School of Medicine and Faculty of Science

PH.D PROGRAM IN TRANSLATIONAL

AND MOLECULAR MEDICINE

DIMET

Differentiation and reprogramming of human mesenchymal stromal cells: insights from epigenetic assessments and preclinical studies

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1.1 Preface

Since 1970 the heart has been considered a post-mitotic organ, unable to guarantee myocyte turnover. In this old view, the total number of cardiomyocytes was established at birth and remained invariable during the organism lifespan. This paradigm has been recently challenged by several evidences showing the existence of small, dividing cardiomyocytes particularly evident after infarction (1). The ability for myocytes to undergo karyokinesis and kitokinesis has become more convincing year after year, arising the question of the origin of dividing cardiomyocytes and suggesting the possibility for the existence of a Cardiac Stem Cell (CSC) pool (2).

1.2 Ischemic Heart Failure and Myocardial Infarction

Myocardial ischemia refers to a lack of oxygen due to inadequate perfusion of the myocardium, causing troubles to the mechanical, biochemical and electrical function of this organ, with an imbalance between oxygen demand and supply . When ischemia is transient, it may be associated with angina pectoris; while when it is prolonged it

can lead to acute myocardial infarction (MI) with the consequences of myocardial necrosis and scar formation.

After MI or ischemia/reperfusion injury, there's an inflammatory reaction that 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, heart failure (3).

Myocardial infarction is the most common cause of death in the civilized world. However, thanks to the improvement in clinical practice, two thirds of the patients after myocardial infarction now survive. The tough problem of these patients after surgery is to face heart failure for the rest of their life. This is a direct consequence of the incapability of the mammalian heart to activate an effective regeneration program due to the myocardium inability to produce a sufficient number of new myocytes.

Cardiac fibroblasts are the main players in infarct healing secreting the collagen that leads to scar formation that finally results in ventricular remodeling and dysfunction. New strategies mainly based on the use of different stem cell types are currently being studied to enhance myocytes and vascular growth, promoting partial restoration of cardiac function after infarction.

1.3 Ischemic Heart Failure Therapy

Current pharmacologic (i.e. ACE inhibitors, diuretics and β-blockers) and interventional (i.e. coronary angioplasty and heart transplantation)

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 therapies. For these reasons the need for alternative therapeutical approach strongly emerged in recent years: cellular therapy have been proposed as the solution for damaged myocardium. Although their use in clinical trials has been limited by their relative inaccessibility and by the difficulty of their *ex vivo* amplification.the first cell type identified as the best potential candidate for cardiomyoplasty has been the resident CSCs. In fact, the feasibility of cellular therapy is influenced by at least three different variables: the efficiency of stem cells recovery from their natural environment; the efficency of *in vitro* propagation; the efficacy of tissue repair after re-injection into the damaged host tissue. An efficient stem cell-mediated-cardiac repair will be then the result of the combination of two different approaches: 1) autologous cell transplantation; 2) activation of resident and circulating stem cells by endogenous cytokine and growth factors.

1.3.1 Stem Cells based therapy

As anticipated the postnatal human heart is unable to restore cardiac function in case of extensive injury. Nevertheless, some recent experimental studies obtained promising results in the therapeutically induced restorative healing of injured myocardium using stem cells.

Currently a variety of stem cell populations are under scrutiny for their capacity to induce cardiac regeneration.

a) *Embryonic Stem Cells:*

The *in vitro* differentiation of ES into cardiomyocytes and other lineages is a spontaneous event which follows the formation of aggregates called embryoid bodies. Members of several growth factor families known to be involved in cardiac mesoderm induction such as Transforming Growth Factor-β (TGF-β), Bone Morphogenetic Proteins (BMPs), FGFs and WNTs, are effective in stimulating the cardiomyogenic differentiation of ES lines and have been employed to commit ES cultures into the cardiogenic lineage (4). Yet the ethical problems raised by the use of embryonic stem cells, along with their *in vivo* tumorigenicity and immunogenicity have currently hampered the enthuasiam about the potentiality of this cells and moved the attention of the scientific world toward adult stem cells.

Although the implantation of different adult stem cell types has been shown to improve cardiac function in MI animal models, no methods however have been established to induce a functional cardiac phenotype in adult stem cells *in vitro* by the use of physiological growth factors or non-toxic chemical compounds.

b) *Skeletal Myoblasts:*

Easily obtainable from biopsies, these cells are differentiation committed, proliferation competent and function as precursors in skeletal muscle. Although their effect in improving ventricular functions in animal experiments has been statistivcally relevant, their

application in clinical trials revealed the risk of developing dangerous arrhythmias (5), probably in relation to the lack of an appropriate electrical coupling between skeletal muscle cells and cardiomyocytes (6).

c) *Bone Marrow Mesenchymal Stem Cells*:

Bone marrow (BM) contains a heterogeneous assortment of stem cells, including hematopietic stem cells (HSCs), and mesenchymal stem cells (BMSC). Remarkably, a global improvement of left ventricular function in patients with BMSC has been shown only in few clinical trials (7). It's important to underline that, as the evaluation of benefits was based on hemodynamic parameters, is still unclear whether the improvement was due to effective myocytes regeneration rather than to increased angiogenesis.

d) *Endothelial Progenitor Cells (EPCs):* EPCs are circulating endothelial progenitors derived from BM and characterized by the expression of the stem cell markers CD34, CD133 and VEGF-Receptor-2 (VEGFR-2). Their ability to improve cardiac function was attributed both to their angiogenic potential and to the induction of myocardial regeneration (8).

e) *Cardiac Stem Cells (CSCs):*

CSCs are considered the most efficient cell type for cardiac repair and have been demonstrated to repair infarcted myocardium in rats and dogs and very recently in humans (9). The identification of regenerating cardiomyocytes after acute (10, 11) and chronic

infarction (12) as well as in failing heart (2), changed, in fact,the dogma of the heart as terminally differentiated organ and supported the view of the heart as a dynamic organ constituted by myocytes that can be replaced. The origin of cycling myocytes has been attributed to cardiac stem cells resident in the heart (10). The first attempt to isolate and characterize the endogenous myocardial stem cell population was made by Hierlihy and co-workers in 2002 (13). They isolated from mouse heart a methylcellulose colony forming population of cells able to extrude Hoechst dye, but unable to differentiate spontaneously into cardiomyocytes. Whether these cells represented a similar population to the so-called Side-Population (SP) isolated from other organs, such as bone marrow and skeletal muscle, is unknown.

One year later Anversa and coworkers published a work where a stem cell pool was isolated from the adult rat heart (10). These cells were found positive for the stem-cell markers c-Kit, Mdr-1 and Sca-1 and negative for the expression of common blood lineage marker (Lin-). Particularly c-Kit+/Lin- cells were self-renewing and, if cultured under proper conditions, they differentiated into cardiomyocytes, endothelial and smooth muscle cells. Most importantly, if injected in situ after coronary legation, they were able to sustain myocardial regeneration, improving ventricular function.

Recently also Sca-1+ cells isolated from mouse heart were shown by Schneider's group able to home into damaged myocardium areas if injected intravenously after ischemia. CSCs expressing Sca-1 represent about the 20% of non-cardiomyocyte heart cells (14). From this fraction, a subpopulation of cells has been isolated for their capacity to exclude the Hoechst dye 33342 (side population of cells,

SP) (14). Together with Sca-1 these cells are positive for the transport protein Abcg2. It is still unknown whether these cells somehow overlap with the Side Population isolated by Hierlihy and colleagues. Moreover, although Beltrami and colleagues excluded fusion between c-Kit+ cells and myocytes part of donor Sca- 1+ cells was demonstrated to fuse with host myocardium cells (14). This aspect is still controversial and requires further investigations.

The isolation and the identification of the primitive cardiac stem cells is complicated by the fact that also more differentiated/committed progenitors, expressing early cardiac markers, such as GATA4 and Nkx2.5, also express stemness markers.

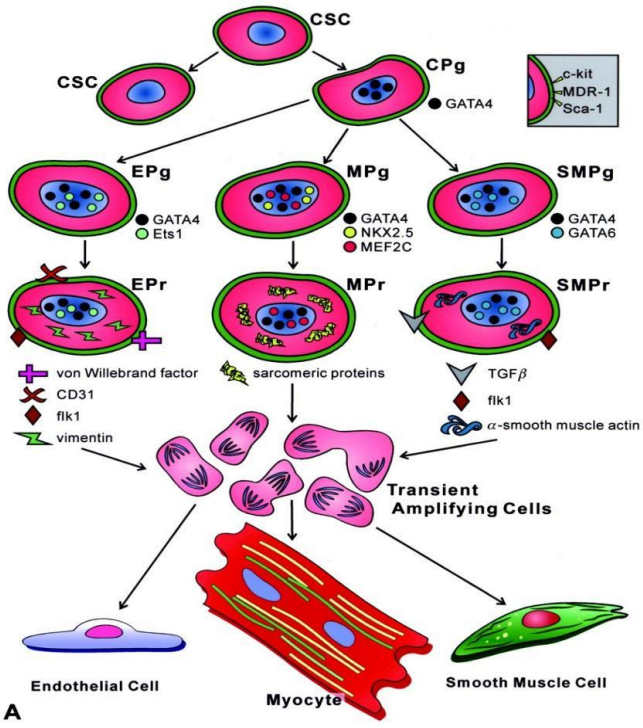

Figure 1. Hierarchy of CSC growth and differentiation

Recently, cardiac progenitors expressing the homeobox gene Islet-1 (Isl-1) have been described in postnatal rat, mouse and human myocardium (16). Isl-1+ cells can be considered cardiomyocyte precursor cells that display the capacity to give origin to fully differentiated cardiomyocytes but it is currently unclear whether they exist in adult hearts.

CSC clusters have been found dispersed through the whole heart, but their frequency appears inversely proportional to the hemodynamical load sustained by the heart region in which they are located. CSC clusters seem to accumulate predominantly in the atria and apex (17).

In summary, different methods of isolation and characterization documented the presence in the heart of several stem cell populations, which have been characterized for the expression of different stem cell markers. Further studies are needed to assess whether they belong to independent pools or represent differentiation steps of the same lineage and also to determine whether technological differences in the detection of a limited number of markers led to unnecessary distinctions. Unfortunately, the use of ES, BMMSC and Cardiac Stem Cells in a therapeutic setting (regeneration of myocardial infarction) have many obstacles, including ethical concerns about the origin of the embryonic material, the immune rejection of the transplanted material and the very poor number of cells reaching the target and actively contributing to regeneration. Alternatively, methods have been developed to “remove” the epigenetic regulation in terminally differentiated somatic cells, thus “reprogramming” these cells toward a more primitive state. The reprogramming of somatic cells may provide a solution to the current obstacles written above. Furthermore,

the reprogrammed cells provide a model to investigate the normal molecular regulation during development and to understand the molecular basis of reprogramming and pluripotency.

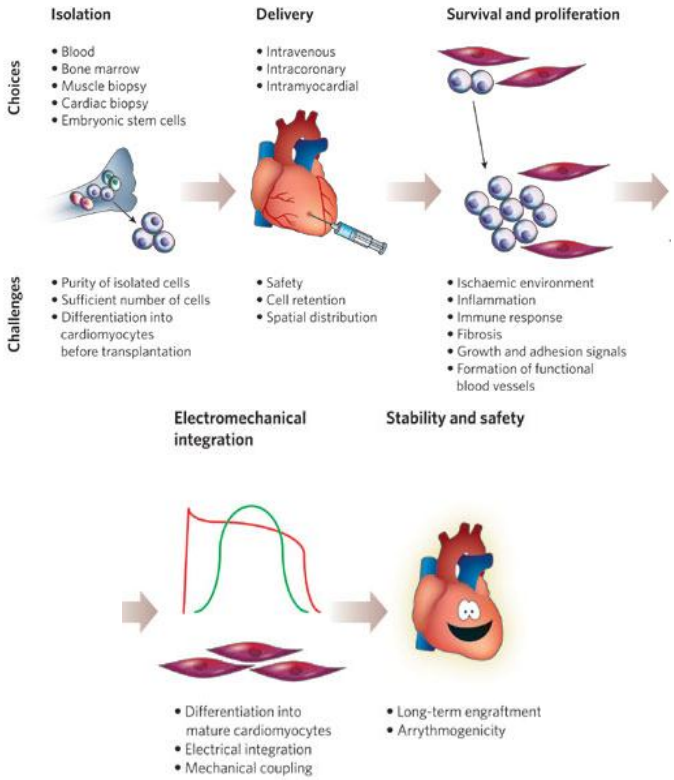

Figure 2. Stem Cell therapy

1.4 Mesenchymal Stem Cells

A stem cell is characterized by its unique ability to self-renew. When self-renewal divisions are symmetrical, the two daughter cells become stem cells, leading to the stem cell pool expansion. During the postnatal life, most stem cell divisions are asymmetrical, yielding one

stem cell and a more differentiated cell, or progenitor cell, which has limited self-renewal ability. The potency of a stem cell is defined by its ability to divide and produce one or many different cell types. The zygote, for

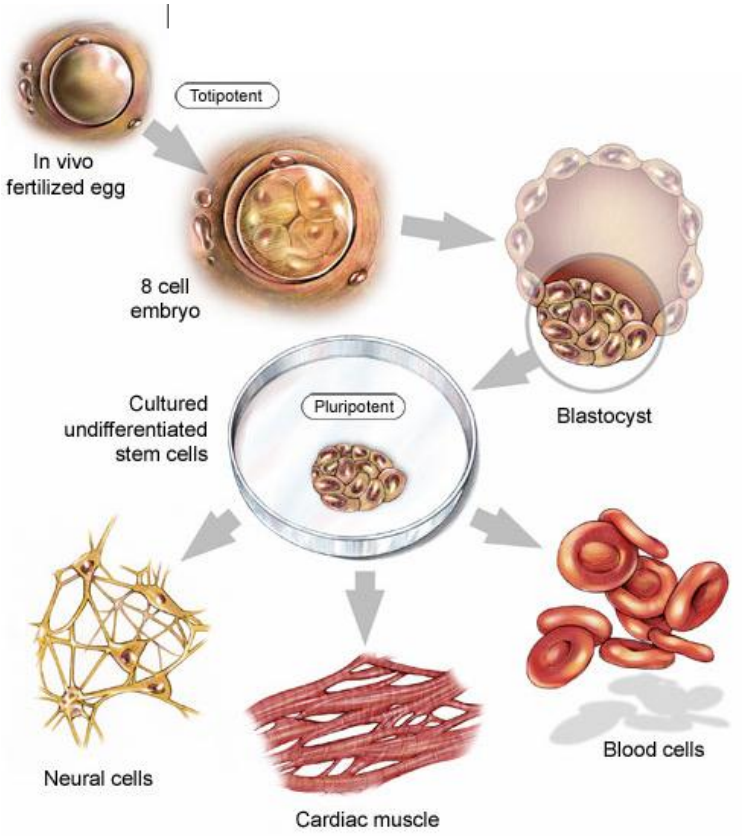

Figure 3. Pluripotency of adult stem cells

example, is totipotent as it can contribute to the cells of embryonic as well as extra-embryonic tissues. An embryonic stem cell (ESC), derived from the inner cell mass (ICM) of the embryo, is pluripotent as it differentiates into all the embryonic cell types,. The most extensively studied adult stem cell is the hematopoietic stem cell (HSC) – isolated from bone marrow (BM) (18, 19), from peripheral

blood following mobilization from the BM or from umbilical cord blood (UCB), which can replenish the entire blood lineage of lethally irradiated organisms upon transplantation. Since the characterization of HSC, many other adult stem cells have been defined, including neural stem cells (NSC) in the hippocampus and subventricular zone of adult brain (20), gastrointestinal stem cells (21), epidermal stem cells (22), among others.

Friedenstein and colleagues demonstrated in 1970 that a population of fibroblast like cells, that can be isolated from BM, formed bone,cartilage and reconstituted the bone marrow microenvironment. These cells were later called mesenchymal stem cells or marrow stromal cells (MSC) (23,24).

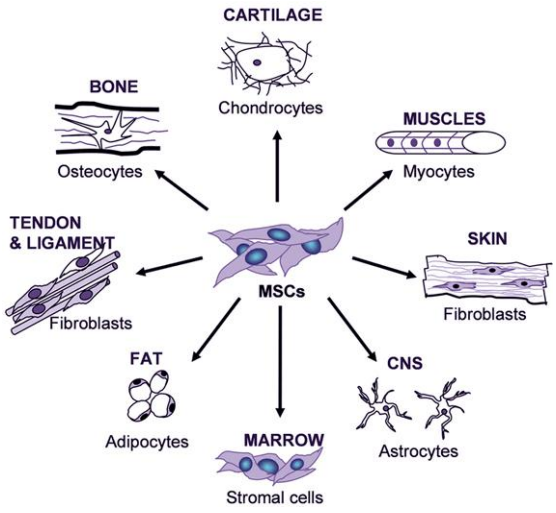

Figure 4. Mesenchymal Stem Cells

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 (25).

The initial interest was encouraged by studies suggesting that these cells could become cardiomyocytes in vitro (26), although the necessity of using inducing treatments such as 5-azacytidine limit the clinical applicability of future strategies. However, several lines of evidence indicate that direct injection of noninduced MSCs into the heart improves ventricular postinfarction recovery in rats and pigs. These experiments shown that MSCs can attenuate the pathological ventricular remodeling (27). Mesenchymal stem cells are also known to produce paracrine growth factors that likely support vascular regeneration and cardiomyocyte protection in the injured myocardium. Moreover these cells 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 verify their properties in preclinical trials. Despite some studies obtained promising results, the clinical application of MSCs still needs to address important questions. Firstly, 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 (28). A second

important point arose from the demonstration that total BM cell injection in a myocardial infarction rat model induced extensive intramyocardial calcifications, while injection of BM multipotent selected cells did not (29)

In a recent study by the Bianco group, it was shown that MSC isolated freshly from BM can recreate bone and the bone marrow microenvironment, from which MSC can again be isolated, demonstrating for the first time that MSC indeed have stem cell properties (28).

Also chorion, amnios and villi are reservoirs of MSC suggesting the hypothesis that MSC from foetal tissues retain higher plasticity compared to adult.

Although it is generally accepted that this type of adult stem cells have restricted differentiation ability, many studies suggested that under some conditions, adult tissue derived stem cells may have broader differentiation potency, also termed stem cell plasticity.

2. Two “dumb” characters

Human heart is composed by many different cell populations including cardiomyocytes, smooth muscle cells, endothelial cells, which are object of investigation in several research laboratories. Surprisingly a reduced attention has been focused on two other populations, which their function is important and not yet completely understood: cardiac fibroblasts and cardiac mesenchymal stromal cells.

2.1 Cardiac Fibroblasts

Fibroblasts are traditionally defined as cells of mesenchymal origin able to produce interstitial collagen. They are associated with the various form of connective tissue where the main role is to maintain Extra Cellular Matrix (ECM) homeostasis, keeping a balance between synthesis and degradation of structural proteins, like collagen and fibronectin. One problem is the definition of the fibroblasts population: the lack of a truly specific marker that has been a limiting factor for studying fibroblasts in tissue sections, while their quick adhesion properties has represented a useful method to prepare virtually pure cultures. Cardiac fibroblasts (cFbs) are the most numerous cellular component of the heart. Cardiomyocytes occupy approximately 75% of cardiac volume, but they only account for 30-40% of cell number. The majority of remaining cells are fibroblasts, the endothelial and vascular smooth cells representing smaller populations (30) . In adult and healthy heart every myocyte is virtually in contact with at least one fibroblast. One of the main role for cFbs is

therefore to maintain optimal spatial placement of contractile myocytes, synthesizing collagen and other ECM component necessary for efficient contraction in health.

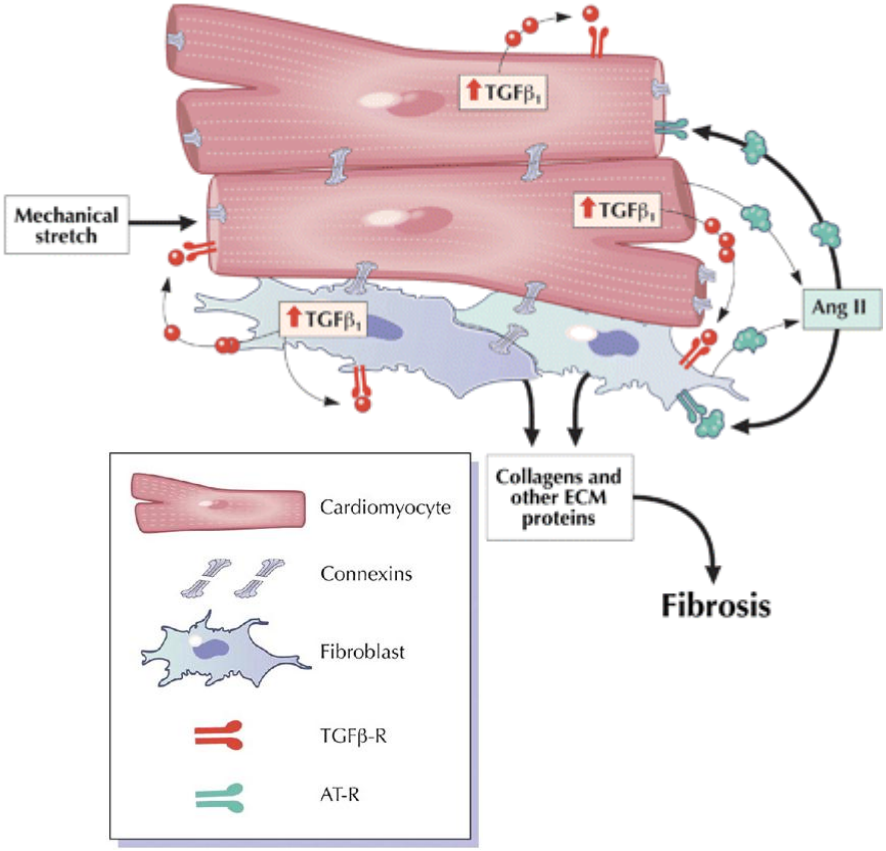

Figure 5. Connection between fibroblast and cardiomyocytes

The classical idea of cardiomyocytes and fibroblast forming *in vivo* two isolated network with only ECM as contact point has recently been challenged by a report showing the existence of functional gap-junctional coupling between cardiomyocytes and fibroblast in rabbit sino-atrial node (31). Nevertheless nothing is known about the myocyte/fibroblast coupling in ventricle and even if co-cultured

neonatal rat cardiac myocytes and fibroblasts readily form functional gap junctions (32), the presence of functional gap junction between myocyte and non-myocyte cells *in vivo* is still on debate (33).

Thanks to the reports quoted above, studies of cFb role in cardiac physiology are currently receiving more interest and there are growing evidences of cFb contribution not only to structural but also to biochemical and electrical properties of the cardiac muscle.

cFbs are notably efficient mechano-electric transducers. They sense mechanical stress via integrins, ions channels and second messenger pathways, which activate ion channels permeable to Na^+, Ca^{2+} and K^+ and result in a net change in the membrane potential (34).

This mechanism, along with the possible direct gap-junctional communication with cardiomyocytes, makes fibroblasts an active component of cardiac electrophysiology. Recently Urbanek and co-workers (35) have also shown the existence of functional gap junctions between cardiac stem cells and fibroblasts, suggesting for cFbs the role of nurse cells in the cardiac stem cells niche.

2.2 Cardiac Stromal Cells (CStCs)

Stromal cells can be isolated from a variety of adult tissues and organs (36), the most extensively characterized being those of bone marrow origin, called bone marrow mesenchymal stromal cells (BMStC).

Although BMStC are currently being evaluated for their capacity to regenerate both skeletal tissues and unrelated tissues, such as the

heart, up to now, poor attention has been given to the therapeutic potential of cardiac-specific stromal cells.

Recently has been published that both *in vitro* and *in vivo* cardiac stromal cells (CStC) isolated from specimens of adult human auricles were more oriented towards the cardiovascular phenotype compared with BMStC. In spite of a remarkable similarity in growth, morphology, and immunophenotype, CStC and BMStC differed significantly in gene, microRNA (miR), and protein expression and exhibited tissue-specific responses to differentiating stimuli. Notably, CStC revealed higher efficiency in the acquisition of a cardiovascular phenotype both *in vitro* and following intramyocardial injection in a rat model of chronic myocardial infarction (MI). Furthermore, CStC persisted longer within the tissue, migrated into the scar, and differentiated into cardiomyocytes more efficiently than their bone marrow counterpart.

In a recently published study by Rossini et al. (37) from our laboratory, the unselected population of CStC has been analysed in comparison with BMStC derived from the same patient and examined whether they could be more oriented to reconstitute cardiac tissue than bone marrow cells.

Regarding mesenchymal-associated antigens expression (i.e. CD105 and CD44) CStC and BMStC are remarkably similar and revealed positivity for both, pericytes (i.e. CD146) and fibroblast markers (i.e. vimentin and human fibroblast surface antigen). This finding is not surprising considering that human bone marrow mesenchymal cells have recently been reported to show a broad structural and functional overlap with dermal fibroblasts (38). Importantly, skin fibroblasts

also differentiate into osteocytes and adipocytes (39) and show immunoregulatory properties (38). On the other hand, marrow mesenchymal cells *in vivo* exhibit fibrogenic potential (40). Therefore, based on phenotypical criteria, it is difficult to clearly discriminate between fibroblasts and mesenchymal cells (41) of cardiac and bone marrow origin. Taking in account these considerations in the present study we defined these cells as 'stromal cells'. Nevertheless significant differences between CStC and BMStC emerged at the biological and molecular level: CStC were, in fact, less competent than BMStC in producing adipocytes and osteocytes but more efficient in the expression of cardiovascular lineage markers.

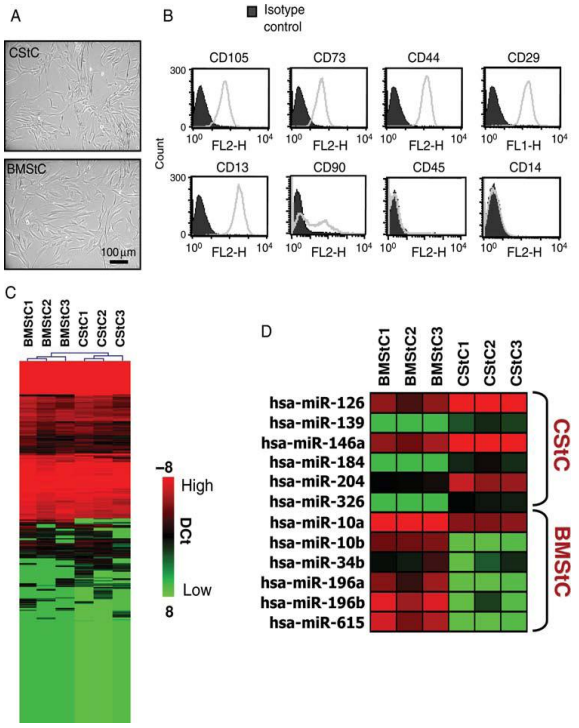

Figure 6. *In vitro* molecular characterization of CStC and BMStC.

In addition, CStC and BMStC significantly diverged at transcriptional level. In fact, CStC expressed early (GATA4) and late (MYLC-2a) cardiac mRNAs and were identified by a specific miR signature unmodified by differentiation stimuli, suggesting that the expression of these miR is only minimally affected by the extracellular environment. A growing body of evidence indicates that many miR play important roles during development (42) highlighting their causal role in establishing cell fate. Consequently, not only miR represent biomarkers useful to discriminate between stromal cells of cardiac or bone marrow origin, but they may also be involved in establishing their tissue-specific plasticity and therapeutic properties.

Notably, some omiRs up-regulated in CStC (miR-126 and -146a) are highly expressed in proliferating cardiomyocyte precursors isolated from the foetal human heart (CMPC (43)), suggesting potential analogies between CMPC and CStC. miR-126 is also known for its role in angiogenesis and angiogenic response (44).

The results obtained transplanting CStC/BMStC in the border zone of rat chronic MI are in line with prior works, describing that the injection of skin fibroblasts (45) bone marrow cells (37), and ventricular cells (46) ameliorates the performance of infarcted hearts.

In the work of Rossini et al. has been demonstrated that CStC not only improve cardiac function, but participates to tissue regeneration more efficiently than their bone marrow counterpart. Importantly, CStC persisted longer within the cardiac tissue and migrated within the scar, promoted angiogenesis, and differentiated into adult-like cardiomyocytes more efficiently than BMStC.

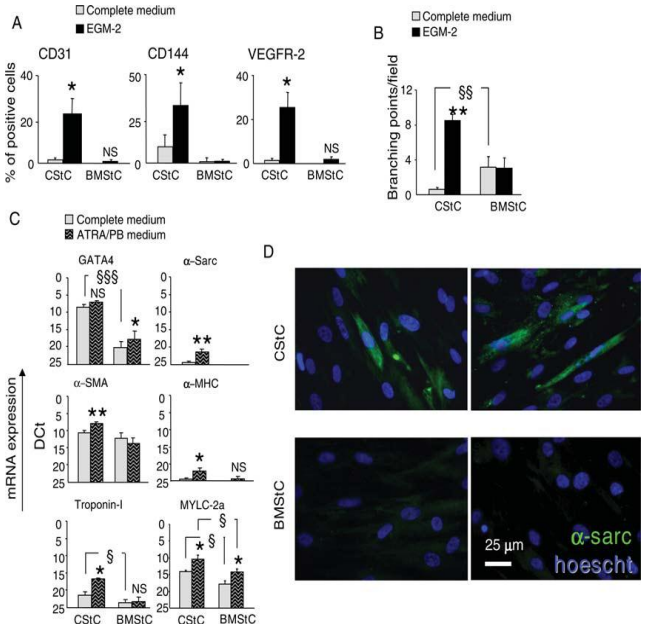

Figure 7. In vitro endothelial and cardiomyogenic differentiation.

In vivo, the evidence in favour of CStC/BMStC differentiation into endothelial/smooth muscle cell lineages was minimal, suggesting that paracrine mechanisms had a higher importance than direct differentiation in the formation of new vessels. The observation that CStC production of IL-6 and LIF was higher than that of BMStC is in agreement with their more efficient ability to promote angiogenesis within the infarcted region (47).

Interestingly, only in CStC-derived cardiomyocytes, a clear evidence of sarcomere formation and volumetric dimensions compatible with those of adult cardiomyocytes could be observed.

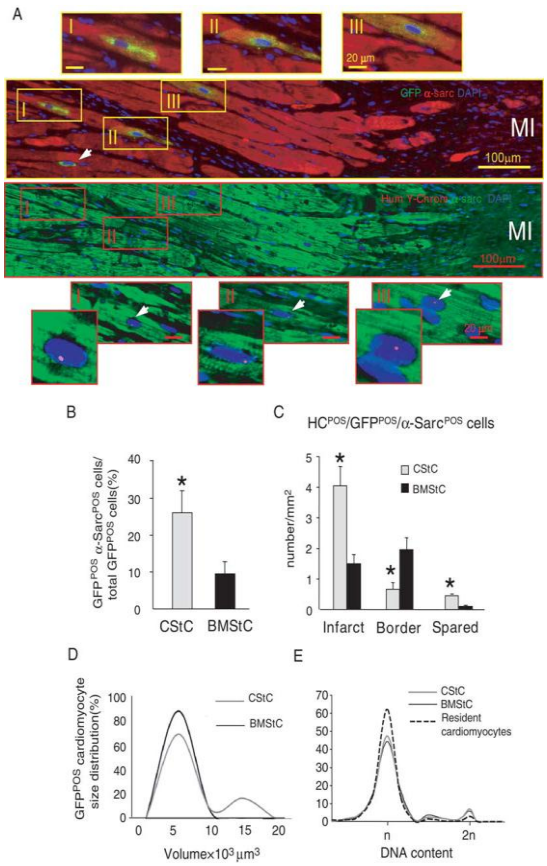

Figure 8. Human CStC and BMStC differentiation in vivo.

The observation that the cardiomyogenic response induced *in vitro* from CStC/BMStC is a very rare event is in agreement with recent reports (48) and at least in part attributable to the fact that our limited knowledge about the mechanisms by which cardiomyogenic differentiation occurs makes it difficult to define standardized *in vitro* efficient differentiation protocols. These findings are only apparently in contrast with the relative high efficiency of CStC in generating cardiomyocytes *in vivo*, as the environment of the healing infarct is likely more prone to sustain tissue-specific differentiation than unspecific epigenetic agents (49).

In conclusion, cardiac-derived stromal cells appear to be a population of potentially relevant clinical interest, being able to acquire the cardiac phenotype and to promote angiogenesis via paracrine mechanisms more efficiently than their bone marrow counterpart, thus indicating the importance of tissue specificity when planning for cell therapy treatments.

2.2.1 CStCs: a new population for cell therapy?

In this study a special attention has been given to the cardiac stromal cell population because CStCs revealed, as described extensively above, high level of plasticity, exerted many known and yet unknown functions, and can be simply isolated from human auricles. The further characterization of CStCs will be then the main topic of this PhD thesis including how it could be possible manipulating them to generate cardiovascular precursors *in vitro* to potentially implement their efficacy and impact in future clinical applications.

3. Cell Plasticity

The standard model of hierarchical and unidirectional cell development and differentiation was determined in early experiments where cells from a part of the embryo were transplanted to another part where they adopted the terminally differentiated state appropriate to the new position. More recent molecular biology studies provided the mechanistic underpinning these observations. In fact, it was demonstrated that progressive repression of gene expression was required for the opening of the new differentiation pathway (50).

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

In brief, the cytosine methylation pattern 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 and maintained at each DNA synthesis cycle.

In mammals, the 80% of DNA methylation occur in the so called CpG islands, often located at promoter sites, and generally leads to restriction of DNA accessibility by transcription complexes thus preventing gene expression.

Histone tails modification is another relevant mechanism by which gene expression is regulated. There are different histone modifications including methylation, phosphorylation, ubiquitylation, sumoylation and acetylation. These post-transduction modifications may occur on different residues with various consequences on gene expression, and the combinatorial effect of all the modifications present in a specific

genomic locus may have a very important effect on its expression. For these reasons, the existence of a histone code superimposed to the standard genetic codehas been recently proposed.

Conrad Hal Waddington brightly depicted the embryonal 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 its 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) (51). It is evident from the picture that the ball cannot grow up the hill, underlying the belief of that differentiation is an irreversible process. Nevertheless, in 1985 this hierarchical model was challenged by the seminal experiment of H. Blau and colleagues. They placed the nucleus of a terminally differentiated cell into the cytoplasm of a different differentiated cell and observed that the donated nucleus was able to change the gene expression pattern of the recipient cell. This led Blau to suggest that differentiation is an actively maintained state rather than a rigidly programmed state acquired during embryonic development (52). About ten years later the publication of "The plasticity of the differentiated state" defined stem cell plasticity as the breakthrough of the year. In fact three original manuscripts demonstrated as bone marrow cells could become skeletal muscle and liver, and neural stem cells could give rise to blood when transplanted in lethally irradiated 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 (53)". This new idea of cell development culminated with cloning embryo experiments which began with the cloning of of the sheep Dolly which demonstrated that epigenetic modifications were completely reversible.

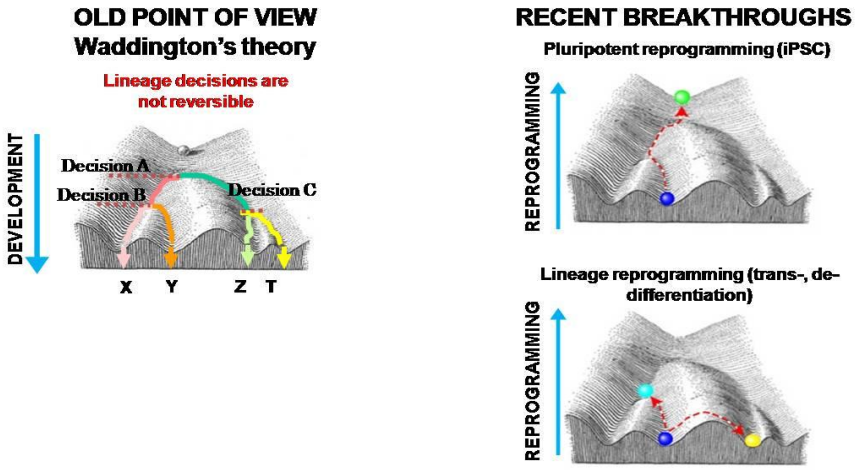

Figure 9. Waddington's theory.

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

1- Standard cell lineage differentiation. All the canonical lineages are generated through plasticity events occurring at defined steps during development, i.e. cells of one type give rise to cells of another and more mature type, althoigh functionally anf phenotypically closely related.

2- Common plasticity. Cell of one lineage become cell of a different lineage apparently going across embryonic tissue barriers. This

occurrence implies 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 the target cell becoming tetraploid. This new cell type may be bi-nucleated or have one nucleus following a nuclear fusion event (54).

3.1 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 phenomena. These alternatives comprise:

A. Trans-differentiation. A stem cell could potentially contribute to cell types of different lineages at the same time.

B. De-differentiation. De-differentiation 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 for tissue specific SCs. These rare cells could be responsible for unexpected phenomena.

E. Cell fusion. Fusion is a common phenomenon in cell development (for example, fusion between sperm and the egg, or fusion that

naturally happens in the generation of multinucleated skeletal myofibers from myoblasts). For this reason various authors think that fusion is a different event, distinct from true plasticity (55).

Considering all these alternatives, a true plasticity event must be demonstrated by clonal analysis, using minimally manipulated cells, excluding cell fusion, and the results must be independently replicated by other laboratories. On the basis of these criteria, Wagener concluded that no presently published study exists which demonstrate true plasticity (55).

Terminally differentiated cells maintain 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 in this direction is given by work of 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) (56). 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 (51). Moreover, two recent works have demonstrated the direct reprogramming of fibroblasts into functional neurons (57) using Ascl1, Brn2 and Myt1, and into functional cardiomyocytes (58) using Gata4, Mef2c and Tbx5. 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 (51).

In conclusion, recent evidences suggest that an expansion of the traditional view of a stem cell is to be considered: staminality does not belong necessarily to a specific cellular entity, but may be rather a function that can be assumed by numerous diverse cell types.

3.2 Epigenetics

The term epigenetics was coined in 1942 by C.H. Waddington in the context of studies on development. Since then, the meaning of epigenetics changed over time. In the beginning, epigenetics was viewed as a phenomenon above and beyond genetics. Epigenetic explanations were invoked when genetics could not explain a phenomenon. From the mid-seventies, the state of understanding started changing, as previously described. Epigenetics has now morphed from a phenomenon to a branch of science whose molecular underpinnings are well understood. The early history has also been emphasized in order to underscore the transformation of the science of epigenetics from a phenomenon to a modern field of intense research.

3.2.1 Induced Pluripotent Stem Cells (iPS)

Due to their wide potency, human embryonic stem cells (hESCs) could be useful to treat a multitude of diseases. However, there are ethical difficulties regarding their use, as well as the problem of tissue rejection following transplantation in patients. Yamanaka and colleagues sought to solve these issues by generating pluripotent cells directly from the patients' own cells. 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 (59) and human fibroblast cultures (60). They called this new cell type "induced pluripotent stem cells", or iPS. Nevertheless, cMyc is an oncogene and the reactivation of cMyc retrovirus could induce tumorigenity. In 2008 they modify their protocol demonstrating that cMyc is not essential (61). Recenlty, iPS were generated with only 2 factors (62) or just with Oct4 (63) from umbilical cord blood (UCB) or neural stem cells (NSC). These papers showed that reprogramming an adult stem cell to a pluripotent state is easier than reprogramming a mature differentiated cell. Notably, iPS generation can be improved using HDAC inhibitors, such as Valproic acid (VPA) (64) or Butyrate (65). Although iPS and ESCs are very similar, some relevant differences, however, still exist, especially comparing ESCs to early passages iPS. These differences were found in gene and miRNA expression and also at apigenetic level (66). In fact, chromatin immunoprecipitation in combination with microarray experiments revealed that trimethylation levels of lysine 27 on histone 3 (H3K27, providing repression signals) and H3K4 (underlying transcriptional activation) were similarly distributed, while trimethylation on H3K9 was different. Further significant differences were detected at the level of DNA methylation, and reprogramming of female human fibroblasts failed to reactivate the somatically silent X chromosome (67).

In theory iPS may have a large variety of applications. It is possible, in fact, to obtain mature cells directly from patient's biopsies for autologous tissue transplantation. Moreover, iPS could be used as an *in vitro* model to study differentiation in normal or pathological

conditions and to study how to treat genetic defects. They also could be used to study drugs toxicity, pharmacological targets and therapeutic potential. Functional cardiomyocytes (CMs) were derived from murine and human fibroblasts (65, 68). They were phenotypically and electrophysiologically similar to CMs derived from ESCs or to fetal CMs. 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 infarction (69).

In 2010 Ieda at al. further expanded this field by directly generating functional cardiomyocytes (the so called iCM) from murine fibroblasts with 3 factors: Gata4, Mef2c and Tbx5 (GMT). This work may have important 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 transduced. Third, this work opened the route to the possibility to introduce the defined factors directly into the heart to reprogram endogenous fibroblasts (58).

Despite these positive results, it is important to notice that viruses, largely used in these studies as delivery agents, 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, several laboratories are actively working on reprogramming methods independent on integrating viral vectors, such as non-integrating adenoviruses or small molecules, which could partially or completely replace the reprogramming factors (70).

4. “The New World”: Small Molecules

Reprogramming somatic cells into pluripotent cells that closely resemble ESC by introduction of defined transcription factors holds great potential in biomedical research and regenerative medicine (59, 60). A lot of different strategies have been developed to generate iPSCs with fewer or not exogenous genetic manipulation, which represent a major problem for iPS applications. With the ultimate goal to generate iPS with a defined small molecule cocktail, substantial effort have been put in identifying chemical compounds that can functionally replace exogenous reprogramming transcription factors and/or enhance the efficiency and kinetics of reprogramming (71,72).

The development and use of small molecules as the sole therapeutic method of choice may of course not be coincidental. Their advantages however, are compelling: A), the current regulatory body remains aligned with that ruling the discovery and development of small molecules as therapeutics; B) Small molecules may be easily manufactured, stored and administered; C) from a biochemical perspective their effects are specific, dose-dependent, rapid and reversible. Small molecules also offer a distinct advantage in development; chemical synthesis of compounds based on biologically active molecular ‘scaffolds’ represent very effective tools to quickly generate large ‘libraries’ of additional small molecules suitable for further development.

In using small molecules to study and address problems in stem cell biology, two distinct approaches can be taken: in the hypothesis-driven approach, prior knowledge of signaling pathways and their

small molecule modulators is used to implicate them in key regulators areas. Conversely, the discovery-driven approach assumes no prior knowledge of the regulatory pathways, and instead uses unbiased high-throughput screening of small molecule libraries to elicit a certain phenotype. For example, using small molecules will be possible to elucidate stem cell signaling niche (focusing the attention on E-cadherin and Rho-ROCK signaling and molecules influencing these pathways), to obtain a directed differentiation of ESCs (avoiding the problems of efficiency, speed, reproducibility and of course therapeutic uses), to modulate the behavior of tissue-specific stem cells (for example regulating GSK3 and Wnt pathway).

Many mature tissues harbour rare populations of multi- and oligopotent adult stem/progenitor cells. These lineage-restricted precursors can play critical roles in tissue homeostasis and, more importantly, the regenerative process following injury and disease.

These qualities make adult stem cells excellent candidates for cell-based therapy approaches, provided their potential can be harnessed as required. This approach was pioneered decades ago with the advent of bone marrow transplants, of which haematopoietic stem cells (HSCs) are the critical component. However, subsequent attempts using other cell types in various organ/tissue systems have typically not met with the same level of success. Aside from issues relating to the histocompatibility of allogeneic cell transplants, difficulties in determining the cell type-specific requirements for the *ex vivo* survival and proliferation of adult stem cells have universally delayed progress. Fortunately, phenotypic screens for compounds mediating survival, proliferation and differentiation of these cells have yielded promising

results. Ultimately, this research may lead to the development of drugs that either facilitate *ex vivo* manipulation of adult stem cells or enable/enhance the regenerative process by exerting precise control over these cells *in vivo*. An excellent example of current drug-development efforts to underscore the immediate clinical relevance of adult stem cell studies that use small molecules is cited below.

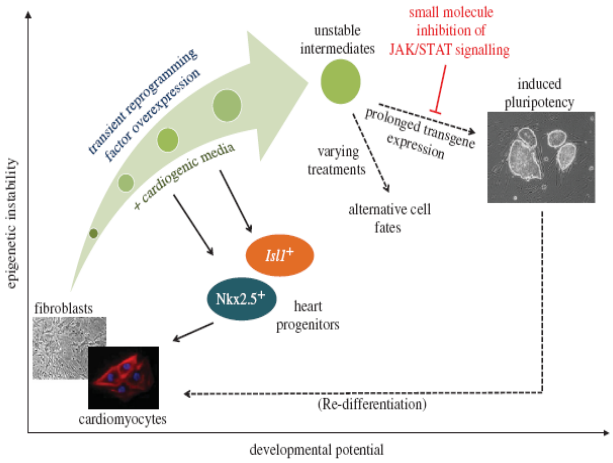

Figure 10. A new transdifferentiation paradigm.

Three years ago, Ding's laboratory performed a study on new small-molecules controlling cardiac precursor cell proliferation and differentiation (73). At this time, it was known that multi-potent Isl1+ cardiac precursors could be expanded in co-culture with cardiac mesenchymal cells following their isolation from post-natal hearts. In order to gain a better understanding of the extracellular signals regulating this expansion, they used genetically-marked (LacZ) Isl1+ precursors in a screen of approximately 15.000 compounds.

They identified three small molecules that could dramatically expand this small population, among which 6-bromoindirubin-30-oxime (BIO) stood out as a known inhibitor of GSK3 and hence a positive

regulator of the canonical Wnt pathway (74). Treatment with BIO reproducibly induced a greater than sevenfold increase in Isl1+ cell number. In fact, the proliferative effect of BIO, and hence that of the canonical Wnt pathway, appear to be conserved in humans.

Small molecules may, indeed, have the potential to functionally replace the Yamanaka factors and to improve speed and efficiency, minimizing the potential for acquisition of undesirable traits in culture, and moreover their use can help to establish a new trans-differentiation paradigm.

They represent a highly desirable alternative to genetic modification in reprogramming and trans-differentiation: chemical reprogramming would be a new frontier in the stem cell biology field applied to translational medicine.

Finally, 'chemical reprogramming' would be easier, more effective and much safer from a disease treatment perspective. This can be the "era" of the small molecules: in fact they may be the center of a profound paradigm shift, like the discovery of antibiotics, many decades ago, uprising the scientific world and not only. They might be considered small in the molecular realm, but their impact on humankind can be huge.

5. Perspectives

The presence of endogenous cardiac progenitors in the rodent adult heart has encouraged several studies into whether similar populations exist in the human adult heart. As was demonstrated in rodents, a heterogeneous population of cardiac cells defined by the expression of the primitive stem cell markers c-Kit, MDR1, or a Sca-1–like epitope has been also found by Anversa laboratory in human cardiac specimens from patients with aortic stenosis,(75) myocardial infarction,(76) and in the postmortem hearts of patients who had undergone cardiac transplantation (76).

Messina et al described the isolation of a heterogeneous population of cells from human atrial and ventricular surgical biopsy specimens that form clonal, multicellular spherical clusters in suspension culture termed “cardiospheres” (77). Cardiospheres contain cells positive for c-Kit, Sca-1, and the KDR (kinase insert domain receptor), are capable of long-term self-renewal, and appear to give rise to both endothelial and smooth muscle cell types (78, 79). When cocultured with neonatal rat cardiomyocytes, they form beating clusters.

Chien’s laboratory has reported the discovery of a “master” heart progenitor cell, marked by the expression of Islet-1, in the mammalian heart, which play a critical role in generating diverse cardiovascular lineages during cardiogenesis (16).

To date, there is no consensus on the best marker for identification of adult cardiac stem cells, and the molecular mechanisms promoting their self-renewal and differentiation into the various lineages of the heart are largely unknown. Ultimately, in vivo lineage tracing studies

in animal models will be required to validate any putative cardiac stem/progenitor cell population both during normal aging and in the setting of cardiac injury.

To play a significant role in the field of cardiac regenerative medicine, cardiac progenitor cells need to be recruited or transplanted to the site of injury in sufficient numbers and directed to differentiate into fully mature and functional cardiomyocytes.

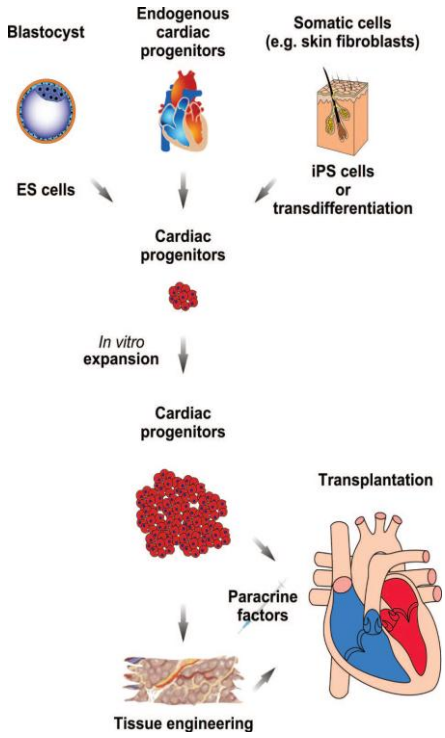

Figure 11. Strategies for delivering cardiac cell therapy

Although the identification of human cardiac progenitor cell-specific surface markers would help to overcome this issue, to date, no single surface marker has been identified that readily distinguishes cardiac

progenitors from other differentiated progenies or their pluripotent precursors.

One solution to overcome this challenge is to directly reprogram exogenous cells such as fibroblasts to a relatively restricted mesodermal or cardiac-restricted progenitor state through induced expression of a defined set of cardiogenic transcription factors. This strategy has already been used successfully to generate pancreatic cells (79), functional neurons (80) and cardiomyocytes (81). Induced expression of two cardiac transcription factors, Gata4 and Tbx5, has been used to direct mesoderm to cardiomyocytes (82), and the combination of Gata4, Tbx5, and Mef2c can direct reprogramming of cardiac or dermal fibroblasts into cells that very closely resemble adult Cardiomyocytes (81). A new system to increase the amount of cardiovascular precursors in a sufficient number to use in a putative cell therapy could be the epigenetic reprogramming of CStCs toward more mature and functional cardiovascular precursors in the presence of epigenetically active drugs.

The re-injection of an adequate number of reprogrammed cells in a mouse model of myocardial infarction or hind limb ischemia will provide new clinical perspective for an efficient cardiac cell therapy.

6. Aim of the study

During my PhD course in Translational and Molecular Medicine at DIMET – Bicocca University, I've worked in the Laboratory of Vascular Biology and Regenerative Medicine at the Centro Cardiologico Monzino-Milan. One of the major focus of the Laboratory is to transfer basic research from bench to bedside.

In this view we have recently isolated and characterized two mesenchymal stromal cell populations: the first one in the heart, that we called Cardiac Stromal Cells (CStC); the second one isolated from first trimester human chorionic villi (CVStC). The full characterization at molecular, biological and physiological level of CStCs and CVStCs will clarify the mechanisms of differentiation and plasticity of these populations and their possible clinical application. Specifically during my my PhD studentship the following objectives were pursued:

1. CStC reprogramming into functionally active cardiovascular precursors via epigenetic interventions by using defined small molecules;
2. CVStCs characterization which exhibited restricted plasticity oriented toward the endothelial lineage.

Both cell populations appear a promising biological source suitable for cell therapy applications in vascular diseases.

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Chapter 2: Epigenetic Reprogramming of Isl1+ Cardiac Mesenchymal Stromal Cells into Functionally Competent Cardiovascular Precursors

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1. Introduction

Cellular cardiomioplasty is a promising therapy to reconstitute injured hearts. Cell based interventions aimed at structurally regenerating the heart imply that transplanted cells graft in the host tissue and adopt the phenotype of dead resident cardiomyocytes, endothelial cells and smooth muscle cells. In this light, cells possessing pluripotency, such as embryonic stem (ES) cells and the so-called induced-pluripotent stem cells (iPS) [1] may be considered a better candidate than adult stem/progenitor cells. In fact, adult cardiac stem cells are well hidden in the heart, which requires sophisticated and time consuming procedures for cell isolation and expansion to therapeutically effective numbers [1]. Furthermore, cardiomyocytes may be relatively simple to obtain from ES and iPS cells [2, 3], but are difficult to obtain ex-vivo from adult stem cells. Yet, although several attempts have been done to simplify iPS cell generation methods avoiding the undesired effect of neoplastic transformation [4], their use still raises safety concerns. Consequently, much effort has been posed in overcoming problems associated with the use of adult cells. Intriguingly, a recent work has shown the possibility to directly convert neonatal or embryonic mouse fibroblasts into cardiomyocytes by transcription-factor based reprogramming strategy [5]. In spite of its potential practical relevance, the efficiency of this procedure is low and genetic manipulation of target cells is still required. In this context, chemical strategies based on the use of small active molecules represent an easier, more effective and safer alternative to genetic methods [6]. Further, achieving terminal differentiation of adult somatic or stem cells into cardiomyocytes may not be the right therapeutic approach,

as multiple cell types (i.e. cardiomyocytes, vascular cells and fibroblasts) should be generated to rebuild damaged heart tissue. In this perspective, reprogramming of adult cells into progenitors, which are less de-differentiated than iPS cells and exhibit lineage commitment restricted to the cell types of interest, appear as a potentially successful strategy.

In addition to adult cardiac stem cells, a different category of heart cells, namely cardiac mesenchymal stromal cells (CStC) deriving from the cardiac parenchyma, have been recently isolated and characterized by our group [7]. These cells are Islet1 positive (this manuscript), c-Kit negative, present surface markers typical of the commonly defined cardiac fibroblast compartment, are easily obtained from small bioptic specimens and may be efficiently grown in vitro. Importantly, CStC exhibit a residual plasticity toward the cardiovascular linage and possess the ability to contribute new cardiomyocytes after heart ischemia [7].

A large number of evidences indicate that biological response modifiers, including epigenetically active small molecules, may redirect adult cellular functions toward stemness [6]. So far no reports, however, described the *in vitro* enhancement of adult cardiac precursors via a defined pharmacological intervention.

In this manuscript, we describe the properties of CStC chemically reprogrammed toward more mature and functional cardiovascular precursors by means of nutrients deprivation in the presence of epigenetically active drugs. Remarkably, retinoids, phenyl butyrate and nitric oxide have been used in the past to stimulate in vitro cardiomyocytes production in different experimental contexts [8-10],

but never combined together on cells isolated from human adult hearts. These compounds have different mechanisms of action including nuclear receptor activation (retinoic acid) [11], inhibition of histone deacetylases (phenyl butyrate) or apoptosis prevention and miRNA up-regulation (Deta/NO) [12] which synergize determining the generation of functionally competent cardiovascular precursor cells.

2. Materials and methods

CStC isolation and culture

Right auricle was obtained from donor patients undergoing cardiac surgery after Local Ethics Committee approval and signed informed consent in accordance with the declaration of Helsinki. CStC were isolated from right auricles and cultured in growth medium (GM) as previously described [7]. CStC at passages 4-8 were incubated for 7 days with Epigenetic cocktail (EpiC), composed by IMDM, 5% Foetal Bovine Serum, Penicillin/Streptomicin (Sigma Aldrich), L-Glutammin (Sigma Aldrich), All trans Retinoic Acid (5 μM), Phenil Butyrate (5 μM), Deta/NO (Sigma Aldrich) 200 um. EpiC medium was changed every 48 hrs.

Western Blot Analysis

CStC in Control and EpiC media were lysed with Laemmli buffer in presence of protease and phosphatase inhibitors (Roche Diagnostic). Proteins were resolved by SDS–PAGE, transferred onto nitrocellulose membrane (BIO-RAD), and incubated overnight at 4°C with antibodies listed in Supplementary Table 1. Subsequently, the appropriate anti-rabbit, anti-mouse or anti-goat horseradish peroxidase-conjugated secondary antibody (Amersham-GE Healtcare) was used for incubation. ECL and ECL plus (Amersham-GE Healthcare) were used for chemiluminescence detection. Each filter was also probed with anti β-actin (or anti-β-tubulin, anti-H3, anti-H4) to verify equal protein loading. Densitometric analyses were performed by NIH image software.

microRNA expression analysis

Total RNA was extracted from cells using TRIzol reagent (Invitrogen) according to manufacturer's instruction. Purity of RNA was assessed by Nano-Drop, and only highly pure RNA (ratio of 260/280 > 1.8 and 260/230 > 1.8) was used for subsequent assays. Individual miR expression was analyzed by using specific single-assay miR primers (hsa-miR1, hsa-miR499, hsa-miR155, hsa-miR126) for retro-transcription and real-time reactions (Applied Biosystem), as previously described [7].

Chromatin Immunoprecipitation (ChIP) assay

ChIPs were performed and DNA fragments analyzed by quantitative Real Time PCR as previously described [13]. Briefly, Standard curves were generated by serially diluting the input (5-log dilutions in triplicate) and qPCR was done in the ABI Prism 7500 PCR instruments (Applied Biosystems) using SYBR Master mix (Applied Biosystems) with evaluation of dissociation curves. The qRT-PCR analyses were performed in duplicate and the data obtained were normalized to the corresponding DNA input. Data are represented as relative enrichment. Primers for human promoters were (from transcriptional starting site, TSS): c-Kit fw: GAGCAGAAACAATTAGCGAAACC (-560bp); c-Kit rev: GGAAATTGAGCCCCGACATT (-468bp); Nkx2.5 fw: TGACTCTGCATGCCTCTGGTA (-198bp); Nkx2.5 rev: TGCAGCCTGCGTTTGCT (-138bp); MDR-1 fw: TTCCTCCACCCAAACTTATCCTT (-93bp); MDR-1 rev:

CCCAGTACCAGAGGAGGAGCTA (-2bp); hGNL3 fw: GAGTTTGTGTCGAACCGTCAAG (-563bp); hGNL3 rev: TCCCTCAGTCCCCAATACCA (-457bp).

Electrophysiology

Patch-clamp analysis was performed on CStC perfused with a normal Tyrode solution containing (mM): 140 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 5.5 D-glucose, 5 Hepes-NaOH; pH 7.4. Patch pipettes were filled with a solution containing (mM): 130 K-Aspartate, 10 NaCl, 5 EGTA-KOH, 2 $CaCl_2$, 2 $MgCl_2$, 2 ATP (Na-salt), 5 creatine phosphate, 0.1 GTP (Na-salt), 10 Hepes-KOH; pH 7.2 and had resistances of 2 to 4 MOhm. Experiments were carried out at room temperature.

The fast Na+ current (I_{Na}) was activated by 50 ms steps to the range -80/+30 mV from a holding potential of -90 mV. Peak I-V relations were constructed by plotting the normalized peak current against test voltages.The time-independent inwardly-rectifying K+ current (I_{K1}) was investigated by applying 4 s voltage-ramps from -100 to 25 mV in Tyrode solution and after addition of Ba^{2+} (2 mM $BaCl_2$), a known blocker of I_{K1}. To record the I_f current, 1mM $BaCl_2$ and 2mM $MnCl_2$ were added to normal Tyrode in order to block contaminating currents. I_f was activated by a standard activation protocol [14]. Hyperpolarizing test steps to the range -35/ -125 mV were applied from a holding potential of -30 mV, followed by a fully activating steps at -125 mV. Each test step was long enough to reach steady-state current activation. Normalized tail currents measured at -125 mV were used to plot activation curves, which were fitted to the Boltzmann distribution function: $y = (1/(1+\exp((V-V_{1/2})/s)$, where V is voltage, y

fractional activation, $V_{1/2}$ the half-activation voltage, and s the inverse slope factor. Measured values are reported as mean ± S.E.M.

Statistics

Statistical analysis was performed using Student's t-test. $P < 0.05$ was considered significant.

3. Results

CstC belong to the Islet1+ progenitor family

Although phenotipically and biologically (i.e. fast growth kinetic) similar to fibroblast-like cells, the human CstC population contains cells positive for cardiomyogenic progenitor markers including Islet1 (Isl1+) and GATA4 (Fig.1), while negative for c-Kit and MDR-1 (Fig.1 and Supplementary Fig.1).

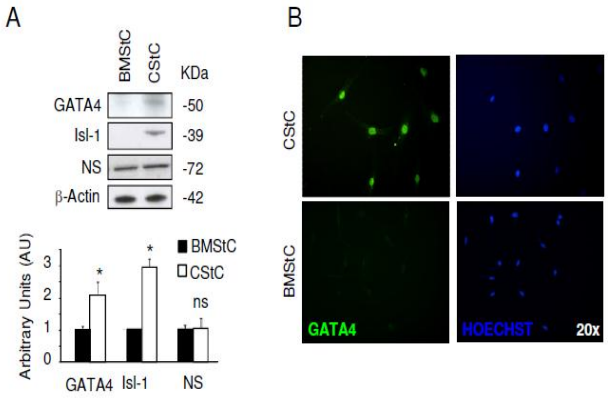
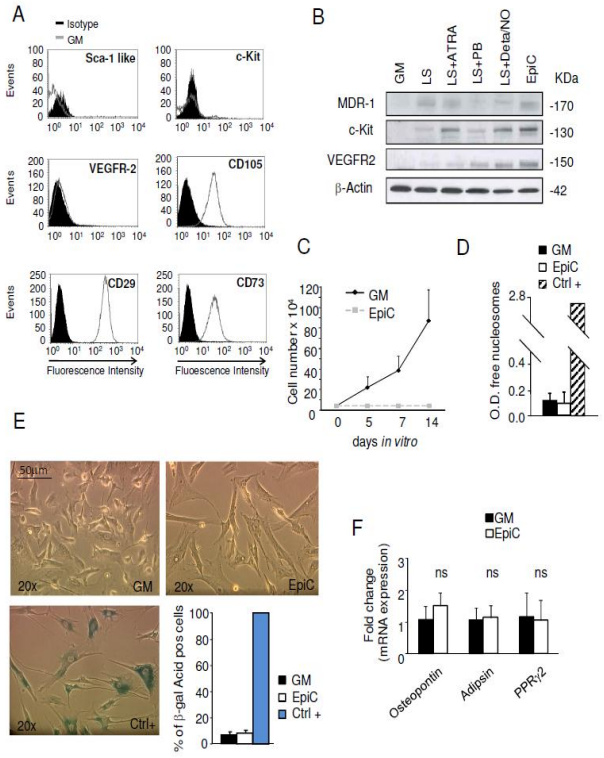


Figure1.

Notably, in a series of parallel experiments syngeneic bone marrow stromal cells have been found negative for Isl1 and GATA4. Both stromal cell populations, however, were positive for the mesenchymal markers CD105, CD29 and CD73 (Supplementary Fig. 1) [7] and Nucleostemin (NS, Fig. 1) a nucleolar protein regulated in stem cell proliferation [15]. Intriguingly, similar to post-natal Isl1+ cardioblasts [16], adult human Isl1+ CStC acquired spontaneous beating activity after 3-5 days of transwell co-culture with neonatal rat cardiomyocytes (Supplementary Video 1 and 2).



Supplementary fig.1

Epigenetic cocktail (EpiC) design

The level of nutrients (i.e. fetal serum) and the presence of selected drugs can modify cell fate and induce functional reprogramming [6, 17]. In this light, after amplification in expansion medium routinely used for mesenchymal cell culture (growth medium, GM), CStC were exposed for 7 days to a differentiation medium (DM) with reduced level fetal bovine serum (5% FBS) either in the presence or in the absence of 5 μM all-trans retinoic acid (ATRA), 5 μM phenyl butyrate (PB) and 200 μM diethylenetriamine/nitric oxide (DETA/NO), alone or in combination. In all these condition the expression of markers

associated with resident cardiac stem cells (c-Kit, VEGFR2, MDR-1) have been evaluated [1, 18]. Our findings indicate that, although serum deprivation alone or a single drug exhibited the ability to up-regulate the expression of one or more markers, only the complete DM formulation, defining an unprecedented "epigenetic cocktail" (EpiC), induced the coincident expression of c-Kit, VEGFR2 and MDR-1 in Isl1+ cells (Fig. 2 and Supplementary Fig. 1). Notably, the EpiC treatment, although stopping cell proliferation, did not induce apoptosis or senescence (Supplementary Fig. 1). Finally, EpiC treatment did not stimulate CStC to differentiate into other mesodermal cells such as adipocytes or osteoblasts (Supplementary Fig. 1).

Effects of EpiC treatment on stem and cardiovascular precursor markers.

EpiC treatment of Isl1 CStC strongly up-regulated the expression of the cardiac-resident adult stem cell markers c-Kit and MDR-1 (Fig. 2). In this condition the MDR-1 transporter was functionally active as indicated by the rhodamine extrusion assay (Supplementary Fig. 2). Other proliferation and differentiation markers including Notch, Jagged-1 and Numb [19, 20] were also increased (Fig. 2), while the expression of the pluripotency factors Oct4 and Nanog remained negative (not shown). In this condition EpiC-treated cells did not

proliferate (Supplementary Fig. 1) and NS was down-regulated (Fig.2)

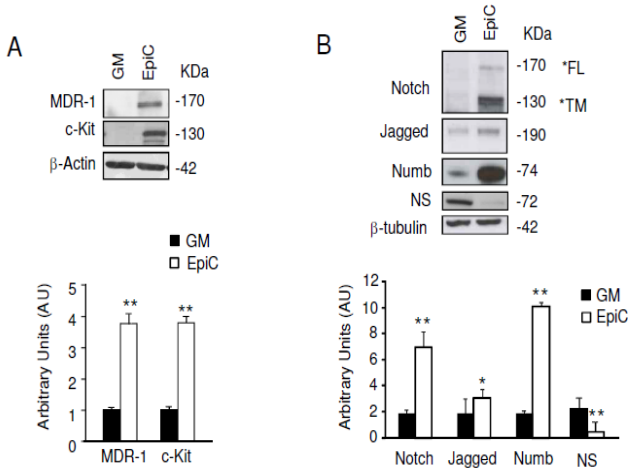
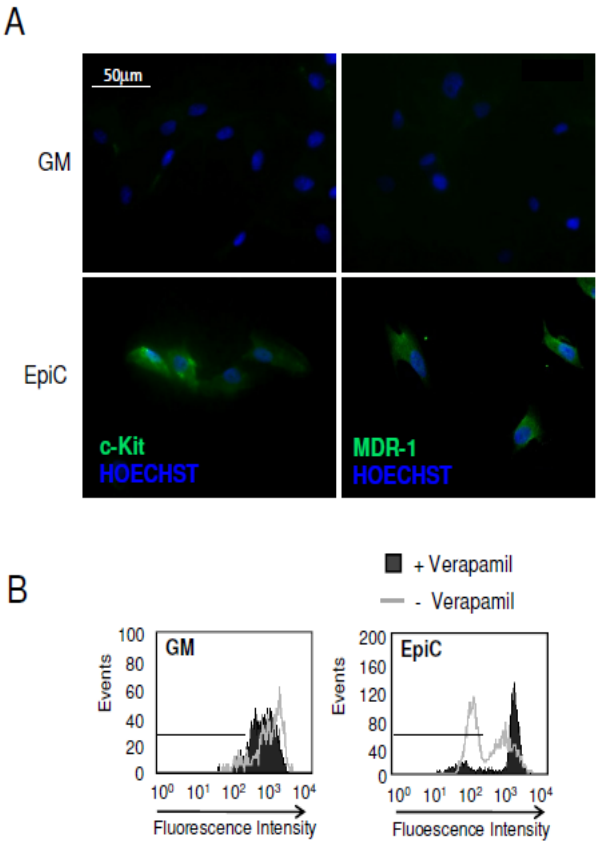


Figure 2.

In addition, EpiC increased the expression of vascular (VEGFR2, GATA6, α-smooth muscle actin) and cardiomyogenic (GATA4, Nkx2.5) precursors markers (Fig. 3), leaving unaltered Isl1 and Mef2C which are already present in untreated CStC (Fig. 3). Although more mature cardiomyogenic markers could be detected in EpiC treated CStC, such as α-sarcomeric actin (α-sarc) and α–myosin heavy chain (α-MHC) neither sarcomere striation nor positivity for cardiac troponin (TnT-C, Fig. 3) were present in EpiC-treated cells. Finally, mir-1 and mir-499, both involved in cardiomyocyte progenitor differentiation [21, 22], were significantly up-regulated after EpiC exposure, while mir-155 and mir-126, whose expression have been associated to proliferating cardiomyocyte precursors, remained unchanged (Fig. 3) [22].



Supplementary Fig 2.

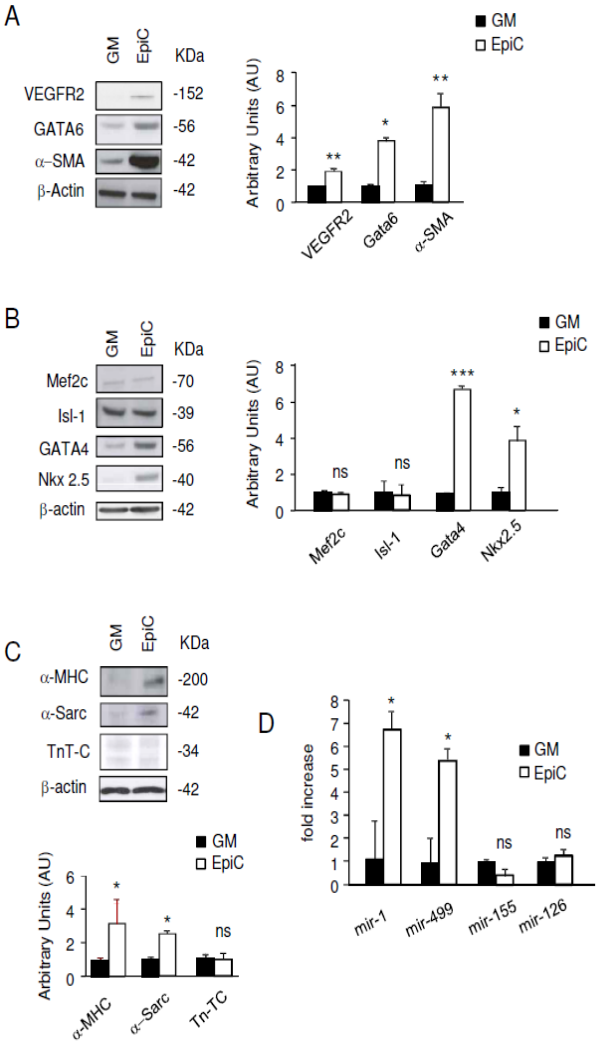


Figure 3.

EpiC treatment changes Isl1+ CStC electrophysiological properties.

EpiC exposure had a profound impact on CStC function, determining the appearance of electrophyisological features typical of cells committed towards the cardiomyocyte lineage. Specifically, a

significant fraction (44.8%, 13 out of 29 cells) of EpiC-treated cells exhibited a fast-activating inward sodium current (Fig. 4) which activated at voltages more positive than -40 mV, peaked around 0 mV and was completely blocked by 10 µM TTX. Expression analyses showed that, in the presence of EpiC, the type V voltage-gated sodium channel (NaV1.5) was significantly up-regulated, both at the mRNA and protein levels.

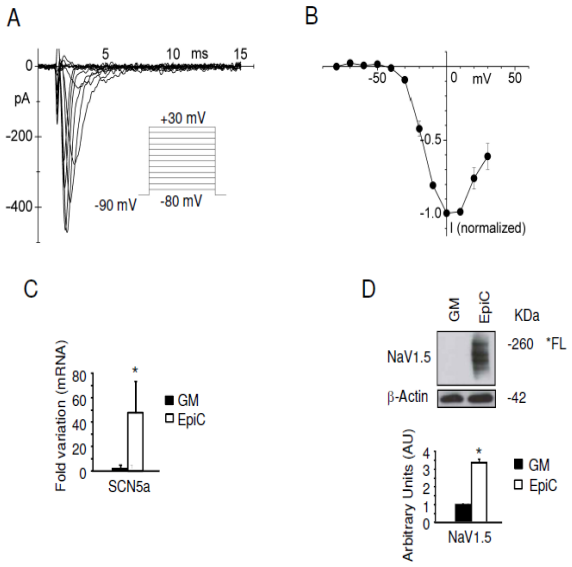


Figure 4.

A pacemaker I_f current (Fig. 4) has also been recorded in 5 out of 33 EpiC-treated cells (15.1%), with kinetic properties compatible with those of native pacemaker cells ($V_{1/2}$: -74.4 ± 5.7 mV, n=3, Fig. 3). Accordingly, EpiC increased the expression of the pacemaker channel subunit HCN4 (Supplementary Fig. 3). Notably, CStC maturation towards the cardiomyocyte lineage was not complete, as demonstrated by the depolarized resting potential characterizing CStC exposed to EpiC (-12.7 ± 2.7 mV, n=7). This is in accordance with the negligible

expression of the inward rectifying I_{K1} current (0.43 ± 0.04 pA/pF at -100mV, n=5), physiologically important in setting the resting potential of working cardiomyocytes.

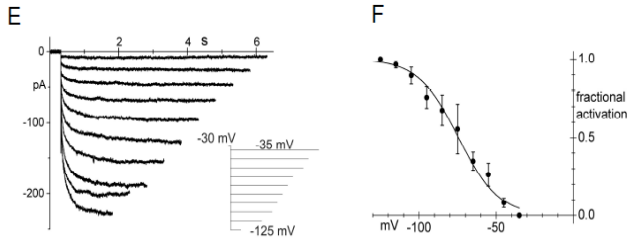
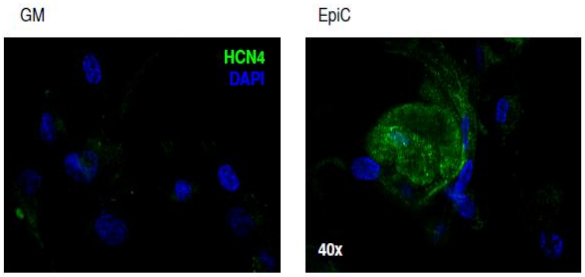


Figure 4 e Supplementary Fig.4.



Effects of EpiC treatment on CStC epigenetic landscape

As expected by EpiC composition described at point 3.2, EpiC-treated CStC revealed a significantly lower total HDAC activity compared to cells kept under control condition (Supplementary Fig. 4) and a number of genome wide histone modifications. Specifically, we observed a global decrease of histone H3 lysine 9 acetylation (H3K9Ac) as well as an increase in histone H4K20 methylation (H4K20Me, Fig. 5). These modifications are compatible with those reported in cells undergoing differentiation or cell cycle arrest [23]. On the other hand, an increased density of the permissive marks

histone H3K4 trimethylation (H3K4Me3) [24] and histone H4K16 acetylation (H4K16Ac) [25] was observed (Fig. 5).

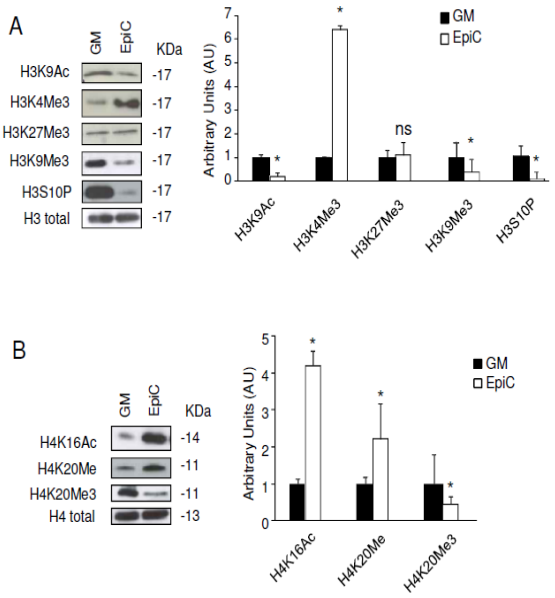


Figure 5.

Intriguingly, these markers are typically associated with the transcriptionally competent chromatin conformation observed in cells with high developmental potential [26]. Along this line of evidence, repressive markers [27] remained stable (H3K27Me3) or were significantly down-modulated (H3K9Me3 and H4K20Me3), confirming that EpiC-treated cells globally underwent to chromatin remodeling and at least partial transcriptional activation. Accordingly to the effect on cell proliferation [28] Epic also induced a significant decrease in Histone H3 phosphorilation at Ser10 (H3S10P, Fig. 5).

Effects of EpiC treatment on specific-gene promoters

To evaluate the transcriptional effects of EpiC treatment, a series of chromatin immunoprecipitation (ChIP) experiments were performed with chromatin extracted by CStC cultured in the presence or the absence of EpiC.

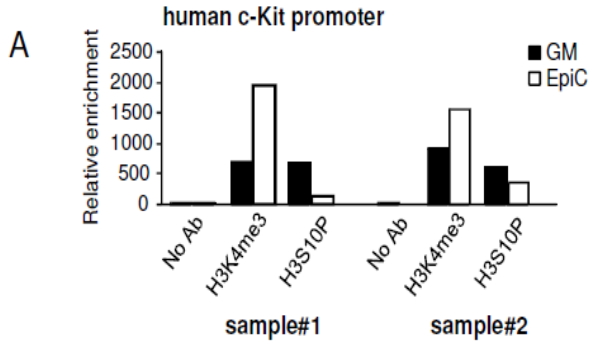


Figure 6A.

Specifically, anti-H3K4Me3 and anti-H3S10P antibodies were used, followed by real time PCR to detect the enrichment of these specific histone modifications in the promoter region of c-Kit, MDR-1, Nkx2.5 and Nucleostemin.

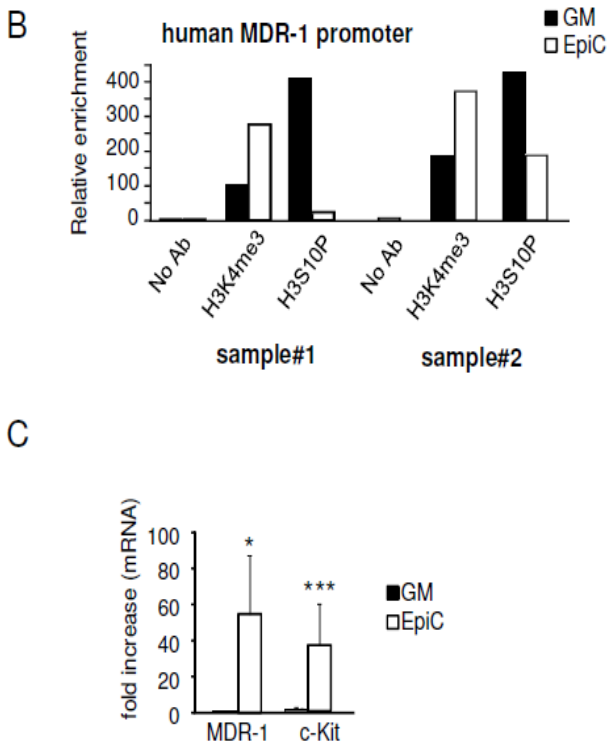
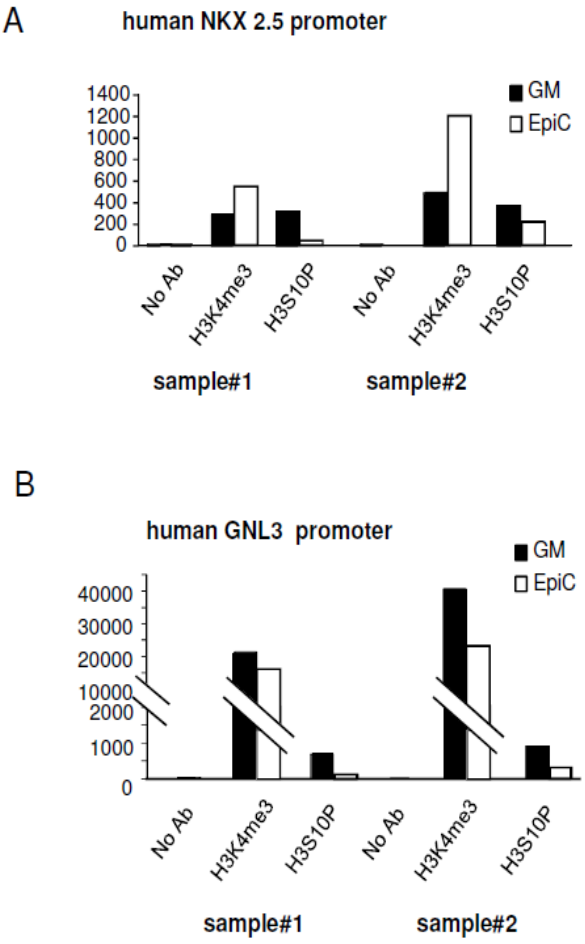


Figure 6B.

As shown in Fig. 6 and in Supplementary Fig. 5, H3K4Me3 association to c-Kit, MDR-1 and Nkx2.5 promoter was increased by EpiC treatment, proving that chromatin conformational modifications account for the expression of these genes. Accordingly, H3K4Me3 association to Nucleostemin (human GNL3 gene product) promoter was reduced, confirming the epigenetic basis of its down-regulation (Supplementary Fig. 5). In agreement with the inhibition of proliferation induced by the EpiC treatment, all the analyzed genomic regions associated to a gene promoter function exhibited a marked decrease in H3S10P level, as expected for cells exiting mitosis.



Supplementary Fig. 6

4. Discussion

A large body of evidence shows the important and efficient contribution of embryonic and inducible stem cells in the process of cardiac regeneration [29, 30]. These pluripotent cells, in fact, contribute to distinct parts of tissue reconstruction including formation of muscular parenchyma and vascular structures. Resident cardiac stem cells, instead, are specialized multipotent cells which, at very low rate, contribute to cardiomyocyte turn-over or cardiac post-ischemic regeneration. They are present in defined cardiac districts, identified as niches, in which these cells are kept quiescent until activation signals will come [1]. Specific markers identify a cardiac stem cell, including the presence of c-Kit and the P-pump (MDR-1) gene products [1, 18]. Other cardiovascular precursors express the LIM-homeodomain transcription factor Isl1 [16]. Although more committed toward the lineage of interest, adult cardiac progenitors are difficult to grow *ex-vivo* and afterwards to effectively differentiate into mature cardiomyocytes. Further, the preparation of near-terminally differentiated cardiomyocytes may not be the best approach for applications aimed at cardiac regeneration where maximum efficiency is required to regenerate all the cell types forming damaged tissues.

Our group has recently isolated, characterized and efficiently amplified a human population of adult cardiac mesenchymal stromal cells (CStC) showing *in vitro* and *in vivo* cardiovascular plasticity [7]. Notably, although CStC proneness to acquire cardiomyogenic markers was higher than that of syngeneic bone marrow cells, *in vivo* CStC efficiency to differentiate into adult cardiovascular cell types remained

low. Our previous work showed that CStC express detectable mRNA level of early cardiovascular markers including c-Kit, GATA4 and GATA6 [7]. In the present work CStC characterization has been refined, demonstrating that these cells, distinct from the cardiac c-Kit+ stem/progenitor population, more closely belongs to the Isl1+ progenitor family described by Laugwitz et al. [16].

Extensive studies on the mouse embryo have demonstrated that Isl1+ cardiovascular progenitors contribute to all cells in atria, right ventricle and conduction system [31, 32]. Therefore, human Isl1+ CStC isolated from right atrium-derived tissue may represent the adult remnant of embryonic Isl1+ cells. Further, based on the hypothesized hierarchy for Isl1+ cells [33] and on their *ex-vivo* and *in vivo* plasticity [7], untreated CStC (Isl1+/Nkx2.5-/VEGFR2-) may be considered similar to adult common primordial cardiovascular progenitors. Of note, adult Isl1+ CStC exhibit also mesenchymal-like properties, which is in agreement with their common mesodermic origin [34]. In the present work, in order to improve CStC cardiomyogenic and vascular potential, CStC were efficiently reprogrammed into multi-lineage cardiovascular precursors, by using an unprecedented combination of small epigenetically active molecules, here called epigenetic cocktail, or EpiC. This cocktail was designed not only to modify CStC chromatin landscape, but also to unmask and drive CStC plasticity possibly towards the cardiovascular lineage. In detail, EpiC was made of: (i) all-trans retinoic acid (ATRA), which has genome-wide regulation properties [35] and whose receptors are known to recruit p300 and CBP acetyl-transferases facilitating their action at the histone level of specific

gene loci [36]; (ii) phenyl butyrate (PB), a drug belonging to the family of histone deacetylase (HDAC) inhibitors, which are known to enhance mesoderm maturation [37]; (iii) diethylenetriamine/nitric oxide (DETA/NO), associated to cell survival growth arrest in vascular cells [38] and increased cardiomyogenesis in mouse ES cells [39]; (iv) a reduced serum content, known to induce spontaneous differentiation in a variety of cell types including C2C12 myoblasts [40] or cardiac mesoangioblasts [17]. Importantly, all drugs used in this study are approved for clinical use or, in case of DETA/NO, currently undergoing clinical trials.

The idea that stem cell fate can be modulated by specific chemicals dates back of decades but recently, an increasing number of new studies showed the potential role of small molecules to promote cardiogenesis in mouse ES including different BMP inhibitors such as dorsomorphin [41] and Wnt pathway modulators [42]. On the other hand, attempts on adult cells were inefficient and the induction of true cardyomyogenesis is still hardly debated. In 1999 Makino and colleagues described the appearance of spontaneously beating MHC, MLC-2v, GATA4, Nkx2.5 positive cells following treatment of immortalized murine bone marrow stromal cells with 5'-azacytidine (5-AZA) [43]. Nevertheless, in these experiments several cells stained positive for adipogenic markers suggesting that this method was not cardiac selective, while other groups were unable to reproduce these findings [44]. Of note, the putative mechanism of 5-AZA induced cardiomyogenesis was firstly attributed to demethylation of cardiac-related genes. However, a study by Cho et al. [45] demonstrated that the effect of 5-AZA was not related to the epigenetic activation of

cardio-specific genes, but rather to the transcriptional inhibition of the glycogen synthase kinase (GSK)-3 gene, a major player in the Wnt signaling pathway [45]. In this light, our findings indicate that EpiC treatment do not simply determines global activation-prone changes in chromatin organization, but directly targets the promoters of c-Kit, MDR-1 and Nkx2.5, inducing their expression without without apparently altering the expression of non-cardiac osteogenic and adipogenic differentiation marks, thus suggesting that CStC response to EpiC may be predominantly cardiovascular oriented [12, 46]. In fact, along with the expression of adult cardiac stem markers, EpiC treatment also induced the up-regulation of specific transcription factor associated with the vascular and cardiomyocyte lineage commitment. In summary, we obtained a heterogeneous population of multi-potent cardiovascular precursors co-expressing at the same time stem cell antigens, transcription factors and membrane/cytoplasmic proteins typical of committed cardiomyocytes, endothelial cells and smooth muscle cells [18]. Interestingly, EpiC-treated cells also exhibited functional, but not yet operational properties typical of differentiating cardiomyocytes as indicated by the presence of the fast sodium current normally responsible for the action potential upstroke, and of the I_f current which, although negligible in the adult ventricle, is present in atria and ventricles during the embryonic and perinatal stage [47]. Of note, in the early embryonic heart the pacemaker channel HCN4 is widely expressed at the venous pole [48], where also Isl1 expression is abundant [49]. Only at later stages of development HCN4 expression becomes predominantly restricted to the sinoatrial

node [50]. Therefore the presence in the same population of Isl1 and HCN4 positive cells is compatible with an early cardiac phenotype

5. Conclusions

In conclusion, it is reported here the first evidence that under the appropriate environmental conditions Isl1 and c-Kit/MDR-1 may co-exist, indicating that the boundaries between these two population are more shaded than previously thought. Further, the chemical reprogramming of adult cells may represent a safe way to generate large amount of committed progenitors cells suitable for therapeutic applications.

6. References

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Chapter 3: Human Chorionic Villus Mesenchymal Stromal Cells Reveal Strong Endothelial Conversion Properties.

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Submitted to *Differentiation*

1. Introduction

Fibroblast-like cells sharing a common immunophenotype and a certain degree of plasticity may be isolated from virtually every adult tissue (da Silva Meirelles et al., 2006) including liver (Herrera et al., 2006), skin (Huang et al.) and heart (Beltrami et al., 2007; Rossini et al.). These cells are widely known as mesenchymal cells and are recognized by defined criteria, including plastic adherence, specific surface antigens (i.e. positive for CD105, CD90, CD73 and negative for the CD45, CD34, CD14, HLA-DR antigens) and the ability to differentiate toward different lineages (Dominici et al., 2006). As their biological properties not always meet the criteria used to define stem cell populations, the International Society for Cellular Therapy (ISCT) guidelines stated to use for these cells the term of "mesenchymal stromal cells". In the present work, the official position statement of ISCT for mesenchymal cell nomenclature has been adopted (Horwitz et al., 2005).

Mesenchymal stromal cells (StC) are considered a promising tool for regenerative medicine and tissue engineering. Currently, the most important source of StC for cell therapy is the bone marrow. Bone marrow stromal cells (BMStC) have been exhaustively described and, at present, they represent the gold standard for StC characterization and application to regenerative medicine.

Recently, also the human placenta raised great interest in the field of regenerative medicine, because of the high residual plasticity of many of the cells isolated from its tissues (Soncini et al., 2007). In addition, in consequence of their origin from a developmentally very young tissue, the possibility that StC from early foetal tissues may retain a

wider differentiation potential compared to adult StC has been suggested (Abdulrazzak et al.). Specifically, although chorion and amnion are a rich source of StC, the largest body of information is available only about StC isolated from term amnion (Bilic et al., 2008; Diaz-Prado et al.; Insausti et al.), whereas only few reports describe chorionic StC derived from first trimester villi (Poloni et al., 2008). Therefore, a series of in vitro experiments were performed with primary mesenchymal stromal cells isolated from first trimester human chorionic villi (CVStC). CVStC were then exposed to differentiation treatments and characterized according to morphological, immunophenotypical and molecular criteria including microRNA expression. Aim of the present work was the evaluation of CVStC plasticity, using StC from adult bone marrow (BMStC) as control. Our study demonstrates that, in analogy with adult stromal cells, CVStC exhibited restricted plasticity, but unlike BMStC, CVStC were preferentially directed toward the endothelial lineage.

2. Materials and Methods

Ethics Statement

Chorionic villus samples were obtained from pregnant women during 11^{th}-13^{th} weeks of gestation after signed informed consent and approval of Local Ethical Committee. Sternal marrow was obtained, according to the principles expressed in the Declaration of Helsinki, from donor patients undergoing cardiac surgery after signed informed consent approved by Local Ethical Committee. All data were analyzed anonymously.

First Trimester Chorionic Villi Stromal Cells (CVStC) Isolation

After washing in Hanks' Balanced Salt Solution (HBSS) without Ca^{2+}-Mg^{2+} (Euroclone, Italy), chorionic villous samples were first incubated in 1mg/ml PronaseE (4000000PU/g) (Merck, Italy) for 4-6 minutes at room temperature (RT) and then in 1 mg/ml Collagenase type II (Sigma-Aldrich, Italy) for 1 hour at 37°C, as modified from (Portmann-Lanz et al., 2006). After digestion, cells were centrifuged for 10 minutes at 1400 rpm and plated in amplification medium. The day after cell plating, cultures were carefully washed twice in Phosphate Buffered Solution (PBS, Lonza, Italy) to remove non-adherent cells. Before digestion, specific attention was paid to remove any decidua fragments by accurate manual selection, in order to avoid maternal contamination (Soncini et al., 2007).

Bone Marrow Stromal Cell Isolation

BMStC were isolated and cultured as previously described (Rossini et al.).

Cell culture and media

Cells were cultured in two different culture media: (i) AmniomaxII® (AM, GIBCO™ Invitrogen Corporation), a medium specifically developed to improve cell attachment and growth (Biddle WC, 1992; Santolaya-Forgas et al., 2005) and routinely used in our laboratory for diagnostic procedures; (ii) a standard mesenchymal medium (MM) (Lee et al., 2004) composed by Iscove's Modified Dulbecco's Medium (IMDM, Lonza) supplemented with 20% foetal bovine serum (FBS, Hyclone, Italy), 10 ng/ml basic Fibroblast Growth Factor (bFGF, R&D, Italy), 10.000 U/ml Penicillin, 10.000 μg/ml Streptomycin (Pen/Strep, Invitrogen) and 20 mM L-Glutamine (L-Glu) (Sigma-Aldrich). All cell cultures were placed in a humidified incubator gassed with 5% CO_2 at 37°C.

Proliferation Assay

CVStC proliferation ability was determined by plating cells at a density of 1500 cells/cm^2 in duplicate in a 6-well plate. After 2, 5, 7 and 9 days in culture cells were harvested by Trypsin-EDTA treatment, stained with Trypan Blue (GIBCO™) and counted in a Burker haemocytometer.

Flow Cytometry

Cells were harvested by treatment with 0.02% EDTA solution (Sigma-Aldrich) and incubated with FITC/PE/APC conjugated primary antibodies for 15 min at room temperature in the dark. The following antibodies were used: (i) CD13, CD29, CD31, CD31, CD34, CD44, CD45, CD73, CD90, CD146, HLA-ABC, HLA-DR and CD117 (BD Bioscience, Italy); (ii) VEGFR2 and CD105 (R&D). Cells were subsequently washed with PBS and analyzed using FACSCalibur (Becton-Dickinson, Italy) equipped with Cell-Quest Software. Isotype control was performed for each experiment.

Real-Time Reverse-Transcription–Polymerase Chain Reaction

Real-Time PCR experiments were performed as previously described (Livak and Schmittgen, 2001; Rossini et al.). The sequences of used primers are reported in Table 1.

Western Blot

CStC and BMStC were lysed with Laemmli buffer containing protease (Sigma-Aldrich) and phosphatase (Roche) inhibitors. Total proteins were resolved by SDS-PAGE and transferred to nitrocellulose membrane (BioRad). After 1 h blocking in Tris Buffered Saline (TBS) containing 0.1% Tween 20, 5% skimmed milk, the membrane was first incubated overnight at 4°C with primary antibody (mouse anti-Oct 4 1:1000; rabbit anti-Sox 2 1:1000; rabbit anti-Nanog 1:1000, Cell Signalling) and then 1 hour at room temperature with the appropriate HRP-conjugated secondary antibody. Bound antibody was detected by

enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech).

Telomere Repeat Amplification Protocol Assay

Telomerase activity was measured by the telomere repeat amplification protocol (TRAP) method (Kim and Wu, 1997). The reaction was carried out adding an internal telomerase assay standard (internal control) for estimation of the levels of telomerase activity and identification of any false-negative samples containing Taq polymerase inhibitors. Assays were repeated at least 3 times with three different preparations of cell lysates. As positive and negative controls, 0.1 μg of protein from telomerase-positive HeLa cells was assayed before and after heat inactivation.

Karyotype analysis

Cytogenetic analysis was conducted on cells at passage zero, after culture expansion (passages 4-6) and after differentiation (passages 5-8), as previously described (Rossini et al.).

***In Vitro* Cell Differentiation**

Chorionic villi stromal cells were plated at a density of 5000 cells/cm^2 and exposed to standardized differentiation-inducing media for 21 days. Medium was changed twice a week. Subsequently, cells were analyzed for the acquisition of lineage-specific properties.

Adipogenic, endothelial and osteogenic differentiation were stimulated as previously described (Rossini et al.).

Spontaneous skeletal myogenic differentiation was evaluated after culturing the cells for 7 days in DMEM High Glucose 4.5 g/l (Dulbecco's Modified Eagle Medium) (Lonza) supplemented with 5% horse serum, Pen/Strep and L-Glu (Di Rocco et al., 2006).The ability to acquire cardiomyogenic markers was evaluated by culturing cells in ATRA/PB medium, able to induce, in vitro, a partial cardiomyogenic differentiation of cardiac stromal cells (Rossini et al.).

Intracellular Lipid Staining by Oil-Red O

Cells were fixed with 4% formaldehyde for 10 min, stained with Oil-red O solution (0.3% in isopropyl alcohol, Sigma-Aldrich) solution for 20 minutes, rinsed twice with water and then examined at the microscope.

Reverse-Transcription–Polymerase Chain Reaction (RT-PCR) analysis

Total RNA was extracted from cells using RNeasy Mini Kit (Qiagen) according to manufacturer's instruction. Total RNA (1 μg) was reversely transcribed using SuperScript III First-Strand Synthesis SuperMix (Invitrogen) and the resultant cDNA was amplified by Platinum® *Taq* DNA Polymerase (Invitrogen) in a BioRad (Italy) ICycler®. PCR from samples with no reverse transcriptase was also performed to exclude genomic DNA contamination. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as normalizer. The sequences of used primers are reported in Table 2.

Von Kossa Staining

The production of mineralized matrix was evaluated by von Kossa staining. Cells, fixed with 4% paraformaldehyde (PFA) were treated with a solution of 1% silver nitrate (Sigma-Aldrich) under UV light for 1 hour, followed by 3% sodium thiosulfate (Sigma-Aldrich) for 3 minutes.

microRNA expression analysis

Total RNA was extracted from cells using TRIzol reagent (Invitrogen) according to manufacturer's instruction. Purity of RNA was assessed by Nano-Drop. Individual miR expression was analyzed by using specific single-assay miR primers (hsa-miR16, hsa-miR21, hsa-miR126, hsa-miR221, hsa-miR-222) for RT and real-time reactions (Applied Biosystem), as previously described (Rossini et al.).

Ac-LDL-DiI uptake

During the last 24 h of EGM-2 treatment, cells were incubated with 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine-labeled acetylated LDL (Ac-LDL-DiI 10 ng/ml, Biomedical Technologies, Inc, USA) as indicator of endothelial cells differentiation (Ewing et al., 2003). After fixation with 4% paraformaldehyde, cells were counterstained with Hoechst 33258 nuclear and observed with a Zeiss microscope equipped for epifluorescence.

Capillary-Like-Structure-Forming Assay on Basement Membrane Extract (BME)

The tube forming ability of the cells was tested by evaluating branchin point number after 3 hours culture on Cultrex® BME, as previously described (Rossini et al.).

Statistical analysis

Statistical analysis was performed with SPSS Statistics v17.0 software using one way ANOVA. Bonferroni's test was used for multiple post-hoc testing. $P < 0.05$ was considered significant.

3. Results

CVStC morphology and proliferation ability

Cells from chorionic villi (CVStC) were obtained from pregnant women undergoing chorionic villus sampling between the 11th and the 13th week of gestation for diagnostic procedures (n=12).

To determine the effect of culture condition on CVStC phenotype and plasticity (Wagner et al., 2006) two different culture media were tested.

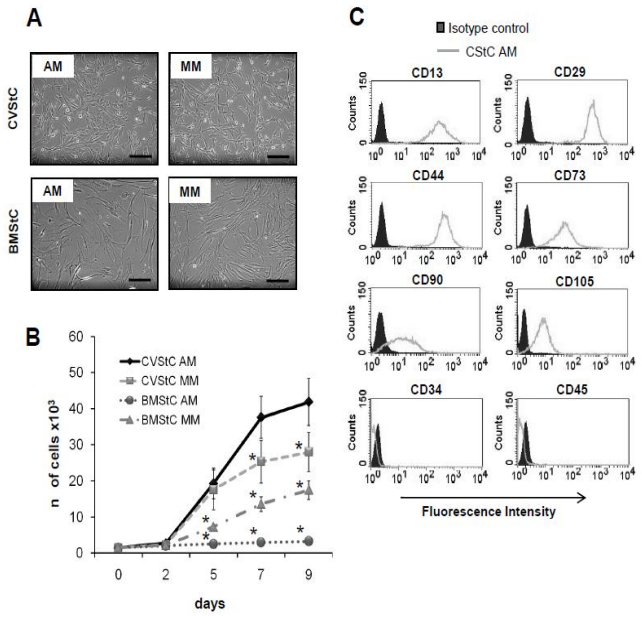

Figure 1.

Specifically, CVStC were isolated and amplified in AmniomaxII® (AM) (Biddle WC, 1992; Santolaya-Forgas et al., 2005), a medium used during prenatal diagnostic techniques, or in a standard mesenchymal medium (MM) (Lee et al., 2004), made of Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 20%

foetal bovine serum (FBS) and 10 ng/ml basic Fibroblast Growth Factor (bFGF) (see methods). The results showed that CVStC cultured in MM or AM presented a fibroblast-like morphology, indistinguishable from that of bone marrow stromal mesenchymal cells (BMStC). Notably, CVStC grown in AM exhibited the highest growth kinetic (Fig. 1), while BMStC did not grow in AM exhibiting morphological signs of senescent cells (Fig. 1). For this reason, BMStC were cultured in MM for all the experiments described below.

Immunophenotyping

CVStC antigenic surface markers expression was found similar to that of BMStC, expressing the mesenchymal membrane markers CD105, CD73, CD90, CD44, CD13, CD29, CD146 while CD34, CD45 and HLA-DR were negative (Fig. 1). No differences were observed in CVStC cultured either in MM or AM (Table 3).

Expression of pluripotency-associated markers

The expression of genes associated to stemness and pluripotency was investigated by qRT-PCR. Although transcripts of Nanog, Oct-4 and Sox-2 were detectable at similar levels (Fig. 2), western blotting analysis revealed that the relative proteins were absent in both CVStC and BMStC (Fig. 2), thus confirming a discrepancy in the expression of pluripotency-associated markers at mRNA and protein level as previously reported for mesenchymal cells of marrow origin (Kaltz et al., 2008).

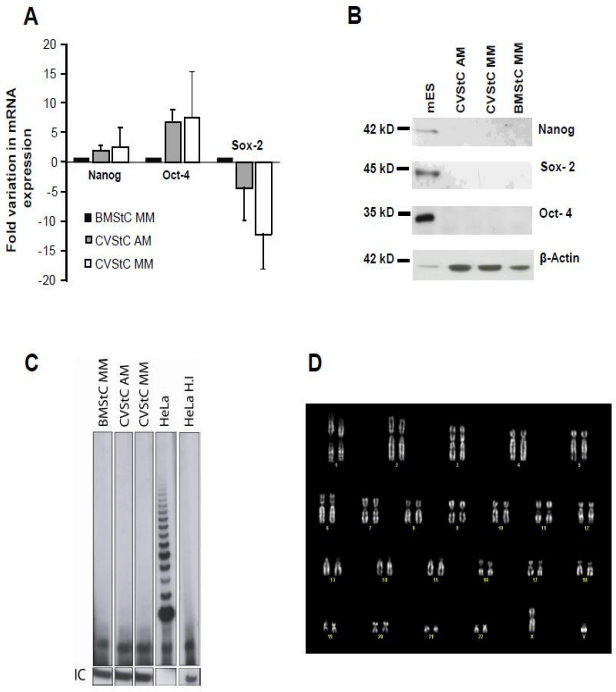

Figure 2.

Telomerase activity

The activity of the human telomerase catalytic subunit (hTERT) was determined by TRAP assay performed on cells at passage 4-6, in absence of differentiation stimuli. hTERT activity was negative in both CVStC and BMStC (Fig. 2), in agreement with previous reports about BMStC (Zhao et al., 2008) and other types of stem cells isolated from adult and foetal tissues, including cord blood-derived hematopoietic cells (Hiyama and Hiyama, 2007).

Karyotype analysis

Karyotype stability in cultured primary cells is one of the most important criteria required for pre-clinical and clinical cell therapy

applications. Cytogenetic analysis was conducted on freshly isolated CVStC (passage 0) and after in vitro expansion (passage 4-10). Importantly, cytogenetic analysis was not conducted on cells after passage 10-12, as at these passages CVStC reached in vitro replicative senescence.

About 25 metaphases from at least two independent cultures, at approximately 300-400 band level, were analyzed. All samples showed a normal karyogram (Fig. 2). This observation, along with the lack of telomerase activity, suggests these cells as genetically stable, not prone to malignant transformation, and potentially suitable for safe therapeutic applications (Poloni et al.).

CVStC differentiation

Residual plasticity of CVStC isolated and amplified either in MM or in AM, was assessed culturing cells for 3 weeks in standard differentiation media and evaluated for the acquisition of lineage-specific properties at morphological, functional and molecular level (see below). Karyotype analysis performed on CVStC after each differentiation treatment, between passages 5-10, confirmed their chromosomal stability (not shown).

- ***Adipogenic differentiation***

  CVStC cultured for 3 weeks in adipogenic medium, stained positive for Oil-Red O. Although adipogenesis in CVStC was less efficient than in BMStC, CVStC expanded in AM accumulated larger lipid droplets than those expanded in MM (Fig. 3).

- ***Osteogenic differentiation***

  In the presence of osteogenic medium (see methods), CVStC pre-cultured in AM accumulated more mineralized matrix than those grown in MM (Fig.3). Nevertheless, osteogenesis was less efficient in CVStC than BMStC, which acquired a typical osteoblast star-like shape and stained more intensely following Von Kossa reaction (Fig. 3)

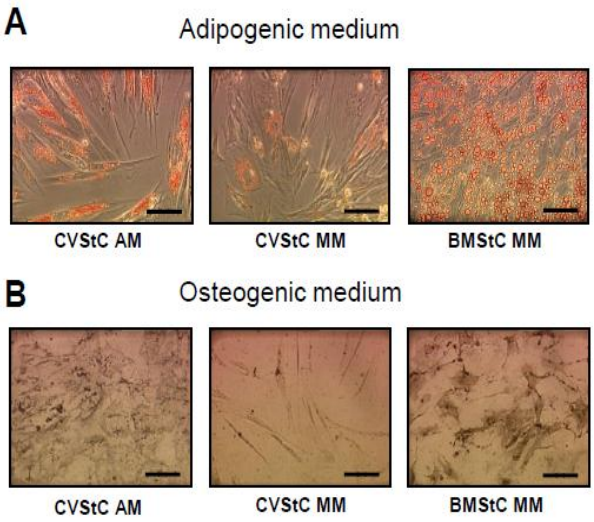

Figure 3.

- ***Myogenic differentiation***

  Myogenic conversion was a sporadic event in CVStC cultured in low serum. Indeed, these cells fused and formed bi- and poly-nucleated myotube-like structures (Fig. 4) up-regulating some myogenic markers including the α-1 nicotinic cholinergic receptor (CHRNA1) and the embryonic isoform of the myosin heavy chain (MYH3, Fig.

4). Nevertheless, the expression of more mature satellite cell markers, such as Pax7, Pax3, Myf5, and that of early (MyoD) or late (α-skeletal actin) skeletal muscle differentiation markers was negative in all the conditions tested (not shown). BMStC did not form bi- and poly-nucleated syncytia (Fig. 4).

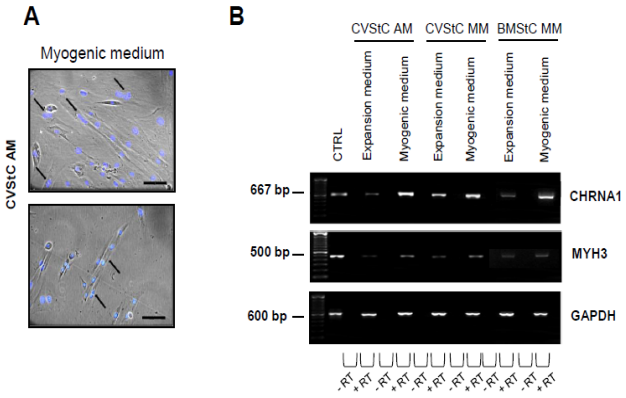

Figure 4A and B.

- ***Cardiomyogenic differentiation***

CVStC cardiomyogenic potential was tested in the presence of all-trans-retinoic acid/ phenylbutyrate (ATRA/PB) medium, which is known to up-regulate the expression of early and late cardiomyogenic markers in cardiac stromal cells (Rossini et al.).

In this condition CVStC assumed a flattened and elongated morphology, similar to cardiac stromal cells (Rossini et al.) (Fig. 4). However they only modestly up-regulated the expression of myosin light chain-2a (MYLC2a), while early (GATA-4 and Nkx2.5) and late cardiac markers (TN-I, α-MHC, α-Sarc) remained unmodulated (not shown). These data are in agreement with prior work (Roura et al.) reporting about the difficulty of cardiomyocyte *in vitro* conversion of non-cardiac stromal cells. Interestingly, in ATRA/PB medium, CVStC

up-regulated the expression of vascular markers including α-smooth muscle actin (α-SMA) and GATA-6 (Fig. 4), a transcription factor highly expressed in vascular smooth muscle cell and important promoting angiogenesis and survival in endothelial cells(Froese et al.).

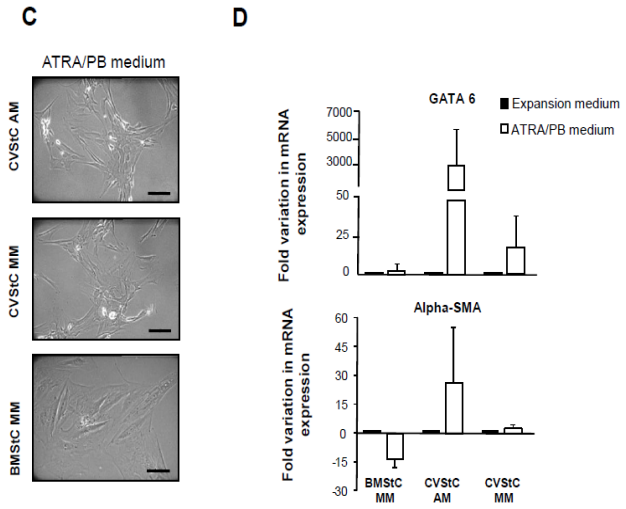

Figure 4C and D.

This observation, along with the higher capacity of CVStC to differentiate into endothelial-like cells (see below), suggests that CVStC exposed to the appropriate differentiation stimuli may activate a vascular reprogramming pathway.

- ***Endothelial differentiation***

  Endothelial differentiation was achieved after 2-3 weeks of cell culturing in the endothelium-specific medium EGM-2. In this condition CVStC, isolated and pre-amplified in AM, significantly changed their morphology showing the characteristic cobblestone-like shape, typical of mature endothelial cells such as human umbilical

vein endothelial cells (HUVEC, Fig. 5). In addition, as previously described after exposure to EGM-2, the number of VEGF-receptor 2 (VEGFR2) positive cells (Fig. 5) considerably increased (Rossini et al.) while BMStC remained negative for VEGFR2 before and after exposure to EGM-2 (Fig.5).

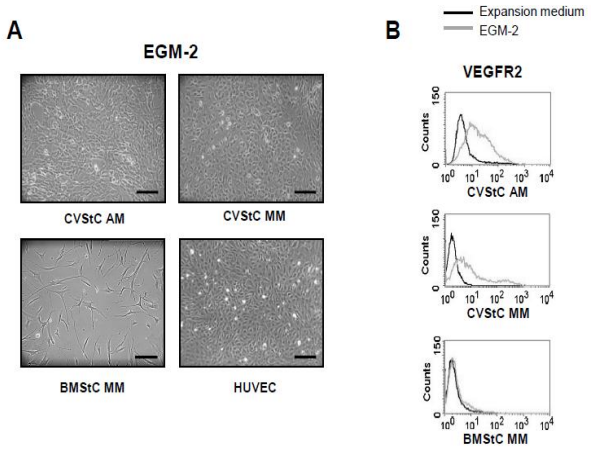

Figure 5A and B.

Prompted by these evidences and considering that several microRNA have been recently shown to play an important role during in vitro and in vivo angiogenesis (angiomiR), the basal angiomiR expression was investigated in CVStC and BMStC (Wang and Olson, 2009) including that of miR-21, -126, -221, -222. While miR-126 expression was comparable between the two cell types, miR-21, -221 and –222, which have been described as anti-angiogenic (Poliseno et al., 2006; Sabatel et al.), were found significantly down-modulated in CVStC compared to BMStC (Fig. 5). The reduced expression of anti-angiogenic miR suggests a possible mechanism explaining the proneness of CVStC to acquire an endothelial-like phenotype.

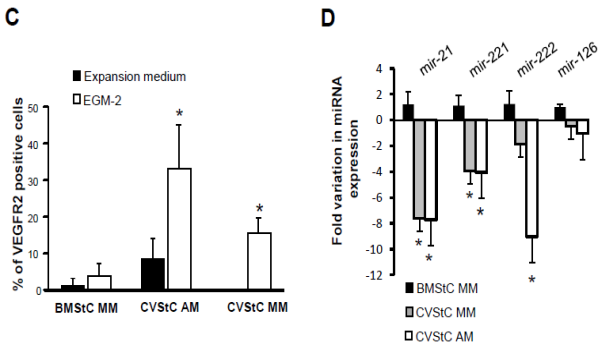

Figure 5C and D.

Intriguingly, although late endothelial markers such as CD31 (Table 1) and von Willebrandt factor (vWF, not shown) were not expressed at the protein level both in CVStC and in BMStC, CVStC ability to form capillary-like structure was enhanced by EGM-2 treatment (Fig. 6). Of note, CVStC isolated and pre-amplified in AM revealed the higher ability to uptake 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine-labeled acetylated LDL (Ac-LDL-DiI, Fig. 6), a commonly used marker to identify endothelial cells in culture (Ewing et al., 2003). Cells positive for Ac-LDL-DiI staining, were negative for CD163, thus indicating that differentiation into macrophages was an unlikely event.

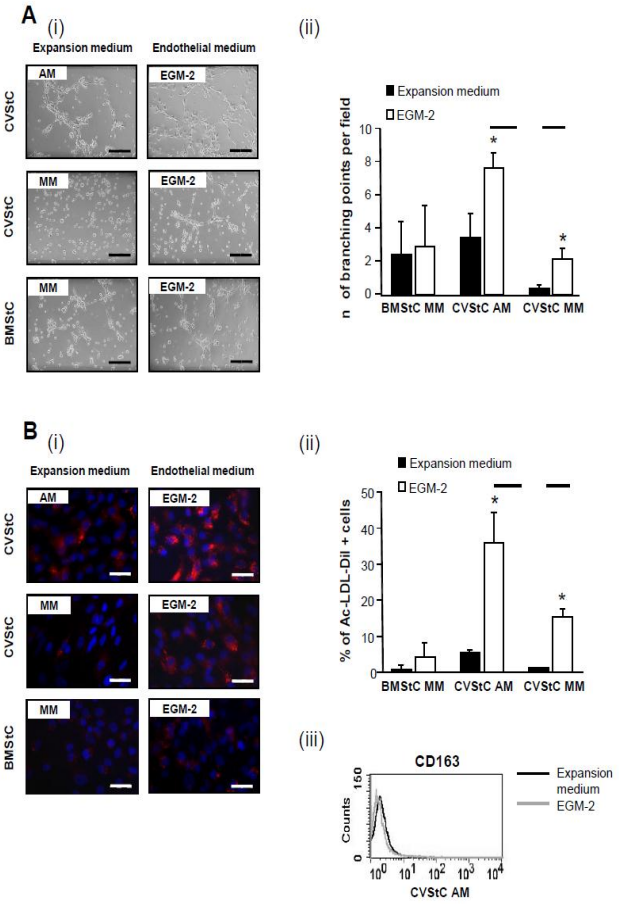
A
(i)
Expansion medium
Endothelial medium
AM
EGM-2
MM
CVStC
BMStC
(ii)
Expansion medium
EGM-2
n of branching points per field
BMStC MM
CVStC AM
CVStC MM
B
% of Ac-LDL-DiI + cells
(iii)
CD163
Counts
Expansion medium
EGM-2
CVStC AM

Figure 6.

4. Discussion

Human cells of foetal origin obtained from placental tissues or from other extra-embryonic structures are potentially promising for therapeutic application in regenerative medicine (Le Blanc et al., 2005). Cells like these are often derived from waste material and appear to be immune-privileged, showing low immunogenicity and high immunosuppressive properties (Dekel et al., 2003; Wang et al., 2009). It is generally accepted that placenta-derived mesenchymal stromal cells display a multi-lineage differentiation ability similar to that of adult bone marrow stromal cells (BMStC) (Fukuchi et al., 2004). Nevertheless, cells of foetal origin have been hypothesized of higher and wider plasticity than stromal cells from adult tissues, but, at present, only few reports addressed this aspect (Abdulrazzak et al.; Guillot et al., 2007). Also, the reported source of foetal cells is often extremely variable, ranging from human amnion epithelium (Miki et al., 2005) to amniotic fluid (Antonucci et al.) and chorion/amnion, a situation which makes it difficult to compare results (Sakuragawa et al., 2004; Soncini et al., 2007). Furthermore, cells isolated from the first, second and third trimester (Portmann-Lanz et al., 2006), as well as those obtained from term placenta (Miao et al., 2006) have been described without taking into account the presence of inherent differences in their regeneration potential. In the present work, we focused on first-trimester villi-derived mesenchymal stromal cells (CVStC) as these cells are easily isolated from waste material generated during early-gestation diagnostic procedures. Chorionic

villus sampling, in fact, is usually performed around the 10^{th}–12^{th} week of pregnancy, while amniocentesis is performed at later time points (14–16 weeks), making CVStC a developmentally younger and relatively abundant cell type that can be obtained from non-abortive human foetal material. In this light, in addition to other non-autologous cell therapy applications, CVStC may be used not only for peri-partum, but also for pre-partum tissue regeneration, potentially serving as autologous grafting either for the foetus and the newborn (Portmann-Lanz et al., 2006).

Our experimental evidence substantiates the higher proliferative potential of CVStC compared to adult BMStC (Poloni et al.). Intriguingly, the immunophenotype, the karyotype stability, the low telomerase activity (Zhao et al., 2008) and the low pluripotency-associated gene-product expression (Kaltz et al., 2008) make CVStC closer to BMStC than to cells of embryonic origin, whose high expression levels of the pluripotency factors Nanog, Oct3/4, Sox-2 and strong telomerase activity are currently well recognized (Hiyama and Hiyama, 2007; Kashyap et al., 2009). On the contrary, the positivity for these specific stem markers is still controversial for cells of adult origin (Avanzini et al., 2009; Hiyama and Hiyama, 2007; Kaltz et al., 2008; Zhao et al., 2008).

In agreement with the hypothesis that stromal cell plasticity is influenced by the tissue of origin (Rossini et al.), differentiation towards unrelated cell types such as cardiomyocytes and skeletal muscle cells was found limited in both CVStC and BMStC. Notably, CVStC ability to acquire osteocyte and adipocyte properties was less efficient than that of BMStC. This finding is in agreement with

previous report showing that osteogenic potential varied depending on sample sources (Guillot et al., 2008). Although CVStC skeletogenic properties were reduced, they expressed endothelial markers and acquired endothelial morphology more efficiently than BMStC. To our knowledge this is the first report in which the angiogenic properties of isolated StC from chorionic villi are described. In this light, the expression analysis of microRNA, selected among those involved in the positive/negative control of angiogenesis, indicated that the anti-angiogenic miR-21, -221 and -222 (Kuehbacher et al., 2008; Wang and Olson, 2009) are significantly down-modulated in CVStC. Specifically, miR-221 and -222 are known to inhibit tube formation in endothelial cells (Poliseno et al., 2006), exhibiting a negative effect on endothelial nitric oxide synthase (eNOS) expression and function (Suarez et al., 2007). Accordingly, miR-21 has been recently shown to have anti-angiogenic properties characterized by its inhibitory effect on endothelial cell proliferation and tube formation determined by RhoGTPase RhoB targeting (Sabatel et al.). Hence, the low expression of miR-21, -221 and -222 may be associated to the enhanced angiogenic response observed in CVStC. Although further experiments are required to elucidate this important point, the observation that CVStC are prone to differentiate along the endothelial lineage is in agreement with previous reports indicating mesenchymal stromal cells with pericytes-like phenotype are present in vascular niches around the blood vessels of term placenta villi, where they may contribute to the regulation of neo-vessel formation and maturation (Castrechini et al.). Along this line of evidence, the progenitors of haemangiogenic cells in early placenta are thought to

directly derive from the villi mesenchyme (Demir et al., 2004). Accordingly, also amnion-derived StC, originating from a virtually a-vascular tissue, have shown the ability to differentiate into endothelial cells (Alviano et al., 2007; Wu et al., 2008), highlighting the importance of vessel homeostasis in placenta development and maturation.

Of note, the isolation and culture of CVStC in AM medium enhanced the angiogenic precursor properties of CVStC, increasing their responsiveness to angiogenic stimuli. AM, in fact, not only improved the response to all the differentiation stimuli tested but maximized the acquisition of endothelial-specific markers. This observation is of therapeutic relevance, as culture conditions are emerging as a major determinant of cell plasticity (Wagner et al., 2006). Therefore, the choice of amplification medium must be considered a critical point in regenerative medicine, where the safety and the efficacy of therapeutic cells should proceed along. In this light, it is of interest that AM, which is the specific medium for CVStC, did not sustain BMStC proliferation, further indicating that environmental conditions influence stromal cells need for specific factors, to express their native proliferative/differentiative potential.

In conclusion, our study demonstrates that, although of foetal origin, CVStC exhibited restricted plasticity preferentially oriented toward the endothelial lineage, and therefore different to that of BMStC, whose potency is primarily directed toward the skeletal lineages (Bianco). Therefore, CVStC appear as a promising biological source suitable for cell therapy applications in vascular diseases.

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Chapter 4: Summary and Conclusions

The view of the heart as a postmitotic organ incapable of generating new myocytes has dominated cardiovascular science in the last decades. The injured heart attempts, ineffectively, to compensate for the loss of functioning myocardium, resulting in a downward spiral of adverse cardiac remodeling, scar formation and, ultimately, congestive heart failure. Likewise, rapid advances in stem cell and regenerative biology have prompted the scientific community to reconsider the assumption that the infarcted myocardium is incapable of self-repair. In this regard, the concept that new myocytes might be generated in the diseased myocardium by administering exogenous stem cells elicited such awesome excitement by the medical community that treatment of cardiac patients with autologous stem cells were expedited into clinical trials. Although the exact benefit of such treatment remains to be defined, it is clear that an effective, safe, and durable therapy for cardiomyocyte replacement will require a detailed understanding of the fundamental biology of cardiac progenitor cells. In this PhD thesis I tried to examine the existing knowledge about cardiovascular progenitor cell biology and adult mammalian heart regeneration, highlighting important questions in particular about efficient cardiac cell therapy.

A comprehensive understanding of developmental cardiovascular progenitor cells will help to inform our future regenerative strategies regardless of the source or type of cell used.

The discovery of cardiac progenitors and the improvement of culture systems to amplify these cells *in vitro* without losing their

differentiation potency opened new perspectives for clinical applications aimed at replacing myocardium lost as a consequence of acute or chronic ischemic disease.

Available treatments do not address the fundamental problem of the loss of cardiac tissue. As a result, interest in attempts to repair the failing heart with the use of stem cells has been increasing, since this approach has the potential to regenerate dead myocardium and thus alleviate the underlying cause of heart failure

We know very well the difficulty to isolate and to obtain a large number of cells for injection in a damaged heart. Also we know that the use of iPS cells in view of their possible therapeutic use is still far from an ending solution, in fact it needs to overcome several key issues, including the ethical concerns. This approach is also not free from challenges like the mechanism of the reprogramming process, which has yet to be elucidated, and the warranties for safety of generated pluripotent cells, especially.

For that reason we must think new ways to manipulate cells already committed and re-program them in precursors able to improve the function of the heart.

The generation of pluripotent cells from adult cells is undoubtedly a keystone achievement in stem cell biology.

A chemical approach for the generation of stem cells from adult cells has been invoked as a safer and more convenient alternative to genetic manipulation, which is widely used to generate iPS cells today. Very recent advances have already shown that the goal is getting closer, and we may imagine the possibility of easily generating stem cells customized to the patient, which will open new perspective in both

regenerative medicine and drug discovery. In fact, the ‘short-term’ application of iPSCs may be to use them for drug discovery or toxicology screens in vitro.

Moreover, they can allow the generation of disease models in culture, starting from patient cells, which would allow to test patient-specific therapies. We are at the beginning of a new exciting era in stem cell biology, there are great expectations and a remarkable potential ahead of us, and the best has yet to come.

Finally, we have also to think and to seek other sources in the human body to obtain, in an easier way, adult stem cells with high plasticity and a possible clinical application.

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Sono quasi passati quattro anni: da quel 5 marzo 2008, giorno della Laurea a oggi, 23 gennaio 2012…dottorato, stento quasi a crederci!
Quanti giorni, quanti ricordi, quante persone sono passate in questi anni…
In queste pagine c'è il riassunto di questi anni di lavoro, delle speranze, delle delusioni, dell'entusiasmo, delle preoccupazioni, delle lacrime e delle risate che mi hanno accompagnato in questo cammino…
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