University of Milano-Bicocca School of medicine and faculty of science

Isolation, characterization and *ex vivo* amplification of heart-derived c-kit progenitor cells for cellular therapy

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Ai miei genitori,

i primi a tifare per me.

TABLE OF CONTENTS

1. INTRODUCTION

1.1 Heart Failure

Cardiovascular diseases (CVD) has emerged as dominant chronic disease in western countries. In the 21st century it is also predicted to become the main cause of disability and death worldwide. In Europe every year CVD kills 2 million of people, 250.000 only in Italy. Heart failure (HF) is a condition in which an abnormality of cardiac structure or function is responsible for the inability of the heart to fill with or eject blood at a rate commensurate with the requirements of the metabolizing tissues. HF is caused by defects in myocardial contraction, that result from a primary abnormality in heart muscle. Atherosclerosis is the primary cause of this: in fact reduction of blood supply to left ventricle results in a chronic ischemia condition that causes progressive ventricular remodelling.

1.2 Ischemic Heart Disease

Ischemic heart disease occurs at virtually any age, but the frequency rises progressively with increasing age (especially men over 40 and women over 50) and with presence of important risk and lifestyle factors that predispose to atherosclerosis, such as hypertension, smoking, diabetes mellitus, obesity, genetic hypercholesterolemia, and other causes of hyperlipoproteinemia. Ischemia refers to a lack of oxygen due to inadequate perfusion of the myocardium, which

causes an imbalance between oxygen supply and demand. The most common cause of myocardial ischemia is obstructive atherosclerosis disease of epicardial coronary arteries. Upon occurrence of inadequate myocardial perfusion caused by coronary atheroslerosis, myocardial tissue oxygen tension falls and causes transient disturbances of mechanical, biochemical and electrical function of the myocardium. The abrupt development of severe ischemia is associated with almost instantaneous failure of normal muscle contraction and relaxation. When ischemia is transient, it may be associated with angina pectoris; when it is prolonged it can lead to acute myocardial infarction (MI) with the consequences of myocardial necrosis and scarring formation. Patients with ischemic heart disease fall into two large groups:

1- patients with stable angina secondary to chronic coronary artery disease

2- patients with acute coronary syndromes (ACS). This group is composed of:

a. patients with acute myocardial infarction (MI) with ST-segment elevation on their presenting electrocardiogram (STEMI)

b. patients with unstable angina (UA) and non ST-segment elevation MI (UA/NSTEMI)

c. patients with unstable angina (UA) and non ST-segment elevation MI (UA/NSTEMI)

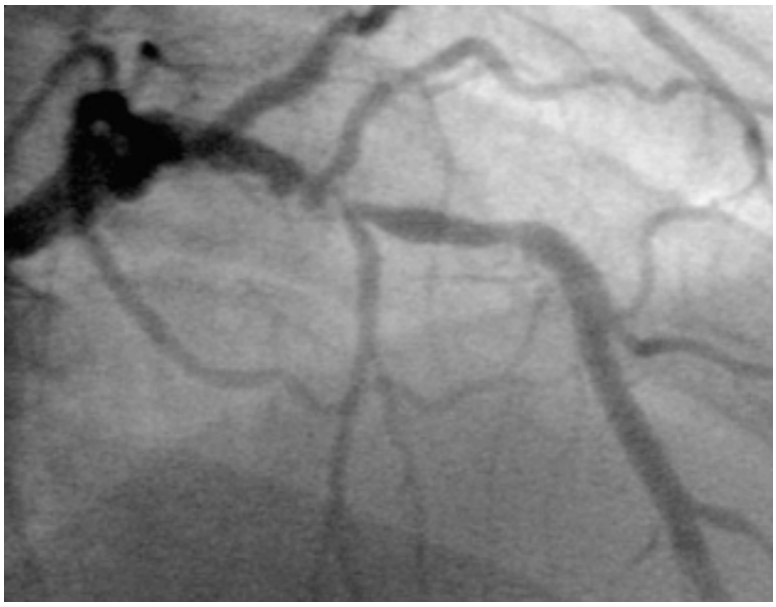


Figure.1 Coronary angiogram showing severe proximal stenosis of the left descending coronary artery due to an atherosclerotic plaque.

UA/NSTEMI is associated to a reduction in oxygen supply and/or an increase in myocardial oxygen demand superimposed on a coronary obstruction. This condition is normally an aggravation of a stable coronary artery disease and requires medical or mechanical interventions.

STEMI generally occurs when coronary blood flow decrease abruptly after a thrombotic occlusion of a coronary artery previously affected by atherosclerosis. STEMI occurs when a coronary artery thrombus develops rapidly at a site of vascular injury. In most cases, infarction occurs when an atherosclerotic plaque fissures, ruptures, or ulcerates and when conditions

favor thrombogenesis, so that a mural thrombus forms at the site of rupture and leads to coronary artery occlusion.

The extension of myocardial damage and scar formation caused by coronary occlusion depends on:

1- the territory supplied by the affected vessel

2- whether or not the vessel becomes totally occluded

3- the duration of coronary occlusion

4- the quantity of blood supplied by collateral vessels to the infarcted tissue

5- the demand for oxygen of the myocardium whose blood supply has been suddenly limited

6- native factors that can produce early spontaneous lysis of the occlusive thrombus

7- the adequacy of myocardial perfusion in the infarct zone when flow is restored in the occluded epicardial coronary artery.

1.3 Post-Infarct Ventricular Remodeling

Scar formation is an essential aspect of rapid wound healing, especially in the injured myocardium, which is under constant wall stress. Without rapid wound healing, the ischemic region would be subject to ropture, which is generally incompatible with life. Scar formation therefore offers protection from immediate danger by providing a rapid mechanical barrier. However scar tissue is largely acellular and lacks the normal biochemical properties if the host cells. This leads to electrical uncoupling, mechanical dysfunction, and loss of structural

integrity, ultimately resulting in a dilated cardiomyopathy. In the remodelling process, cardiac myocytes become longer without a proportional increase in transverse diameter, which explains the increase in chamber diameter without an increase in wall thickness. The end result of these processes is a condition called heart failure: a poorly contractile, dilated ventricular chamber that at some point can no longer adequately support the circulatory requirements of daily living. Limiting scar formation or even reversing the process could thus prove beneficial in maintaining the overall function of the heart.

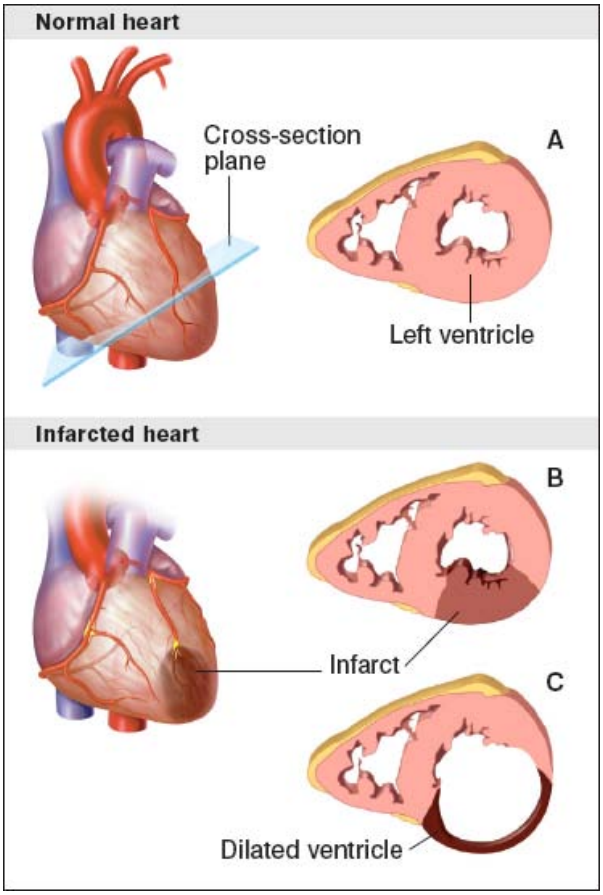


Figure 2. Post-infarct ventricular remodeling.

1.3.1 Menagement Of Ischemic Heart Failure

The ideal therapy would have the following activities: it would minimize cardiomyocytes loss by reducing cell death, promote return of ischemic dysfunctional myocardium to normal function, stimulate revascularization of the ischemic region by enhancing angiogenesis, and regenerate viable tissue by replacing that lost as a consequence of the ischemic event thereby preserving contractile function and reducing the opportunity for scarring.

Current therapies address the process of heart ischemic failure by different approaches: in the acute setting reducing the duration of the ischemia by surgically or pharmacologically removing the vascular blockage; in the chronic phase relieving infarcted heart work with drugs and devices.

LIMITATION OF THE INFARCT SIZE

The quantity of myocardium that becomes necrotic as a consequence of coronary artery occlusion is determined by factors other than just the site of occlusion. While the central zone of the infarct contains necrotic tissue that is irretrievably lost, the fate of the surrounding ischemic myocardium may be improved by timely restoration of coronary perfusion, reduction of myocardial oxygen demand, prevention of the accumulation of noxious metabolites. Up to one-third of patients with STEMI may achieve spontaneous reperfusion of the infarct-related coronary artery within 24h. Parmacological treatments (i.e. by

fibrinolysis) or by mechanical revascularization with coronary angioplasty (PCI) accelerate the reperfusion through the occluded infarct-related artery in those patients in whom spontaneous thrombolysis ultimately would have occurred.

PHARMACOLOGIC THERAPY

ACE-inhibitors decrease afterload by antagonizing the vasopressor effect of angiotensin, thereby decreasing the amount of work the heart must perform. It is also believed that angiotensin directly affect cardiac remodeling and blocking it activity can consequently slow the deterioration of cardiac function. Diuretics: diuretics therapy is indicated for relief of congestive symptoms. Beta blockers: as with ACEI therapy, the addition of a β-blocker can decrease mortality and improve left ventricular function.

SURGICAL THERAPY

Surgery may have a role in ameliorating ischemic heart failure. Patients with dysfunctional but viable myocardium (a condition named hybernation) and diffuse coronaropathy benefit from coronary bypass surgery in terms of ventricular performance and prognosis. Further, a viable option for severe remodeling due to large scars is ventricular cardiomyoplasty, although long-term benefits have been recently challenged.

RESYNCRONISATION THERAPY

More recently, a biventricular pacemaker, also known as CRT (cardiac resynchronization therapy) has been introduced as a type of pacemaker that can pace both the septal and lateral walls of the left ventricle. By pacing both sides of the left ventricle, the pacemaker can resynchronize a heart whose opposing walls do not contract in synchrony, which occurs in approximately 25-50 % of heart failure patients. CRT devices have been shown to reduce mortality and improve quality of life in patients with heart failure symptoms. CRT can be combined with an implantable cardioverter-defibrillator.

VENTRICULAR ASSIST DEVICES AND HEART TRANSPLANTATION

The final options, if other measures have failed, are heart transplantation or implantation of an articifial heart.

THE NEED FOR FURTHER THERAPEUTIC SOLUTIONS.

Despite advances in the management of acute myocardial infarction and ischemic cardiomyopathy, congestive heart failure following coronary artery disease continues to be a major worldwide medical problem. Current pharmacologic and interventional strategies fail to regenerate dead myocardium and are insufficient in a consistent subset of patients to impair ventricular remodelling. As a consequence, many patients with advanced ventricular dysfunction become refractory to

conventional pharmacologic and interventional therapy and non eligible for heart transplantation. Recently, a biological approach to this challenging medical condition has been intensively investigated. The recent discovery of a heart resident stem cells population that maintains a continuous supply of new myocytes during lifetime has raised the fascinating promise that dead myocardium may be eventually regenerated.

1.3.2 The Wound Healing Process (Endogenous Mechanism Of Myocardial Repair After Infarction)

Mammalian heart is incapable of activating an effective regeneration program in case of extensive injuries such as massive infarction. The main reparative process observed in this case is the scar formation aimed to maintain the integrity of the ventricle. Cardiac fibroblasts represent the key cell population involved in collagen deposition and scar formation. During the first period after infarction the myocardium portions affected by ischemia begins to change from an active, generating-force tissue to a passive and relatively un-elastic material. Within hours from the ischemic event, the cardiomyocytes undergo apoptosis and sarcomeric structural proteins are disrupted. Matrix Metallo-Proteinases (MMPs) activity is significantly increased 1-2 hours after infarction, leading to Extracellular Matrix breakdown [1]. After this *acute phase*, a *necrotic phase* takes place. Cardiomyocytes start dying by necrosis and inflammatory cells are recruited in the loosen ECM, releasing

factors able to recruit fibroblasts and stem cells. Particularly activated macrophages elaborate TGF-β thus promoting myofibroblast (MyoFbs) appearance. MyoFbs are a differentiated form of fibroblasts with properties of smooth muscle cells and higher levels of ECM secretion. The dead tissue is then replaced with granulation tissue, a provisional tissue rich in cFbs / MyoFbs, endothelial and inflammatory cells. In addition, angiogenesis is a critical feature at this point, with new blood vessels nourishing the granulation tissue composing cells. The formation of granulation tissue is considered as the beginning of *fibrotic phase*, characterized by high ECM remodeling and by myofibroblasts secreting elevated quantities of collagen. After a number of weeks different for different species, interstitial cells forming the granulation tissue gradually die for apoptosis and leave the chronic stage scar tissue characterized by few cellular components. Thus, the net loss of cell mass, primary due to cardiomyocyte necrosis and then to granulation tissue apoptosis causes the ventricle wall to thin and the chamber to dilate, leading to ventricular remodeling and congestive heart failure. Inhibition of granulation tissue apoptosis was interestingly found to attenuate cardiac dysfunction [2] by a mechanism likely dependent both on the increase of small vessel number and on decreased wall stress due to the presence of a higher number of MyoFbs with contractile properties.

The presence of stem cells does not prevent the inevitable evolution of the infarct with scar formation. Occlusion of a

major conductive artery or large branch results in loss of tissue in the skin, kidney, intestine, brain, liver, and reproductive organs [3], in a manner identical to the heart. This general outcome of ischemic injury is dictated by 2 crucial factors: (1) Stem cells within the infarct die, as do all other cells deprived of oxygen supply, and (2) resident stem cells cannot migrate from the viable tissue to the damaged area, home, grow, and differentiate to replace the dead cell lineages [3]. These observations provide an explanation for the lack of myocardial regeneration within the infarct and the intense myocyte formation in the nonischemic region of the ventricular wall [4-6]. Although myocardial infarcts do not regenerate spontaneously, the endogenous repair mechanisms of the heart can be manipulated and exploited to accomplish this objective.

1.4 Stem Cells And Regenerative Medicine

The clinical limitations of the efficiency of all these approaches justify the search for new therapeutic options. From experimental and human studies it appears clear that the growth reserve capacity of myocytes to respond to functional demands brought about by the loss of cells associated with aging, ischemic injury and chronic overload is limited. [5, 6]. Increasing the generation of new cells and/or preventing myocardial damage may thus represent the major goal to achieve a relevant impact on the onset and development of failure.

Regenerative medicine can be considered the next step in the evolution of organ replacement therapy. It is driven largely by the same health needs as transplantation and replacement therapies, but aims far further than traditional approaches[7]. In fact, it aims not just at replacing the malfunctioning organs, but at providing the elements required for *in vivo* repair, to devise replacements that seamlessly integrate with the living body, and to stimulate and support the body's intrinsic capacities to regenerate and to heal itself [8].

Recent research has indicated that stem cells are likely the best candidate bearing this regeneration activity. In fact, the discovery of the proliferative capacity and plasticity of various adult-derived stem cell population has sparked much interest and debate regarding their use as a potential therapy.

On a functional point of view, stem cells are undifferentiated cells that have the capacity to produce daughter cells with different activities: 1) cells that maintain constant the reservoir of stem cells and 2) progenitors that serve as a replacement for lost cells due to physiologic turnover. The first type of cells produced are, therefore, identical their mother (self-renewal) while the second type are cells having a more restricted, but a more specialized, tissue specific, potential (differentiated cells). Another issue that defines stem cells is potency; this term refers to the ability of stem cells to produce and entire organism (totipotency) multiple differentiated cell types

(pluripotency and multipotency) or only one type of differentiated cell (unipotent).

1.4.1 Self-Renewal And Clonality

The majority of somatic cells, cultured *in vitro*, display a limited number of population doublings prior to undergoing replicative arrest or senescence. This appears to be in contradiction with the seemingly unlimited proliferative capacity of stem cells in culture. Self-renewal and clonal expansion ability of certain adult stem cells can be obtained by single-cell or serial transfer into acceptable hosts. Typical is the example of hematopoietic stem cells (HSCs) that are able, even in single cells, to stably reconstitute the entire hematopoietic and lymphoid tissues of lethally irradiated mice [9] and in patients with leukemia undergoing myelosuppressive therapie [10, 11].

Clonal tracking and analysis of stem cells *in vivo* has evolved from the first work by Wu et al. [12] where radiation-induced chromosome marking was used to follow the fate of all transplanted HSCs-derived cellular clones.

1.4.2 Differentiation Potency

The issue of stem cells potency is currently most contentious part of widely accepted definition for stem cells. A stem cell type can be defined as multipotent when it can generate multiple types of differentiated cells, the latter being cells with distinct morphologies and gene expression patterns.

Progenitors are typically the descendants of stem cells; these are normally more restricted in their differentiation potential or capacity for self-renewal.

Different types of stem cells, distinguished on the basis of their differentiation potency are recognized:

- *Totipotent SCs*: in mammalians, totipotent cells are the first cells formed in a new organisms; they are produced by the initial segmentations steps of the fertilized egg. Such cells construct a complete, viable, organism as well as all embryonic annexes (placenta, umbilical chord...), necessary for embryo implantation and feeding.
- *Pluripotent Scs or Embryonic stem cells (ESCs)* : pluripotent stem cells are the descendants of totipotent cells and differentiate into cells composing the three germ layers. They thus give origin to all the tissues constituting an adult organism, including reproductive germ cells, but not to the extra-embryos tissues. Pluripotent SCs are obtained in culture from the inner mass of blastocysts and expanded *in vitro*, under proper condition (i.e, LIF stimulation), keeping their stemness properties. If re-introduced into host blastocysts they give rise to chimeric embryos.

- *Multipotent SCs*: Multipotent stem cells are mainly found during the adult life; in fact, they differentiate into a number of progenitors and differentiated cells, but only into cells belonging to restricted. An example of a multipotent stem cell is a hematopoietic cell, a blood stem cell that can develop into several types of blood cells, but cannot develop into brain cells or other types of cells.

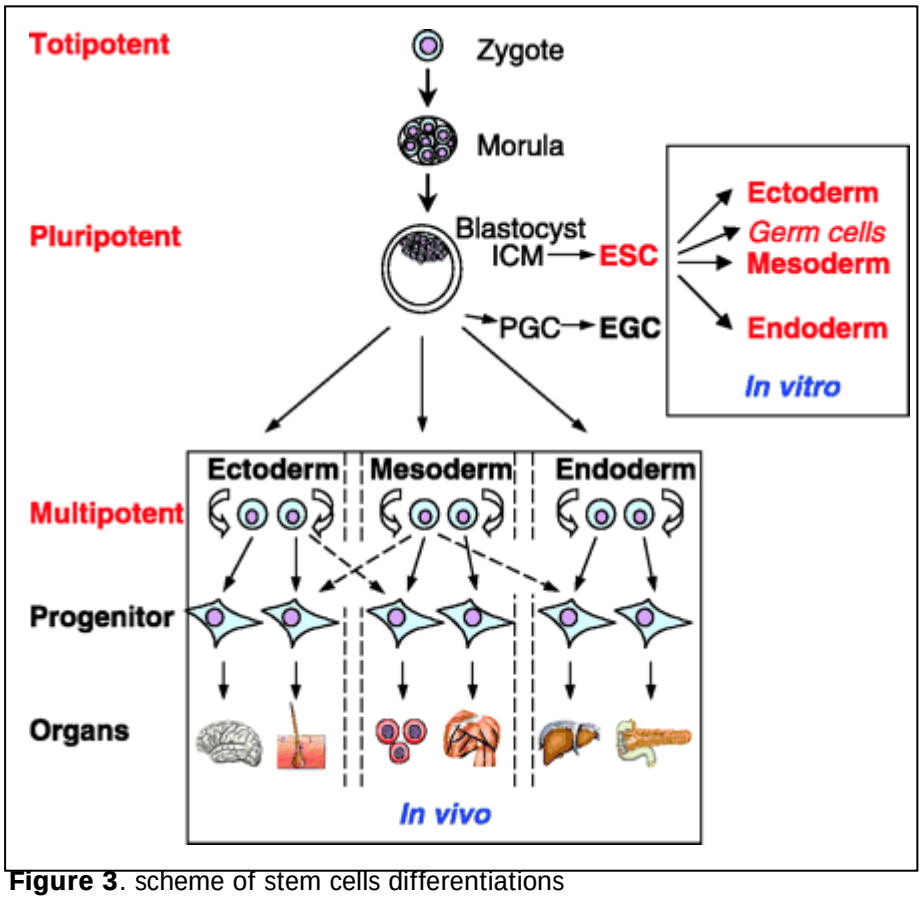


Figure 3. scheme of stem cells differentiations

1.5 Stem Cell Therapy For Cardiac Disease

The biological basis for regenerative medicine of the heart is that, in normal conditions, the heart has the ability to self renew due to the presence of specific stem cells population, called cardiac resident stem cells (CSCs). In the case of ischemic damage, CSCs activity is insufficient to replenish the high amount of lost cardiac myocytes as a consequence of

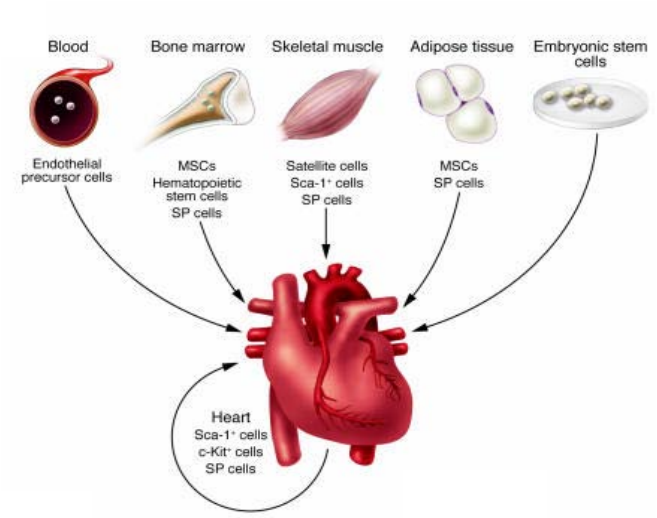


Figure 4. Sources of cells for cardiac repair. Dimmeler et al. jci2005

ischemic disease. This is due to the intrinsic unability of heart-resident stem cells to produce a massive amount of contractile cells to repair the heart, but also to death of the stem cell compartments due to ischemic insult and inflammatory

response to ischemia. Different stem cells types have been proposed for reconstructing the lost myocardium.

1.5.1 Embryonic Stem Cells

CONCEPT

Embryonic stem cells (ESCs) are stem cells directly derived from the undifferentiated cells in the mammalian embryos. As such they fulfill all requirements for stem cells: clonality, self-renewal and multi-lin eage differentiation ability.

When allowed to differentiate, usually as embryoid bodies, ESCs are able to give rise to several somatic cell lineages. Culturing the embryoid bodies in various growth media, one can drive differentiation towards a desired cell type such as cardiomyocytes. ESC-derived cardiomyocytes express cardiac specific genes, proteins and ion channels in a developmentally controlled manner and exhibit characteristics typical for heart cells in early stages of cardiac development.

The differentiation of ESCs into cardiomyocyites has been attributed to the paracrine effects of host signal, including members of the transforming growth factor-superfamily. [13]

BENEFIT-ADVANTAGES

Cardiomyocytes derived from human ESCs resemble fetal or embryonic cardiac myocytes. They exhibit spontaneous contractile activity, express cardiac transcription factors such as

GATA4, myocyte enhancer factor 2C (MEF2C) and Nkx2.5, and sarcomeric proteins such as for α-β and sarcomeric myosin heavy chain (MHC), atrial and ventricular forms of myosin light chain (MLC-2a and -2v) tropomyosin, α-actin and desmin and the hormone atrial naturietic peptide (ANP). Electrophysiological studies have shown that cardiomyocytes with atrial-, ventricular, and pacemaker-like action potentials can be derived from human ESCs *in vitro*. After differentiation into cardiomyocytes, these cells can be implanted into the corresponding organ. This approach to repair cardiac tissue after injury has been tested in preclinical studies with encouraging results [14]. In addition, human ESC-derived cardiomyocytes form structural and electromechanical connections with cultured rat cardiomyocytes [15] and functionally integrate with quiescent, recipient, ventricular cardiomyocytes to induce rhythmic electrical and contractile activities *in vitro* [16]. *In vivo* studies have shown that grafts of transplanted human ESCs- derived cardiomyocytes survive in the rat heart and grow in size seven-fold over a 4-week period [17].

DISADVANTAGES

The mechanical and electrical behavior of beating EBs indicates that human ESCs give rise to cells that display functional properties typical of embryonic human myocardium. Whether

these immature myocytes can reach adult characteristics in vivo remains an unanswered question[18].

hES cells are mainly derived from In Vitro Fertilisation (IVF) surplus embryos meaning that they are of allogenic origin, which may result in rejection by the recipient's immune systems; additionally, the isolation and use of ES cells still are topic of intense ethical debate.

For their immature phenotype and high pluripotentcy, ES cells are by definition tumorigenic; in fact, several reports have shown that transplantation of undifferentiated ESCs into the heart leads to the formation of teratomas; although these teratomas are benign *in vivo*, reasonable concerns have been raised because some cells have been found to express markers similar to those express in malignant tumors [19]. Methods to limit teratoma formation by ES cells include genetic selection of differentiated cells [20] using fluorescent protein expression driven by cardiac promoters followed, by fluorescent-activated cell sorting [21], or differentiation of ESCs *in vitro* into cardiomyocytes or endothelial cells before injection [14]. However, even though these systems is expected to reduce the risk for unwanted teratoma formation, at the moment they rely on genetic engineering methods that are not approved for use in humans. Other limitations to the use of human ESCs for therapeutic purposes include the generation of sufficient numbers of the desired cell type (typically only 1% of a differentiating culture are cardiomyocytes), the definition of

methods to deliver at best the cells in the myocardium (intramural injection, coronary injection, endocavitary injection) [22] [23], and the poor survival of hESCs derived myocytes after transplantation. In this regard, for example, Laflamme et al. [14] identified a cocktail of pro-survival factors, including BMP and activin A which limited cardiomyocyte death after transplantation and enabled consistent formation of myocardial grafts in the infarcted rat heart.

The recent discovery that it is possible to generate ESC-like cells, by reprogramming adult somatic cells with genes regulating ESC pluripotency, may resolve the ethical and immunogenic issue associated to ESCs use. Recently, Takahashi et al, and, independently, Okita et al. opened the way to the generation of pluripotent embryonic-like stem (iPS) cells obtained by a novel protocol consisting in reprogramming of somatic cells with retroviral transfection for the pluripotent genes Oct4, Sox2, C-Myc and klf4 or Lin28 [24, 25]. iPSCs exhibit the two key features of ESCs: pluripotency and self renewal, that allow, at least potentially to the generation of large amounts of differentiated cells without loosing the possibility to maintain a patient-specific undifferentiated stem cell reservoir for repeated and personalized therapeutic applications. Concerning cardiac differentiation, some interesting reports have already demonstrated the promising iPSCs cardiovascular potential. For example Mauritz et al. have described that functional murine cardiomyocytes can be derived

from iPS cells for cellular cardiomyoplasty and myocardial tissue engineering purposes [26]. If the potential advantages of iPSCs may resolve the ethical and the allogenic transplantation issues, the caveats resulting from iPSC reprogramming procedure for deriving cells for clinical use are not of secondary importance. In fact, the protocol to derive iPSCs relies on the use of viruses integrating into the genome of cells. Moreover, some of the virally encoded genes are oncogenes that may be lead to malignant transformation. Very recently, it was shown that human iPSC derivation could be alternatively obtained by transposon [27, 28], episomal [29], and direct protein delivery [30] systems. In this respect and it will be of interest to understand whether iPSCs generated by these methods are equivalent to iPSCs generated with integrating viruses.

1.5.2 Adult-Derived Stem Cells

b. Skeletal Myoblasts

CONCEPT

Among the first approaches to a cell-based cardiac regeneration, the injection of autologous skeletal myoblasts into ischaemic myocardium has been an option that, starting from preclinical studies, has led to clinical trials in humans. This approcah has been based on the well known properties of the skeletal muscle progenitor cells, called satellite cells. Satellite

cells normally lie in a quiescent state under the basal membrane of skeletal muscle fibers, and proliferate following skeletal muscular injury. At this stage, they are referred to as myoblasts, unipotent cells that, by undergoing a well characterized muscle differentiation program, fuse to surrounding myoblasts and form nascent muscle fibers. In the heart they have been supposed to have a double effect on

1- inhibition of scar scar formation by providing a body of viable cells within infarct;
2- improvement of myocardial contractility.

BENEFIT-ADVANTAGES

Skeletal myoblasts have several advatages to be clinically used to improve heart performance after MI in patients. For example, they can be isolated from small muscle biopsy specimens of the patient's own skeletal muscle, expanded in sufficient numbers in culture and, and later injected in donor patients' heart without risk for immune rejection and tumorigenicity [31]. Finally, when implanted in the infarcted heart, myoblasts differentiate into multinuclear myotubes that improve ventricular function and decrease remodelling due to their ability to contract.

DISADVANTAGES

Despite myoblasts provide a major advantage of mechanical supporting the failing heart, the major problems inherent to

their clinical use is that, they do not have the normal electrophysiological behaviour of cardiac myocytes. For example, they are characterized by short action potential durations and lack electrophysiological coupling to the neighbors host cardiomyocytes [32]. This results in a non-synchronous beating with the surrounding myocardium, thus causing a high risk of developing life-threatening arrhythmias. The lack of electromechanical coupling with the surrounding myocardium is due to the lack of specific gap junctions associated proteins such as Connexins.

CLINICAL TRIALS

Clinical studies have been performed to assess the feasibility and the safety of skeletal myoblasts implantation in the heart. These studies have shown that the approach is feasible and that skeletal myoblasts survive in the human heart, although with marginal functional benefits. In addition, there has been considerable concern regarding the potential for arrhytmias. In fact in some clinical myoblast administration was associated with a high rate of ventricular arrhythmias and sudden cardiac deaths. These events might be explained by the establishment of re-entry circuits resulting from the heterogeneity of electrical membrane properties between donor and recipient cells [33, 34]. Another limitation is the relative low engrafment ability of injected myoblasts. For example it has been estimated that myoblast lethality reaches 90% within the first few days after

transplantation in mice [35] and humans [36]. The most notable clinical trial of skeletal myoblasts injection in patients with cardiac ischemia is the MAGIC trial [37]. In this trial randomised patients (that underwent CABG surgery), received either myoblasts or culture medium. Unfortunately, in these patients, myoblast transfer did not show significant improvement of regional of global left ventricular function, compared to placebo-treated patients.

b. Stem Cells In Adult Bone Marrow

The adult bone marrow contains several differentiatiated cell populations. These include stromal cells, vascular cells, adipocytes, osteoblasts, osteoclasts, and hematopoietic cells. BM also contains different undifferentiated cellular sets; these include hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), multipotent adult progenitor cells (MAPCs) and endothelial progenitor cells (EPCs). All these cells can be considered stem cells as they have different plasticity features [38].

✓ Haematopoietic Stem Cells (HSCs)

CONCEPT

In 1963, Till and coworkers [39] discovered clonogenic bone marrow cells giving rise to multilineage hematopoietic colonies in the spleen. They proposed that these cells were multipotent possessing the properties of self-renewal and multilineage

differentiation; HSCs are in fact responsible for the constant and complete renewal of blood. They are rare, perhaps as few as about 1 every 10.000 to 15.000 bone marrow cells. Although HSCs were discovered several decades ago, their isolation has become possible only more recently when surface markers were identified [40, 41]. Different epitopes characterize HSCs in different species. Mouse HSCs correspond to a subpopulation of c-kit^{+} Sca-1^{+}Thy-1low BMCs [42]. In humans, CD34 and, most importantly, CD133 define the most primitive HSCs [43] [44] [45].

BENEFIT-ADVANTAGES

The first evidence that adult bone-marrow-derived progenitor cells participate in the formation of cardiomyocytes in adult human hearts was based on reports of Y-chromosome-positive cardiomyocytes in female donor heart transplanted in male recipients [46]. In a seminal report in 2001, Orlic et al showed for the first time that BM-derived lin^{-} c-Kit^{+} HSCs injected into the infarcted heart, efficiently differentiate into myocytes with no detectable differentiation into haematopoietic cells and improve myocardial function [47]. To test the ability of injected HSCs to promote myocardial regeneration these Authors isolated these cells from mice constitutively expressing enhanced green fluorescent protein (EGFP^{+}) and injected them into the peri-ischemic region of wild-type mice with acute myocardial infarcts. Evidence for regeneration included

colocalization of EGFP fluorescence with immunostaining for cardiomyocyte markers, including sarcomeric actins and myosins, troponin and several transcriptor factors active in cardiomyocytes (GATA-4, MEF2, NKX2.5).

The ability of BM-derived HSCs to give rise to functional myocardium in the infarcted heart has been questionated by later studies (Balsam et al [48], Murry et al [49], Nygren et al. [50]) showing that $EGFP^+$ HSCs injected into infarcted myocardium did not form cardiomyocytes, but differentiated into blood cells or gave rise to cardiomyocytes at low frequency and predominantly through fusion of injected HSCs to host myocytes. These proofs where in turn questionated by the group originally describing conversion of HSCs into myocytes by repetition of the original experiments [51] and calling for metodological artefacts [52]. All these discussions and the hot debate that followed these important reports do not have yet a clear conclusion. In fact, currently still no consensus exists on whether bone-marrow-derived progenitor cells substantially differentiate into cardiomyocytes *in vivo* [53]. On the other hand, the cardiac protection activity of BMCs into infarcted heart may limit ischemia-associated damages by secretion of paracrine factors that act as myocyte *pro*-survival signals and *pro*-angiogenic angiogenic stimuli [54, 55].

DISADVANTAGES

The disadvantage provided by use of HSCs for myocardial repair, is that even though cell progeny derived from c-kit+ cells express cardiac markers these markers (GATA-4, MEF2, NKX2.5), the newly formed myocytes appear to reach a fully differentiated phenotype but resemble neonatal cardiac myocytes; in fact they are small, round to spindle-shaped, and had no sarcomeres.

- ✓ **Mesenchymal Stem Cells (MSCs)**

CONCEPT

Mesenchymal stem cells (MSCs) are a rare population of $CD34^-$ /$CD45^-$/$CD133^-$ cells present in the stromal cell fraction of the bone marrow (10-fold less abundant than HSCs) and other cells having a mesenchymal phenotype [56]. MSCs are typically known to differentiate into osteoblasts, chondrocytes and adipocytes [57]. Further, they can be separated from haematopoietic cells by their ability to adhere to the culture dish [58]. Pioneer studies performed by Huss et al.[59], and, more importantly, by Jiang et al. [60], suggested the existence of multipotent mesenchymal cells (termed Multipotent Adult Progenotor Cells, MAPCs 71) that showed extensive analogy with the phenotype of pluripotent ES cells for their multilineage differentiation ability, their expression of genes regulating totipotency and their ability to give rise to all germ layers in

chimeric embryos pridced by injecting these cells into recipient blastocysts [60].

BENEFIT-ADVANTAGES

The first example of MSCs cardiac differentiation was produced by Makino and co workers [61], showing that the exposure of MSCs to 5-azacytidine induces generation of functional cardiac myocytes *in vitro*. Preclinical studies using MSCs transplantation in post-infarct mice thereafter demonstrated an overall decrease in mortality [62], an improved left ventricular function and reduction in infarct size [63-65]. Non invasive modalities of MSCs administration have also been tested. For example, intravenously administered MSCs, were found to home in the infarcted heart likley in response to a chemotactic gradient by chemokine gradient established by SDF-1, a chemokine that is upregulated in the myocarium due to hypoxia [66]. Mesenchymal stem cells are known to produce paracrine growth factors that likely support vascular regeneration and cardiomyocyte protection in the injured myocardium [67]. Importantly the paracrine activity by MSCs can be also potentiated by genetic engineering approaches. For example, overexpression of *pro*-survival factors (such as AKT), angiogenic factors (such as VEGF), or stem-cell homing factors (such as SDF-1) enhances the preclinical therapeutic efficiency of MSCs [67]. A final advantage of MSCs use for myocardial

repair is offered by MSCs immunosuppressive properties on T-cell function suppression [68, 69]. Furthermore, MSCs appear to have a major advantage over many other cell types for cellular therapy, in that they are immunological privileged and in large animals, they can be transplanted in MHC mismatched recipients without need for immunosuppression [70]. In fact MSCs express low levels of human MHC class I and lack human MHC class II [63, 71], which at least potentially may alleviate rejection of allogenic MSCs in the tranplanted hearts.

DISADVANTAGES

Preclinical *in vivo* studies have shown that MSCs injection improves ischemic heart function despite relatively low number of injected cells showed evident signs of cardiomyocyte differentiation [65]. In fact, despite MSCs express several cardiac muscle markers, their morphology mostly resembled that of fibroblasts more than that of cardiomyocytes [72]. In addition, no electromechanical junctions bridging grafted and host cells were observed. On the other hand, MSC-engrafted hearts had much thicker infarct scars and a reduced ventricular dilatation, indicating that these cells at least attenuated pathological ventricular remodeling. Among the major contraints of the MSCs use for cardiac repair, a major role has the lack of defined sets of markers defining these cells and, thus, the lack of protocols to isolate specific MSCs subsets having cardiovascular repair activity. In addition studies in mice

[73] and rats [74] infarct models have clearly shown that implanted MSCs were not impaired from undergoing typical mesenchymal differentiation program into osteoblasts inside ventricular tissue, thereby causing significant intramyocardial calcification. This is an obvious cause for concern and needs to be addressed prior to full-scale therapy in humans.

CLINICAL TRIALS

Improvements in ventricular function following BM-derived cells administration in preclinical models have prompted several clinical trials using autologous bone marrow cells to treat heart failure patients or patients who had suffered acute myocardial infarction. Most of these studies have focused on circulating hematopoietic progenitor cells or bone marrow mononuclear cells (MNCs), a heterogeneous population of hematopoietic and mesenchymal cells containing a very low amount (<0.1%) of cells having stem cell features [75].

This experimental approach was criticized because, even though it avoided a “more-than-minimal *in vitro* manipulation” of the injected cells, it did not investigate the function of specific BM cells fraction that may have different cardiac regeneration and consequently cells able to differentiate into unwanted cell types were also co-administrated [76].

Two types of general experimental design have been used to deliver stem cells in the ischemic heart; these are intracoronary delivery to patients with recent myocardial infarctions and

intramural injection into patients with chronic ischemic heart disease and old infarcts. These studies have shown that injection of bone marrow cells to the heart is feasible and safe. However, larger, randomised, placebo-controlled and blinded studies have shown mixed results.

For example, the **REPAIR-AMI** (Reinfusion of Enriched Progenitor Cells and Infarct Remodelling in Acute Myocardial Infarction) trial was positive in that it not only showed improved left ventricular function (at 3-5%) and ejection fraction versus control (placebo), but also showed a reduction in the combined clinical endpoint of death, myocardial infarction or revascularization at one year [77]. It was suggested that patients with more severe reduction of ventricular function experienced the greatest benefits [78]. The **TOPCARE-AMI** [75] and The **BOOST** (Bone marrOw transfer to enhance ST-elevation infarct regeneration) trial also showed improved left ventricular function after 4 months compared with control patients; however, at 18 months differences disappeared as control patients caught up with those who received cells [79, 80]. In contrast, a double-blind, randomised controlled study, using autologous bone marrow MNCs in patients with myocardial infarction 24h after successful percutaneous coronary intervention, showed no benefit in left ventricular ejection fraction, but a significant reduction in infarct size and improved regional left ventricular function [81].

The **STEMI** (ST-Elevation acute Myocardial Infarction) and the **ASTAMI** (Autologous Stem Cell Transplantation in Acute Myocardial Infarction) trials also showed no overall improvement in global cardiac function[82, 83]. Perin et al. delivered autologous bone marrow cells to patients with chronic refractory myocardial ischemia by direct transendocardial NOGA-guided injection into hibernating myocardium. Although this study was small, significant improvements in symptoms and in ventricular function [84] and it is noteworthy because it is one of few studies having its major endpoint in induction of re-vascularization of the chronically ischemic myocardium. Different meta-analysis studies showing that the improvement in heart function observed in progenitor cell-treated patients compared with placebo-treated patients is still modest [85, 86].

✓ Endothelial Progenitor Cells (EPCs)

CONCEPT

In 1997, Asahara and colleagues [87] reported evidences for the presence of circulating cells giving rise to endothelial cells in culture; these cells were named endothelial progenitor cells (EPCs). EPCs were found to proliferate, express endothelial-specific markers and genes and to incorporate into sites of *neo*-vascularization in ischemic limbs in mice and rabbits. Thereafter, other laboratories showed that these cells can be mobilized from the BM with growth factors and/or cytokines [88], and home to areas of ischemic injury, where they integrate into

growing vessels [89]. In the adult life, EPCs, are supposed to derive from the hemangioblasts and can be expanded *ex vivo* from $CD34^+/CD133^+/KDR^+/CD45^{+/-}$ cells from BM peripheral blood or cord blood[90]. EPCs are distinguished in "early" and "late" EPCs based on the different timing of their appearance and differences in the clones shape [91]. Yoon and colleagues [92] demonstrated the *neo*-vascularization induction by early EPCs *in vivo* occurs through paracrine stimulation, while late EPCs directly contribute to formation of novel vessels [92]. Another distinction between early and late EPCs has been established with the finding that early EPCs, also named colony forming unit-endothelial cells (CFU-ECs), originate from $CD34^+/CD133^+/KDR^+/CD45^+$ cells in the MNCs cellular fraction while late EPCs, also named endothelial colony forming cells (ECFCs) originate from $CD34^+/CD133^+/KDR^+/CD45^-$ cells [93-96]. Other cell types present in BM and PB mononuclear fractions are considered EPCs. For example, it has been shown that $CD14^+$ monocytes have angiogenic activity [97] and that certain subsets of T-lymphocytes also behave as EPCs [87, 98]. There is evidence that patients with risk factors (diabetes, hypertension, high cholesterol) or with chronic coronary artery disease, have dysfunctional endothelial progenitors [99, 100]; in fact their numbers are reduced in the circulation, they have a reduced migratory activity, impaired clonogenicity and survival and, thereby, a reduced *in vivo neo*-vascularization capacity. It has been found that EPCs dysfunction may be restored by *ex*

vivo treatment with drugs and compunds able to “rejuvenate” these cells before transplantation into patients hearts. These strategies are complexively called EPCs “ehnancement strategies”. Several different approaches have been devised to this aim. Gene transfer represents a first example of an enhancement strategy to restore or even enhance EPC *pro*-angiogenic activity; preclinical studies have shown that progenitor cells infected with adenoviral vectors carrying $VEGF_{165}$ cDNA have an enhanced *pro*-angiogenic effect compared to empty vector infected cells [101]. Another approach is incubation of human EPCs in the presence of defined cytokines that are known to enhance endothelial differentiation ability of these cells *in vitro*, and thereby increases their *neo*-vascularization effect *in vivo*. An example is chemokine stromal derived factor-1 (SDF-1). SDF-1 increases commitment of BM-derived c-kit^+ cells into endothelial-like cells and their ability to form vascular-like structures in matrigel plugs assays [102]. Pretreatment with SDF-1 has been also suggested as a strategy to increase EPCs migratory ability [103], while *in vivo* gene transfer of this chemokine has been shown to enhance EPCs homing and survival into ischemic tissues [104-106].

BENEFIT-ADVANTAGES

The advantage of EPCs therapeutic use depends on their ability to integrate into newly forming vessels (ECFCs) or to activate

neo-vascularization by pararcine mechanisms (CFU-ECs). The two distinct, direct and indirect, ways of human EPC types participation to *neo*-vascularization process may represents two different modalities for biologically treating ischemic disorders at the heart or peripheral levels. In fact, while CFU-ECs have a predominantly paracrine angiogenic activity, ECFCs have a modest paracrine effect and may be thus useful for long term engrafting into ischemic tissues or promote re-endothelization of injured vessels [107]. The positive contribution of EPCs to adult *neo*-vascularization has been considered an useful approach in order to attenuate myocardial ischemia in coronary artery disease. For example, when EPCs were delivered in animal models of myocardial ischemia *via* either systemic administration or direct intramyocardial injection, they were found in the infarcted tissue and contributed to *neo*-vascularization, thereby diminishing the infarct size [108]. An important feature of EPCs is their ability to promote rapid re-endothelialization of carotid vessels denuded as a consequence of balloon-injury [109]. One of the principal mechanisms in this framework appears to be the release of vasculoprotective molecules, such as nitric oxide (NO). In particular, the endothelial-specific NO Synthase (eNOS) exerts pleiotropic cytoprotective effects in the vessel wall, reduces oxidative stress, modulates vascular tone and platelet adhesion, and impairs the development of atherosclerosis. It has been shown that EPCs overexpressing eNOS have an enhanced

antiproliferative *in vivo* effect that significantly reduced the neointimal hyperplasia [110]. A study by Werner at al showed that blood levels of $CD34^+$ KDR^+ EPCs are inversely correlated with cardiovascular events and death from cardiovascular causes. These findings implied that EPCs support the integrity of vascular endothelial cells [111]. EPCs also exert a significant reduction in collagen deposition, apoptosis of cardiomyocytes and cardiac remodelling [112]. Finally, Hinkel and co-workers [113], showed that Embryonic EPCs (eEPCs) exert postischemic cardioprotection by paracrine factors activating the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway in cardiomyocytes *in vitro* and *in vivo* (after ischemia and reperfusion in a preclinical pig model). eEPCs were capable of reducing the amount of adhesive inflammatory cells. In particolar they found that Tβ4, one of the most highly expressed AKT-activating factors in their eEPC population, is indeed responsible for cardiomyocyte protection.

CLINICAL TRIALS

A definitive consensus about the clinical use of one or the other EPC type has not been reached. In fact, despite preclinical studies have highlighted lineage distinctions and different biological properties of CFU-ECs and ECFCs, the CD34 and CD133 antigens remain the reference markers to be used to isolate EPCs for clinical trials, irrespective of their CD45 expression. Significant clinical improvements by purified $CD34^+$

and $CD133^+$ cells administration have been reported, for example, in relatively small cohorts of patients affected by acute or chronic myocardial ischemia. In particular Douglas et al. showed in a randomized trial in patients with intractable angina, feasibility, safety and bioactivity of intramyocardial injection of autologous $CD34^+$ cells. Other reports of different groups, (Stamm et al. [114], Pompilio et al. [115], Losordo et al.[116]) showed that intramyocardial delivery of purified $CD133^+$ cells is safe; if associated with coronary artery bypass grafting (CABG) surgery, it provides beneficial effects and if used for refractory myocardial ischemia improves heart perfusion.

c. Resident Myocardial Progenitors

Since the 1970s, the mammalian heart has been considered as a post-mitotic, static organ without regeneration ability and largely unable to sustain myocytes turnover. According to this classical view, the total number of cardiomyocytes was supposed to be established at birth and to remain fairly constant throughout the life-span. This view has been challenged by seminal reports showing the presence of small cells within the non-injured myocardium that closely resemble young myocytes and the evidence of active myocyte cells divisions in response to ischemic injury [5, 6, 94, 117]. These findings have led to the conclusion that the human heart is a normal, self-renewing, organ. Recently, Bergmann et al.

provided an estimation of cardiomyocytes age in humans using ^{14}C detection. They demonstrated that DNA syntesis in cardiac myocytes is not restricted to a limited period in childhood, but continues during the entire life. They have also calculated that DNA synthesis gradual decreases from 1% annual amount in the total heart mass at the age of 25 to 0.45% at the age of 75 and that about 50% cardiomyocytes are replaced during a normal lifetime [118].

The notion that myocyte division in the heart could result from recruitment of stem cells derived from the BM was strongly supported by a *post-mortem* study of male patients transplanted with female donor hearts. In these hearts, a number of undifferentiated cells containing Y-chromosomes expressing the stem cell markers Sca-1, c-kit and MDR-1 were found, suggesting that male host cells had invaded myocardial tissue, thereby contributing to myocardial cells turnover [46].

Several candidate progenitors that sustain the myocardium self-renewing activity have been found; these include: 1) side population (SP) stem cells, identified on the basis of their ability to extrude Hoechst33342 dye by MDR-1 gene product, 2) progenitor cells characterized by expression of Sca-1/Sca-1-like antigen, 3) cells derived from the so called “cardiospheres”, 4) Isl-1+ cardioblasts and 5) c-kit$^+$ cardiac progenitor cells.

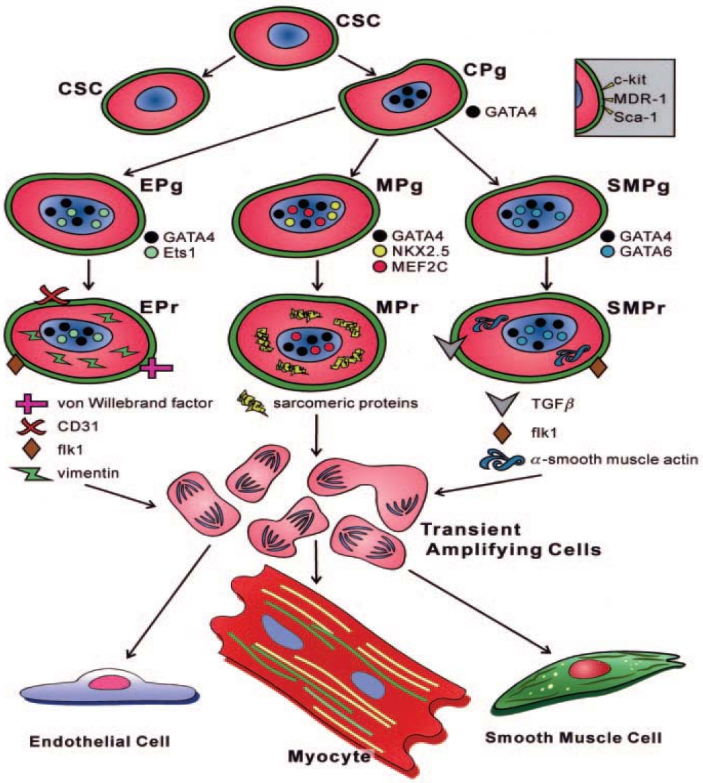


Figure 5. Hierarchy of CSC growth and differentiation. Anversa et al.

Subpopulation of CPCs

✓ Sca-1 positive cells

Expression of the Stem Cell Antigen-1 Sca-1 defines primitive stem cells population in adults [119]. Sca-1 is a member of the ly-6 family, which was originally reported as one of the cell surface marker of hematopoietic stem cells [120]. Like SP cells, Sca-1 cells possess telomerase activity, and undergo self-renewal [121]. Undifferentiated Sca-1 cells do not express cardiac structural genes (NKX2.5, MEF-2C, α/ β-MHC,

MLC2a/v), blood cell lineage markers CD45 and haematopoietic stem cells marker CD34. By contrast, they express cardiac master genes GATA-4, MEF-2C and TEF-1 and, after treatment with oxytocin [122] and the cytosine analog 5-azacytidine *in vitro*, these cells showed spontaneous contractions and differentiate into cells expressing cardiac specific markers NKX2.5, α,β-myosin heavy chain and bone morphogenetic protein (BMP) receptor 1A. When injected intravenously into a mouse with MI, these cells were able to home to the infarcted myocardium and generate cardiomyocytes. The Sca-1^+ cell subpopulation, which does not express CD31, was shown to differentiate into both cardiomyocytes and endothelial cells in culture [123]. Transplantation of these cells cells in mice after myocardial infarction improved cardiac function and promoted new blood vessel formation [123]. An the increase of the Sca-1^+CD31^- cell number present in the LV after MI was also shown; this was likely caused by proliferation of the Sca-1^+CD31^- cell population endogenous to the heart rather than from recruitment of bone marrow-derived cells. The phenotype of undiffentiated Sca-1 cells was confirmed by studies describing results of culture in single–cells condition to isolate proliferating heart stem cells population. For example, Oh and colleagues [124] identified clonogenic cells strongly expressing Sca-1 antigen but not hematopoietic and endothelial progenitor cell specific surface antigen CD45, CD34 and CD31. In addition to these markers, interestingly, these cells expressed the

typical mesenchymal stem-cell surface antigens CD90, CD105, CD29, CD44 and telomerase-specific enzyme TERT.

In humans, Sca-1^+ cells have been also described [125]. Like in mice, these cells are negative for CD45, CD34, CD133 and CD14, express CD105 mesenchymal marker and, when treated with TGF-β and 5-azacytidine, they give rise to mature cardiomyocytes *in vitro*. [126]. Transplantation of human Sca-1^+ progenitors into immunodeficient mice after MI, resulted in a higher ejection fraction and a reduced left ventricular remodelling up to 3 months after MI, suggesting their potential use in patients.

✓ **Cardiac Side Populations (CSP)**

SP cells have been identified in several stem cells-containing districts including the bone marrow, the skeletal muscle and adipose tissue [127] by their ability to extrude the Hoechst 33342 dye [128]. This ability is attributable to expression of ATP-binding drugs transporters ABCG2 and MDR-1. The first demonstration of a cardiac-specific SP was provided by Hierlihy and colleagues [129] suggesting, the existence of a putative CPCs population in the adult heart. These Authors showed that these cells, represent ~1% of the total cell number in the adult heart, have the ability to efflux Hoechst or Rhodamine dyes through ATP-binding transporter and to differentiate into the cardiomyocyte lineage. Subsequently, Martin et al. [130], isolated ABCG2-expressing SP cells from embyonic as well as

adult mouse hearts. These cardiac SP (CSP) cells were found to express Sca-1 antigen and, at lower levels, c-kit, CD34 and CD45; these cells were further shown to proliferate and differentiate to form mesoderm derivatives (e.g., cardiac and hematopoietic lineages) and capable of differentiating into cardiomyocyte phenotype after co-culturing with cardiac cells. It has been shown that CSP cells are >93% Sca-1^+ and are enriched 100-fold in the Sca-1^+ population [119], for this reason CSP can be considered Sca-1^+ cells subpopulations.

Pfister et al [131] showed that cardiac SP are immunophenotypically distinct from bone marrow SP cells. They demonstrated that, among cardiac SP cells, the greatest potential for cardiomyogenic differentiation was restricted to cells negative $CD31^-$Sca-1^+ cells that represent the 10% of cardiac SP; conversely $CD31^+$ Sca-1^+ SP (that represent approximately 78% of SP cells within the heart) did not have such potential. The ability of these cells to differentiate into cardiac myocytes was witnessed by expression of the early cardiac transcription factors NKX2.5 and GATA-4, the early cardiac myofilament proteins desmin and smooth muscle actin; additionally, when co-cultured in the presence of adult cardiomyocytes, they expressed the gap junction protein connexin-43, differentiated into cardiomyocytes, with maturation of contractile apparatus and sarcolemma.

One study documented that CSPs from neonatal rat hearts were treated with oxytocin or trichostatin A and showed after 3

weeks of treatments spontaneous beating. In this study, it was also demonstrated differentiation of transplanted SP cells to cardiomyocytes, endothelial cells and smooth muscle cells in injured heart [132]. Liang et al.[133] showed that cells Sca-$1^{+}CD31^{-}$ are able to migrate from non-ischemic to ischemic areas through up-regulation of CXCR4 and chemotactic guidance by chemokine SDF-1α. The CSP turnover in the heart following MI has been also investigated. Mouquet et al [134], for example, showed that while under normal conditions the cardiac SP cells pool in the heart was maintained without any significant contribution from bone marrow cells. By contrast, shortly after MI, CSPs were massively depleted; however, the CSPs population was thereafter restored to baseline levels both via proliferation of resident CSPs cells, but also by homing of $CD45^{+}$ BM cells. Interestingly, homing to the infarcted heart apparently caused a phenotypic conversion of bone marrow-derived cells which was evidenced by loss of CD45 expression.

- ✓ **Islet positive cells**

In 2005, Laugwitz et al. reported the presence of primitive cells in the heart that express the LIM homeodomain transcription factor Islet-1 (Isl-1). The phenotype of these cells does not overlap with that of the Sca-1+/CSPs. In fact Ils-1^{+} cells do not extrude Hoechst 33342 and do not express c-kit and Sca-1 [135]. The Isl-1^{+} cells have been termed “cardioblasts” as they likely represent a subpopulation of cells constituting the

embryonic heart and disappear soon after birth [135]. These cells exhibit a cardiomyocytic phenotype when co-cultured with neonatal cardiomyocytes in *vitro* and show electrical, as well as, contractile properties reminiscent of neonatal cardiomyocytes. In the embryo, the primary location of these cells is the atria, the right ventricle and the outflow tract regions, where Isl-1 is most prevalently expressed during cardiac organogenesis.

Isl-1^+ cells have been reported to express early cardiac differentiation markers Nkx2.5 and GATA-4; however they lack transcripts typical of mature myocytes. A wider differentiation ability compared to cardiac SP cells or Sca-1^+ cells has been reported for Isl-1^+ cells. Indeed these cells undergo mutipotential differentiation into smooth muscle and endothelial cells [136]. Although originally described in the morphogenesis of the mouse heart, Isl-1^+ cells have been also isolated from hearts of rats, mice and 1-5 days old human newborns. It is unknown whether cells with the same characteristics can be isolated from the adult heart as well. In addition, Isl-1^+ cells have not been yet shown to be able to repair the damaged heart.

- ✓ **Cardiospheres (CSs)**

The term “cardiosphere” has been introduced to describe the derivation in culture of spheroid cellular aggregates likely resulting from clonal expansion of cardiac progenitors,

occurring in a similar fashion to the formation of embryoid bodies from undifferentiated embryonic stem cells or the formation of neurosphereas obtained from culturing neural stem cells. In the first report describing derivation of cardiospheres [137], it was suggested that they are composed of clonally-derived, undifferentiated cells, that express c-kit transmembrane receptor in the core of the sphere and differentiated cells expressing cardiac and endothelial cells markers (KDR, CD31), which are mostly located at their periphery. Human cardiospheres can be considerably expanded in culture and, when ectopically transplanted in the dorsal subcutaneous connective tissue, they yield the major specialized cell types in the heart: cardiomyocytes and vascular endothelial or smooth muscle cells. Unlike mouse CSs, human CSs do not beat spontaneously but begin to beat within 24 hours, when co-cultured with postnatal rat cardiomyocytes. Finally, following plating of cardiospheres, cardiospheres-derived cells (CDCs), were found to express CD105, c-kit, CD90, CD34 and CD31; by contrast they did not show MDR-1, CD133 and CD45 expression [138]. CDCs can be induced to differentiate into electrically functional myocytes *in vitro* using a co-culture method onto neonatal rat ventricular myocytes. This process can be enhanced by exposing CSs or CDCs to extremely low frequency electromagnetic fields (ELF-EMFs) for 5 days [139]. Oh et al. [140] further showed that the proliferation of hCSCs appears to be dependent on the

activation of Akt in response to EGF/bFGF stimulation, and that inibition of Akt pathway impairs cell growth and survival. Injection of CDCs into infarcted hearts of mice, promotes cardiac regeneration and improves heart function. In addition, using a pig model, it has been shown that controlled delivery of bFGF modulates the post-ischemic microenviroment to enhance human cardiosphere-derived cell (hCDC) engraftment and differentiation [141]. The additive effect of bFGF was observed in hCDC-injected animals, but not observed in animals receiving human bone marrow-derived mesenchymal stem cells indicating a specificity for bFGF effect on the activity of hCDCs [141]. Recently, an alternative origin for cardiospheres has been supposed. In fact two independent groups reported recently that CSs may result from aggregation of fibroblasts or small contaminating myocardial tissue remnants instead of putative CSCs [142, 143]. Both reports concluded that similarly to fibroblasts, CSs and CDCs might improve cardiac function even in the absence of cardiomyogenesis and angiogenesis due to their supportive function in the heart.

Our experience with Cardiospheres

During the initial phase of the present work, we have attempted at characterizing cardiosphere-derived cells using high throughput flow cytometry (FACS). Given that

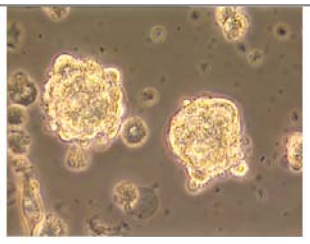
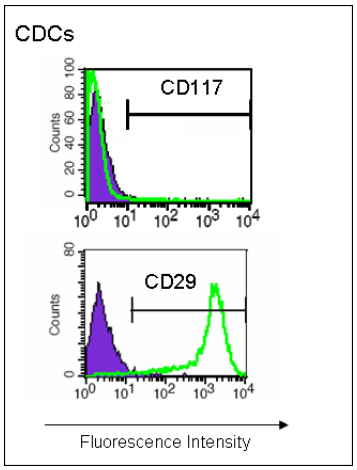


Figure. 6 Cardiospheres

c-kit receptor appears as one of the most considered markers to identify CPCs, we proceeded with analysis of cell suspensions obtained from cardiospheres enzymatic digestion. Our results showed that the c-kit$^+$ cells percentage in cardiospheres-derived cells was 0.26 ± 0.08 % (mean ± SE n=9). To understand whether the small c-kit$^+$ cell fraction contained in cardiospheres is a clonogenic cell population, we sorted these cells by flow cytometry and cultured them under conditions described in [144, 145]. As a control, c-kit$^-$ cell populations and un-fractionated cells (cardiospheres derived cells, CDCs) obtained from cardiospheres enzymatic digestion were cultured. Although c-kit$^-$ and CDCs could be grown for a number of passages, c-kit$^+$ cells from cardiospheres did not grow, suggesting that the absence of intrinsic clonogenic ability and the need for supporting c-kit$^-$ cells to sustain c-kit$^+$ cells self-renewal. To assess whether this was the case, we analyzed the c-kit- and the CDC populations deriving from cardiospheres. After expansion, c-kit$^-$ and CDCs cells were analyzed for the expression of several surface markers by flow cytometry and by q-RT-PCR for the expression of early and late cardiac genes. The results showed that CDC population contained a small fraction of c-kit$^+$ cells; in addition c-kit$^-$ cells gave rise to a similarly abundant c-kit$^+$ cell population (see table above), suggesting the presence of a progenitor for c-kit$^+$ cells themselves into c-kit$^-$ fraction. However, we did not verify this hypothesis.

No differences were found between the two cellular populations concerning expression of mesenchymal-specific markers, as evidenced by high and comparable CD29, CD105 and CD44 antigens expression and low or no expression of cardiac genes. These initial findings, according to two other groups [142, 143] suggested that cardiosphere-derived c-kit^{+} cells are non-clonogenic at least under our experimental conditions, and that cardiospheres contain a mesenchymal mixed cellular population.



MARKER	CDCs % ± S.E.	c-kit - % ± S.E.
C-KIT	0,40 ± 0,40%	0,63 ± 0,21%
CD90	35,19 ± 10,23%	35,27 ± 10,18%
CD105	95,54 ± 2,47%	98,82 ± 0,39%
CD29	99,16 ± 0,18%	99,16 ± 0,30%
CD73	86,8 ± 4,56%	91,76 ± 7,00%
CD44	96,95 ± 2,25%	98,53 ± 0,69%
CD45	0,0 ± 0,0%	0,11 ± 0,08%
CD34	2,26 ± 1,28%	2,35 ± 2,21%
CD71	17,82 ± 6,60%	26,84 ± 5,58%
HLA-DR	0,0 ± 0,0%	0,0 ± 0,0%

Figure 7. Facs analysys

✓ c-kit positive cells

The current best characterized group of stem cells resident in the heart constitutes a pool of cells that express the receptor for stem cell factor known as c-kit (CD117) [4, 111, 139, 140]. This receptors has been widely used as a cell surface marker of pluripotent long-term reconstituting murine stem cells and has

previously been used to define a population of cells from bone marrow that seemed to be capable of differentiating into functional myocardium when injected into infarcted mouse heart [141]. *In vitro* data suggest that the growth potential of c-kit^{+} CSCs in greater than that of Sca-1like or MDR-1^{+} cells, although these cell categories give rise to all cardiac cell lineages [140]. Moreover, information obtain from available transgenic mice showed that Sca-1-/- mouse and MDR-1-/- [142, 143] mouse have essentially a normal phenotype with modest defect of the hematopoietic system and other organ; in contrast, mutation of the c-kit receptor led to dies shortly after birth, while the heterozygous has defect of the hematopoietic system but reaches adulthood, and the heart shows alterations in cardiac anatomy and function [144, 145].

The documentation of myocardial specification of embryonic stem cells, in particular c-kit^{POS} Nkx2.5^{POS} cells [146], support the view that a pool of resident c-kit^{POS} progenitors is implicated in cardiac morphogenesis. These findings are consistent with the existence of a pool of primitive cells in the adult human heart.

Among CPCs resident in the heart only the c-kit^{+} cells have so far fulfilled all criteria for being stem cells. In fact the c-kit^{+} cells from the heart with stem cell abilities, characterized by a high nucleus to cytoplasm ratio, do not express cell surface markers of other cell lineages, making them lineage negative, they scored negative for proteins specific for myocytes,

endothelial smooth muscle cells or fibroblast, are clonogenic, able to self-renew, multipotent (they are able to differentiate into myogenic, endothelial or smooth muscle cell lineages *in vitro*) and have been shown to reconstitute the heart after an acute ischemic event by differentiating into smooth muscle, endothelial and cardiac lineages, with the characteristic of young cells in the absence of cell fusion. However, even c-kit$^+$ CPCs are not a homogeneous population since around 10% of freshly isolated cells express transcriptional factors from early myocyte lineages Nkx2.5, GATA-4 and MEF2 [139]. It is of particular clinical importance that c-kit$^+$ CPCs have been found resident in the adult human heart [138, 147] and seem to be upregulated under pathological conditions including aortic stenosis and infarction [4, 111], and furthermore, that a depletion of CPCs in chronic infarcts compromise myocardial regeneration and leads to ventricular dilatation and reduce cardiac performance. The functional competence of CPCs *in vivo* was first demonstrated by injecting CPCs into the border zones of experimentally induced myocardial infarcts in rat model leading to a substantial band of regenerating myocardium [139]. In addition, in a model of ischemia-reperfusion the CPCs homed to the injured rat myocardium after intravenous infusion [148]. In both studies, CPCs turned into phenotipically differentiated cells including capillaries, arterioles, and cardiac myocytes, and treated animals exhibited significant improvementsin cardiac function as validated by

echocardiographic and hemodynamic parameters [139, 148]. Injection of survival factors like hepatocite growth factor (HGF) and insuline-like growth factor 1 (IGF-1) into he border zones of the infarcted myocardium in mice [149], dogs [140] and rats [150, 151], respectively, boosted the repair of damaged cardiac tissue. Cardiac repair was mediated by the ability of CPCs to synthesize matrix metalloproteinases (MMP-9) that degraded collagen proteins, forming tunnels within the fibrotic tissue during their migration across the scarred myocardium [150]. CPCs, activated by HGF and IGF-1, regenerate conductive, intermediate-sized and small coronary arteries and arterioles together with capillary structure in vivo, replacing partly the function pf the occluded coronary artery and its distal branches [151]. Anyway the new myocytes remained immature with cell sizes only a fraction of normal adult cardiac myocytes. This suggest that the myocytes need additional factors to fully differentiate [113]. Multiple autocrine and paracrine factors like TGF-β, VEGF and FGF secreted from host myocardium could be important for differentiation and maturation of CPCs into functional myocardium and this might also be exploited in order to obtain a higher survival among donated cells and more complete regeneration [152, 153]. CPCs found in the infarcted area were small and not fully differentiated, while those found in non-infarcted ventricular tissue were similar to surrounding healthy cardiomyocytes suggesting that the infarcted myocardium is an inadequate milieu for the maturation of CPCs

[140]. Nuclear Akt accumulation expands the cardiac myocyte progenitor population by a combination of proliferative and survival signaling mediated by intracellular signaling and paracrine factors [154]. Tallini and colleagues [155] developed a transgenic mice in which enhanced green fluorescent protein (EGFP) is placed under control of the c-kit locus; with this technique they estimate that c-kit^{+} cells constitute < 1% of the cells in the neonatal mouse heart and that these populations includes cardiovascular progenitor cells in several stages of development and lineage commitment.

✓ Cardiac Progenitor Cell Niches

Stem cells are stored in niches that are located deep in the tissue for protection from damaging stimuli [156-158]. The niche constitutes the microenvironment in which primitive cells divide, differentiate and die. The recognition of stem cells within their natural milieu is of crucial importance; stem cells do not exist in the absence of supporting cells within the niche [157, 159]. Stem cells divide rarely, and cell replication is most confined to the niches [157]. Stem cells undergo symmetric or asymmetric division [158, 160]. When stem cells divide symmetrically, two self-renewing daughter cells are formed, and the purpose of this mechanism of growth is the expansion of the stem cell compartment [158]. When stem cells engage in asymmetric division, one daughter stem and one daughter amplifying cell are obtained. The objective of this division is cell

differentiation, the production of a committed progeny. Stem cells can also divide symmetrically into two committed amplifying cells, decreasing the number of primitive cells [158]. The developmental choice made by stem cells at any given time has a direct impact on the stem cell pool size, the number of progenitors and precursors and, ultimately, the number of mature cells [157, 158]. The niche characteristics are applicable to all organs whether bone marrow, brain, or heart. The niches define the growth potential of an organ, and the recognition of their microenvironment is more important than the requirements postulated to be fundamental for definition of stem cells [37]. Recently, CSC cluster have been found in the adult heart [4, 139, 161, 162]. Although CSC cluster are scattered through the myocardium, their distribution appears to be conditioned by the distinct levels of wall stress. In fact, the frequency of CSC cluster is inversely related to the hemodynamic load sustained by the anatomical regions of the heart: they accumulate in the atria and apex and are less numerous at the base and midportion of the left ventricle. Physical forces, mechanical deformation, and high wall stress can be transduced in intracellular responses that regulate cell behavior and fate. Bearzi et al. [138] identified in the normal human myocardium stem cell niches and hCSCs that divide symmetrically and asymmetrically and give rise to differentiating and lineage-negative cells. Urbanek and co-workers [159] identified some of the components of the cardiac niche,

particularly they found that CSCs and cardiac progenitor form cell to cell contact with myocytes and fibroblasts. The latter are thus though to act as nurse cells. Stem cells and supporting cells in the niches interact structurally and functionally through specialized gap and adherent junction proteins [156]. Connexins are gap junction channel proteins that mediate passage of small molecules and signals involved in cell-to-cell communication [163]. Survival factors and mitogens traverse gap junctions to oppose cell death and favor cell growth. CSCs are also found to be positive for $\alpha_4\beta_1$-integrins, which binds to fibronectin and α_2-chain of laminin. In analogy to what happen in other systems, the binding between integrins and ECM is considered involved in the preservation of CSCs stemness. BrdU retaining assay have permitted to distinguish more primitive stem cells (long term label) from the progeny (progressive dilution of BrdU label) and to define the slow kinetic of CSC divisions. As the number of CSCs/progenitor within the niche remains constant, asymmetric division is though to be the most used mechanism of CSC proliferation during physiological turnover. How and when this mechanism is modified, for example in response to stressful stimuli, is not yet known and must be adressed in future studies. Recent evidence has also shown that there is a marked increase in the number and migration of such cells to injury areas following an ischaemic insult [51]. Thus, the recognition of cardiac niches and the identity of the supporting cells constitute a major challenge for the definitive proof that the

heart is a self-renewing organ in which cardiac homeostasis is regulated by a stem cell compartment.

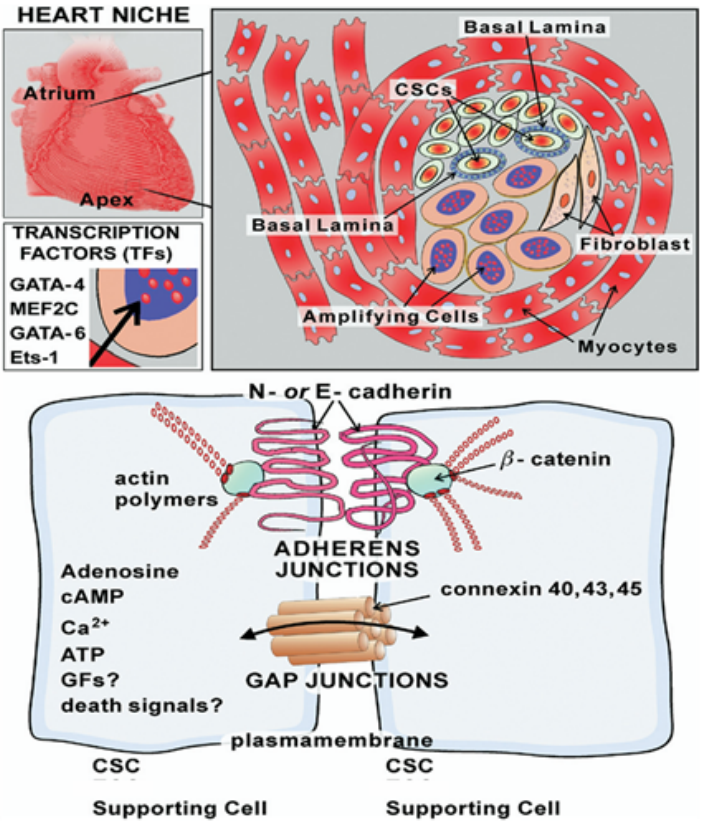


Figure 8.
Representation of CSC niche elements. Cardiac niches are represented by differentiated myocytes that surround cluster of CSCs and higly dividing amplifying cells. Contact among stem cells and supporting cells occurs via junctional proteins (modified from Leri at al., Physiol Rev 2005

✓ Origin of cardiac stem cells

Following the recognition of a tissue-specific stem cell in the adult heart, the question concerned whether this primitive cell

population originates, lives, and dies within the myocardium or whether other organs continuously replenish the heart with an undifferentiated stem cell pool that subsequently acquires cardiac characteristics. The bone marrow constitutes the main reservoir of primitive cells in the organism, and these cells can egress from the marrow niches, enter the systemic circulation, and chronically repopulate the heart and other organs [164-166]. Furthermore, the fact that HSCs and the heart have the same mesodermal origin and that cardiac SP cells form hematopoietic colony *in vitro* raised questions about their actual derivation and suggest possible colonization of the heart by the hematopoietic system. As in embryonic development, HSCs colonize organs that express SCF and the heart is one of them, one of the hypothesis suggests that CSCs may home in the fetal heart [167]. However, this possibility has never been definitely proven. The contribution of bone marrow cells to cardiac chimerism has been proposed. Interestingly, a comparison has been made between the degree of chimerism in cardiac allografts and in hearts of patients who received allogeneic bone marrow transplantation [168]. In the latter case, only 2–5% chimeric myocytes were detected, while 14–16% of chimeric myocytes and endothelial cells were found in transplanted hearts. These observations suggest the intracardiac origin of the recipient cells in the donor heart and the extracardiac origin of chimeric cells in the resident heart following bone marrow transplantation. In the first case, host cells may have migrated

from the residual atrial stumps to the donor heart [45] and, in the second, donor cells may have reached the myocardium because of the high level of blood chimerism [168]. If the bone marrow continuously replenishes the heart with new functionally competent HSCs, the decline in myocyte number with cardiac diseases would not occur, and the poorly contracting myocytes would be constantly replaced by a bone marrow-derived progeny [169]. It is reasonable to assume that only a minor fraction of bone marrow derived cells is eligible for surveillance of organs like the heart and recruitment in the case of injury [113]. At least one study indicates that a distinct population of 'tissue committed' stem cells existing within the bone marrow exhibits early cardiac markers (transcription factors),is mobilized to the blood stream after myocardial infarction, and is recruited to the site of injury by locally secreted chemokines such as SDF-1[170]. Myocardial infarction results in an almost immediate mobilization of mononuclear cells expressing specific cardiac, endothelial and muscle markers [170]. They reside in the non-adherent and non-hematopoietic $CXCR4^{+}$, $Sca\text{-}1^{+}$, lin^{-}, $CD45^{-}$ mononuclear cell fraction in mice and $CXCR4^{+}$, $CD34^{+}$, $AC133^{+}$, lin^{-}, $CD45^{-}$ mononuclear cell fraction in humans [171].

To date, no information is available concerning the repopulating ability of individual CSCs in the depleted heart. Stem cell depletion is complex in solid organs, and currently, there are no protocols available eliminate resident CSCs in the

absence of extensive parenchymal damage and myocardial scarring [169]. So far, only limited characterization of the in vitro properties of CSCs has been performed. The clinical utility of CDC technology remains conjectural, but some phase 1 clinical trial with intra-coronary administration of autologous CDCs has begun, with evaluation of safety as the initial goal.

In summary, different methods of isolation and characterization documented the presence on the heart of several stem cell populations, which have been characterized for the expression of different stem cell markers. Further studies are needed to assess whether they belong to independent pools of represent differentiation steps of the same lineage and also to determine whether technological differences in the detection of a limited number of markers led to unnecessary distinctions.

1.6 Translational Medicine And Stem Cell Therapy

The definition of Translational Medicine is that of a two-way discovery path involving a strict communication between benchside (basic research) and bedside (clinical research and patients care). The tight complementation of these two worlds ensures that biological phenomena are correctly introduced into clinical frameworks and that, conversely, important clinical issues obtain comprehensive biological interpretation. In this way, basic evidences providing potential therapeutic advancements come to the rigorous validation level imposed by clinical practice and, in turn, receive further refined questions.

For these reasons translational medicine should be defined as a discipline transversally encompassing:

1. Basic science studies which define the biological background for potential treatments of a given disease as well as investigations in human samples to define the diseases biological bases and provide the scientific foundations for development of new or improved therapies
2. preclinical studies allowing to provide relevant “proofs of principle” of the benefits provided by basic findings.
3. Optimization and adaptation of treatment protocols defined in preclinical setting to use in humans
4. Organize clinical trials of validated therapeutic protocols where to assess their safety (phase 1 clinical trials) and verify their efficacy (phase 2-on clinical trials)

Cell therapy has emerged as a possible translational option to treat ischemic heart disease. However, the bulk of clinical data obtained thus far in patients suggest still puzzling results in term of clinical benefit. Several different issues may contribute to uncertainty in the detection of real stem cells potential in heart repair. The lack of a clear indication about the optimal cell type to be used in defined subsets of patients; the lack of a consensus about the methods to prepare and store stem cells; the absence of clear evidences for a method of choice and the ideal timing for delivering stem cells in the ischemic heart and

lately, the definition of conditions to promote full stem cells conversion into mature cardiac or endothelial cells and the appropriate alignment and electrical integration with the recipient myocardium. Only further studies addressing all these issues will be of help to set up universally recognized guidelines to proceed with stem cells clinical use for myocardial repair.

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1.7 Aims Of The Project

The aims of the present project were to characterize the c-kit^{+} stem cells population from the human heart and to provide a defined protocol to amplify them in culture under condition that may be translated to the clinical practice.

To this aim we devised a co-culture system that represent an absolute novelty and provide significant innovation over existing protocols.

2. Ex Vivo Amplified C-Kit^{+} Cardiac Progenitor Cells Differentiate Into Mesenchymal Cells Having A Preferential Cardiovascular Commitment

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Submitted

2. MATERIALS AND METHODS

2.1 Cell isolation and culture

2.1.1 Isolation of myocardial c-kit$^+$ cells

The procedure to obtain primary and secondary cultures of human CPCs from auricolae fragments is a modification of the methods published in[12]. Isolated myocardial tissue was cut into 1-to 2 mm^3 pieces, washed with PBS (Lonza, Italy) and incubated four times for 20-30 minutes at 37°C with 3mg/mL collagenase NB 4 (Serva, Germany). After digestion, the solution was filtered using 70µm mesh nylon filters (BD-biosciences, Italy). The cells were finally seeded into uncoated Petri dishes (Corning, Italy) containing Ham's F12 medium (Lonza, Milan, Italy) supplemented with 10% FBS (Hyclone, USA), 2mM L-Glutathione (Sigma, Italy), $5x10^{-3}$U/ml Human Erythropoietin (Sigma, Italy), 10ng/ml bFGF (Peprotech), and antibiotics (Lonza, Italy). At 48h after the beginning of the culture, the medium was completely changed to remove dead non-adherent cells. When reaching a 70% confluence, cells were split 1:10 and seeded into 10 cm uncoated Petri dishes (Corning, Italy) until reaching 70% confluence. At this time, the cells were detached using a non enzymatic cell dissociation solution (Sigma, Italy) to avoid reduction of c-kit antigen immunoreactivity, and incubated using an anti-c-kit monoclonal Ab, (BD Bioscences, Italy) followed by flow

cytometry. Sorted c-kit$^+$ cells (10^4/well) were sub-cultured into transwell inserts (pore size 0.4 µm, Corning, Italy), alone or onto un-selected cells from the same patient without physical contact. Under these conditions, c-kit$^+$ CPCs were amplified for 4 passages, after which they were analyzed for the expression of c-kit and different surface markers by flow cytometry and/or were plated in culture media promoting adipogenic, osteogenic, endothelial and cardiac differentiation. Fold enrichment of c-kit$^+$ cells at P4 was calculated by normalizing the actual number of c-kit$^+$ cells at P4 to the number of these cells at T0.

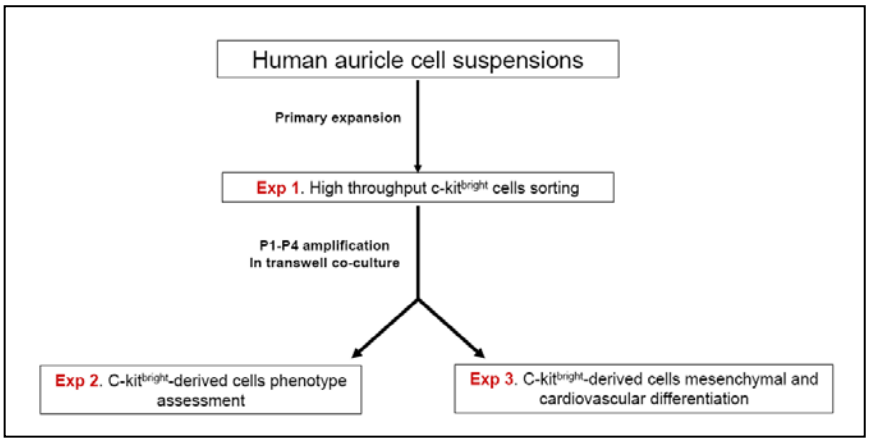

Figure 1A. Experimental design, results of c-kit$^+$ cells sorting from primary culture and effects of culture conditions on c-kit protein expression. A) Work included three main experiments that allowed to obtain purified c-kit$^+$ cells (Exp 1), phenotype assessment of P4 c-kit$^+$-derived cells (Exp 2) and differentiation of P4 c-kit$^+$-derived cells under conditions promoting mesenchymal, vascular and cardiac lineages (Exp 3). These experiments were performed in two subsequent periods, designated as primary culture" and T0-P4 periods.

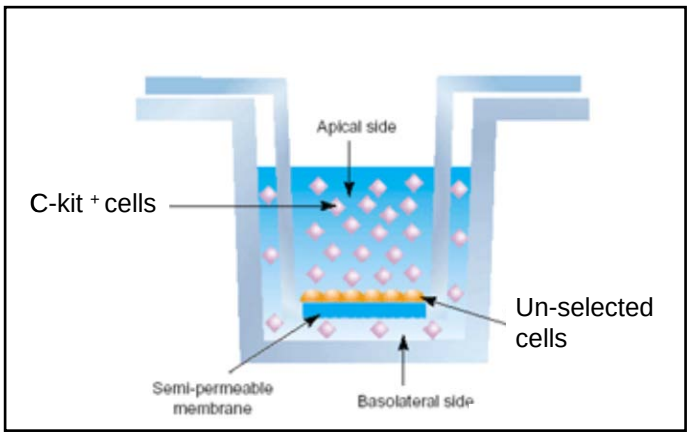

Figure 2. Cell co-culture by the use of a Transwell permeable support. The microporous membrane allowed free diffusion of soluble factors between the upper and the lower chamber. Un-selected cells were plated in the lower chamber, while the c-kit$^+$ cells was cultured in the upper compartment. The use of polyester (PET) Transwell-Clear inserts permitted to have a good cell visibility under phase contrast microscopy.

2.1.2 Isolation of human BM-MSCs

Heparinized BM samples were diluited 1:3 in PBS, layered onto a density gradient (Lymphoprep, Nycomed Pharma AS) and centrifuged (830xg/30min/25°C). Cells at the interphase were collected and plated at a 1x10^6 cells/cm^2 density in IMDM supplemented with 20% FBS (Euroclone, USA), 10ng/ml bFGF (Peprotech), 200mM L-Glutamin (Lonza, Italy) and antibiotics (Lonza, Italy). After 3 days, the medium was completely changed to remove non-adherent cells.

2.2 High troughput cell sorting

A FACSAria (Beckton-Dickinson) flow cytometer/cell sorter was used to identify and separate c-kit$^+$ from c-kit$^-$ cells. Primary amplified cells were grown for two passages as

adherent monolayers before being detached using a non enzymatic cell dissociation solution (Sigma, Italy). Cells were then incubated in PBS containing 0.1% BSA (Gibco, USA) and 2mM EDTA (Gibco, USA) for 15 min in the presence of Allophycocyanin (APC)-conjugated monoclonal antibodies directed against human c-kit receptor (anti-CD117, clone YB5.B8, BD Pharmingen, Italy), at 10 µg/mL concentration. Sorting setup and appropriate gating was established each time using cells appropriately labeled with APC-conjugated isotype control antibody at the same concentration. To minimize cell death and maximize recovery, cells were sorted at low pressure (20 PSI) using a FACSAria Flow cytometer and sorter (BD Biosciences, USA).

2.3 Flow Cytometry

Analysis of mesenchymal, endothelial, HLA and hematopoietic markers was performed by multicolour analysis using the same flow cytometer platform. After detachment from transwell inserts using the non enzymatic method, cells were incubated in PBS containing 0.1% BSA (Gibco, USA) and 2mM EDTA (Gibco, USA) for 15 min with suitable combinations of the following monoclonal antibodies or isotype-matched control monoclonal antibodies: c-kit-APC (clone YB5.B8), CD34- FITC (clone 581), CD45-PE (clone HI30), CD29-PE (clone MAR4), CD44-PE (clone 515), CD90-FITC (clone 5E10), CD130-PE (clone

AM64), HLA-DR-FITC (clone G46-6), (BD Pharmingen, Italy), CD146-FITC (clone 128018), CD200-FITC (clone 325516), KDR-PE (clone 89106), (R&D Systems, USA), CD14-FITC (clone TUK4, MiltenyiBiotec, Italy), CD105-Alexa700 (cloneMEM-229), HLA-G-PE (clone MEM-G/9), (Exbio Praha, Czech Republic), CD144-Alexa700 (clone 16B1, eBioscience, UK).

2.4 Differentiation methods

2.4.1 Adipogenic differentiation

Adipogenic differentiation was induced by culturing P4 c-kit^{+}-derived cells for 3 weeks in adipogenic differentiation medium, consisting of IMDM supplemented with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich), 1 µM hydrocortisone (Sigma-Aldrich), 0.1 mM indomethacin (Sigma-Aldrich), and 10% rabbit serum (Sigma-Aldrich)[22]. The presence of adipocytes was assessed using staining with Oil Red-O stain as an indicator for intracellular lipid accumulation: after fixation, cells were washed in PBS, rinsed with 60% Isopropanol for 1 min and stained with Oil Red-O reagent for 15 min at room temperature. Excess stain was removed by washing with 60% Isopropanol, followed by several washes using PBS. The cells were finally counterstained for 1 min with hematoxylin. As a positive control, mesenchymal stem cells obtained from human bone marrow plated under identical conditions were used.

2.4.2 Osteogenic differentiation

Osteogenic differentiation was induced by culturing P4 c-kit$^+$-derived cells for 3 weeks in osteogenic differentiation medium consisting of IMDM supplemented with 0.1 μM dexamethasone (Sigma-Aldrich), 10 mM β-glycerol phosphate (Sigma-Aldrich), and 0.2 mM ascorbic acid (AsA; Sigma-Aldrich) [22]. The deposition of minelarized matrix was assessed by von Kossa staining: the cells were fixed with 4% paraformaldehyde for 60 min at room temperature, rinsed with distilled water and then incubated using a 1% silver nitrate solution in the dark for 30 min. Cells were thoroughly washed with distilled water and exposed to UV light for 60 min to visualize the crystals (to detect the presence of calcium deposition in osteocyte precursor). After the exposure, the unbound silver was removed by extensive washing. Finally, the cells were counter-stained using 0.1% eosin in ethanol. As a positive control, BM-MSCs plated under identical conditions were used.

2.4.3 Cardiogenic differentiation

Cardiac commitment was tested by culturing c-kit$^+$-derived cells at P4 for 3 weeks in two different differentiation media. Differentiation medium #1 [25] consisted in IMDM containing 5% FBS (Euroclone, Italy), penicillin 0.1U/ml-

streptomycin 0.1ug/ml (Lonza, Italy), 1% L-glutamine (Lonza, Milan, Italy), 5µm All-*trans* retinoic acid (ATRA, Sigma, Italy) and 5µm phenyl-butyrate (Sigma, Italy); differentiation medium #2 [11] consisted in IMDM containing 10% FBS (Euroclone, Italy), penicillin 0.1U/ml–streptomycin 0.1ug/ml (Lonza, Milan, Italy) and 10^{-8} M dexamethasone (Sigma, Milan, Italy).

2.4.4 Endothelial functional assay and differentiation

To assess the ability of P4 c-kit$^+$-derived cells to form vascular structures *in vitro*, they were seeded onto Cultrex (Trevigen, USA) artificial cell basal membrane. Cultrex (250µl) was allowed to polymerize into 24-well plates at 37°C, 5% CO_2 for 30 min. C-kit$^+$-derived cells at P4 were detached by incubating for 3 min at 37°C, 5% CO with trypsin/EDTA (Sigma, Italy) and re-suspended in 7 ml F12H medium. The cells were counted, diluted to $4x10^4$/ml in Endothelial Growth Medium-2 (EGM-2, Lonza, Italy) and transferred to each well containing Cultrex matrix. The plates were incubated at 37°C, 5% CO_2 for a maximum of 24h after which the number of capillary-like structures [23] and the number of their branching points per 2.5x microscopic field were counted. As a positive control in these experiments HUVEC cells (Lonza, Italy) were used under identical culture condition.

Endothelial commitment was analyzed by culturing P4 c-kit$^+$-derived cells at P4 for 3 weeks in Endothelial Growth Medium-2 (EGM-2, Lonza Italy), after which the immunophenotype was determined by flow cytometry.

2.5 Phenotype analysis by q-RT-PCR

Total RNA from non selected cells, c-kit$^+$, P4 c-kit$^+$-derived cells and differentiated cells was extracted using RNeasy Mini kit and RNase-Free DNase Set (Qiagen, CA, USA). Total RNA was eluted using 20-40 µl of RNase-free water. 1 µg of total RNA was treated with DNase I. After DNase I heat inactivation, RNA was reverse transcribed (RT) using SuperScriptIII cDNA synthesis kit (Invitrogen, CA). RNA was incubated for 30 min at 50°C. Synthesized cDNA was finally treated with 2U Rnase H at 37°C for 20min. Real time RT-PCR analysis was performed on iQ5 Real Time PCR System (Bio-Rad, Italy). Each primer pair was tested in duplicate using 10ng of the cDNA. Primers were designed from available human sequences by using the primer analysis software Primer Express v3.0 (Applied Biosystems, CA). The PCR-reaction included 10ng of template cDNA, 0.2µM of each (forward and reverse) primers, and 12.5 µl of iQ SYBR Green Supermix (Bio-Rad, Italy), conjugated with the fluorescent dye FAM in a total volume of 25 µl. Cycling conditions were as follows: 95°C enzyme activation for 10 min, followed by 40 cycles of amplification (95°C 15''

denaturation, 60°C 1 min annealing/elongation). Quantified values were normalized against the input determined by the housekeeping human gene β-2-microglobulin. Data are expressed as mean of the differences between the threshold cycle (CT) for each amplified transcript and the housekeeping gene (ΔCT). After reverse transcription, primers specific for cardiac, stem cell, differentiated mesenchymal cells and vascular cells were used to assess cell phenotype of human CPCs by real time PCR.

Human β-2-microglobulin fw	CATTCCTGAAGCTGACACCATTC
Human β-2-microglobulin rev	TGCTGGATGACGTGAGTAAACC
Human c-kit forward	GCTTTTCTTACCAGGTGCCAAA
Human c-kit reverse	GAGGATATTTCTGGCTGCCAAGT
Human MDR-1 forward	GGCTCCGATACATGGTTTTCC
Human MDR-1 reverse	CCAGTGGTGTTTTTAGGGTCATC
Human GATA-4 forward	AGCCTGGCCTGTCATCTCACT
Human GATA-4 reverse	GGCCAGACATCGCACTGACT
Human Myo-C forward	CAGGGTCTCCTCGCCCAT
Human Myo-C reverse	CTTATCAGAAGAGTGTAAACCACGCA
Human Tbx-5 forward	TAGCAGTGACTTCCTACC
Human Tbx-5 reverse	ACGGGATATTCTTTACTTT
Human Mlc2a forward	CTTGTAGTCGATGTTCCCCG
Human Mlc2a reverse	TCAAGCAGCTTCTCCTGACC
Human PPAR-γ2 forward	TCCTTCACTGATACACTGTCTGC
Human PPAR-γ2 reverse	CATTACGGAGAGATCCACGGA
Human Adipsin forward	GACACCATCGACCACGACC
Human Adipsin reverse	GCCACGTCGCAGAGAGTTC
Human Osteopontin forward	GCCGAGGTGATAGTGTGGTT
Human Osteopontin reverse	TGAGGTGATGTCCTCGTCTG
Human α-SMA forward	GCTCCCCTAAATCCCAAGGC
Human α-SMA reverse	ATCACCTGAATCCAGGACGAT
Human α-MHC forward	GACTGTTGTGGCCCTGTACCA
Human α-MHC reverse	GACACCGTCTGGAAGGATGAG
Human α-sarcomeric actin fw	TGTCCTGAGACACTCTTC
Human α-sarcomeric actin rev	TGATGCTATTGTAAGTTGTT

Table 1. Sequences of primers pairs that were used

2.6 Immunofluorescence

P4 c-kit^{+}-derived cells were immediately fixed or cultured for 1-3 weeks into cardiac differentiation media; the cells were fixed in PBS 4% paraformaldheyde. After blocking with PBS containing 5% serum and 0.3% Triton-X100 for an overnight at 4°C; cells were incubated with primary antibodies for alpha-Smooth Muscle Actin (α-SMA) or cardiac markers. The following primary antibodies were used: anti-Tropomyosin, anti-Troponin, anti-Actinin, anti-Atrial Natriuretic Peptide (ANP), anti-Desmin (all from Cardiomyocite characterization kit, Millipore, USA), anti-Connexin 43 (clone GJA1, abcam, UK), phalloidin-TRITC (Sigma-Aldrich). Alexa 488-conjugated secondary antibodies (Invitrogen) were used. Nuclear staining was performed by incubating cells with Hoechst 33342 (Sigma-Aldrich). Cells Images were acquired with Zeiss Axio Observer Z1 microscope equipped with Apotome image deconvolution system and Axiovision software (Carl Zeiss, Germany).

2.7 Cytokine, chemokine and growth factor detection

Bio-Plex assay (Bio-Rad Laboratories, Italy), a bead-based multiplex immunoassay, was used to quantify cytokines, chemokines and growth factors released in the culture

supernatant conditioned for 24 hrs by similar amounts of primary cells at T0 and supporting stromal cells at P4. Supernatants were collected after 24h of conditioning and analysed by Bio-plex assay.

2.8 Statistical analysis

Comparison of results was performed by using paired or unpaired Student's t-test using GraphPad statistical software. A $P<0.05$ value was chosen as the significance level for considering results different on a statistical basis.

3. RESULTS

3.1 High throughput sorting of c-kit$^+$ cells and co-culture onto parental unselected cells allows the propagation of a CPC-enriched cellular population

In the mouse and the human heart, c-kit$^+$ CPCs have been defined as clonogenic cells arising from cell suspensions obtained by myocardium enzymatic digestion. Two different methods have been used, both of which have been successfully applied to obtain these cells: 1) direct purification of c-kit$^+$ cells from primary cell suspensions obtained by enzymatic digestion of myocardial specimens using immunobeads [11, 12] or 2) cellular outgrowth of cells by the primary explants technique[12], followed by immunosorting c-kit$^+$ cells and culture under clonal dilution conditions. To maximize stem cells growth before sorting, we plated the total cellular population from heart specimens for a two passages amplification step. This phase was designated as "primary" expansion period (Figure 1A). The time to reach 70% confluence in 10 cm cell culture dishes and the number of cells that were collected at the end of the primary culture period are shown for each patient in Table 2; except for two cases, the time to reach this stage was shorter than ≈3 wks. When reaching the established

confluence condition, cells were detached and stained with an APC-conjugated monoclonal antibody directed against the c-kit receptor followed by flow cytometry (Figure 1A). Flow cytometry analysis allowed the recognition of cells stained with c-kit antibody; this population was gated to separate CPCs from differentiated cells using flow cytometry-based cell sorting (Figure 1B). The percentage of cells gated as c-kit$^+$ cells for cell sorting is indicated in Table S1 for each patient; the purity of these cells was 81.1%±4.1% (mean±SE, n=4, Figure 1B).

After sorting, c-kit$^+$ cells were sub-cultured into transwell inserts in the same medium that was used to amplify the primary cellular suspensions. Surprisingly, the percentage of cells expressing c-kit at passage 4 was reduced, suggesting *c-kit* downregulation during *ex vivo* expansion (Fig. 1 C, D). In a previous report from our laboratory it was shown that heart-derived fibroblasts support the propagation of c-kit$^+$ CPCs *via* a paracrine action[18]. To assess whether unselected primary cells supported CPCs propagation, we seeded c-kit$^+$ cells into transwells placed into culture wells containing unfractionated cells from the same patient. In this way c-kit$^+$ CPCs were allowed to grow in conditioned medium by unselected cells, but without physical contact with them for four passages (T0-P4 expansion period; Fig. 1A). To assess c-kit expression in these cells, the percentage of c-kit$^+$ cells and the c-kit mean fluorescence

intensity (MFI) in these cells was determined by flow cytometry. The determination of the c-kit MFI was performed in order to evaluate, although indirectly, the c-kit protein expression level. The results showed that in coculture, the percentage of c-kit$^+$ cells and the MFI relative to anti-c-kit antibody staining were both significantly higher compared to cells cultured in the absence of supporting cells (Figure 1 C, D). This suggested that conditioning by heart stromal cells prevented, at least in part *c-kit* downregulation, and maintained CPCs self-renewal even in the absence of physical cell contact. To characterize factors that are possibly implicated in the maintenance of the stem cell pool in co-culture, we screened for cytokines expressed by unselected cells at T0 and supporting stromal cells recovered from the lower transwell chambers at P4, using multiplex technology. To this aim these cells were cultured alone under adhesive conditions and allowed to release factors in the medium for 24hrs. The results showed that unselected cells from primary expansion period yielded significantly lower amounts of several cytokines and growth factors compared to supporting stromal cells at P4 (Table 3). G-CSF, IL-8, VEGF and IL-6 were the mostly upregulated cytokines.

PATIENT NO	DAYS TO T0	Number of cells @ T0 ($x10^6$)	% c-kit$^+$ cells in sorting gate
13	21	9.60	3,3
14	28	1.10	2,4
15	28	1.00	2,4
16	21	3.00	0,2
17	18	14.80	0,9
20	19	32.60	1,9
21	11	14.60	0,1
22	14	30.50	3,2
23	22	5.50	1,1
24	22	16.20	1,9
25	11	17.30	0,8
26	12	6.70	1,0
27	17	17.50	4,0
28	21	18.30	0,4
29	14	9.40	4,0
30	18	36.00	2,0
31	21	1.70	1,1
32	13	11.50	3,5
34	14	5.50	2,4
35	12	4.25	2,2
36	7	7.60	3,4
37	8	37.70	3,7
38	10	18.60	2,2
39	20	2.27	1,4
41	17	8.70	2,0
42	13	10.90	3,8
43	16	6.62	0,7
45	18	16.40	0,4
MEAN ± SE	**16.64 ± 1.01**	**13.07 ± 1.96**	**2.01 ± 0.23**

Table 2 Parameters relative to primary cells amplification procedure. Days necessary to perform the initial expansion of primary cells, the number of cells obtained at the end of this period and available for c-kit$^+$ cells sorting and percentage of sorted c-kit$^+$ cells are shown for each patient enrolled in the study.

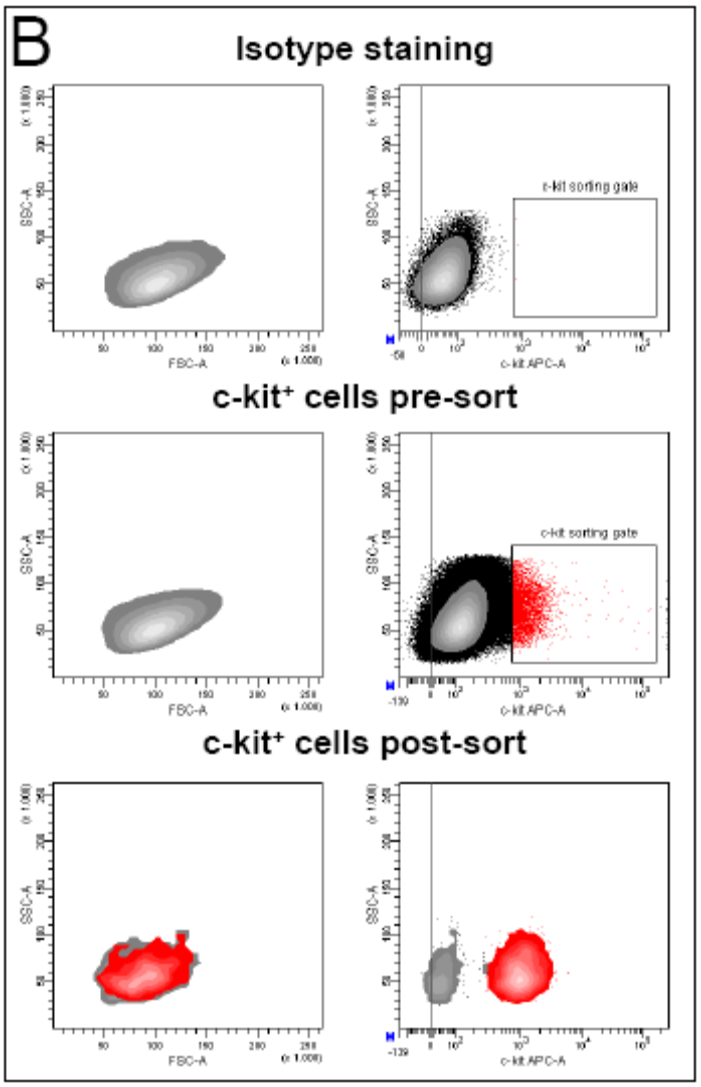

Figure 1B. C-kit$^+$ cells sorting procedure. The appropriate gating to separate c-kit$^+$ from c-kit$^-$ cells was established each time using ACP-conjugated isotype antibody (upper panels). C-kit$^+$ cells were then sorted using this gating (middle panels). C-kit$^+$ cells purity was checked by analyzing sorted cells immediately after separation using flow cytometry (lower panels). Sorted cells where 81.1±4.1% positive for c-kit (mean±SE, n=4).

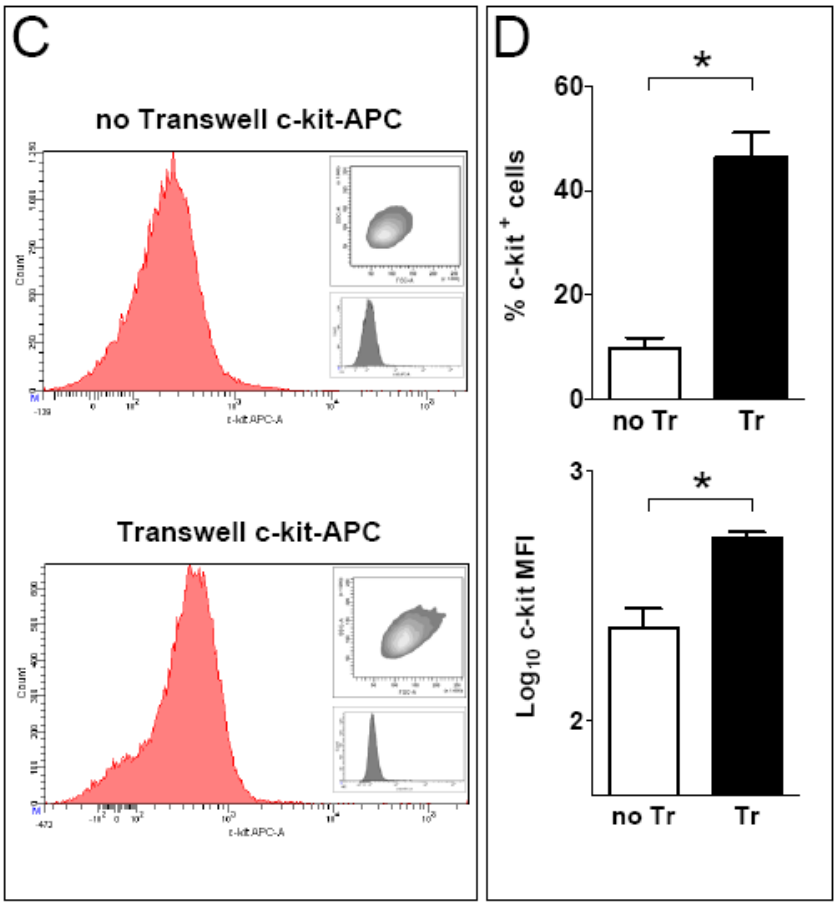

Figure 1C. Coculture in transwells onto unselected cells maintained expression of c-kit in a greater percentage of cells with a higher c-kit fluorescence MFI. Cells were cultured alone or in transwells for 4 passages in the same culture medium, after which they were analyzed for c-kit expression by flow cytometry. The panels show the analysis of cells from the same patient cultured in the two conditions. Insets show the forward scatter/side scatter plots and the APC-conjugated isotype antibody histogram plots, used as a reference to calculate c-kit$^+$ cells percentages.

Figure 1D. Quantification of c-kit$^+$ cells percentage and MFI (expressed as log10) in c-kit$^+$-derived cells at P4 cultured in transwell vs. no transwell conditions. * indicates $P<0.05$ by Student's t-test (n=3).

CYTOKINES	Primary culture cells (n=4)	P4 c-kit$^-$ supporting cells (n=5)
Angiopoietin-2	0,148±0.049	0.0±0.0
Follistatin	0,734±0.231	1.29±0.13
G-CSF (32)	0,003±0.0006	23.77±6.77 *
HGF	0,069±0.029	1.36±0.73
IL-8	0,295±0.023	30.84±5.21 *
Leptin	0,017±0.001	0.15±0.02 *
PDGF-BB	0,012±0.001	0.43±0.04 *
PECAM-1	0,183±0.016	4.51±0.39 *
VEGF	0,101±0.025	8.16±1.57 *
LIF	0,0007±0.0004	0.4±0.08 *
SDF-1 α	0,0646±0.015	0.51±0.17
SCF	0,000	0.0025±0.0016
IL-6	0,511±0.106	35.66±3.93 *
GM-CSF	0,001±0.0003	0.13±0.02 *
IFN-γ	0,052±0.008	0.99±0.10 *
IL-2	<0.0001	0.029±0.003 *
IL-4	<0.0001	0.009±0.0009
TNF-α	<0.0001	0.019±0.002 *

Table 3. Expression of growth factors and cytokines in unselected cells compared to supporting stromal cells recovered from the transwells lower chambers. Table indicates the expression of each cytokine in ng/ml/10^5 cells/24hrs[18] and the results of statistical comparison of cytokine yielded by the two cell types by Student's t-test. * indicates $P<0.05$.

3.2 Immunophenotype of c-kit$^+$-derived cells reveals a mesenchymal phenotype of human heart-derived CPCs.

After 4 passages in transwell co-culture, the absolute number of c-kit$^+$ CPCs was increased by 15.59±3.5 folds (mean±SE; n=13; Table 4).

PATIENT NO	Number of sorted T0 c-kit$^+$ cells	DAYS to P4 in transwell	Number of P4 c-kit$^+$-derived cells (x10^6)	CPCs Fold enrichment
22	150000	27	2,9	5,80
23	40000	28	3,5	11,39
24	160000	31	3,4	3,17
26	40000	37	3,6	8,28
27	120000	27	9,6	21,44
28	160000	21	3,5	1,90
29	80000	31	7,2	21,98
30	61505	27	3,6	10,20
32	62079	27	5,0	15,57
37	90000	27	5,5	33,37
38	60000	39	5,8	10,05
41	29100	21	1,0	13,13
42	40000	21	4,0	46,40
MEAN ± SE	**8.4 X10^4 ± 1.33 X10^4**	**28 ± 1.54**	**4.5 ± 0.59**	**15.59 ± 3.5**

Table 4. Parameters relative to T0-P4 c-kit+ cells expansion period in transwells onto unselected cells. Number of sorted c-kit$^+$ cells, days necessary to reach P4, number of P4 c-kit$^+$-derived cells and the CPCs fold enrichment, are indicated.

To characterize the immunophenotype of P4 c-kit$^+$-derived cells, different antibody panels to detect hematopoietic, endothelial and mesenchymal antigens expression, alone or

in combination with c-kit were used in flow cytometry experiments. A high percentage of P4 c-kit$^+$-derived cells expressed mesenchymal markers CD29, CD44, CD105, CD130 and CD200[13, 14, 19] (Fig. 3A) . CD90, another marker canonically defining MSCs[14] was expressed in smaller percentages of cells with higher variability. The MFI analysis confirmed these findings and identified CD29, CD44 and CD105 as the mostly expressed MSCs markers in c-kit$^+$-derived cells at P4 (Fig. 3B). The expression of the hematopoietic markers CD14, CD34, CD45, the endothelial markers VEGFR-2/KDR, CD144, CD146, and major histocompatibility complex (MHC) HLA-DR and HLA-G was also analyzed in P4 c-kit$^+$-derived cells. The results showed the absence of hematopoietic markers (not shown) and HLA-DR (Fig. 4) and a low percentage of cells expressing endothelial and human HLA-G antigen (Fig. 3 C, D and 4).

The multiparametric flow cytometry analysis performed at P4 showed that, with the exception of CD90, MSCs markers were co-expressed in a fraction >92% of c-kit$^+$ cells after coculturing onto primary unselected cells (Figure 5A). By contrast, only a minimal percentage of c-kit$^+$ cells at this stage expressed endothelial markers (Fig 5B). The unexpected high expression level of MSC markers in cells at P4 may result from changes in the c-kit$^+$ stem cell activity due to the *ex-vivo* amplification conditions in coculture. To address this possibility, a flow cytometry analysis was

performed on c-kit$^+$ cells at T0. As shown in Figure S3, c-kit$^+$ cells at T0 co-expressed high levels of mesenchymal markers CD29, CD44 and CD105, suggesting that expression of mesenchymal markers was not a consequence of co-culture onto differentiated cells in transwells.

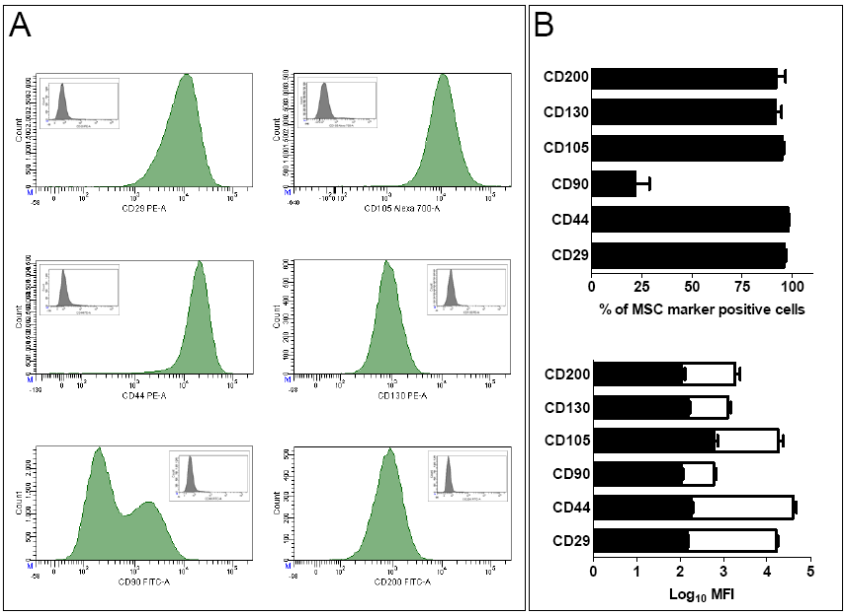

Figure 3. Expression of mesenchymal and endothelial markers in P4 c-kit$^+$-derived cells in transwell coculture.
A-B) Histogram plots represent the expression of CD29, CD44, CD90, CD105 (all n=7) CD130 (n=3) and CD200 (n=5) mesenchymal (MSC) markers in P4 c-kit$^+$-derived cells. Percentage bar graph indicates the quantification of the MSC marker+ cell percentage. Insets in each histogram plot represent the fluorescence profile of cells stained with appropriate isotype antibodies conjugated with the same fluorochromes. Log10 MFI bar graph indicates the fluorescence intensity of cells stained with appropriate isotype antibodies conjugated with the same fluorochromes. Plotting was performed by overlapping the log10 MFI values scored with each marker-specific antibody (open bars) to the log10 MFI values scored using appropriate isotype antibodies for each marker (closed bars).

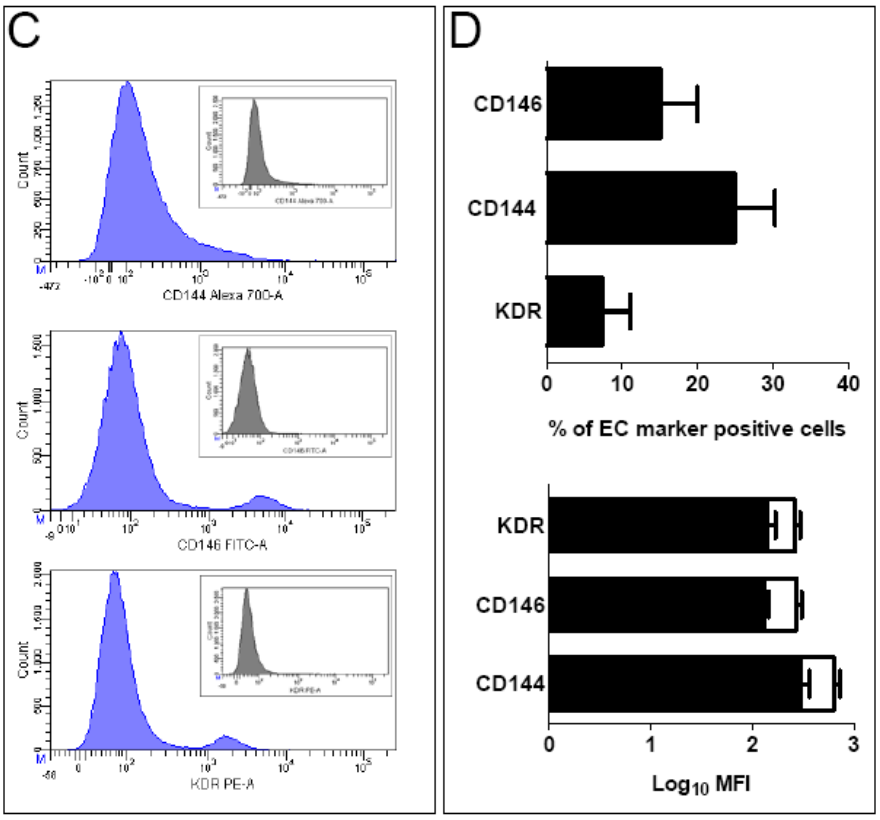

Figure 3. C-D) Histogram plots representing the expression of endothelial cell (EC) VEGFR2/KDR, CD144 and CD146 markers in P4 c-kit^{+}-derived cells. Graph bar indicate quantification of the EC marker^{+} cell percentage and log10 MFI by flow cytometry. Insets in each histogram plot represent the fluorescence profile of cells stained with appropriate isotype antibodies conjugated with the same fluorochromes. Percentage bar graph indicates the quantification of the MSC marker+ cell percentage. Log10 MFI bar graph indicates the fluorescence intensity of cells stained with appropriate isotype antibodies conjugated with the same fluorochromes. Plotting was performed by overlapping the log10 MFI values scored with each marker-specific antibody (open bars) to the log10 MFI values scored using appropriate isotype antibodies for each marker (closed bars).

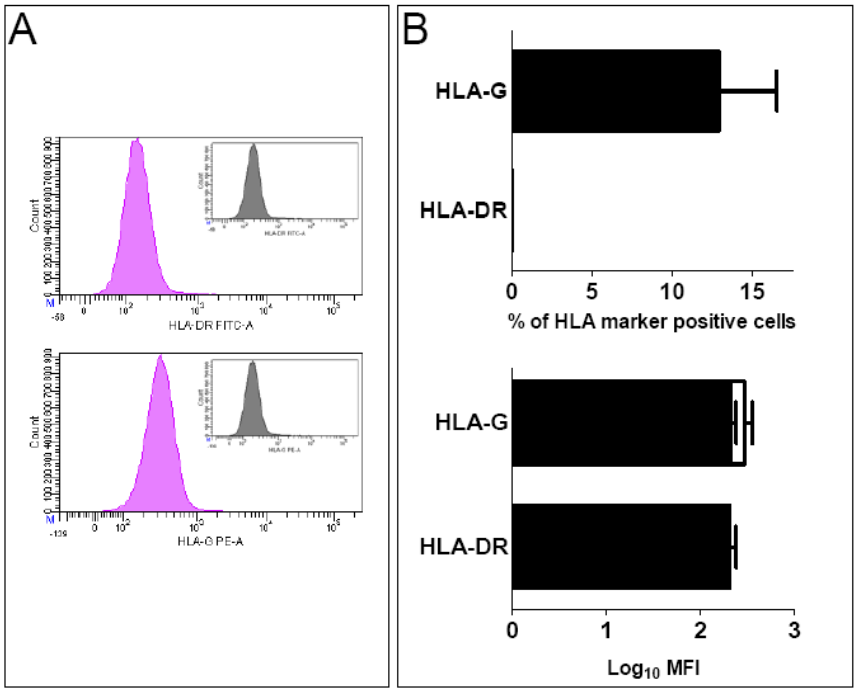

Figure 4. Expression of HLA-DR and HLA-G in P4 c-kit$^+$-derived cells in transwell coculture.
A-B) Histogram plots represent the expression of of HLA-DR and HLA-G (n=5) in P4 c-kit$^+$-derived cells. Percentage bar graph indicates the quantification of the MSC marker$^+$ cell percentage. Insets in each histogram plot represent the fluorescence profile of cells stained with appropriate isotype antibodies conjugated with the same fluorochromes. Log_{10} MFI bar graph indicates the fluorescence intensity of cells stained with appropriate isotype antibodies conjugated with the same fluorochromes. Plotting was performed by overlapping the log_{10} MFI values scored with each marker-specific antibody (open bars) to the log_{10} MFI values scored using appropriate isotype antibodies for each marker (closed bars).

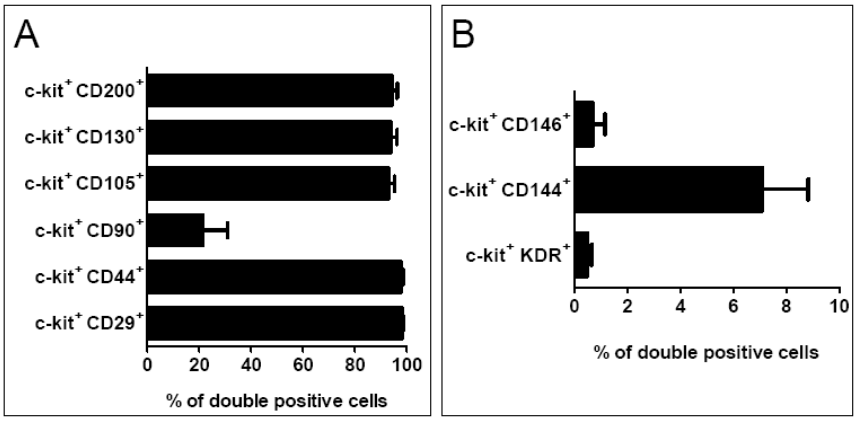

Figure 5. Co-expression of c-kit with mesenchymal and endothelial cells markers in P4 c-kit$^+$-derived cells.
A) P4 c-kit$^+$-derived cells were analyzed for the co-expression of the indicated markers with c-kit by multicolour flow cytometry analysis. The bar graph shows the percentage of double positive c-kit$^+$/MSC marker$^+$ in 5 independent cellular preparations (n=3 for CD130).
B) Co-expression of EC cell markers CD144, CD146 and VEGFR-2/KDR and c-kit in 7 independent cellular preparations. Bar graph shows the percentage of double positive c-kit+/EC marker+.

3.3 Phenotype stability of c-kit+-derived cells

To assess the CPCs phenotype stability during the culture procedure, we performed q-RT-PCR on cDNA exctracted from primary cells, sorted c-kit$^+$ at T0 and P4 c-kit$^+$-derived cells. Primers to amplify genes expressed in undifferentiated (*c-kit*, *MDR-1*) or early differentiating cardiac progenitors (*GATA-4*, *Tbx-5* and *Mlc2A*)[20, 21] were used. The cells expressing highest *c-kit* levels were T0 c-kit$^+$ cells. Interestingly, *c-kit* expression in P4 c-kit$^+$-derived cells was significantly higher compared to unselected primary cells. All the other tested genes were expressed at comparable

levels, indicating phenotype stability of c-kit$^+$-derived cells (Fig. 6). To test whether major changes occurred in the cardiac genes expression in undifferentiated stem cells compartment during the coculture period, c-kit$^+$ cells from the same individuals were sorted at T0 and further resorted at P4. The expression of the same genes analyzed in Figure 6 were thus compared by q-RT-PCR. Figure 7A shows the results of sorting experiments performed at the two subsequent times using cells from the 5 same individuals; in these experiments, the percentage of sorted c-kit$^+$ cells were similar and not statistically different. No differences were found in the gene expression level in c-kit$^+$ cells sorted at the two different culture steps (Figure 7B), suggesting CPCs self-renewal and phenotype stability over the time of *ex vivo* amplification procedure.

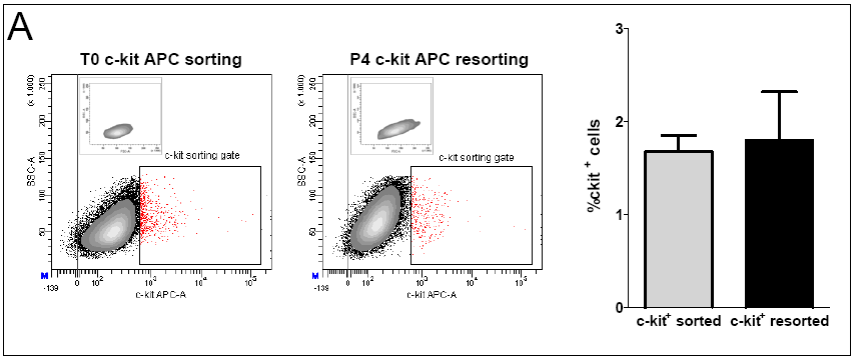

Figure 6. Comparison of c-kit$^+$ cells sorting at T0 and P4 and results of cardiac genes expression at in c-kit$^+$ cells sorted at T0 and P4. **A)** Comparison between the sorting gating protocols adopted to separate c-kit$^+$ cells from the same individuals after primary culture (T0) and after 4 passages in transwell coculture onto unselected cells (P4). The plots indicate the two logical gates that were adopted to identify and separate c-kit$^+$ cells, while the bar graph indicates the percentage of c-kit$^+$ cells sorted at the two different stages in amplification procedures from 5 individuals. No difference were found neither in the percentage of cells, nor in the MFI values relative to c-kit staining (not shown).

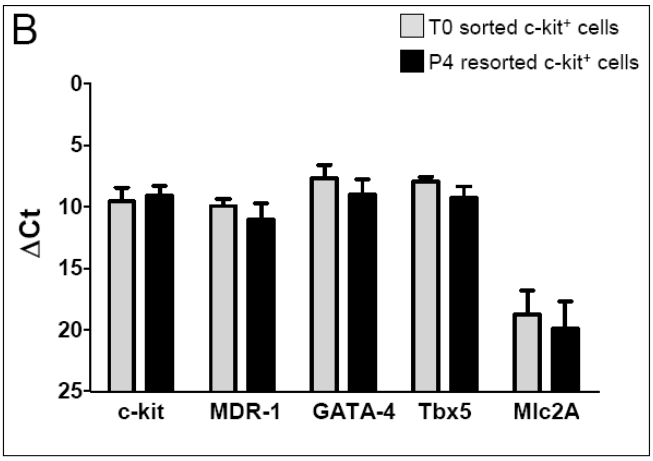

Figure 6 B) q-RT-PCR expression analysis of the same cardiac markers analyzed as in Figure 3. No differences were found between T0 sorted and P4 resorted c-kit$^+$ cells.

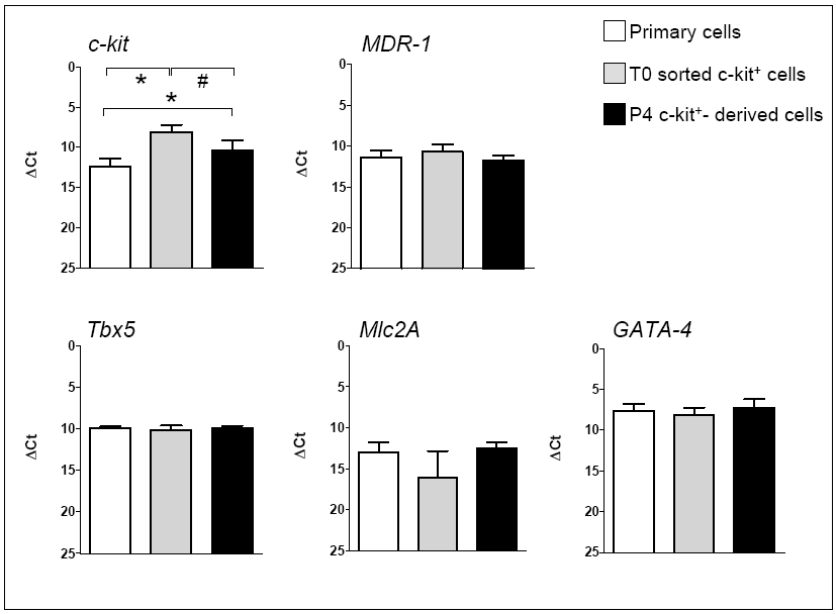

Figure 7. q-RT-PCR analysis of cardiac markers expression throughout the c-kit$^+$ CPCs sorting/amplification procedure. Total RNA from primary cultured unselected cells, T0 c-kit$^+$ cells, and P4 c-kit$^+$-derived cells (all n=4) were extracted and, after reverse transcription, amplified using primers pairs specific to detect human cDNAs for *c-kit*, *MDR-1*, *Tbx5*, *GATA-4* and *Mlc2A* genes. Results, that were compared with the ΔCT method, were normalized using β-2-microglobulin as a reference housekeeping gene. The analysis showed significantly different expression levels only in the case of *c-kit*. The expression of the other genes was not changed indicating phenotype stability of cells throughout the amplification process. * indicates P<0.05, #indicates P=0.05, by Student's t-test.

3.4 Multilineage differentiation of P4 c-kit$^+$-derived cells

Among the major criteria that have been adopted to recognize cells having a mesenchymal phenotype, the ability to differentiate into osteoblasts, adipocytes and chondroblasts is a prerequisite[14]. The expression of mesenchymal markers at high levels in P4 c-kit$^+$-derived cells suggested a possible mesenchymal differentiation ability. Adipogenic and osteogenic differentiation were thus tested using BM-derived MSCs as a positive control[22] (Fig. 9). Plating P4 c-kit$^+$-derived cells under *pro*-adipogenic conditions produced cells that were capable of accumulating lipid droplets similarly to BM-MSCs, as assessed by Oil-red-O staining (Fig. 9A). A morphological differentiation of the two cells types into osteocyte-like cells was also recognized by von Kossa staining when P4 c-kit$^+$-derived cells and BM-MSCs were plated under *pro*-osteogenic conditions (Fig. 9B). Significant differences were noticed by q-RT-PCR analysis on key genes involved in adipogenic and osteogenic differentiation. In fact, P4 c-kit$^+$-derived cells expressed *pro*-adipogenic genes *PPAR-γ* and *Adipsin* and *pro*-osteogenic gene *Osteopontin (OPN)* at significantly lower levels compared to BM-MSCs.

Vascular commitment of c-kit$^+$-derived cells was then assessed by functional assays and phenotype analysis by flow cytometry, immunofluorescence and q-RT-PCR. As a

functional test, the ability of P4 c-kit$^+$-derived cells to form capillary-like structures was tested[23]. Figure 8A shows that formation of tubular-like structures onto artificial basal lamina, a typical hallmark of endothelial cells, was quantitatively not different from human umbilical vein endothelial cells (HUVEC). To further assess the endothelial commitment of c-kit$^+$-derived cells at P4, these cells were sub-cultured into endothelial-specific EGM-2 medium for three weeks. At the end of this period, expression of endothelial–specific antigens was assessed by flow cytometry. Results showed a statistically significant VEGFR2/KDR expression increase (Figure 8 B). CD144, another marker expressed in mature endothelial cells, was increased, although with a high variability that did not allow to reach statistical significance, while CD146 was not changed. Interestingly, culture into EGM-2 medium caused a significant c-kit downregulation (Fig. 8B), suggesting commitment of *ex vivo* amplified CPCs towards a mature endothelial phenotype. It has been recently shown that c-kit expression characterizes two different heart resident progenitor populations, the myocyte progenitor cells (MPCs) and the vascular progenitor cells (VPCs)[24]. These two CPCs types have a preferential commitment to differentiate into myocytes and vascular cells, respectively. The expression of α-SMA was investigated in unselected, sorted T0 c-kit$^+$ cells and P4 c-kit$^+$-derived cells by q-RT-PCR (Fig.

9C). Consistently with the more immature phenotype revealed by higher *c-kit* expression level (Fig. 7), the q-RT-PCR analysis revealed a lower, although not significant, α-*SMA* expression in T0 sorted c-kit$^+$ cells (Fig. 8C); immunofluorescence for α-SMA revealed a mixed phenotype of P4 c-kit$^+$-derived cells, consisting in small cells expressing low or intermediate α-SMA levels, and larger cells expressing α-SMA at high levels and showing, at least in some cases, partially polymerized α-SMA (Fig. 8C).

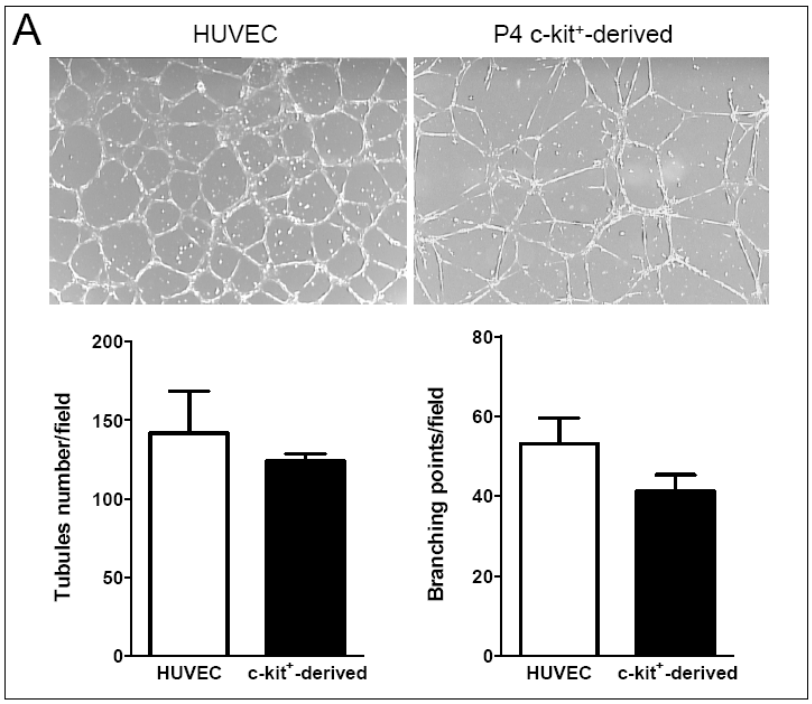

Figure 8. Vascular commitment of P4 c-kit$^+$-derived cells. **A)** Capillary-like structures formation by HUVEC EC cells and P4 c-kit$^+$-derived cells onto Cultrex basement membrane. Micrographs show the morphology of these structures formed by both cells types, as observed at low magnification. Plots indicate the quantification of the numbers of these structures/microscopic field (left plot) and the number of branching points between them (right plot) at 2.5X magnification. Statistical comparison of these values revealed no significant differences (n=3 for each parameter).

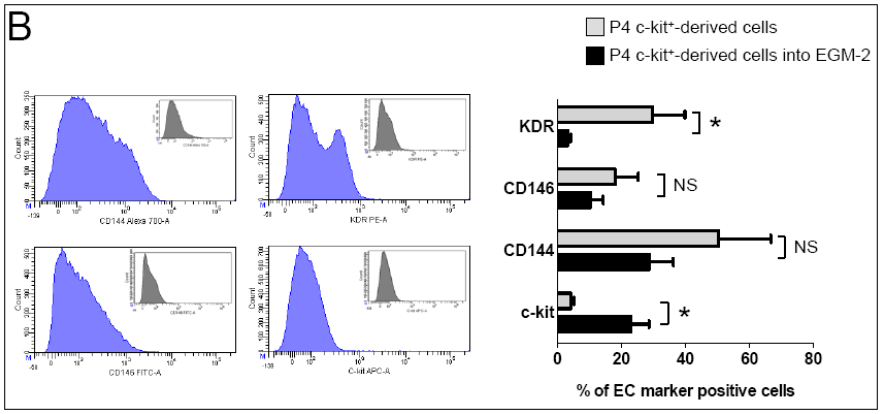

Figure 8 B) Differentiation of P4 c-kit$^+$-derived cells into EC cells. Flow cytometry was performed to determine expression of VEGFR-2/KDR, CD144 and CD146 mature EC cell markers (all n=4) after culturing P4 c-kit$^+$-derived cells for three weeks into EC cell-specific EGM-2 medium. Histogram plots show the fluorescence profile of each antigens after EGM-2 culture; inserts show the reference fluorescence plots obtained by staining cells using isotype antibodies. Bar graph on the right side shows the comparison between expression of c-kit, CD144, CD146 and VEGFR-2/KDR markers before and after culturing P4 c-kit$^+$-derived cells into EGM-2 medium. * indicates P<0.05 using Student's t-test.

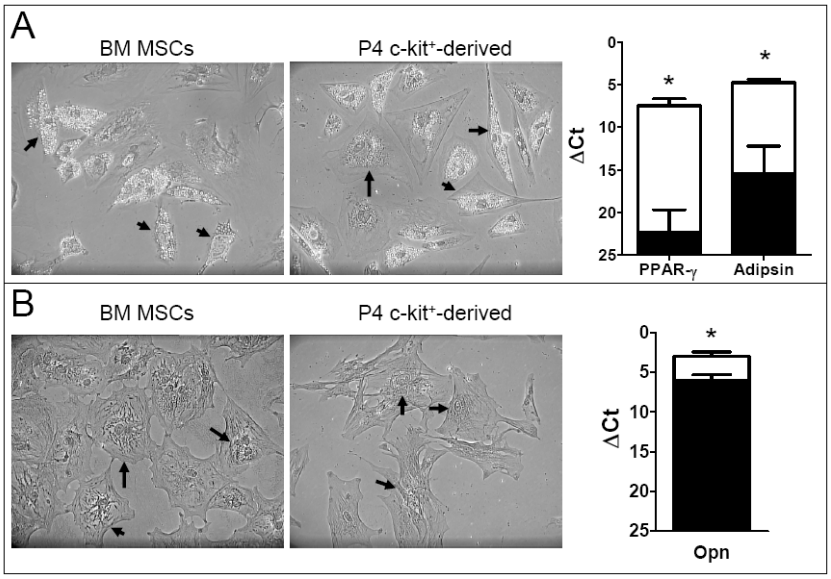

Figure 9. Mesenchymal differentiation of P4 c-kit$^+$-derived cells and BM-MSCs. **A)** The results of culture into *pro*-adipogenic conditions favoured, in both cell types, the accumulation of lipid droplets in the cytoplasm (arrows), as revealed by oil-Red-O staining (left panels). Morphological differentiation was not, however, associated to similar upregulation of the two *pro*-adipogenic genes *PPAR-γ* and *Adipsin*, that resulted to be significantly higher expressed in BM-MSCs compared to P4 c-kit$^+$-derived cells. Results, that were compared with the

ΔCT method, were normalized using β-2-microglobulin as a reference housekeeping gene. Data are plotted by overlapping the ΔCT values calculated for *PPAR-γ* (n=4) and *Adipsin* (n=3) in BM-derived MSCs (open bars) and P4 c-kit$^+$-derived cells (closed bars). * indicate P<0.05 for statistical comparison of ΔCT values for each gene expressed in each cell type under adipogenic differentiation conditions by Student's t-test.

B) Osteoblast differentiation of BM-derived MSCs and P4 c-kit$^+$-derived cells. von Kossa staining revealed the presence of intracellular Ca crystals (arrows), although at higher levels in BM-derived MSCs compared to P4 c-kit$^+$-derived cells. The significantly lower expression of *Osteopontin* (*OPN*) gene suggests a lower ability of P4 c-kit$^+$-derived cells to differentiate into osteocytes. *OPN* expression data (n=4) were normalized, plotted and statistically analyzed as in panel A.

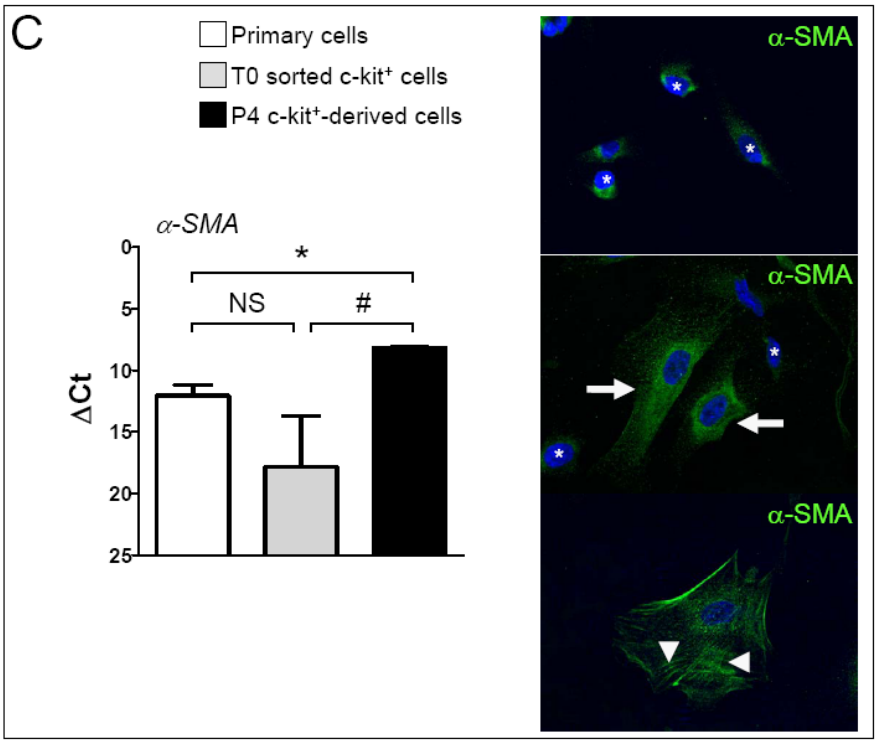

Figure 9 C) Smooth muscle cells phenotype of P4 c-kit+-derived cells. Bar graph on the left side indicates the comparison of the α-SMA gene expression in primary unselected cells, T0 c-kit$^+$ and P4 c-kit$^+$-derived cells. α-SMA expression data (n=4; ΔCT method) are plotted and statistically analyzed as in Figure 3. * indicates P<0.05, #indicates P=0.05, by Student's t-test. Panels on the right indicate three micrographs showing immunofluorescence staining of P4 c-kit$^+$-derived cells using an anti α-SMA antibody. Different morphologies of these cells and different α-SMA expression levels were detected. Some cells (asterisks) were small and expressed low or intermediate α -SMA levels. Larger cells expressed higher α-SMA levels (arrows); in some cases cells showing partially polymerized α -SMA in the cytoplasm were also observed (arrowheads).

3.5 Cardiac commitment of P4 c-kit$^+$-derived cells

Cardiac differentiation was assessed by culturing P4 c-kit$^+$-derived cells into two distinct media that, according to previous reports[11, 25], induce cardiac commitment of pluripotent stem cells and heart-derived CPCs, respectively. As shown in figure 7A, both media induced the expression of the two late cardiac markers alpha myosin heavy chain (αMHC) and alpha sarcomeric actin (αSarc-Actin); these markers were not expressed in P4 c-kit$^+$-derived cells (Fig. 10A). To further explore cardiac differentiation ability, P4 c-kit$^+$-derived cells were plated for 1-3 weeks in cardiac differentiation medium #1 and were analyzed for the expression of late cardiac markers by immunofluorescence. Atrial Natriouretic Peptide (ANP), gap junction-associated protein Connexin 43 (CX43), and Tropomyosin (T-Myo) were specifically detected in P4 c-kit$^+$ cells (Fig. 10B). Staining by phalloidin revealed the formation of sarcomeric structures. Other markers such as Troponin-I (TnI), Actinin and Desmin were not detected (not shown). Altogether, these findings suggest that P4 c-kit$^+$-derived cells are committed into cardiac phenotype, although they did not undergo a complete differentiation program into mature cardiac myocytes.

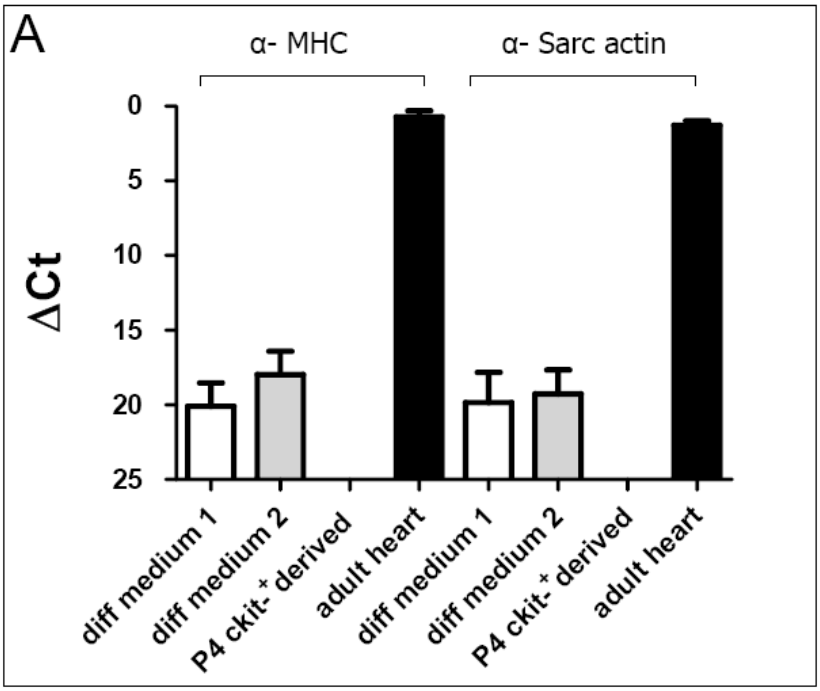

Figure 10. Cardiac commitment of P4 c-kit^{+}-derived cells. **A)** P4 c-kit^{+}-derived cells were plated into two different cardiac differentiation media both of which induced expression of late cardiac markers α-Myosin Heavy Chain (α-MHC) and α -Sarcomeric Actin at similar levels. The induction of these markers (n=5) was quantified by q-RT-PCR and quantified using the ΔCT method as in Figure 3. The expression of these genes in P4 c-kit^{+}-derived cells was lower compared to positive control (adult human myocardium).

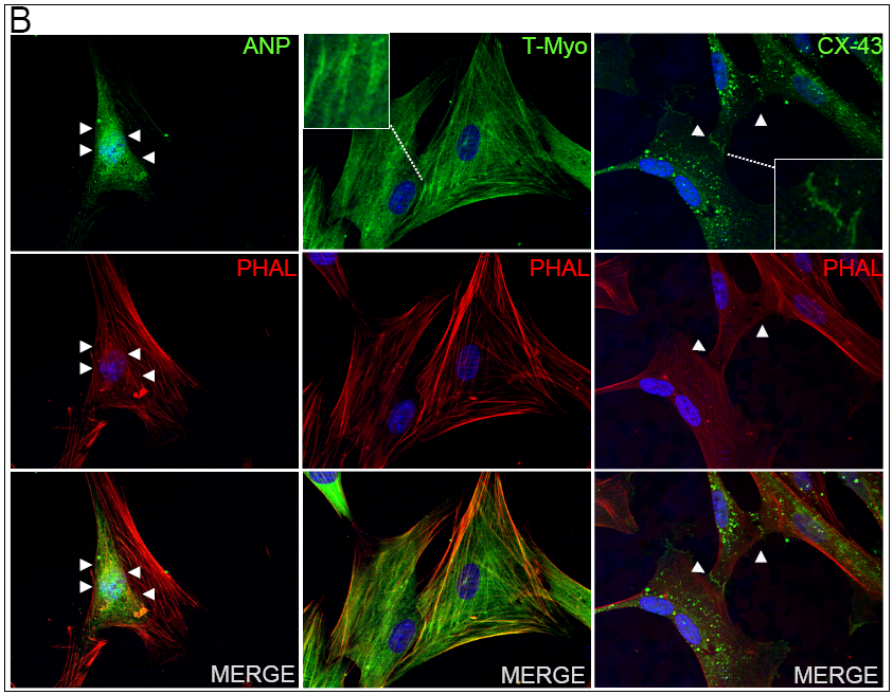

Figure 10. B) Immunofluorescence staining using antibodies specific for Atrial Natriouretic Peptide (ANP), Tropomyosin (T-Myo) and gap junction-associated protein Connexin 43 (CX43). Green fluorescence shows the specific detection of each primary antibody using Alexa488-conjugated secondary antibodies; red fluorescence indicates staining with Rhodamine-conjugated phalloidin, recognizing sarcomeric structures; blue fluorescence indicates nuclear staining by Hoechst 33342. Arrowheads in the ANP staining panels set indicate that ANP immunoreactivity was confined in the perinuclear region; in the CX-43 panels set, they indicate gap junctions visible at the intercellular contacts between adjacent cells. Insets in the upper panels of the T-Myo and CX-43 panels indicate 10X magnifications of cytoplasm zones likely containing sarcomere-associated tropomyosin and one of the intercellular contact zones showing the presence of gap junctions, respectively.

4. DISCUSSION

The definition of culture procedures to amplify CPCs in sufficient numbers to be used in patients, is one of the primary endpoints in translation of stem cell therapy to treat ischemia. However, the precise definition of protocols to amplify these cells in culture still has to be provided. The only example of a defined procedure to obtain CPCs in culture from human adult heart specimens and to direct their efficient differentiation into myocardial cells has been recently provided for Sca-1-like^{+} human CPCs [5, 26]. By contrast, despite the enormous amount of work performed on the understanding of c-kit^{+} CPCs basic biology[20, 27-30] and potency to repair the ischemic mycardium [11, 12, 24, 31], to date no systematic study of the behaviour of these cells in culture has been performed, with the aim of providing minimal criteria for their amplification for cellular therapy.

The methodology followed in the present study was therefore specifically designed to address the following questions: 1) establish minimal and maximal temporal limits to obtain sufficient numbers of cells to proceed with efficient sorting of c-kit^{+} cells; 2) to check for c-kit expression after sorting and subculturing for a definite passages number and under defined conditions; 3) calculate the CPCs enrichment achieved by the culturing process, 4)

analyze the phenotype stability of the cells throughout the amplification process and 5) assess their differentiation potency.

4.1 Towards the definition of a c-kit$^+$ CPCs amplification procedure

The definition of temporal thresholds during the execution of stem cells amplification procedures for clinical application is not trivial. In fact, it has been shown that long term culture for many passages of primary stem cells, obtained by repeated subculturing or culturing under clonal dilution conditions determines the risk for chromosomal abnormalities[32]. Our conditions were different from the first of the two methods reported by Bearzi et al[12], consisting in immunosorting of c-kit$^+$ cells immediately after dissociation of myocardial tissue. In fact, we established a method that maximized primary amplification of heterogeneous cells obtained from enzymatic dissociation of auricolae fragments in a reduced number of passages. This method is more similar to the second method proposed by Bearzi et al[12] describing sorting of c-kit$^+$ CPCs from cells outgrown from small fragments of myocardial tissue let to adhere into tissue culture dishes. In fact, the percentage of c-kit$^+$ cells in the primary amplified cells (2.01±0.23%, Table 2) was similar to percentage of c-kit$^+$ CPCs obtained in that study[12], suggesting a similarity between the efficiency of

the two primary culture methods concerning maintenance of the c-kit$^+$ cell population. The secondary amplification method adopted to expand c-kit$^+$ cells after sorting produced different results form what reported by Bearzi et al [12]. In fact, culture of high throughput-sorted c-kit$^+$ CPCs for 4 passages in plastic adherence conditions, gave rise to a cellular population that almost completely lost c-kit expression. This difference may be explained by the lack, in our method, of a step performed by seeding c-kit$^+$ cells at a clonal dilution, allowing growth of single cell-derived c-kit$^+$ CPCs colonies. In our view, this step was not necessary for the reason that amplification of single c-kit$^+$ CPCs clones would require too long time to derive sufficient amounts of cells to treat patients, it would increase the risk for unwanted cellular transformation and that, according to Bearzi et al., non-clonogenic c-kit$^+$ have a comparable activity to that of clonogenic c-kit$^+$ cells in myocardial repair [12].

Self-renewal of c-kit$^+$ CPCs in cardiac stem cell niches[20] is promoted by paracrine factors released by supporting cells in the niche. For example, it has been reported that Notch ligands regulate CPCs fate *in vivo* and *in vitro*[30] and that cardiac fibroblasts sustain proliferation of c-kit$^+$ CPCs by release of multiple cytokines in the medium[18]. In the present study, we were able to greatly improve the efficiency of c-kit$^+$ expansion process by growing sorted c-

kit$^+$ cells in transwells containing unselected cells from the same individuals. In fact, the comparison of the c-kit$^+$ cell percentage after 4 passages under these conditions produced strikingly different results showing maintenance of c-kit expression in a significant cell percentage (Fig. 1D) and at significantly higher levels (MFI data, Figure 1D). This led to a 15.59±3.5 folds increase in c-kit$^+$ cells in 28±1.54 days after seeding in coculture (Table 4). Interestingly, the quantification of cytokine release in conditioned medium by supporting stromal cells at P4 showed enhanced expression of several factors compared to unselected cells. G-CSF, IL-8, VEGF and IL-6 were the most upregulated cytokines in these cells at P4 (Table 3). Different hypotheses may be raised to explain the increase in cytokine production by supporting cells at P4. For example, it is possible that the non-selected cells undergo a phenotypic maturation into a mesenchymal-like cell type producing higher cytokines amounts compared to the starting stromal population; another explanation is that, analogous to mouse Sca-1$^+$ CPCs[33], c-kit$^+$ CPCs are themselves producing an array of paracrine factors determining selective growth of specific cell types in the lower transwell chambers that, in turn, support CPCs growth.

In summary, the results in the present study identify the crucial role of myocardial-derived supporting cells in the maintenance of CPCs stem cell phenotype stability and

define an array of cytokines that are likely implicated in this effect. Future studies are needed to understand whether addition of these cytokines, alone or in combination, will be sufficient to replace the paracrine action of supporting cells, thus allowing the formulation of a culture medium tailored to optimize *ex vivo* expansion of non-clonogenic human c-kit$^+$ CPCs.

4.2 MSCs features of human c-kit$^+$ CPCs: overlapping or just heterogeneity?

Besides myocytes, vascular and resident progenitor cells, the adult heart contains stromal cells. Under normal conditions, myocytes loss is compensated by continuous renewing by stem cells pools that produce new contractile cells throughout the adult life [1, 2]. In the case of ischemia, massive death of myocytes is not compensated by an efficient replacement, but it is associated to extensive fibrosis due to inflammation-related fibrovascular tissue formation[17]. Although it is well established that the lack of myocyte replacement after ischemic insult is the consequence enhanced stem cell death and/or stem cells pools exhaustion[28], it is not clear whether a relationship exists between the stem cell compartment and the stromal component in the heart. The demonstration of a heart-specific mesenchymal cell population has not been formally provided. However, the expression of mesenchymal

markers in heart-derived clonogenic and non-clonogenic progenitors, has been already documented. For example, expression of MSCs markers was reported in clonally amplified mouse Sca-1$^+$ CPCs[33, 34], human cardiosphere-derived cells[6, 35, 36], human Sca-1-like$^+$ cells[5, 26] and epicardial CD34$^+$ cells[10]. Interestingly, in some of these reports, c-kit was found to be expressed in these cells [5, 6, 26, 35] while in others the expression of c-kit and MSCs markers was not observed [33, 34] or not determined[10]. Work performed in mammalian embryos has shown a possible heterogeneity of CPCs populations. For example, postnatal cardioblasts characterized by expression of transcription factor Isl-1 do not express c-kit[8]. Conversely, progenitors residing in the neonatal heart expressing the fluorescent protein EGFP controlled by c-kit regulatory sequences were not found to express Isl-1 [37]. This may result, in the adult, in the generation of separate stem cell compartments such as the recently reported myocyte progenitor cells and vascular progenitor cells, both characterized by c-kit expression[24].

In the present report, we show that *ex vivo* amplified non-clonogenic c-kit$^+$ cells from the human heart express some of the most common mesenchymal markers such as CD29, CD44, CD105, CD130 and CD200. Compared to BM-derived MSCs, these cells had a reduced expression of genes promoting typical mesenchymal differentiation such as

adipocytes and osteocytes, while they gave rise to endothelial, smooth muscle cells and, although incompletely, to myocardial cells suggesting a preferential cardiovascular lineage commitment. Whether or not the expression of mesenchymal markers belongs to undifferentiated c-kit^{+} cells in the stem cell niche or whether it defines a mesenchymal lineage derived from them *in vitro* is matter of current investigation in our laboratory.

5. SUMMARY, CONCLUSIONS AND FUTURE PERSPECTIVES

The myocardium contains a resident progenitor cells, named cardiac progenitor cells, that maintain the heart homeostasis by replacing lost myocytes. The c-kit tyrosine kinase receptor is one of the principal markers defining cardiac progenitors in the adult mammalian heart, including human. C-kit$^+$ cardiac progenitors have been recently identified as a multipotent cells population able to give rise to myocardial, endothelial and smooth muscle both *in vitro* and *in vivo*. The discovery of cardiac progenitors and the devise of culture systems to amplify these cells in culture without loosing their differentiation potency has opened new perspectives for clinical applications aimed at replacing myocardium lost as a consequence of acute or chronic ischemic disease. While cardiac progenitors have been deeply investigated for their stem cell activity, susceptibility to stress conditions and aging, and their ability to repair the infarcted heart, their origin, phenotype and biological features have been disclosed only in part. In the present study, we report that c-kit$^+$ cardiac progenitors derived from the human heart express markers in common with mesenchymal stem cells. Our *in vitro* analyses revealed that, compared to bone marrow-derived mesenchymal cells, c-kit$^+$ cardiac progenitors have a reduced ability to differentiate into canonical mesenchymal cellular derivatives

such as osteoblasts and adipocytes; by contrast, under appropriate differentiation conditions, these cells gave rise to tubular-like structures at the same extent as endothelial cells, expressed smooth muscle cells markers, and expressed early and late myocardial markers.

In summary, by providing evidences that c-kit^{+} cells from the human heart express mesenchymal markers, our study suggest lineage relationships between the heart mesenchymal component and heart resident stem cells. This opens up new perspectives for the definition of the human CSCs ontogeny and establishes a novels basis for their use in human ischemic heart repair.

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