Dimet

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Translational and Molecular Medicine

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

# Analysis of differentiation, plasticity and biological activity of human endothelial progenitor cells (hEPC): Insights from epigenetic assessment and clinical applications

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# ***CHAPTER 1: GENERAL INTRODUCTION***

# 1. CARDIOVASCULAR DISEASES

## 1.1 Cardiovascular Diseases (CVDs)

CVDs are responsible for the inability of the heart to fill with or eject blood at a rate commensurate with the requirements of the metabolizing tissues. The total direct an indirect cost of CVDs in the United States for 2010 is estimated in $503.2 billion and they are now considered the primary cause of disability and death in western countries. In fact, mortality data for the 2006 show that CVDs accounted for 1 of every 2.9 death in United States, that is equal to an average of 1 death every 38 seconds. Several risk and lifestyle factors have been identified, such as smoking, hypertension, obesity and diabetes mellitus. Moreover, although CVDs can virtually occur at any age, the frequency rises progressively with age, especially for men. In fact the average annual rates of first CVD events rise from 3 per 1000 men at 35-44 years of age, to 74 per 1000 men at 85-94 years of age. Instead, for women the same rates occur ten years later in life [1].

## 1.2 Ischemic Heart Failure

Myocardial ischemia refers to a lack of oxygen due to inadequate perfusion of the myocardium, which causes disturbances of mechanical, biochemical and electrical function of the myocardium and an imbalance between oxygen supply and demand. 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 unstable angina (UA) and non ST-segment elevation MI (UA/NSTEMI). This condition is normally an aggravation of a stable coronary artery disease and requires medical or mechanical interventions

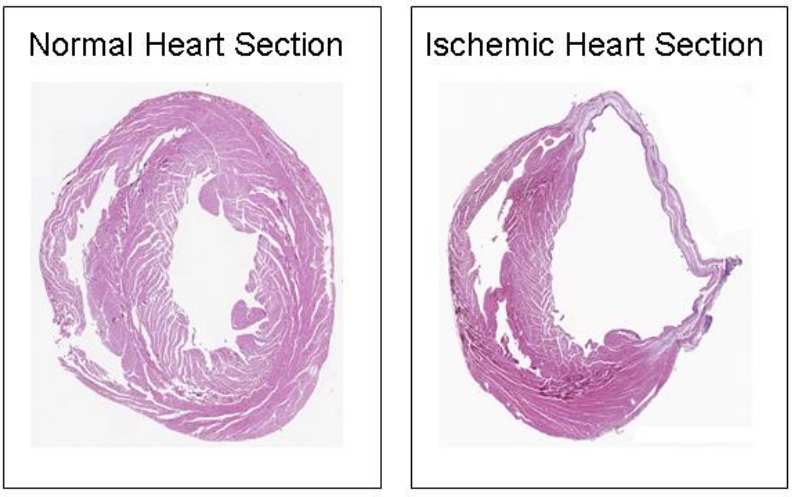
b. Patients with acute myocardial infarction (MI) with ST-segment elevation on their presenting electrocardiogram (STEMI). It generally occurs when coronary blood flow decrease abruptly after a thrombotic occlusion of a

coronary artery previously affected by atherosclerosis. 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 [2]. Under normal immune function, the inflammatory response occurs immediately after tissue injury. In fact, after MI or ischemia/reperfusion injury, dead cells activate a series of inflammatory chemokines or cytokines, which recruit leukocytes, neutrophils and monocytes/macrophages to clear the infarct zone of dead cells and matrix debris. This inflammatory reaction is aimed to repair and heal damage tissues, thus avoiding the ischemic region rupture, which is generally incompatible with life. However, for myocardium, the infarct healing results in a severe molecular and structural remodeling, with scar formation and, ultimately, it can produce heart failure [3].

Cardiac fibroblasts represent the key cell population involved in scar formation and collagen deposition. During the first period after infarction, the myocardium portions affected by ischemia begin 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 (ECM)

breakdown [2]. After this *acute phase*, a *necrotic phase* takes place. Cardiomyocytes start dying by necrosis and inflammatory cells are recruited in the loosen ECM. They release factors able to induce fibroblasts migration into the wound area, where they synthesize and remodel newly created extracellular matrix (ECM). These specialized fibroblasts express α–Smooth Muscle Actin (α-SMA) and are called Myofibroblasts (MyoFbs). Abnormal persistence of the MyoFbs is a hallmark of fibrotic disease. Proteins such as transforming growth factor (TGF)β , andothelin-1, angiotensin II (AngII)connective tissue growth factor (CCN2/CTGF) and platelet-derived growth factor (PDGF), appear to act in a network that contributes to MyoFbs persistence [4].

After a number of weeks interstitial cells forming the granulation tissue gradually die for apoptosis and leave the chronic stage to 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 remodelling and congestive heart failure.



Inhibition of granulation tissue apoptosis was interestingly found to attenuate cardiac dysfunction 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 [5].

## 1.3 Ischemic Heart Failure Therapy

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*

1. ACE-inhibitors decrease afterload by antagonizing the vasopressor effect of angiotensin, thereby decreasing the amount of work the heart must perform. Moreover, angiotensin directly affects cardiac remodeling and blocking its activity can consequently slow the deterioration of cardiac function.
2. Diuretics: diuretics therapy is indicated for relief of congestive symptoms.

3. 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 and diffuse coronaropathy benefit from coronary bypass surgery in terms of ventricular performance and prognosis.

*Resynchronization therapy*

Cardiac resynchronization therapy (CRT) consists in the implantation of a biventricular pacemaker, 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 can be combined with an implantable cardioverter-defibrillator.

*Heart transplantation*

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

## 1.4 The need for further therapeutic solutions: Stem Cell Therapy

Despite advances in the management of CVDs, they continue to be a major worldwide medical problem. Current pharmacologic and interventional strategies fail to regenerate dead myocardium and are often insufficient to avoid 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. For these reasons the need for alternative therapeutical options is emerged.

Today we know that bone marrow (BM) contains niches of primitive progenitor cells that are mobilized during the ischemic injury [6][7]. These cells can migrate to the damage site following chemotactic gradients, for example stromal derived factor-1 (SDF-1) gradient [8]. So, these circulating progenitors may constitute a reparative mechanism to ischemic injury, controlled by BM through circulating cytokines and soluble receptors or adhesive molecules. Moreover Bearzi and colleagues recently demonstrated the existence of self-renewing, clonogenic, and multipotent human cardiac stem cell (hCSCs) population, raising the fascinating promise that dead myocardium may be eventually regenerated [9]. Despite these mechanisms are spontaneously activated in response to ischemic injury, they are not sufficient to prevent the inevitable

evolution of the infarct with scar formation. Although myocardial infarct does not spontaneously regenerate, the endogenous repair mechanisms can be manipulated and enhanced to accomplish this goal.

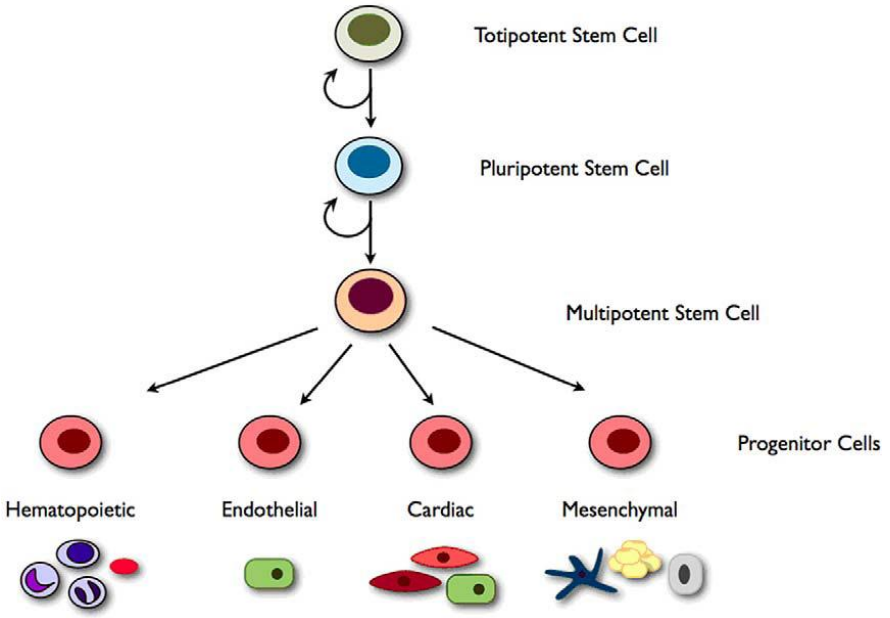
# 2. STEM CELLS

Stem cells (SCs) are present in all multi cellular organisms.
On a functional point of view, stem cells are undifferentiated cells with two distinguishing properties:

*Self-renewal* – Stem cells and progenitors have the ability to give rise to differentiated cells while, at the same time, maintaining a pool of immature cells. One way in which cells can balance renewal with commitment is *via* control of asymmetric and symmetric division. During asymmetric division, one daughter cell remains a stem cell, while the other becomes committed. In contrast, during symmetric divisions, a stem cell divides to become two stem cells (symmetric renewal) or two committed cells (symmetric commitment). Stem cell asymmetric division maintains a constant pool of undifferentiated cells

while, at the same time, it allows a progressive increase in the number of differentiated cells. Conversely, symmetric renewal division exerts the expansion of the immature stem or precursor pool, whereas symmetric commitment increases the number of differentiated cells [10].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 [11][11] and in patients with leukemia undergoing myelosuppressive therapy [12, 13].

*Potency* - This term refers to the ability of stem cells to produce all the cell types composing an entire organism (totipotency), multiple differentiated cell types (pluripotency), all the cell types belonging to a specific germ layer (multipotency), or only one type of differentiated cell (unipotent).



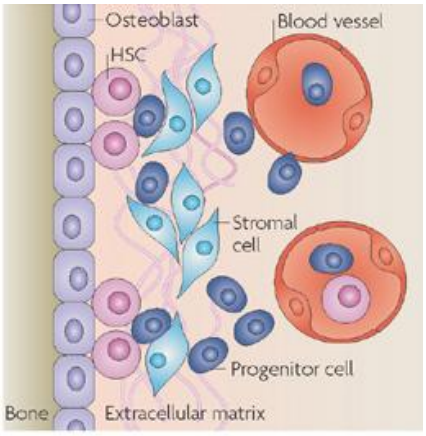

## 2.1 Stem cell self-renewal: the role of the niche

In 2006 Scadden published a good definition of niche:

"Stem-cell populations are established in 'niches' — specific anatomic locations that regulate how they participate in tissue generation, maintenance and repair. The niche saves stem cells from depletion, while protecting the host from over-exuberant stem-cell proliferation. It constitutes a basic unit of tissue physiology, integrating signals that mediate the balanced response of stem cells to the needs of organisms." [14]

Because hematopoietic stem cells (HSCs) are historically the most characterized type of adult stem cells, even the concept of HSC niche was already proposed in the '70s.

Today, we know that osteoblastic cells on the endosteal surface serve as the primary support for HSCs, as they secrete molecules regulating SC proliferation and differentiation. Moreover, adhesion between SCs and supporting stromal cells, and/or extracellular matrix, anchors SCs within the niche providing polarity cues. Thus the SC niche provides structural and trophic support, topographic information and appropriate signals to regulate HSC functions [15].




In particular, osteoblasts produce a vast array of cytokines important for the expansion of HSCs, such as interleukins (IL3, IL6, IL11), thrombopoietin (TPO), Stem cell factor (SCF) that is a growth factor that interacts with its receptor c-kit, Flt-3 ligand (Flt3L) and granulocyte-colony stimulating factor (G-CSF) [16]. Additionally, osteoblasts also produce CXC-chemochine ligand 12 (CXCL12) (also named stromal-derived factor 1, SDF1) which mediates the retention of HSCs in the niche by interacting with its receptor CXCR4 expressed on HSC surface. The cleavage of this interaction has been deeply studied to produce stem cell mobilization for therapeutic purposes (see also Chapter 7) [17].

This various factors stimulate HSC pool expansion but they tend to do so at the cost of losing the undifferentiated state. This

could explain why in vitro culture of HSCs, even with multiple factors, results in limited long-term expansion and maintenance. Therefore, extrinsic factors should not be considered as regulators each unto themselves, but as a whole used collectively by niche to control stemness [18].

## 2.2 Signaling pathways governing stem cell fate

In recent years, some relevant signaling mechanisms regulating SC fate have been deeply characterized, in particular for HSCs. In the following paragraphs I would like to give an overview of those pathways I found most involved in my research projects.

### a) Numb and Notch

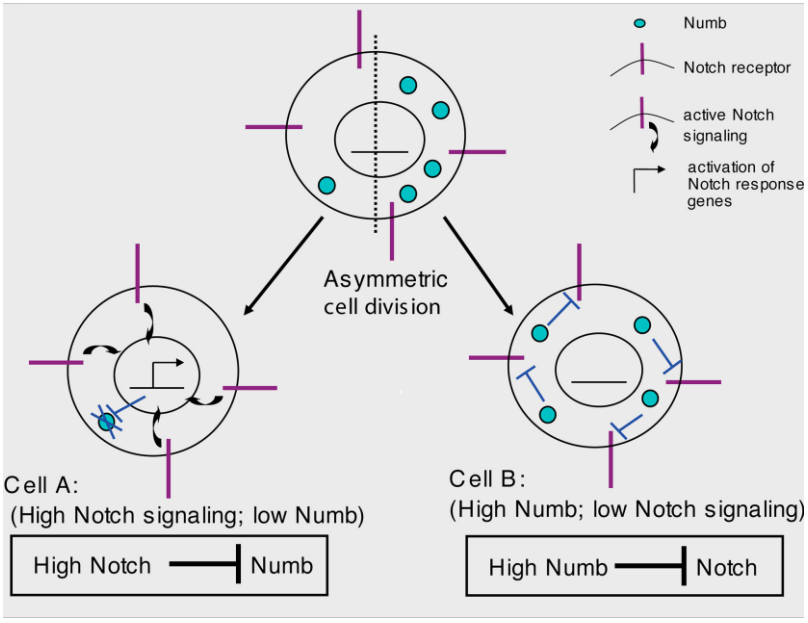
Numb is a membrane-associated protein that asymmetrically segregates in dividing cells and determine distinct cell fates by interacting with and inhibiting Notch activity. It has been initially identified in *Drosophila* (dNumb) and only after in mammalian (mNumb) where it encodes for four alternatively spliced transcripts generating four isoforms. The complexity of mNumb phenotypes and its prominent function suggests that its expression is finely regulated. A major regulatory control acts at the post-transcriptional level, including alternative splicing and translational and post-translational control. A microRNA-mediated control has also been recently described by the role of miR146a in inhibiting Numb expression. An additional critical control is represented by ubiquitin-dependent proteolytic

degradation. Finally, we know that post-synthetic protein modifications (e.g. phosphorylation) have more subtle consequences on Numb, by affecting protein subcellular distribution (from membrane to cytosol) rather than intracellular levels [19]. The asymmetric segregation of Numb is mediated by the polarized distribution of its partners: Pon and the PAR3-PAR6-aPKC complex. Moreover, the kinases Aurora A and Polo seem to be required for the asymmetric localization of Numb [20].

During asymmetric cell division, Numb is asymmetrically localised only in one daughter cell. The cell receiving high levels of Numb inhibits the function of Notch, which is a key gene in maintaining stem cells in an undifferentiated state, so the cell becomes committed to differentiation. In particular, Notch is a cell-surface receptor capable of binding two ligands: Delta and Jagged. Binding of either ligand results in proteolycitc cleavage of the receptor and release of the cytoplasmatic domain. This domain can translocate to the nucleus where it activates the transcription of several gene targets.

On the other hand, the cell receiving low levels of Numb preserves Notch activity and remains undifferentiated [21]. Interestingly, the effects of Numb on cellular differentiation are also dependent on Notch expression levels. In fact, at lower levels of Notch, Numb is able to inhibit Notch signalling and it promotes differentiation, while at higher Notch levels differentiation is anyhow blocked and Numb expression

decreases (see figure). This feedback mechanism would reduce the requirements on the asymmetric segregation machinery to perfectly distribute Numb to only one daughter cell, as small amounts of Numb segregating to the Notch-signalling cell would be eliminated. It may also be particularly important to reduce Numb-like protein levels in this cell, as Numb-like appears not to be asymmetrically localized [22].




The microenvironment might also influence the SC growth or differentiation by regulating Notch/Numb pathway. This point was addressed by Wu et al. by using transgenic Notch reporter

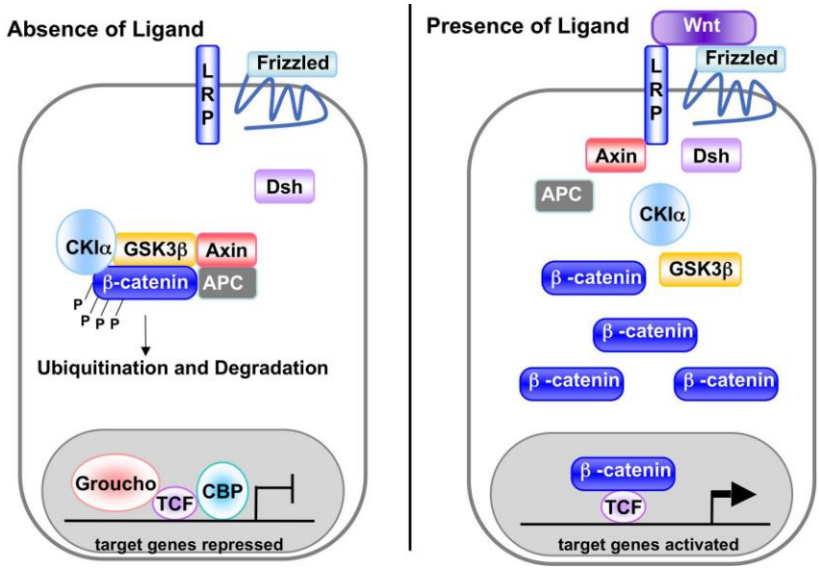
mice in which Notch signaling status was evaluated in real time imaging thanks to GFP fluorescence and time-lapse microscopy. HSCs placed in a prodifferentiation environment prefentially did asymmetric and symmetric divisions, whereas cells placed in a prorenewal environment preferentially divide through symmetric renewal divisions. These data indicate that different environments induce different outcomes, not by governing rates of division, but rather by acting on the balance between asymmetric and symmetric cell division [10].

## b) Wnt pathway

At least three different Wnt pathways are recognized: the canonical Wnt pathway, which is mediated via β-catenin, the plenar cell polarity (PCP) pathway and the Wnt-$Ca^{2+}$ pathway. However, the vast majority of hematopoietic studies involve the canonical Wnt signaling; they showed that Wnt could have a role in maintaining the undifferentiated phenotype of HSCs. In fact, HSCs transduced with β-catenin give rise to sustained reconstitution of myeloid and lymphoid lineages *in vivo*, when transplanted in limiting numbers [23].

In the canonical pathway, in absence of Wnt, β-catenin is recruited in a complex with tumor suppressor proteins APC, Axin, GSK-3β and CK-1 kinases. Phosphorylation on β-catenin specific residues creates a recognition site that leads to ubiquitination and proteosome degradation. In the nucleus,

members of the T cell factor (Tcf) family form a repressor complex with Groucho/TLE and histone deacetylases (HDACs) that silence Wnt target genes (see figure). Instead, when Wnt binds to its cell-surface receptor complex made by Frizzled (Fz) and LRP5/6, β-catenin degradation is prevented and it can translocate to the nucleus. Here, it forms a transcription complex with Tcf members and it allows Wnt target genes expression [24].

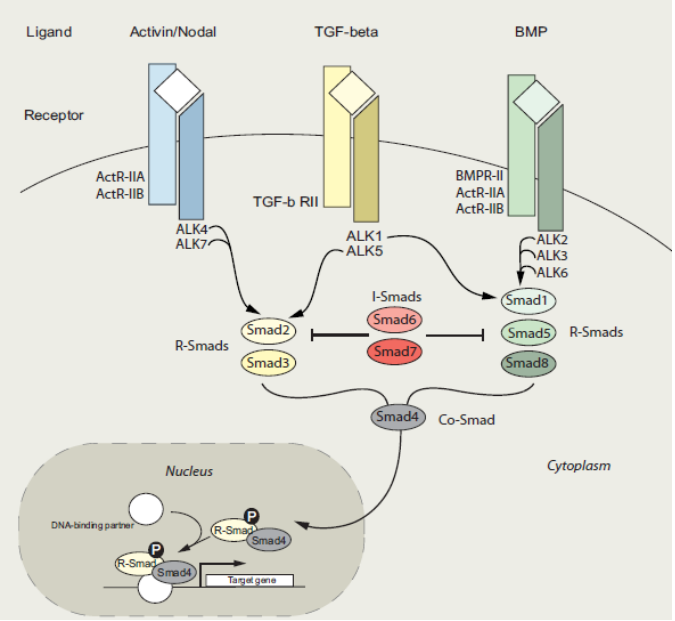



The main consequence of Wnt signaling is the stabilization of β-catenin allowing a cross-talk between Wnt and other self-renewal pathways, such as Sonic Hedgehog, Bone Morphogenic Protein (BMP) and Notch. In particular Reya et al. demonstrated that overexpression of activated β-catenin upregulates Notch in HSCs [23]. Even though Wnt and Notch

are both involved in self-renewal, it is proposed that they do so through quite different mechanisms. In fact, Wnt seem to induce proliferation (symmetric renewal) and it supports cell viability, whereas Notch is imperative in maintaining the undifferentiated stem cell state [16].

### c) Transforming growth factor-β superfamily

The Transforming growth factor-β (TGF-β) superfamily consists of some ligands which regulate a bewildering number of biological processes, including SC self-renewal. Apart from TGF-β, Activin, Nodal and BMPs also belong to this family. For all these ligands the downstream signal is transduced to the nucleus by the Smad signaling pathway. The ligands bind to cell-surface type I and type II receptors with threonine/serine kinase activity. In particular, upon ligand binding, the type II receptor phosphorylates the type I receptor, which in turns phosphorylates intracellular receptor-Smad (R-Smad). R-Smad is now activated and it can associate with Smad4 and they localize to the nucleus where they activate target genes transcription.




TGF-β has stem cell growth inhibitory properties; in fact neutralization of TGF-β *in vitro* was shown to release HSCs from quiescence. This was probably due to TGF-β effect on the expression of cyclin-dependent kinase inhibitors, such as p21 and p57 [16].

BMPs are a family of 15 different growth factors. Among these, 6 proteins also belong to the TGF-β super-family: BMP2-BMP7. In the niche the undifferentiated phenotype of hESCs is maintained by the activity of Growth and Differentiation Factor 3 (GDF3) that antagonizes BMP-2 and, therefore, suppresses the activity of Smad1. On the other hand, BMP-2 expression induces differentiation and antagonizes stem cell self-renewal [25]. Instead, in the context of adult hematopoiesis, BMP-4 was

shown to promote maintenance of HSCs in culture, whereas lower concentrations of this factor induced proliferation and differentiation of HSCs. The key molecule in transducing this self-renewing signal is Smad5, as demonstrated by *in vitro* disruption of the gene [16].

Sonic hedgehog (Shh) is a transmembrane protein which upon binding to its receptor Patched, blocks the inhibition of Patched on another transmembrane protein, Smoothened, thereby freeing it to regulate transcription of target genes. The characterization of Shh pathway was particularly difficult to study as the inactivation of just one key component produces embryonic lethality. In 2001 Bhardwaj et al. demonstrated that HSCs express all the pathway components and that HSCs proliferation is regulated by Shh activity. Moreover they provided evidences that Shh acts upstream of the BMP pathway and requires intact BMP signaling to act [26].

### d) Hypoxia and Reactive Oxigen Species (ROS)

A peculiar property of the BM microenvironment is its hypoxic nature, indicating that the level of oxidative stress influences HSC function. Interestingly, ROS appear to activate the p38/MAPK pathway inducing quiescent HSCs to cycle more frequently [16]. Moreover, members of the FoxO family of transcription factors up-regulates genes involved in SC detoxification, thus protecting HSCs from oxidative stress. So it is conceivable that the hypoxic environment of the HSC niche

may serve to protect them from oxygen radicals keeping them quiescent [27].

## 2.3 Relationship between self-renewal and cell cycle control

Although stem cells have the highest potential to produce a large number of progeny, they are slowly cycling, or quiescent, at least in the adult organism and during homeostasis. In the case of HSCs, two different subpopulations have been described: slow-dividing (or dormant) HSCs that have the highest self-renewal potential, and fast- dividing (or active) HSCs. This distinction is experimentally based on fluorescent labeling assays. In fact, since the label dye halved at each division, it is possible to calculate the division history on the base of the label fluorescence decay over time [28].

Due to their extremely slow cycling, slow-dividing HSCs are unlikely to significantly contribute to blood cells homeostasis, but they are able to enter the cycle, suggesting they can serve as a reservoir which can be activated in response to injury signals. So, the balance between slow and fast-dividing cells is an outstanding parameter within the SCs and must be cautiously regulated.

Why is it advantageous for a SC to remain dormant during homeostasis? First of all HSC self-renewal potential appears to be limited, as demonstrated by functional exhaustion of HSC after 5 to 6 times serially BM transplantations. This indicates

that there is an inverse correlation between cell proliferation and self-renewal capacity. The second reason is that keeping cell division low, and therefore DNA replication, minimizes the number of DNA mutations that can accumulate in the SC reservoir and may cause oncogenic transformations. The third reason is that slow-dividing protects SCs from toxic drugs such as anti-proliferative chemotherapeutic agents [29].

In the niche, HSCs interact with numerous adhesion molecules and activate different signaling pathways maintaining the quiescent state. Besides the already mentioned Notch, Wnt, TGF-β, ROS and FoxO, another key gene in regulating the balance between slow and fast-dividing SCs is Bmi. Bmi is a positive regulator of the self-renewal and it is also an inhibitor of $p21^{CIP1}$, $p16^{INK4}$ and $p19^{ARF}$. Moreover it is able to restrict the ROS production by acting on the mitochondrial function [30].

## 2.4 Stem cell plasticity

The standard model of hierarchical and unidirectional cell development and differentiation derived from early experiments where cells from a part of the embryo were transplanted to another part and they adopt the terminally differentiated state appropriate to the new localization. Contemporary molecular biology studies provided the mechanistic underpinning of these observations. In fact, it was demonstrated that progressive

repression of gene expression was not required for the new differentiation pathway [31].

This repression is carried out by two main mechanisms: DNA methylation and histone tails modifications.

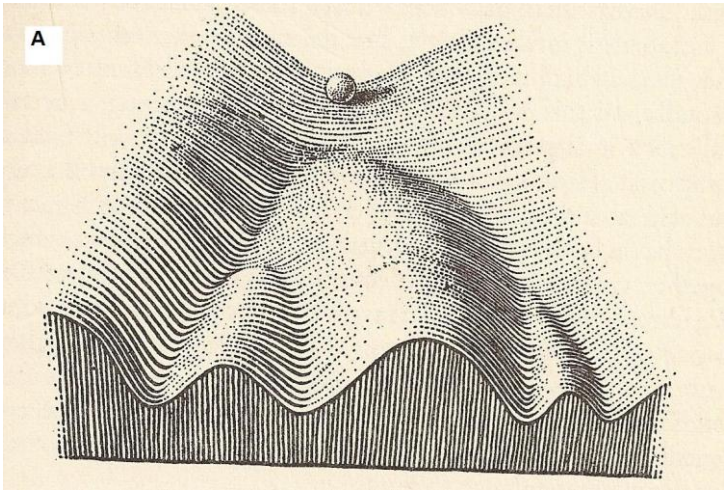
In brief, the cytosine methylation is established after DNA synthesis and it alters DNA conformation and DNA interaction with other proteins. It is mediated by tissue-specific DNA methyl-transferases (DNMT) and it represents a specific epigenetic marker that is copied at each DNA synthesis cycle. In mammals, the 80% of the DNA methylations are in the so-called CpG islands, often located at promoter sites, and generally leads to restriction of DNA accessibility thus preventing gene expression.

Histone tails modification is another relevant epigenetic mechanism by which gene expression is regulated. There are different histone modifications: methylation, phosphorylation, ubiquitylation, sumoylation and acetylation. These modifications can be on different residues with different consequences on gene expression, and even the combination of all the modifications in a site has a relevant role. For these reasons it has been proposed the existence of a histone code besides the genetic code.

Lysine acetylation covers the residue positive charge thus reducing the link between the histone tail and the negatively charged DNA. So, histone tails acetylation enhances the formation of euchromatin regions that are more prone to be

transcribed. This modification is triggered by the balance of two specific classes of enzymes: the histone acetyl-transferases (HATs) and the histone deacteylases (HDACs).

Conrad Hal Waddington vividly depicted this process of cell differentiation as a ball rolling down valleys in an epigenetic landscape. The cell, represented by a ball, arrive at a stable position by a progressive restriction in their options, beginning as a pluripotent embryonic cell (on the top of the hill) and ending up at the mature terminally differentiated state (on the ground) [32]. It is evident from the picture that the ball cannot grow up the hill, thus illustrating the belief of that tome that differentiation is an irreversible process.




Nevertheless, in 1985 the standard hierarchical model was revolutionized thanks to seminal experiments by H. Blau and colleagues. They placed the nucleus of a *terminally* differentiated cell into the cytoplasm of a different differentiated cell finding that the donated nucleus was able to

change gene expression pattern of the recipient cell. This led Blau to suggest that differentiation is an actively maintained state rather than the rigidly programmed one set during embyonic development [33]. Only ten years after the publication of "The plasticity of the differentiated state" Science defined stem cell plasticity as the breakthrough of the year. In fact three papers demonstrated bone marrow cells could become sketal muscle and liver, and neural stem cells could give rise to blood when transplanted in lethally irradieted mice. Adult stem cell plasticity seemed to be the answer to ethical and economic issues stood out by research on ESCs, leading Vogel concluding: "If it lives its early promises, it may one day restore vigor to aged and diseased muscles, hearts and brains, perhaps even allowing humans to combine the wisdom of old age with the potential of youth" [34]. This new idea of cell development was further supported by cloning experiment which began, famously, with the cloning of Dolly and which demonstrated that epigenetic modifications are completely reversible.

Three different pathways were proposed to explain the observed stem cell plasticity.

1- *Canonical cell lineage differentiation*. Even all the canonical lineages are true plasticity events in that at every step of lineage development, cells of one type give rise to cells of another type, however closely related.

2- *Common plasticity.* Cell of one lineage become cell of another lineage, across embryonic tissue barriers, by changing gene expression in response to environmental cues.

3- *Cell fusion.* A circulating cell is recruited by cytokine/chemokine signaling to the engrafting site. Here it merges with target cell to become a tetraploid cell. This new cell type may be binucleated or have one nucleus if nuclear fusion follows cell fusion [35].

## 2.5 Plasticity *vs.* Reprogramming

In spite of the initial enthusiasm, a growing number of authors criticized the concept of plasticity, proposing other possible explanations for the observed phenomenons. These alternatives comprised:

A. *Transdifferention.* A stem cell could potentially contribute to cell types of different lineages at the same time.

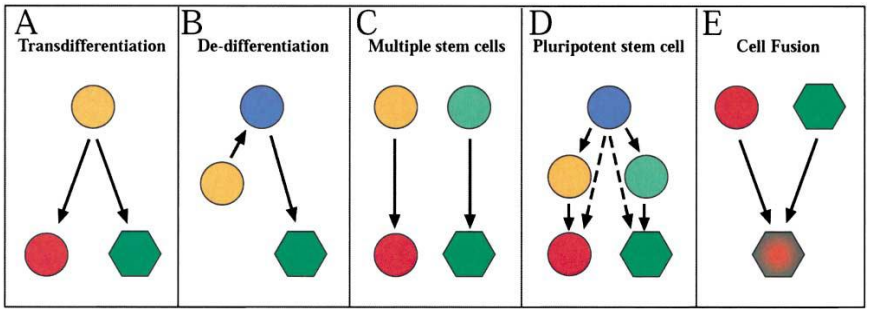
B. *Dedifferentiation.* Dedifferentiation of a progenitor to a more primitive and multipotent cell, followed by re-differentiation along a new lineage pathway.

C. *Heterogeneity.* It is essential to exclude heterogeneity in the tested population, to avoid the possibility that multiple distinct SCs could contribute to the observed outcome.

D. *Rare pluripotent SCs.* In several tissues were identified rare pluripotent cell which possibly co-purify in protocols designed to

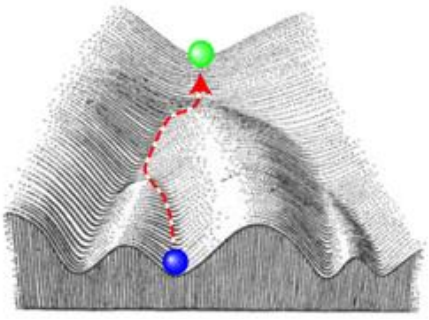
enrich fotr tissue soecific SCs. These rare cells could be responsible for the unexpected phenomenons observed.

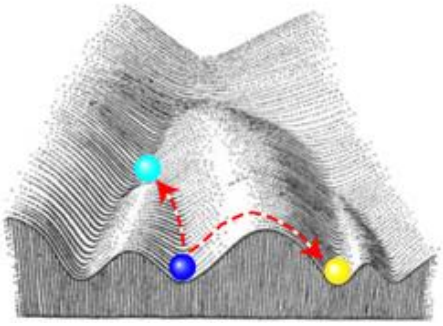
E. *Cell fusion.* Fusion is a common phenomenon in cell development. For example, fusion between sperm and the egg is the initiating event in vertebrate development, moreover fusion naturally occurs in the generation of multinucleated sketal myofibers from myoblasts. For this reason various authors think that fusion is a different event, distinct from true plasticity [36].




Considering all these alternatives, a true plasticity event must be demonstrated by clonal analysis, using minimally manipulated cells, excluding cell fusion, and results must be independently replicated by other laboratories. On the basis of these criterions, Wagener concluded that no presently published study exists on plasticity [36]. So, it is fundamental to distinguish between *normal development*, in which plasticity does not appear to happen at an appreciable rate and/or it doesn't have a physiological relevance, and *experimental*

*manipulation*. In other words, terminally differentiated cells maintains their inherited potency in heterochromatin regions of their genome but, because it is nowadays well established that epigenetic modifications are completely reversible, experimental manipulation could be used to reprogram the cell fate. An excellent example is given by work by Yamanaka and colleagues who demonstrated that adult skin fibroblasts can be induced to become pluripotent stem cells (iPS) in culture by a small subset of reprogramming factors (Sox2, Oct4, cMyc and Klf4) [37]. This work completely overturned the Waddington vision, because it means that the right combination of transcription factors could induce a cell to go back up the hill by erasing its acquired epigenetic modifications [32]. Moreover, two recent works have demonstrated the direct reprogramming of fibroblasts into functional neurons [38] using Ascl1, Brn2 and Myt1, and into functional cardiomyocytes [39] using Gata4, Mef2c and Tbx5 (see also Chap 3.4). These results introduce a new version of





the Waddington figure, in which the cell can operate a "flip" from a "terminally differentiated valley" direct into another by modifying its gene expression profile [32].

In conclusion, recent evidences suggest that an expansion of the traditional view of a stem cell is to be considered.
A stem cell is not necessarily a specific cellular entity, but rather a function that can be assumed by numerous diverse cell types [40].

# 3. STEM CELLS FOR CVDs THARAPY

Over the past decade, several different stem cell types have been studied in an effort to find the best source for cardiac repair. Each stem cell population has its own advantages and disadvantages, and most of them have also been tested in animal models an even in clinical trials.
Here I would like to give an overview of the most relevant stem cell types utilized for CVD therapy, mainly focusing on those involved in my research.

## 3.1 Sketal myoblasts

The injection of autologous skeletal myoblasts into ischemic myocardium has been one of the first approaches to a cell-based cardiac regeneration. This approach 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 as to myoblasts, unipotent cells that can fuse to surrounding myoblasts and form nascent muscle fibers. In the heart, they have been supposed to have a double effect: inhibition of scar formation and improvement of myocardial contractility. Skeletal myoblasts can be isolated from small muscle biopsy specimens of the patient's own skeletal muscle, expanded in culture and injected in donor patients' heart without risk for immune rejection and tumorigenicity. Moreover, when implanted in the infracted heart, myoblasts differentiate into multinuclear myotubes that improve ventricular function and decrease remodeling [41].

Despite myoblasts provide the major advantage of a mechanical support for the failing heart, the major problems inherent to their clinical use is that they don't have the normal electrophysiological behavior of cardiac myocytes. Therefore, the lack of electromechanical coupling with the myocardium results in a non-synchronous beating with the surrounding tissue. This is mainly due to the lack of specific gap junctions associated proteins such as Connexins and it can cause a high risk for developing life-threatening arrhythmias. [42]. Another limitation of injected myoblasts is their relative low engraftment ability. For example it has been estimated that myoblasts lethality reaches 84% within the first 24 hours after

transplantation in mice and similar results were confirmed in humans [43]. For these reasons the MAGIC trial, the most notable clinical trial on skeletal myoblasts injection in patients with cardiac ischemia, did not show a significant improvement of regional or global left ventricular function beyond that seen in control group. Moreover they observed an increased number of early postoperated arrhytmic events [44].

## 3.2 Resident Myocardial Progenitors

Since recent years, the mammalian heart has been considered as a post-mitotic organ without regeneration ability. 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 divisions in response to ischemic injury [45][46]. Moreover, Bergmann et al. demonstrated that DNA syntesis in cardiac myocytes is not restricted to a limited period in childhood, but continues during the entire life [47]. These findings demonstrated that the human heart is a normal, self-renewing organ and they opened new unexplored opportunities for myocardial repair.

Several candidate progenitors that sustain the myocardium self-renewing activity have been found; these include:

**1)** c-kit$^+$ and Sca-1(like)$^+$ cells

**2)** Isl-1$^+$ cells

**3)** epicardial-resident stem cells

**4)** "cardiosphere" derived cells

**5)** "cardiac explants"-derived stem cells that greatly resemble mesenchymal cells.

It seems paradoxical that an organ known for its lack of regenerative capacity would harbour multiple sets of cardiomyocyte progenitors [48]. However, the lack of specific markers to identify different cardiogenic lineages still makes unfeasible their precise molecular characterization and their tracing in the postnatal heart. As a consequence, it is still not clear whether these cellular pools are functionally redundant or whether they have specific roles in heart morphogenesis and subsequent cardiac homeostasis (Pesce et al. 2011).

Anyway, the best characterized resident myocardial progenitor cells are those identified by the expression of c-kit, the SCF receptor. This marker was initially used to define a population of stem cells from the BM. In 2001 Orlic et al. demonstrated that injection of BM Lin$^-$/c-kit$^+$ cells can generate de novo myocardium and it can ameliorate the outcome of coronary artery disease [49]. Subsequently, the same laboratory demonstrated the presence of Lin$^-$/c-kit$^+$ cells resident in rat and human heart. They also isolate and characterized these cells, finding that they are self-renewing, clonogenic, and multipotent, as they gave rise to myocytes, smooth muscle, and

endothelial cells. Nevertheless, activation of human cardiac stem cells (CSCs) occurs in response to ischemic injury [50] [51]. Given the great amount of evidences for CSCs possible relevance in CVDs therapy, some groups planned to develop GMP procedures for CSCs isolation and they enrol patients in clinical trials to evaluate the safety and efficacy of the treatment (for references see http://clinicaltrials.gov/ct2/results?term=cardiac+stem+cell).

## 3.3 Mesenchymal Stem Cells (MSCs)

MSCs reside in the non-hematopoietic, stromal compartment of the BM. Due to the lack of a single marker specific for MSCs isolation, the Mesenchymal and Tissue Stem Cell Committee proposed to adopt three identifying criteria:

1- Adherence to plastic

2- Antigen expression. MSCs don't express (<2% positive) typical hematopoietic markers, such as CD45, CD34, CD14, CD11b, CD19, CD79α and HLA class II. Instead, MSCs must express (>95% positive) CD105, CD90 and CD73.

3- Multipotent differentiation. MSCs are able to differentiate into osteoblasts, adipocytes and chondroblasts [52].

Initial interest was stimulated by studies suggesting that these cells could become cardiomyocytes *in vitro* [53], although the necessity of using inducing regimens such as 5-azacytidine limits the clinical applicability of such strategies. However,

several lines of evidence indicate that direct injection of non-induced MSCs into the heart improves ventricular function postinfarction in rats and pigs. These experiments shown that MSCs can attenuate the pathological ventricular remodelling [54] [55]. Mesenchymal stem cells are also known to produce paracrine growth factors that likely support vascular regeneration and cardiomyocyte protection in the injured myocardium. 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 [56]. Another particularly useful property of these cells is that they express low levels of MHC class I and lack human MHC class II, so they can be allo-transplanted without need for immunosoppression.

The ability of MSCs to enhance left ventricular (LV) function and their allo-tollerance led several groups to test them in preclinical trials. Despite some studies obtained promising results, the clinical application of MSCs still needs to address two important points. First of all, to easily and thoroughly isolate MSCs it would be better to have at disposal a specific subset of antigens, for example to sort MSCs in accordance with GMP guide lines [52]. The second point arise from the demonstration that total BM cell injection in a myocardial infraction rat model induced extensive intramyocardial

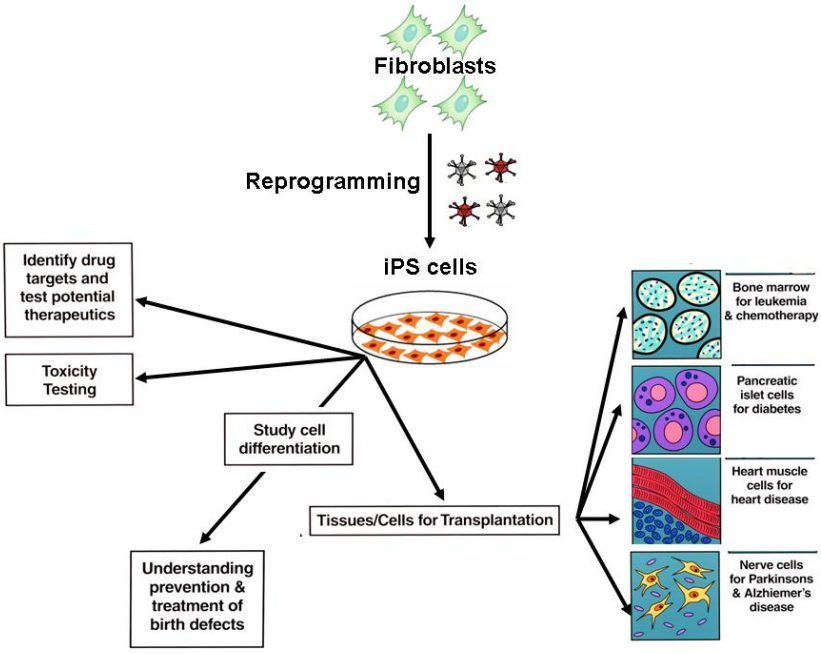
calcifications, while injection of BM multipotent selected cells does not [57].

## 3.4 Induced Pluripotent Stem Cells (iPS)

Due to their wide potency, human embryonic stem cells (hESCs) could be useful to treat a host of diseases. However, there are ethical difficulties regarding their use, as well as the problem of tissue rejection following transplantation in patients. Yamanka and colleagues hypothesized to solve these issues by generating pluripotent cells directly from the patients' own cells. In fact in 2006 they published a seminal report showing that pluripotent stem cells can be directly generate by overexpressing four factors (Sox2, Oct4, Klf4 and cMyc) in mouse [58] and human fibroblast cultures [59]. They called this new cell type "induced pluripotent stem cells", or simply iPS. Nevertheless, cMyc is an oncogene and reactivation of cMyc retrovirus could induce tumorigenity. So, in 2008 they modify their protocol demonstrating that cMyc is not essential [60]. Moreover, in next years iPS were generated with only 2 factors [61] or just with Oct4 [62] from umbilical cord blood (UCB) stem cells or neural stem cells (NSC). These papers shown that reprogramming an adult stem cell to a pluripotent state is easier than reprogramming a mature differentiated cell. Moreover, iPS generation can be improved using HDAC inhibitors, such as Valproic acid (VPA) [63] or Butyrate [64].

Although iPS and ESCs are very similar, some relevant differences still exist, especially comparing ESCs to early passages iPS. First of all there are some differences in gene expression that could be explained by the residual misexpression from the cell type of origin. Consistent differences were also reported in miRNA expression and at the level of the epigenome [65]. In fact, chromatin immunoprecipitation in combination with microarrays experiments showed that trimethylation levels on histone 3 lysine 27 (H3K27, repression) and H3K4 (activation) were similar, while trimethylation on H3K9 was different. Moreover, significant differences were be detected at the level of DNA methylation, and reprogramming of female human fibroblasts failed to reactivate the somatically silent X chromosome [66].

Induced pluripotent SCs might have various applications. First of all, it is possible to obtain mature cell types directly from patient's biopsy for autogenic tissue transplantation. These cells could also be genetically engineered. Moreover, iPS could be used as an *in vitro* model to study differentiation in normal or pathological conditions and to study how to treat defects. They also could be used to study drugs toxicity, target and therapeutic potential.




Functional cardiomyocytes (CMs) were derived from murine and human fibroblasts [64] [67]. They were phenotipically and electrophysiologically similar to CMs derived from ESCs or to fetal CMs. Instead, cardiac development of iPS cells is delayed compared with maturation of native fetal or ESCs-derived CMs. This difference may indicate an incomplete reprogramming of iPS. In addition to cardiomyocytes, also endothelial cells and smooth muscle cells were derived from iPS and their ability to promote myocardial repair has been successfully tested in preclinical models of myocardial infraction [68].

In 2010 Ieda at al. further implement this field by direct obtaining functional cardiomyocytes (the so called iCM) from

murine fibroblasts with 3 factors: Gata4, Mef2c and Tbx5 (GMT). Their work has many therapeutic implications. First of all iCMs have a lower risk of tumor formation than CMs obtained from iPS. Moreover, because cardiac fibroblasts comprised over 50% of all the cells in the heart, large amounts of individual's fibroblast can be cultured and transducted. Third, this work open the route to the possibility to introduce the difined factors directly into the heart to reprogram the endogenous fibroblasts [39].

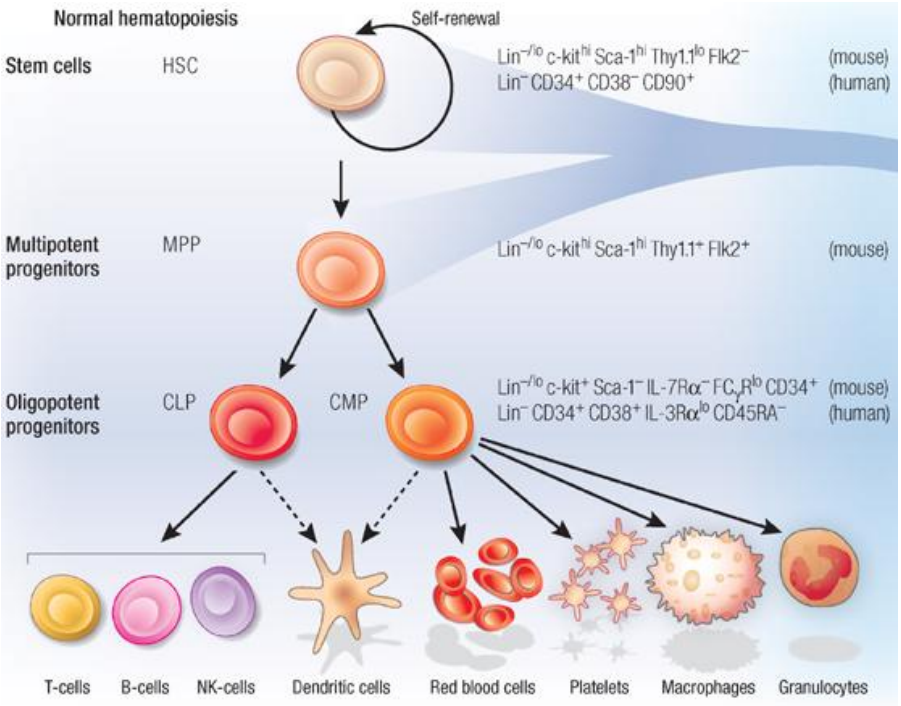
Despite all these excellent results, it is important to notice that viruses have the disadvantage of random integration into the host genome. This can lead to alteration in the expression of endogenous genes and even cause cancer mutations. For these reasons, some labs are know working on reprogramming methods independent from integrating viral vectors, such as non-integrating adenoviruses or small molecule which could replace the reprogramming factors [69].

## 3.5 Hematopoietic and Endothelial Progenitor Cells

Hematopoietic stem cells (HSCs) are probably the best characterized adult stem cell population, since first groundbreaking experiments published in the '60.

*In vivo* dilution series analysis identified the so-called Long-term repopulating cells (LT-HSCs) and the Short-term HSCs

(ST-HSCs). These cells normally give rise to the common lymphocyte progenitor (CLP) and the common myeloid progenitor (CMP), from which derives all the mature blood cells. Moreover, when transplanted to secondary hosts, HSCs reconstitute all blood cell lineages.[70].




Understanding HSC biology has led to relevant medical advances, especially in cancer therapy, because BM transplantation has become a routine practice to regenerate the hematolymphoid system. Additionally, as already discussed, the discovery that HSCs can also differentiate in unexpected lineages giving rise, for example, to sketal muscle and liver,

opened the route to a wide field of research. For what concern the CVD therapy, in 2001 Orlic and colleagues reported that BM-derived $lin^-/c\text{-}Kit^+$ HSCs injected into the infracted heart efficiently differentiate into myocytes with no detectable differentiation into hematopoietic cells and improve myocardial function. They also isolated these cells from mice constitutively expressing enhanced green fluorescent protein ($EGFP^+$) and injected them at the border zone of wild-type mice with acute myocardial infarcts. Evidence for regeneration included co-localization of EGFP fluorescence with immunostaining for cardiomyocyte markers, including sarcomeric actin, myosin, troponin and several transcriptor factors active in cardiomyocytes (GATA-4, MEF2, NKX2.5) [49].

However, several groups questioned and failed to reproduce these results [71][72]. Moreover, Cre-*lox* recombination experiments provided evidences that cardiomyocytes obtained after HSCs transplantation are products of cell fusion rather than transdifferentiation [73]. Anyhow, even if HSCs were not able to induce myocardial regeneration, they can be of functional benefit by inducing angiogenesis and/or by secreting relevant cytokines and factors.

Angiogenesis is the process of blood vessel formation operated by progenitor cells that home to sites of *neo*-vascularization and differentiate into endothelial cells *in situ*. Until one decade ago this process was believed to occur only during development of blood vessels in the embryo [7]. In 1997 Ashara and colleagues

isolated and characterized human peripheral blood-derived cells that formed endothelial cells clusters in culture [6]. These cells, called endothelial progenitor cells (EPCs) induced *neo*-vascularization and became incorporated into the wall of newly formed vessels when injected into animal models of hind limb ischemia [37]. Cells with EPC activity have next been identified in Mononuclear cells (MNCs) obtained from BM and umbilical cord blood (UCB) and they co-purify with HSCs due to common antigenic marker expression. [74].

These seminal studies demonstrated that induction of *neo*-vascularization in postnatal ages is feasible and raised important implications for translational use of EPCs to regenerate blood vessels in patients affected by ischemic disease.

While after the original discovery EPC referred to only one cell type, today, distinct cellular populations named EPCs are recognized based on their ability to proliferate and on their ability to contribute directly or indirectly to *neo*-vascularization process *in vitro* and *in vivo*. The distinction between different EPCs populations began in 2004 when Hur and colleagues described two distinct EPCs types in human PB [75]. These cells were called "early" and "late" EPCs based on the different timing of their appearance and differences in the clones shape. Early EPCs, also named colony forming unit-endothelial cells (CFU-ECs), originate from $CD34^{+}/CD133^{+}/KDR^{+}/CD14^{+/-}/CD45^{+}$ cells in the MNCs cellular fraction while late EPCs, also named

endothelial outgrowing cells (EOCs) or endothelial colony forming cells (ECFCs) originate from $CD34^+/CD133^-/KDR^+/CD45^-$ cells [76][77][78][79]. Different biological activities have been found in the two subsets. ECFCs show a greater proliferation rate: they can grow up to at least 100 population doublings, give rise to secondary and tertiary colonies and maintain high levels of telomerase activity [80]. Moreover they directly participate to *neo*-angiogenesis by giving rise to endothelial cells *in situ* [81]. On the other hand, early EPC promote angiogenesis by a paracrine interaction with existing endothelial cells and, likely, by cross-talk with inflammatory cells in the ischemic environment [82].

Definition of EPCs is still a matter for debates due to the expression in both EPCs types of endothelial-specific markers, such as CD31, CD146, von Willebrand Factor (vWF), VE-Cadherin and CD105, and the absence of a marker allowing, in conjunction to CD45, to unambiguously distinguish between ECFCs ($CD45^-$) from CFU-ECs ($CD45^+$). At present, in summary, there are only two major functional criteria that allow discriminate between the two EPCs types: their *in vivo* potential and their clonogenic ability.

The overlap in surface marker expression and the contemporary presence of haematopoietic stem cells (HSCs) and also EPCs at sites of *neo*-vascularization supported the hypothesis of a bipotent precursor for both. Despite the existence of the "hemangioblast" has been clearly demonstrated in specific

regions of the developing embryo [83][84] and in cellular cohorts derived from ES cells [85], it is not clear whether rare cells with a similar differentiation ability and a specific antigenic repertoire reside in the BM in postnatal stages, and may thus represent progenitors for both lineages also in the adult life.

# 4. EPCs FROM A TRANSLATIONAL POINT OF VIEW

## 4.1 Translational medicine

Translational medicine emerged some years ago to fill the gap between basic *in vitro* and *in vivo* research and clinical application. In fact, despite an explosion of knowledge in stem cell biology, this is not being translated into a corresponding increase in new treatments. Therefore, the aim of translational medicine is to convert all these preclinical basic research discoveries into social benefits, which are medical but also economical [86]. This concept is very well summarized in the famous slogan "from bench-to-bedside". Moreover, translational medicine involves different professionals that look at different aspects of it. From an academic point of view, translational medicine responds to the need of identifying novel scientific hypothesis on human pathology. For clinical professionals, it responds to need to accelerate the capture of research benefits

and converting them in daily medical practice. Finally, for the commercial sector translational medicine can develop new products. Of course, all these aspects are not mutually exclusive, but can overlap. [87].

## 4.2 Translational aspects of stem cell therapy

The bench to bedside path leading stem cells towards clinical application requires several steps having each a specific endpoint.

1- *Which stem cell for which pathology?*

It is essential to derive from preclinical experience safe and reproducible means of stem cells isolation/production. Standardization of procedures and ability to work according to quality requirements are also required. Perform a suitable screen of eligible patients in order to choose the best cellular product to be used in a given pathology.

2- *Enhancement strategies*

Implement and validate procedures to obtain clinical grade cells with enhanced qualities.

3- *How to reach the damage site?*

Define the best delivery mode for a specific stem cell type and a specific application.

4- *Test the therapeutic potential in Clinical trials*

PHASE I TRIALS: Initial studies to determine the metabolism and pharmacologic actions of drugs in humans, the side effects associated with increasing doses, and to gain early evidence of effectiveness; may include healthy participants and/or patients.

PHASE II TRIALS: Controlled clinical studies conducted to evaluate the effectiveness of the drug for a particular indication or indications in patients with the disease or condition under study and to determine the common short-term side effects and risks.

PHASE III TRIALS: Expanded controlled and uncontrolled trials after preliminary evidence suggesting effectiveness of the drug has been obtained, and are intended to gather additional information to evaluate the overall benefit-risk relationship of the drug and provide and adequate basis for physician labeling.

PHASE IV TRIALS: Post-marketing studies to delineate additional information including the drug's risks, benefits, and optimal use.

# 5. SCOPE OF THE THESIS

During my PhD fellowship I've worked in "Laboratrio di Biologia Vascolare e Terapia Genica" at Centro Cardiologico Monzino. One of the aims of the lab is to dissect different aspects concerning EPCs and their translation path to a possible clinical application.
In particular, we have developed three main themes:

1) We established standard operating procedures (SOPs) to isolate $CD133^+$ cells from human BM samples. Their full biological characteristics were validated *in vitro* and *in vivo*, and our SOPs were applied to obtain autologous $CD133^+$ cells to support use of these cells in an ongoing trail in patients affected by chronic cardiac ischemia [88].

2) We assessed the efficacy of a preconditioning strategy of human EPCs with histone deacetylase inhibitors in an attempt at enhancing their heart repair potency.

3) We assessed the biological effect of pharmacological mobilization of BM-derived cells in the PB with Granulocyte-Colony Stimulating Factor (G-CSF) on circulating EPC differentiation and on myocardial performance recovery in patients with acute myocardial infarction.

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# ***CHAPTER 2: GMP-BASED CD133$^{+}$ CELLS ISOLATION MAINTAINS PROGENITOR ANGIOGENIC PROPERTIES AND ENHANCES STANDARDIZATION IN CARDIOVASCULAR CELL THERAPY***

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# 1. Introduction

In the cardiovascular field, the use of adult bone marrow cells (BMCs) having angiogenic activity and myocardial regeneration potential has yielded promising results *in vitro* and in animal models [1, 2]. This opened the way to several clinical trials and, in recent years to some meta-analysis. Altogether, these studies were in agreement that administration of autologous cells in the heart is safe, and that it causes an improvement, although modest, in primary clinical endpoints such as ejection fraction (EF), end-systolic volume (ESV) and infarct scar size.

A possible explanation for the difference in the clinical readout between pre-clinical models and trials on BMCs may arise from the use of non selected cellular fractions. In fact, it has been hypothesized that administration of non selected BMCs may have a "Janus-like" effect. During ischemia a lot of cytokines are expressed. These cytokines exert a local pro-angiogenic effect, but also a systemic effect by activating the immune response and inflammatory cells. These cells home to activated tissue and so they can induce atherogenesis. Therefore, Epstein et al. define the "Janus phenomenon" by posit that when an intervention benefits the collateral development, it contemporary has the potential to provide insights into atherosclerotic mechanism, and vice versa [3]. However, the use of selected progenitors may prevent possible adverse consequences, such as inflammatory and atherogenic response,

and can also exert a greater effect. These was clearly demonstrated by comparing in a rat model the administration of: total MNCs, $CD34^+$ cells, and a higher dose of total MNCs containing the same number of $CD34^+$ cells of the stem cell-treatment group. The $CD34^+$ cell receiving group was the best in terms of capillary density, fibrosis area, shortening fraction and echocardiographic measurements [4].

For what concerns EPCs, a definitive consensus about the clinical use of one EPC type, and about which antigen identifies the best cell type has not been reached. Despite this, a growing body of evidences suggests that CD133 could be a useful marker that identifies a more primitive human progenitor subpopulation compared to CD34. Moreover, in addition to haematopoiesis, $CD133^+$ cells have been shown to possess endothelial capacity [5]. Another study by Freund and co-workers directly compared $CD34^+$ and $CD133^+$ cells isolated from 10 individual healthy donors. Although they did not find differences in terms of cell expansion properties, they found a greater subpopulation of more committed cells in the CD34+ group and a lower long term colony-forming units (LTC-FU). Moreover, $CD34^+$ cells contained a higher proportion of erythroid colony-forming cells, whereas the highest content of myeloid colony-forming cells were in the $CD133^+$ selected cells [6]. From all these results we can conclude that CD133 could be a useful antigen to select progenitor cells for a therapeutic purpose.

The use of purified stem cell populations in therapy needs a careful evaluation concerning safety, quality and efficacy of cells. To provide an answer to the growing need for improved standardization of cell preparation protocols, the major regulatory agencies worldwide, i.e. the US Food and Drug Administration (FDA) and the European Medicines Agency (EMEA), have established regulatory frameworks to ensure high safety standards and biological quality of cell-based medicinal products (CBMPs), which must comply with good manufacturing practice (GMP) specifications. Cell preparation has to be performed in dedicated laboratories institutionally certified as "Cell Factories" and following specific criterions. The first criterion is to assure the microbiological purity of the cellular product; this maximizes safety into patients. Since CBMPs manufacturing process cannot include a terminal sterilization or virus inactivation steps, the entire production process not only must comply to principles established for work in sterile conditions under good manufacturing practice (introduced by Directive 2003/94/EC), but also needs stringent approval for human use requirements for all the reagents and materials to be used. The application of this principle is of course subject to a complete validation process by appropriate validation runs that have to be conducted before release of the final cellular product; these provide demonstration of CBMP safety, conduct to an established knowledge of the production process as safe

by itself and set precise Quality Control (QC) steps to be repeated before release of every cellular lot.

The second principle underpinning the EMEA regulatory framework on CBMPs is based on the concept that cells are viable entities that interact with the surrounding environment. Therefore a primarily important issue that has to be taken into account is the analysis of the potential risks/benefits related to their *in vivo* administration. Accordingly, a comprehensive analysis should precisely identify the phenotype of cells, set minimal phenotypic release criteria according to basic experience, assess whether stem cells administration determines risks impairing their therapeutic use for a given pathology, and describe the potential benefits provided by these cells according to their intended use, through suitable *in vitro* and/or *in vivo* models [7]. Concerning this point, in a recent study, F. Seeger and co-workers demonstrated that the different outcome of two clinical trials, the REPAIR-AMI and the ASTAMI, could be due to the different protocols used to isolate the BMCs. In particular, the Lymphoprep protocol (used in the ASTAMI trial) allowed obtaining a lower recovery of cells compared to the Ficoll protocol (REPAIR-AMI). In addition, the ASTAMI included an additional overnight incubation of the cells. Although this did not further reduce the overall cell number, it significantly reduced their migratory capacity by decreasing CXCR4 expression. Therefore, different isolation protocols have a profound impact on cell function, and even small changes can

influence the clinical outcome [8]. This example suggests that definition of clear and standardized procedures to prepare stem cells for cardiovascular cell therapy represents a key issue to obtain optimal clinical results.

Therefore, the goal of our study was to develop and test GMP-compliant conditions to obtain purified $CD133^+$ cells (called here CD133 CBMPs) to be used in patients with cardiac or lower limb ischemia. We established standard operating procedures (SOPs) to purify human CB $CD133^+$ cells using CliniMACS and applied these procedures to obtain BM-derived CD133 cells for an ongoing clinical trial in patients affected by chronic cardiac ischemia. Here we thus describe a fully GMP-compliant CD133 CBMPs production and quality control process. We also provide a demonstration that manipulated cells maintain a full biological potency using *in vitro* and *in vivo* assays.

## 2. Materials and Methods

### Samples

UCB collection was performed after written approval by mothers. The age of neonates ranged between 36 and 42 weeks of gestation. BM samples were obtained from patients undergoing cardiac surgery upon approval of the study by local ethical committee and Italian national regulatory agencies (Istituto Superiore di Sanità, authorization no 63163-PRE.21-869, 30-03-2006).

### Isolation of CD133 CBMPs

Cord blood was recovered in EDTA-containing bags immediately after delivery. Blood samples from at least 3 different donors were pooled and used in each experiment. UCB mononuclear cell fraction was obtained by Ficoll-Histopaque density gradient. Isolation of $CD133^+$ cells was performed by CliniMACS™ (Miltenyi) system using the direct CD133 isolation kit (Miltenyi Biotech, catalog No. 177-01) according to Manufacturer's instructions. Briefly, after recovery from gradients, mononuclear cells were washed using Dulbecco's phosphate-buffered saline containing 1% (v/v) human serum albumin (DPBS-HSA). This was followed by two centrifugations at 600g for 10 minutes. Cells were subsequently resuspended into 100 ml volume with DPBS-HSA containing 7.5 ml of CliniMACS™ CD133 reagent (Miltenyi Biotec) and 1.5 ml of human IgG and incubated for 45

min at room temperature under gentle agitation. After two washing steps (550 g, 10 minutes), mononuclear cells were resuspended in 100 ml of DPBS-HSA and thereafter loaded into a column into CliniMACS device, where $CD133^+$ cells were separated and collected. Experiments performed to assess KDR expression in $CD133^+$ cells were performed using cells isolated from small BM aliquots (5-7ml) of patients undergoing heart surgery using miniMACS™ device (Miltenyi) and the indirect $CD133^+$ cells isolation kit (Miltenyi), according to manufacturer's instructions.

**GMP process and Quality Control (QC) testing**

Sterility and mycoplasma tests were performed according to European Pharmacopeia (EP) guidelines (chapter 2.6.27 and 2.6.7, respectively) in a GMP-certified laboratory. Endotoxin test were performed by Limulus Amebocyte Lysate (LAL) method according to EP (2.6.14) by using the Pyrogent Plus kit (Lonza, Basel, Switzerland).

*Environmental control and monitoring*

Cell manufacturing was carried out in a cell factory (Laboratorio Stefano Verri) authorized by the competent authority (Agenzia Italiana del Farmaco, AIFA) for the production of cellular-based medicinals for advanced therapies. All cell manipulation were performed in a Grade A safety cabinet and surronding Grade B clean room according to Eu-GMP guidelines (Eu GMP Vol. 4 Annex 1). Further information about environmental monitoring

is described in the supplementary online information. For Grade A zones a continuous sampling particle monitoring system was used (Airnet 510, A. &L.CO Ind), while for B and lower sterility grade zones, a regular frequent monitoring program was carried out. Microbiological monitoring was performed during and after every critical operations: surfaces, devices, and operating personnel were tested by contact plates. Air was monitored by sediment and volumetric air aspiration sampling (EU GMP Vol. 4 Annex 1). Filtered air supplies were present in all the premises in order to maintain a pressure gradient by maintaining a 10–15 Pascal positive pressure to surrounding areas of a lower sterility grade under all operational conditions. Temperature, humidity and all environmental parameters were set and recorded by a continuous remote control system (Desigo Insight™, Siemens Building Technologies LTD).

*GMP facility Quality Program*

A formal quality program evaluating all aspects of operations to assure GMP compliance was adopted, in particular this included: personnel qualification and training, and adoption of SOPs during all steps of manufacturing. The majority of the reagents used in the facility were GMP-manufactured. In all cases a quality certification of all products was available.

*Transportation and release of CD133 CBMP*

After the isolation procedure in the GMP facility (Laboratorio Stefano Verri, Monza, Italy), the UCB $CD133^+$ cells were packaged in appropriate refrigerated containers allowing the

maintenance of a controlled temperature comprised between +2 and+6 °C. Data loggers recording the temperature over the time of storage and transportation were placed in the package. A fully compliant GMP transportation was set by car overnight so that the cellular product reached the animal facility during the following morning before 8 a.m (within 18 hrs after QC tests in cell factory). A responsible person took out the cells from the package and, after performing the procedures for the QC (visual inspection of package, temperature monitoring, double checking of cellular lot number), injected the CD133 CBMP into the mice. By the same transportation modalities (after storage at 4°C for the same time period spent to send cells to animal facilty), CBMP-CD133 cells were sent from Laboratorio Stefano Verri to Centro Cardiologico Monzino-IRCCS (Milan, Italy) in order to perform the *in vitro* angiogenic tests.

### ***In vitro*** **experiments**

CD133 CBMPs transported from the cell factory were stored in different solutions: saline containing 1mg/ml human albumin, Stem Span (Stem Cells Technologies) or X-VIVO-15 medium (Lonza). Shortly after arrival, cells were counted. They were seeded in Stem Span containing a cytokine mixture supplemented with IL-3 and IL-6 (both at 20 ng/ml), Flt3-Ligand and Stem cell factor (SCF) (both at 100 ng/ml) to allow cell proliferation. In these experiments cells were seeded at $10^5$/well in 96 well plates. After 5 days under these conditions,

cells were counted. To assess endothelial differentiation, CD133 CBMPs expanded for 5 days were seeded onto Fibronectin (Sigma)-coated dishes using M199 medium (Gibco) supplemented with 20%FBS, 100 units/ml penicillin/streptomycin and 2mM L-Glutamine, and cultured for 7 days. After 7 days in these differentiation-promoting conditions, cells were fixed with 4% paraformaldehyde (Sigma) for 20 minutes and incubated overnight with 2 μg/ml of dioctadecyl-tetramethylindo-carbocyanine perchlorate (DiI)-labeled acetylated LDL (DiIAcLDL; Biomedical Technologies). After washing with PBS, cells were stained with 40 μg/ml of FITC-labelled Lectin from Ulex europaeus I (Lectin UEA I; Sigma-Aldrich) for 1 hour. Nuclei were stained with Hoechst 33258 (Sigma). For vWF immunofluorescence, cells were fixed with 4% paraformaldehyde and permeabilized using PBS containing 0.2% Triton-X 100 and 1% BSA. Anti human vWF antibody (Chemicon) was diluted at 10μg/ml in the same medium. Cells were incubated with primary antibody overnight at 4°C and, following extensive washing, were incubated with Alexa-488 conjugated anti rabbit secondary antibody for 1 hr at room temperature. Cells were observed under a Zeiss Axio Observer.Z1 fluorescence microscope equipped with Apotome image deconvolution hardware.

Flow cytometry analysis of CliniMACS-sorted cells was performed by incubating cells with fluorochrome-conjugated mouse anti-human monoclonal antibodies cocktail (CD45-FITC;

CD133-PE; CD34-APC, all from Miltenyi) for 20 minutes at 4°C in PBS containing 5% FCS. PE- or FITC-conjugated mouse IgGs were used as isotype control in FACS analysis at the same concentration as primary antibodies. Immediately before analysis, Propidium Iodide (Sigma) at final concentration of 5 μg/ml was added to each tube. At least $10^4$ cells were analyzed using FACScalibur flow cytometer (Becton Dickinson). Marker expression was analyzed using the FACSDiva (Becton Dickinson) software.

Flow cytometry analysis of BM-CD133$^+$ cells (KDR detection) and BM-CD133$^+$ cells-derived CFU-ECs was performed by using the following antibody cocktails: 1) anti CD133 PE (Miltenyi) and anti KDR-APC (R&D Systems) for CD133/KDR staining; 2) anti CD14-FITC (Miltenyi), CD31-FITC (Miltenyi), CD34-FITC (Miltenyi), CD45-APC-Cy7 (Beckton-Dickinson), CD48-Alexafluor700 (Exbio), CD105-PE (R&D Systems), CD144-PE (R&D systems) and CD146-PE-Cy5 (Beckman Coulter), for detection of CFU-EC endothelial myeloid and hematopoietic stem cells markers. $10^4$ cells were gated in each analysis performed by FACSAria cell sorter (Becton Dickinson). Appropriate isotype antibodies conjugated to each fluorochrome were used to set negative controls. Marker expression was analyzed using either Cell Quest™ or the FACSDiva™ (Becton Dickinson) software.

**Cytokine quantification by multiplex analysis**

Angiogenic cytokines expression in CFU-ECs obtained from BM-derived CD133 CBMPs was measured by analyzing their concentration (expressed as pg/ml/$10^5$ cells) in conditioned medium. M199 containing 20% FBS was conditioned for 24 hours. The concentration was measured by Bio-Plex Pro-Angiogenesis assay (Bio-Rad). As a negative control the same, non-conditioned, medium was used.

***In vivo* experiments**

Swiss CD1 male mice, 2 months old (Charles River, Italy), were used in this study. Immunosuppression was performed by injecting Cyclosporin-A (Cs-A) at 20 mg/kg weight for 2 days before, and daily after surgery, for the entire period of the experiment. To produce hind limb ischemia, the left femoral artery was excised with an electrocoagulator from its proximal origin as a branch of the external iliac artery till the bifurcation into saphenous and popliteal arteries as described [10]. Mice were anesthetized with an intra-peritoneal injection of 2.5% Avertin (Sigma) (100% Avertin: 10 g 2,2,2-tribromoethyl alcohol in 10 ml tert-amyl alcohol). Injection of CD133 CBMPs ($10^5$/animal) resuspended in saline solution ($10^5$ cells /50□l) was performed at the time of surgery; 10 □l of cell containing solution were injected at five levels (from proximal to distal) into the adductor muscle, along the femoral artery site after its removal. Laser Doppler Perfusion Imaging (LDPI; Lisca) was

used to monitor tissue immediately after and at 7 and 14 days after surgery.

**Histology and morphometric analysis**

Anesthetized mice were perfused with saline solution containing 1000 U/ml heparin (Roche Molecular Biochemical) followed by 10% buffered formalin for 10 min via the left ventricle at 100 mm Hg. Adductor muscles were removed, fixed in formalin for 48 hours and embedded in paraffin (Bio-plast special; melting point 52–54°C). Sections from each sample were cut at a 3 µm thickness and both capillary number and arteriole number was analysed as described[11, 12].

**Statistical Analysis**

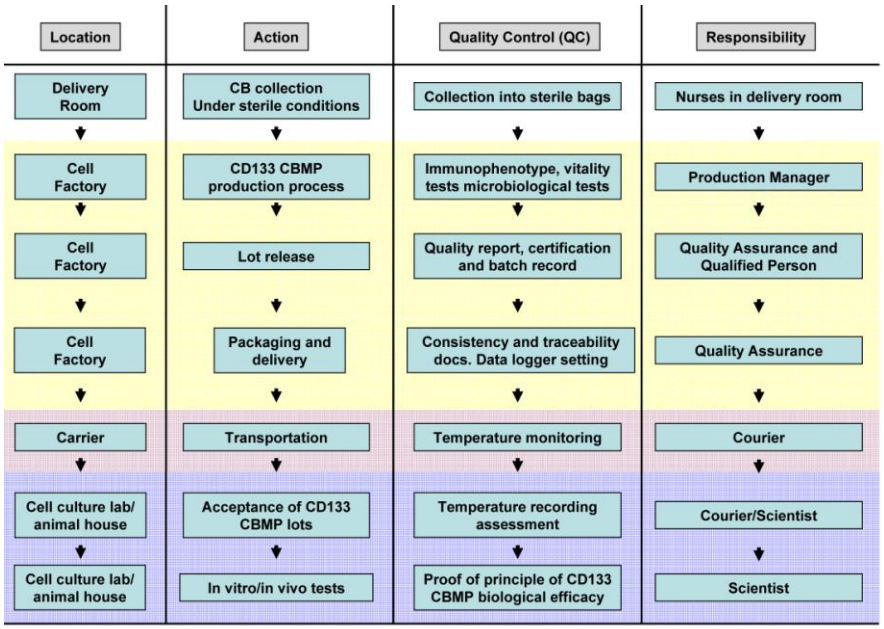
All data are expressed as mean±SEM; 2-tailed unpaired student's *t* test was performed using GraphPad statistical software, and a probability value of $P<0.05$ was considered statistically significant.

# 3. Results

**From purification to quality control and certification of CB CD133 CBMPs production procedure**

Implementation of currently available laboratory procedures to large scale GMP setting into the cell factory required appropriate validations in order to: 1) establish detailed standard operating procedures (SOPs), 2) identify and monitor critical steps during the production process and 3) define final product release specifications. In order to develop SOPs for $CD133^+$ cells isolation from human cord blood we used laboratory protocols already established during our previous experience. Magnetic purification by CliniMACS was chosen as it is performed using clinical grade reagents that are routinely used for purification of hematopoietic stem cells in haematological transplantation settings [13, 14], and because it was safely used in previous studies to produce $CD133^+$ cells for the treatment of patients with cardiac ischemia [15-17].

The GMP-compliant CD133 CBMPs production process was organised and accomplished according to the scheme presented in Fig. 1. In this scheme, the QC testing actions, the locations of each action and the responsibility of the actions at each step are represented.

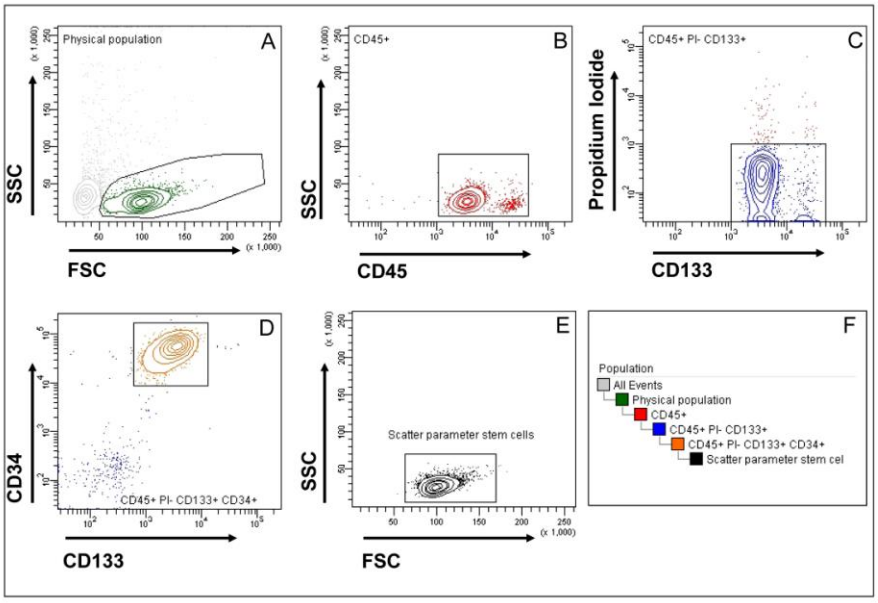



Gaipa et al., Figure 1

Table 1 describes the tests, the methods according to European Pharmacopeia and the product specifications that were utilized to proceed with CD133 CBMPs lot certification and release. Specifications were intended as threshold values to be met for lot release.

| Test | Method | Specification |
|---|---|---|
| Purity | Flow cytometry (% of CD133+) | ≥50% |
| Vitality | Flow cytometry (Propidium Iodide staining) | ≥70% |
| Number of total nucleated Cell | automated/manual cell counting | $\geq 10^6$ |
| Sterility | Sterility test (European Pharmacopeia) | negative |
| Mycoplasma | Cultural test (European Pharmacopeia) | negative |
| Endotoxin | LAL test (European Pharmacopeia) | <0,5 EU/mL |

Figure 2 represents a typical example of flow cytometric assessment of CD133 CBMP vitality and purity. To this purpose a hierarchical gating strategy was followed; specifically: cell purity was evaluated by first gating the $CD45^+$ cells and then by determining the percentage of $CD45^+/PI^-$ living cells, to which was finally applied the appropriate gate to identify $CD133^+/CD34^+$ cells. Results obtained by this testing are shown for each CB-derived CD133 CBMPs lot in table 2.




Gaipa et al., Figure 2

| Lot # | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | mean±SD |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Viability before isolation | 89.76% | 90.00% | 95.00% | 92.00% | 95.90% | 92.76% | 84.80% | 92.30% | 92.00% | 97.18% | 92.17%± 3.53% |
| Viability post isolation | 78.70% | 85.44% | 80.00% | 93.00% | 91.00% | 90.90% | 93.54% | 87.70% | 98.48% | 99.32% | 89.81%±6.96% |
| Number of nucleated cells recovered from CliniMACS | 4.32x $10^6$ | 4.40x $10^6$ | 1.65x $10^6$ | 5.80x $10^6$ | 4.20x $10^6$ | 2.39x $10^6$ | 11.20x $10^6$ | 16.32x $10^6$ | 2.11x $10^6$ | 2.61x $10^6$ | 5.50x $10^6$±4.69x $10^6$ |
| $CD133^+$ cells purity | 66.90% | 84.70% | 82.70% | 83.50% | 82.50% | 61.74% | 90.40% | 64.90% | 77.97% | 75.28% | 77.06%± 9.59% |
| $CD133^+$ cells recovery | 67.30% | 90.00% | 58.87% | 48.40% | 58.90% | 28.80% | 90.00% | 71.10% | 65.00% | 80.73% | 65.91%±18.83% |

Gaipa et al., Table 2

It is to be noted that in all these preparations, threshold values were successfully met. Finally, as shown in table 3, sterility, endotoxin and mycoplasma tests were negative in all CD133 CBMPs lots preparation. Altogether, these results show high reproducibility of the CD133 CBMPs production process under aseptic conditions.

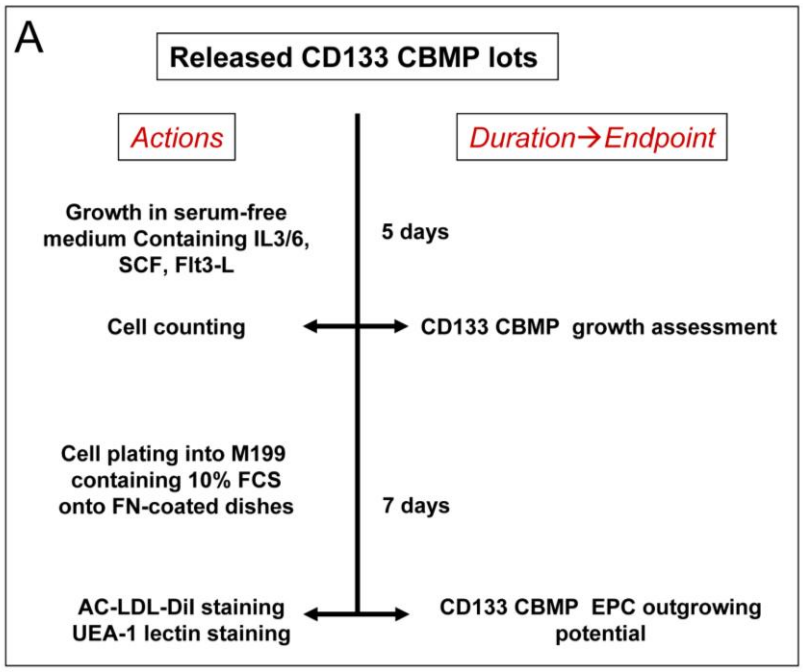
| Lot # | Sterility | Endotoxin | Mycoplasma |
|---|---|---|---|
| 1 | Neg | <0.24 EU | Neg |
| 2 | Neg | <0.24 EU | Neg |
| 3 | Neg | <0.24EU | Neg |
| 4 | Neg | <0.24 EU | Neg |
| 5 | Neg | <0.24 EU | Neg |
| 6 | Neg | <0.24 EU | Neg |
| 7 | Neg | <0.24 EU | Neg |
| 8 | Neg | <0.24 EU | Neg |
| 9 | Neg | <0.24 EU | Neg |
| 10 | Neg | <0.24 EU | ND |

Gaipa et al., Table 3

**Proof of principle of CB CD133 CBMPs biological quality maintenance (1): *in vitro* experiments**

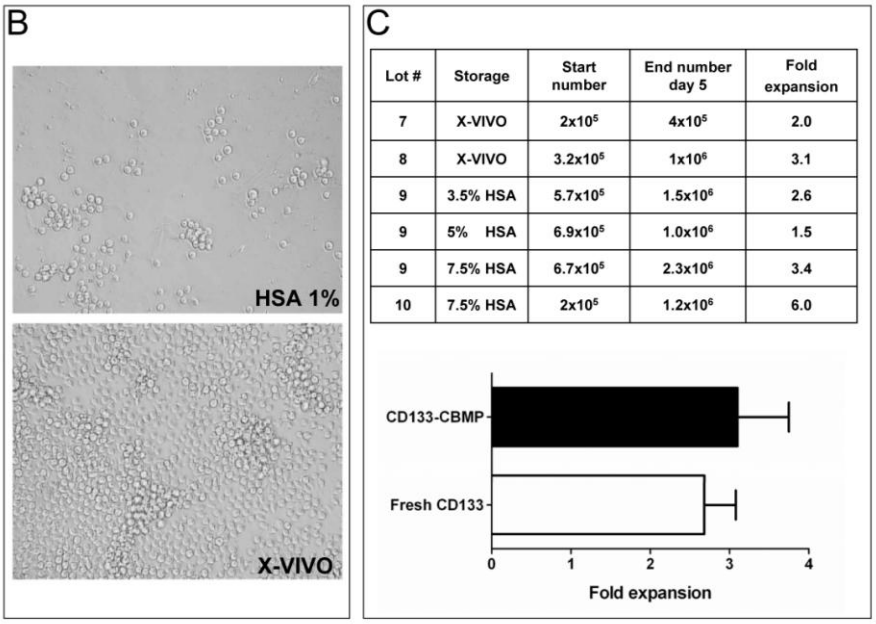
A key issue to be addressed in the assessment of CBMPs quality is, according to EMEA guidelines (Guideline on Human Cell-Based Medicinal Products, doc. ref. emea/chmp/410869/2006), the demonstration of "proofs of principle" that CBMPs maintain a full biological potential by appropriate "potency" tests. This ensures that the production process does not modify the ability of stem cells to engraft in recipient tissues and that they maintain the expected and desired regenerative effect.

We thus performed *in vitro* experiments according the scheme shown in Figure 3A.




Gaipa et al., Figure 3

Briefly, the cells were grown in culture with mitogenic cytokines[18] for a period of five days, after which they were counted. After counting, cells were plated under differentiation conditions to assess the formation of EPC (CFU-EC) early colonies [9, 19, 20]. According to Seeger et al. [21], the choice of the storage medium represents an important step toward the optimization of cells to be transplanted. In our *in vitro* assays, we tested different storage media in order to identify the best condition to maintain fully viable and biologically active CD133 CBMPs for 18 hrs, i.e. the time which would elapse between lot release and delivery of the cellular product at the clinical centre. As a storage medium during initial tests, we used saline solution supplemented with human albumin (HSA) at a 1% concentration (HSA 1%). CD133 CBMPs, stored overnight into HSA 1% were cultured in serum free-medium containing mitogenic cytokines (see materials and methods). To our surprise, we found that purified cells stored in this medium failed to proliferate (Figure 3B), likely due to cell damage during the overnight storage, despite PI exclusion tests, performed before and after the overnight period, did not indicate substantial cell death (not shown).



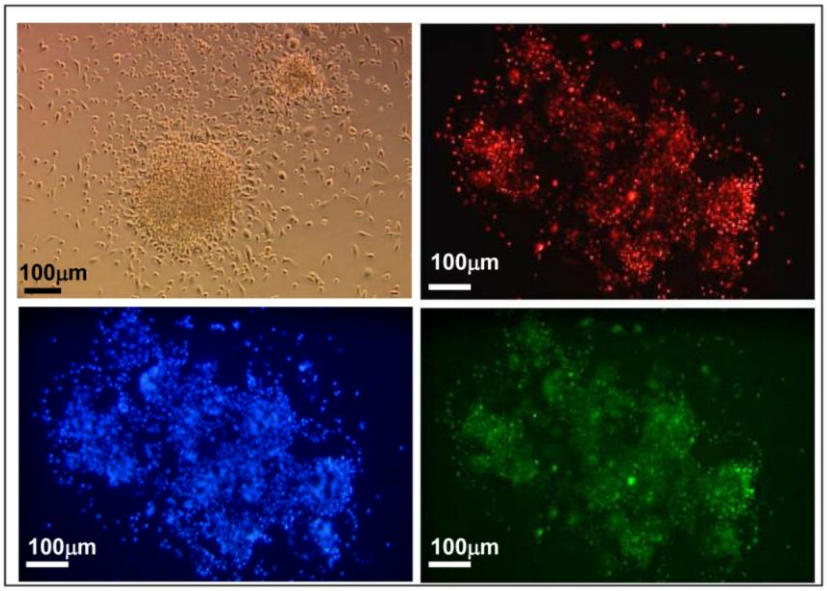

| Lot # | Storage | Start number | End number day 5 | Fold expansion |
|---|---|---|---|---|
| 7 | X-VIVO | $2x10^5$ | $4x10^5$ | 2.0 |
| 8 | X-VIVO | $3.2x10^5$ | $1x10^6$ | 3.1 |
| 9 | 3.5% HSA | $5.7x10^5$ | $1.5x10^6$ | 2.6 |
| 9 | 5% HSA | $6.9x10^5$ | $1.0x10^6$ | 1.5 |
| 9 | 7.5% HSA | $6.7x10^5$ | $2.3x10^6$ | 3.4 |
| 10 | 7.5% HSA | $2x10^5$ | $1.2x10^6$ | 6.0 |

Gaipa et al., Figure 3

To better preserve CD133 CBMPs vitality, we thus set different storage strategies: we used X-VIVO-15 (X-VIVO) without serum and saline solution containing different amounts of human albumin (HSA 3.5%, HSA 5% and HSA 7.5%). The results (Figure 3 B, C) showed that CD133 CBMPs stored in these media had a proliferation rate that was comparable to that shown by CB-derived freshly isolated $CD133^+$cells under the same culture conditions (Figure 3C).

Both in animal models[22] and in patients[16, 23], $CD133^+$ cells have been found to have an angiogenic function. As a preliminary *in vitro* screen for CD133 CBMPs angiogenic activity, we tested formation of CFU-EC colonies by plating pre-expanded CD133 CBMPs (stored into X-VIVO or HSA 7.5%) into fibronectin-coated wells in high serum conditions[9, 20]. The

results showed the development of large cellular clusters taking up Ac-LDL-DiI and stained with UEA-1 lectin[19] (Figure 4), typically resembling CFU-EC colonies[20].

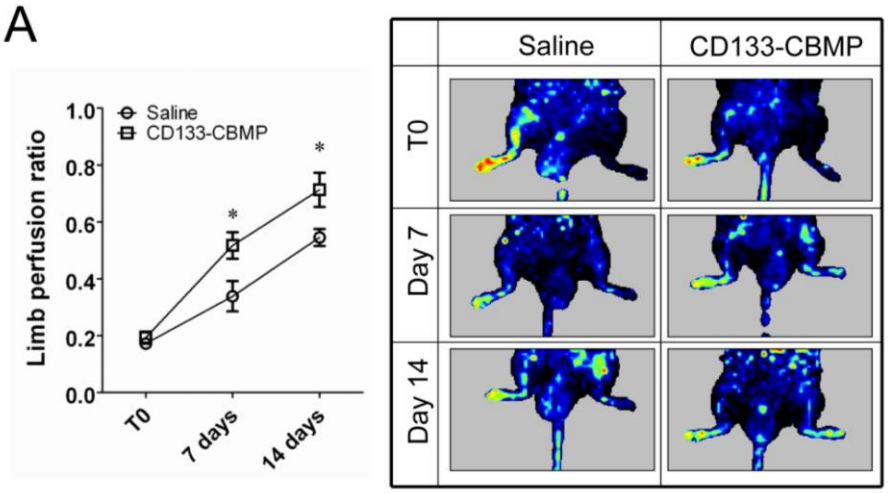


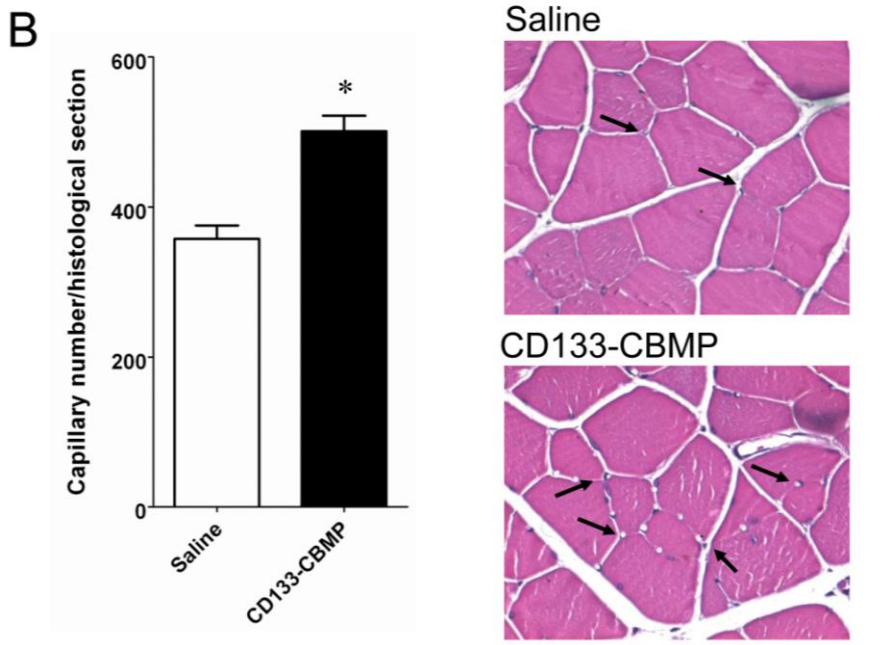

Gaipa et al., Figure 4

**Proof of principle of CB CD133 CBMP biological quality maintenance (2): Induction of angiogenesis in ischemic limbs**

Previous studies have shown the ability of human $CD34^+$ and $CD133^+$ cells to induce myocardial repair following infarction in animal models of myocardial infarction or injury [22, 24-27]. Additionally, we and others have shown the *pro*-vasculogenic effect of $CD34^+$ progenitor cells using hind limb ischemia models in mice [18, 28-30]. In the present study the latter approach was used to demonstrate that GMP-produced CD133

CBMPs maintain a *pro*-vasculogenic potential and contribute to ischemic tissue repair, thus providing direct evidences that GMP production process of these cells preserves their biological activity. The ability of CD133 CBMPs stored in X-VIVO and 7.5% HSA to improve recovery of limb perfusion was evaluated by LASER-Doppler perfusion imaging (LDPI) and by capillaries and arterioles density determination. As shown in Figure 5A, LDPI imaging showed that in all animals blood flow was drastically reduced immediately after femoral artery dissection. In saline-injected animals, a progressive recovery was detected between day 7 and day 14 after femoral artery dissection. When CD133 CBMPs were injected in the ischemic limbs, a significant increase in perfusion ratio compared to saline-injected mice was observed at the same time points. Morphometric analysis was performed on adductor muscle sections at day 14 after femoral artery dissection. Both the capillary and the arterioles density (Figure 5B and 5C, respectively) were significantly increased by CD133 CBMP injection.




Gaipa et al., Figure 5

**Translation of isolation procedures to BM samples for autologous transplantation in patients affected by myocardial ischemia: release quality control and *in vitro* proofs of principle.**

SOPs established to produce CB-derived CD133 CBMPs were translated to production of clinical grade CD133 CBMPs from bone marrow of three patients undergoing a phase II clinical trial of $CD133^+$ cells intramyocardial administration, which has been recently approved by Italian Regulatory Agency. This trial is currently being carried at Centro Cardiologico Monzino.

Translation of GMP procedures to obtain BM-derived CD133 CBMPs from CB was not immediate and required suitable validation runs. In fact, differences in the stem cells sources may cause 1) a different recovery of MNCs after ficoll gradient centrifugation and 2) a different purity of yielded cells due to differences in the CD133 expression level in BM compared to CB stem cells. To address these issues we calculated the percentage of MNCs loss after CB and BM ficoll gradient centrifugation. As shown in the table below, a higher cellular loss was observed using BM compared to CB.

| Source of cellular product | MNCs recovery after ficoll | T-test |
|---|---|---|
| BM | 18.74%±13.70% (n=3) | P=0.0723 (ns) |
| CB | 47.48% ±23.42% (n=10) | |

The difference in MNCs recovery was not statistically significant and a high variability was observed between different samples. We also calculated the mean fluorescence intensity (MFI) relative to CD133 expression in CB and BM stem cells by flow cytometry.

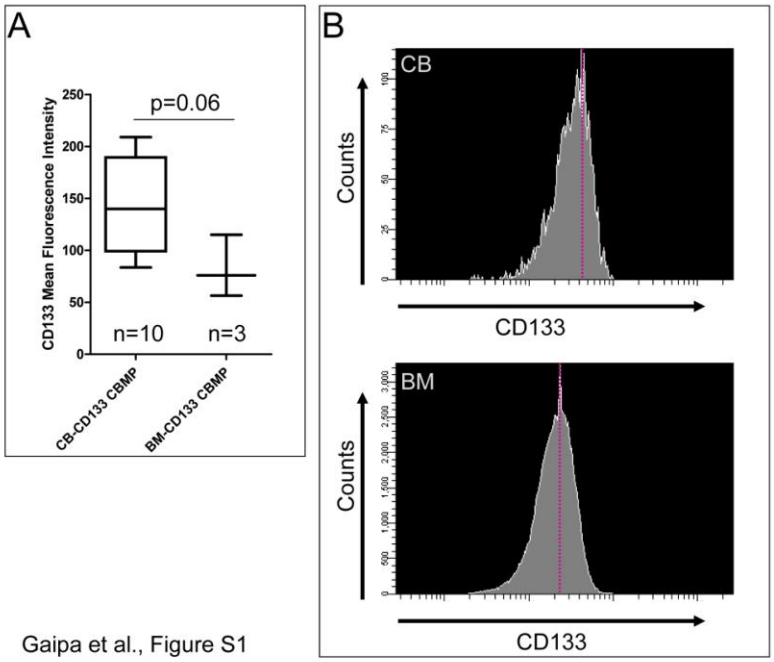
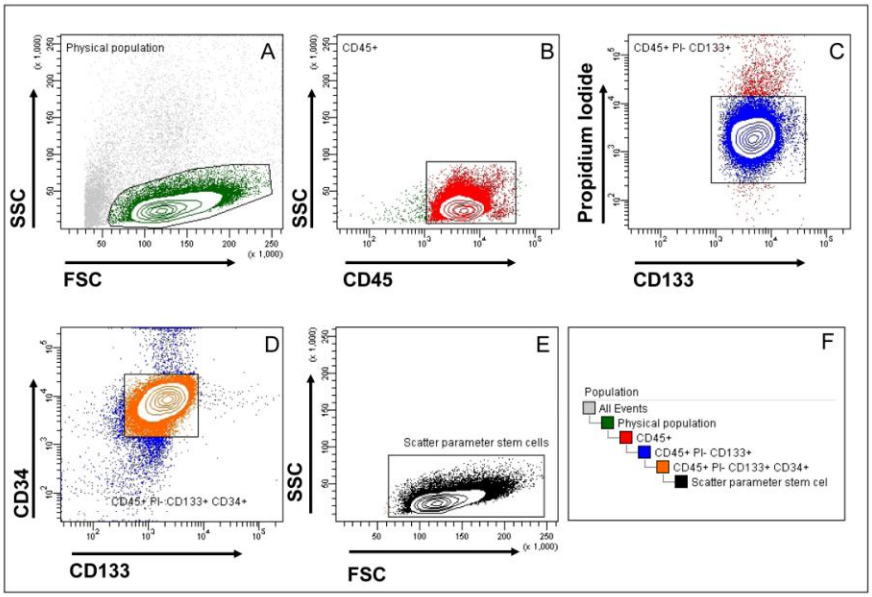



Figure S1 shows a relatively lower MFI in BM compared to CB stem cells; again comparison of the MFI values did not reveal a statistically significant difference. Finally, to assess whether lower MNCs recovery after Ficoll gradient centrifugation and CD133 expression affect the quality of the final products, we compared CB and BM CD133 CBMPs release quality parameters such as purity of $CD133^+$ cells (mean±SD: 73.52%±20.22% *vs.* 77.06%±9.59%, BM vs. CB; P>0.05, Student's t-test), recovery of nucleated cells after cliniMACS$^{TM}$ (mean±SD:

$5.70x10^6 \pm 3.18x10^6$ cells *vs.* $5.50x10^6 \pm 4.69x10^6$ cells BM vs. CB; P>0.05, Student's t-test) and post-isolation vitality (mean±SD: 98.77%±1.02% vs. 89.91%±6.96%, BM vs. CB; P>0.05, Student's t-test). All these comparisons did not reveal statistically significant differences (Figure 6, see also Tables 2 above and Table 4), showing that translation of SOPs developed for CB $CD133^+$ cells to GMP production of BM-derived CD133 CBMPs produced similar results to those obtained using CB.

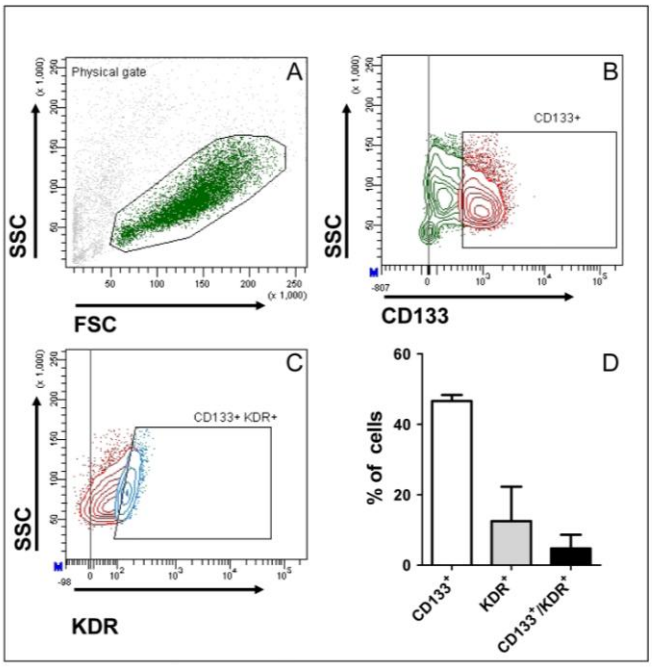



Gaipa et al., Figure 6

| Test | Method | Specification | Result | | | mean±SD) |
|---|---|---|---|---|---|---|
| | | | patient #1 | patient #2 | patient #3 | |
| Purity | Flow cytometry (% of $CD133^+$) | ≥50% | 51.31% | 90.87% | 78.38% | 73.52±20.22 |
| Viability post isolation | Flow cytometry (Propidium Iodide staining) | ≥70% | 98.37% | 99.93% | 98.02% | 98.77±1.02 |
| Number of nucleated cells recovered from CliniMACS | automated/manual cell counting | $\geq 10^6$ | $4.46 \times 10^6$ | $9.31 \times 10^6$ | $3.32 \times 10^6$ | $5.70 \times 10^6 \pm 3.18 \times 10^6$ |
| Sterility | Sterility test (European Pharmacopeia) | negative | negative | negative | negative | - |
| Mycoplasma | Cultural test (European Pharmacopeia) | negative | negative | negative | negative | - |
| Endotoxin | LAL test (European Pharmacopeia) | <0,5 EU/mL | <0,24 EU/mL | <0,24 EU/mL | <0,24 EU/mL | <0,24 EU/mL |

Gaipa et al., Table 4

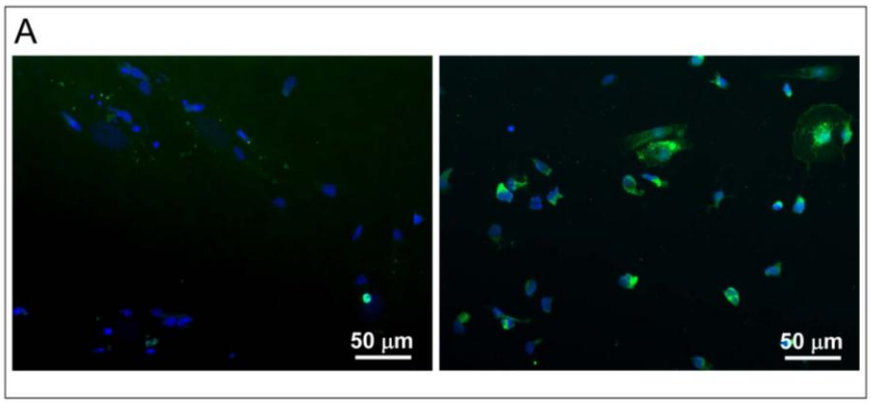
A second step was to provide the suitable proofs of principle that BM-derived CD133 CBMPs have a similar phenotype to that of CB-$CD133^+$ cells. As an initial biological quality control test, patients-derived $CD133^+$ cells first tested for the expression of VEGFR-2/KDR antigen, an important marker defining progenitors having an *in vivo* angiogenic activity [25, 29] (Figure 7).

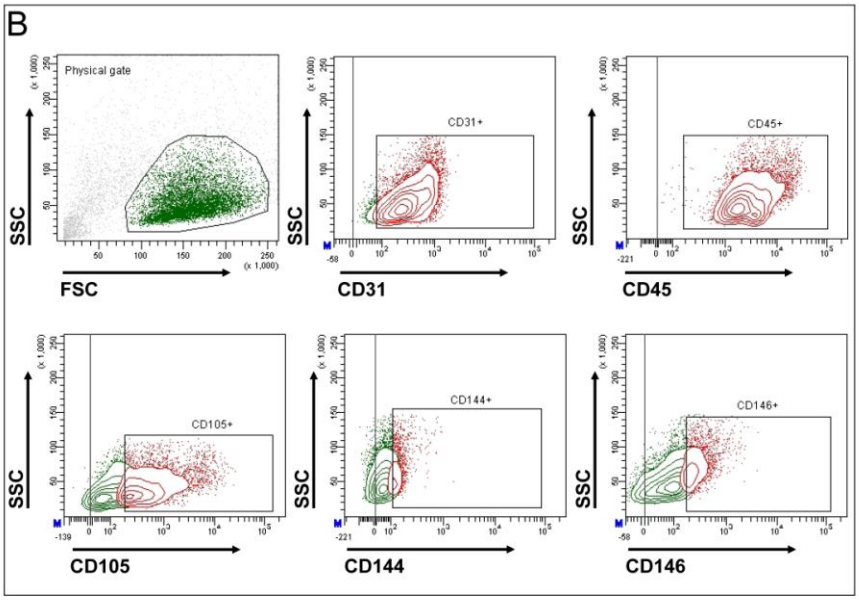



Gaipa et al., Figure 7

The biological quality of BM CD133 CBMPs was then functionally assessed by *in vitro* tests that were performed according to the procedure established for CB-derived $CD133^+$ shown in Figure 3A above. In these experiments it was found that, analogous to CB, BM-derived CD133 CBMPS the formed CFU-EC colonies (not shown). The endothelial-like phenotype of cells composing these colonies was confirmed by detection of the endothelial marker vWF by immunofluorescence (Figure 8A) and by multicolour flow cytometry analysis, which revealed that these cells were $CD45^+$ and expressed, although at a lesser extent, $CD31^+$ and $CD105^+$ endothelial cells markers (Figure 8 B and C). By contrast, the expression of CD144 and CD146 antigens,

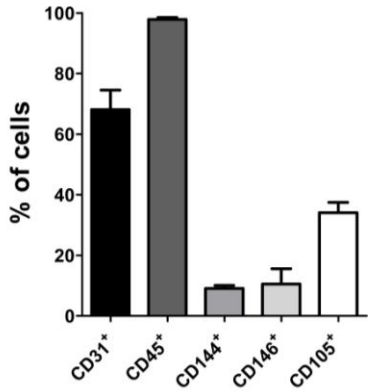
that are expressed in terminally differentiated endothelial cells, was expressed in a minority of cells (Figure 8 B and C).




Gaipa et al., Figure 8

C

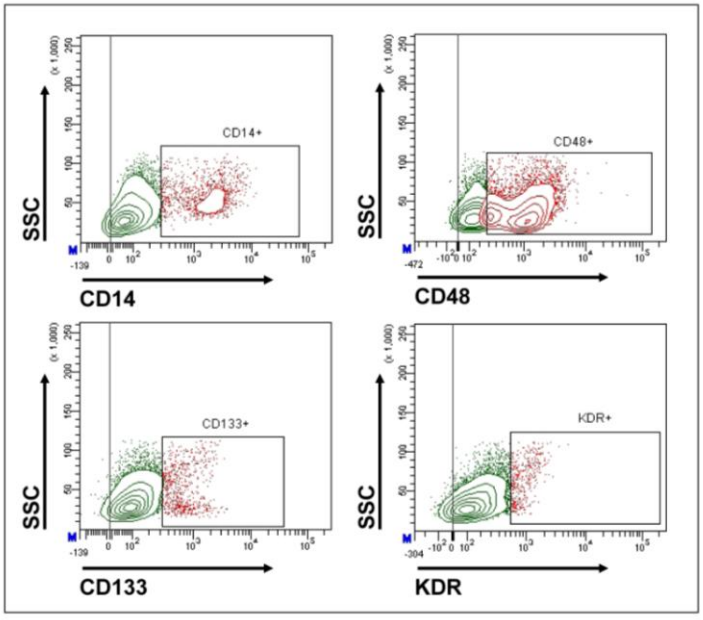



D

| Cytokine | Amount (pg/ml/$10^5$ cells) mean±SE |
|---|---|
| HGF | 50.37±7.7 |
| IL-8 | 599.4±336 |
| PDGF-BB | 16.07±8.04 |
| VEGF | 5.61±2.59 |

Gaipa et al., Figure 8

Cells were also assayed for expression of monocyte marker CD14, the pre-B/pre-T antigen CD48 and CD133 and KDR stem cell markers (Figure S2).




Gaipa et al., Figure S2

Finally, to functionally characterise the putative *pro*-angiogenic activity of BM-derived CD133 CBMPs, we tested the expression of angiogenic cytokines in CFU-EC conditioned media by multiplex analysis. Results showed that these cells expressed HGF, IL-8, PDGF-BB and VEGF cytokines (Figure 8D above).

# 4.Discussion

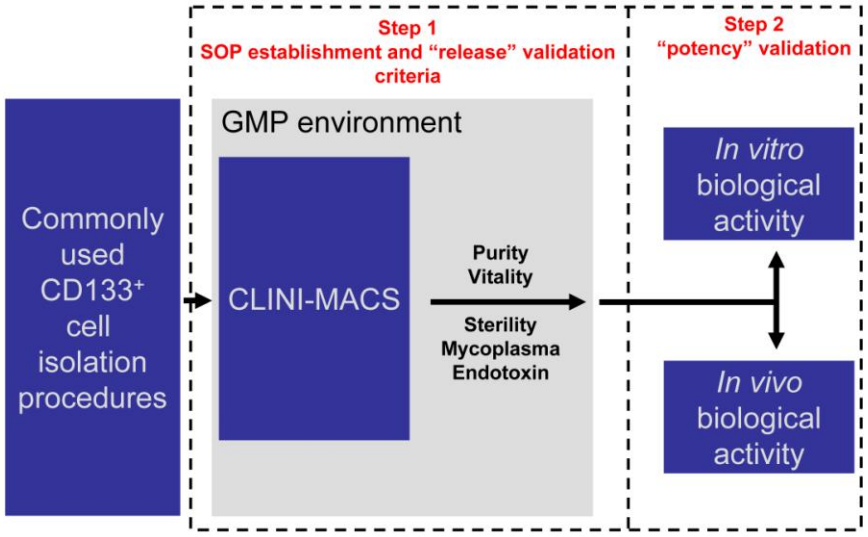
Therapeutic use of EPCs has been suggested as a promising approach to induce *neo*-vascularization in ischemic diseases[31]. Despite a large number of preclinical reports describing marked EPCs beneficial effect on ischemic tissues perfusion, data obtained from meta-analyses of clinical trials have provided evidence of only a modest clinical benefit[32-36]. So far, the reason for such a modest increase in heart function following stem/progenitor cells administration is not clear. In fact, it is still a matter of debate whether it may be related to a decreased biological function of cells obtained from patients with CVD risk factors (diabetes, hypercholesterolemia, hypertension) and/or to a low survival/engraftment of the cells due to a hostile microenvironment such as that present into the ischemic myocardium (discussed in [37]).

Different possible strategies to overcome the limited biological function of EPCs from subjects at risk have been suggested. A first option is provided by the possibility to use *ex-vivo* manipulation strategies such as culture in the presence of factors that enhance ability of EPCs to migrate, proliferate/survive and adhere [38-40] (also discussed in [40-42]). A second possibility, not necessarily alternative to the previous, is the use of strategies aimed at maximizing CBMPs reproducibility, safety and efficacy[21].

**From GMP compliance to proofs of principle: "release" and "potency" validation levels for standardization of CBMPs production process for cardiovascular therapy**

Today, GMP compliance requirement is one of the major tasks in translational application of stem cells in cardiovascular disease. A central component of GMP is the establishment of standard operational procedures for clinical grade manufacturing of cells, ranging from the systematic checking and traceability of materials to microbiological and efficacy validation of final products. Guidelines for GMP application in stem cell therapy have been established by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). The European Community has adopted these guidelines to introduce GMP regulations in the EU Member States. According to the Directive 2006/86/EC "validation means establishing documented evidence that provides a high degree of assurance that a specific process, piece of equipment or environment will consistently produce a product meeting its predetermined specifications and quality attributes; a process is validated to evaluate the performance of a system with regard to its effectiveness based on intended use".

From these considerations CBMPs certification for use in ischemic patients essentially requires a two-step validation program (Fig. 9).




Gaipa et al., Figure 9

**Step 1.** During a preliminary step of GMP compliant CBMP preparation procedure setup, tests are performed are in order to check for sterility and absence of mycoplasma and endotoxin contamination and to set CBMPs purity/vitality thresholds. These experiments enabled us to establish criteria to ensure the reproducibility of the purification procedure based on the CliniMACS device (purity and vitality) and the safety of manipulation procedures in sterility controls (mycoplasma, endotoxin and sterility tests), to establish quality thresholds for the production process of CD133 CBMPs as well as the lot-to-lot consistency. They also led us to the important result of defining media (X-VIVO-15 and 7.5% HSA containing saline) for an optimal storage of the cells.

**Step 2.** A second but not less important validation step is the assessment of CBMPs biological properties. These studies demonstrate the CBMP potency (biological quality) in a suitable model *in vitro* or *in vivo*. The demonstration provided by step 2 is necessary to demonstrate that stem cells produced according to the GMP-compliant procedure maintain the intended biological activity which should be related to the expected clinical effect. While step 1 validation tests are necessary at every CBMP lot release, potency validation tests should not to be repeated. In fact, once validated and suitably performed without amendments, the procedure used for CBMPs preparation is recognized itself as a compliant procedure to meet requirements needed for CBMP human use. The experiments performed during this second phase in the present study showed that the isolation procedure followed by storage and transport under optimal conditions, allowed to maintain a biological potency of CB $CD133^+$ cells similar to that supposed for native cells (angiogenic potential) anticipated in several preclinical studies.

**Release and potency validation steps of BM CD133 CBMPs for cardiovascular repair**

The use of CD133 CBMPs in patients affected by myocardial ischemia required translation to BM samples of isolation procedures set using CB. Validation of procedures translated from CB to BM firstly required appropriate testing in order to

ensure that product specification and minimal threshold parameters established during CB isolation runs were met. This testing led us to establish minimal starting quality thresholds (Table 5) such as 1) a vitality higher than 90%, 2) a starting number of mononuclear cells higher than $5x10^9$, 3) a total number of $CD133^+$ cells higher than $15x10^6$ and 4) execution of $CD133^+$ cells purification process within 24 hrs after BM collection.

We then considered possible major deviations that may arise from the different cellular composition of BM compared to CB and possible differences in the expression levels of the CD133 stem cell marker, which may affect recovery and purity of the final cellular product. Comparison of MNCs loss after Ficoll centrifugation and the analysis of MFI relative to CD133 expression in CB and MB cells did not reveal statistically significant differences even if, as shown in Fig. S1 and Table S1, for both parameters we noticed a possible deviation that might reach statistically significance by increasing the experimental replicates. On the other hand, it has to be noted that purity, number of MNCs recovered from cliniMACS$^{TM}$ and vitality of $CD133^+$ cells did not show the same trend, suggesting that translation of isolation procedures from CB to BM was feasible with a comparable efficiency. In this respect, the adoption of improved strategies to minimize cellular loss during Ficoll centrifugation is under study.

A final step required to assess the feasibility of BM-derived CD133 CBMPs GMP production was to demonstrate the biological potency of these cells by providing suitable proofs of principle. Due to the limited amounts of BM that we could use for this purpose, we performed only *in vitro* tests aimed at demonstrating the phenotype and the potential *pro*-angiogenic function of these cells. These tests showed the endothelial-like phenotype of CFU-ECs grown from these cells in culture and, in line with existing literature, the mixed endothelial/monocytic/myeloid phenotype of these cells[20, 43-48]. Furthermore, these cells produced, although at different levels, HGF, IL-8, PDGF-BB and VEGF cytokines. Altogether, these data suggest that BM-derived CD133 CBMPs possess a *pro*-angiogenic paracrine activity.

In summary, our work shows that GMP implementation of currently available protocols to produce $CD133^+$ stem cells for cardiovascular repair is feasible and that it maintains the innate angiogenic properties of these cells. This work represents the first example of protocol standardization that goes in the still unexplored direction of optimizing quality and enhancing reproducibility of cellular products application into patients affected by ischemic heart disease.

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# ***CHAPTER 3: MODIFICATION OF THE EPIGENETIC LANDSCAPE BY HISTONE DEACETYLASE INHIBITION INDUCES GLOBAL GENE EXPRESSION CHANGES AND ENHANCES CARDIOPROTECTION BY EX VIVO CULTURED HUMAN CORD BLOOD-DERIVED CD34$^{+}$ CELLS.***

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# 1. Introduction

As also discussed in the previous chapter, despite the great promises offered by preclinical studies in animal models clinical trials in humans, recent published meta-analysis gathering together results of different clinical trials have delineated a different scenario.

Lipinski et al. analyzed 10 clinical trials, corresponding to 698 patients, with a mean follow-up of 6 months. They found that intracoronary cell therapy was significantly superior to standard medical treatment. In fact it induced a 3% increase in LVEF; it also increased the LVESV (average difference -7.4ml) and decreased the infarct size (average difference -5.6%). Moreover, a trend in enhancing the LVEDV was also observed.

Even statistically significant, these results are of modest entity compared to what observed in animal models [1]. A similar conclusion was depicted by Jiang et al. They analyzed results from 980 patients enrolled in 18 independent trials. They grouped the studies on the basis of the length of the follow-up period. After 3 months they found a significantly increase in LVEF (cell treatment *vs.* placebo group) that was further sustained at 6 months and still present, although at a lower extent, after one year. The same beneficial effect was also observed on LVESV and MI size at each time point. Interestingly the authors also performed a subgroup analysis by breaking down the patient data according to the disease status

(acute MI (AMI) or chronic MI (CMI)), time from PCI to cell infusion, sample storage duration and dose of cells received.

Even if only a few CMI trials were included in this analysis, it seems that cell treatment is effective only in AMI patients. Moreover, prompt infusion was more beneficial than overnight cell storage, and the best BMCs dosage seemed to be lower than $10^9$ [2].

In addition to what delineated in this papers, several causes may explain the reduced ability of patient-derived EPCs to induce cardiovascular repair. These include:

- the lack of refined and fully validated methods to prepare cells for transplantation purposes that, still today, represents a hurdle in the way toward standardization not only in cardiovascular cell-based regenerative medicine [3].
- an incomplete characterization of the stem cell properties of cells to be clinically used.
- cell autonomous defects caused by cardiovascular risk factors that greatly reduce EPCs tolerance to stress conditions [4] with consequent reduction of the ability to produce differentiated progenies and to survive into recipient tissues [5] [6].

The acknowledgment of intrinsic and extrinsic factors limiting efficacy of cardiovascular repair by patient-derived EPCs has prompted the birth of various strategies to restore innate EPC biological properties and maximize their clinical efficacy.

For example, the paracrine EPCs activity is believed as one of the main ways of action whereby EPCs induce *neo*-vascularization and it is significantly reduced by risk factors. Studies showing the preclinical efficacy of progenitor cells infected with adenoviral vectors carrying the $VEGF_{165}$ cDNA under the control of ubiquitous promoters have proposed that a gene transfer approach may represent a possible enhancement strategy to restore or even enhance EPC *pro*-angiogenic activity. In addition, ELISA experiments showed a systemic increase in VEGF level in animals treated with $Ad.VEGF_{165}$ infected EPCs, indicating a possible indirect effect of $VEGF_{165}$ expression on the improvement of *neo*-vascularization into ischemic tissues [7].

Moreover, several reports in the literature have shown that culture of human EPCs in the presence of defined cytokines enhances endothelial differentiation ability of these cells *in vitro*, and increases their participation to *neo*-vascularization *in vivo*. Typical is the example of the chemokine stromal derived factor-1 (SDF-1). Work from our laboratory has shown that 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. Furthermore, we found that responsiveness of c-$kit^+$ cells to SDF-1 was lost when c-$kit^+$ cells were obtained from mice treated with streptozotocin to induce diabetes mellitus, and that it was restored after culture

for one week in normoglycemic conditions in the presence of mitogenic cytokines IL-3/6, SCF and Flt3L [6]

Compounds known to stimulate the AKT signal transduction pathway have been used to pre-treat EPCs in culture, thereby enhancing their potential efficacy. For example, HMG-CoA reductase inhibitors (statins) are potent activators of AKT signaling in endothelial cells that promote cell survival and angiogenesis [8].

In addition, it is well known that 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 [9].

These examples, together with several other published works, have paved the way to enhance the EPCs repair function in the ischemic context. Nevertheless, relatively fewer studies have addressed the relationship between the potency of selected stem cells and their in vivo repair ability. This latter issue is of great interest as still not unanimous definition of EPCs identity currently exist [10].

Stem cell potency, differentiation and memory are all driven by epigenetic modifications.

In paragraphs 2.4 and 2.5 we have already discussed the relationship between epigenetic modifications, such as DNA methylation and histone tails modifications, and stem cell potency/plasticity. In recent years has also been discovered a role of small non coding RNAs in post-trascriptional regulation. In fact, miRNAs have been shown not only to regulate the stability and translation of mRNAs but also to induce the formation of inactive chromatin structures of target genomic sequences [11].

Chromatin structure is another relevant factor that modulates gene expression. Folding and the level of condensation determine the basic types of chromatin. Euchromatin is defined as gene-rich regions that are less condensed and, therefore, more accessible to transcriptional complexes, instead heterochromatin domains are inaccessible to DNA binding factors. Heterochromatin is further divided in constitutive heterochromatin, that include centromeres, pericentromeric regions and telomeres, and facultative heterochromatin. Constitutive heterochromatin often causes the inactivation of genes that are moved into its proximity by chromosomal rearrangement, while facultative heterochromatin is formed by the stable inactivation of genes during development and differentiation (for example the inactivation of one X chromosome) [12]. Of course, histones can be modified simultaneously at different sites giving rise to a cross-talk among different marks and even different histone tails. Thus, a

single modification does not determine the outcome alone, but it is the combination of all marks in a region that specify the outcome. From these observations have been postulated the existence of an "Epigenetic code" beside the "genetic code" to drive gene expression and thus cell functions [13].

The importance of epigenetics in maintaining normal development and biology is reflected by the observation that many diseases develop when the wrong type of epigenetic mark is introduced, or it is added at the wrong time during cell differentiation, or it is added in the wrong site.

Moreover, epigenetics determine the cell identity and cell memory. In fact, all embryonic stem cells begin with identical genotypes and phenotypes but, during commitment, external signals drive them to become phenotipically distinct although they still remain genotipically identical. Of course these changes are not mediated by DNA sequence itself, but they are mediated by epigenetic modifications that define the cell identity [13]. Due to the fact that these epigenetic marks are hereditable, cell identity is perpetuated across generation, and this process is called epigenetic memory. A good example are T cells that form long-lasting immunological memory in response to a transient signal from the environment [14]. Recent findings suggested that epigenetic memory is maintained by chromatin marking at discrete sites by histone variants, like the histone H3.3, that are inherited through cell divisions [15].

Based on this new understanding of epigenetic phenomena as fundamental triggers of (stem) cell differentiation and plasticity, it would be of interest to study the effects of HDAC blockade on stem/progenitor cells.

A few published works have yet analyzed the effect of different HDAC inhibitors (HDACi) on $CD34^+$ cells isolated from UCB and BM. In summary, they found that this treatment maintains a subpopulation of primitive slow-dividing HSCs in culture, and these cells have greater engraftment ability *in vivo* [16] [17].

We focused our attention on a specific HDACi: Valproic acid (VPA). VPA is an established drug for the treatment of epilepsy due to its potent inhibiting activity on the neurotransmitter GABA in human brain. As an HDACi, it is able to affect class I and II HDACs, except for HDAC 6 and 10 [18]. HDAC inhibitors, and also VPA of course, have been deeply studied for their anti-neoplastic potential. In fact it is well known that they can induce differentiation in leukemia cells [19].

Starting from this literature background, we decided to analyze VPA effect on UCB-derived $CD34^+$ cell growth, stemness, phenotype and gene expression. Moreover we wanted to assess whether the treatment could enhance cell regeneration capacity in a mouse model of myocardial ischemia.

## 2. Matherials and Methods

### Cord blood collection, selection and expansion of $CD34^+$ cells

Mononuclear cells (MNCs) were separated by Ficoll density gradient centrifugation and $CD34^+$ cells were selected by immunomagnetic separation using direct MiniMACS (Miltenyi Biotec) CD34 isolation kit according to the manufacturer's instruction. Cells ($1x10^5$/well) were seeded into 96-well plates and expanded in Stem Span (StemCell Technologies Inc.) containing a cytokine mixture with IL-3 (Biodesign) and IL-6 (Endogen; both at 20 ng/ml), Flt3-Ligand (FLT3L; Meridian LifeScience) and Stem cell factor (SCF; Thermo Scientific; both at 100 ng/ml). Cells were incubated at 37° C and 5% $CO_2$; culture medium was changed every second day with fresh complete medium. In VPA and TSA (both from Sigma Aldrich, Italy) cultures the drug was added at each medium change, while in the VPA priming (VPA-p) experimental setting the drug was added only at day 0. To assess HDACi effect on cell expansion we performed a set of experiments comparing VPA (1mM, 2.5mM and 5mM) and TSA (5ng/ml, 12.5ng/ml and 25ng/ml) to control (CTR) condition. After 3 and 5 days of expansion we evaluated cell number, viability and immunophenotype. Using VPA 2.5mM cells were plated for 7, 14 and 21 days to evaluate the drug effect over a longer period.

**Immunophenotype, cell growth and stem cell activity analysis by flow cytometry**

The antibodies used to perform immunophenotype of control and HDACi-treated $CD34^+$ cells were as follows: CD29-PECy5, CD31-FITC, CD34-PE, CD45-PE, CD49b-FITC, CD73-PE, CD90-PE, CD146-PE (all from BD Pharmingen, Italy), CD14-PE, CD133-PE (from Miltenyi Biotec), KDR-PE, CD105-PE (from R&D Systems, USA), CD144-PE (from eBioscience, UK), CD49d-FITC and CD49e-FITC (from Beckman Coulter). For dioctadecyl-tetramethylindo-carbocyanine perchlorate (DiI)-labeling, DiIAcLDL (Biomedical Technologies, 2 μg/ml) uptake assay was performed by incubating cells for 4 hours at 37°C. After staining procedures, cells were immediately analyzed using a FACSCalibur cytometer (Becton Dickinson) and then analyzed. Data plotting was performed using FACSDiva software (Becton Dickinson).

CFSE staining was performed using the CFSE Cell Proliferation kit (Invitrogen) according to manufacturer instructions. Briefly cell ($4x10^6$/ml concentration) were stained with 1μM CFSE for 10 minutes in the dark at RT. The reaction was stopped by adding 1/5 of the volume of FBS and 10 volumes of complete medium. After 5 minutes, cells were washed and seeded. Due to CFSE bright fluorescence intensity immediately after staining day 0 FACS analyses were performed 12 hours after labelling. To detect CD34 expression in CFSE stained cells, co-staining with CD34-PE was performed as previously described. The

analyses were repeated after 5 and 7 days of expansion. Data were analyzed with software CellQuest and ModFit; they were plotted with FACSDiva.

Cell cycle analysis of CTR and VPA-treated cells was performed by using a conventional propidium Iodide stainin procedure. Cells ($2x10^5$) cells were washed and resuspended in 500 µL of PI/RNase Staining Buffer (Becton Dickinson). After 20 minutes samples were transferred into FACS tubes and immediately analyzed using FACScalibur flow cytometer. Data were finally analyzed by ModFit software. To discern the different distribution of $CD34^+$cells between G0 and G1 phases, a co-staining with a Ki67-specific antibody and CD34-PE antibody was performed. Staining for kKi67 was performed as follows: cells were first incubated with with CD34 PE antibody and then fixed in 2% PFA for 10 minutes at room temperature. After washing they were permeabilized with Perm/Wash solution (staining buffer, Becton Dickinson) for $10^{min}$. Cells were resuspended in 100 µl of staining buffer at concentration of $1x10^6$ cells/ml and finally stained with 10 µl of anti-human Ki67 FITC (Beckton Dickinson) for 1h at RT. After a washing in staining buffer and before analysis in a FACScalibur flow cytometer (Beckton Dickinson), cells were suspended in 300 µl PBS.

To assess the activity of *MDR1* gene product, cells ($1x10^6$cells/ml) were incubated with Rhodamine123 (Sigma-Aldrich), at a 0.2 µg/ml for 2 hours at 4°C in the dark in

complete culture medium. After incubation, cells were washed and resuspended in fresh complete medium. To detect specific extrusion of the fluorescent dye, one half of the cells in each replicate was incubated with the MDR1 Pgp channel inhibitor Verapamil (150 μM; Sigma Aldrich Italy). All samples were then incubated at 37°C in the dark for $2^{h}15^{min}$. Before analysis in FACSCalibur flow cytometer, cells incubated with Rho123 ± Verapamil were finally co-stained with isotype-APC or anti CD34-APC (Becton Dickinson) antibodies for 40 minutes in ice to avoid extrusion of the Rho123 dye.

For ALDH activity detection, the Aldefluor staining kit was used (Stem Cell Technologies). Briefly, up to $1x10^{6}$ cells were re-suspended in 1 ml Aldefluor Staining Buffer and incubated with 5 μL activated Aldefluor reagent. Half of the sample volume was transferred in a tube containing 2.5 μL the reaction inhibitor, DEAB. All samples were incubated for 1 hour at 37°C in the dark and then stained with anti CD34-APC or the relative isotype control as described for Rho123.

## qRT-PCR for small Cyclin/CDK inhibitors detection

Total RNA was purified from day 7 CTR and VPA-treated cells using Trizol protocol (Invitrogen, CA). For each sample, 1 μg of total RNA was treated with DNase I and it was reverse transcribed (RT) using SuperScriptIII cDNA synthesis kit (both from Invitrogen, CA).

Real time q-RT-PCR analysis was performed using iQ5 Real Time PCR System (Bio-Rad, Italy). Each primer pair was tested in duplicate using 5ng 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 5ng 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-actin. Data are expressed and plotted as relative expression. The primer sequences are:

**$p14^{ARF}$** Fw GTTTTCGTGGTTCACACT

and Rv CCTCAGTAGCATCAGCAC

**$p16^{INK4}$** Fw ATGGAGCCTTCGGCTGACT

and Rv CGTAACTATTCGGTGCGTTG

**$p21^{Cip1/Waf1}$** Fw CAGCATGACAGATTTCTACCACTCC

and Rv ATGTAGAGCGGGCCTTTGAG

**p27** Fw ACCTGCAACCGACGATTCTTC

and Rv GGGCGTCTGCTCCACAGA

**Transcript and miRNA profiling**

For $RT^2$ Profiler PCR Arrays, total RNA from cells cultured for 7 days (n=4, Control and VPA-treated) was isolated using TRIzol (Invitrogen). RNA was cleaned up using RNeasy Mini Kit (Qiagen). RNA quality was evaluated by capillary electrophoresis (Experion System, Bio-Rad Laboratories). Before reverse transcription (RT), 150 ng RNA/sample was subject to a second genomic DNA elimination step. Quantitative PCR (qPCR) cocktails were loaded using provided 384EZLoad Covers (SABiosciences) and performed using into 7900HT Fast Real-Time PCR System (Applied Biosystems).

MicroRNAs profiling was performed in $CD34^{bright}$ cells. Cell sorting was performed by incubation of control and VPA-treated cells with isotype-APC and CD34-APC antibody followed by sorting into a FACSAria flow cytometer (Beckton Dickinson). A purity checking was performed after cell sorting to control the purity of isolated cells before mRNA extraction. Total RNA from high throughput-sorted cells (n=4, n=4, Control and VPA-treated cells) using TRIzol protocol (Invitrogen). RNA quality was evaluated by capillary electrophoresis using Experion System (Bio-Rad Laboratories). TaqMan Human MicroRNA A and B Arrays, version 2.0 (Applied Biosystems, USA), were used for the contemporary detection of 733 human miRNAs. According to the manufacturer's protocol, (RT) reaction were performed using 98.4 ng total RNA from each sample, while in pre-amplification steps, 2.5 µl of each RT product were used.

All steps were performed using a 7900HT Fast Real-Time PCR System (Applied Biosystems).

**Analysis of stem cells-related transcripts**

$RT^2$ Profiler PCR Arrays were analyzed using the software ABI Prism SDS version 2.3. Optimal baseline was determined automatically by the software algorithm for each amplification curve and threshold for Ct (cycle threshold) was set manually at the same value in all plates, in order to obtain closely similar Ct for RT and PCR positive controls across the different plates. This allowed comparison of multiple arrays. All Ct values reported as greater than 35 or as not detected were changed to 35 and considered a negative call. Three sets of replicate control wells (genomic DNA, RT, and positive PCR controls) were used to assess each groups' level of genomic DNA contamination, reverse transcription efficiency, and PCR reproducibility, as well as to test for inter-well and intra-plate consistency.

Raw expression intensities of target mRNAs were normalized for differences in the amount of total RNA added to each reaction using a set of five reference genes (β-actin, ACTB; β-2-microglobulin, B2M; glyceraldehyde-3-phosphate dehydrogenase, GAPDH; hypoxanthine guanine phosphoribosyl transferase 1, HPRT1; and ribosomal protein L13a, RPL13A). Analysis of gene expression stability and selection of the best

reference genes were performed using the NormFinder version 0.953 Excel Add-In[20].

Relative quantitation of gene expression was performed using the comparative Ct method (ΔCt). ΔCt value was defined as the difference between the Ct of a gene (either target or housekeeping) in the calibrator sample (the sample with the highest expression, i.e. lowest Ct value) and the Ct of the same gene in experimental sample. The Ct values were transformed to raw, not-normalized quantities using the formula $\eta^{\Delta Ct}$, where amplification efficiency (η) was set arbitrarily to 2 (100%). Gene expression normalization factors were obtained calculating the geometric mean of the raw quantities of the three most stable reference genes (ACTB, GAPDH, and RPL13A). The normalized expression of each target mRNA was finally calculated by dividing the raw gene-of-interest quantity by the appropriate normalization factor. Finally, the mean fold change of VPA-treated $CD34^+$ *vs.* control cells was calculated, by dividing the normalized mean expression value in $CD34^+$ cells by the mean expression value in control cells.

The MultiExperiment Viewer (MeV) software version 4.6[21] was used for high-level analysis. Genes were deemed as non informative and filtered out from all plates when the percent of negative calls exceeded 50%. Unsupervised hierarchical cluster analysis was performed to assess the similarity and differences in gene expression profiles among the samples and whether expression profiles discriminates treatment groups.

The similarity of gene expression among arrays and probes was assessed by calculating Pearson's correlation coefficient using an algorithm first described by Eisen *et al* [22]. Normalized gene expression values were log transformed (log base 2), mean centered by gene mean and clustered by correlation (centered) average linkage, using leaf order optimization. Differentially expressed genes among treatment groups were identified using a multivariate paired *t*-test, using Welch approximation (assuming unequal group variances), computing p-values based on 100 permutations with a confidence level of 80% (1-α), and a limiting the false discovery rate (FDR) proportion to <0.1. Differences in gene expression were considered statistically significant if their p-value was less than 0.05. Differentially expressed genes were then clustered using the same distance metric and linkage method, in order to visualize and confirm the existence of the gene signature.

**Gene enrichment analysis**

Gene functional classification was performed using the web-based application Database for Annotation, Visualization and Integrated Discovery (DAVID 6.7, http://david.abcc.ncifcrf.gov)[23]. DAVID functional annotation clustering and chart tools were used to query Biocarta and KEGG pathway databases, in order to identify significantly non-redundant over-represented biological themes in the differentially expressed gene set. Gene-enrichment analysis was

performed using the EASE algorithm, which compares the representation of functional classes for genes within differentially regulated subset to the entirety represented on the array. EASE performs a conservative adjustment of the Fisher's exact test to calculate the probability that the composition of the differentially expressed set occurs by chance. A pathway was considered differentially regulated if the significance level was < 0.05 .

**Analysis of miRNA profiles**

Low level analysis and quality control of TaqMan Human MicroRNA A and B Arrays were performed with the software ABI Prism SDS version 2.3. All Ct values reported as greater than 35 or as not detected were changed to 35 and considered a negative call.

Raw expression intensities of target miRNAs were normalized for differences in the amount of total RNA added to each reaction using the mean expression value of all expressed miRNAs in a given sample, following the method recently described by Mestdagh *et al* [24]. Relative quantitation of miRNA expression was performed using the comparative Ct method (ΔCt) described above. The mean fold change of VPA-treated $CD34^+$ *vs.* control cells was calculated as well.

The BRB-ArrayTools software package version 3.81, developed by Dr. Richard Simon and BRB-ArrayTools Development Team, was used for high-level statistical analysis and the MeV 4.6

software for clustering analysis. Normalized gene expression values were $\log_2$ transformed. Genes were deemed as non informative and filtered out from all plates when the percentile of negative calls exceeded 50 and when the percentile of the log-ratio variation in less than 10. Unsupervised hierarchical cluster analysis was performed to assess whether expression profiles discriminates treatment groups. The distance metric used for calculating the similarity of gene expression among arrays and probes was the Spearman rank correlation. $\text{Log}_2$ transformed gene expression values were mean centered by gene mean; gene and sample vectors were normalized and clustered by average linkage, using leaf order optimization. Differentially expressed genes among treatment groups were identified using a random-variance model for univariate significance paired *t*-test. The random-variance *t*-test is an improvement over the standard separate *t*-test as it permits sharing information among genes about within-class variation without assuming that all genes have the same variance[25]. A univariate permutation test was also performed to confirm the statistical significance of the findings, based on all available permutations. The maximum proportion of FDR was <0.2. Differences in gene expression were considered statistically significant if their p-value was less than 0.05. Differentially expressed genes were then clustered using Pearson's correlation (centered) and average linkage method.

## Analysis of cell secretome

Unsupervised hierarchical clustering and statistical analysis of the two bead-based multiplex immunoassay data were performed using the MeV 4.6 software. $Log_2$ transformed protein concentrations were centered by mean or median values, and clustered using Pearson's correlation (centered) and average linkage method, with leaf order optimization. Differentially expressed proteins among treatment groups were identified using a paired *t*-test, controlling for the FDR proportion (<0.1). Differences in gene expression were considered statistically significant if their p-value was less than 0.05.

## Animal model

130 SCID Beige male mice with an initial body weight of 20-23 g, were anesthetized with Tribromoethanol (Avertin®, 250 mg/Kg). After thoracotomy, the left coronary artery was ligated (CAL). 15 minutes after ligation, PBS, CTR or VPA cells were injected in the left ventricular (LV) wall in the infarct border zone ($1.5X10^5$ cells/2.5 µl). In sham-operated animals the suture was loosely placed around the left coronary artery for 15 minutes. All mice received buprenorphine (0.1mg/kg s.c. q12h for 1 day) as post surgical analgesia and were followed for 6 weeks.

**Echocardiography**

Four weeks after CAL, transthoracic ultra-imaging echocardiography (VisualSonics, Vevo 770) was performed on anesthetized mice (isoflurane: 0.5-1.5% in $O_2$). Animals were positioned on a rail system for maintaining body temperature and scan head position under ECG and respiration monitoring.

A parasternal long-axis B-mode image was acquired with appropriate positioning of the probe to obtain the maximum left ventricular (LV) length, necessary for LV volumes measurements and calculations. Short-axis B-mode images of the LV were recorded from the base to the apex by translating the mouse platform by 1 mm increments along the LV long-axis. M-Mode tracings were recorded at the mid papillary level with two-dimensional image guidance. All images were stored in DICOM format to be processed off-line. LV measurements and calculations were performed as recommended by the American and European Societies of Ecocardiography. LV internal diastolic and systolic diameters (LVIDd and LVIDs), LV wall thicknesses (septal, antero-septal and posterior), and fractional shortening (FS) were evaluated at the mid papillary level from the M-Mode modality in both PLAX and PSAX views; LV volumes (LVEDV and LVESV) were measured and calculated from the PLAX view with the modified Simpson method (ComPACS software, Medimatic S.R.L), as usually performed in echocardiography in small rodents[26]. Serial consecutive slices (1mm each) were obtained from the SAX view and volumes

were calculated from manually drawn diastolic and systolic areas using the disk summation method[27].

**Perfusion fixation and tissue sampling**

Six weeks after surgery, animals were anesthetized with an i.v. Avertin® injection and sacrificed by iv injection of 0.5 ml of 2.5N KCl, to arrest the heart in diastole. Retrograde perfusion fixation of the heart via the abdominal aorta was performed using 2% buffered paraformaldehyde solution. The hearts were then excised, quickly rinsed in saline, blotted; ventricles were weighted, and put in 4% buffered formalin for 24 h before processing for inclusion in paraffin (Sakura, Tissue-Tek VIP ).

**Morphometric analysis**

Infarct size and ventricular dimensions were measured immediately below the coronary ligation on a 5 μm section stained with hematoxylin-eosin, using image analysis software (*Image Tool software,* freely available online). Infarct size was calculated by manually tracing perimeters and expressed as percentage of the left ventricle as follows:

*[(external infarct perimeter+internal infarct perimeter) x 100]/(total external LV perimeter+total internal LV perimeter)*

External and internal perimeters, and LV wall and septum thickness were traced manually. Perimeters were assumed to be the circumference of a circle and used to calculate

transverse chamber diameter, LV chamber area and mean ventricular wall thickness.

**Capillary density**

Staining for capillaries was carried out with Rhodamine-labelled Griffonia simplicifolia Lectin 1 (Vector Laboratories, Peterborough, UK). Nuclei were counterstained with bisbenzimide. Capillaries were counted in the zone bordering the infarction at a magnification of 600x (Olympus IX51), and expressed as capillary number /$mm^2$.

**Engraftment analysis**

DNA from Saline, CTR and VPA hearts was extracted using DNEasy blood and tissues kit (Qiagen); 465 ng of DNA/reaction were used. The DNA amplification was performed by RT PCR using a TaqMan Genotyping protocol (Applied Biosystem). Specific primers and VIC and FAM fluorescent TaqMan probes were purchased. These primers were designed on the human genomic sequence (Xq13.1b) containing the SNP C/T (rs6625561 Reference: NCBI SNP). This sequence is highly conserved in humans and does not have homologues within mouse genome. The amplification protocol consisted in a hot start step for 10 minutes at 95° C, followed by 50 cycles including: 1) denaturation for $15^{sec}$ at 92°C and 2) annealing/amplification for $1^{min}$ at 60°C. Calibration curve was performed by amplifying under these conditions a 10-folds

dilution series starting from 6 ng and ending to 0.6 pg of human DNA extracted from CD34 cells, mixed with a 465 ng of HL1 mouse cardiomyocytes DNA.

# 3. Results

## HDAC inhibition by TSA and VPA causes growth inhibition and phenotype change in CD34$^+$ cells.

In our previous investigations we established a serum-free culture method preserving ischemic tissue repair ability of human CB-derived CD34$^+$/CD133$^+$ cells [28]. In the present study, this method was used to culture CD34$^+$ cells in the presence of increasing amounts of Trichostatin A (TSA) and Valproic Acid (VPA), two wide range HDAC inhibitors. Morphology of HDACi-treated cells was dose-dependently affected by treatment with both drugs. This was evident by the different appearance of Control vs. HDACi-treated cells (Figure S1), as well as by the dose-dependent increase in side scatter value in flow cytometry (Figure S2).

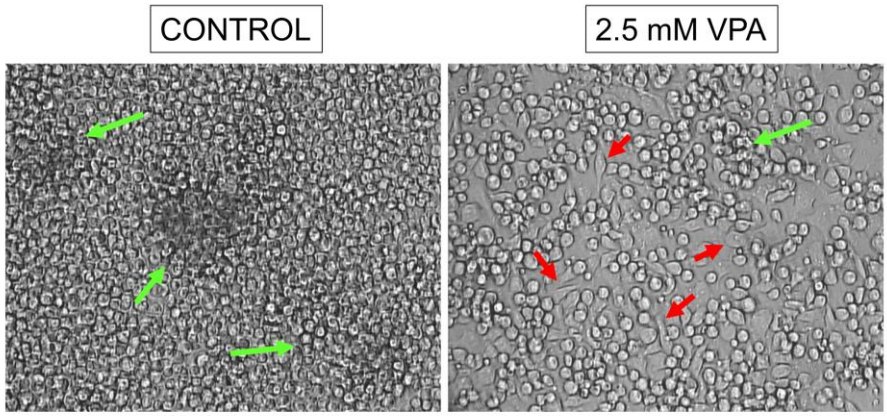



Figure S1

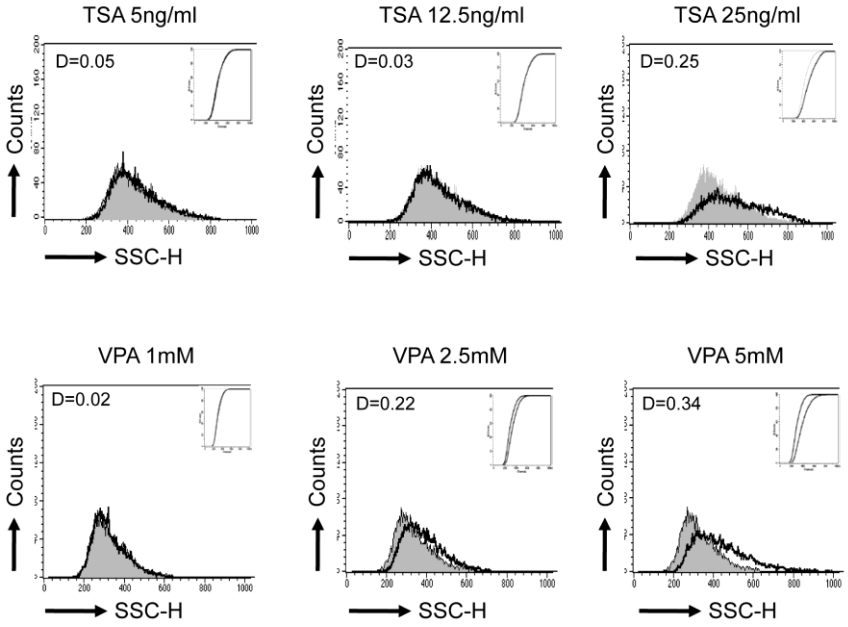



Figure S2

Previous reports showed that the percentage of primitive hematopoietic stem cells (HSCs) is increased by HDAC inhibition under culture conditions similar to those employed in the present study [29, 30]. To identify whether specific subpopulations in the $CD34^{+}$ cells were affected by treatment with increasing VPA and TSA concentrations, a flow cytometry analysis was performed. The percentage of $CD34^{+}$ cells was determined by establishing three sub-regions: R1, corresponding to $CD34^{neg}$ cells, R2, corresponding to $CD34^{dim}$ and R3, corresponding to $CD34^{bright}$ cells; in parallel, cellular growth was assessed by cell counting at discrete time points. Figure 1A and B show that increasing doses of TSA and VPA

decreased cellular growth in the presence of mitogenic cytokines with a dose-dependent shift toward a homogeneous $CD34^{bright}$ cell population. Dose-response treatments were of help to define the 2.5 mM VPA as the best HDACi dose to be used in all the following experiments. To assess the time extension of HDAC inhibition on stem cell marker expression, a flow cytometry analysis of CD34 and CD133 was performed at 5, 14 and 21 days with appropriate quantification of the cells in the three analysis gates (Figure 1C and D, Fig S3). This experiment revealed a striking modification of the two stem cells markers in the three gating regions and identified 5 and (although at lesser extent) 14 days as the time points of maximum divergence of control *vs.* HDACi-treated cells phenotype.

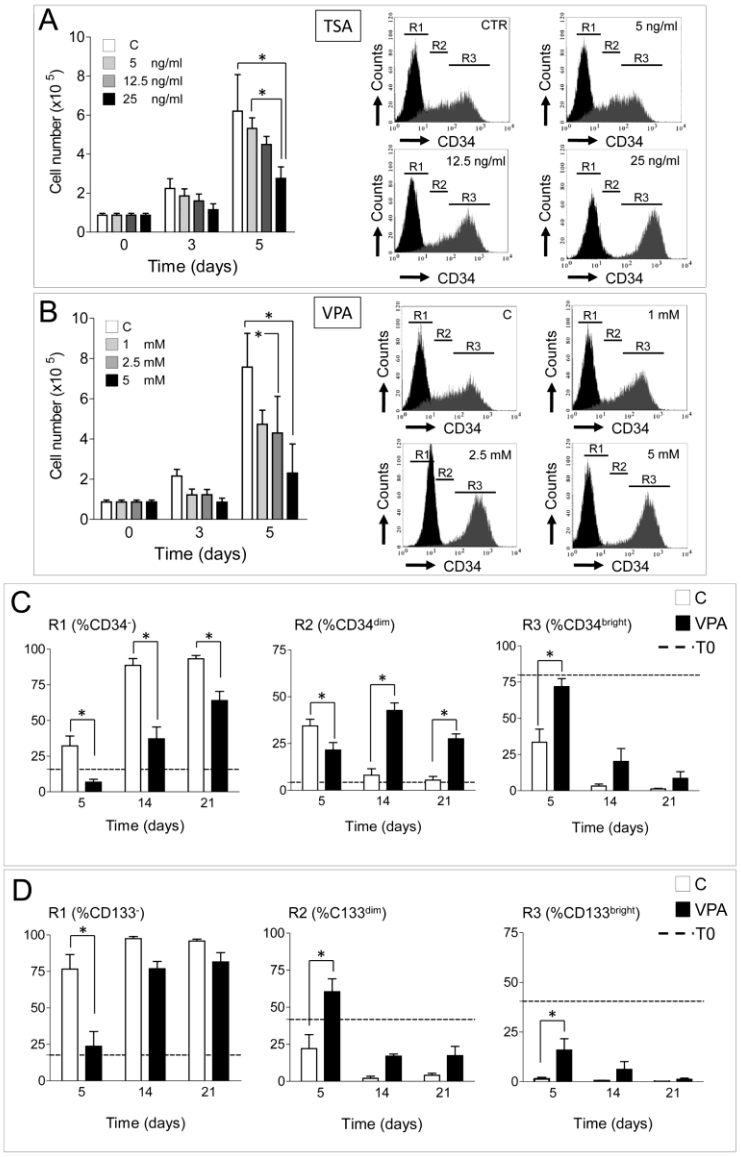

A
TSA
C
5 ng/ml
12.5 ng/ml
25 ng/ml
Cell number (x10 5)
Time (days)
CTR
5 ng/ml
12.5 ng/ml
25 ng/ml
R1
R2
R3
Counts
CD34
B
VPA
C
1 mM
2.5 mM
5 mM
Cell number (x10 5)
Time (days)
1 mM
2.5 mM
5 mM
C
R1 (%CD34-)
R2 (%CD34dim)
R3 (%CD34bright)
VPA
T0
Time (days)
D
R1 (%CD133-)
R2 (%C133dim)
R3 (%CD133bright)
VPA
T0
Time (days)

Figure 1

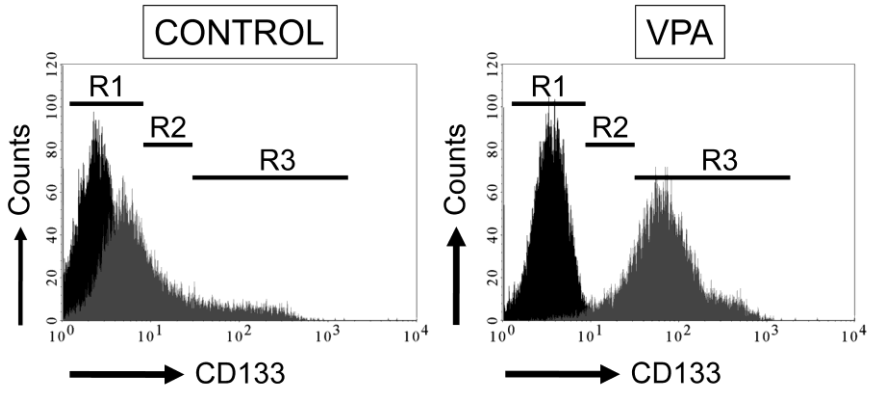



Figure S3

**HDAC blockade reduces immature cells proliferation.**

Previous investigations have shown that increase in self renewal of human HSCs exposed to HDACi is characterized by a delay in stem cells generation time [31] and retardation in cell cycle progression [32]; however, the direct effect of HDAC blockade on the dynamics of immature stem cells is still relatively unaddressed. To this clarify this point, we exploited the staining method with Carboxyfluorescein Succinimidyl ester (CFSE) dye to calculate proliferation index and cellular generations number at 5 and 7 days in the presence and absence of VPA [33]. Figure 2A and B, respectively, show that proliferation index was significantly reduced at 7 days in VPA *vs.* CTR cells and that this coincided with an increase in $CD34^{bright}/CFSE^{bright}$ cells fraction after VPA treatment. Furthermore, mathematical deconvolution of CFSE fluorescence signals showed, on average, a 1-2

generations delay in the presence of VPA (Figure S4). In keeping with these results, cell cycle analysis and double staining with antibodies recognizing CD34 and Ki67 showed that VPA treatment caused a significant elongation of G1 phase and a slight, but significant, increase in G0 cells ($CD34^{+}/Ki67^{-}$; Figure 2 C and D). Finally, it was investigated whether expression of CDK inhibitors ($p14^{ARF}$, $p16^{INK4}$, $p21^{Cip1/Waf-1}$ and p27) was modified in the presence of VPA; except for p27, all these genes were increased (Figure 2 E).

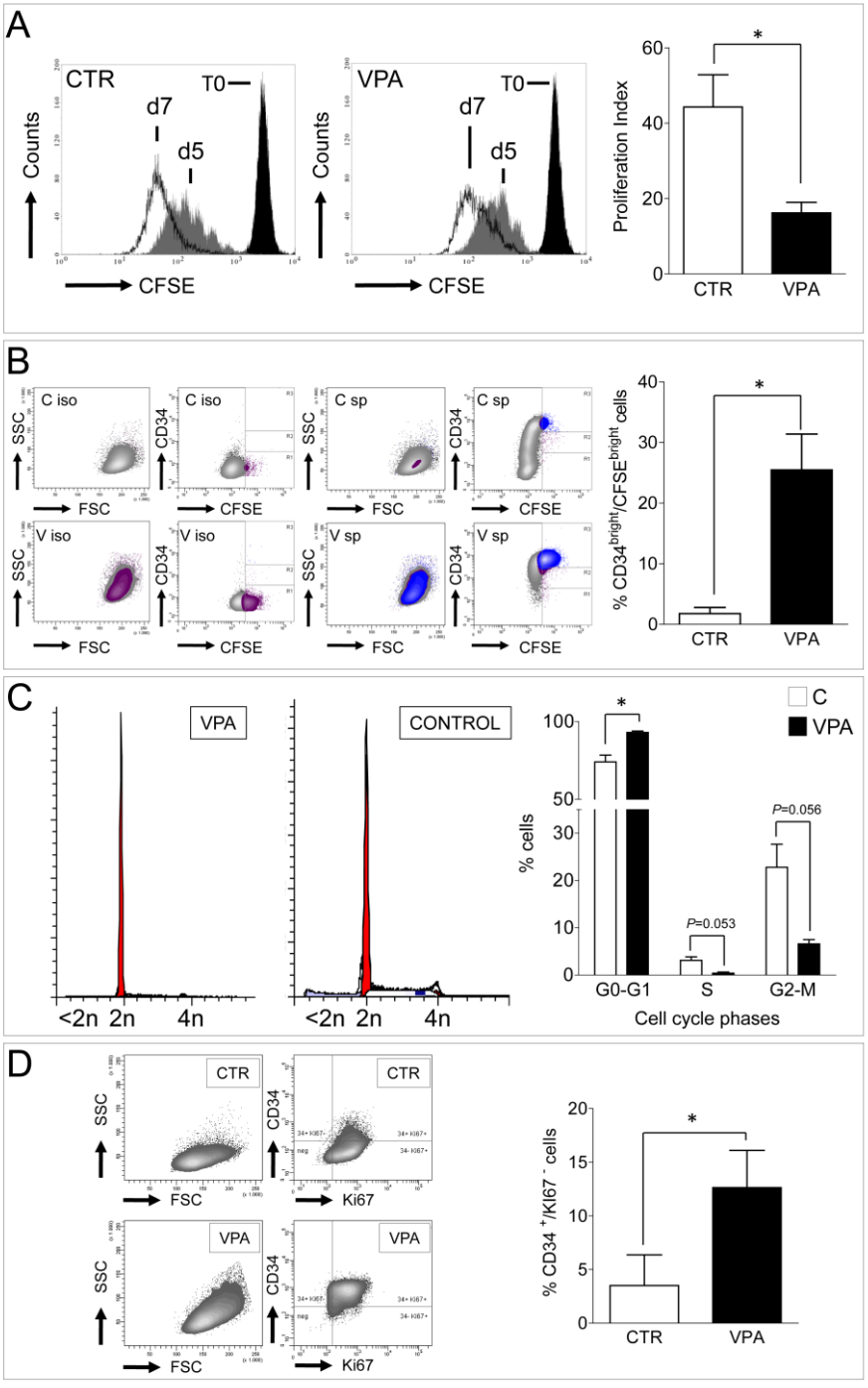

A
CTR
T0
d7
d5
Counts
CFSE
VPA
Proliferation Index
60
40
20
0
*
CTR
VPA
B
C iso
C sp
V iso
V sp
SSC
CD34
FSC
CFSE
% CD34bright/CFSEbright cells
C
VPA
CONTROL
<2n
2n
4n
% cells
100
50
30
20
10
P=0.056
P=0.053
G0-G1
S
G2-M
Cell cycle phases
D
Ki67
% CD34 +/KI67 - cells

Figure 2

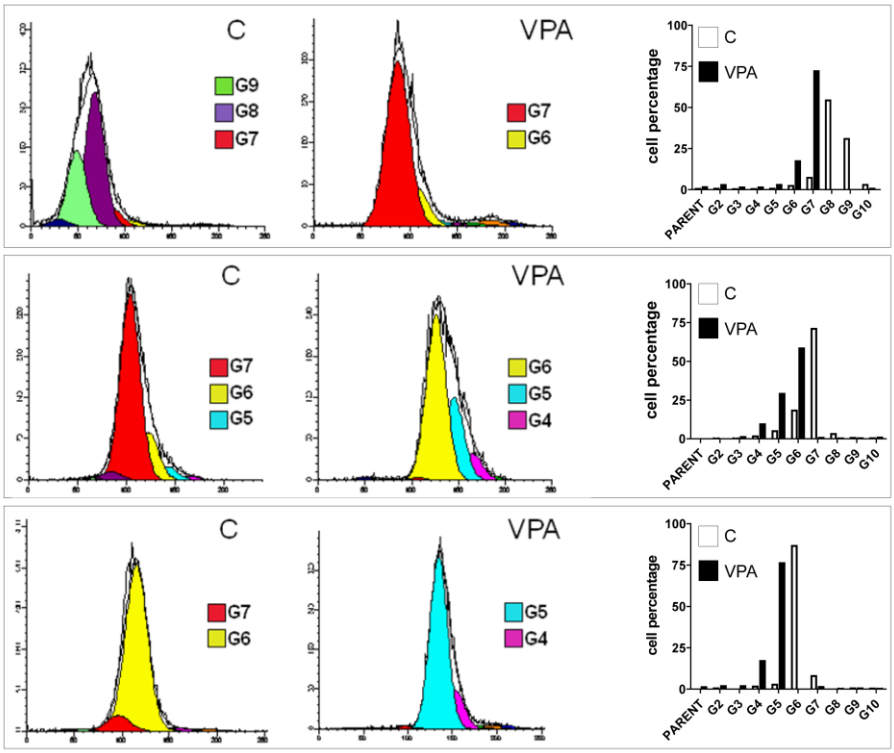

C
G9
G8
G7
VPA
G7
G6
C
VPA
cell percentage
100
75
50
25
0
PARENT G2 G3 G4 G5 G6 G7 G8 G9 G10
C
G7
G6
G5
VPA
G6
G5
G4
C
VPA
cell percentage
100
75
50
25
0
PARENT G2 G3 G4 G5 G6 G7 G8 G9 G10
C
G7
G6
VPA
G5
G4
C
VPA
cell percentage
100
75
50
25
0
PARENT G2 G3 G4 G5 G6 G7 G8 G9 G10

Figure S4

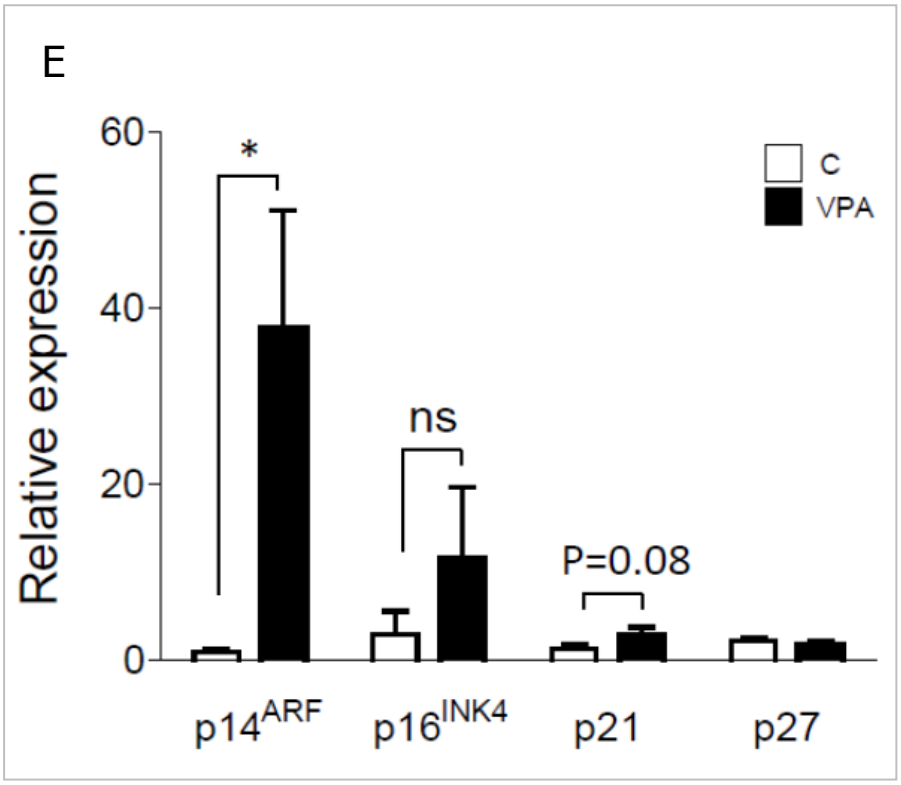



Figure 2

**Histone hyperacetylation, stemness and epigenetic "memory" in CD34$^{+}$ cells.**

Experiments were performed to determine whether shifting toward a homogeneous CD34$^{bright}$ phenotype is consequent to histone hyperacetylation resulting from HDAC inhibition. To determine the specific effects of HDAC inhibition on growth and CD34 expression, Valpromide (VPM), a VPA structural analogue lacking HDAC inhibition activity [34], was used at the same concentration in parallel to VPA. As shown in Figure 3A, VPA substitution with VPM inhibited at intermediate levels cellular

growth, while it produced a totally overlapping CD34 expression profile in R1, R2 and R3 regions. Further, it was investigated whether HDACi preconditioned cells maintain a memory of their phenotypic and functional changes after VPA withdrawal. To this aim, a protocol was devised to expose $CD34^+$ cells to a single time-limited HDACi priming with VPA (see methods); this treatment was defined VPA priming (VPA-p). Figure 3B shows that the growth of cells cultured under this condition was significanty higher compared to cells cultured in constant presence of VPA; by contrast, CD34 expression in VPA-p cells was not different compared to cells continuously exposed to VPA. Western blotting *pan*-acetylated histone H4 (H4Ac) and lysine 9 acetylated histone H3 (H3K9Ac), confirmed that VPA and VPA-p treatments enhanced histone tails acetylation, while VPM had only a minimal effect (Figure 3C).

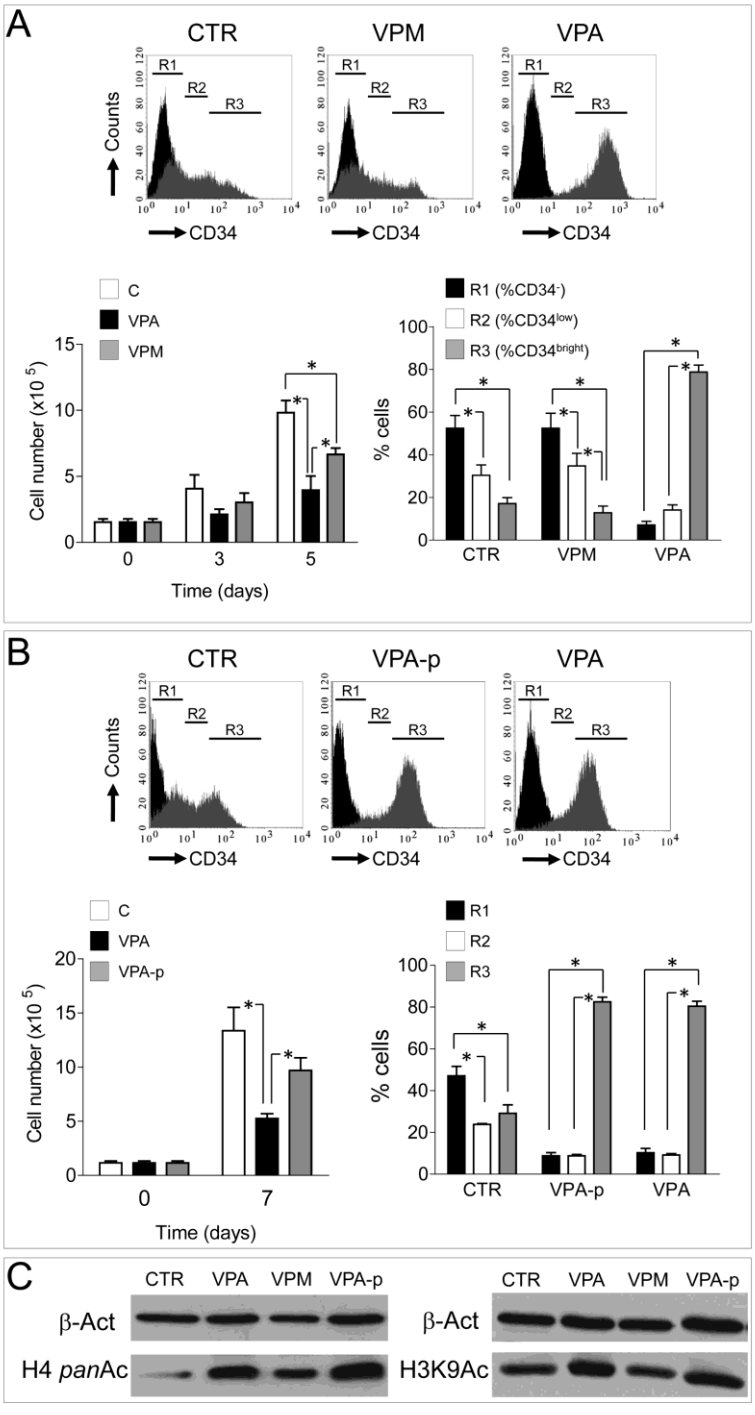
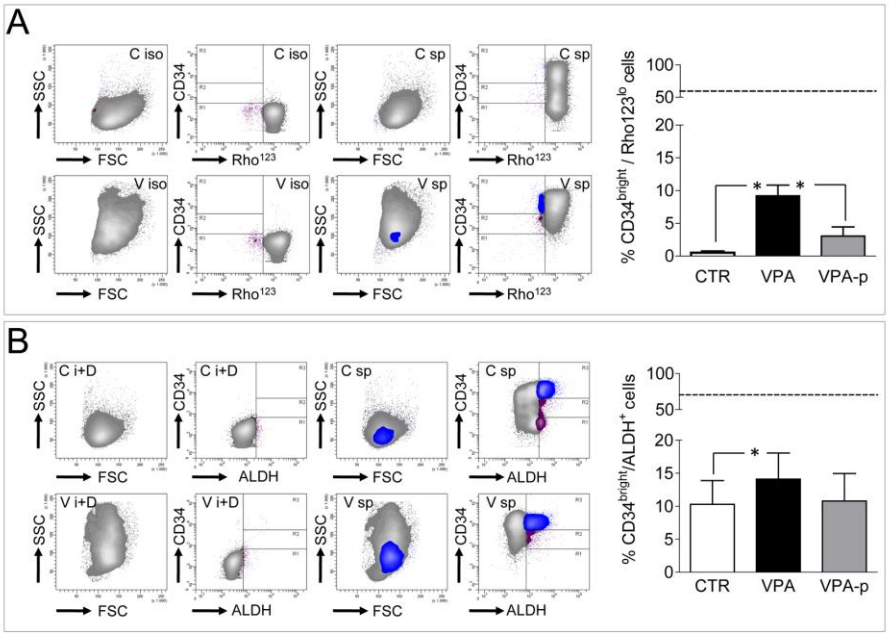

A
CTR
VPM
VPA
R1
R2
R3
Counts
CD34
C
VPA
VPM
Cell number (x10 5)
Time (days)
R1 (%CD34-)
R2 (%CD34low)
R3 (%CD34bright)
% cells
B
VPA-p
VPA
C
VPA
VPA-p
Cell number (x10 5)
Time (days)
R1
R2
R3
% cells
C
CTR
VPA
VPM
VPA-p
β-Act
H4 panAc
H3K9Ac

Figure 3

**Assessment of stem cell function in HDACi-treated CD34$^{+}$ cells.**

To investigate whether HDACi-treated cells maintained stem cell properties, experiments were performed to measure the ability to extrude drugs, a typical primitive stem cell feature relative to the *MDR-1* gene product expression[35], and verify expression of Aldehyde Dehydrogenase (ALDH) enzyme expression, typically detected in immature EPCs [36]. Rhodamine123 (Rho123) dye extrusion and ALDH enzyme activity were assessed by flow cytometry (Figures 4 A and B). These tests showed that VPA treatment maintained higher the percentage of CD34$^{bright}$/Rho123$^{lo}$ and CD34$^{bright}$/ALDH$^{+}$ cells compared to controls. Remarkably VPA-p did not produce similar results.



**Phenotype and transcriptome analysis of HDACi treated CD34$^+$ cells.**

Figure S7 shows the experimental design adopted for phenotypic and molecular characterization of HDACi-treated CD34$^+$ cells.

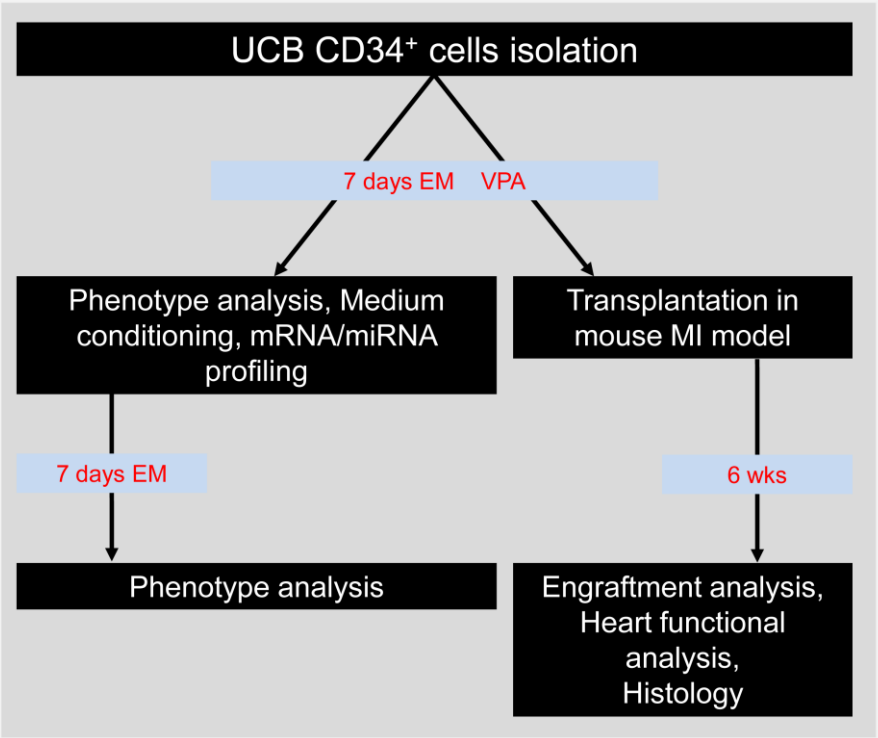



Figure S7

A panel of antibodies was used to detect expression of stem cell (CD34, CD133, KDR), hematopoietic (CD14, CD45), endothelial (CD31, CD105, CD144, CD146, LDL uptake), hematopoietic/mesenchymal (CD90, CD73) and integrin ($\alpha_2\beta_1$,

$\alpha_4\beta_1$, $\alpha_5\beta_1$) markers by multiparametric flow cytometry in control, VPA and VPA-p cells at 7 and 14 days. Results (Figure 4C and D) indicate that VPA treatment was the most effective preconditioning protocol, as indicated by **1)** CD133 and KDR stem cell marker upregulation compared to control cells, **2)** *de novo* expression of CD90 and CD146 at both time points, **3)** increased CD31 expression at day 14 and **4)** a higher LDL uptake ability at both time points. According to previous results showing inhibition of myeloid differentiation by HDACi [37], expression of CD14 was significantly inhibited. Notably, VPA-p treatment produced comparable results to VPA condition only at day 7, while at day 14 *de novo* CD90 and CD146 expression or sustained CD34 expression were not observed, suggesting that VPA-p is not sufficient to achieve a stable memory.

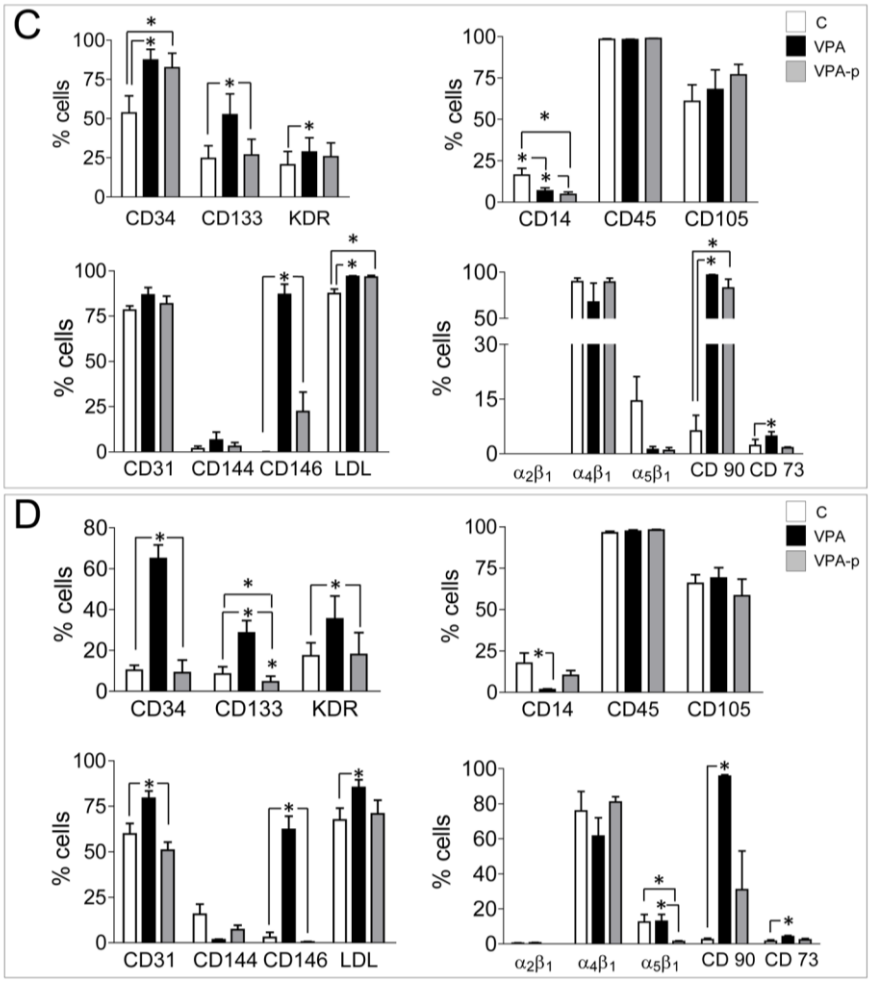



Figure 4

To investigate possible gene expression reprogramming in HDACi conditioned cells, a survey of stem cells-specific transcripts expression was performed in control and VPA-treated $CD34^+$ cells using qRT-PCR low density arrays (Figure 5A). Unsupervised clustering analysis recognized coherently upregulated or downregulated stem cell-associated genes,

allowing to discriminate between control *vs.* VPA treatments. Statistical analysis revealed a total of 34 transcripts significantly modified in VPA *vs.* control cells (Table in figure 5A). Of these, 6 were down-modulated while 17 were up-regulated. Finally, 10 genes that were not detectable or were at detection limit by qRT-PCR, were expressed *de novo,* although at different levels, in VPA-treated *vs.* control cells.

**Modification of microRNAs (miRNAs) signature in HDACi-treated CD34$^{+}$ cells.**

In embryonic stem cells, microRNAs (miRNAs) are important components of the epigenetically-controlled transcriptional circuitry which regulates pluripotency [38]. In addition, treatment with HDACi causes rapid shift of miRNA signature in cancer cells [39]. Since the major differences in growth characteristics and stemness were correlated with high CD34 expression (cells enclosed in the CD34$^{bright}$ gate in Figures 1, 2 and 4), miRNA profiling was performed in high throughput FACS-sorted CD34$^{bright}$ cells populations after culturing CD34$^{+}$ cells for 7 days in the presence or the absence of VPA (Figure S8).

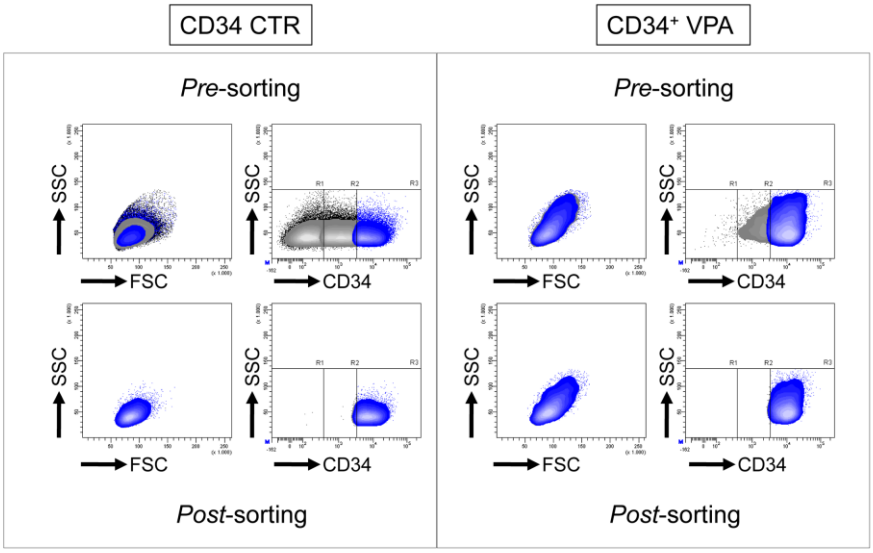
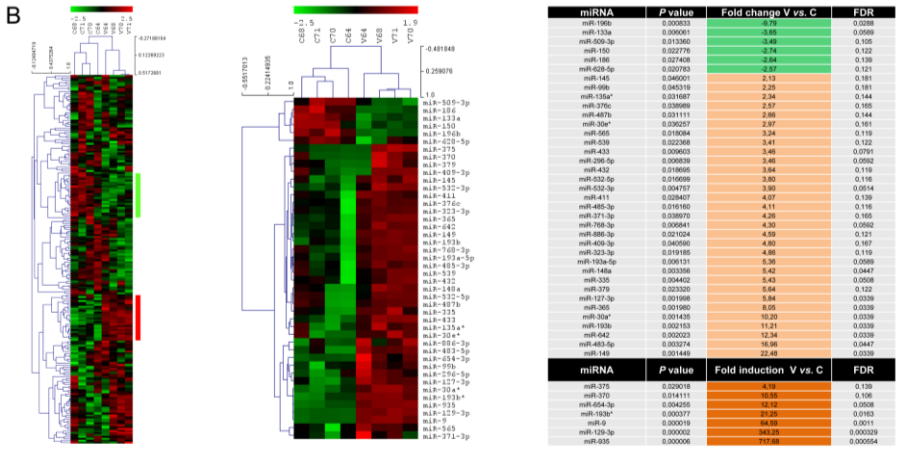



Figure S8

Remarkably, unsupervised clustering clearly distinguished miRNA profiles of CD34$^{bright}$ cells in VPA *vs.* CTR culture conditions (Figure 5B). Results from statistical analysis identified a total of 43 miRNAs (out of 733 tested) whose expression was significantly modified. Of these 6, were downregulated, 30 were upregulated, while 7 were *de novo* expressed.




| miRNA | *P* value | Fold change V vs. C | FDR |
|---|---|---|---|
| miR-190b | 0,000833 | -9,79 | 0,0288 |
| miR-133a | 0,000061 | -3,65 | 0,0089 |
| miR-509-3p | 0,013360 | -3,49 | 0,105 |
| miR-150 | 0,022776 | -2,74 | 0,122 |
| miR-186 | 0,027408 | -2,64 | 0,139 |
| miR-628-5p | 0,020783 | -2,57 | 0,121 |
| miR-145 | 0,046001 | 2,13 | 0,181 |
| miR-99b | 0,045319 | 2,25 | 0,181 |
| miR-135a* | 0,031687 | 2,34 | 0,144 |
| miR-376c | 0,038989 | 2,57 | 0,165 |
| miR-487b | 0,031111 | 2,66 | 0,144 |
| miR-30e* | 0,036257 | 2,97 | 0,161 |
| miR-565 | 0,018084 | 3,24 | 0,119 |
| miR-539 | 0,022368 | 3,41 | 0,122 |
| miR-433 | 0,009603 | 3,46 | 0,0791 |
| miR-296-5p | 0,006839 | 3,46 | 0,0592 |
| miR-432 | 0,018695 | 3,64 | 0,119 |
| miR-532-5p | 0,016699 | 3,80 | 0,116 |
| miR-532-3p | 0,004757 | 3,90 | 0,0614 |
| miR-411 | 0,028407 | 4,07 | 0,139 |
| miR-485-3p | 0,016160 | 4,11 | 0,116 |
| miR-371-3p | 0,038970 | 4,26 | 0,165 |
| miR-768-3p | 0,006841 | 4,30 | 0,0592 |
| miR-886-3p | 0,021024 | 4,59 | 0,121 |
| miR-409-3p | 0,040590 | 4,80 | 0,167 |
| miR-323-3p | 0,019185 | 4,86 | 0,119 |
| miR-193a-5p | 0,006131 | 5,36 | 0,0589 |
| miR-148a | 0,003356 | 5,42 | 0,0447 |
| miR-335 | 0,004402 | 5,43 | 0,0508 |
| miR-379 | 0,023320 | 5,64 | 0,122 |
| miR-127-3p | 0,001998 | 5,84 | 0,0339 |
| miR-365 | 0,001980 | 8,05 | 0,0339 |
| miR-30a* | 0,001435 | 10,20 | 0,0339 |
| miR-193b* | 0,002153 | 11,21 | 0,0339 |
| miR-642 | 0,002023 | 12,34 | 0,0339 |
| miR-483-5p | 0,003274 | 16,96 | 0,0447 |
| miR-149 | 0,001449 | 22,48 | 0,0339 |

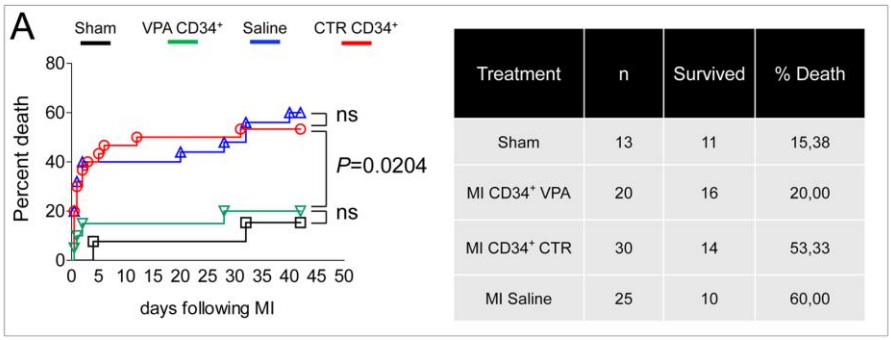
| miRNA | *P* value | Fold induction V vs. C | FDR |
|---|---|---|---|
| miR-375 | 0,029018 | 4,19 | 0,139 |
| miR-370 | 0,014111 | 10,55 | 0,106 |
| miR-654-3p | 0,004255 | 12,12 | 0,0508 |
| miR-193b* | 0,000377 | 21,25 | 0,0163 |
| miR-9 | 0,000019 | 64,59 | 0,0011 |
| miR-129-3p | 0,000002 | 343,25 | 0,000329 |
| miR-935 | 0,000006 | 717,68 | 0,000554 |

Figure 5

**Heart repair potency of control and HDACi-treated CD34$^+$ cells.**

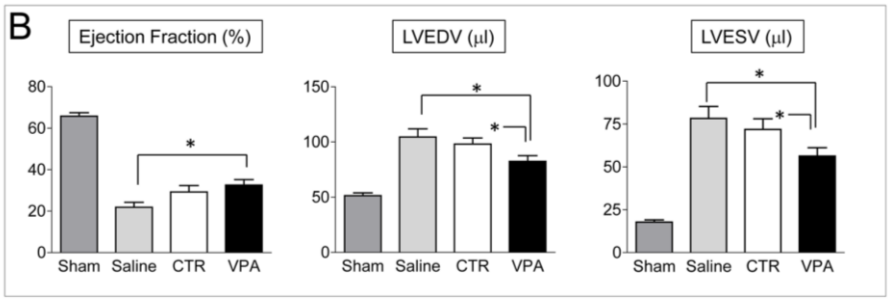
We and others have shown that *ex vivo* cultured human cord blood-derived CD34$^+$ or CD133$^+$ progenitors differentiate into endothelial-like cells *in vitro* or induce *neo*-vascularization in ischemic tissues (see e.g. [28, 40-42]. On the other hand, since stem cells markers expression and *in vivo* engraftment ability decline as HSCs are cultured in the presence of mitogenic cytokines [31], we sought to determine whether HDACi preconditioning modifies CD34$^+$ cells ability to protect or to induce repair of the ischemic heart compared to control cells. As illustrated in Figure S7, control and HDACi-preconditioned cells were injected in the ischemic left ventricle of SCID$^{beige}$ mice 15 min after coronary artery ligation (CAL). Six weeks after CAL and cell injection, mice that survived and reached the end of the follow up period were analyzed for heart function

and morphometry. Mortality data during the follow up period (Figure 6A) showed a dramatic difference between animals treated with $CD34^+$ cells preconditioned with VPA compared to those receiving control cells.




| Treatment | n | Survived | % Death |
|---|---|---|---|
| Sham | 13 | 11 | 15,38 |
| MI $CD34^+$ VPA | 20 | 16 | 20,00 |
| MI $CD34^+$ CTR | 30 | 14 | 53,33 |
| MI Saline | 25 | 10 | 60,00 |

Echocardiography-based heart functional assessment revealed that in animal injected with HDACi-treated cells Left Ventricular Ejection Fraction (LVEF) was significantly increased (33% improvement) compared to saline-injected mice; in addition VPA-treated cells caused a reduction in Left Ventricular End Diastolic/Systolic volumes (LVEDV, LVESV) compared to CTR cells-injected and saline-injected animals (Figure 6B and S9).



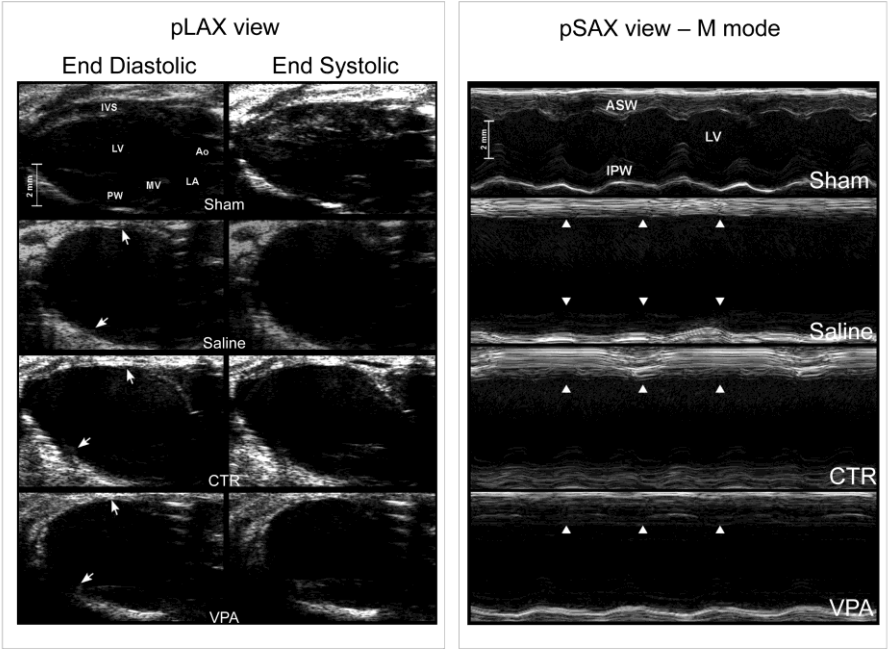
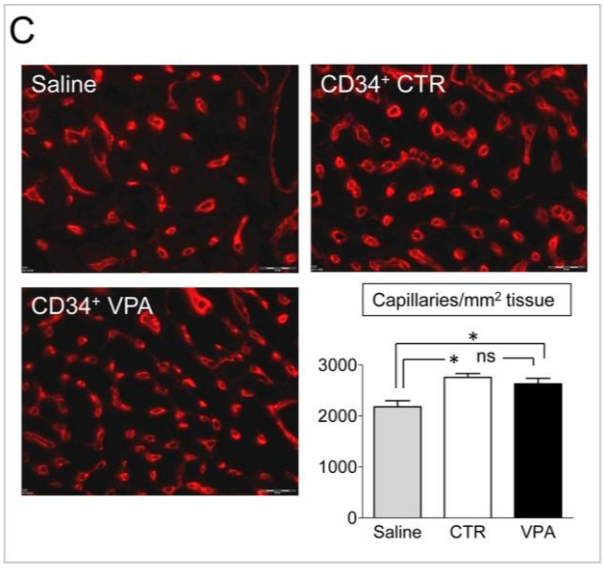



Figure S9

Consistent with a decrease in stem cell activity as a result of *ex vivo* expansion, control cells injection led to a moderate heart functional improvement compared to saline-injected animals; although this improvement corresponded to an overall net 25% LVEF increase over saline injected animals, it did not reach statistical significance (Figure 6B above). To further assess therapeutic efficacy of control and HDACi-treated cells, hearts from sham operated, saline-injected, control and HDACi-treated $CD34^{+}$-injected mice were analyzed by immunofluorescence and histology. Figure 6C shows the result of capillary density calculation at the infarct border zone by fluorescent lectin staining, while figure 6D shows the results of morphometric analysis. Surprisingly, these analyses showed that HDACi-

treated cells did not show a significantly superior efficacy in inducing angiogenesis or reducing heart scarring compared to control-treated cells.



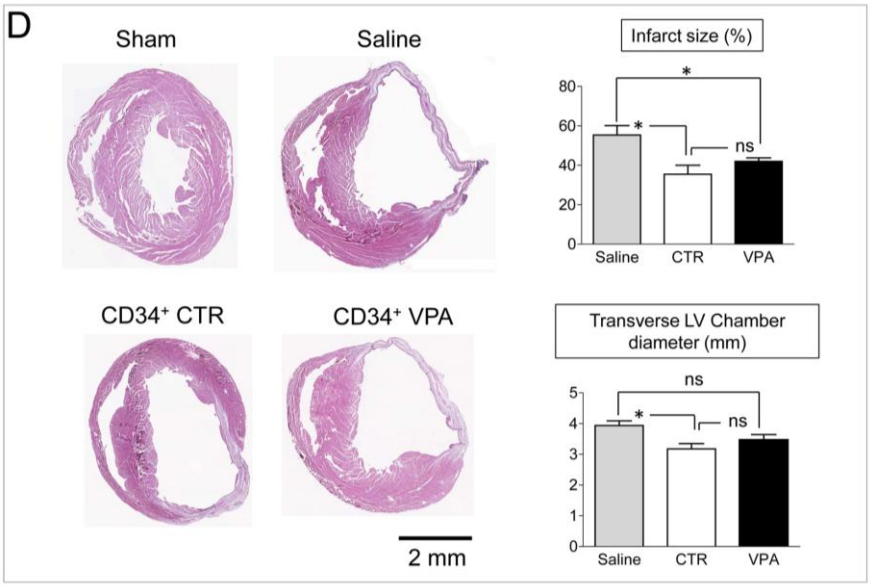




Figure 6

To ascertain whether human cells survived until 6 wks after CAL, the presence of human DNA was evaluated by qPCR method tailored to detect a human polymorphism with an efficiency of 0.1-1 equivalent human cell genome (0.6-6 pg) in an unrelated DNA mixture (Figure S10). Using this method, it was found that the overall survival of human cells in the ischemic mouse myocardium during the 6 wks follow up period was low (estimated in about $10^2$ cells out of the $1.5x10^5$ injected cells) and similar in control *vs.* VPA-conditioned cells injected mice.

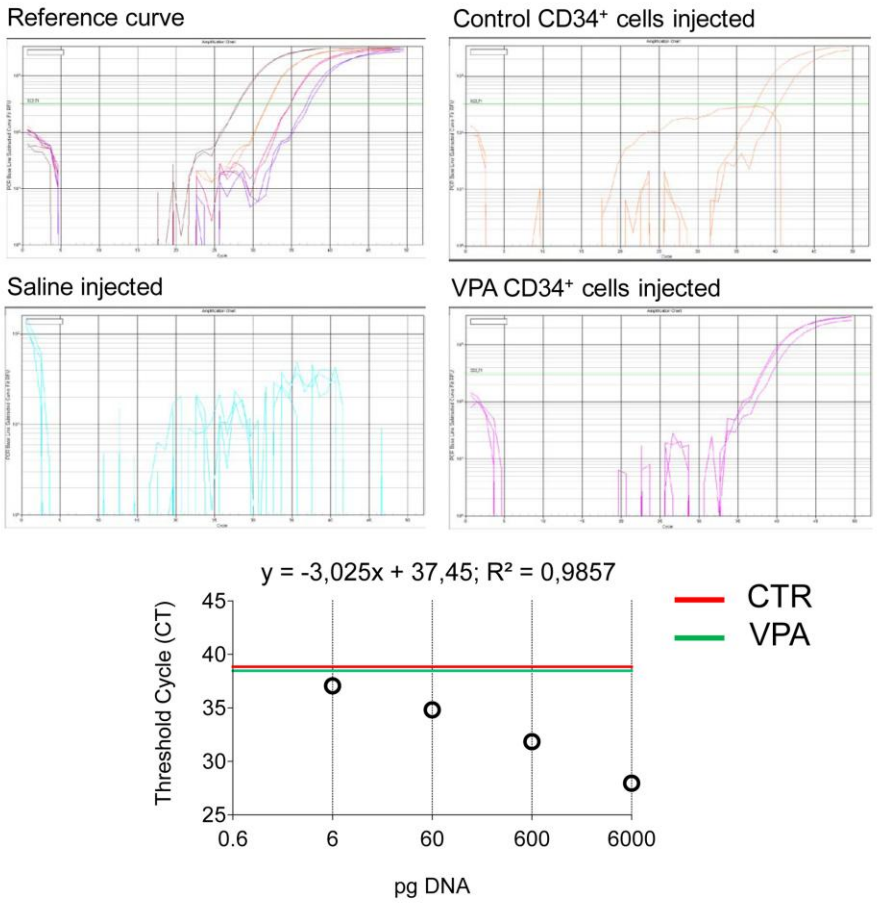



Figure S10

Finally, HDACi-treated *vs.* control cells were evaluated for release of selected inflammatory/*pro*-angiogenic cytokines in conditioned medium by using Bioplex technology. This latter experiment showed a significantly higher expression of 11 out of 15 tested cytokines in HDACi-treated cells *vs.* control cells (Figure 7), suggesting a potent enhancement of their *in vivo* paracrine effect.

| Factor | mean CTR (pg/ml/$10^5$ cells) | SE CTR | mean VPA (pg/ml/$10^5$ cells) | SE VPA |
|---|---|---|---|---|
| IL-4 | 0,62 | 0,2173 | 2,062 | 0,6579 |
| TNFa | 3,298 | 0,9923 | 13,01 | 4,577 |
| bFGF | 6,19 | 2,239 | 16,72 | 5,305 |
| GM-CSF | 6,718 | 2,327 | 23,56 | 7,051 |
| G-CSF | 11,63 | 5,19 | 33,11 | 12,54 |
| IFNg | 13,64 | 4,771 | 49,23 | 16,66 |
| PDGF-BB | 17,98 | 5,617 | 37,9 | 10,73 |
| FolI | 31,40 | 11,73 | 328,00 | 82,83 |
| PECAM-1 | 78,9 | 35,05 | 224,6 | 136,4 |
| Leptin | 96,61 | 33,73 | 132,6 | 81,22 |
| IL-2 | 134,9 | 62,93 | 292,7 | 124,2 |
| Ang-2 | 161,80 | 44,36 | 267,90 | 82,91 |
| HGF | 548 | 144,9 | 2819 | 541 |
| IL-8 | 1185 | 453,5 | 2293 | 1030 |
| VEGF | 3505 | 1512 | 7739 | 3255 |

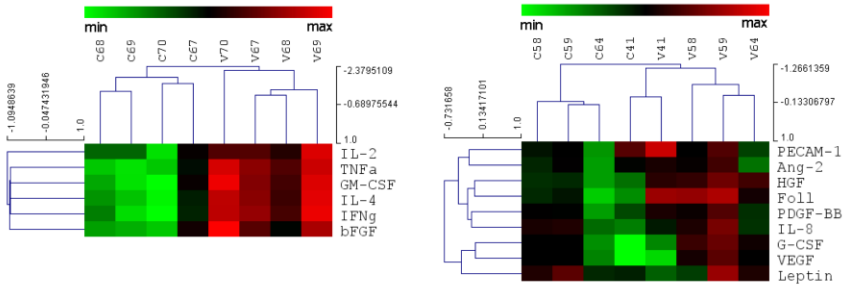



Figure 7

# 4. Discussion

The role of epigenetics as a force balancing differentiation *vs.* self renewal has recently emerged by studies where the potency of various adult-derived stem cells were assessed after treatment with inhibitors of histone acetylation/deacetylation. For years recognized as an useful treatment for inducing differentiation of transformed cells, histone deacetylase (HDAC) pharmacologic inhibition has been more recently indicated to promote stem cells self renewal and/or to favor phenotype reprogramming. Typical is the example of neuronal stem cells (NSCs), where the use of VPA and TSA represses glial commitment and promotes neuronal differentiation by an ERK-dependent intracellular signalling cascade, or that of differentiated cells, where use of epigenetic active drugs was found to facilitate *de*-differentiation into iPS [43-47].

**Direct and pleiotropic roles of HDACi blockade on $CD34^+$ cells stemness, cell cycle and phenotype**

In the present report, in line with previous studies [29-31, 33, 48], we show that $CD34^+$ cells were functionally modified by HDACi pretreatment and induced to maintain a more immature phenotype compared to cytokine-only-treated cells. This is witnessed by **1)** dose-dependent inhibition of cellular growth and increase of CD34 protein expression by TSA and VPA, **2)** reduced downregulation of CD34/CD133 markers at different

times of culture by VPA, 3) reduction of $CD34^+$ cells proliferation index and, finally, **4)** an increase in the so called "slow dividing" stem cells fraction [49]. In addition, HDACi treatment also increased the percentage of $CD34^{bright}$ cells actively extruding Rhodamine123 dye, as a consequence of MDR-1 expression, and those expressing ALDH, another marker defining immature HSCs and EPCs[35, 36, 50-53]. The effects of HDAC inhibition were likely dependent on direct effects of VPA on histone enhanced acetylation: this was evident by detection of higher *pan*-Acetylated H4 and K9-Acetylated H3 histones levels paralleled by the absence of substantial effects on CD34 upregulation by the non-functional VPA analog VPM, as well as by the only partial effect of VPM on cellular growth retardation (Figure 3).

Increased immaturity and retarded growth were not the unique consequences of HDAC inhibition in $CD34^+$ cells. In fact, our results showed negative and positive modulation of various markers in treated *vs.* control cells. According to the enhanced immaturity of HDACi-treated cells, monocyte marker CD14 was downregulated, while immature HSC/EPC marker VEGFR2/KDR receptor, endothelial marker CD31 and uptake ability of Ac-LDL were significantly increased (Figure 4). Strikingly, VPA treatment induced *de novo* expression of mesenchymal (CD90) and "late" EPCs/mature endothelial cells (CD146)[54, 55] markers. Taken together, these results suggest that HDAC inhibition caused a general phenotype reprogramming of $CD34^+$

cells due to activation of different, but likely interdependent, molecular pathways targeting at the same time cell proliferation, stemness, and markers expression.

**mRNA and miRNA profiling of HDACi-treated CD34$^+$ cells reveals a streamlined epigenetic supervision of HSCs self renewal**

The clusterization of mRNAs and miRNAs regulated by VPA in CD34$^+$ cells (Figure 5) allowed to clearly recognize specific signatures distinguishing the two cells types, thus enabling identification of transcriptional circuitries which may be involved in enhanced self renewal, phenotype change and, possibly, *in vivo* function. For mRNAs, for example, it was possible to derive functional annotation charts describing occurrence of HDACi-regulated mRNAs into various BIOCARTA/KEGG categories. This helped us to categorize the function of mRNAs which were positively or negatively modulated by VPA in CD34$^+$ cells and identify them as crucial nodes of the canonical Wnt- (FZD1, WNT1), Notch- (NOTCH1, DLL3, DLL4) and Hedgehog-activated signaling (DHH, BMP2). Interestingly, other genes which act as active components in these pathways such as CDH1 (E-cadherin) and CCND1, or genes involved in early mesoderm/mesenchymal commitment such as T (Brachyury), GDF2 and MSX1, stemness maintenance such as GDF3, ALDH1A1, TERT and ABCG2, angiogenesis such as FGF2, FGFR1, CXCL12/SDF-1 and cell cycle CCNA2, CCNE1 were also

modulated, with consequences for $CD34^+$ cells phenotype, growth, differentiation and function.

Particular importance for the interpretation of our results may have the role of Wnt- and Notch-activated pathways. In fact, it has been recently proposed that canonical-Wnt and Notch signaling converge to maintain HSCs self renewal in bone marrow stem cell niche [56-61], even though it is still matter for discussions how the niche protects stem cells from aberrant cycling in an immature state (malignant transformation) or from proliferation-related massive differentiation (stem cells exhaustion). The observed downregulation of CDH1 (E-cadherin) and upregulation of FDZ1, WNT1, CCND1, DLL1/3 and mir-9 in VPA-treated $CD34^+$ cells (Tables 1 and 2) support the hypothesis that modification of the epigenetic landscape by HDAC blockade has a direct effect on hyper-activation of the Notch-pathway and on the nuclear β-catenin/TCF-LEF1-dependent transactivation. In tumor biology, constitutive activation of the Wnt/β-catenin signaling is a hallmark of malignant transformation leading to unrepressed cellular growth [62]. This notion is, however, in striking contrast with the results of the present and previous reports [29-31] showing growth retardation and increased immaturity in VPA-stimulated HSCs. This latter consideration suggests, that global epigenetic changes induced by VPA treatment also establish negative loops maintaining immature HSCs in a slow-dividing state even under the "pressure" of positive proliferative stimuli. How this

"balancing" loop may be established? A first possibility comes from the potent anti proliferative effect exerted by VPA through the known p53 upregulation [63], as well as of the small cyclin/CDK inhibitors such as $p14^{ARF}$, $p16^{INK4}$ and $p21^{Cip1/Waf1}$ gene products, known to cause G0-G1 arrest of several cancer cells types. A second option, not in contrast with the first, is that epigenetically driven miRNAs upregulation comes to reinforce the direct anti-proliferative effect of VPA. In line with this hypothesis, we found that several miRNAs known to act as tumor suppressors/inducers or deleted in various cancers were significantly and coherently modulated by VPA. This is the case, for example, of mir-129-3p, a miRNA with a potent anti proliferative effect by targeting proto-oncogene SOX4 and cell cycle associated CDK6, mir-193b (clustered with mir-365 at the 16p13.12), found to directly interfere with CCDN1 expression, mir-196b, a positive regulator of leukemic cells immortalization, mir-370, a member of the large miRNA cluster located at the 14q32.31 and regulated by IL-6, that has been shown to have an antiproliferative effect by targeting MAP3K8 proto-oncogene, or, finally, mir-335 that directly cooperates with p53 in cell cycle arrest by targeting pRB. Reinforcement of negative effects on cell proliferation may also depend on an HDACi-related modification of the DNA methylation status. This is suggested by the finding that VPA induced mir148, a miRNA specifically targeting DNMT3b methyltransferase, and by the evidence that several miRNAs over- or under-expressed by VPA in $CD34^{+}$ cells

are known to be transcriptionally regulated by CpG islands methylation [64-72].

**Cardiac protection by HDACi-treated CD34$^+$ cells is independent of CD34$^+$ cells regeneration enhancement; insights into the "paracrine effect" in the ischemic heart**

HDAC inhibitors are known to potently reduce *in vitro* and *in vivo* angiogenesis by repressing the ability of mature endothelial cells to form vascular structures [73] or by inhibiting primary EPCs maturation into endothelium [74]. In addition, HDAC genes targeting (e.g. SIRT-1 or HDAC4,7) impairs vascular development [75-77]. Therefore, HDACi therapeutic use has been proposed as a feasible strategy to keep under control tumor angiogenesis [78, 79]. According to cell autonomous inhibition of EPCs endothelial commitment and angiogenesis, preconditioning with HDACi should reduce and not increase CD34$^+$ cells *pro*-angiogenic function into ischemic tissues. On the other hand, the pleiotropic effects of HDAC blockade on multiple signaling pathways, might convert these cells into more immature progenitors having a modified or enhanced ischemic tissue repair function.

The results of *in vivo* administration of CTR and VPA-treated cells in the ischemic heart (Figure 6) clearly indicated that the most remarkable effect of HDACi-preconditioned cells was to improve the survival of treated mice at a similar level to sham operated mice. This was likely mediated by enhancement of

cardiac function but, surprisingly, was neither associated to a more efficient reduction of the infarct size, nor to a significant improvement of myocardial tissue regeneration compared to control $CD34^+$ cells, as assessed by quantification of LV scar size or by capillary counting (Figure 6). Furthermore, as shown by survival of similar but low amount of living human cells in the host myocardium (Figure S10), this effect was not due to a higher engraftment of VPA-treated $CD34^+$ cells.

How to reconcile these data? Our results call for a generalized enhancement of the $CD34^+$ cells cardioprotection ability or an enhanced "paracrine effect" that may sustain cardiac contractility or interfere with myocardial cells apoptosis as short times after infarction and, hence, explain the remarkable effect on survival and preservation of the heart mechanical function. Moreover, VPA-treated cells showed an enhanced expression of several *pro*-angiogenic/*pro*-inflammatory cytokines at mRNA (FGF2, CXCL12/SDF-1) and/or protein (bFGF, IL-8, VEGF, Ang-2, IFNγTNFα) levels, cardioprotective factors such as Follistatin [80] and cardiac progenitors activating factors such as HGF [81]. The generalized upregulation of all these gene products in the secretome of $CD34^+$ cells may lead to the functional preservation of the left ventricle by sustaining cardiac metabolism and contractility even in the absence of a net reduction in the infarct scar size compared to control $CD34^+$ cells (discussed in [82]). It is also possible that [52] enhanced secretion of Wnt-1 and Notch ligands DLL1/3 also has a

important role in this, for example, by controlling expression of Connexin-43 [83] and thus contributing to attenuate gap junction remodeling in the ischemic heart [84], or by cooperating with cardiac myocytes *pro*-survival signaling [85]. Lastly, it is also possible that higher mice survival in animals injected with VPA-treated cells might have resulted in a positive selection of more ill mice reaching the final stage of the follow up period thus leading to confounding results on infarct size, capillary density and LV remodeling.

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# ***CHAPTER 4:*** ***GRANULOCYTE COLONY-STIMULATING FACTOR ATTENUATES LEFT VENTRICULAR REMODELLING AFTER ACUTE ANTERIOR STEMI. RESULTS OF THE SINGLE-BLIND, RANDOMIZED, PLACEBO-CONTROLLED MULTICENTER STEM CELL MOBILIZATION IN ACUTE MYOCARDIAL INFARCTION (STEM-AMI) TRIAL.***

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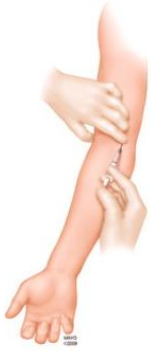
## 1. Introduction

Once identified the best cellular product to be used in a given pathology, it is also essential to identify which is the best delivery strategy. Moreover, the timing of administration in relationship to the onset of the disease is also a key element.

At date, different delivery routes have been developed, but none has emerged as the preferred technique [1].

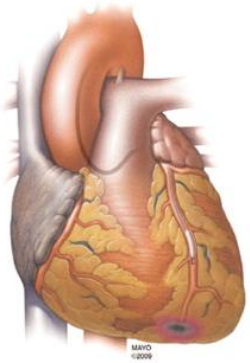
For patients with ischemic heart disease three main delivery strategies are available:

1 – *Intravenous cell infusion*



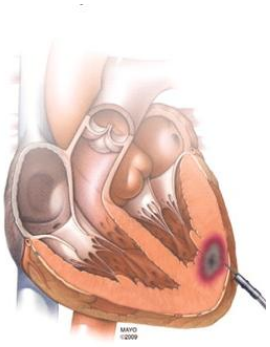
This technique involves cell infusion through a central venous catheter. This method is simple and non invasive but it has two main disadvantages. First of all, a large number of cells become trapped in lungs, liver and spleen, so only small numbers can reach the myocardium. Another disadvantage is that large cells may create microemboli in the vasculature [2].

2 – *Intracoronary cell infusion*



This technique involves cell infusion through the central lumen of a balloon catheter which is positioned into a coronary artery. The main advantage of this method is that cells can rapidly reach a myocardial region with preserved oxygen supply. This method is particularly effective for the delivery of cells to a specific coronary territory, and for the treatment of recently infracted myocardium, when homing signals are highly expressed. The main disadvantage is the risk of micro-infarctions due to the injection of large or sticky cells [2].

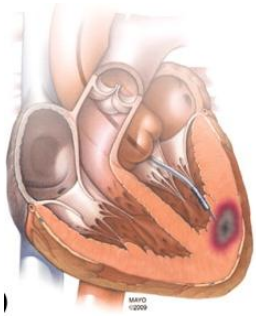
3 – *Intramyocardial cell injection*



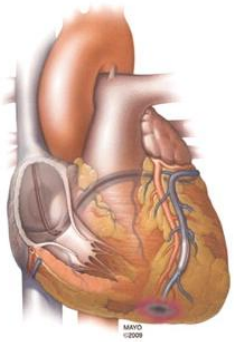
This technique involves cell infusion directly into the myocardium. At date, three routes of injection have been proposed.

*Trans-epicardial* injection has the advantage that it can be performed during coronary artery bypass grafting (CABG). However, it has a high degree of invasiveness and cells injected into a necrotic tissue may not survive.

*Trans-endocardial* injection involves infusion of cells through a needle inserted in the myocardium and guided by a three dimensional electromechanical mapping system (NOGA) which can distinguish between viable, hibernating and infracted tissue. Even it is a very precise technique, its main disadvantage is the risk of tissue damage and ventricle perforation.



*Transvenous* technique involves the injection of cells through the coronary sinus to the myocardium. This method requires cell infusion with a catheter system incorporating an ultrasound tip as a guide. This route allow to inject cells deep into the myocardium but it is technically challenging, it doesn't allow to reach all the myocardial territories and it is not possible in all patients [2].



In addition to these strategies, pharmacological cell *mobilization* has emerged as a great alternative. Mobilization is defined as the pharmacological recruitment of cells from BM into the PB. This process mimics the physiological release of cells from BM reservoir during injury and inflammation, and it is less invasive than above mentioned strategies. Moreover, it doesn't need to

manipulate cells *ex vivo*. In the early 1980s chemotherapy was discovered as a mobilizing strategy and first experiments of autologous cell transplantation of mobilized PB cells were conducted. Today cytokine mobilization has become the standard care, and several drugs have been studied for their mobilization potential and clinical application. Despite this, the unique cytokines approved by the food and drug administration (FDA) for cell mobilization are granulocyte-colony-stimulating factor (G-CSF) and grnulocyte-macrophage colony-stimulating factor (GM-CSF) [3].

In BM niche, G-CSF induces the release of a number of proteases, such as neutrophil elastase (NE), cathepsin G (CG) and matrix metalloproteinase-9 (MMP9). These proteases cleave some key molecules that anchor HSCs in the BM niche. Among these cleaved molecules there are adhesion proteins, trafficking epitopes and molecules enrolled in cell migration. For example, G-CSF induces the cleavage of the extracellular domain of CXCR4, which maintains HSCs in the niche through its interaction with Stromal-derived Factor-1 (SDF-1, or CXCL12). Nevertheless, CXCR4 is a key regulator of cell migration and retention in the infracted tissue, so mobilized cells seem to be impaired in their biological activity [4]. Despite these disappointing observations, fgood results were obtained in pre-clinical animal models. For example, in a coronary occlusion and reperfusion rabbit model, G-CSF injection increased left ventricular ejection fraction (LVEF) and reduced

the necrotic, granulation and scar tissue areas. This led the authors to conclude that G-CSF can accelerate the healing process [5]. Moreover, in a dominant-negative STAT3 transgenic mouse model (dnSTAT3-tg) of myocardial infraction, G-CSF enhanced cardiomyocytes survival, reduced endothelial cell apoptosis and increased vascularization in the infracted heart. Lastly, in infracted C57BL/6 mice, cytokine-mediated translocation of BMCs resulted in tissue regeneration. In fact new myocytes, arteries and capillaries were identified and they also seemed functionally connected to the unaffected ventricle [6].

Taken together, these pre-clinical results supported clinical trials of G-CSF administration in patients with myocardial infraction (MI). Results of seven independent clinical trials have been published between 2004 and 2007 [7-13]. Among these, the MAGIC trial was the only one in which investigators decide to stop the enrollment due to a higher rate of in-stent restenosis in the treatment group. However, the safety concern expressed by the MAGIC trial was refuted on the basis of recent data meta-analysis and, nowadays, it appears unjustified [14]. In 2008 another meta-analysis on data obtained from 328 patients was published. Overall results showed that G-CSF does not increase EF and it does not has a significant effect on infarct size, LVEDV and LVESV decrease. So, G-CSF therapy failed to significantly improve any of the primary surrogate endpoints. Nevertheless, a stratified analysis showed that the

therapy could have a great effect on patients with a baseline EF lower than 50% and when the drug is administered at early time points (≤37 hours). Therefore, the authors concluded that G-CSF administration in unselected patients is not effective, but they suggested some criterions useful to design future studies [15].

On this basis we conducted a perspective, randomized, single-blind, placebo-controlled clinical trial on 60 patients with:

- Anterior STEMI with EF below 45% after reperfusion
- Symptoms-to-balloon time of 2-12 hours
- Very early administration of high-dose G-CSF (mean of 9.2 hours after the onset of symptoms).

## 2. Materials and Methods

The **St**em C**e**ll **M**obilization in **A**cute **M**yocardial **I**nfarction **(STEM-AMI)** trial was approved by the local Ethics Committee of "A. Manzoni" Hospital in Lecco (November 9th, 2005) and by the Ethics Committee of each hospital involved in the study (see Appendix S1). Patients were enrolled in four Italian centres between July 2006 and May 2009, and 6-months follow-up was completed in November 2009. The study protocol conformed to the Declaration of Helsinki. Written informed consent was obtained from each patient. This trial is filed as "STEM-AMI" trial on www.emea.europa.eu with the Eudra-CT Code 2005-004706-90.

### Study population

Sixty consecutive patients, admitted to the hospital with first acute STEMI, undergoing primary PCI between 2 and 12 hours after symptoms onset, with ejection fraction (EF) ≤ 45% detected by echocardiography (ECHO) within 12 hours after successful revascularization (TIMI flow score ≥ 2), were randomized to G-CSF or placebo (1:1). The patients allocated in the study were treated with the standard pharmacological therapy according to STEMI and Heart Failure Guidelines [16-17]: ACE inhibitors, betablockers, statin, aspirin, clopidogrel and abciximab used in the acute phase. Anterior STEMI was diagnosed by typical chest pain lasting > 30 minutes, evidence of ST elevation in at least 2 consecutive anterior leads or

presence of left bundle branch block at 12-leads ECG and significant increase of plasma Troponin I.

Inclusion and exclusion criteria are summarized in Table 1. Patients were excluded from the study if one of the following adverse events occurred following G-CSF injection: new acute coronary syndrome requiring urgent surgical revascularization, any other adverse life-threatening clinical event, patient consent withdrawal. White blood cells (WBC) count > 50.000/$mm^3$ was the cut-off for discontinuation of G-CSF therapy in the treatment arm.

**Table 1 Inclusion and exclusion criteria**

| |
|---|
| Inclusion criteria |
| Signed informed consent |
| Acute anterior STEMI undergoing PCI |
| Time symptom-to-balloon >2 and <12 h |
| Ejection fraction post-PCI ≤45% |
| TIMI flow post-PCI 2–3 |
| Exclusion criteria |
| Previous myocardial infarction |
| Leukaemia, myeloproliferative, or myelodysplastic disorders |
| Immune system disease |
| Malignant disease |
| Age >75 years |
| Life expectancy <6 months |
| Previous cardiac surgery or stenting within 12 months |
| Coronary lesions needing CAB |
| Platelet count <50 000 $mm^3$ |

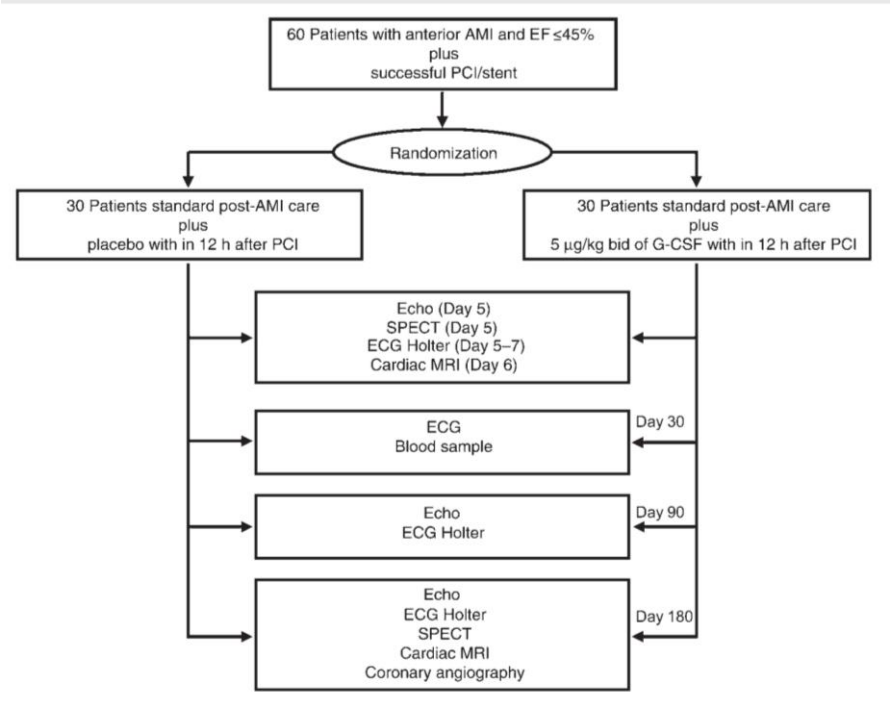
STEMI, ST-elevation myocardial infarction; PCI, percutaneous coronary intervention; CAB, coronary artery bypass; TIMI, thrombolysis in myocardial infarction.

## G-CSF treatment

Five (5) µg/Kg of G-CSF (Lenograstim, Sanofi-Aventis, Milano, Italy) or placebo were administered subcutaneously *bis in die* (*bid*) for 5 days (from day 0 to day 4), starting within 12 hours after PCI and reperfusion.

## Study design

The STEM-AMI trial was a multicenter, prospective, single-blind, randomized, placebo-controlled study; patients with acute anterior STEMI were randomized in a 1:1 ratio, after the primary PCI, to G-CSF or placebo, as a supplement to standard treatment (Figure 1).



Within 7 days from STEMI onset, laboratory tests, 2-D ECHO, ECG Holter, gated $^{99m}$Technetium Sestamibi Single-Photon Emission Computed Tomography (SPECT) and cardiac magnetic resonance imaging (MRI) were performed by experts blinded to treatment allocation and clinical information. Imaging interpretation was performed by core laboratories, with physicians masked to treatment. Randomization was computed-generated, in blocks of 10, using sequentially numbered, sealed envelops.

**Primary and secondary end-points**

The pre-specified primary endpoint was a change from baseline to 6-months of 5% in LV EF, as measured by MRI. Co-primary end-point was a difference of 20 ml of end-diastolic volume (EDV) at MRI. Secondary end-points were infarct size reduction evaluated by MRI and improvement of myocardial perfusion by SPECT at 6-months. Safety end-points were survival, major cardiac adverse events (MACE) and rate of restenosis. MACE were defined as death, reinfarction, acute heart failure, urgent revascularization and stroke.

**Cardiac imaging**

*Cardiac magnetic resonance imaging.*

Cardiac MRI was performed on day 6 and 180. MRI studies were obtained for evaluation of LVEF, end-diastolic (EDV) and systolic (ESV) volumes, and infarct size assessed by late

gadolinium enhancement (LGE). (More details are available in the Supplemental Data, Appendix S2).

*Echocardiography.*

Two-dimensional ECHO was performed before randomization within 12 hours from revascularization (day 0) and on day 5 (baseline), 90 and 180. (More details are available in the Supplemental Data, Appendix S2).

*SPECT.*

Rest gated $^{99m}$Technetium Sestamibi SPECT was performed on day 5 and 180 in order to evaluate global and regional myocardial perfusion by quantitative perfusion SPECT (QPS). (More details are available in the Supplemental Data, Appendix S2).

*Angiography.*

All patients were submitted to coronary angiogram and primary PCI plus stenting of the culprit lesion before randomization. Coronary angiogram was repeated at 6-months of follow-up to evaluate restenosis of the target lesion, new vessel disease and myocardial blush.

**Isolation and Culture of peripheral blood and Flow cytometry analysis**

On days 0 (enrolment), 5 and 30 after MI, mononuclear cells (MNCs) were isolated from peripheral blood (PB) using Ficoll density gradient centrifugation, and counted. The total $CD34^+$, $CD133^+$ and $CD14^+$ total cell number was then calculated.

**EPCs staining**

The number $LDL^+/Lectin^+/Hoechst^+$ positive clusters was determined by fluorescence microscopy.

**Blood analysis**

Complete blood count, serum lactate dehydrogenase, creatine kinase, alkaline phosphatase, C-reactive protein (CRP), interleukin 6 (IL-6) and pro-brain natriuretic peptide (BNP) measured before and, daily after randomization until hospital discharge, as well as at 1, 3 and 6 months.

**Statistical analysis**

The STEM-AMI trial was designed to randomize 50 patients 1:1 to G-CSF therapy or placebo in addition to standard therapy to obtain, with 85% power and an SD of 5% in the 2 groups, a possible significant difference of 5% in LVEF and/or a difference of 20 ml of EDV as measured by MRI [18]. To allow for 20% drop-out and claustrophobia or difficulties with breath-holding during MRI, sample size was increased to 60 patients.

All analyses were performed on the basis of the intention-to-treat principle using data from all patients as randomized. Categorical data are presented as counts or proportions (percentages). Continuous data are presented as mean ± SD. Normal distribution was tested by Skewness-Kurtosis test. Between group comparison for continuous variables were performed by *t* test or the corresponding nonparametric

alternative (Mann-Whitney test), as appropriate. Discrete variables were compared by chi-square or Fischer exact test when appropriate. Within group comparison for continuous variables were performed using repeated measures ANOVA with Holm-Sidak post-hoc analysis or Friedman repeated measure ANOVA on ranks with Tukey post-hoc analysis, when appropriate. Two-way multivariate analysis of covariance (MANCOVA) was performed to assess changes of LVEDV, LVESV and EF from baseline to 6-months follow-up between the 2 groups, adjusted for relevant baseline variables. The MANCOVA included the changes from baseline to 6-months for LVEDV, LVESV and EF as dependent variables, and the associated baseline values (pro-BNP, Troponin I, $CD34^+$, time-to-reperfusion) and two factors for Killip score and treatment as independent variables. Treatment effects were estimated by computing the differences between the adjusted means of the G-CSF and placebo treated patients and their corresponding 95% confidence intervals. An ANCOVA analysis was performed to assess the significance of the changes of infarct size from baseline to 6-months, including the same covariates and independent variables as above.

The level of statistical significance was set at $p<0.05$. We used IBM-SPSS Statistics software version 17.0 (SPSS, Chicago, IL, USA).

# 3. Results

## Clinical, biochemical and angiographic findings

Demographic, clinical characteristics, and co-morbidity profiles were homogeneous and revealed a typical distribution of risk factors and evidence-based medications (Table 2).

**Table 2 Demography, and clinical and biochemical characteristics of the study population**

| Variable | G-CSF ($n$ = 29) | Placebo ($n$ = 27) | $P$ |
|---|---|---|---|
| Age, y | 61 ± 8 | 61 ± 10 | 0.97 |
| Male sex, $n$ (%) | 29 (100) | 24 (89) | 0.21 |
| Hypertension, $n$ (%) | 14 (48) | 18 (67) | 0.26 |
| Diabetes, $n$ (%) | 6 (21) | 3 (11) | 0.47 |
| Dyslipidaemia, $n$ (%) | 17 (59) | 16 (59) | 0.82 |
| Smoking, $n$ (%) | 17 (59) | 13 (48) | 0.60 |
| Family history of CAD, $n$ (%) | 12 (41) | 9 (33) | 0.73 |
| Blood analysis | | | |
| C-reactive protein (mg/dL), Day 0 | 1.4 ± 3.2 | 0.89 ± 1.00 | 0.39 |
| NT-proBNP (pg/mL), Day 0 | 2094 ± 3969 | 1749 ± 2039 | 0.69 |
| NT-proBNP (pg/mL), Day 6 | 2595 ± 1858 | 1370 ± 1378 | 0.01 |
| NT-proBNP (pg/mL), 6 months | 940 ± 1390 | 787 ± 1304 | 0.68 |
| WBC count ($\times 10^3/mm^3$), Day 0 | 13 ± 5 | 12 ± 2 | 0.33 |
| Maximum WBC count ($\times 10^3/mm^3$) | 40 ± 9 | 12 ± 2 | <0.001 |
| WBC count ($\times 10^3/mm^3$) at discharge | 6 ± 2 | 7 ± 2 | 0.32 |
| Maximum platelet count ($\times 10^3/mm^3$) | 231 ± 64 | 249 ± 49 | 0.30 |
| Platelet count ($\times 10^3/mm^3$) at discharge | 196 ± 49 | 211 ± 51 | 0.28 |
| $CD34^+/\mu L$, Day 0 | 56.69 ± 61.10 | 31.15 ± 19.07 | <0.04 |
| Creatinine (mg/dL), Day 0 | 0.87 ± 0.21 | 0.94 ± 0.22 | 0.23 |
| Medication at discharge, % | | | |
| Aspirin | 100 | 100 | 1.0 |
| Clopidogrel | 100 | 100 | 1.0 |
| Statins | 100 | 100 | 1.0 |
| ACE inhibitors | 100 | 100 | 1.0 |
| Beta-blockers | 100 | 100 | 1.0 |
| Diuretics | 35 | 37 | 0.93 |
| Medication at 6 months of follow-up | | | |
| Statin, % | 100 | 100 | 1.0 |
| Atorvastatin median dose (mg) | 35.0 ± 9.00 | 27.5 ± 11.4 | 0.12 |
| ACE inhibitors, % | 100 | 100 | 1.0 |
| Ramipril median dose (mg) | 6.6 ± 3.5 | 7.4 ± 3.0 | 0.62 |
| Beta-blockers, % | 100 | 100 | 1.0 |
| Carvedilol median dose (mg) | 25.4 ± 12.8 | 23.0 ± 14.7 | 0.63 |
| Diuretics, % | 40 | 25 | 1.0 |
| Furosemide median dose (mg) | 31.2 ± 22 | 28.1 ± 15.7 | 0.81 |

Values are expressed as mean ± SD. y, year; BNP, brain natriuretic peptide; WBC, white blood cell; ACE, angiotensin-converting enzyme.

Angiographic and infarction-related features (Table 3) were also not significant different between the two groups. Moreover, time from symptoms onset to PCI was similar in the G-CSF arm and in the placebo group (277 ± 166 minutes and 238 ± 124 minutes, respectively). No differences were noted in

inflammatory parameters. Platelet count was not different during hospitalization in the two groups. As expected, G-CSF treatment was associated with more than tripling WBC count at day 6. Notably, a worse clinical profile of the G-CSF group was observed, as expressed by a significantly higher pro-BNP plasma value at day 6, and by an unfavourable trend in Killip class at admission, intra-aortic balloon pump (IABP) use and prevalence of multivessel disease. Notably, although pro-BNP values at the end of 6-months follow-up were not significantly different between the two groups, a marked reduction between day 6 vs day 180 was seen in the treatment group only, whereas placebo group did not show a significant difference (2595 ± 1858 vs 940 ± 1390 pg/mL ($p$=0.001) and 1370 ± 1378 vs 787 ± 1304 pg/mL ($p$=0.12) in G-CSF and placebo groups, respectively).

G-CSF was administered 9.2 ± 2.7 hours after PCI. Cytokine treatment was prematurely stopped in only one patient for excessive WBC rise without any adverse event. Four patients withdrew consent before completion of G-CSF/placebo treatment, therefore they were excluded from the analysis and their characteristics are not reported in tables 2-4.

**Table 3 Angiographic and infarction-related characteristics**

| Variable | G-CSF ($n$ = 29) | Placebo ($n$ = 27) | $P$ |
|---|---|---|---|
| ECG involved leads, $n$ (%) Pts >5 leads | 14 (48) | 13 (48) | 0.79 |
| Medium ST elevation (mm) | 6 ± 4 | 5 ± 2 | 0.30 |
| Creatine kinase max (U/L) | 4207 ± 2767 | 3052 ± 1721 | 0.09 |
| Troponin I peak | 205 ± 265 | 117 ± 83 | 0.11 |
| EF, % | 39.1 ± 6.2 | 38.6 ± 7.4 | 0.85 |
| Killip class, $n$ (%) Pts ≥3 | 7 (24) | 3 (11) | 0.29 |
| IABP, $n$ (%) | 6 (21) | 1 (4) | 0.10 |
| Onset of AMI to PCI (min) | 277 ± 166 | 238 ± 124 | 0.32 |
| TIMI flow 0 pre-PCI, $n$ (%) | 22 (76) | 15 (56) | 0.18 |
| TIMI flow III post-PCI, $n$ (%) | 24 (83) | 25 (93) | 0.47 |
| PCI plus stent, $n$ (%) | 29 (100) | 27 (100) | 1.0 |
| Drug eluting stent, $n$ (%) | 10 (35) | 5 (19) | 0.29 |
| Multivessel disease, $n$ (%) | 15 (52) | 8 (30) | 0.15 |
| PCI to G-CSF administration (min) | 553 ± 166 | — | |
| ST resolution within 90 min after PCI | | | |
| <30% | 4 (14) | 4 (15) | 1.0 |
| >70% | 13 (45) | 12 (44) | 0.81 |
| Abciximab, Clopidogrel, Aspirin, % | 100 | 100 | 1.0 |

Values are expressed as mean ± SD. Pts, patients; EF, ejection fraction; IABP, intra-aortic balloon pump.

## Effects of G-CSF on progenitor cells mobilization and function

As shown in Figure 2, the number of circulating MNCs and that of $CD34^+$, $CD133^+$ and $CD14^+$ cells was significantly enhanced by the G-CSF treatment and achieved its highest level at day at day 5. Interestingly, the number of $CD34^+$ cells, but not that of $CD14^+$ and $CD133^+$ cells, was significantly enhanced in G-CSF-treated patients compared to controls at 0 and 30 days.

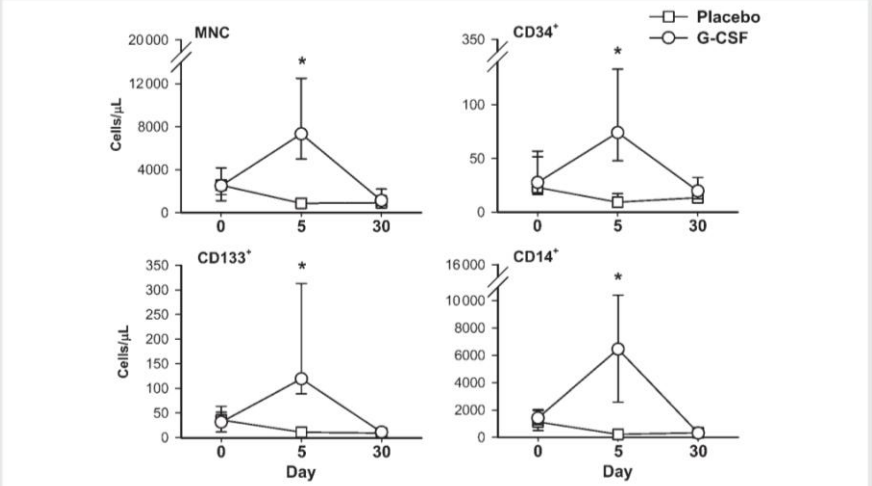
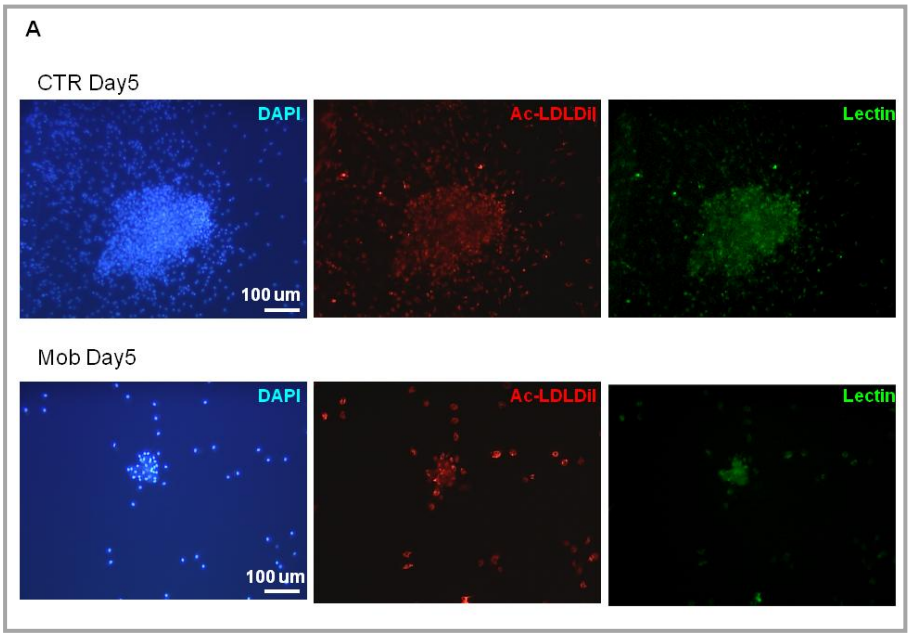



**Figure 2** Effects of treatment on mobilization of mononuclear cells (MNCs), and $CD34^+$, $CD133^+$, and $CD14^+$ cells in placebo and granulocyte colony-stimulating factor-treated groups. Data are expressed as cells/μL of peripheral blood. *$P < 0.001$.

Figures S1 A and B show the results of CFU-EC clusters staining and counting after culture for 7 days.



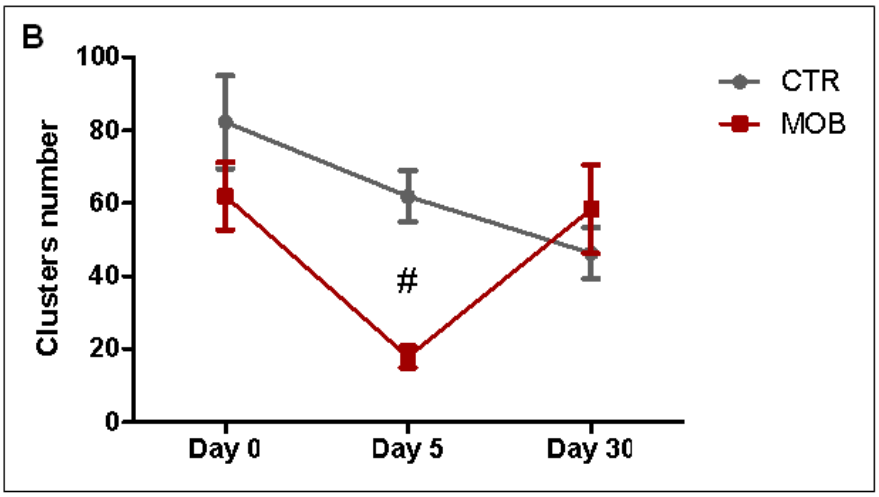



Figure S1

**Safety of GCS-F administration**

After 6-months of follow-up, 54 patients could be examined for clinical outcome (2 patients died and 4 were withdrawn from the study). G-CSF was generally well tolerated. No patients were removed from the trial because of early complications. Table 4 summarizes all in-hospital and 6-months follow-up cumulative events in both the G-CSF and placebo groups. There were two in-hospital reinfarction for acute stent thrombosis in the G-CSF group ($p$=0.49), due to device malapposition in one case. Acute heart failure (AHF), defined as Killip Class ≥3 and/or necessity of IABP and/or i.v. diuretics and inotropic drugs lasting at least for 48 hours from PCI, was seen in 9 patients in the G-CSF group vs 3 cases in the placebo group. However, an higher prevalence of AHF was already present in

the G-CSF population at hospital admission (7 vs 3 in the treatment and placebo groups, respectively).

Finally, a low incidence of MACE within 6 months with no significant differences between groups was observed.

**Table 4 Clinical events over 6 months of follow-up**

| Events | G-CSF ($n = 29$) | Placebo ($n = 27$) | *P* |
|---|---|---|---|
| In hospital events, *n* | | | |
| Death | 0 | 0 | 1.0 |
| Re-infarction | 2 | 0 | 0.49 |
| Ventricular arrhythmia | 0 | 0 | 1.0 |
| AHF[a] | 9 | 3 | 0.10 |
| Stroke | 0 | 0 | 1.0 |
| Events from discharge to 6 months, *n* | | | |
| Death | 1 | 1 | 1.0 |
| Re-infarction/ischaemia | 0 | 0 | 1.0 |
| Late stent thrombosis | 0 | 0 | 1.0 |
| Target vessel restenosis | 12 | 9 | 0.73 |
| Ventricular arrhythmia | 0 | 0 | 1.0 |
| Heart failure | 4 | 1 | 0.35 |
| Stroke | 1 | 0 | 1.0 |
| Urgent revascularization | 0 | 1 | 0.48 |
| MACE[b], *n* | 6 | 3 | 0.47 |

[a]AHF, acute heart failure was defined as Killip Class $\geq$3 and/or need for intra-aortic balloon pump and/or i.v. diuretics and inotropic drugs lasting at least 48 h after PCI.

[b]defined as death, re-infarction, acute heart failure, urgent revascularization, and stroke.

## Treatment effect of G-CSF on LV function and volumes

Table 5 summarizes MRI and ECHO parameters at day 0 (enrolment), 5 (baseline) and 180 (follow-up). MRI data were obtained from 49 patients (24 and 25, treatment and placebo group, respectively).

**Table 5 Echocardiography and magnetic resonance imaging data at Days 0, 5, and 180**

| Treatment | | Day 0 | Day 5 | Day 180 | *P* (Day 0 vs. 5) | *P* (Day 5 vs. 180) | *P* (Day 0 vs. 180) |
|---|---|---|---|---|---|---|---|
| ECHO | | | | | | | |
| LVEF, % | G-CSF | 39.4 ± 6.0 | 47.3 ± 10.6 | 46.2 ± 10.1 | 0.006 | 0.498 | 0.016 |
| | Placebo | 39.6 ± 7.0 | 45.1 ± 11.1 | 42.8 ± 12.1 | 0.013 | 0.207 | 0.170 |
| LVEDV (mL/m$^2$) | G-CSF | 48.7 ± 9.2 | 58.9 ± 11.1 | 60.8 ± 20.4 | 0.003 | 0.659 | 0.024 |
| | Placebo | 48.5 ± 10.7 | 55.2 ± 14.8 | 68.8 ± 24.0 | 0.006 | 0.001 | 0.002 |
| LVESV (mL/m$^2$) | G-CSF | 28.3 ± 6.5 | 31.1 ± 9.8 | 34.0 ± 16.4 | 0.280 | 0.273 | 0.144 |
| | Placebo | 29.0 ± 9.2 | 31.5 ± 14.3 | 41.6 ± 21.2 | 0.164 | 0.001 | 0.010 |
| MRI | | | | | | | |
| LVEF, % | G-CSF | | 45.2 ± 11.5 | 45.2 ± 11.2 | | 1.0 | |
| | Placebo | | 44.6 ± 9.9 | 45.6 ± 10.7 | | 0.459 | |
| LVEDV (mL/m$^2$) | G-CSF | | 82.2 ± 20.3 | 85.7 ± 23.7 | | 0.40 | |
| | Placebo | | 81.7 ± 24.4 | 94.4 ± 26.0 | | 0.00005 | |
| LVESV (mL/m$^2$) | G-CSF | | 46.0 ± 18.2 | 48.4 ± 20.8 | | 0.338 | |
| | Placebo | | 45.2 ± 20.0 | 53.2 ± 23.8 | | 0.016 | |

Values are expressed as mean ± SD. LVEF, left ventricular ejection fraction; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; ECHO, echocardiography; MRI, magnetic resonance imaging.

At enrolment (day 0), the two groups were similar for EF and volumes as measured by 2-D ECHO. A significant improvement in EF was observed from day 0 to day 5 in both groups and from day 0 to day 180 in G-CSF group only (from 39.4 ± 6.0% to 46.2 ± 10.1%, $p$=0.016). MRI analysis was limited to day 5 and 180 and, in agreement with the ECHO results, neither group exhibited a significant change in EF in this time frame (Fig. 3B and Table 5).

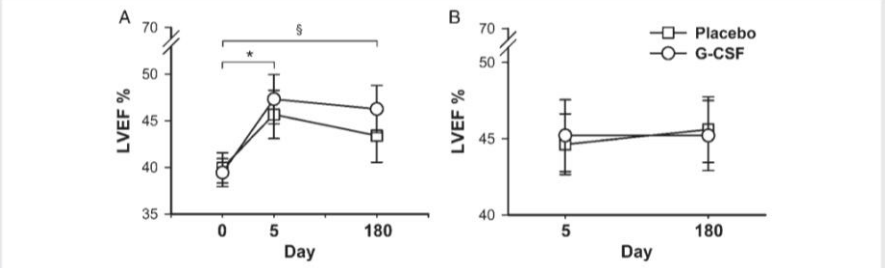



**Figure 3** Changes in the left ventricular ejection fraction (LVEF) by echocardiography (*A*) and cardiac magnetic resonance imaging (*B*) from enrolment (echocardiography only) to baseline and 6 months in the granulocyte colony-stimulating factor and placebo groups. Data are reported as mean ± SEM. *Increase of LVEF from enrolment to baseline for granulocyte colony-stimulating factor ($P = 0.006$) and placebo ($P = 0.013$); §Increase of LVEF from enrolment to 6 months for granulocyte colony-stimulating factor ($P = 0.016$).

Both MRI (Fig.4) and ECHO (Table 5) showed a significant LVEDV and LVESV increase at 6months in the placebo group, whereas no significant changes were observed in the G-CSF group. By MRI, LVEDV and LVESV significantly increased at 6 months from baseline in placebo arm (from 81.7 ± 24.4 ml/m$^2$ to 94.4 ± 26.0 ml/m$^2$, $p$=0.00005; and from 45.2 ± 20 ml/m$^2$ to 53.2 ± 23.8 ml/m$^2$, $p$=0.016, respectively); in contrast, LVEDV and LVESV did not significantly change in the G-CSF group (from 82.2 ± 20.3 ml/m$^2$ to 85.7 ± 23.7 ml/m$^2$, $p$=0.40; and from 46.0 ± 18.2 ml/m$^2$ to 48.4, ± 20.8 ml/m$^2$, $p$=0.338, respectively) (Figure 4 A and B). By ECHO, LVEDV and LVESV significantly increased at 6 months from baseline in placebo arm (from 55.2 ± 14.8 ml/m$^2$ to 68.8 ± 24.0 ml/m$^2$, $p$=0.001; and from 31.5 ± 14.3 ml/m$^2$ to 41.6 ± 21.2 ml, $p$=0.001, respectively) whereas did not significantly change in the G-CSF group (from 58.9 ± 11.1 ml/m$^2$ to 60.8 ± 20.4 ml/m$^2$, $p$=0.659; and from 31.1 ± 9.8 ml/m$^2$ to 34.0 ± 16.4 ml/m$^2$, $p$=0.273, respectively). MANCOVA analysis showed that the change of

LVEDV from baseline to 6 months at MRI is significantly lower in G-CSF vs placebo group ($p$=0.004), and that this effect is independent from the following baseline variables: pro-BNP, Troponin I, number of CD34+ cells, time-to-reperfusion and Killip score (see Figure 4C). The changes of LVESV and EF did not show a significant between-group difference ($p$=0.099 and $p$=0.328, respectively). Diastolic function, expressed as E/E' ratio, significantly improved from baseline to 6-months in the G-CSF group (from 12.0 ± 3.4 to 10.2 ± 2.8 m/s, $p$=0.02), whereas placebo group did not show significant changes (from 9.2 ± 2.5 to 9.0 ± 3.5 m/s, $p$=0.39).

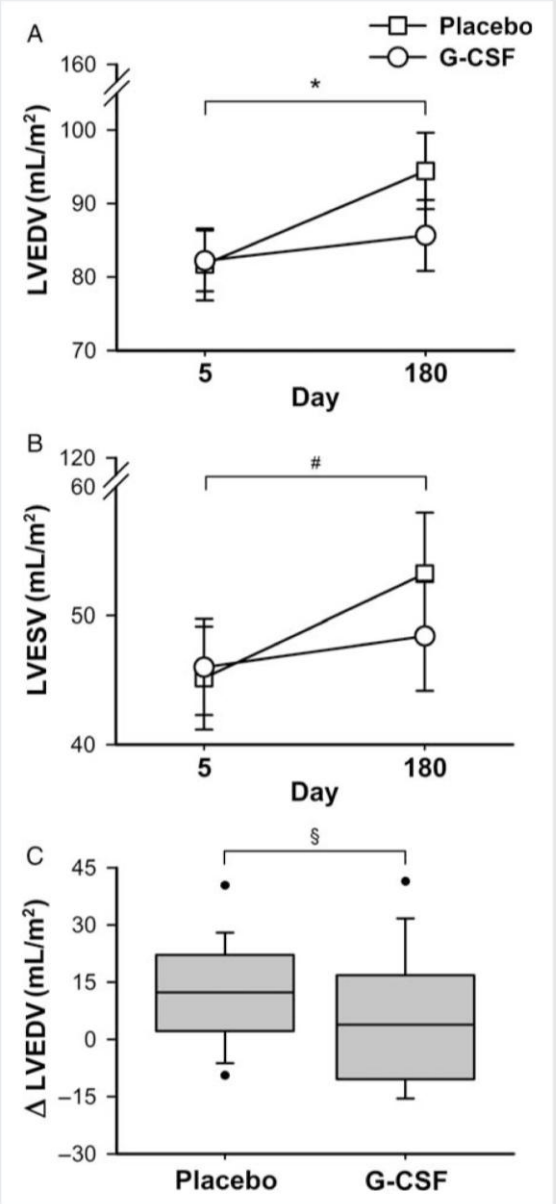



**Figure 4** Changes in normalized left ventricular end-diastolic (LVEDV) (*A*) and normalized end-systolic (LVESV) (*B*) volumes by cardiac magnetic resonance imaging from baseline to 6 months in the granulocyte colony-stimulating factor and placebo groups. Data are reported as mean ± SEM. (*C*) shows the difference between the Δ increment of LVEDV in placebo and granulocyte colony-stimulating factor-treated patients. *Changes in normalized LVEDV from baseline to 6 months for placebo ($P = 0.00005$); #Changes in normalized LVESV from baseline to 6 months for placebo ($P = 0.016$). §$P = 0.004$.

The analysis of the relationship between time-to-reperfusion and extent of remodelling showed that significantly more patients of the placebo group exhibited a change of LVEDV above the median (9.3 ml/$m^2$) when reperfused beyond 180 minutes (median of time-to-reperfusion) ($p$=0.0123). Conversely, no significant difference in the distribution of patients by treatment was present when both time-to-reperfusion and change of LVEDV were lower than the median (see Figure 5).

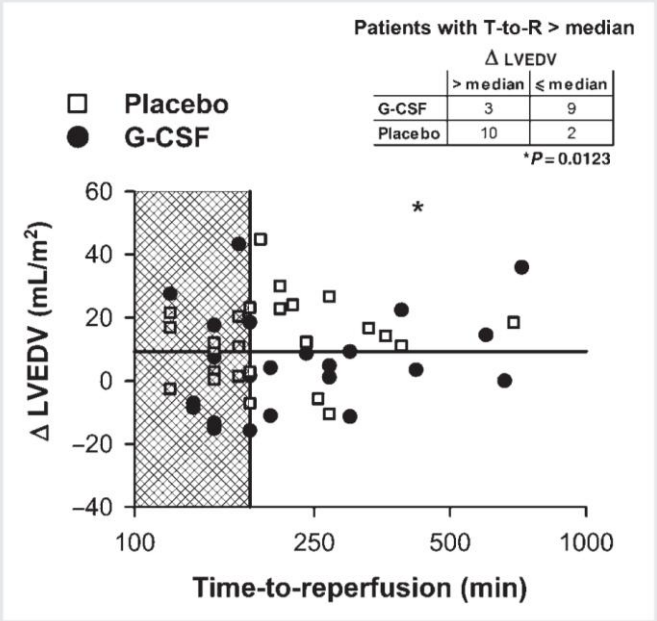



**Figure 5** Scatter plots showing the change in left ventricular end-diastolic volume from baseline to 6 months (Δ left ventricular end-diastolic volume) vs. time-to-reperfusion in the placebo (open square) and granulocyte colony-stimulating factor (solid circles) groups. Solid lines represent the median of Δ left ventricular end-diastolic volume (9.3 mL/$m^2$) and time-to-reperfusion (180 min). *Upper right panel: patients with time-to-reperfusion (T-to-R) >180 min and Δ LVEDV >9.3 mL/$m^2$ ($P = 0.0123$).

**Treatment effect of G-CSF on infarct size**

Infarct size assessed by MRI at 5 days and at 6-months exhibited a reduction of the number of transmural LGE segments in the G-CSF and not in the placebo group (from 4.38 ± 2.9 to 3.3 ± 2.6, $p$=0.04 and from 4.2 ± 2.6 to 3.6 ± 2.7, $p$=0.301, respectively) (Figure 6). An ANCOVA confirmed that the change of the infarct size from baseline to 6-months is significantly affected by G-CSF treatment only, independently from the following baseline variables: pro-BNP, Troponin I, number of CD34+ cells, time-to-reperfusion and Killip score ($p$=0.025).

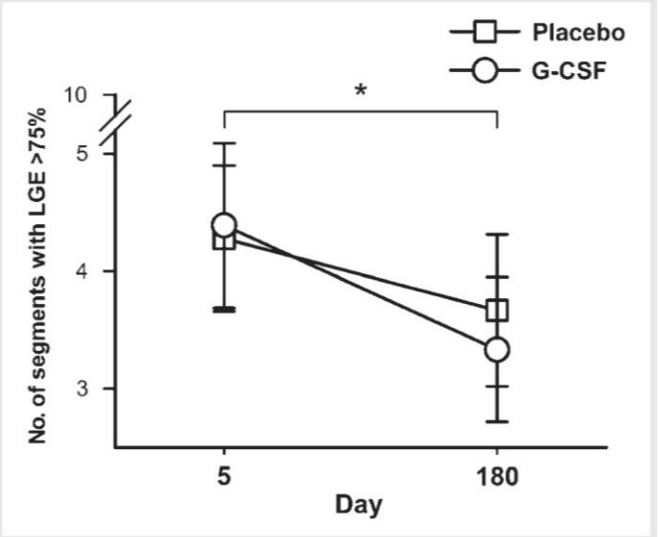



**Figure 6** Change in the mean number of segments with late gadolinium enhancement >75% by cardiac magnetic resonance imaging from baseline to 6 months in the granulocyte colony-stimulating factor and placebo groups. Values of late gadolinium enhancement are median (lines) ± SEM. *Changes of mean number of segments with late gadolinium enhancement >75% for granulocyte colony-stimulating factor ($P = 0.04$).

### Treatment effect of G-CSF on LV perfusion

Perfusion by SPECT in the infarct-related territory, expressed as mean signal intensity (MSI), exhibited a significant and comparable improvement in both groups from day 5 to 6 months (from 47.7 ± 11.1% to 51.4 ± 11.0% and from 49.7 ± 13.5% to 52.9 ± 11.5% in G-CSF and placebo group, $p$=0.005 and $p$=0.008, respectively) (Figure 7).

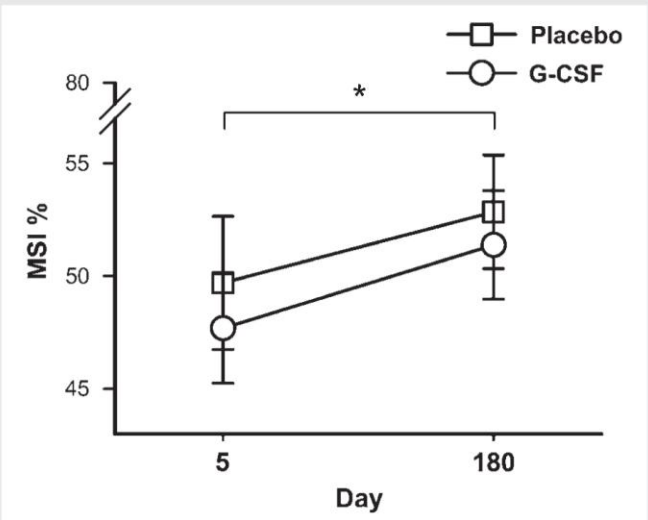



**Figure 7** Change in the mean signal intensity (MSI) in the infarct-related territory by single-photon emission computed tomography imaging from baseline to 6 months in granulocyte colony-stimulating factor and placebo groups. Values of MSI are mean ± SEM. *Changes of MSI for granulocyte colony-stimulating factor ($P = 0.005$) and placebo ($P = 0.008$).

## 4. Discussion

The main finding of this multicenter, randomized, single-blind, placebo-controlled trial was that G-CSF therapy attenuates left ventricular remodelling 6 months after mechanical reperfusion of a large anterior STEMI.

Up to now, evidences available on efficacy of G-CSF treatment for STEMI have been cumulatively disappointing. In a recent meta-analysis, Zohlnhöfer and colleagues [19], including 445 patients from 10 randomized trials, did not find a beneficial effect of G-CSF treatment in patients with STEMI after reperfusion. No differences were found in EF and reduction in infarct size between cytokine treatment and placebo. Similarly, Latif et al. [15] failed to demonstrate any positive effect of G-CSF in EF, infarct size and left ventricular volumes including in their meta-analysis 385 patients from 8 randomized studies. However, patient subgroups evaluation revealed a modest but statistically significant increase in EF with cytokine therapy in trials that enrolled patients with EF <50% at baseline and with relatively early administration of G-CSF after STEMI. The authors concluded that G-CSF therapy may be effective if initiated early after the onset of symptoms and in STEMI patients with LV dysfunction.

The three main characteristics of our study were: anterior STEMI with EF below 45% after reperfusion, symptoms-to-balloon time of 2-12 hours, high-dose G-CSF administration initiated after reperfusion and within a mean of 9.2 hours after

the onset of symptoms. To our knowledge, patient's profile of this trial is unique, since no other randomized study using G-CSF after STEMI has fulfilled these enrolment criteria altogether.

First, it has been stressed that patients enrolled in previous negative trials with G-CSF were at low-risk, with preserved ejection fraction (>50%), small volumes and low percent anterior localization [12]. Notably, the greater benefit in ventricular function recovery after intracoronary delivery of bone-marrow mononuclear cells in STEMI was described in patients with an impaired EF after reperfusion [20,21]. In this regard, our pure population of anterior STEMI with an entry level of EF <45% differs from any previous published trial.

Furthermore, the recovery of left ventricular function after primary angioplasty is well known to be time-dependent, since the major determinant of infarct size and transmurality is early reperfusion [22]. Nevertheless, only two trials with G-CSF in STEMI [7,13] reported, as exclusion criteria for enrolment, a time-to-reperfusion less than 2 hours, although the powerful effect of early reperfusion is clearly one of the main factors that may mask the efficacy of cell-based cardiac repair [23].

Finally, a higher likelihood of improvement in EF was observed when G-CSF administration was initiated early after the acute event [15]. The mean time from PCI to G-CSF administration differs considerably among the published trials [19]. In the FIRSTLINE-AMI trial only cytokine treatment started within 12

hours from PCI [4], similarly to our study. Such an administration strategy promotes a peak of circulating progenitors after 5-6 days from reperfusion [19]. This may be of relevance because a favourable milieu is likely to affect progenitor cell recruitment in the infarct area [24]. Accordingly, the major benefit of EF improvement was noted when intra-coronary bone-marrow-derived cell infusion was administered later than 7 days after STEMI.

In our study, we thus enrolled patients homogeneous in baseline characteristics and at highly probability of unfavourable remodelling. Left ventricular function 5 days after reperfusion, when most of the spontaneous recovery had already taken place [25], was similar in both groups, and significantly depressed. We have observed, at MRI, a marked increase in EDV and ESV during follow-up in the placebo group, whereas the G-CSF group did not experience significant unfavourable remodelling. Further, this difference was found not to be affected by any relevant baseline variable correlated with the acute STEMI. G-CSF, among baseline adjusted factors, is the only variable that appears to influence LV remodelling. Cardiac MRI data were paralleled by those obtained with 2-D ECHO. The analysis of the diastolic function further confirmed a more pronounced amelioration of baseline ventricular dysfunction in the G-CSF population. Interestingly, our results on LV remodelling are similar to those of the trials of Takano et al [10] and Leone et al. [12], which enrolled patients with anterior

STEMI only. The ability of G-CSF to attenuate ventricular remodelling may be of importance for patients survival, since LV volumes are well known to be the main determinants of survival in patients with heart failure. Furthermore, the analysis of the relationship between extent of remodelling and to time-to-reperfusion has shown that the protective effect of G-CSF seems even more relevant when reperfusion time exceeds 3 hours. This data have not been previously reported and can be helpful for the identification of patients who may particularly benefit from cytokine treatment.

Concerning EF, a significant improvement was noted by ECHO early after reperfusion in both groups (from enrolment to day 5), as expected [25]. However, G-CSF treatment group only exhibited a persistent and significant improvement in EF from day 0 to 6-months whereas no such improvement was seen in the placebo group. Further, both MRI and ECHO demonstrated that neither group showed a significant increase in EF from day 5 to 6-months.

Although our data do not support any undisputable physiopathologic explanation of the benefits observed in ventricular dimensions in the G-CSF group, the number of ventricular segments with transmural infarct was found to be significantly reduced in treated patients vs placebo, suggesting that cells homing into the infarct zone may have played a role in reducing scar dimensions through the release of factors promoting infarct healing, as previously observed in animal

models and in humans. [2,27] Another possible mechanism might be related to the cytokine gradient which attracts the mobilized BMC as shown by Lee et al. [28] Moreover, G-CSF is able to directly interfere with the acute ischemic injury by increasing NO bioavailability and reducing apoptosis [12].

In patients with STEMI, CD34+ stem cell mobilization occurs naturally, peaking after 1 week [29]. In our study, early G-CSF administration after STEMI provoked a peak in circulating progenitors in treated patients occurring at day 5, as previously reported [19]. More interestingly, CD34+ levels at baseline in both groups were higher than in any other previous study with STEMI patients. This may be related to the severity of myocardial damage in our population.

Finally, cytokine treatment was well tolerated in this trial. We did not observe during follow-up a higher incidence of clinical and angiographic adverse events in the treatment group with respect to placebo, accordingly with previous reports [30]. Specifically, no differences were noted in target vessel restenosis and stent thrombosis.

**Study Limitations**

The major limitation of our study is the lack of an indisputable mechanistic explanation of the changes in ventricular remodelling between the two groups, although the observed greater reduction of infarct size in G-CSF treated patients may partially explain our results. We also recognize the relatively limited sample size and the relatively short follow-up time, but long-term follow-up is currently ongoing. Further, although a slight unbalance of baseline characteristics between the two groups was present, we were able to exclude that a selection bias could interfere with the effect of G-CSF on ventricular remodelling.

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# ***CHAPTER 5: SUMMARY AND CONCLUSIONS***

## 1. SUMMARY AND CONCLUSIONS

Despite advances in the management of CVDs, they continue to be a major medical problem in western countries. Current pharmacologic and interventional strategies fail to regenerate dead myocardium and are often insufficient to avoid ventricular remodelling. In this context cell therapy is emerging, and in particular stem cell therapy, due to the great potential of these cells.

Besides basic research, aimed at better understanding molecular and biological pathways governing stem cell abilities, some clinical trials have already been performed worldwide. Meta-analysis revealed that, despite exciting preclinical results, the benefit in patients with cardiovascular disease is significant albeit modest. Another relevant point emerging from recent these studies is that it is essential to better identify the specific cohorts of patients.

This is what emerged, for example, by analyzing BMC infusion in AMI *vs.* CMI patients. In fact, it appears that cell therapy is less effective in chronic condition than in the acute disease. Moreover, it is relevant to isolate a specific subset of cells, to findi the best time point for cell infusion, the best cellular

delivery strategy and, finally, to isolate and maintain the cells according to standardized protocols [1] [2].

In this framework we attempted to provide a contribution in three main issues relative to EPC therapy:

1) We established standard operating procedures (SOPs) to purify human CB $CD133^+$ cells using CliniMACS and applied these procedures to obtain BM-derived CD133 cells for an ongoing clinical trial in patients with chronic cardiac ischemia. In this respect, we described a fully GMP-compliant CD133 CBMPs production, quality control process and also provided a demonstration that manipulated cells maintain a full biological potency using *in vitro* and *in vivo* assays [3].

2) We assessed the effect of VPA, a chromatin remodelling agent, to improve human $CD34^+$ cells repair potential in a context of acute myocardial ischemia. Our data showed that VPA-treated cells have an improved myocardial protection ability through epigenetic regulated enhancement of stem cells self renewal capacity. Since VPA is a widely used drug in humans, this protocol has real chances to be adopted to enhance stem cell function and myocardial repair ability in the clinical context.

3) We studied the biological properties of mobilized EPCs in a clinical trial that was designed to evaluate the effect of G-CSF as a non-invasive cell delivery strategy in AMI patients [4]. Our trial suggest that in patients with large anterior STEMI, with EF after reperfusion <45% and a time to reperfusion more than 2 and less than 12 hours, early administration (<12 hours) of high-dose G-CSF may attenuate 6-months unfavourable remodelling. This occurred despite the biological function of the mobilized progenitors was depressed likely due to G-CSF effects on EPC clonogenic activity.

**Concluding Remarks**

The fact that several positive effects observed with cell-based therapy have been attributed to paracrine effects, rather than to direct differentiation of engrafted cells, may seem discouraging (gnecchi). However, cell therapy has been suggested to protect cardiac cells from dying, modulate inflammation, induce angiogenesis, promote endogenous repair and attenuate fibrosis. Therefore, cell therapy may activate the heart's endogenous healing mechanisms [5].

Moreover, as observed by Lipinski and co-workers in their meta-analysis, despite cell therapy after AMI has resulted in a modest, yet significant, benefit, it has to be considered that that even small increase in the heart function may have clinically relevant consequences, and so it could be an important issue. For example, many interventions with an

established life-saving effect after AMI provide a moderate increase in LVEF, but they are of course clinically meaningful [6].

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