE Healthcare Bio-Sciences Corp., Piscataway, USA). RNA from both extractions was quantified by Qubit® RNA Assay Kits (Life Technologies). 5 ng total

RNA per reaction was reverse-transcribed using ImProm-II Reverse Transciptase Kit (Promega Corporation, Madison, Wisconsin, USA) and GoTaq Flexi DNA Polymerase (Promega Corporation) following manufacturer's instructions. cDNA was amplified using primers listed in Table1. Beta-actin was used as a positive control. RT-PCR products were separated on 3% agarose gel and visualized with UV light. The gel was stained with SYBR Safe DNA gel stain (Life Technologies). ImageJ Free software (NIH, Bethesda, MD) (http://rsb.info.nih.gov/ij/) was used to obtain a numeric value for each band intensity (Mean intensity of color x Number of pixels). Values between 1 and 150,000 were considered +/-; values between 15,000 and 50,000 were considered +; values between 50,000 and 120,000 were considered ++; values between 120,000 and 200,000 were considered +++; 0 was considered – (Table 3). The gel was stained with SYBR SafeDNAgel stain (Life Technologies). PCR fragments purified using the Nucleospin PCR and Gel Clean-up Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) were sequenced by the MWG Biotech Sequencing Service (Edersberg, Germany). Sequences were then analyzed with the BLASTn Web Tool on the NIH website (http://www. ncbi.nlm.nih.gov/BLAST/). All PCR-amplified fragments corresponded to the desired target.

Table1.Primers used for semiquantitative reverse-transcription-polymerase chain reaction

Primer	Target Sequence	Forward	Reverse
Beta actin	Actb NM_031144	5'-AGCCATGTACGTAGCCATCG-3'	5'-CTCTGAGCTGTGGTGGTGAA-3'
Laminin	Lamb3 NM_001100841.1	5'-TGTAGTTCTGCAGCCATTCG-3'	5'-CATAGGGCTGGAAGCAAGAG-3'
Integrin alpha V	Itgav_XM_003749496.1	5'-GCTGCCGTTGAGATAAGAGG-3'	5'-TGCCTTGCTGAATGAACTTG-3'
Integrin alpha 6	Itga6 NM_0537251	5'-GGTGACTTCAAAGCCTGCTC-3'	5'-AGCCAGATCAAAAACCAAGG-3'
Integrin alpha 7	Itga7 NM_030842	5'-ACCTGTGCACACCGATATGA-3'	5'-GCAGAACCCAAATTGTTCGT-3'
Integrin Beta 1	Itgb1 NM_017022	5'-GAACAGCAAGGGTGAAGCTC-3'	5'-CACAGTTGTCACGGCACTCT-3'
Fibronectin 1	Fn1 NM_019143	5'-GAAAGGCAACCAGCAGAGTC-3'	5'-CTGGAGTCAAGCCAGACACA-3'
Ilk	Ilk NM_133409	5'-AAGGTGCTGAAGGTTCGAGA-3'	5'-CAGTGTGTGTATGAGGGTTGG-3'
Fak	PtK2 NM_013081	5'-CGTGAAGCCTTTTCAAGGAG-3'	5'-TCCATCCTCATCCGTTCTTC-3'
CgA	Chga NM_021655.2	5'-GCCACCAATACCCAATCACC-3'	5'-CTTTAGGCCCAGCCTTCTCT-3'
Collagen 1 α1	Col1a1 NM_RATC1A1PR	5'-GGGTGCTAGATCAGGAGCAG-3'	5'-ATGCCCACTCCCTAACAGTG-3'
Vitronectin	Vtn NM_019156	5'-ACCCTGATTATCCCCGAAAC-3'	5'-CAAACACGGCTGACAGAGAA-3'
eNos	NOS3 NM_021838.2	5'-TGACCCTCACCGATACAACA-3'	5'-CTGGCCTTCTGCTCATTTTC -3'
Hsp90	HSP90ab1 NM_001004082	5'-GATTGACATCATCCCCAACC-3'	5'-CTGCTCATCATCGTTGTGCT-3'
Trop T2	Tnnt2 NM_012676.1	5'-CGTGAGGAGGAGGAGAACAG-3'	5'-CGGCCTCTAGGTTGTGGATA-3'
Myosin HC6 Cardiac Muscle α	Myh6 NM_017239.2	5'-CTGCTCAAGGGTCTGTGTCA-3'	5'-CGAACATGTGGTGGTTGAAG-3'
Akt	Akt2 NM_ 017093.1	5'-CAGCATCGGTTCTTCCTCAG-3'	5'-AGAACTGGGGGAAGTGTGTG-3'
Calmodulin	Calm1 NM_031969	5'-ACTGGGTCAGAACCCAACAG-3'	5'-CTTGACCTGTCCGTCTCCAT-3'
Caveolin	Cav3 NM_019155.2	5'-AGACCACTTTCACCGTCTCC-3'	5'-GCTGATGCACTGGATCTCAA-3'
CD73	Nt5e NM_021576	5'-AGAGCAAACCAGCGATGACT-3'	5'-CATTTCTGAGGAGGGGATCA-3'
CD14	Cd14 NM_021744	5'-CTTGTTGCTGTTGCCTTTGA-3'	5'-CGTGTCCACACGCTTTAGAA-3'
CD90	Thy1 NM_012673	5'-CGAACTTCACCACCAAGGAT-3'	5'-AAGGAGAGGGAAAGCAGGAG-3'

18. Real-time quantitative RT–PCR analysis

Quantitative RT–PCR (qRT–PCR) analysis was performed using GoTaq qPCR Master Mix (Promega). mRNA levels were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyl transferase 1 (HPRT1), ribosomal protein S6 kinase (S6) and b-glucuronidase (GUSB). Changes in the transcript level were calculated using the – ΔΔCT method [20]. cDNA was amplified using the primers indicated in Table S2 (see Supporting information). cDNA was amplified using the StepOnePlus™ Real-Time PCR System (Life Technologies). PCR fragments purified using the Nucleospin PCR and Gel Clean-up Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) were sequenced by the MWG Biotech Sequencing Service (Edersberg, Germany). Sequences were then analyzed with the BLASTn Web Tool on the NIH website (http://www.ncbi.nlm.nih.gov/BLAST/). All PCR-amplified fragments corresponded to the desired target.

Table2. Primers used for Real-time quantitative RT–PCR analysis

Primer	Target Sequence	Forward	Reverse
Ribosomal Protein S6	Rps6 NM_017160	5'-TCTTGTTACTCCCCGTGTC-3'	5'-CAGCCTCCTCCTTGTTTTTC-3'
GADPH	Gapdh-ps2 NG_0283015'-GAAACCCATCACCATCTTCC-3'		5'-TCCACGACATACTCAGCAC-3
Hprt1	Hprt1 NM_012583	5'-TGTCATGAAGGAGATGGGAG-3'	5'-ATCCAGCAGGTCAGCAAA-3'
Glucoronidase β	Gusb NM_017015	5'-ACCACCCCTACCACCTATATC-3'	5'-ATCCAGTAGTTCACCAGCCC-3'
Cardiac α Actin 1	Actc1 NM_019183	5'-TACCAGGATGTGTGACGACGAG-3'	5'-ATCATCACCCGCAAAGCCAG-3'
Muscle Z-Line Capping Protein α3	Capza3 NM_017164	5'-ATGAGAAGCTCATGCACCAC-3'	5'-TCAAAGAACCGGAAGTCACC-3'
Myosin-Binding Protein H	Mybph NM_031813	5'-AGCCCAGCCCTTTTGATTC-3'	5'-ACCTCCAACCGACAATCCAC-3'
Nebulette	Nebl XM_225644	5'-GAAAGGCCTCTCCGATTATG-3'	5'-TTACTCTGGTGCCTGTTGACC-3'
Telethonin-Like	Titin cap protein XM_001081394	5'-TGTCAAGTGTCTGAGGAGAACC-3'	5'-AGGTCTCATGCCTCTGTGTATC-3'
Titin	Ttn XM_575155	5'-TGGCGAGTATACCTGCAAAG-3'	5'-AGAAACTTGGCTGCCTTCC-3'
Cardiac Troponin I Type 3	Tnni3 NM_017144	5'-AGATTGCGAAGCAGGAGATG-3'	5'-AGCCCATCCAACACCAAG-3'
Tropomodulin 1	Tmod1 NM_013044	5'-TCTCCCCCACAACACTTCTC-3'	5'-CCATTTCCACTTTGTTGCCC-3'
Cardiac Troponin T Type 2	Tnnt2 NM_012676.1	5'-GCGGGCTGAACAGCAGCGTA-3'	5'-TTCAGCCAGGCGGTTCTGCC-3'
Myosin HC7 Cardiac Muscle β	Myh7 NM_017240	5'-GCGGACATTGCCGAGTCCCAG-3'	5'-AAGGCTCCAGGTCTCAGGGCTTC-3'

19. Preparation for TEM

The 3D cultures were grown in M-199 medium (Life Technologies) supplemented with 20% FBS (Life Technologies) for 21 days and then fixed twice. In the first step, scaffolds were fixed with 2.5% glutaraldehyde (Electron Microscopy Sciences) in 100mM sodium cacodylate buffer, pH 7.4 (Life Technologies), for 30 min and rinsed three times with 100mM sodium cacodylate buffer. A second fixation step was performed with 2% OsO_4 (Electron Microscopy Sciences) in 100mM sodium cacodylate buffer. After fixation, the samples were rinsed twice with 100mM sodium cacodylate buffer, pH 7.4, and dehydrated with an ethanol series (25%, 50%, 70%, 95% and 100%). After dehydration, the scaffolds were embedded in EPON resin (Electron Microscopy Sciences) with passages of 1:3 resin:70% ethanol for 2 h, 3:1 resin:70% ethanol for 2 h, pure resin overnight, pure resin with 0.1 ml DMP-30 for 3 h before inclusion and for 48 h at 65°C. Semi-thin sections were prepared and stained with methylene blue (Sigma-Aldrich) or used for immunohistochemistry.

20. Immunohistochemistry on EPON semi-thin sections

EPON-embedded TEM samples were cut into 1 mm semithin sections and dried over poly-D-lysin-coated slides unstained. The slides were then treated with 10% sodium ethoxide for 6 min at room temperature under a laboratory chemical hood to dissolve the EPON resin. After two 5 min rinses in 100% ethanol, the sections were rehydrated with an ethanol series (95%, 70%, 50%, 30%) and distilled water. After rehydration, the sections were rinsed in Tris buffer saline (TBS), pH 7.3, for 5 min and heated in a microwave oven at 700W for 5 min in a hot unmasking sodium citrate buffer, pH 6. After 30 min at room temperature, the sections were rinsed twice for 5 min in TBS and endogenous peroxidase blocked, treating the slides with 3% H_2O_2 for 5 min. Then the sections were blocked with 3% BSA in TBS for 30 min and incubated for 1 h at room temperature with the antititin antibody (SC-8724, Santa Cruz Biotechnology; diluted 1:50 in 1% BSA in TBS). After two rinses in TBS, the sections were incubated with Goat Probe (GP613G, Goat AP-Polymer Kit, Biocare Medical LLC, Concord, CA, USA) for 15 min, rinsed twice in TBS and incubated with Goat Polymer AP (GAP628G, Goat AP-Polymer Kit, Biocare Medical) for 15min. After three 5 min rinses in TBS, the sections were incubatedwith Warp Red™ chromogen (WR806 H, Biocare Medical) for 10 min and the reaction was stopped under tap water. The sections were then counterstained with haematoxylin for 30 min at 60°C; the reaction was stopped with tap water and samples kept at 60°C for 20 min. The slides were mounted with Vectamount permanent mounting medium (H-5000, Vector Laboratories, Burlingame, CA, USA).

21. Immunocytochemistry

Cells cultured on poly-D-lysine (Sigma-Aldrich)-coated chamber slides were fixed first with 4% paraformaldehyde for 30 min and then with ice-cold methanol for 30 min. Immunocytochemistry was performed using a MACH 1 Universal HRP–Polymer Detection kit (M1U539 G, Biocare Medical) for rabbit primary antibodies and Goat APPolymer Kit (GAP514 G, Biocare Medical) for goat primary antibodies, according to the manufacturer's instructions. Betazoid DAB chromogen (BDB2004 H, Biocare Medical) and Warp Red™ chromogen (WR806 H, Biocare Medical) were used as chromogens. The cellswere counterstained with haematoxylin for 2 min. Anti-c-Kit, [KAP-TK005 (Stressgen Bioreagents)] and anti-titin, SC-8724 (Santa Cruz Biotechnology) were used as primary antibodies.

22. Statistical analysis

All data are expressed as meanstandard deviation (SD). qRT–PCR data in the form of cycle thresholds (C) were exported to Microsoft Excel. The data were analysed as $2^{-\Delta CT}$ and $2^{-\Delta\Delta CT}$ compared to the level of the same mRNAs in the heart tissue. Value comparisons of the genes between treatments were calculated with one-way ANOVA with Bonferroni's multiple comparison test. Values were considered significantly different at $p<0.05$. These analyses were performed using GraphPad PrismW software (GraphPad Software, La Jolla, CA, USA).

Results

1. Material characterization

3D scaffolds Poly-lactic Acid-N and U with random porosity are prepared by dissolving the salt in order to obtain a useful combination of high compressive strength and uniform interconnected pores. The morphological and structural characteristics of the scaffolds produced by dissolution of salt depend on a number of variables including the concentration of P(d,l)LA, loading of solid particles of salt, particle size and use of the process aqueous. The distribution were observed in the porous structure with pore sizes in the range 150 and 224 μm (Figure 1 b-c). Silk porous structures with different morphologies were prepared using freeze-drying and electrospinning techniques (Figure 1d-f). To obtain porous sponges, a silk fibroin–water solution (5% w/v) was frozen under different conditions, thus affecting pore size and distribution. A relatively uniform pore morphology and distribution were observed in the porous structure obtained by freezing at –80 C (Figure 1d), with pore sizes in the range 28–85 mm. The scaffold produced by inducing a temperature gradient demonstrated a well-orientated sheet-like structure, with a bimodal pore size distribution in the range 200–400 mm (largest pores), as shown in Figure 1f. The electrospun net showed fibres that were randomly distributed in the mats, with an average fibre diameter of 33274nm (Figure 1e).The water up-take capability of the porous scaffolds was also evaluated. The scaffold with orientated lamellae showed higher water uptake (728%) compared to the sample with randomly distributed pores (503%).

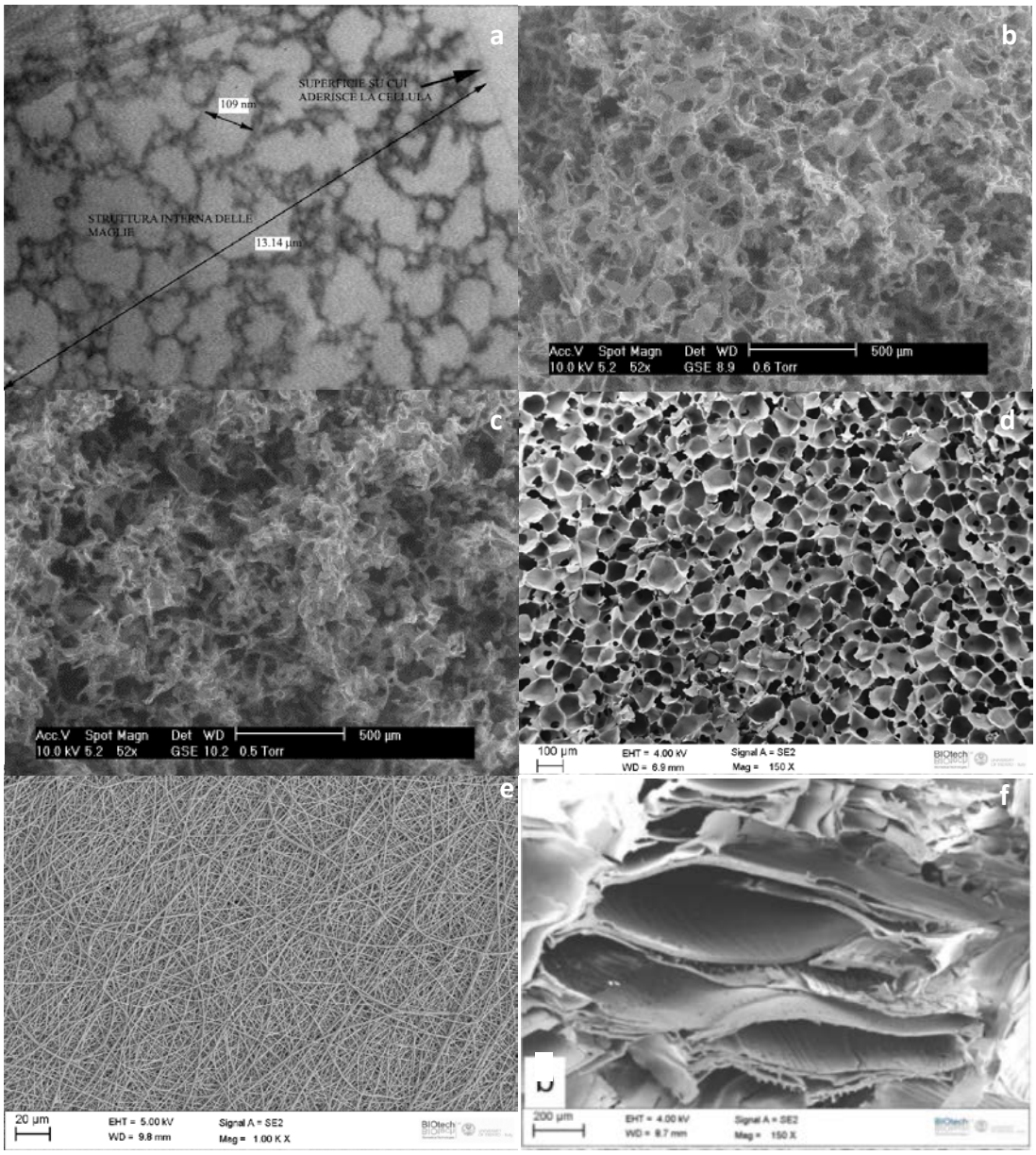


Figure 1. SEM micrographs of BD™ Three Dimensional OPLA® Scaffold 150x (a), Poly-lactic Acid -N and U random porosity Magnification = 52x (b-c). Silk fibroin scaffolds used in the in vitro and in vivo experiments: (d) scaffolds with random porosity (RP), magnification = 150x; (e) electrospun nets (F) magnifications = 5000x; (f) partially orientated scaffolds (O), magnification = 150x

2. Cardiac stem cell isolation and characterization

Cardiac immature cells were isolated from adult rats using a differential adhesion method (Figure 2). Two weeks after cell isolation, a good amount of proliferating cells was obtained. Isolated cells were characterized by flow cytofluorimetric analysis and, as shown in Figure 3a, the fraction of c-Kit positive cells varied from 61.0% to 96.7% (mean 83.23 ± SD 19.3) as previously described [101], Sca-1 positive cells varied from 74.6% to 98.2% (mean 86.93% ± 11.83), MDR-1 positive cells varied from 61.1% to 72.3% (mean 63.13% ± 5.68). The expression of the three markers was also confirmed by immunofluorescence analysis (Figure 3b-d).

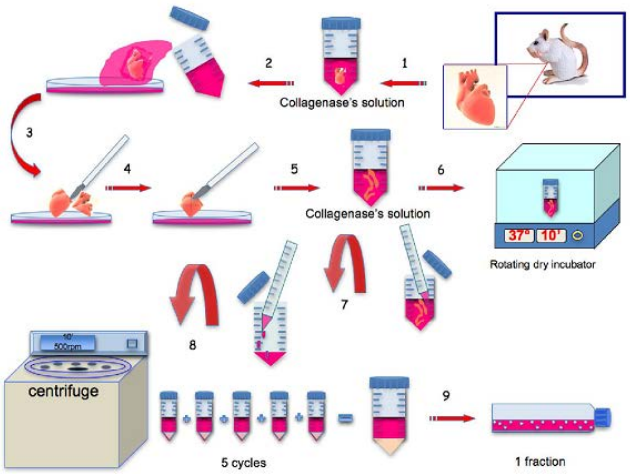


Figure 2. Digestion tissue and cell preparation

In order to investigate the tumorigenic capacity of isolated cells, specific in vivo and in vitro tests were performed. 7-10 days after the beginning of the in vitro tumorigenicity test, Hep-2 (positive control) started to replicate producing multicellular agglomerates, while the negative cells (VERO) showed atrophy. Both the in vitro and in vivo test revealed that CSCs: There were no evidences of tumors in the several organs analyzed in mice inoculated with VERO and cardiac progenitor cells (Figure 4). Plates were kept in incubator at 37°C for 3 weeks: no clusters formation, negative test (Figure 5)

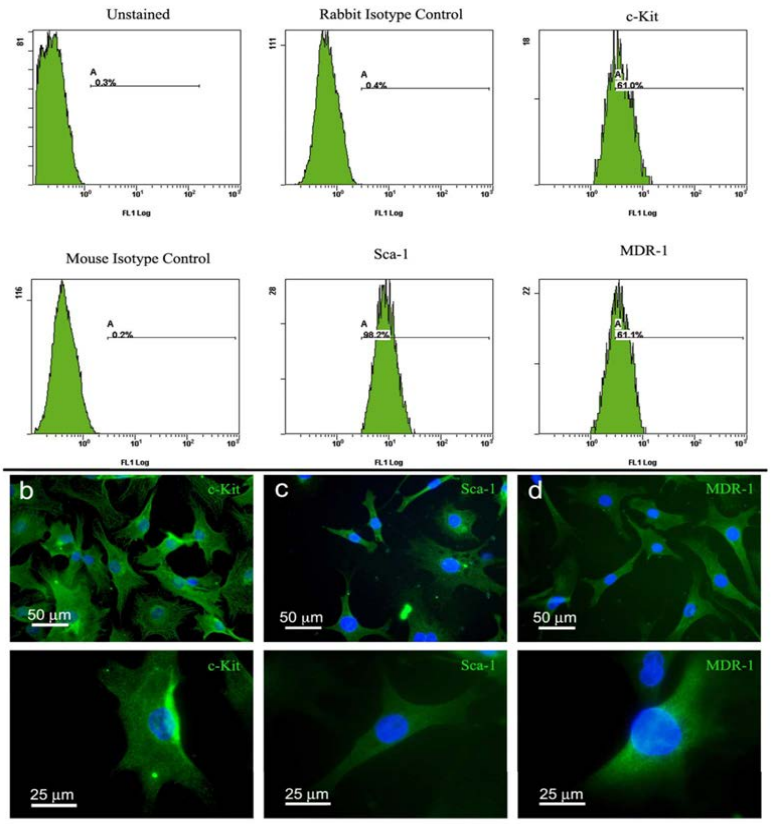


Figure 3. Cardiac progenitor cell characterization. Cytofluorimetric analysis to evaluate the percent of c-Kit, Sca-1 and MDR-1 positivity (a). Immunofluorescence analysis to c-Kit (b), Sca-1 (c) and MDR-1 (d) [111].

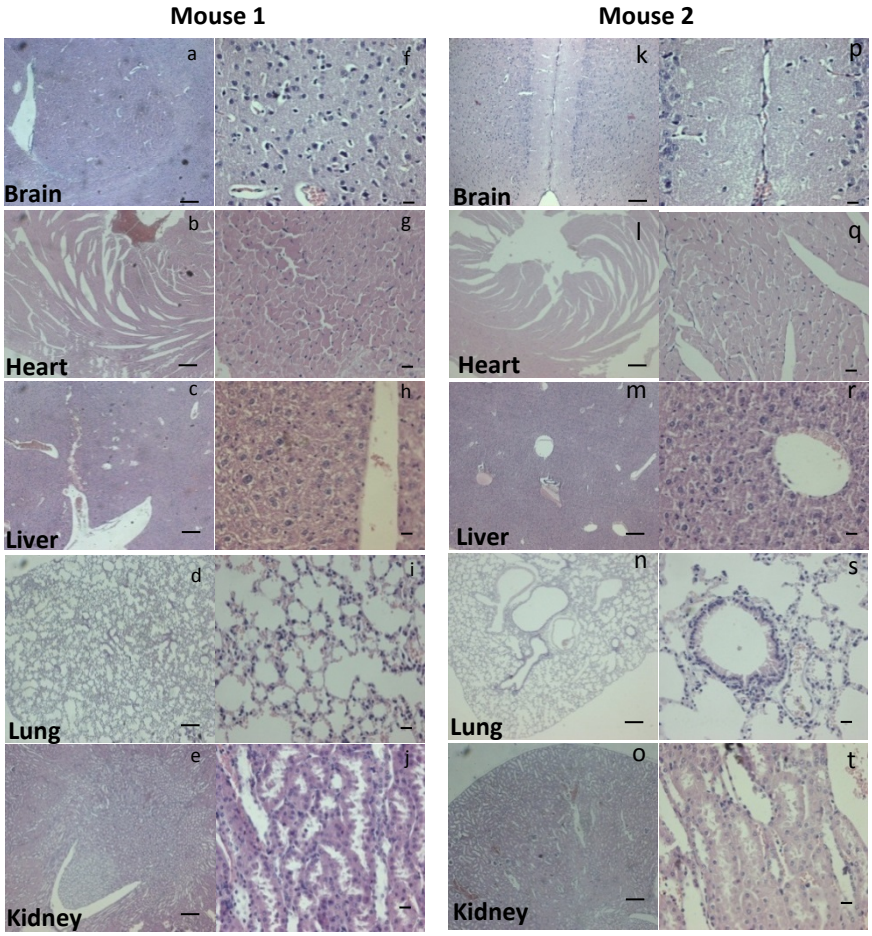


Figure 4. Histological analysis of organs after CSCs inoculation in mice. Organs of two different mice were analyzed (A-J and K-T). Bar = 200µm (a-e and k-o); bar = 20µm (f-j and p-t)

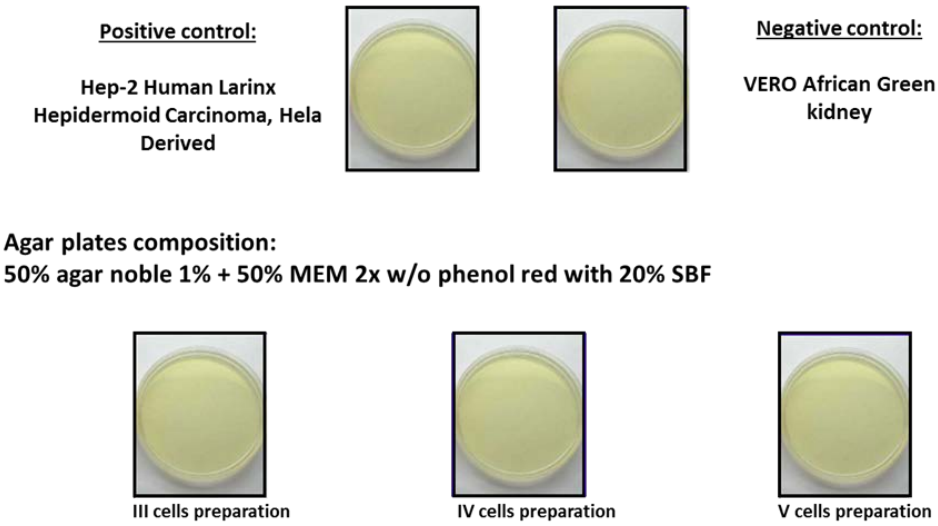


Figure 5. Tumorigenicity Test in *vitro*.

Cells selected with anti-Sca-1 antibody-coated magnetic beads showed the same expression levels of Sca-1, c-Kit and MDR-1 surface markers as non-selected cells. The surface markers CD73, CD14 and CD90 were tested by RT–PCR for each experiment to verify that there were no mesenchymal stem cells in the preparation, as previously described. These three markers were not expressed in our samples. Cells were seeded by capillarity in porous scaffolds and layered on electrospun nets. For porous scaffolds in P(d,l)LA and silk fibroin, a small drop of medium–collagen–CSCs (600000 cells/20 ml) was added to two small dry pieces of the matrices (two samples with approximately the same drop volume). For electrospun nets, the same drop was put on the top of the sheet and layered with 150 ml collagen I diluted in M-199 medium (1:8). Both porous and electrospun nets were placed into BD inserts and covered with medium. This method insured a homogeneous distribution of cells inside the scaffolds (Figure 6).

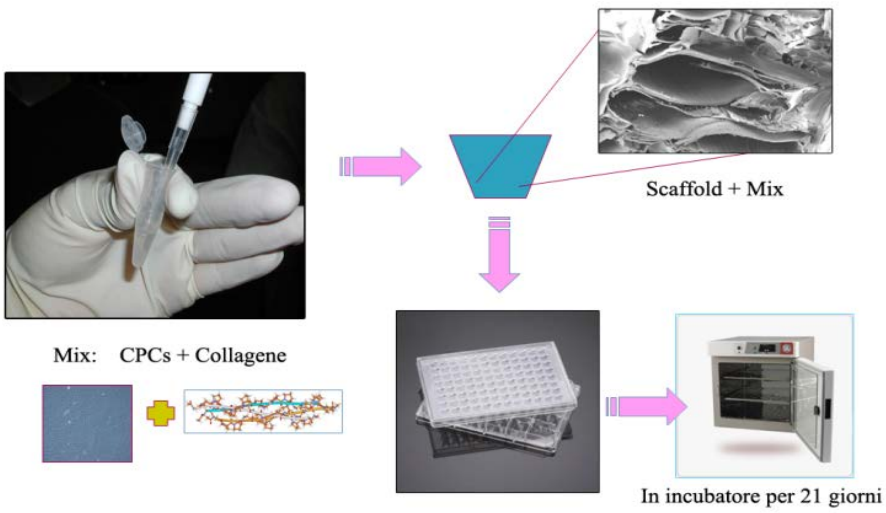


Figure 6. Preparation of the insert in CSC, Collagen and biomaterial.

3. Cells in three-dimensional culture are partially differentiated in vitro: morphological evaluations

In a previous study performed in our laboratory, scaffolds were used to study the differentiation potential of CSCs [97]. In order to understand which was the better condition for cell differentiation induction, three-dimensional cultures with BD OPLA scaffolds, customized P(d,l)LA and Silk fibroin scaffolds were performed. Morphologic features of CSC grown bi-dimensional culture are shown in Figure 7a; as displayed in the Figure 7b, cells were equally distributed inside the scaffolds and perfectly embedded in the scaffold matrix (Figure 7d). CSCs were let grow into the scaffolds

for 7, 14 and 21 days in their normal growing conditions ; as shown in Figure 7e-f after 21 days they expressed great amount of troponin T. A more accurate TEM observation showed the presence of caveolae on the cell membrane (Figure 7c). Since 3D cultures were performed embedding cells in collagen I, in order to understand whether cell differentiation was depending on the presence of the three-dimensional structure offered by scaffold or not, CSCs were cultured for 21 days in 3D matrix made by collagen I, without scaffolds. Fascinatingly, the cytoplasm appeared more granular and organized proposing a higher degree of differentiation (Figure 7d)

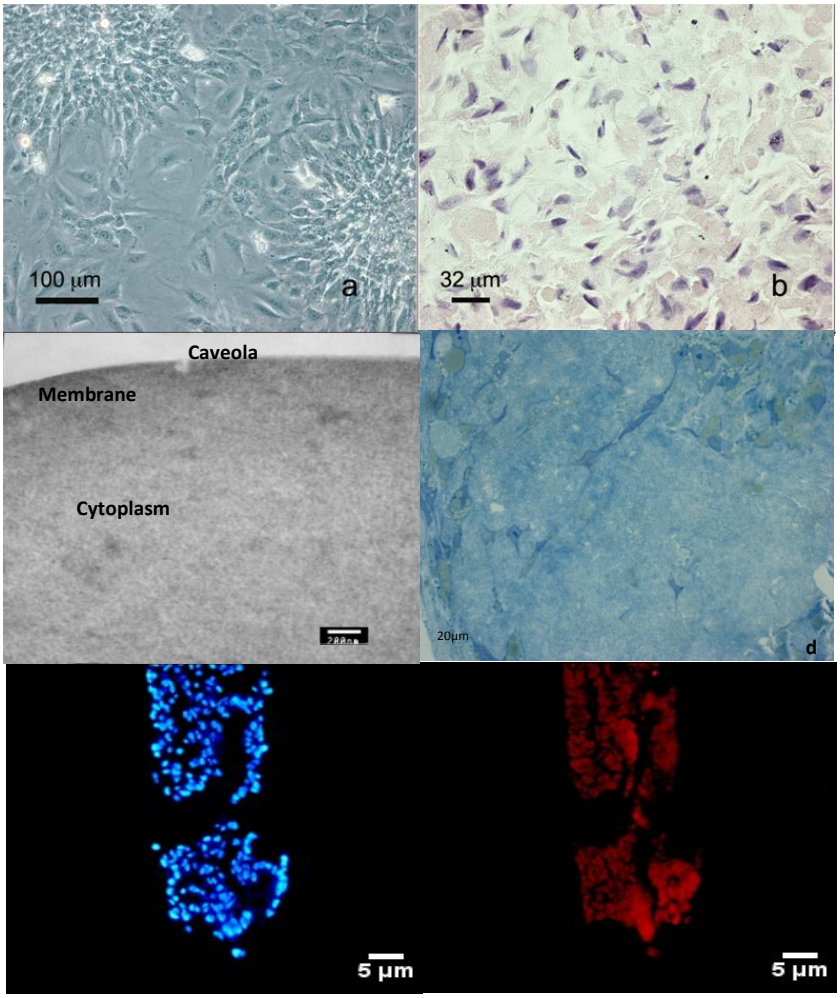


Figure 7. Phase contrast image of isolated cells in 2D cultures (a), ematoxilin/eosin staining of isolated cells cultured for 21 days inside BD OPLA scaffold (b). TEM image of the OPLA scaffold showing the cellular membrane, the cytoplasm and one caveola (c). E). Methylene blue stained semi-thin section of 21days 3D cultures inside collagen I (d). Confocal scanning microscopy showing the expression of cardiac troponin T in 21 days 3D cultures inside inside OPLA

In morphological analysis of semithin sections after 21 days, these cells grew inside the scaffold BD, P(d,l)LA and Silk fibroin scaffolds were uniformly distributed and synthesized a great quantity of ECM revealed that cells appeared huger and full with material similar to unorganized sarcomeres

or cytoskeletal proteins. They changed their morphology, adapt their cell growth to the internal structure of the scaffold (Figure 8a-e).

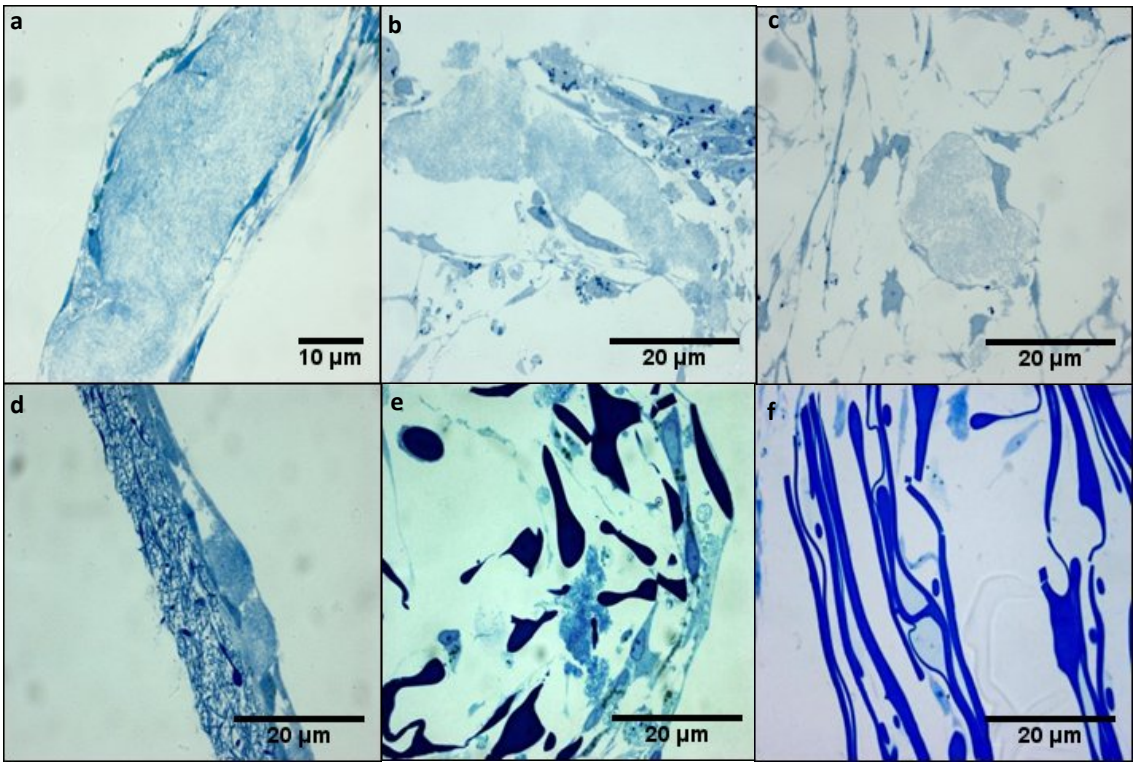


Figure 6. Cardiac stem cells partially differentiate in vitro inside porous scaffolds and collagen gel. Morphological analysis of 3D in vitro cultures. BD OPLA, P(d,l)LA-U, P(d,l)LA-N, Silk fibroin F, RP and O.

4. Testing of biomaterials: In vivo reaction to biomaterials

To investigate the possibility to use customized P(d,l)LA and fibroin scaffolds in cardiac tissue engineering, we tested the immunogenic properties of porous and partially oriented scaffolds prepared with different protocols, by injecting small pieces embedded in rat collagen I in the dorsal region of athymic Nude-Foxn1^{nu} mice for 45 day. We used collagen I as the embedding medium, because cardiac stem cells showed a higher degree of cardiac differentiation in in vitro three-dimensional cultures as previously described [97]. As shown in Figure 8, all scaffolds both P(d,l)LA and fibroin (Figure 8b-e), gave a FBR similar to the BD OPLA® scaffold (Figure 8a) previously used in our studies [97]. This FBR was characterized by the activation of macrophages with the formation of giant cells, neovascularization and encapsulation. No giant cells were visible on the surface of the F nets (Figure 8d).

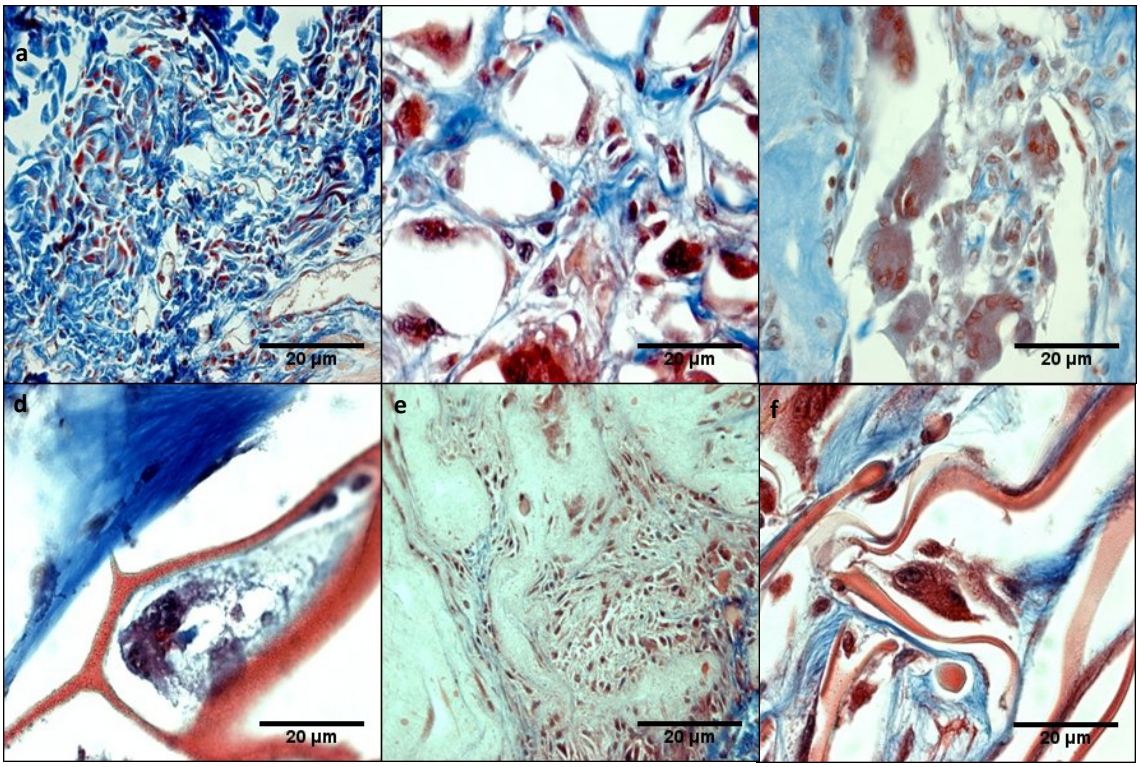


Figure 8. *In vivo* reaction to biomaterials: a morphological analysis. Small pieces (2 mm^2) of biomaterials alone were implanted subcutaneously in Nude mice to evaluate the immune response. Masson's trichrome staining of BD (a), P(d,l)LA-U (b), P(d,l)LA-N (c) RP (d), F (e), O (f).

5. Interleukin-mediated foreign body reaction

The expression of CD11b, IL-4 and IL-13 was analyzed in immunofluorescence and confocal microscopy (Figure 8a-c), confirmed the presence of giant cells and the induction of a foreign body reaction when biomaterials alone were implanted. CD11b (Figure 9a), a surface marker of giant cells [171], was expressed on the surface of macrophages adhered to the chords of the porous P(d,l)LA and fibroin samples. IL-4 and IL-13 are two interleukins that are expressed during a foreign body reaction and induce the fusion of macrophages on the surface of the scaffolds. Our confocal analysis showed the expression of both interleukins in the presence of porous P(d,l)LA and fibroin samples (Figure 9b-c).

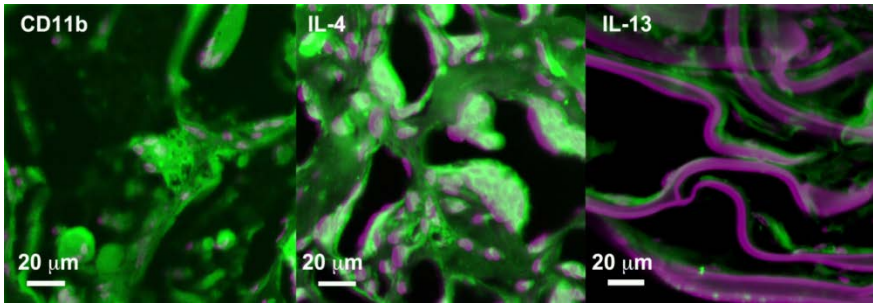


Figure 9. Foreign body reaction to biomaterials and CSCs. BD, RP and O scaffolds induced an IL-4 /IL-13-mediated foreign body reaction with CD11b+ giant cells. One image per reaction is shown because the results were identical for all three mentioned scaffolds

6. Testing of biomaterials and CSC *in vivo*

To understand the behavior of the immune system against CSC seeded within the scaffold was put in place a specific protocol where the construct with CSC, Medium and Collagen I was kept in culture within the same scaffold for a few days using medium M199 culture media supplemented with 20% FBS and then inoculated in three different animal models.

Haematoxylin/eosin stained sections of BD OPLA scaffold (A), porous P(d,l)LA-N scaffold (Figure 10b), porous P(d,l)LA-U (Figure 10c), scaffold silk fibroin porous scaffold RP (Figure 10d), silk fibroin porous partially oriented scaffold O (Figure 10e), silk fibroin partially oriented nets F (Figure 10f) show a foreign body reaction and the presence of giant cells is evident. After a 45-day incubation in vivo, the implanted silk fibroin scaffolds did not appear to have undergone any substantial biodegradation (Figure 10d-f).

Scaffold F shows a different behavior than porous scaffolds Although the material is compact and difficult to deliver in the subcutaneous region, after 45 days, there was only a light capsule and macrophages were not visible. Also fibroblasts colonized the surface of the net, but not seem to be present lymphocytes (Figure 10f).

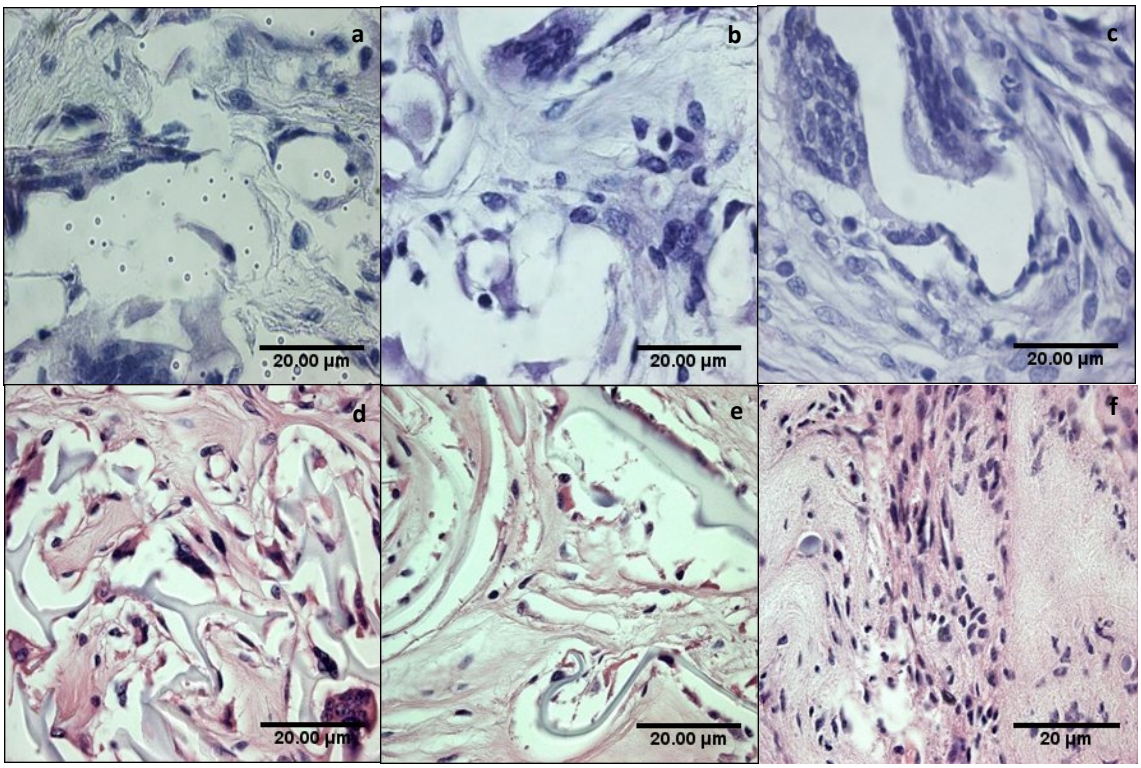


Figure 10. Testing of biomaterials and CSC *in vivo.* Histological analysis of skin biopsies of athymic Nude-Foxn1^{nu} mice for 45 day. Haematoxylin/eosin stained sections: BD OPLA scaffold (a), porous P(d,l)LA-N scaffold (b), porous P(d,l)LA-U (c), scaffold silk fibroin porous scaffold RP (d), silk fibroin porous partially oriented scaffold O (e), silk fibroin partially oriented nets F (f).

Sections counterstained with hematoxylin/eosin in nude rats (Figure 11a, b) and SCID mice (Figure11c, d) confirmed the presence of a strong degradative action against the scaffolds with random porosity in P (d, l) LA (Figure 11a, c) unlike the silk fibroin scaffolds (Figure11b, d) where we note the presence of a dense cellular infiltrate. Porous scaffolds induced the same foreign body reaction with encapsulation, dermal fibroblast invasion and giant cell formation subcutaneously in SCID mice and Nude rats.

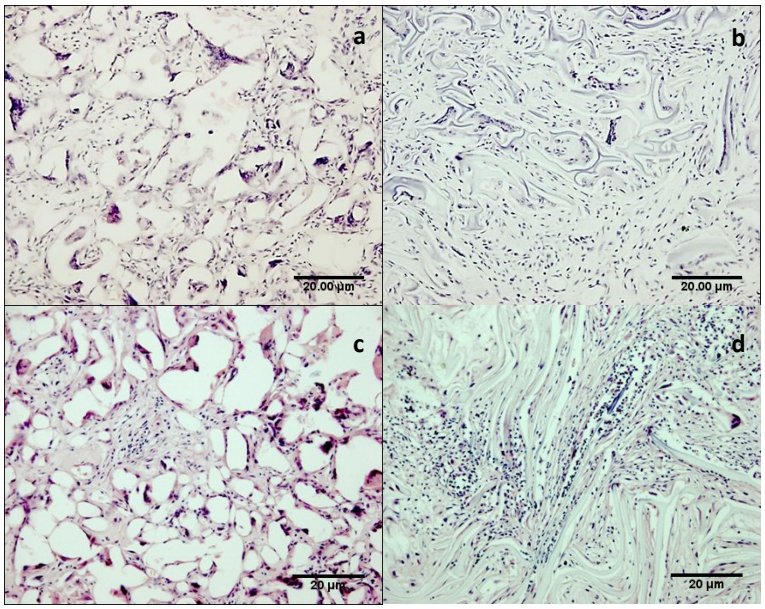


Figure 11. Testing of biomaterials and CSC in vivo. Haematoxylin/eosin stained sections of skin biopsies of athymic Nude-Foxn1nu rats in P(d,l)LA scaffold (a) and silk fibroin (b). SCID mice in P(d,l)LA scaffold (c) and silk fibroin (d) for 45 day.

7. Characterization of the immune response to the CSC *in vivo*: fate of rat cells after implantation

When CSCs were injected in the subcutanous region of Nude mice, as cells and collagen constructs (Cell+coll), they induced a nodule formation and T-cell mediated immune response (Figure 12a-d). The results were the same at 21 and 45 days. Samples underwent encapsulation (Figure 12a, c). Collagen residues were still visible in the center of the encapsulated area (Figure 12a, black arrow). Masson's trichrome staining showing the massive presence of lymphocytes (Figure 12c). and IL-4, an inflammatory cytokine, was detected inside nodules (Figure 12f)

Immunohistochemistry with the anti-CD3 antibody confirmed this hypothesis and revealed that a subpopulation of T lymphocytes was still active in Nude mice, as previously reported (Figure12d). When CSCs were implanted in the same manner in Nude rats we note the same behavior with T-cell mediated immune response (Figure 12f).

FISH provided a reliable method for characterizing the fate of the implanted Cell+coll (Figure 12h-i) and Biomaterial+CSC (Figure 12g) constructs. The mouse centromere probe reveals the presence of mouse cells, while the rat cells are unstained. After 21 and 45 days only cells positive to the

FISH probe were present, while rat donor cells were not detected in both conditions (Fig. 1G). Rat cells were destroyed by the adverse immune response before any differentiation process could star.

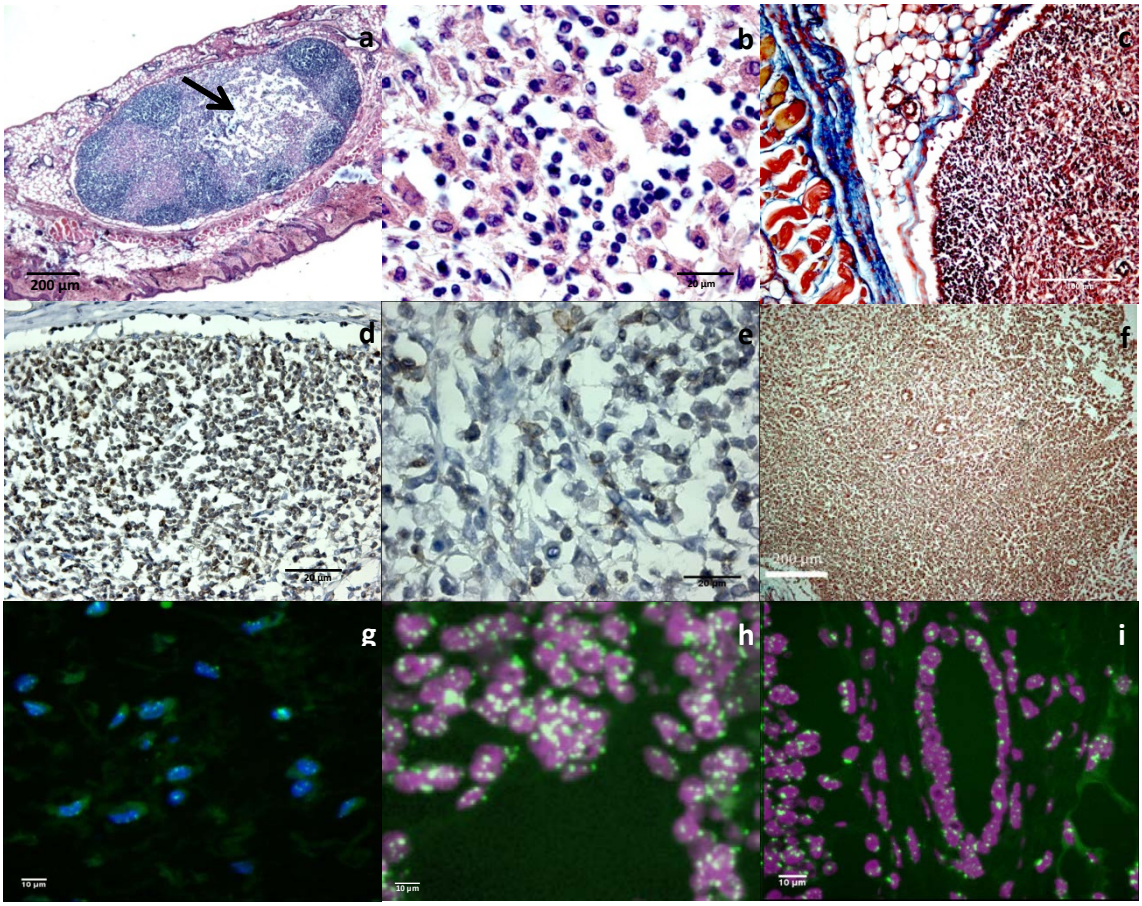


Figure 12. Fate of rat cells after implantation. (a-b), Haematoxylin/eosin staining of CSC and collagen I constructs after 45 days from implantation into Nude mice. (c), Masson's trichrome staining CSC and collagen I constructs after 45 days from implantation into Nude mice.(d), CD3 immunostaining of the subcutaneous biopsy.(e), IL-4 immunostaining of the subcutaneous biopsy. Masson's trichrome staining of CSC and collagen I. (f) after 45 days from implantation into Nude rats.(g), FISH analysis demonstrating that no rat cells survived 45 days after implantation in biomaterial+CSCs and (h-i) Cell+coll costruct

8. Extracellular matrix molecules expression in bi-dimensional and threedimensional cultures

To evaluate the differentiation state and the level of expression of mRNAs encoding ECM molecules, adhesion proteins, eNos pathway members, and structural proteins typically expressed in cardiomyocytes, we performed several RT-PCR reactions in CSCs cultured in bi and three-dimensional cultures and cardiac tissue. The expression of extracellular matrix proteins and their receptors is essential for the proper development and for keeping myocardium functional. As shown in Table 3, cells cultured for 21 days in commercial OPLA scaffolds show a low expression pattern of almost all considered genes compared with the expression pattern of cells

cultured in customized P(d,l)LA scaffolds. On the other hand, although cells in three-dimensional scaffolds have been cultured in presence of collagen I while CSCs cultured in collagen I alone expressed many proteins at a level similar to the cardiac tissue. Particularly, extracellular matrix proteins like laminin and fibronectin are more expressed in 3D-culture with collagen I compared to 3D-cultures with commercial and customized scaffold and 2D-cultures; the same happens in the case of almost all the ECM receptors and downstream signaling molecules as integrin classes, AKT, ILK and FAK. CSCs cultured in flasks expressed mainly fibronectin, calmodulin and heat shock protein 90 (HSP90). As shown in Table 1, CSCs cultured inside the different scaffolds expressed several mRNAs, but the O sample expressed the most mRNAs compared to the other constructs in particular integrin α7β1 provides a mechanical link between muscle fibres and the basement membrane in skeletal muscle, contributing to costamere assembly and eNos known to be implicated in signal transduction mechanisms affecting myocardial contractility [169]. Samples with many positive signals are most likely the most differentiated ones.

Table 3. Expression of ECM molecules and receptor, cardiac-specific genes by RT-PCR

	In vivo	*In vitro*	*Scaffold Control*		*CSC and Scaffold Natural*			*CSC and Scaffold Natural*	
	Rat cardiac tissue	2D Culture	Collagen I only	Collagen I and BD	Collagen I and RP	Collagen I and F	Collagen I and O	Collagen I and P(d,l)LA-N	Collagen I and P(d,l)LA-U
Beta-actin	+++	++	+++	+	+	+	+++	++	++
Laminin	+	-	++	-	-	-	-	-	+
Fibronectin	+	++	+++	++	+	+	++	+++	+++
Coll V alpha	++	-	+	+	-	-	+	+	-
Vitronectin	++	-	-	-	-	+	-	+	-
Integ alpha V	+	-	+++	-	-	-	++	++	+
Integ alpha 6	++	-	++	+	-	-	+	+	+
Integ alpha 7	++	-	++	+/-	+	+	++	+	-
Integ beta 1	++	-	+++	+/-	-	-	+/-	++	++
ILK	++	-	+++	+	+	+	++	+++	++
FAK	+	-	+++	-	-	-	-	+	+
AKT	++	+	+++	-	+	-	+	++	++
Calmodulin	+++	+++	+++	+++	+/-	-	+/-	+++	+++
Cav3	+++	-	+++	-	+/-	+	+/-	+	+
CgA	++	-	-	-	-	+	-	+	-
eNOS	+	-	-	-	-	-	++	-	-
HSP90	+++	++	+++	+	-	-	+	++	++
Trop T2	+++	+	+++	+++	-	-	+	+	+
Cardiac MHC	+++	-	-	-	-	-	-	-	-

9. Expression of sarcomeric proteins

To understand the degree of differentiation in the different constructs, we analysed the gene expression of ten known rat sarcomeric proteins comparing bi-dimensional (2D) samples and/or heart tissue to Cell+coll, BD OPLA (BD, our control), random porosity (RP) and partially oriented (O) fibroin porous matrices, fibroin electrospun nets (F), P(d,l)LA-U and P(d,l)LA-N with random porosity (Fig. 4).

The data were analyzed as $2^{-\Delta Ct}$ (Table 4) and $2^{-\Delta\Delta Ct}$ (Table 5). With respect to $2^{-\Delta Ct}$, only cardiac α-actinin 1 was considerably expressed in the 2D and P(d,l)LA samples. When BD and P(d,l)LA were the scaffold, the lower expression of all ten mRNAs was observed. Silk fibroin scaffolds induced good expression of all the mRNAs, Cell+coll and Sample O expressed higher levels of titin and muscle Z-line capping protein. Cell+coll and Scaffold F show higher levels Cardiac Troponin T type 2. Telethonin-like mRNA was expressed only by O. Cardiac troponin I type 3 was expressed only by silk fibroin sponges. Among the synthesized scaffold, the sample O showing the expression of all the genes analysed probably due to the mechanical properties of the scaffold that is capable of driving the differentiation process.

For the $2^{-\Delta\Delta Ct}$ method, in Table 5, only muscle Z-line capping protein, myosin-binding protein H, and titin showed a level of expression comparable to the heart tissue sample. Among these three mRNAs, titin was well expressed in silk fibroin scaffolds and Cell+coll constructs (Fig. 4b). P(d,l)LA scaffold no show significant results.

Table 4. Expression of sarcomeric proteins. Normalization with the reference genes only $2^{-\Delta Ct}$

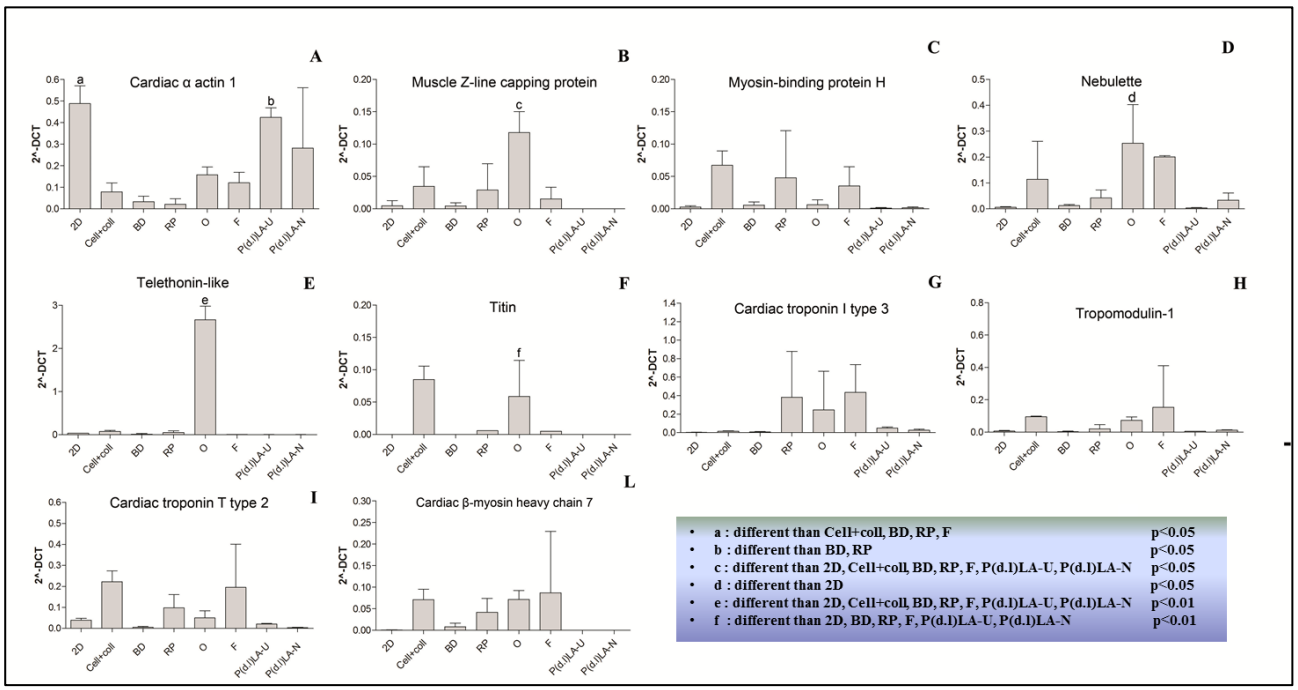
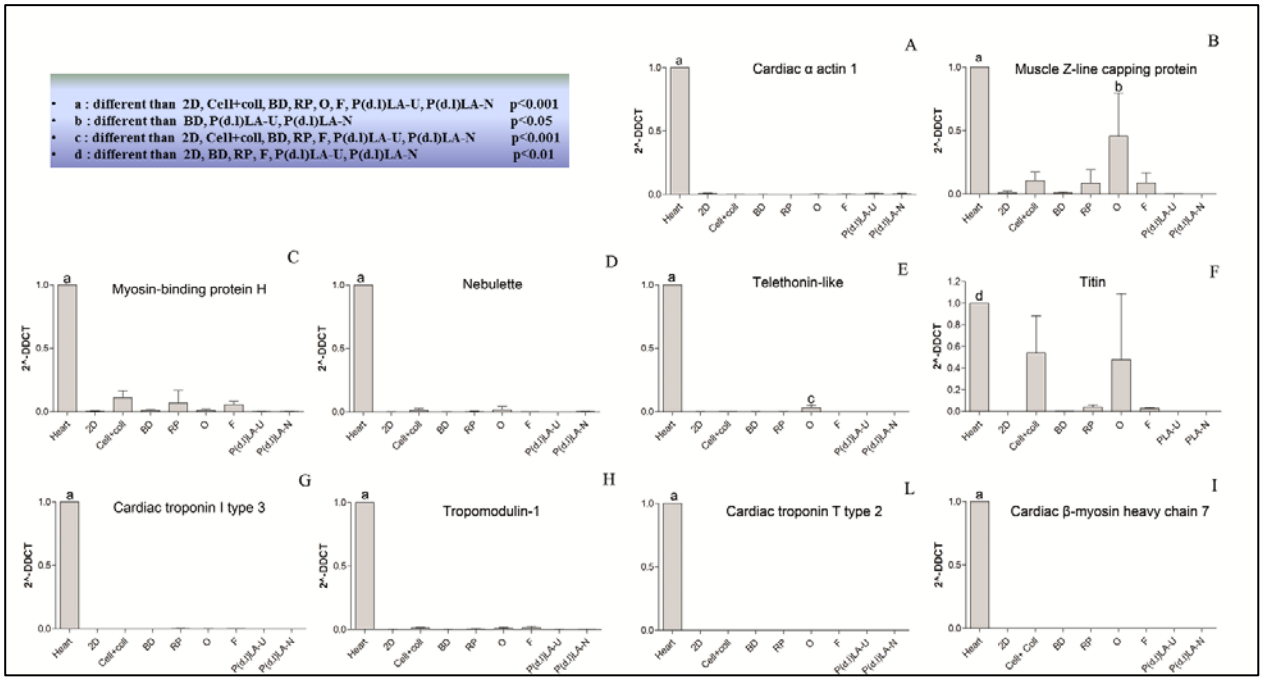


Table 5. Expression of sarcomeric proteins. Normalization with the reference genes only $2^{-\Delta\Delta Ct}$



10. Expression of Titin and characterization of Z bodies

Real-time PCR data are confirmed by the expression of titin in Cell+coll, RP, O and F, we performed immunohistochemistry on TEM–EPON embedded samples. As Figure 6 shows, titin was not expressed in 2D samples (Figure 13a) compared to c-Kit (our positive control) (Figure 13b), while it was expressed in Cell+coll (Figure 13d), RP (Figure 13f), O (Figure 13g-i) and F (Figure 13h) in 21-day samples. Negative controls of 2d and Cell+coll samples are shown in Figure 13c and e. EPON embedded constructs have been created in parallel with Real-time PCR samples. The choice of silk fibroin samples and Cell+coll is due to expression levels higher in real-time PCR analysis highlighted respect to P(d,l)LA samples. In the samples Cell+coll and scaffold O where titin was expressed, dense bodies with aligned fibres (forming Z-bodies) were visible inside the cytoplasm of the cells. In 21-day samples (Figure 14b), forming Z bodies with aligned and similar thick fibres were visible in 21 day O and Cell+coll samples; while in 72 h samples (Figure 14a) only disorganized fibres were noted both samples.

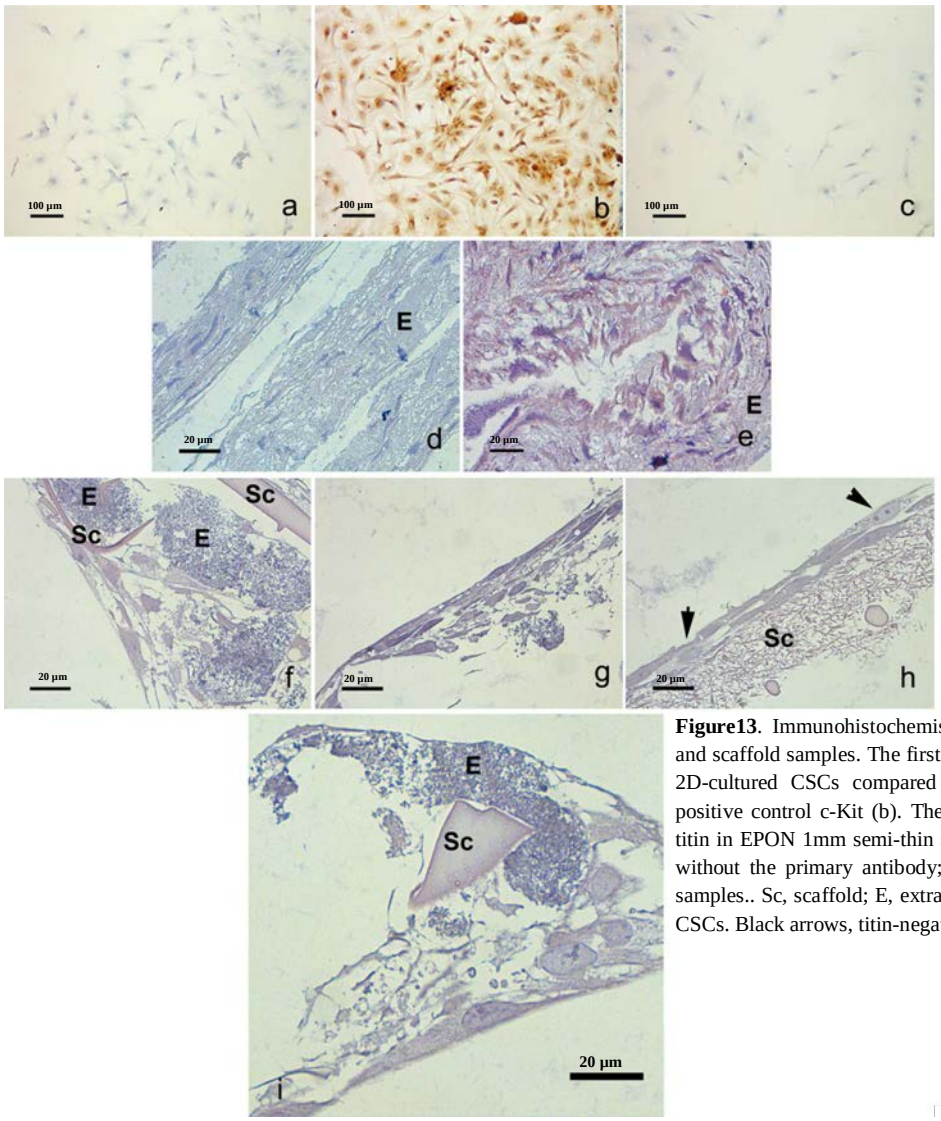


Figure13. Immunohistochemistry showing titin expression in CSCs and scaffold samples. The first row shows the expression of titin (c) in 2D-cultured CSCs compared to the negative control (a) and our positive control c-Kit (b). The other pictures show the expression of titin in EPON 1mm semi-thin sections: (d) Cell+coll, negative control without the primary antibody; (e) Cell+coll; (f) RP; (g, i) O; (h) F samples.. Sc, scaffold; E, extracellular matrix synthesized by the same CSCs. Black arrows, titin-negative cells in the F sample [111].

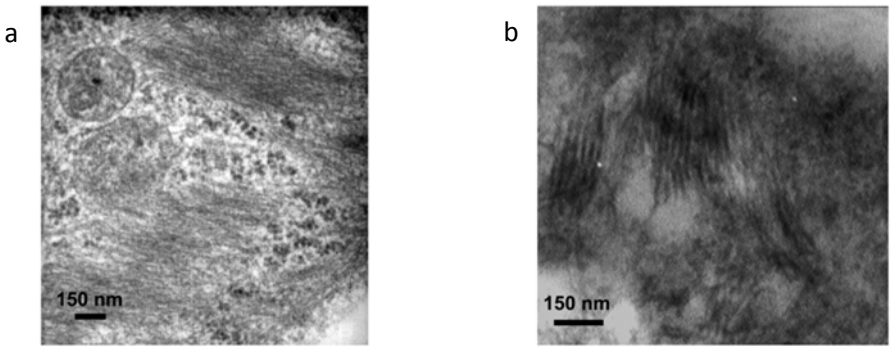


Figure14. TEM image of disorganized fibres in 72 h Cell+coll samples. (b) TEM image of forming Z-bodies in 21 day Cell+coll samples. Fibres similar to (a) and (b) were also visible in 72 h and 21 day O [111].

Discussion

The old dogma of a heart post-mitotic with myocytes formed during embryonic and fetal cardiac development and they responsible for the preservation of myocardial performance in the young, adult, old, and senescent heart has profoundly conditioned basic and clinical research in cardiology for the last three decades [68, 51]. Based on this paradigm, cardiomyocytes undergo during their vital cycle cellular to hypertrophy, cannot be replaced by the entry into the cell cycle of a subpopulation of nonterminally differentiated myocytes or by the activation of a pool of primitive cells that become committed to the myocyte lineage. However, the important work done from many researches in this field, introduce a highly dynamic perspective of the heart, where it is been possible the identification and characterization of a resident pool of stem cells that can generate myocite, and ECs and SMCs organized in coronary vessels [72]. This discovery has created a new, heated debate concerning the implementation of adult cardiac stem cells in the treatment of heart failure of ischemic and nonischemic origin. As described, the first experiment in which CSC were isolated from rat hearts 9 years ago by Anversa et al., using, as a marker, the Stem Cell Factor Receptor, c-Kit [72]. Few years later, the same marker resulted to be effective in isolating CSC also from human hearts [170]. The therapeutic effectiveness demonstrated by both human CSC in preclinical models of cardiac damage [170, 171], have given the start to the phase I clinical trials whose preliminary and promising results have been recently published [109,110]. With the current knowledge, to get a myocardial tissue in vitro from progenitor or stem cells it is considered as a hope for the future, currently the only chance of recovery of cardiac function in damaged heart is the transplantation of heart, but very often this opportunity is precluded by the lack of organ donors. The use of cardiac stem cells to repair a damaged myocardium, it is vital to find a surgical protocol that reduces the number of cells used, which maximizes the efficiency of the differentiation process and can be easily applied by surgeons. Effective treatment of heart failure is the regenerative cardiology, where the conceptual framework regarding this protocol is simple, indeed stem cells could be used in the synthesis of new myocardial tissue lost as a result of disease[172]. There are important barriers that need to be investigated such as the number of cells that needs to be replaced and the loss of cardiomyocytes, that is accompanied by the formation of a fibrous scar, resulting in decreased pumping capacity of the heart. In a typical myocardial infarct it is estimated that one billion cardiomyocytes are lost. However, so far no study has been able to find an appropriate protocol to demonstrate an equal repopulation with transdifferentiated cardiomyocytes after cell therapy. Experimental studies have noted such as transferred cells are destroyed in large part from the heart between the first 24 to 48 hours after intracoronary infusion [173]. Indeed, in work analyzed the immunological characteristics after that the cells are inoculated in vivo and adverse immune response.

Owing to the complexity and physiological requirements of functional myocardial tissue, an abundant source of cardiac myocytes alone is not enough to build a clinically useful graft. To enhance the pumping capacity of the failing heart, the cells able to remain in the heart after transplantation and survive, integrating into the native cardiac tissue. How individual differentiated cells come together to form a three-dimensional structure where the cardiomyocytes are subsequently coupled to each other in an end-to-end fashion by intercalated discs. These specialized structures, containing gap junction proteins that facilitate the spreading of the electrical impulse from one fiber of cardiomyocytes to another. Thus, the distinct cellular architecture of contractile tissue permits two key features of the physiology of the ventricular myocardium: rapid and coordinated spreading of the electrical impulse controlling the frequency of the heartbeat, and a coordinated contraction allowing the blood pumping to the tissues of the organism. Indeed, to improve cardiac function, transplanted cells must reach the correct structure with the myocardium of the host in specific way. How this organization is orchestrated isn't completely known, but published transplantation experiments show that integration does occur, raising the probability that ECM or resident cells in adult heart provide directional organization. As discussed above, the tissue engineering with grafts in myocardium with surgical implantation can prove an alternative option differently to injection of single cell suspensions into the damaged myocardium. Given the great importance of cardiac area it may prove fruitful to develop tissues structures able to integrate in anatomic damaged site. The greatest challenge in tissue engineering is provide an adequate supply of oxygen and nutrients. One approach has been to engineer thin cardiac sheets, which can then be stacked for in vivo delivery [174]. Although these layered sheets indicated some degree of electromechanical coordination and neovascularization in vivo, it isn't no clear if this approach can be optimized to yield full-thickness myocardium with an adequate blood supply. The addition of non-myocyte cellular components, such as fibroblasts and endothelial cells, leads to the formation of primitive vascular structures within engineered grafts, but the electromechanical properties are been seen not sufficient for normal functionality[175]. The scaffolds can be the alternative for increase the efficiency because a fewer cells are needed contrary to injected cells directly in the myocardial tissue that are showed dispersed and regeneration is very poor. The use of biomaterial may reduce the risk of thrombi indeed the construct could be fixed in the damaged area with specifics chirurgical sutures. It is been indicated such as the complete differentiation of CSCs with formation of myofibrils organized is very difficult, few groups of research have had succeed in the differentiation in vitro, where the integration of these cells into the damaged myocardium is been suggested such as an artefact due an effect of fusion of cells that are already present in vivo, instead of a real regeneration of the myocardium. Experiments of co-colture showed the complete

differentiation of CSCs, with the formation of organized sarcomeres reached coupled to a feeding layer of neonatal cardiomyocytes, as previously demonstrated. It has also been suggested that the particular architecture of a scaffold can be able to drive the structural cardiac organization , where the concomitant effect of soluble factors, cell–cell interaction signals and 3D structure can be determinate for complete differentiation [176]. When we proceed to use biomaterials to deliver stem cells to the site of implantation, it is opportune think to possible reactions of the immune system and the possible foreign body reaction (FBR). This phenomenon also known as the host response to implanted biomaterials, involves a complex cascade of immune modulators, including various cell types, soluble mediators, and cellular interactions [159]. The FBR is considered disastrous for patients, also if in other cases it is been studied from another point of view such as initial inflammation whence it starts a phase of tissue regeneration. The two major problems with FBR are a fibrotic capsule and the promotion of catabolic enzymes and reactive intermediates, with the activation from the systemic circulation of macrophages and giant cells, which derive from the fusion of macrophages. The presence of giant cells is driven by the release of interleukin-4 (IL-4) and IL-13 in the site of inflammation. Lymphocytes transiently appear at the implant site during the inflammatory response but the lymphocyte response to biomaterials is still unclear [162]. Lymphocytes have been shown to influence macrophage behavior at biomaterial surfaces in vitro through enhancement of monocyte/macrophage adhesion, macrophage fusion, and macrophage activation.2,3 These effects are mediated by indirect and direct lymphocyte interactions with monocytes, macrophages, and foreign body giant cells. In study of Chang et al., they demonstrate that CD4+ and CD8+ T lymphocytes were the predominant lymphocyte subtypes adhering to biomaterial surfaces and to macrophages and foreign body giant cells. The reduced of CD56+ NK cells on the biomaterial surfaces does not rule out the fact that NK cells may be interacting at the surface. Other studies have shown a predilection for CD3+ T lymphocytes to adhere onto surfaces pre-adsorbed with particular extracellular matrix proteins. The differences about the composition of scaffolds surfaces utilized has demonstrated such as the biomaterial is important for the percentage of CD4+ and CD8+ T lymphocytes that interact with adherent macrophages and foreign body giant cells. for all these reasons in the present study we focused on the induction of myogenic differentiation of adult cscs in vitro using biosynthetic and natural biomaterials such as the poly-lactic acid and the silk fibroin, in porous matrices and an electrospun net. Another important studied aspect, is the mechanisms that regulate the immunological behaviour of rat CSC and their fate after injection in animal models of nude mice, nude rats and SCID mice [163].

Cardiac stem cells from adult rat myocardium were isolated and after 10 days characterized for the expression of some stem cell markers on the cell membrane. Flow cytometric analysis revealed

such as these cells are largely positive for all the three considered markers: c-Kit, Sca-1 and MDR-1. The isolated cells did not induce cancer or masses in the several organs analysed and did not induce colony forming units even in vitro. These c-Kit+ CPCs are able to grow inside a commercially available poly-lactic acid scaffold (BD OPLA) and may synthesise cardiac proteins, such as cardiac troponin T [97]. As observed by transmission electron microscopy, these cells had a cytoplasm that engulfed with proteins even though the plasma membrane was still intact. In order to stimulate CSC differentiation, three-dimensional cultures in P(d,l)LA and silk fibroin porous scaffolds, electrospun net and collagen I matrix alone were performed. Morphological analysis of CSCs in 3D cultures showed a higher state of differentiation of CSCs in 3D cultures compared with 2D cultures; particularly, cytoplasm of CSCs grown in a collagen I matrix seemed to be more granular than cytoplasm of CSCs grown in P(d,l)LA, silk fibroin or BD scaffolds. Since 3D cultures in P(d,l)LA and silk fibroin scaffolds were prepared first embedding cells in collagen I, this evidence let us suppose that collagen I itself is responsible of differentiation. To investigate the possibility of a possible FBR to customized P(d,l)LA and fibroin scaffolds in cardiac tissue engineering, we tested porous and partially oriented scaffolds prepared with different protocols, by injecting small pieces embedded in rat collagen I in the dorsal region of athymic Nude-Foxn1nu mice, we used collagen I as the embedding medium [97]. A FBR is common for poly-lactic acid biomaterials used in orthopedic surgery, we show in our study that this reaction is undesired, because it could have a negative effect about our stem cells significantly, influencing the initiation of any differentiation process. Fibroin scaffolds derives from the silk, used to obtain structures tolerated by the host immune system with an implant able to mechanically to rule the hydrodynamic environment, critical to support cell growth in vitro and especially in vivo [177]. In our study only fibroin partially oriented fibres (sample F) did not induced a FBR. In partially oriented nets, cells from the dermis colonized the scaffold without entering the structure itself, because the spaces between fibres are of nanometric size. Only fibroblasts colonized the surface of the scaffold, and there were neither fibrous capsule nor neovascularization.

Both P(d,l)LA and silk fibroin porous scaffolds synthesized gave a FBR, characterized by giant cells, a fibrous capsule and neovascularization. The material preparation common to P(d,l)LA three-dimensional scaffolds is the salt leaching method. Probably this method may create an immunogenic film over the surface of the scaffold. A FBR for fibroin scaffolds has been reported only once by Dal Pra and colleagues [178], where they studied the immunogenicity of disorganized degummed fibroin fibres, reporting a mild FBR. When the scaffolds were implanted inside the nude mice, a foreign body reaction with CD11b+, giant cell formation, and IL-4 and IL-13 release seem to prevail in P(d,l)LA scaffold. The first experiment about the FBR induced in nude mice, it show

that silk fibroin is a possible biodegradable non-immunogenic biomaterial which can be used to deliver our cardiac stem cells to the site of injection not activating a violent inflammatory reaction. In the present study, we provide the first description of an adverse immune response activated by the adult CSC isolated and inoculated in Nude-Foxn1^{nu} mice, athymic Nude-Foxn1^{nu} rats and SCID mice for 45 where the presence of macrophages and giant cells is high. The fish experiment with CSC and biomaterial and only CSC only embedded in rat collagen, show as the cells are destroyed during this inflammation process, both in Nude mice and rats. Even in athymic models animal, a peripheral subpopulation of positive T-cell for CD3 it is indicated to create a massive infiltrate in the site of injection with the induce IL-4 synthesis and the complete destruction of injected adult CSC. These cells generate a T-cell-mediated immune response that is similar to that induced by HLA bearing cells [179]. Although it has been shown that the memory component of the adaptive immune response is not present in the FBR,[180] it is been showed as the presence of T and B lymphocytes at the implant site has a role for these cells.6 Indeed, Th2-polarized T cells were originally theorized to be the source of the cytokines IL-10, IL-4, and IL-13 during the FBR [181]. However, a recent report demonstrated that T-cell-deficient mice were still able to develop a FBR and that the levels of IL-13 and IL-4 were not affected by the T-cell deficiency [180].It has been demonstrated in vitro that lymphocytes that activated the development of foreign body giant cells through both indirect and direct cell-cell interactions [163]. Molecular analysis of the expression pattern of ECM coding genes, ECM receptors, downstream signaling molecules and cardiac specific genes confirmed this hypothesis. Comparison between the expression pattern of cells grown in bi-dimensional culture and in 3D BD, P(d,l)LA and silk fibroin scaffolds, cells in collagen I 3D-matrix revealed that genes encoding for ECM molecules, integrins, cardiac-specific proteins were more expressed in presence of collagen I only and inside partially orientated porous scaffolds (O). In particular, they expressed high levels of integrin subunits α7 and β1, specific for laminin, which is one of the main constituents of the cardiac ECM [182]. Moreover, it is known that integrin a7b1 provides a mechanical link between muscle fibres and the basement membrane in skeletal muscle, contributing to costamere assembly[183]. Upon maturation of myofibers, the α7β1 integrin is localized along the sarcolemma and it is concentrated at costameres [184]. The integrin is important to maintaining stable connections at these specialized sites of contact between myofibers and the extracellular matrix that are needed to receive and transmit signals and withstand contractile forces. The α7β1 integrin also functions in signal transduction as a mechanoreceptor, and it protects muscle against exercise-induced damage [185]. Thus, the α7β1integrin is essential for maintaining muscle integrity and stabilizing connections between the sarcolemma and extracellular matrix. The importance of β1 integrin expression in cardiac cell cycle has been demonstrated using a

monoclonal β1-blocking antibody, which inhibits cell cycle progression in fetal but not neonatal cells [186]. In cardiomyocytes, the effects on the cell cycle may be related to the change in the β1 isoform from A to D during the fetal to neonatal transition. The switch in integrin isoforms results from alternative processing of the cytoplasmic domain, and has been correlated with terminal differentiation in skeletal muscle cells [187]. Another β1 cytoplasmic domain variant, β1C, also has been shown to inhibit DNA synthesis and block cell cycle progression near the G1/S boundary. In cardiac muscle, little information is available on the expression pattern of integrins during the transition from active cell proliferation to terminal differentiation. Brancaccio et al. [188] studied changes in the cytoplasmic spliced variants of β1 integrin by immunostaining. Both β1A and β1D were present in fetal mouse cardiomyocytes, but in adult cardiomyocytes only the β1D isoform could be demonstrated. Inactivation of integrin β1A and β1D subunits in knockin/knockout studies has been shown to mildly affect cardiac morphology but provided no information related to effects on the cell cycle. The work of Maitra et al. [189]defines the pattern of β1 and α integrin isoforms in developing rat primary cardiomyocytes and correlating the changes to the expression with cell cycle exit. As a consequence of the switch in β1 isoforms from A to D, there is a change in the associated α partners. Immunoprecipitation with β1 antibody from heart extracts showed that the β1-subunit is associated with α3, α5, α7B, and αV monomers [187]. When immunoprecipitation was carried out with an antibody specific for β1D, however, the β1 isoform was only associated with α7B [187]. The eNos gene expression is evaluated in O sample only, this result is important because eNos generate of NO and it is activated by various physiological mediators, including mechanical shear stress [190], estrogens [191], insulin [192], acetylcholine, and other receptor-dependent agonist. The expression pattern sarcomeric proteins is bene analyzed in all 3D cultures analysed, and when comparing the real-time data with the expression levels in the cardiac tissue, only muscle Z-line capping protein (CapZ) and titin were expressed at considerable levels in both the Cell+coll and O samples. The presence of assembled Z-bodies of nascent premyofibrils support our hypothesis that c-Kit+ CSCs 3D cultured in a collagen I gel, with or without scaffold, they are able to differentiation in cardiac cell. Differences in the values of titin levels among samples probably depended on both the levels of the mRNA in the single cell and the number of titin-expressing cells in the sample. The z-discs (z-lines, z-bands) are the lateral boundaries of the basal contractile unit of the myocyte, the sarcomere. Three of the four filament systems of the sarcomere, filamentous F-actin, titin, and nebulin/nebulette, interact with Z-disc structures. the function of the z-disc is in relation its position able to sense, integrate, and transduce biomechanical stress signals [193]. The z-disc is structurally linked to the sarcolemma via the costameres, which circumferentially surround the z-disc. Both CapZ and titin are involved in the cardiac Z-disc signalling network. CapZ is the

capping molecule found on the barbed ends of F-actin filaments in the sarcomere at the Z-line. It interacts with α-actinin and regulates actin dynamics [193]. The cardiac sarcomere contains, in addition to actin-based thin filaments and myosin-based thick filaments, the giant protein titin (also called connectin). While the proteins that compose the thin and thick filaments are responsible for generating active force in the sarcomere, titin provides passive elasticity to muscle. This elastic property of titin, in addition to the extracellular matrix, defines the passive stiffness of muscle that resists sarcomere stretch. A single titin molecule spans the half sarcomere, binding to the Z-disk and to the thin filament at its N-terminus, and binding to the thick filament and M-band towards its C-terminus. Between thin and thick filament, binding domains of titin acting as a molecular spring that extends during sarcomere stretch[194]. This study demonstrates such as physicochemical stimuli, provided by 3D structure of biomaterial, in particular, about the behaviour showed by partially orientated fibroin scaffolds and biological stimuli provided by the collagen gel and by the propensity to produce ECM that cells autonomously synthesize, it is been noted that adult CSC are able differentiate to cardiac function, that properly combined in vitro to resemble the stem cell niche rules CSC commitment. Moreover, the obtained data about fibroin orientated scaffolds and collagen gels may help overcome the issues concerning cell dispersion obtained when stem cells are directly injected in the damaged myocardial area. Future investigations of the full spectrum of genes involved in sarcomeric formation and how these functionally genes interact, could improve the understanding of their role in the CSC biology also the pathways underlying to the averse immune reaction is relevant for the production of new biomaterial able to provide the correct 3D structure for myocardial regeneration.

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