**University of Milano-Bicocca**
**School of Medicine and Faculty of Science**

**PH.D PROGRAM IN TRANSLATIONAL AND MOLECULAR MEDICINE**
**DIMET**


# Human mesenchymal stromal cells: how the tissue of origin influences plastic properties and microRNA expression profile

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**XXVI CYCLE**
**Academic Year 2012-2013**

*“Learn from yesterday, live for today, hope for tomorrow.*

*The important thing is not to stop questioning”*

**Albert Einstein**

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### 1.1 Preface

The ability to generate an adult organism from a single fertilized oocyte or to regenerate tissues upon physiological turnover or injury depends on the activity of stem cells, which represent one of the most intriguing fields of modern biology. The discovery of the proliferative capacity and plasticity of various adult-derived stem cell population has sparked much interest and debate regarding their use as a potential therapy. In fact, the unexpected plasticity of adult stem cells to generate cells for autologous tissue grafts, have created a new interest in looking for alternative therapeutic approaches in adult organs that were previously thought to be unable to regenerate. Recent research has indicated that stem cells are likely the best candidate bearing this regeneration activity and regenerative medicine can be considered the next step in the evolution of organ replacement therapy [1] [2].

Mesenchymal cells are currently very much in the news because they represent a potential therapeutic tool for various

diseases such as muscular dystrophy [3], systemic lupus erythematosus [4], heart failure [5] and bone and joint disorders [6] [7].

The most well characterized mesenchymal cells are those obtained from bone marrow, which have been identified for the first time more than 40 years ago by Friedenstein and colleagues. They have been described a fibroblast-like population, adherent to plastic and able to differentiate *in vitro* into bone, cartilage, and adipose tissue [8] [9].

The current concept of mesenchymal stem cells was coined for the first time by Caplan in 1991 [10] as a result of the reported experiment. Bone marrow transplantation in heterotopic anatomical sites led to the development of ectopic bone. According to Caplan, adherent bone marrow non-hematopoietic cells could be defined as stem cells because they were able to self-renew and to differentiate into different cell types. However, the use of the term "stem" in relation to bone marrow non-hematopoietic cells has been criticized because of their heterogeneous nature and their replicative senescence (i.e. inability to stay in culture indefinitely). Moreover, there are no assays to determine bone marrow non-hematopoietic cell potential *in vivo*, in contrast to bone marrow hematopoietic cells which are able to reconstitute hematopoiesis in lethally irradiated mice [11] and in patients with leukemia undergoing myelosuppressive therapies [12] [13]. Convincing data to support the “stemness” of these cells were not forthcoming, and now most investigators recognize that *in vitro* isolated MStCs

are not a homogenous population of stem cells; for these reasons, it was recently introduced the term *mesenchymal stromal cells* (MStCs), referring to a fibroblastoid cell population with residual plasticity that can be isolated not only from bone marrow but virtually from the connective tissue of all adult [14] and fetal organs [15].

## 1.2 Stem Cells: an Overview

The concept of stem cell originated in the late XIX century as a theoretical postulate to explain the ability of certain tissues (blood, skin...) to self renew for the entire life of the organism. Although the question of what exactly is a stem cell remains controversial even after years of debate [16], the most widely accepted definition implies that stem cells must possess two essential properties:

- ✓ *Self-renewal*: is the ability of the cells to go through numerous cycles of cell division while maintaining the undifferentiated state.

- ✓ *Potency*: indicates the ability of stem cells to go through asymmetric division and is the capacity to differentiate into specialized cell types and terminally differentiated progenitors.

Usually between the primitive stem cell and differentiated descendants, there are intermediate populations of cells with a progressive proliferative potential and differentiation, classified as immature progenitors and precursors. Normally stem cells are mainly quiescent (in the G0 phase of the cell cycle), while the progenitors are highly proliferating; stem cells are characterized by a limited self-renewal in order to maintain a storage.

In relation to their differentiation capacity, stem cells can be classified into different groups (Figure 1):

- ✓ *Totipotent stem cells*: are the first cells formed in a new organisms and they are produced by the initial segmentations steps of the fertilized egg. Totipoten stem cells can construct a complete, viable organism and also differentiate into all embryonic annexes (for example placenta, umbilical cord), necessary for embryo implantation and feeding [17].

- ✓ *Pluripotent stem cells or embryonic stem cells* (ESCs) are the descendants of totipotent cells and can give rise to all the tissues of an adult organism (i.e. cells composing all the three germ layers), including the reproductive germ cells, but not extra-embryonic tissues [18]. The pluripotent cells are obtained from the inner cell mass of the blastocyst and can be grown indefinitely *in vitro*, under appropriate conditions (for example, stimulation with LIF,

leukemia inhibitory factor), maintaining their stem cell properties without karyotype aberrations. If reintroduced into a host blastocyst can reform a new viable embryo.

- ✓ *Multipotent stem cells:* are mainly found during the adult life and they are cells that possess more limited potential and can differentiate into only a few types of differentiated cells. Whether a stem cell is able to give rise to a single cell type terminally differentiated is defined unipotent cell. Unipotent cells can produce only one cell type, but have the property of self-renewal, which distinguishes them from non-stem cells (e.g. progenitor cells).

Multipotent and unipotent stem cells are present in the adult organism (Somatic or Adult Stem Cells), usually in tissues where the differentiated cells do not divide and have a short life span. The most known adult multipotent stem cells are Hematopoietic Stem Cells, a subpopulation of Bone Marrow Stem Cells able to generate all blood lineages.

Anyway, also organs that do not require frequent tissue regeneration must have a mechanism to replenish cells as they die, if not by injury, at least by natural homeostasis. Multipotent stem cells have in fact been found also in tissues that undergo limited regeneration, such liver [19], brain [20] and heart [21].

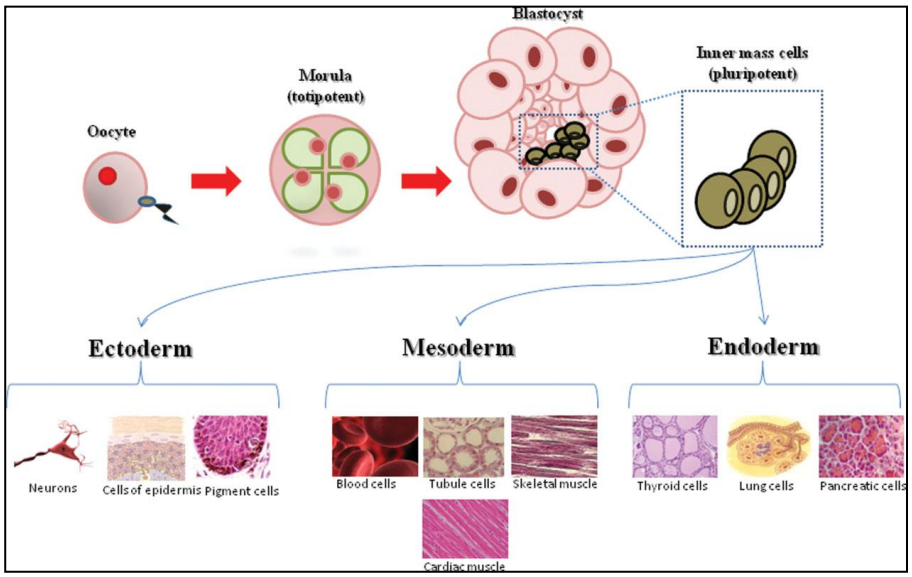


**Figure 1: Hyerarchy of stem cells *(H.K. Salem. Stem Cells, 2010)***

## 1.3 Definition and Features of Mesenchymal Stromal Cells

Friedenstein and colleagues demonstrated in 1970 that a population of fibroblast like cells, that can be isolated from bone marrow, formed bone, cartilage and reconstituted the bone marrow microenvironment. These cells were later called mesenchymal stem cells or marrow stromal cells (MStCs) [22] [23].

MStCs were originally isolated from bone marrow, but similar populations have been studied from different sources. Several studies have described the isolation of MStCs from various sources, such as: peripheral blood [24], umbilical cord [25],

bone [26], adipose tissue [27], skin [28], muscle [29], lung [30], brain [20], liver [19], heart [21]. Recent work has also shown considerable phenotypic and functional similarities between mesenchymal cells and bone marrow stromal cells isolated from fetal organs [15]. However, different laboratories employ different isolation and *in vitro* culture methods which could be responsible for the phenotype and function of resulting cell populations. In addition, the lack of any single unique specific cell surface marker to identify MStC population, coupled with differences in terminology have hindered the progress of MStC research. In order to overcome these issues, the International Society for Cellular Therapy Mesenchymal and Tissue Stem Cell Committee proposed the following minimum criteria for defining multipotent human mesenchymal stromal cells [31]:

- ✓ *Plastic-adherence* under standard culture conditions.

- ✓ *Expression of specific surface antigens*: MStCs must be positive (>95%) for CD105, CD73, and CD90, and lack the expression (<2% positive) of typical hematopoietic markers CD34, CD14, CD45, CD19, and HLA-DR.

- ✓ *Multipotent differentiation*: under specific stimulus, MStCs are able differentiate into osteocytes, adipocytes, and chondrocytes *in vitro*.

These criteria are designed not only to define the MStCs, but also to exclude hematopoietic cells, which is important since, as stated above, MStCs are most commonly isolated from bone marrow. Also, the minimum criteria used for the definition of MStCs do not allow to discriminate between stromal cells derived from different tissues. Furthermore, the properties of MStCs described previously are also related to other stromal cells such as fibroblasts [32]. By the current definition provided by the ISCT, a precise distinction from MStCs and fibroblasts is impossible [33]; also discrimination of subpopulations of stromal cells with specialized functions is really difficult. Nevertheless, there is still a general consensus in accepting that MStCs constitute a cell population distinct from fibroblasts [33]. This hypothesis is supported by recent studies in which was established a hierarchy of mesenchymal cells during differentiation; these results have proposed SSEA-1 as a possible marker at the beginning of the hierarchy [34].

## 1.4 From Bench to Bed: MStCs for Regenerative Medicine

MStCs are able to differentiate into osteocytes, adipocytes, chondrocytes and skeletal myoblasts. In addition, it has also been demonstrated that MStCs present a significantly higher differentiation plasticity leading, both *in vitro* and *in vivo,* to the

commitment not only to multiple skeletal cell types, but also to endothelium, neural cells, epatocytes and cardiomyocytes [35] [36] [37] [38] [39]. These observations have formed the basis for most of the current studies of MStCs because they have shown a great clinical interest in regenerative medicine and cell therapy to induce tissue repair and restore organ functionality. In order to induce the regeneration of different injured tissues, such as the heart [40], skeletal muscle [3], bone [6], cartilage [7], nervous tissue [41], liver [42], lungs [43], kidney [44], numerous pre-clinical studies on animal models have been carried out and also first clinical trials on human patients have started. Up until now, cell therapy has demonstrated benefits and efficacy in pre- clinical studies on animals, while results on human clinical trials are still limited and in most cases preliminary data are related to the safety of cellular product [45].

### 1.4.1 MStCs and their differentiative potential in cell therapy

Although isolation of MStCs has been reported from several adult [14] and fetal tissues [15], bone marrow remains the principal source of MStCs for most preclinical and clinical studies and this is mainly due to the fact that MStCs from bone marrow (BMStCs) are the most well characterized.

MStCs are of great interest as potential therapeutic tools for bone and cartilage regeneration in metabolic diseases or genetic disorders, such as osteogenesis imperfecta or hypophosphatasia [6]. Horwitz and colleagues performed the first transplant of therapeutic BMStCs in patients with osteogenesis imperfecta , a genetic disorder responsible for the production of defective collagen type I, resulting in a delay of bone growth, high frequency of fractures and progressive bone deformity [46]. Results obtained seem to be encouraging and treated patients showed an improvement in bone structure and general clinical conditions [31]. Moreover, new clinical strategy has been reported in which improved bone quality was obtained by applying BMStCs with platelet rich plasma [47].

MStCs transplantation is a new clinical approach to induce the regeneration of skeletal muscle due to degenerative diseases such as Duchenne muscular dystrophy (DMD). Pre-clinical and clinical studies showed that BMStCs increase the regenerative ability of degenerating muscles in DMD patients [3]. After intra arterial transplantation, MStCs were able to migrate in the host damaged muscle tissue by chemotactic signals not fully elucidated [48]. Also, it has been observed that MStCs transplantation, coupled with exercise, plays an important role in improving the regeneration of muscle fibers and in the acquisition of contractile capacity in distrophic murin model [49].

Some studies showed that BMStCs or MStCs from umbilical cord have a limited ability to differentiate into neural-like cells, expressing typical neural markers such as neurofilament and beta-tubulin [50]. Kopen and colleagues demonstrated that MStCs injected into the central nervous systems of newborn mice migrate throughout the brain and adopt morphological and phenotypic characteristics of astrocytes and neurons [51]. These findings were confirmed by other reports, which prompted efforts to identify conditions that induced neural differentiation of MStCs *in vitro* as a means to investigate their observed plasticity *in vivo* [52] [53] [54]. Moreover, it has also been reported a neuroprotective effect of MStCs mediated by their ability to produce various trophic factors that contribute to functional recovery, neuronal cell survival, and stimulation of endogenous regeneration [41]. Because of their ready availability, their ability to be easily expanded, and reports of neural potential, MStCs populations have generated considerable interest cell-based therapies for neurodegenerative diseases. However, review of *in vitro* studies indicates that the methods used to promote neural cell differentiation and assess the biology of the differentiated cells are fragmented and inconsistent. Furthermore, obtaining neural fate to MStCs is further confounded by the lack of specificity of neural markers employed, the heterogeneous nature of the MSC populations under examination, and artifacts associated with methods used to culture-expand cells *in vitro* [55].

Regarding the regeneration of the heart, pre-clinical and clinical studies have demonstrated that BMStCs induce an improvement in cardiac function in the treatment of acute or chronic heart failure; in fact, these experiments showed that MStCs can attenuate the pathological ventricular remodeling in rats [56] [57]. Nevertheless, the mechanisms underlying improvement in ventricle function are still unclear and object of a strong debate. In fact, the possibility that BMStCs may trans-differentiate into non-skeletal tissues has been recently criticized and the differentiation of BMStCs into cardiomyocytes is being revaluated [58]. It has been proposed that the secretion of cytokines and angiogenic factors, rather than differentiation into cardiovascular cell types, could be responsible for the beneficial effects of MStCs on the heart function [59]. In addition, major safety concerns have been raised following the observation that BMStCs injected in a mouse myocardial infarction (MI) model induced intra-myocardial calcification [60].
Recent studies have focused their attention on the role of MStCs derived from other sources to regenerate heart; numerous studies have demonstrated that transplantation of cardiac mesenchymal cells in animal models of post-myocardial infarction (MI) limits left ventricular (LV) remodeling and improves LV function, in the setting of both an acute and a chronic MI [61] [62]. Moreover, the first Phase I clinical trial of cardiac mesenchymal cells (SCIPIO) is currently ongoing on patients with heart failure of ischemic etiology undergoing coronary artery bypass grafting (CABG) [63], but recent results

shows that infusion of these cells produces a striking improvement in both global and regional LV function, a reduction in infarct size, and an increase in viable tissue that persist at least 1 year and are consistent with cardiac regeneration [64].

### 1.4.2 MStCs and transdifferentiation

According to the dogma of embryonic specification of adult stem cells, either multi or unipotent stem cells may only acquire cell lineages present in the organ they belong to. This classical view has been challenged by different works showing the ability of BMStCs to differentiate into unexpected cellular types, like neurons [65], muscle cells [66] or myocardial-like cells [67] if inserted into a proper microenvironment. The discovery of this property, called *plasticity* or *transdifferentiation*, opened a new era for stem cells research. Many works described this phenomenon in other cells; neuronal stem cells were demonstrated to be able to generate blood [68], stem cells from adipose tissue differentiated into skeletal myotubes [69] and mesenchymal stem cells from placenta may give rise to components of neurogenic lineage [70]. However, *in vitro* cell plasticity may be a result of epigenetic modifications that do not occur in normal tissue repair processes *in vivo* [71]. Nevertheless, despite the initial enthusiasm, the plasticity of MStCs is today the object of an open debate. Notably since 2001 different authors have been questioning the concept of

transdifferentation, pointing out particularly the difficulty to reproduce the results shown in the early works and demostrating that *in vitro* transdifferentiation may be at least in part the result of fusion events between the stem cells and the differentiated cells [72]. Also, in vitro transdifferentiation may be derived by an incomplete differentiation; for example, it has been observed that BMStCs co-cultured with rat embryionic cardiomyocytes stained positively for sarcomeric proteins (troponin I and alpha-actinin) and expressed selected cardiac-specific genes (atrial natriuretic factor, Nkx2.5, and alpha-cardiac actin), but they did not generate action potentials or display ionic currents typical of cardiomyocytes. In fact, detailed immunophenotyping of differentiated BMStCs demonstrated expression of all antigens used to characterize MStCs, as well as the acquisition of additional markers of cardiomyocytes. Retention of a stromal cell phenotype could explained the incomplete differentiation observed [73].

### 1.4.3 MStCs and their immunomodulatory properties

A large number of studies have assessed the fate of MStCs *in vitro* and *in vivo* and their effect on disease progression in experimental animal models and human clinical trials. Despite some initial encouraging results, these *in vivo* studies have shown that MStCs typically exhibit low levels of engraftment and transdifferentiation within diseased or injured tissues and therefore do not contribute physically to tissue regeneration to a

significant extent [55]. These findings initially cast doubt on the prospect of harnessing MStCs plasticity to treat diseases. However, more recent reports have revealed that MStCs promote tissue repair by secretion of factors that enhance regeneration of injured cells, stimulate proliferation and differentiation of endogenous stem-like progenitors found in most tissues, decrease inflammatory and immune reactions. Therefore, the ability of such cells to alter the tissue microenvironment may contribute more significantly than their capacity for transdifferentiation in effecting tissue repair. The increase in inflammatory chemokine concentration at the site of inflammation is a key mediator of trafficking of MSC to the site of injury. Chemokines are released after tissue damage and MSC express several receptors for chemokines [74]. MStCs are known to produce paracrine growth factors that likely support vascular regeneration and cardiomyocyte protection in the injured myocardium [75]. Importantly, the paracrine activity by MStCs can be also potentiated by genetic engineering approaches. For example, overexpression of *pro*-survival factors (such as AKT), angiogenic factors (such as VEGF), or stem-cell homing factors (such as SDF-1) enhances the preclinical therapeutic efficiency of MStCs [75]. Furthermore, MStCs appear to have a major advantage over many other cell types for cellular therapy because they are immunological privileged (i.e. escape immune recognition) and in large animals, they can be transplanted in MHC mismatched recipients without need for immunosuppression [76]. In fact

MStCs express low levels of human MHC class I and lack human MHC class II [77], which at least potentially may alleviate rejection of allogenic MStCs in the tranplanted organs. Also, another advantage of MStCs use for tissue repair is offered by MStCs immunosuppressive properties on T-cell function suppression [38] [78]; in particular studies have been demonstrated that the effects of MStCs on T-lymphocyte proliferation suppression is not associated with induction of apoptosis, but it is due to a block of cell division, which maintains T cells in a quiescent state [79]. MStCs immunoregulatory potential has also been observed on other immune cells , such as B lymphocytes [80], natural killer cells (NK) [81] and dendritic cells [82] (Figure 2). *In vivo*, it has been shown that co- infusion of MStCs and HSCs promotes and facilitates the recovery of hematopoiesis in the host, reducing the risk of adverse reaction after graft [83].
In particular, in a paper published in the Lancet in 2004 , the authors have shown that MStCs injection is able to dramatically reduce and/or reverse the evolution severe graft versus host disease (GVHD, a syndrome that occurs after transplantation of tissues or organs, in which the newly transplanted donor cells attack the transplant recipient body and provide an exaggerated immunological response against the host immune system) in a significant proportion of patients [84]. Although the mechanisms of immunomodulation *in vivo* are still unfolding and currently under investigation, MStCs represents also a source for clinical application in inflammatory [85] and autoimmune disorders [86].

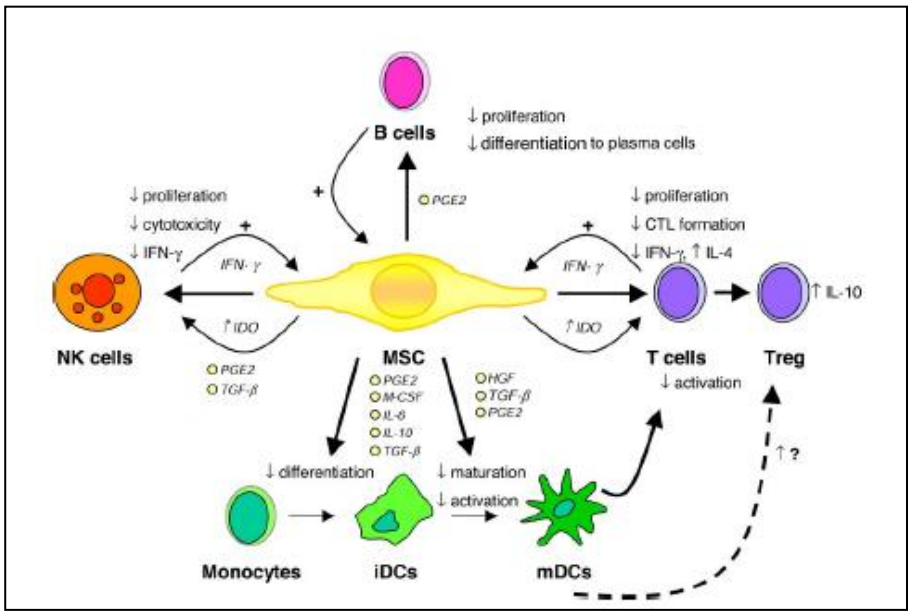


**Figure 2: Immunomodulatory properties of MStCs *(A.J. Nauta. Blood, 2007)***

## 1.5 Some examples of MStCs obtained from different tissues

Stromal cells can be isolated from a variety of adult tissues and organs [14] [21], but recently it has been observed the presence of MStCs also in fetal tissues, such as placenta [87]. In particular, a special attention has been given to MStCs isolated from three different sources in our laboratory: bone marrow-derived mesenchymal stromal cells (BMStCs), cardiac mesenchymal stromal cells (CStCs) and chorionic villi-derived mesenchymal stromal cells (CVStCs).

### 1.5.1 Bone Marrow Mesenchymal Cells (BMStCs)

BMStCs are the most extensively characterized mesenchymal stromal cells and they represent the gold standard for MStCs characterization and application to regenerative medicine. Adult bone marrow contains a heterogeneous population of cells, including hematopoietic stem cells, macrophages, erythrocytes, fibroblasts, adipocytes, and endothelial cells. In addition to these cell types, bone marrow also contains a subset of nonhematopoietic stem cells that posses a multilineage potential [35], commonly called bone marrow mesenchymal stromal cells. More than 40 years ago, Friedenstein and colleagues [8]. first described BMStCs as hematopoietic supportive cells of bone marrow. It has been described a population of multipotential stromal precursor cells that were spindle-shaped and clonogenic in culture conditions, defining them as colony-forming unit fibroblasts (CFU-F) [88]. These cells were able to differentiate into adipocytes, chondrocytes, osteocytes, and myoblasts, both *in vitro* and *in vivo*. In addition, it has also been demonstrated that BMStCs are capable of differentiating into cardiomyocytes, neurons, and muscle cells. However, convincing data to support the “stemness” of these cells were not forthcoming, and now most investigators recognize that *in vitro* isolated BMStCs are a heterogeneous population of stem cells, although a bona fide mesenchymal stem cell may reside within the adherent cell compartment of marrow [89]. In fact, a study published by Bianco’s group

showed 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 [90].

### 1.5.2 Cardiac Mesenchymal Cells (CStCs)

Although BMStC are currently being evaluated for their capacity to regenerate both skeletal tissues and unrelated tissues, such as the heart [91], up to now, poor attention has been given to the therapeutic potential of cardiac-specific stromal cells (CStCs). CStCs are in theory the most suitable cells for cardiac repair and have been demonstrated to repair infarcted myocardium in rats [92], dogs [93] and very recently in humans [63]. CStCs represent an heterogenous population of MStCs; 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. For example, the first attempt to isolate and characterize an endogenous myocardial stem cell population (so-called Side-Population) was made by Hierlihy and co-workers in 2002 [94]: these cells were characterized by the ability to extrude Hoechst dye, attributable to expression of ATP-binding drugs transporters ABCG2 and MDR-1, but these cells were unable to differentiate spontaneously into cardiomyocytes. Anversa's group published a work where a stem cell pool was isolated from the adult rat [92] and human

heart [95], positive for the stem-cells markers c-Kit. C-kit positive cells were self-renewing and, if cultured under proper stimulation condition, they differentiated into cardiomyocytes, endothelial and smooth muscle cells. Also, Sca-1+ cells isolated from mouse heart were shown by Schneider's group to home to damaged myocardium if injected intravenously after ischemia [96]. Recently, cardiac progenitors expressing the homeobox gene Islet-1 (Isl-1) have been described in postnatal rat, mouse and human myocardium [97]. Isl-1+ cells can be considered cardiomyocyte precursor cells that display the capacity to give origin to fully differentiated cardiomyocytes but it is currently unknown whether they exist in adult hearts.

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. In a recently published study by Rossini and colleagues [98] 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. This study has been demostrated that both *in vitro* and *in vivo* CStCs isolated from specimens of adult human auricles were more oriented towards the cardiovascular phenotype compared with BMStCs.

### 1.5.3 Chorionic Villi Mesenchymal Cells (CVStCs)

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 membranes, such as amnion and chorion [87]. Specifically, although chorion and amnion are a rich source of MStCs, the largest body of information is available only about cells isolated from term amnion [99] [100], whereas only few reports describe chorionic stromal cell-derived from first trimester villi [101]. MStCs derived from first-trimester villi (CVStCs) are easily isolated from waste material generated during early-gestation diagnostic procedures. Chorionic villus sampling, in fact, is usually performed around the 11–13th week of pregnancy, making CVStC a developmentally younger and relatively abundant cell type that can be obtained from non-abortive human fetal material. In consequence of their origin from a developmentally very young tissue, the possibility that MStCs from early fetal tissues may retain a wider differentiation potential compared to adult MStCs has been suggested, but, at present, only few reports addressed this aspect [102] [103]. In fact, it is generally accepted that placenta-derived MStCs, including CVStCs, display a multi-lineage differentiation ability similar to that of adult BMStC [104]. However, CVStCs and placenta-derived MStCs exhibited a greater proliferative capacity compared to BMStCs [105]. Moreover, the fetal origin of placental cells may be responsible for the greater immunosuppressive action

compared to MStCs from adult tissues, such as BMStCs [106]; in fact, MStCs from placenta may generate a lower immune response, with decrease of immunological complications relating to allograft transplants [107].

Due to their differentiation ability and immunomodulatory properties, CVStCs represent an attractive source for potential clinical applications in cell therapy and regenerative medicine.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 fetus and the newborn [70].

## 1.6 MStCs and distinctive differentiative properties

MStCs obtained from several adult and fetal tissues have morphology, immunophenotype and growth properties similar to the most well characterized BMStCs (Figure 3). They 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, HLADR antigens) [31]. However, numerous studies have reported that MStCs differentiation potential was related to their tissue of origin [14].

For example, Kern and colleagues [108] demonstrated that MStCs obtained from umbilical cord blood (UCB-MStCs), adipose tissue (AT-MStCs) and BMStCs exhibited a

comparable differentiation ability toward osteogenic (Von Kossa staining and expression of alkaline phoshatase) and

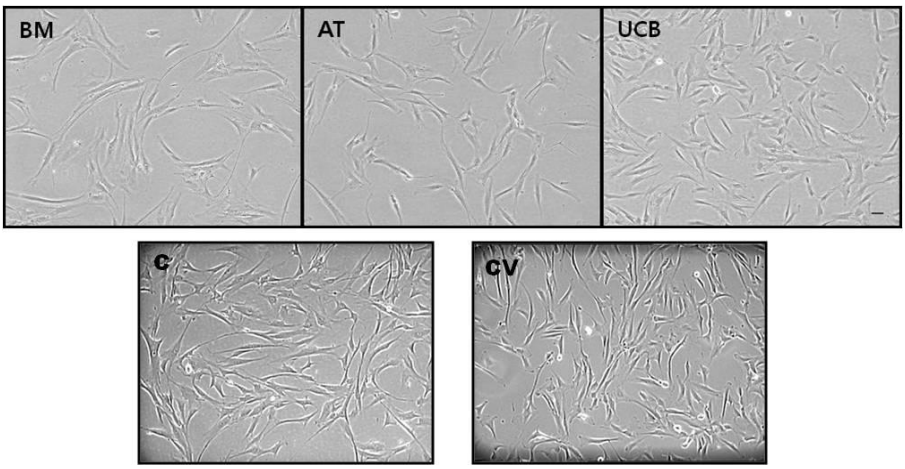


**Figure 3: MStCs exhibit the same morphology, independently by different tissue of origin** (BM, bone marrow; AT, adipose tissue; UCB, umbilical cord blood; C, cardiac stromal tissue; CV, chorionic villi)

chondrogenic (Safranin O staining) lineages. However, UCB-MStCs showed no adipogenic differentiation capacity (Oil Red O), in contrast to BM- and AT-MStCs. These results have been confirmed by another work [109] which showed that all three sources (UCB-MStCs, AT-MStCs and BMStCs) presented a similar capacity for chondrogenic and osteogenic differentiation and they differed in their adipogenic potential (Figure 4). Adipogenic differentiation showed that UCB-MStCs produced few and small lipid vacuoles in contrast to those of BMStCs and AT-MStCs; moreover, the mean area occupied by individual lipid droplets BMStCs was higher than those of AT-MStCs, a

finding indicating more mature adipocytes in BM-derived MStCs than in treated cultures of AT-MStCs. However, there are conflicting data concerning the adipogenic differentiation capacity of UCB-MStCs [110] [111].

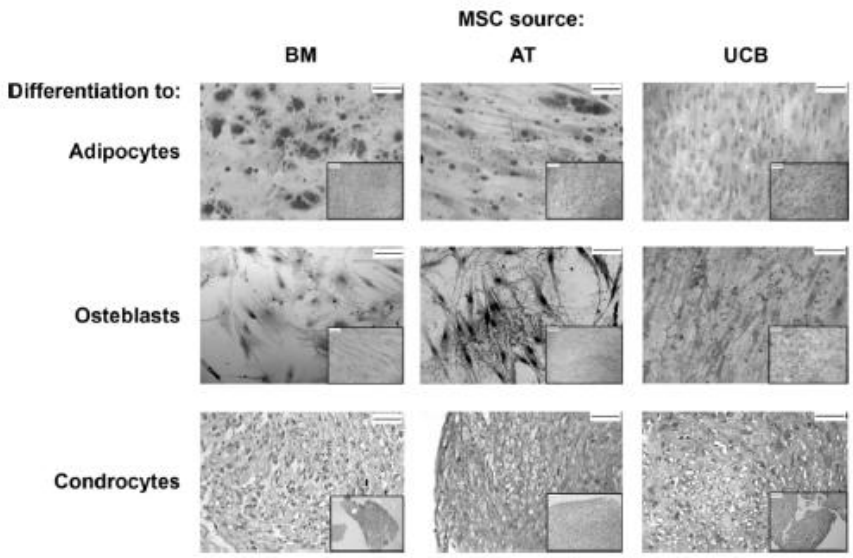


**Figure 4: Differentiation of BMStCs, UCB-MStCs, and AT-SCs Cells after 21 days in the presence of specific media for adipocytes, osteoblasts, and chondrocytes (C.K. Rebelatto. *Exp Biol Med, 2008*)**. Differentiation into the adipocyte lineage was demonstrated by staining with Oil Red O. Alizarin Red S staining shows mineralization of the extracellular matrix. Toluidine Blue shows the deposition of proteoglycans and lacunae. Untreated control cultures without adipogenic, osteogenic, or chondrogenic differentiation stimuli are shown on the bottom right corner of each image.

In the study of da Silva Meirelles and colleagues, MStCs were obtained from different tissues or organs of adult mice: aorta, brain, bone marrow, kidney, liver, lungs, muscle, pancreas, spleen, thymus and vena cava [14]. When subjected to

osteogenic or adipogenic differentiation conditions, MStCs populations confirmed their mesenchymal characteristics by depositing a calcium-rich mineralized matrix as evidenced by Alizarin Red S staining, or by acquiring intracellular lipid droplets, evidenced by Oil Red O staining. Differences in the frequency of differentiated cells, as well as in the degree of differentiation, could be observed among the cultures originating from different tissues. For instance, vena cava derived-MStCs were very efficient at depositing mineralized matrix, whereas muscle-derived MStCs showed little efficiency. On the other hand, muscle-derived MStCs were easily induced to differentiate into mature adipocytes whereas the vena-cava-derived cultures presented small, poorly developed lipid vacuoles. The adipogenic differentiation observed in lung-, brain- and kidney-derived MStCs seemed to be less efficient even though the degree of adipogenic differentiation presented by kidney- and lung-derived MStCs was comparable to that of BMStCs. However, these populations required a longer induction period to differentiate into adipocytes as compared with BMStCs. In addition to differentiation induced as described above, in some cases spontaneous differentiation was seen in primary cultures. For example, in cultures derived from aorta and muscle, myotube-like cells and adipocytes were often observed. This phenomenon could be caused by the presence of myogenic- and adipogenic-committed progenitors in the primary culture. Spontaneous differentiation was not observed

in primary cultures originating from the other organs and tissues studied.

In a recently published study by Rossini and colleagues from our laboratory, it has been reported that both *in vitro* and *in vivo* cardiac stromal cells (CStCs) isolated from specimens of adult human auricles were more oriented towards the cardiovascular phenotype compared to BMStCs [98]. In spite of a remarkable similarity in growth, morphology, and immunophenotype, CStCs and BMStCs differed significantly in gene, and protein expression and exhibited tissue-specific responses to differentiating stimuli. Notably, CStCs revealed higher level of c-Kit, GATA4, GATA6, KLF5 and myosin light chain-2a (MYLC-2a) mRNA than those of BMStCs; however, Nkx2.5, α-myosin heavy chain (α-MHC), α-sarcomeric actin (α-Sarc) and cardiac Troponin-I (Tn-I) were either absent or present at very low level in both cell populations. *In vitro* differentiation assays showed that CStCs, compared to BMStCs, were less competent in acquiring the adipogenic and osteogenic phenotype but more efficiently expressed cardiovascular markers. Under specific endothelial stimuli, CStC significantly up-regulated the expression of CD31, CD144 and VEGFR-2, whereas BMStCs remained negative. Additionally, only CStCs ability to develop capillary-like structures on Cultrex BME increased over 8-fold, while BMStCs response was unchanged. In an attempt to improve CStC cardiogenic potential, we tested a low serum medium supplemented with All-Trans Retinoic Acid and

phenylbutyrate (ATRA/PB medium) [112]. Under these conditions CStC significantly up-regulated the expression of α-Sarc, α-MHC, Tn-I and MYLC-2a, as well as α-SMA. In contrast, BMStC modestly up-regulated only GATA-4 and MYLC-2a (Figure 5).

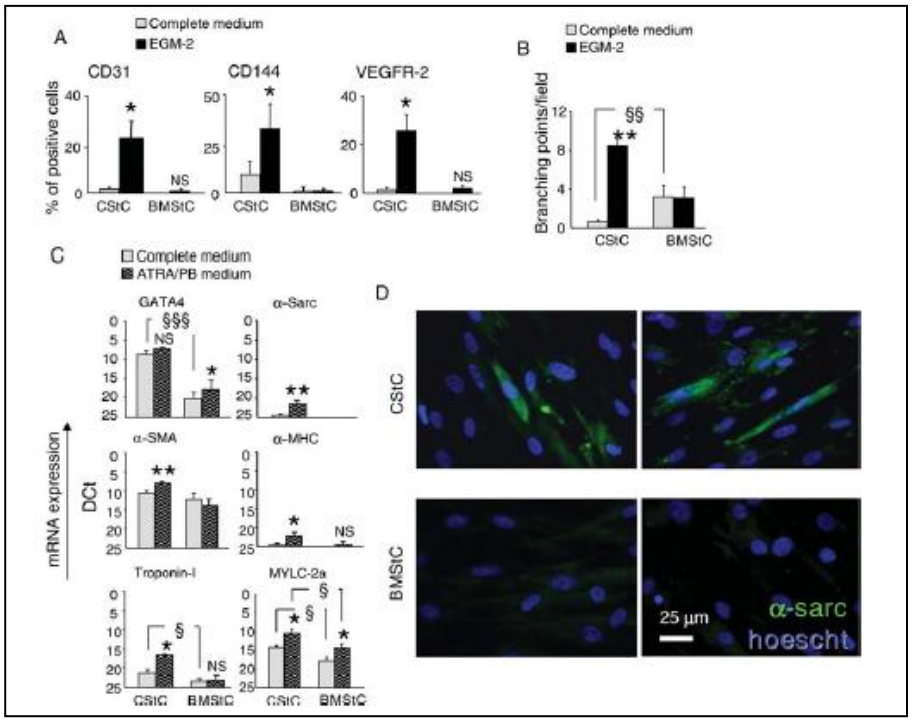


**Figure 5: *In vitro* endothelial and cardiomyogenic differentiation (A. Rossini. *Cardiovasc Res, 2011*).**
(A) After 3 weeks in EGM-2, only CStCs acquired positivity for CD31, CD144, and VEGFR-2. (B) Bar graph depicting average results for the tube formation assay on Cultrex. (C) Cardiomyogenic differentiation was evaluated in a medium containing ATRA and PB, where CStCs up-regulated the expression of α-Sarc and α-SMA transcripts as well as of α-MHC, Tn-I, and MYLC-2a. GATA4 expression was unvaried. BMStCs exhibited a modest enhancement in GATA4 and MYLC-2a expression, whereas α-Sarc, Tn-I, and α-MHC transcripts remained absent or low. (D) In ATRA/PB medium, cells acquired an elongated shape and CStCs, but not BMStCs, expressed the protein a-Sarc (FITC, green).

In light of these *in vitro* observations CStCs and BMStCs therapeutic potential was challenged *in vivo* following intramyocardial injection in a rat model of chronic myocardial infarction (MI). It has been demonstrated that CStCs not only improve cardiac function, but participates to tissue regeneration more efficiently than their bone marrow counterpart. Importantly, CStCs persisted longer within the cardiac tissue and migrated within the scar, promoted angiogenesis, and differentiated into adult-like cardiomyocytes more efficiently than BMStCs. 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 6). *In vivo*, the evidence in favour of CStCs/BMStCs 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 CStCs production of IL-6 and LIF was higher than that of BMStCs is in agreement with their more efficient ability to promote angiogenesis within the infarcted region [113]. 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.

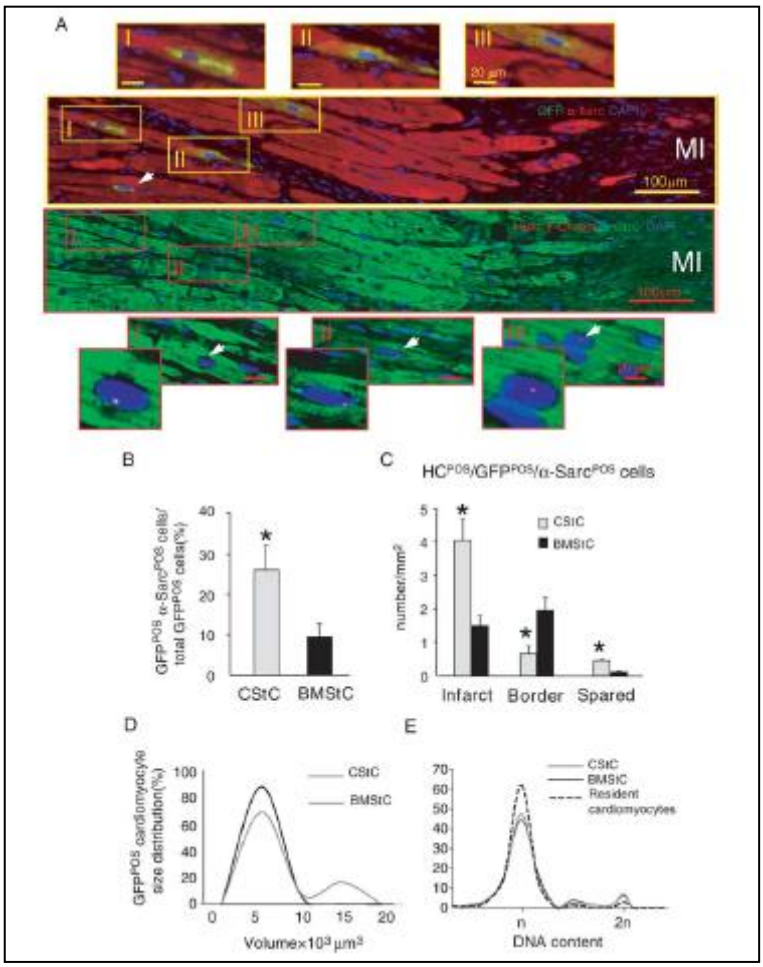


**Figure 6: Human CStC and BMStC differentiation *in vivo* (A. Rossini. *Cardiovasc Res, 2011*).**

(A) The upper central panel shows GFP+ (green)/α-Sarc+ (red) cardiomyocytes with defined sarcomere striations derived from CStCs and located in the infarct (MI) border zone. The lower central panel corresponds to the serial section confirming, by FISH for human chromosome Y (HC, red), the human origin of α-Sarc+ (FITC)-labelled cells. The areas in the rectangles (I, II, and III) are shown at higher magnification. (B) The percentage of GFP+/α-Sarc+ cardiomyocytes over the total counted GFP+ cells. (C) The density of HC+/ GFP+/α-Sarc+ cardiomyocytes. (D) CStC- and BMStC-derived cardiomyocytes size distribution. Notably, a significant fraction of CStC-derived cardiomyocytes reached volumes 15 000 $mm^3$. (E) DNA content distribution of CStC-, BMStC-derived, and rat resident cardiomyocytes.

A recent study proposed the hypothesis that MStCs from the heart and epicardial fat would be better cells for infarct repair. To test this hypothesis MStCs derived from epicardial fat, pericardial and subcutaneous fat, bone marrow and atrial tissue were compared [114]. Angiogenic effect of MStCs from different sources were evaluated both *in vitro* and *in vivo; s*ignificantly, the number of capillary-like structures in BMStCs was the lowest compared with right atrial and epicardial fat MStCs. To assess the angiogenic effect of MStCs transplantation, cell survival and vessel density 27 days after cell transplantation in rats infarcted myocardium. Consistent with the findings of *in vitro* angiogenic assays, right atrial MStCs induced the highest capillary density (Figure 7). To test the effect of different MStCs on cardiac remodeling and function after myocardial infarction (MI), rats were evaluated through serial echocardiography studies. Surprisingly, in contrast to *in vitro* and *in vivo* findings, which highlighted the angiogenic potential of right atrial and epicardial fat MStCs, their effect of on cardiac remodeling and function was inferior to subcutaneous fat MStCs. In fact, the advantage of MStCs from subcutaneous fat was reflected in attenuation of LV dysfunction after MI (Figure 8). The major new findings of the present study include several unexpected results that could be relevant to the use of MStCs for cardiovascular regenerative medicine because it has been demonstrated that MStCs from the right atrium and epicardial fat of cardiac patients could impair heart function after myocardial infarction; moreover this work extend the knowledge

regarding the involvement of adipose tissue MStCs in the pathogenesis and progression of various cardiovascular disease.

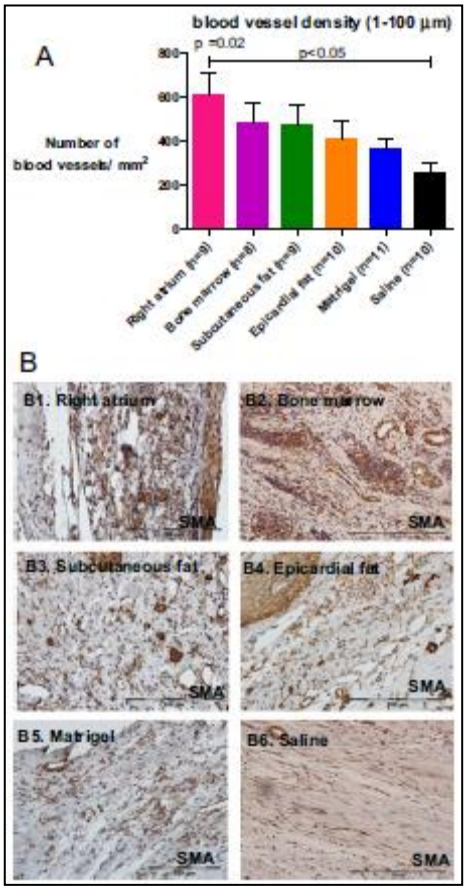


**Figure 7: Diverse vascularization effect of human MStCs from different locations after transplantation in rats (N. Naftali-Shani. *J Am Heart Assoc, 2013*)**. A) Vessel density was determined by the number of smooth muscle actin positive vessels in the infarct zone of the different groups. B) Representative microscopic images from each group.

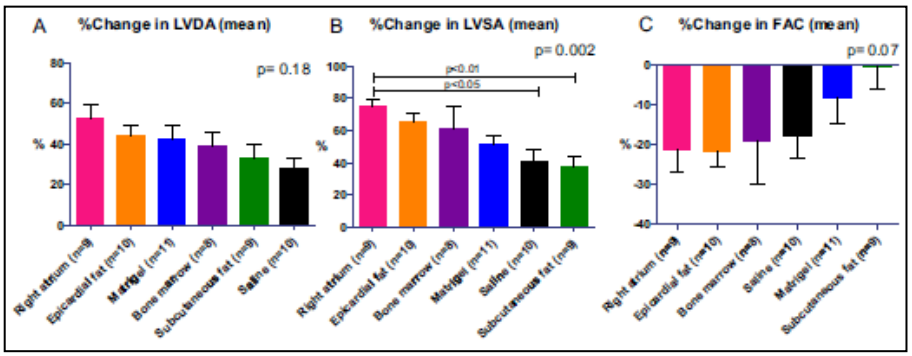


**Figure 8: Diverse effects of MStCs from different locations on LV remodeling and function in a rat model of MI based on changes in echocardiography measurements (N. Naftali-Shani. *J Am Heart Assoc, 2013*)**. While subcutaneous fat MStCs preserved LV diastolic (A) and systolic (B) area, right atrial MStCs increased it (B). Significantly, MStCs from subcutaneous fat attenuated LV dysfunction as indicated by change in fractional area change, FAC (C).

# 2. What are microRNAs?

MicroRNAs (miRNAs) comprise a large family of small, single-strand, non-coding RNAs (21-23 nucleotides) that have emerged as key post-transcriptional regulators of gene expression in animals and plants, and have revolutionized comprehension of the post-trascriptional regulation of gene expression [115]. Encoded by eukaryotic nuclear DNA, miRNAs are thought to control gene expression at the post-transcriptional level by degrading or repressing target messenger RNAs (mRNAs). The human genome may encode over 1000 miRNAs [116] and are predicted to control the activity of more than 50% of all protein-coding genes [117]. Functional studies indicate that miRNAs have important roles in a wide range of biological processes, including development, determination of cell identity, differentiation and also changes in their expression are associated with many human pathologies.

## 2.1 Hystory

The first miRNAs were characterized in the early 1990s, specifically in 1993 by Victor Ambros, Rosalind Lee and Rhonda Feinbaum during a study of the gene *lin-14* in C. elegans development [118]. They found that LIN-14 protein

abundance was regulated by a short RNA encoded by the *lin-4* gene. A 61-nucleotide precursor from the *lin-4* gene matured to a 22-nucleotide RNA that contained sequences partially complementary to multiple sequences in the 3' UTR of the *lin-14* mRNA. This complementarity was both necessary and sufficient to inhibit the translation of the *lin-14* mRNA into the LIN-14 protein. However, miRNAs were not recognized as a distinct class of biological regulators with conserved functions until the early 2000s, when a second short RNA was characterized: let-7, which repressed gene expression during developmental stage transitions in C. elegans. let-7 was soon found to be conserved in many species, indicating the existence of a wider phenomenon [119].

## 2.2 Biogenesis and Processing of microRNAs

MiRNAs are individually encoded by their own set of genes and are an integral component of the genetic program. Some are independent genes located in non-coding regions of the genome, whereas others occur in the introns of protein-coding genes [120]. MiRNAs originate from longer precursor RNAs called primary miRNAs (pri-miRNAs) that are regulated by conventional transcription factors and transcribed by RNA polymerase II. Pri-miRNAs are hundreds to thousands of nucleotides long which fold into harpins and are processed in the nucleus into an ~70- to 100-nucleotide hairpin-shaped

precursor miRNA (pre-miRNA) by the RNase III enzyme Drosha and the double-stranded RNA binding protein DGCR8 (DiGeorge syndrome critical region gene 8) [121].

Pre-miRNAs that are spliced directly out of introns, by-passing the Drosha-DGCR8 step, are known as "Mirtrons" and are produced as a result of splicing and debranching [122]. Pre-miRNAs are then transported into the cytoplasm by the nuclear export factor, Exportin 5 and further processed into an ~19- to 25-nucleotide double-stranded RNA by the RNaseIII enzyme Dicer, assisted by the double-stranded RNA binding protein TRBP (transactivation-responsive RNA-binding protein). Following processing, one strand of the miRNA duplex is preferentially incorporated into an miRNA-induced silencing complex (miRISC) and becomes the “mature” miRNA, whereas the other strand can be rapidly degraded or processed to become a “mature” miRNA itself [121]. In the latter case, names like miR-xx-5p (from the 5' arm) and miR-xx-3p (from the 3' arm) are used [123]. On being loaded into the miRISC, the mature miRNA associates with target mRNAs and acts as a negative regulator of gene expression (Figure 9). Argonaute (AGO) proteins, which directly interact with miRNAs, and glycine-tryptophan protein of 182 kDa (GW182) proteins, which act as downstream effectors in the repression, are key factors in the assembly and function of miRISCs [124]. However, the ratio of miR-5p to miR-3p can vary in different tissues or developmental stages, which probably depends on specific properties of the pre-miRNA or miRNA duplex, or on the activity

of different accessory processing factors [125] [126]. Moreover, the ratio might be modulated by the availability of mRNA targets as result of enhanced destabilization of either miR-5p or miR-3p occurring in the absence of respective complementary mRNAs [127].

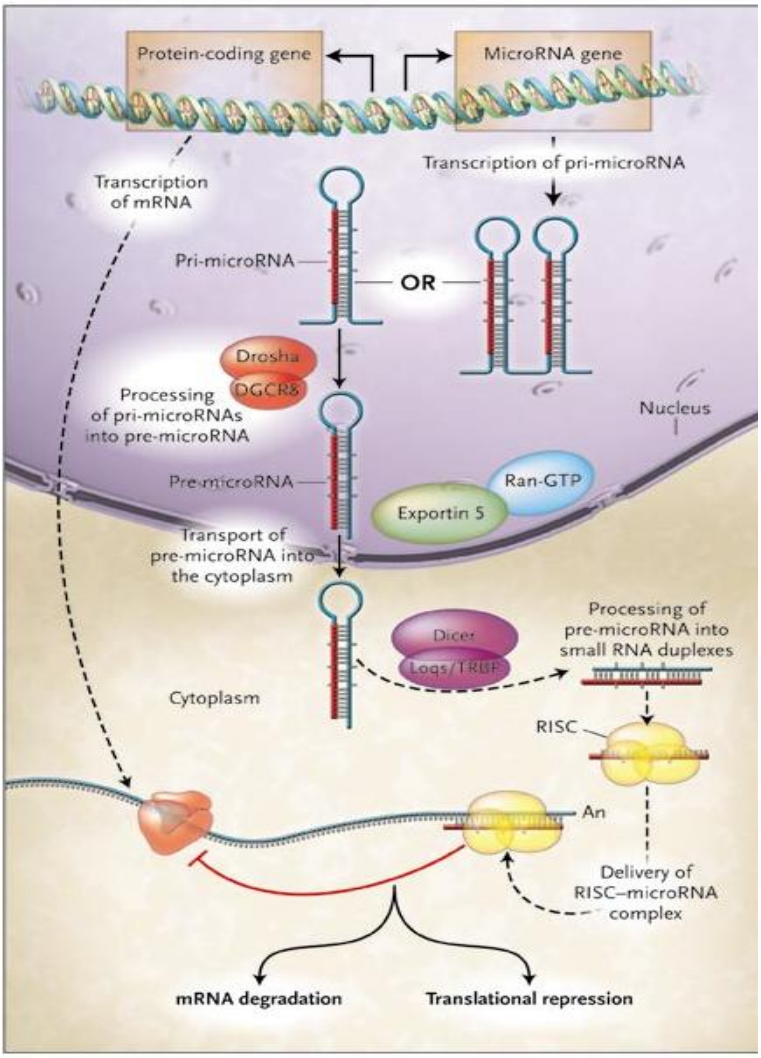


**Figure 9: Biogenesis of microRNA (*C.Z. Chen. N Eng J Med, 2005).***

## 2.3 Regulation of microRNA gene transcription

Transcription of miRNA genes is regulated in a similar manner to that of protein-coding genes, and is a major level of control responsible for tissue-specific or development-specific expression of miRNAs [128]. The promoter regions of autonomously expressed miRNA genes are highly similar to those of protein-coding genes; the presence of CpG islands, TATA box sequences, initiation elements and certain histone modifications indicate that the promoters of miRNA genes are controlled by transcription factors, enhancers, silencing elements and chromatine modifications, which is similar to protein-coding genes [129]. Control of gene expression by autoregulatory feedback loops is a common regulatory mechanism that is particularly important during cell fate determination and development. Indeed, many examples have been described of miRNAs regulating their own transcription through single-negative or double-negative feedback loops with specific transcription factors [130] [131]. By fine-tuning miRNA expression and adjusting it to physiologically optimal levels, these have a strong impact on the precise spatio-temporal expression of miRNA targets. Up to 1000 miRNAs are predicted to exist in the human genome, each of which could potentially target hundreds of mRNAs. Each mRNA target may contain potential binding sites for a large number of individual miRNAs, allowing redundancy or cooperative interactions between various seemingly unrelated miRNAs. Furthermore, the targets

of many miRNAs can modulate the expression of additional miRNAs or groups of miRNAs. For these reasons, it is easy to appreciate that most biological processes are, at least in part, under the influence of miRNAs.

## 2.4 MicroRNAs function: mechanisms of miRNA-mediated gene regulation

MiRNAs typically regulate gene expression via an association with the 3' untranslated region (UTR) of an mRNA with complementary sequence of 6-8 nucleotides, called "seed" region [132] [124]. MiRNAs with high sequence homology and identical seed region are commonly grouped into miRNA families that are likely to target similar sets of mRNAs [117]. Recent evidences suggest that miRNAs may also target 5'UTRs [133] or exons [134] and may potentially even undergo base pairing with promoter regions [135] or the sequence of long non-coding RNA [136].

The function of miRNAs appears to be in the regulation of gene expression, but there are multiple mechanisms of miRNA-mediated repression and there could be possible connections between these mechanisms [137] (Figure 10).

### 2.4.1 Translational inhibition

The first mechanistic analyses of miRNA function were carried out using *C. elegans*: it was found that the abundance of miRNA-regulated mRNAs was not substantially changed, but the abundance of proteins encoded by those mRNAs was markedly reduced. Of note, the regulated mRNAs seemed to be present in polysomes, i.e. mRNAs occupied by several actively elongating ribosomes [138]. There are numerous examples in which miRNA-mediated reduction of protein production is not accompanied by corresponding changes in mRNA abundance. It has been demonstrated miRNA-mediated inhibition at the level of initiation of protein synthesis, which results in prevention of ribosome association with the target mRNAs. In particular, some studies present evidences that initiation of translation is impaired by miRNAs at level of 5'-cap recognition [139] [140].

Other reports provide evidence for repression of protein production after protein synthesis has been initiated [141] Several lines of evidence showed that polysomes were actively translating but nascent polypeptides could not be detected, suggesting a protein degradation concomitant with translation [142]. In other case, evidence suggested that the deficit in protein production resulted from premature termination of translation (ribosome drop off). Interestingly, it has also been observed the ability of miRNAs to suppress translation promoted by internal ribosome entry sites IRESs (nucleotide

sequences that allow translation initiation in the middle of a mRNAs) [143].

### 2.4.2 MicroRNAs degradation or de-adenylation

There are also numerous examples of miRNAs destabilizing their target mRNAs and miRNAs accelerate mRNA decay by two distinct mechanisms. Those that are fully complementary to their mRNA targets (or nearly so) direct endonucleolytic cleavage within the base-paired region [144] [145].

This event leads to rapid decay of the entire message by generating a pair of RNA fragments, each bearing an unprotected end that is susceptible to 5’- or 3’-exonuclease attack [146]. Although miRNAs share the potential to mediate internal mRNA cleavage, they rarely do so in animal cells due to the inadequate complementarity of nearly all mRNAs [145]. Instead, miRNAs accelerate mRNA turnover by directing removal of the 3’ poly(A) tail from messages to which they are partially complementary [147] [148]. Deadenylation and the consequent loss of poly(A)-binding protein trigger 5’ decapping, thereby exposing the message to exonucleolytic digestion from the 5’ end [149] [150]. The ability of miRNAs to hasten mRNA decay has two important consequences. First, by diminishing the concentration of targeted transcripts, it supplements the decreased efficiency with which each message is translated, resulting in a greater overall reduction in protein synthesis. Second, by inducing message degradation, miRNAs render

irreversible their inhibitory influence on gene expression, an outcome not achievable by translational downregulation alone [151].

### 2.4.3 MicroRNAs sequestration

It is clear that miRNA-mediated repression can be manifested by inhibition of translation and/or by increased mRNA degradation. A significant fraction of translationally silent mRNAs, including those repressed by miRNAs, are found concentrated in cytoplasmic foci known as P bodies (processing bodies) [152] [153]. These ribonucleoprotein aggregates contain high concentrations of miRNAs, miRISC-associated proteins (AGO, GW182) and RNA degradative enzymes, including deccaping enzymes, de-adenylases and exonucleases [154] [155] [156]. Although lines of evidence initially raised the possibility that P body localization might be important for silencing by miRNAs [157] [158], these observations did not prove that the ability of miRNAs to direct targeted messages to P bodies contributes to translational repression. Indeed, recent evidence suggests that P bodies likely play a less pivotal role either as graveyards where miRNA-associated messages that have already been translationally inactivated are sent to decompose or as depots where messages transiently repressed by miRNAs can be store until needed [159] [150] [158].

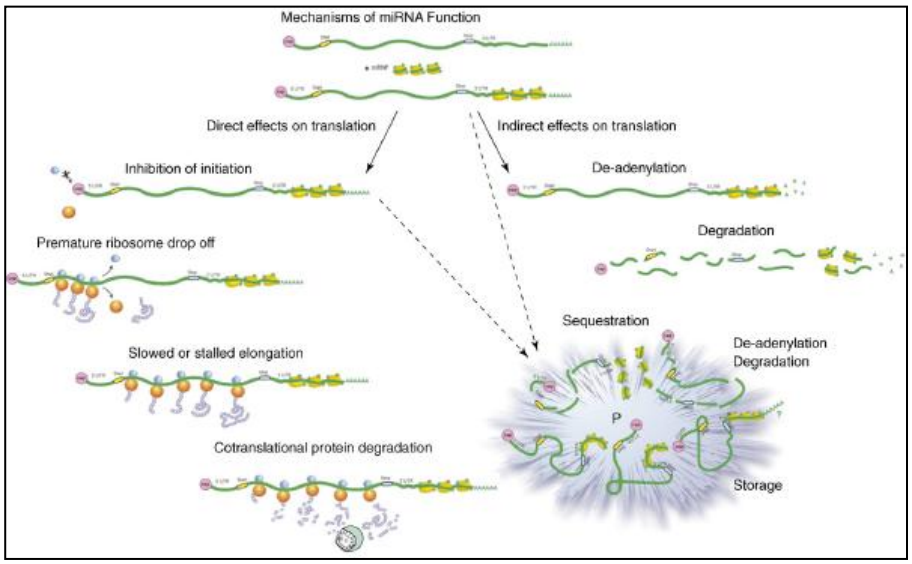


**Figure 10: Mechanisms of miRNA-mediated repression *(T.W. Nilsen. TRENDS in Genetics, 2007)***

## 2.5 MicroRNAs and their role in different biologic processes

Multiple miRNA target prediction tools are now available and the identification and validation of miRNA targets are essential to better understand miRNA biology and function. Indeed, direct examination of candidate miRNAs has validated their participation in various processes, such as development [160] and diseases [161, 162]. Importantly, miRNA have been involved in pluripotency maintenance [163], cell proliferation and differentiation [164], epithelial to mesenchymal transition [165], senescence [166], and apoptosis [167]. Due to their wide role in cell process regulation, miRNAs have gained popularity also as tool able to promote adult cell reprogramming into induced pluripotent stem cells (iPSCs) or direct cell to cell phenotypic conversion.

### 2.5.1 MicroRNAs and pluripotency

Recently, highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency was reported [168]; this study showed that the expression of the miR302/367 cluster using integrating viral vectors rapidly and efficiently reprograms mouse and human somatic cells to an induced pluripotent stem state without a requirement for exogenous transcription factors (Figure 11).

Also, it has been observed that it is possible to reprogram mouse and human cells to pluripotency by direct transfection of mature double-stranded miRNAs, using a combination of mir-200c plus mir-302 s and mir-369 s family miRNAs [169].

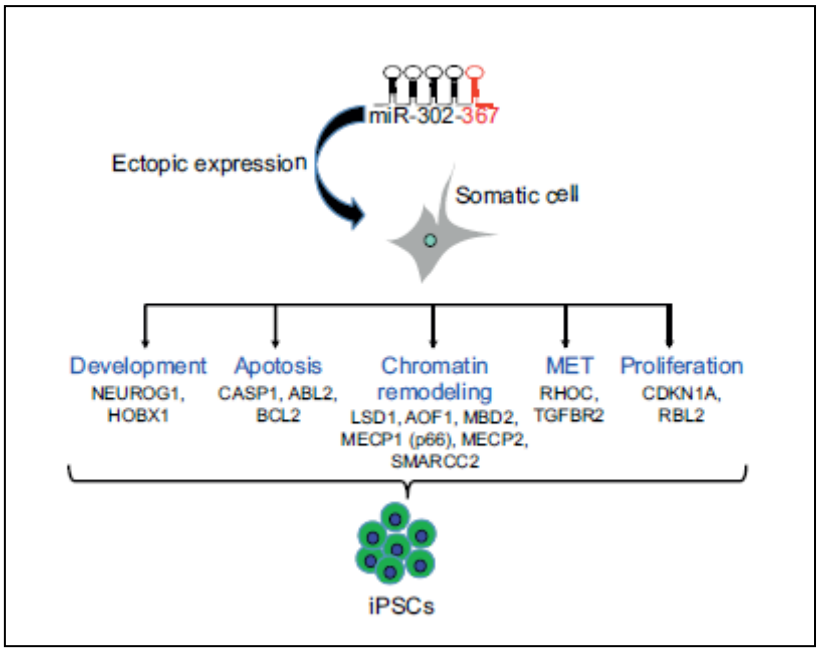


**Figure 11: Effects of miRNA in the induction of pluripotency *(F. Anokye-Danso. Cell Stem Cell, 2011).*** The diagram shows how the miR-302-367 cluster coordinates multiple cellular processes that are important for reprogramming of somatic cells into pluripotent stem cells as well as maintaining the pluripotent stem cell phenotype. Potential miR-302-367 and other related targets that could affect a global change in cell proliferation, epigenetic state, mesenchymal-epithelial transition and suppression of developmental factors, which leads to reprogramming in iPSCs are shown.

### 2.5.2 MicroRNAs and differentiation

Interestingly, it has been recently demonstrated that miRNAs have the possibility to promote direct cell to cell phenotypic conversion. For example, Jayawardena and colleagues have been developed a strategy capable of inducing reprogramming of cardiac fibroblasts directly to cardiomyocytes in vitro and in vivo, using a combination of specific miRNAs (miR-1, -133, -208, and -499) and JAK inhibitor I treatments, which enhanced miRNA-mediated reprogramming of 10 fold [170]. Detailed studies of the reprogrammed cells demonstrated that a single transient transfection of the microRNAs can direct a switch in cell fate as documented by expression of mature cardiomyocyte markers, sarcomeric organization, and exhibition of spontaneous calcium flux characteristic of a cardiomyocyte-like phenotype. Another study have determined the optimal combination of factors necessary and sufficient for direct myocardial reprogramming in vitro and in vivo of neonatal and adult human fibroblasts, using four human cardiac transcription factors, including GATA4, Hand2, Tbx5, and myocardin, and two microRNAs, miR-1 and -133 [171]. After maintenance in culture for 4-11 wk, human fibroblasts reprogrammed with these proteins and miRNAs displayed sarcomere-like structures and calcium transients, and a small subset of such cells exhibited spontaneous contractility. These phenotypic changes were accompanied by expression of a broad range of cardiac genes and suppression of nonmyocyte genes (Figure 12).

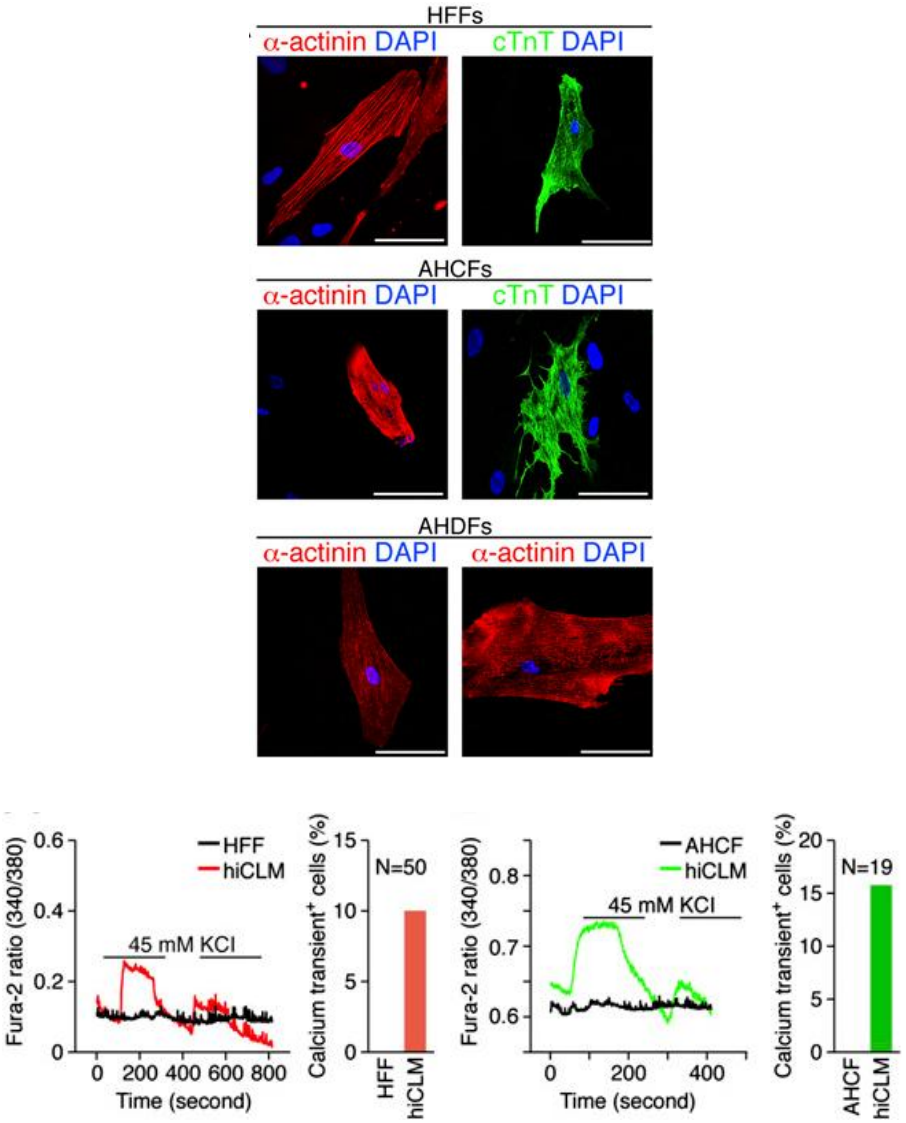


**Figure 12: Conversion of adult fibroblasts into functional cardiomyocytes using GATA4, Hand2, Tbx5, myocardin, miR-1 and -133 (modified from *Y.J. Nam. PNAS, 2013*).**

Immunostaining of cardiac markers α-actinin (red) or cTnT (green) performed on induced cardiac-like myocytes (iCLMs) obtained from human foreskin fibroblasts (HFFs), adult human cardiac fibroblasts (AHCFs), or adult human dermal fibroblasts (AHDFs). Scale bar, 100 μm (upper panel). Measurement of calcium transient in hiCLMs derived from HFFs and AHCFs (lower panel).

Phenotypic conversion experiments are also reported for induction of functional neurons from fibroblasts. Recently, it has been demonstrated that a combination of a microRNA (miR-124) and two transcription factors (MYT1L and BRN2) was sufficient to directly reprogram postnatal and adult human primary dermal fibroblasts (mesoderm) to functional neurons (ectoderm) under precisely defined conditions [172] (Figure 13). These human induced neurons (hiNs) exhibited typical neuronal morphology and marker gene expression, fire action potentials, and produce functional synapses between each other.

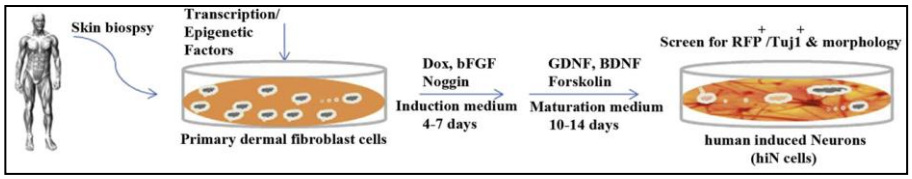


**Figure 13. Schematic experimental protocol used for conversion of postnatal human dermal fibroblasts to hiN cells using defined factors under defined conditions (*R. Ambasudhan. Cell Stem Cell, 2011*).**

Yoo and colleagues proposed that the expression of miR-9, miR-124 (miR-9-124) and NEUROD2 in human fibroblasts were able to induce their conversion into neurons. Further addition of neurogenic transcription factors ASCL1 and MYT1L enhanced the rate of conversion and the maturation of the converted neurons, whereas expression of these transcription factors alone without miR-9-124 was ineffective [173] (Figure 14).

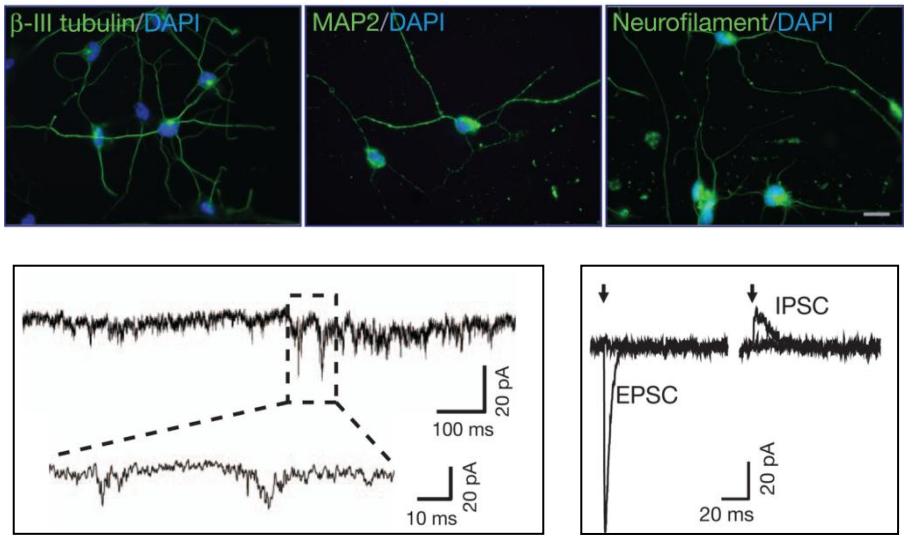


**Figure 14: Conversion of adult fibroblasts into functional neurons by miR-9-124 together with NEUROD2, ASCL1 and MYT1L (DAM) (modified from *A.S. Yoo. Nature, 2011*).**

Immunostaining of β-III tubulin (upper left), MAP2 (upper middle) and neurofilament (upper right) in human adult dermal fibroblasts converted by miR-9-124-DAM. Scale bar, 20 μm.

A representative trace demonstrating spontaneous excitatory post synaptic potentials (EPSCs) (lower left). Representative traces of evoked postsynaptic currents (EPSC and IPSC) obtained in response to local field stimulation with single current pulses (lower right).

# 3. Aim of the study

During my PhD program in Translational and Molecular Medicine at DIMET – University of Milano-Bicocca, I've worked in the Laboratory of Vascular Biology and Regenerative Medicine at the Centro Cardiologico Monzino-Milano. One of the major focus of our Laboratory is transferring basic research to clinical application. In this view we have recently isolated and characterized mesenchymal stromal cell populations obtained from different districts: bone marrow (BMStCs), heart right auricle (Cardiac Stromal Cells, CStCs), and first trimester human chorionic villi (CVStCs). Specifically during my my PhD studentship, my work has focused on:

- ✓ The analysis of miRNA expression profiling obtained from syngenic CStCs and BMStCs cultured in amplification medium and differentiation media in order to better understand the role of miRNAs molecular mechanisms in different cell types and during differentiation;

- ✓ CStC reprogramming into functionally active cardiovascular precursors via epigenetic interventions by using defined small molecules;

- ✓ CVStCs characterization which exhibited restricted plasticity oriented toward the endothelial lineage.

The full characterization at molecular, biological and physiological level of BMStCs, CStCs and CVStCs will clarify the mechanisms of differentiation and plasticity of these populations and their possible clinical application in cardiovascular diseases.

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# Chapter 2: Syngeneic Cardiac and Bone Marrow Stromal Cells Display Tissue-specific microRNA Signatures and microRNA Subsets Restricted to Diverse Differentiation Processes

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*Submitted*

# 1. Introduction

MicroRNAs (miRs) are 21-23 nucleotide non-coding RNA molecules, which modulate the stability and/or the translational efficiency of messenger RNAs (mRNA). miRs act upon imperfectly binding their mRNA to the so called “seed sequence” a 6-8 region usually located in the 3’ untranslated region (3’UTR) of the repressed target [1,2]. Recent evidences actually show that the specific binding sequence in target mRNAs can be also located in the coding region [3] or even in the 5’UTR [4]. Since miRs may target multiple transcripts and individual transcripts may be subject to multiple miR regulation, it is easy to appreciate that most biological processes are, at least in part, under the influence of miRs. Interestingly, evidences have been provided that miRs can have binding motifs also located into promoter regions [5] or into the sequence of long non-coding RNA [6], thus enormously extending their possible functions. It has also been shown that, for some genes, the level of expressed protein is more dependent on the amount of regulatory miRs rather than on the level of the encoding mRNA.

Direct examination of candidate miRs has validated their participation in various processes, such as development [7] and diseases [8,9]. Importantly, miR have been involved in pluripotency maintenance [10], cell proliferation and

differentiation [11], epithelial to mesenchymal transition [12], senescence [13], and apoptosis [14].

Due to their wide role in cell process regulation, miR have gained popularity also as tools able to promote direct cell to cell phenotypic conversion as well as adult cell reprogramming into pluripotent stem cells. In fact, it has been recently demonstrated that miRs have the possibility to induce fibroblast differentiation into cardiomyocyte-like cells [15] and to facilitate, in concert with specific transcription factors, the conversion of adult human fibroblasts into neurons [16] or cardiomyocyte-like cells [17]. In addition, miRs might promote adult cell reprogramming into pluripotent cells [18,19], although further work has to be done to understand whether miRs alone are sufficient to reprogram somatic cells into stem cells or other type of specialized cells. Nevertheless, being able to regulate and, possibly, to fine tune cell fate, miRs appear as a new frontier for application in regenerative medicine.

In order to clarify miR role in the definition of cell identity and fate we decided to: (1) isolate, from syngeneic donors, two different types of adult human cell potentially useful in cardiac regenerative medicine (namely cardiac and bone marrow mesenchymal stromal cells, CStC and BMStC respectively), (2) expose them to five media standardly used in literature to promote their differentiation into adipocyte-, osteocyte-, endothelial, cardiomyocyte-like cells and (3) analyze their miR profile before and after differentiation treatments.

Specific aims of the present work were to: (1) identify a tissue-specific miR expression signature which was not influenced *in vitro* by differentiation media; (2) identify miR subsets specifically modulated by each differentiation medium, independently from the cell type of origin; and (3) identify those miRs that are differently modulated by the media between the two cell types.

To do so, we used a two-factor experimental approach that allowed us to ascertain miRs that unequivocally discriminated the cell type of origin, miRs that are similarly modulated by differentiation media in both cell types, and miRs that are differentially modulated by the media in the cell types. In addition, bioinformatics tools were used to relate miR expression to their predicted and/or validated mRNA targets in order to propose an interpretation of the results in terms of functional consequences on cell function, stemness and regenerative potential.

# 2. Materials and Methods

## 2.1 Ethics Statement

CStC and BMStC were obtained respectively from right auricle and sternal marrow samples of the same donor patients (*n* = 4) undergoing cardiac surgery, after approval by the Centro Cardiologico Monzino (Milano, Italy) Local Ethics Committee and signed informed consent. Experiments were conducted in accordance to the principles expressed in the Declaration of Helsinki. All data were analyzed anonymously.

## 2.2 CStC and BMStC isolation and culture

CStC and BMStC were isolated as described in [20]. Briefly, CStC were enzymatically isolated from small auricle fragments using 3 mg/ml collagenase (Serva) and cultured in standard growth medium (GM), composed of Iscove's Modified Dulbecco's Medium (IMDM, Lonza) supplemented with 20% fetal bovine serum (FBS, Hyclone), 10 ng/ml basic Fibroblasts Growth Factor (bFGF, R&D), 10.000 U/ml Penicillin/Streptomycin (Invitrogen), 20 mM L-Glutamine (Sigma-Aldrich). BMStC obtained from 5 ml of heparinized bone

marrow were separated by stratification on Ficoll gradient and cultured in the same GM.

## 2.3 *In vitro* cell differentiation

CStC and BMStC were plated at a density of 5000 cells/cm$^2$ and exposed to standardize differentiation-inducing media for 21 days. Media were changed twice a week. Adipogenic and osteogenic differentiation were achieved following standard *in vitro* protocols [21]. Endothelial (EGM-2, Lonza) and cardiomyogenic differentiation were induced as previously described [20].

## 2.4 Intracellular lipid staining by Oil-Red O

The accumulation of lipid droplets was evaluated by Oil-Red O staining [22].

## 2.5 Von Kossa staining

The production of mineralized matrix was evaluated by von Kossa staining as previously described [22].

## 2.6 Ac-LDL-DiI uptake

Cells were incubated with 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine-labeled acetylated LDL (Ac-LDL-DiI, Biomedical Technologies) as indicator of endothelial cells differentiation [22,23]. After fixation with 4% PFA, cells were counterstained with Hoechst 33258 nuclear and observed with a Zeiss microscope equipped for epifluorescence.

## 2.7 Immunofluorescence

α-sarcomeric actin expression was detected by incubation with specific primary antibody (AbCam) and FITC-conjugated secondary antibody (Jackson Immunoresearch). Nuclei were counterstained with Hoechst 33258 (Sigma-Aldrich) [20].

## 2.8 MicroRNA profiling

Total RNA was extracted from cells using TRIzol reagent (Invitrogen) in accordance to the manufacturer's instruction. Total RNA concentration and purity were evaluated by a NanoDrop 1000 spectrophotometer (Thermo Scientific), while RNA integrity was assessed with an Experion electrophoresis system and RNA High Sense Analysis Kit (Bio-Rad). Only high

quality RNAs, with A260/A280 and A260/A230 ratios > 1.8 and a RQI > 9.5/10, were used for subsequent investigations. Comparative miR expression profiling was carried out using the TaqMan Low Density Array Human MicroRNA Panel (Applied Biosystems), according to the manufacturer's instructions, using a 7900TH Real Time PCR System (Applied Biosystems).

Prior to the analysis, probes were renamed and reannotated according to miRBase Release 20 (http://www.mirbase.org) [24]. This allowed us identifying 360 target sequences unique to human miRs, discarding probes for tRNA, snoRNA, and misannotated sequences. Expression analysis and quality control of TaqMan Arrays were performed using the ExpressionSuite Software v1.0.3 (Applied Biosystems). All Ct values reported as greater than 40 or as not detected were changed to 40 and considered a negative call. Raw expression intensities of target miRs were normalized for differences in the amount of total RNA added to each reaction using the mean expression value of all expressed miRs in a given sample [25]. Relative quantitation of miR expression was performed using the comparative Ct method ($\Delta\Delta$Ct). MiRs were deemed as non informative and filtered out when the percentile of negative calls exceeded 6 (20% of the samples): thus, the number of miRs considered for subsequent analysis was 306.

## 2.9 Reverse Transcription – Real-time Polymerase Chain Reaction Analysis (RT-qPCR)

To validate array-derived expression data, individual miR expression was analyzed using specific single-assay miR primers and target probes (Applied Biosystems) for miR-126-5p, 146a-5p, 155-5p, and 222-3p. Reverse-transcription and real-time reactions were performed according to the manufacturer's instructions, using a 7900TH Real Time PCR System (Applied Biosystems). Raw expression intensities of target miRs were normalized using the mean expression value of RNU44 and U6 RNA in any given sample. Relative quantitation was performed using the $\Delta\Delta$Ct method.

## 2.10 Statistical analysis

The MeV v4.9.0 software [26] was used for the primary statistical and for unsupervised hierarchical clustering analyses. The GraphPad Prism v5.03 software was used for post-hoc analyses. Array data were analyzed by 2-factor ANOVA, calculating the *P*-values based on the F-distribution. In order to control for the false discovery rate (FDR), a *q*-value was estimated for each gene [27], both for the effects of the two factors and for the interaction *P*-value, using the QVALUE v1.36.0 implemented in Bioconductor v2.13 software package.

The *q*-value was used as a FDR-based measure of significance and the threshold α was set to ≤ 0.01. Bonferroni post-hoc test was used to compare the effect of each medium to GM and adjusted *P*-values < 0.05 were considered statistically significant. Linear regression analysis and calculation of Pearson correlation coefficient were performed to relate array to singleplex qPCR expression data.

## 2.11 Bioinformatics analysis

Queries for miR target prediction with three different algorithms (miRanda, PITA and TargetScan) was performed using the web-based tool MAGIA [28], applying stringent score filters (-12 for PITA, 500 for miRanda). Queries for miR validated targets were performed on the database miRTarBase Release 4.4 [29]. The gene lists generated by these queries were exploited for gene-annotation (Gene Ontology, GO, terms and KEGG pathways) enrichment analysis using the web-based application DAVID 6.7 [30]: the EASE score (a conservative adjustment of the Fisher Exact *P*-value) threshold was set to 0.005. Redundant GO terms were removed using the web-based tool REViGO [31], with an allowed similarity threshold of 0.5.

# 3. Results

Stromal cells obtained from different tissues show similar morphology and immunophenotype but different plastic properties [32]. Consistent with our previous results [20], we observed that CStC [33] displayed greater propensity to differentiate into cardiomyocyte-like and endothelial-like cells when compared to BMStC [34]. This is demonstrated by higher expression of α-sarcomeric actin and a more efficient accumulation of Ac-LDL after 21 days of cardiomyogenic and endothelial medium exposure, respectively (Figure 1). On the contrary, CStC exhibited lower ability to gain both adipocyte and osteocyte features, esteemed by Oil-red O and Von Kossa staining, respectively (Figure 1). This different behavior is likely due to distinct molecular networks activated or repressed in the two cell populations despite their phenotypical similarity. To prove this hypothesis, miR expression profiles were evaluated by low-density microRNA Taqman array in both CStC and BMStC exposed for 21 days to standard culture conditions (GM) or to five differentiation media, namely Adipogenic (AM), Osteogenic (OM), Cardiomyogenic (CM), and Endothelial (EM) Media. Differentially expressed genes were sought by performing a 2-way ANOVA, being the two factors the tissue of origin and the medium to which cells were exposed. Results obtained by this analysis led us to identify a grand-total of 115

significantly modulated miRs (after correction for multiple comparisons with a FDR ≤ 0.01) either by the tissue of origin, or by the differentiation media, or by the interaction between the two factors. An unsupervised hierarchical clustering revealed that the expression profile of these 115 miRs was able to fully discriminate medium-differentiated cells, *i.e.* cells of the same origin cultured in the same conditions were grouped in distinct nodes of the sample dendrogram. Interestingly, also CStC and BMStC cultured in GM belonged to the same main cluster, but a deeper insight revealed that only cells #1, 3, and 4 clustered according to the tissue of origin: CStC and BMStC from patient #2 grouped together in a distinct sub-cluster. This might be due to specific characteristics or different genetic background of patient #2, although the survey of his clinical records did not evidence anything that could explain his different behavior.

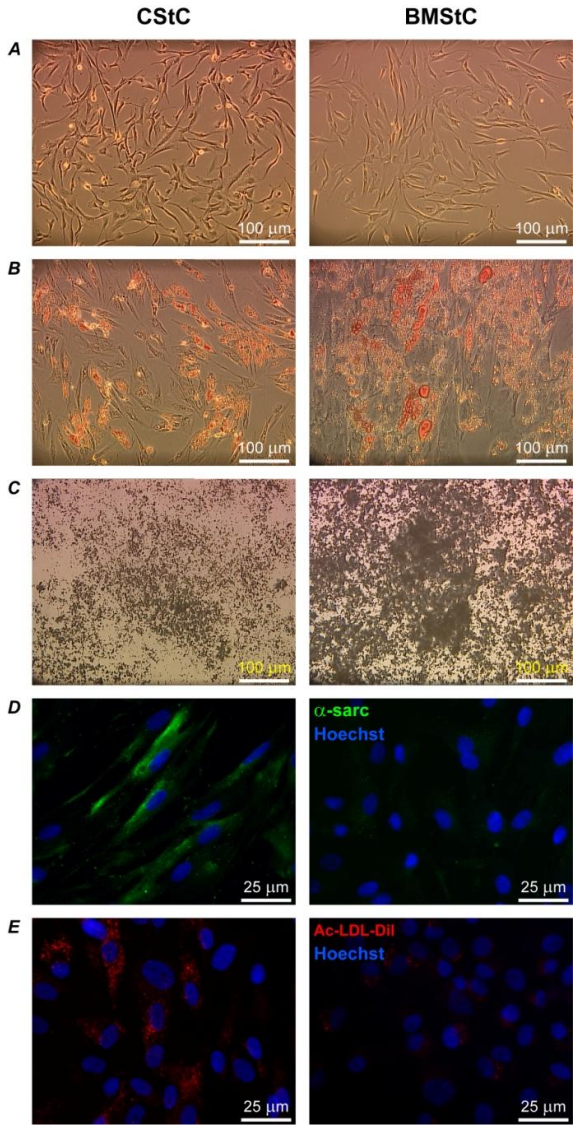


**Figure 1. Morphology and response to *in vitro* differentiation.** (***A***) CStC BMStC cultured in standard growth medium (GM). (***B***) CStC and BMStC exposed to adipogenic media show intracellular lipid accumulation as evidenced by Oil-red O staining. (***C***) Von Kossa staining of CStC and BMStC after osteogenic treatment. (***D***) Immunostaining for α-sarcomeric actin, a marker of cardiomyogenic differentiation. (***E***) Ac-LDL uptake assay: red in cytoplasm represents DiI-labeled acetylated LDL. Original magnifications: 10× for panels A, B, and C, and 40× for panels D and E.

## 3.1 Tissue-specific miR profiles

Among the 115 differentially modulated miRs, 41 miRs were dependent on the tissue of origin. Accordingly, the unsupervised hierarchical clustering showed that these 41 miRs were able to divide CStC and BMStC into two different clusters independently of the culture conditions. We then used a Venn diagram (Figure 2A) to visualize which miRs were exclusively influenced by the tissue of origin, excluding those miRs that were modulated also or solely by the culture medium and/or by the interaction between the two factors. The remaining subset was composed of 19 miRs that were independent from and un-modulated by any differentiation stimuli. To further refine this tissue-specific miR profile, we excluded 3 miRs that showed a mean fold difference between CStC and BMStC ≤ |±2| (*i.e.* miR-214-3p, 324-3p, and 365a-3p). This allowed us to identify two tissue specific miR signatures (Figure 2B), which included: 4 miRs that were significantly overexpressed in CStC (miR-146a-5p, 211-5p, 532-5p, and 660-5p); 8 miRs overexpressed in BMStC (miR-10a-5p, 199a-3p, 199a-5p, 224-5p, 299-5p, 376a-5p, 497-5p, and 618) plus 4 BMStC-specific miRs that were virtually absent in CStC (miR-10b-5p, 196a-5p, 196b-5p, and 615-3p).

Importantly, gene-annotation enrichment analysis, conducted on both predicted and validated targets of the two miR signatures, showed that several pathways and gene categories were targeted by both CStC and BMStC specific miRs, *e.g.*

calcium, insulin, MAPK, ErbB, Jak-STAT, mTOR, and Wnt signaling pathways (not shown). Conversely, this analysis (Figure 2C and 2D) revealed a number of distinct unique GO biological processes (blue-colored bars), molecular functions (green), and KEGG pathways (red), which are potentially targeted by the signature miRs in either cell populations.

## 3.2 Medium-specific miR profiles

Two-way ANOVA identified 95 miRs that were significantly modulated by the differentiation media (Supplementary Table 1). The Venn diagram analysis (Figure 3A) showed that 13 miRs were influenced by both the tissue of origin and the media (upper intersection area), *i.e.* that they were differentially expressed between BMStC and CStC and that one or more media modulated their expression similarly in both cell types. In addition, the Venn diagram revealed 67 miRs that were exclusively influenced by differentiation media, independently from the tissue of origin (*i.e.* no significant differential expression between the cell types, but a significant modulation in the same direction and of a similar extent in both CStC and BMStC). Importantly, unsupervised hierarchical clustering indicated that these 80 miRs were able to fully discriminate cells exposed to the same medium, independently from their tissue of origin (Figure 3A).

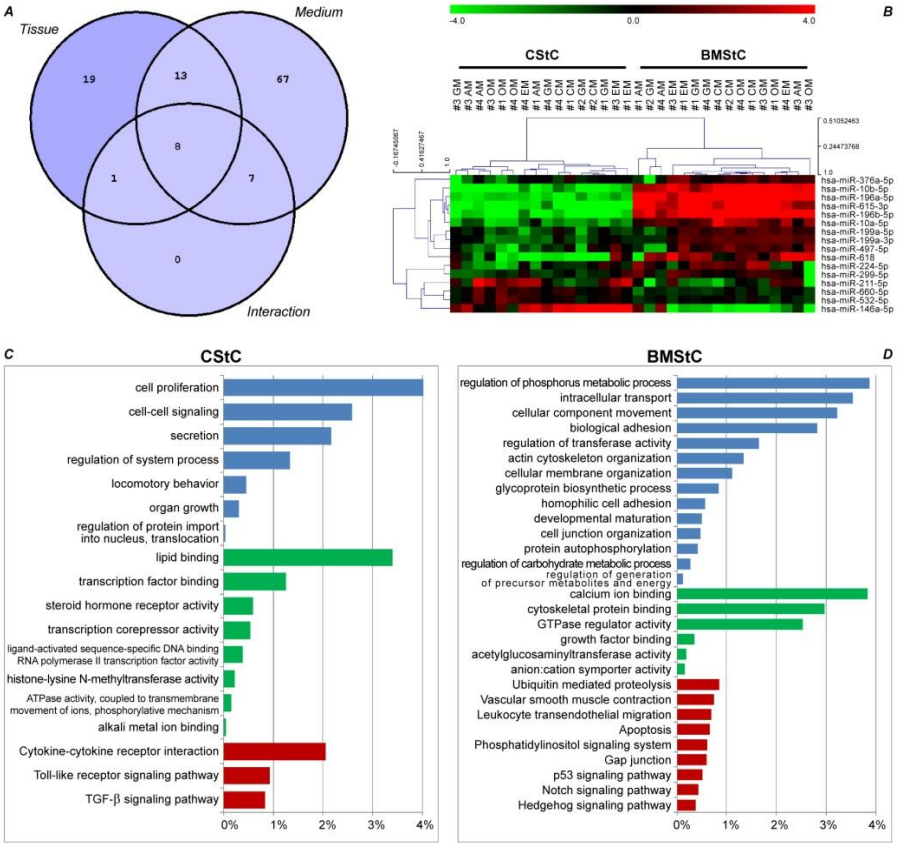


**Figure 2. miR tissue signature and its potential functional implications.** (***A***) A Venn diagram of the 115 significantly modulated miRs. (***B***) Unsupervised hierarchical clustering defining the miR tissue signature composed by 16 miRs, distinctive of the tissue of origin and with at least a 2-fold difference. The dendrogram above shows that this signature is able to divide CStC and BMStC in two distinct clusters, irrespective of the culture media exposure. (***C***) Gene-annotation enrichment analysis revealed GO biological processes (blue bars), molecular functions (green), and KEGG pathways (red) potentially and exclusively targeted by CStC tissue-specific miRs. (***D***) Gene categories potentially targeted by BMStC tissue-specific miRs, as revealed by gene-annotation enrichment analysis.

Post-hoc tests, conducted comparing each differentiation medium with the GM, combining BMStC and CStC data, allowed identifying smaller subsets of miRs specifically modulated in each condition (Table 1). Among them, miR-7-5p, 15b-5p, 18a-5p, 20a-5p, 31-5p, 155-5p, and 629-3p were significantly down-regulated by all media in both cells, while other subsets were specifically modulated by each differentiation condition, with a fold change (FC) ≥ |±2| in most cases (Table 1).

In agreement with the expected effect on cell phenotype, a look-up of gene-annotation enrichment analysis revealed that miRs exclusively modulated (*i*) by AM targeted pathways related to the lipid metabolism, (*ii*) by OM phosphate homeostasis and mineralization, (*iii*) by CM processes linked to cytoskeleton organization and calcium handling, and (*iv*) by EM vessel and endothelial cell proliferation (Figure 3B, 3C, 3D, 3E).

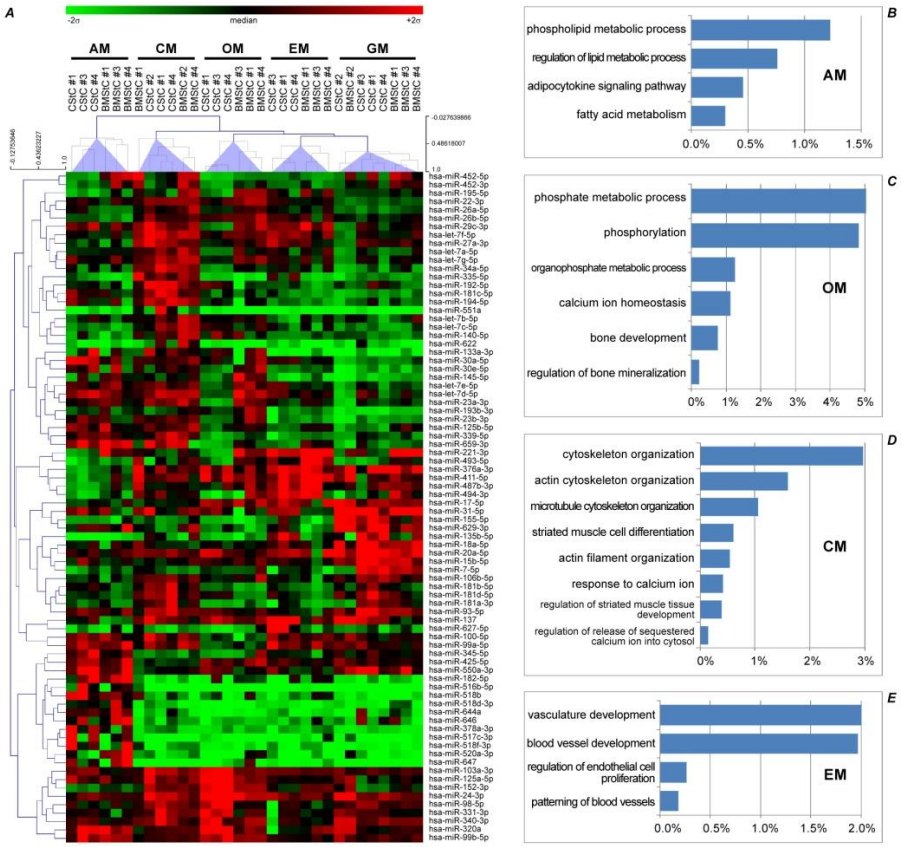


**Figure 3. miRs specifically influenced by differentiation media.** (***A***) Heatmap representing the expression of 80 miRs significantly modulated by differentiation stimuli (FDR ≤ 0.01) independently from the tissue of origin. Unsupervised hierarchical analysis groups in five distinct clusters both Cardiac (CStC) and Bone Marrow (BMStC) Stromal Cells exposed to the same medium, as highlighted by translucent purple wedges drawn from the five main nodes. Clustering was done using Pearson's correlation (centered) and average linkage method. The relative expression level of each miR is represented with a green, black, and red color scale (as in Figure 3). Gene-annotation enrichment analysis showed relevant GO biological processes and/or molecular functions potentially targeted by miRs modulated by (***B***) Adipogenic Medium (AM), (***C***) Osteogenic Medium (OM), (***D***) Cardiomyogenic Medium (CM), and (***E***) Endothelial Medium (EM).

| microRNA | FC *vs.* GM | | | | *P*-values† | | | |
|---|---|---|---|---|---|---|---|---|
| | AM | OM | CM | EM | AM | OM | CM | EM |
| **hsa-miR-629-3p** | -1.7 | -5.5 | -2.6 | -3.9 | * | *** | *** | *** |
| **hsa-miR-15b-5p** | -2.2 | -1.8 | -2.4 | -2.3 | ** | * | *** | *** |
| **hsa-miR-20a-5p** | -2.4 | -1.8 | -1.8 | -1.6 | *** | ** | ** | * |
| **hsa-miR-18a-5p** | -2.7 | -2.7 | -2.5 | -2.1 | *** | *** | *** | ** |
| **hsa-miR-31-5p** | -2.9 | -3.7 | -2.3 | -2.8 | *** | *** | *** | *** |
| **hsa-miR-7-5p** | -4.4 | -2.6 | -3.3 | -4.4 | *** | ** | *** | *** |
| **hsa-miR-155-5p** | -6.6 | -11.3 | -4.5 | -4.2 | *** | *** | *** | *** |
| **hsa-miR-516b-5p** | 11243 | | | | * | ns | ns | ns |
| **hsa-miR-647** | 1995 | | | | *** | ns | ns | ns |
| **hsa-miR-518f-3p** | 42.1 | | | | *** | ns | ns | ns |
| **hsa-miR-518b** | 29.6 | | | | *** | ns | ns | ns |
| **hsa-miR-517c-3p** | 15.3 | | | | ** | ns | ns | ns |
| **hsa-miR-182-5p** | 13.7 | | | | *** | ns | ns | ns |
| **hsa-miR-378a-3p** | 13.4 | | | | *** | ns | ns | ns |
| **hsa-miR-520a-3p** | 7.8 | | | | ** | ns | ns | ns |
| **hsa-miR-30a-5p** | 4.3 | | | | *** | ns | ns | ns |
| **hsa-miR-644a** | 3.5 | | | | * | ns | ns | ns |
| **hsa-miR-137** | -2.5 | | | | * | ns | ns | ns |
| **hsa-miR-376a-3p** | -5.0 | | | | *** | ns | ns | ns |
| **hsa-miR-193b-3p** | | 8.6 | | | ns | * | ns | ns |

| | | | | | | |
|---|---|---|---|---|---|---|
| **hsa-miR-145-5p** | 6.6 | | ns | ** | ns | ns |
| **hsa-miR-152-3p** | 2.9 | | ns | *** | ns | ns |
| **hsa-miR-125a-5p** | 2.3 | | ns | *** | ns | ns |
| **hsa-miR-320a** | 1.8 | | ns | * | ns | ns |
| **hsa-miR-335-5p** | | 5.4 | ns | ns | *** | ns |
| **hsa-miR-181c-5p** | | 5.0 | ns | ns | *** | ns |
| **hsa-miR-23b-3p** | | 4.5 | ns | ns | * | ns |
| **hsa-miR-34a-5p** | | 4.1 | ns | ns | *** | ns |
| **hsa-let-7c-5p** | | 3.7 | ns | ns | ** | ns |
| **hsa-miR-194-5p** | | 3.6 | ns | ns | *** | ns |
| **hsa-miR-140-5p** | | 2.9 | ns | ns | * | ns |
| **hsa-miR-339-5p** | | 2.8 | ns | ns | * | ns |
| **hsa-miR-23a-3p** | | 2.7 | ns | ns | * | ns |
| **hsa-let-7a-5p** | | 2.7 | ns | ns | * | ns |
| **hsa-miR-192-5p** | | 2.6 | ns | ns | ** | ns |
| **hsa-let-7f-5p** | | 2.5 | ns | ns | *** | ns |
| **hsa-let-7g-5p** | | 2.4 | ns | ns | * | ns |
| **hsa-let-7d-5p** | | 2.4 | ns | ns | * | ns |
| **hsa-miR-550a-3p** | | -2.2 | ns | ns | * | ns |
| **hsa-miR-627-5p** | | | 4.0 | ns | ns | ns | *** |
| **hsa-miR-493-5p** | | | 3.6 | ns | ns | ns | *** |
| **hsa-miR-494-3p** | | | 2.6 | ns | ns | ns | *** |

| | | | | | |
|---|---|---|---|---|---|
| **hsa-miR-411-5p** | 1.9 | ns | ns | ns | *** |
| **hsa-miR-221-3p** | 1.9 | ns | ns | ns | *** |
| **hsa-miR-93-5p** | -2.3 | ns | ns | ns | * |

**Table 1.** miR subsets specifically modulated by differentiation media *vs.* growth medium in both CStC and BMStC. † Bonferroni corrected *P*-values. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. FC: fold-change.

## 3.3 Interaction effects

Two-way ANOVA identified a subset of 16 miRs, which showed a statistically significant interaction effect of the media on the cell type (Figure 4), *i.e.* miRs for which the effect of the differentiation conditions (difference between differentiation media and control GM) differed between CStC and BMStC. This subset comprises miRs that were differentially modulated by one or more media between the two cell types. In detail, the Venn diagram (Figure 2A) shows that the expression of one miR (362-5p) is influenced by both cell type and interaction, 8 by both factors (tissue and medium) and interaction (1, 133b, 184, 204-5p, 216a-5p, 222-3p, 29a-3p, 503-5p), and 7 by media and interaction (130a-3p, 135a-5p, 142-5p, 24-1-5p, 27b-3p, 30d-5p, 511-5p). Figure 5 depicts the heatmap of the mean fold differences of these miRs between CStC and BMStC cultured with either GM or each differentiation medium. For instance, miR-1 was not different between the two cell types when cultured in GM, but showed a significant overexpression induced by OM and CM and a virtually complete repression by EM in CStC. miR-113b was slightly higher in CStC than in BMStC, and upregulated by OM and downregulated by EM in CStC. miR-184 was virtually absent in BMStC; the AM, CM, and EM induced it in BMStC so that the FC compared to CStC was greatly reduced or suppressed, whereas OM induced it in CStC enhancing the difference with BMStC. miR-204-5p was overexpressed in CStC and induced by AM and EM in both cell

types, so that the fold difference remained similar; CM induced it in BMStC while OM induced it in CStC, so that the fold difference between CStC *vs.* BMStC, respectively, decreased and increased.

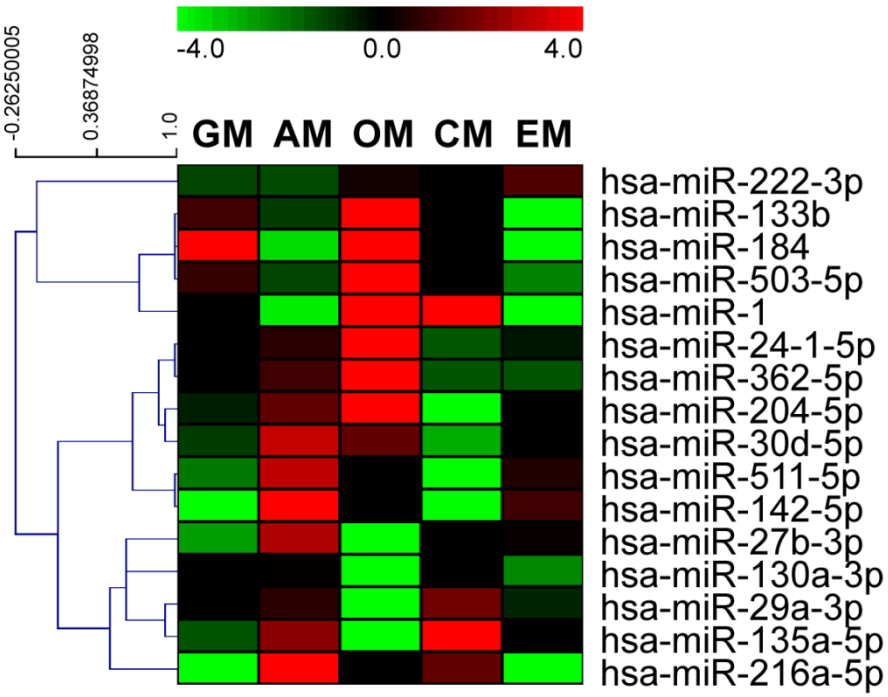


**Figure 4. miRs for which the effect of the media differed between CStC and BMStC.** The heatmap shows the mean fold differences between CStC and BMStC exposed to diverse culture media for miRs significant for the interaction effect.

## 3.4 Singleplex RT-qPCR validation

Single RT-qPCR experiments were performed for 4 miRs to validate data derived from the arrays (Figure 5). To cover all possible combinations, we chose one miR from the tissue signature (miR-146a-5p), one modulated by all media but not differentially expressed between the cell types (155-5p), one with a significant interaction *q*-value and influenced by both tissue and medium (222-3p), and one miR that was not significantly different between cell types and among differentiation media (126-5p). Results fully confirmed the array data, as the Pearson coefficient was ≥ 0.70 and *P*-values < 0.001 for every correlation analysis.

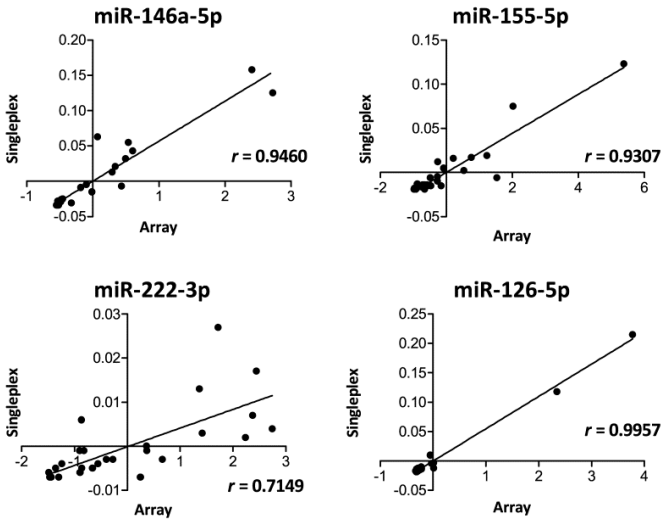


**Figure 5. Validation by single assay RT-qPCR.** Four miRs (three significant for any of the effects, miR-146a-5p, 155-5p, and 222-3p, and one, 126-5p, not significant) were validated by single-assay real-time PCR. All correlations were significant with $P < 0.001$.

# 4. Discussion

MicroRNAs have been suggested to be part of the molecular network responsible for cell identity regulation [20,35]. In the present work, by applying a 2-way ANOVA, we confirmed that the microRNA expression profile of stromal cells obtained from different tissues is at least in part dependent on their origin [20], but we are also highlighting significant influences of the culture conditions and of the differentiation strategies put in place. Further, our results showed that the interaction between tissue of origin and environmental factor had a strong impact in determining miR expression of a given cell type.

MiRs included in the tissue signature remained unmodified after *in vitro* standard differentiation treatment. This observation has important consequences, giving confirmatory evidences that miR-regulated pathways are involved in cell identity and fate determination. Among the 16 miRs constituting the tissue specific signature, miRs-10a, 10b, 196a, 196b, 199a and 615 have a role in controlling cell cycle, proliferation and development. Interestingly, miR-10, 196 and 615 are encoded in genes embedded in the HOX cluster [36], which are recognized as main players acting early in the development of embryo morphology and symmetry [37]. MiR-199a-3p and 199a-5p may have a role in transcriptional regulation by targeting the SWI/SNF complex [38]. Also, miR-146a and 532

are implicated in proliferation and survival [39-41]. Of note, miR-146a, which is in the regulation of IFN and NF-κB pathways [42], is highly expressed in proliferating cardiovascular precursors [43], as confirmed by its higher expression in CStC compared to BMStC. Importantly, GO analysis revealed that miR specifically overexpressed in BMStC might target processes involved in phosphorous metabolism, which is in line with the increased osteogenic ability of BMStC compared to CStC. On the other hand, miR specifically up-regulated in CStC can actually target cell proliferation, transmembrane ion movement and toll-like receptor signaling pathways. These findings are in agreement with the higher proliferation rate of CStC that we previously described [20], as well as with their ability to acquire some excitable cell properties [33] and to respond to HMGB-1 stimulation [44], respectively.

MiRs modulated only by differentiation stimuli independently from tissue origin were mostly expressed at similar levels in CStC and BMStC cultured in GM and significantly modulated at a comparable extent and direction between the two cell populations after differentiation treatment. Within this group, a smaller subset of 7 miRs was significantly downregulated by all the differentiating conditions. Among them, miR-155, miR-20a-5p and miR-18a-5p are associated to cell proliferation and to apoptosis suppression [45,46]. In our findings, the down-modulation of miR-155, 20a-5p, and 18a-5p is in agreement with the reduced proliferation ability observed during differentiation processes. Also, miR-20a-5p and 18a-5p are

negative regulators of angiogenesis [47] and their down-regulation is known to promote endothelial differentiation *in vitro* and *in vivo* [48]. Further, suppression of miR-7-5p and miR-31-5p is related to differentiation processes, such as osteogenesis [49,50], myogenic differentiation [51] and vascular development [52]. Interestingly, among the microRNAs modulated by the differentiation treatment independently of the cell type, we found the let-7 family, significantly upregulated, together with miR-23a and 23b, after cardiomyogenic treatment. This is in agreement with literature reporting that those miRs are related to cardiac differentiation [53] and cardiovascular processes [54]. Similarly, miR-320, 193 and 125a have been associated to osteogenesis of mesenchymal cells in vitro [55,56] and appear significantly up-regulated after osteogenic treatment. Among miRs modulated only by endothelial medium, it is interesting that the exposure of both cell types to EM resulted in a down-regulation of miR-93-5p. Indications are available that this miR is related to angiogenesis, although there are conflicting evidences showing both that its up-regulation enhance capillary-like structure formation [57] and that its down-regulation increases VEGF release [58].

Further, in line with their known role in adipogenesis, miR-30a-5p and 378a-5p were up-regulated by adipogenic medium in both cell types. In particular, miR-30a-5p overexpression has been demonstrated to induce PPARγ activation during adipogenic differentiation of human adipose-tissue derived stem cells [59]. This is consistent with PPARγ increase observed in

CStC exposed to adipogenic media [20]. Intriguingly, it seems that the increase miR-378a-5p observed in CStC after adipogenic treatment might depend on PPARγ increase, this transcription factor being able to enhance miR-378 transcription [60]. This observation actually underlines the complexity of miR role in differentiation processes, showing the impossibility to establish, only by miR expression analysis, a cause-effect relationship between miR profile and a given phenotype.

MiRs that showed an interaction between the cell type and the differentiation media are those that differentially responded to the differentiation stimuli comparing CStC and BMStC, starting or not from different expression levels when the two cell types were cultured in GM. For example, miR-204 expression is approximately 20 fold higher in CStC *vs.* BMStC. This ratio remained almost unchanged in endothelial and adipogenic medium, was diminished in cardiomyogenic medium and increased to up 400 fold in osteogenic medium, giving evidences that microRNA differentially expressed between the two cell-types can be also differentially modulated by the medium. In this specific case, the fact that miR-204 is higher in CStC after osteogenic treatment is in line with the lower observed osteogenic ability of these cells compared to BMStC. It is in fact documented that miR-204 may act through Runx-2 inhibiting osteogenesis in mesenchymal progenitor cells [61]. Interestingly, the expression of myomiRs like miR-1 and miR-133b [62] was decreased in endothelial medium and stable or increased in CstC compared to BMStC after cardiomyogenic

and osteogenic treatment. The observation that also osteogenic treatment can induce myomiR up-regulation in CStC is not surprising considering that this medium contained Dexamethasone, a drug known to partially induce cardiomyogenic differentiation of adult cardiac stem cells [63].

The existence of a tissue molecular signature untouched by *in vitro* treatment has potentially an impact on the development of new reprogramming strategies and is in line with evidences demonstrating that, when a specific combination of miRs efficiently act in reprogramming one type of somatic cells, it might not be so efficient for another cell type. Myoshi et al [18], in fact, demonstrated that the combination of mir-22c, 302s and 369 was able to reprogram adipose stromal cells into iPSC, but the same combination was useless for skin fibroblast reprogramming. This observation means that the exact miR combination, successful for cell transdifferentiation, possibly needs to be individually determined. Further, the finding that different, potent differentiating stimuli are not able to erase the expression of signature miRs has potentially great consequences for regenerative medicine, implying that the molecular network sustaining the identity of adult cells is apparently stronger than environmental factors, thus imposing a barrier for the concept of adult cell transdifferentiation. In fact, if this observation is validated by *in vivo* injection, it will possibly limit the use of adult cell only to tissue-specific application, or at least indicates that more drastic strategies should be put in place before injection in order to achieve “true” cell

transdifferentiation. In this light, our evidences strongly suggest that reaching the goal of fully overcoming lineage boundaries should be based necessarily on the knowledge of the main molecular determinants of the cell type at both the starting point and the arrival of the process.

In conclusion, our results demonstrated the existence of a tissue-specific miR signature, which survived to any differentiation stimuli, suggesting that miRs play as main factors in determining cell identity related to tissue origin. Importantly, our results imply that the key factor able to *in vitro* abolish the tissue specific differences still have to be discovered. Moreover, we identified miR subsets modulated by different culture conditions irrespective of tissue origin, pointing out their importance during differentiation processes.

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# Chapter 3: In vitro Epigenetic Reprogramming of Human Cardiac Mesenchymal Stromal Cells into Functionally Competent Cardiovascular Precursors

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*PLoS One. 2012;7(12):e51694*

# 1. Introduction

Cellular cardiomyoplasty 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 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 good candidate. Yet, although several attempts have been made to simplify iPS cell generation methods avoiding the undesired effect of neoplastic transformation [2], their use still raises safety concerns. Consequently, much effort has been put into promoting cardiovascular differentiation of adult cells. Intriguingly, a recent work has shown the possibility of directly converting neonatal or embryonic mouse fibroblasts into cardiomyocytes by a transcription-factor based reprogramming strategy [3]. 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 [4]. 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 cardiac cells into progenitors, which are less de-differentiated than iPS cells and exhibit lineage commitment restricted to the cell types of interest, may represent a 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 [5]. CStC are c-Kit negative, reveal positivity for both pericytes (i.e. CD146) and fibroblast markers (i.e. vimentin and human fibroblast surface antigen) and share similarities with syngeneic bone marrow mesenchymal stromal cells (BMStC), showing comparable morphology and expression of mesenchymal-associated antigens (i.e. CD105, CD73, CD29, and CD44). Despite their similarities, significant differences between CStC and BMStC emerged. In fact, CStC may be identified by the expression of a specific microRNA signature and exhibit a residual plasticity toward the cardiovascular lineage, possessing the ability to contribute new adult-like cardiomyocytes after heart ischemia with higher efficiency than BMStC [5]. Of note, CStC are easily obtained from small biopsyspecimens and may be efficiently grown *in vitro*.

A large number of evidences indicate that biological response modifiers, including epigenetically active small molecules, such as histone deacetylase inhibitors (HDACi), may facilitate the

redirection of adult cellular functions toward stemness [4]. So far , however, no reportsdescribed the *in vitro* enhancement of adult cardiac precursors via a defined cocktail of drugs.

In this report, we describe the properties of CStC chemically converted into functional cardiovascular precursors by means of nutrients deprivation in the presence of retinoids, phenyl butyrate and nitric oxide drugs. Remarkably, these drugs have been used in the past to stimulate *in vitro* cardiomyocyte production in different experimental contexts [6,7,8], but they were never combined together on cells isolated from human adult heart. These compounds have different mechanisms of action including nuclear receptor activation (retinoic acid) [9] and inhibition of histone deacetylases (phenyl butyrate). Further, nitric oxide donors such as DETA/NO, have been shown to play a role in the prevention of apoptosis, microRNA up-regulation [10] and cardiac commitment [11]. Therefore it is possible that these drugs may synergize in determining the generation of functionally competent cardiovascular precursor-like cells.

# 2. Materials and methods

## 2.1 Ethics Statement

Cardiac stromal cells were obtained from right auricle samples of donor patients undergoing valve substitution or by-pass surgery after signed informed consent and approval by the Centro Cardiologico Monzino (Milano, Italy) Ethical Committee. Investigations were conducted according to the principles expressed in the Declaration of Helsinki. Data were analyzed anonymously.

## 2.2 CStC isolation and culture

CStC were isolated from right auricles and cultured in growth medium (GM) as previously described [5]. CStC at passages 4-8 were incubated for 7 days with an Epigenetic cocktail (EpiC), composed by Iscove's Modified Dulbecco's Medium (IMDM), 5% Foetal Bovine Serum (FBS), 10.000 U/ml Penicillin/Streptomicin , 10 mg/ml L-Glutamine , 5 µM All trans Retinoic Acid (ATRA), 5 µM Phenyl Butyrate (PB), and 200 µM diethylenetriamine/nitric oxide (DETA/NO), all purchased from Sigma Aldrich. The EpiC medium was changed every 48 hrs.

## 2.3 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 membranes (Bio-Rad Laboratories), and incubated overnight at 4°C with primary antibodies listed in Supplementary Table S1. Subsequently, the blots were incubated with the appropriate anti-rabbit, anti-mouse, or anti-goat horseradish peroxidase-conjugated secondary antibody (Amersham-GE Healthcare). ECL or, when appropriate, ECL plus (Amersham-GE Healthcare) were used for chemiluminescence detection. Each filter was also probed with anti-β-actin, anti-β-tubulin, anti-H3, or anti-H4, to verify equal protein loading. Densitometric analyses were performed by NIH ImageJ software, version 1.4.3.67.

## 2.4 Real-time reverse transcription–polymerase chain reaction analysis

Total RNA was extracted from cells using the TRIzol reagent and 500 ng of RNA were reverse-transcribed using Superscript III reverse transcriptase (Invitrogen). cDNA was amplified by SYBR-GREEN quantitative PCR in an iQ5 Cycler (Bio-Rad Laboratories). Primer sequences are reported in Supplementary

Table S2. Relative expression was estimated using the DeltaCt (ΔCt) method. ΔΔCt were calculated using average ΔCt for each gene expression in GM. Fold changes in gene expression were estimated as $2^{(-\Delta\Delta Ct)}$. ΔCt = 25 was arbitrarily assigned to non-expressed genes.

## 2.5 MicroRNA profiling analysis

Total RNA was extracted from cells using TRIzol reagent (Invitrogen), according to the manufacturer's instructions. The concentration and purity of RNA were determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific), and only highly pure preparations (ratio of 260/280 > 1.8 and 260/230 > 1.8) were used. The integrity of total RNA was assessed using an Experion electrophoresis system and the RNA high sense Analysis Kit (Bio-Rad Laboratories) and only highly quality RNA (RQI > 9.5/10) was subjected to subsequent analysis. Comparative microRNA expression profiling was carried out using TaqMan Low Density Arrays Human MicroRNA A+B Cards Set v3.0 (Applied Biosystems). All procedures were performed according to the manufacturer's instructions, on a 7900HT Real-Time PCR System (Applied Biosystems).

Quality control and low level analysis of TaqMan Arrays were performed using the software ABI Prism SDS v2.4. Optimal baseline and Ct (threshold cycle) were determined

automatically by the software algorithm. All Ct values reported as greater than 35 or as not detected were changed to 35 and considered negative calls. Raw expression intensities of target microRNAs were normalized for differences in the amount of total RNA added to each reaction using the mean expression value of all expressed microRNAs in a given sample, following the method described by Mestdagh and co-workers [12]. Relative quantitation of microRNA expression was performed using the comparative Ct method ($\Delta$Ct). $\Delta$Ct values were defined as the difference between the Ct of any microRNA in the calibrator sample (the sample with the highest expression, i.e. lowest Ct value) and the Ct of the same microRNA in experimental sample. The Ct values were transformed to raw quantities using the formula $\eta^{\Delta Ct}$, where amplification efficiency ($\eta$) was set arbitrarily to 2 (100%). The normalized relative expression quantity of each microRNA was calculated by dividing its raw quantity by the normalization factor. MicroRNAs that had missing values in greater than 50% of the samples (i.e. that were not present at least in all cells on one treatment) were deemed as uninformative and removed from the dataset.

## 2.6 Chromatin Immunoprecipitation (ChIP) assay

ChIP assays were performed and DNA fragments analyzed by quantitative real-time PCR (qPCR) as previously described [13]. Briefly, standard curves were generated by serially diluting the

input DNA (5-log dilutions in triplicate) and qPCR was done on an ABI Prism 7500 PCR instrument (Applied Biosystems), using a SYBR Master mix (Applied Biosystems) and evaluating the dissociation curves. The qPCR analyses were performed in duplicate and the data obtained were normalized to the corresponding DNA input. Data are represented as relative enrichment of specific histone modifications in EpiC-treated cells compared to growth medium (GM). Primers for human promoters were (position from transcriptional starting site, TSS): c-Kit fw: GAGCAGAAACAATTAGCGAAACC (-560 bp); c-Kit rev: GGAAATTGAGCCCCGACATT (-468 bp); Nkx2.5 fw: TGACTCTGCATGCCTCTGGTA (-198 bp); Nkx2.5 rev: TGCAGCCTGCGTTTGCT (-138 bp); MDR-1 fw: TTCCTCCACCCAAACTTATCCTT (-93 bp); MDR-1 rev: CCCAGTACCAGAGGAGGAGCTA (-2 bp); hGNL3 fw: GAGTTTGTGTCGAACCGTCAAG (-563 bp); hGNL3 rev: TCCCTCAGTCCCCAATACCA (-457 bp).

## 2.7 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, 1 mM $BaCl_2$ and 2 mM $MnCl_2$ 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 step 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 ± SEM.

## 2.8 Statistics

Statistical analysis of real-time PCR, Western Blot, and HDAC Class I activity data was performed using Student's *t*-test. A $P < 0.05$ was considered significant.

Statistical analysis of the TaqMan Arrays was performed using the MultiExperiment Viewer (MeV) software v4.8.1 [15]. microRNAs with a fold change between the two treatment groups less than ±2 were filtered out. Normalized expression values were $\log_2$ transformed and differentially expressed microRNAs were identified using a paired *t*-test computing p-values based on all available permutations with a confidence level of 95% and limiting the false discovery rate (FDR) proportion to <0.15. Differences in microRNA expression were considered statistically significant if their P-value was $< 0.05$. Unsupervised hierarchical cluster analysis was performed to assess whether differential profile discriminates the EpiC from the control treated cells. The similarity of microRNA expression among arrays and probes was assessed by calculating the Pearson's correlation (uncentered) coefficient. Normalized $\log_2$ transformed expression values were mean centered and clustered by correlation average linkage, using leaf order optimization.

# 3. Results

## 3.1 Epigenetic cocktail (EpiC) design

Human cardiac stromal cells (CStC) cultured in standard medium for mesenchymal cells (GM) are positive for the mesenchymal markers CD105, CD29 and CD73, but negative for adult cardiac stem cell markers Sca-1, c-Kit and VEGFR2 (Figure S1A).

The level of nutrients (i.e. foetal serum) and the presence of selected drugs can modify cell phenotype and fate inducing functional reprogramming [4]. In light of this, after expansion in a medium routinely used for mesenchymal cell culture (growth medium, GM), CStC were exposed for 7 days to a medium with reduced level of foetal 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 conditions, the expression of markers associated with resident cardiac stem cells (c-Kit, VEGFR2, and MDR-1) [16,17] has been evaluated. Our findings indicated 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 formulation, defining a novel “epigenetic cocktail” (EpiC), induced the coincident expression of c-Kit,

VEGFR2, and MDR-1 in CStC (Figure S1B). Notably, EpiC treatment, while stopping cell proliferation, did neither induce apoptosis or senescence (Figure 1), nor stimulate CStC to differentiate into other mesodermal cells such as adipocytes or osteoblasts (Figure S1C), nor modulated the expression of Sca-1 and typical mesenchymal markers such as CD105 (not shown).

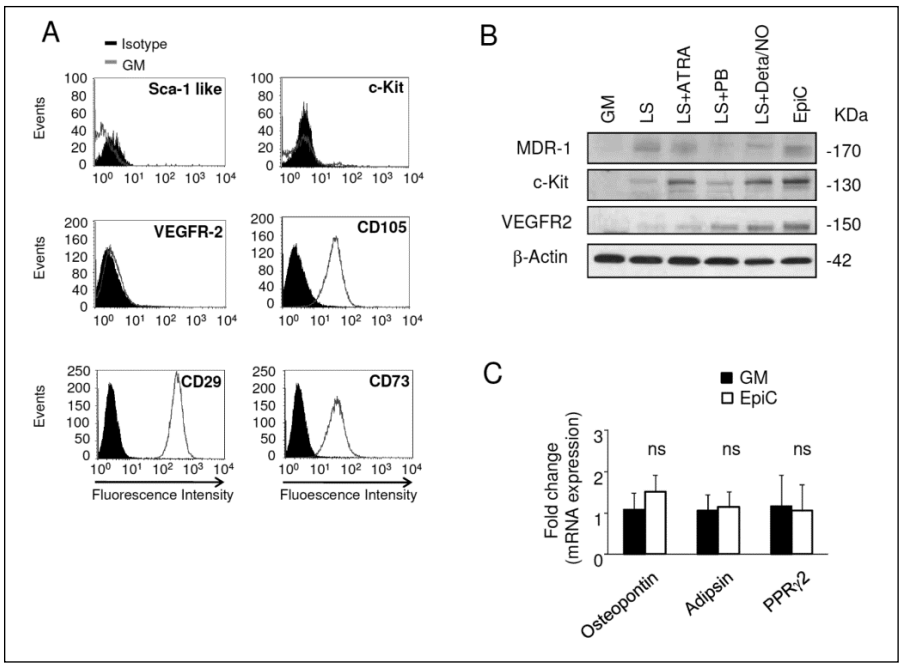


**Figure 1 Supplementary. CStC characterization and Epigenetic Cocktail (EpiC) design. (A)** Representative FACS analysis of CStC surface markers. **(B)** Western blot showing MDR-1, c-Kit, and VEGFR-2 expression of CStC cultured in growth medium (GM) or in low serum (LS) with or without epigenetic drugs for 7 days. ATRA = all-trans-retinoic acid; PB = phenyl butyrate; DETA/NO = diethylenetriamine/nitric oxide; EpiC = LS+ATRA+PB+DETA/NO. **(C)** Real-Time RT-PCR analysis demonstrates no up-regulation of adipogenic (Adipsin and PPRγ2) and osteogenic (Osteopontin) markers (n = 3). ns = not significant.

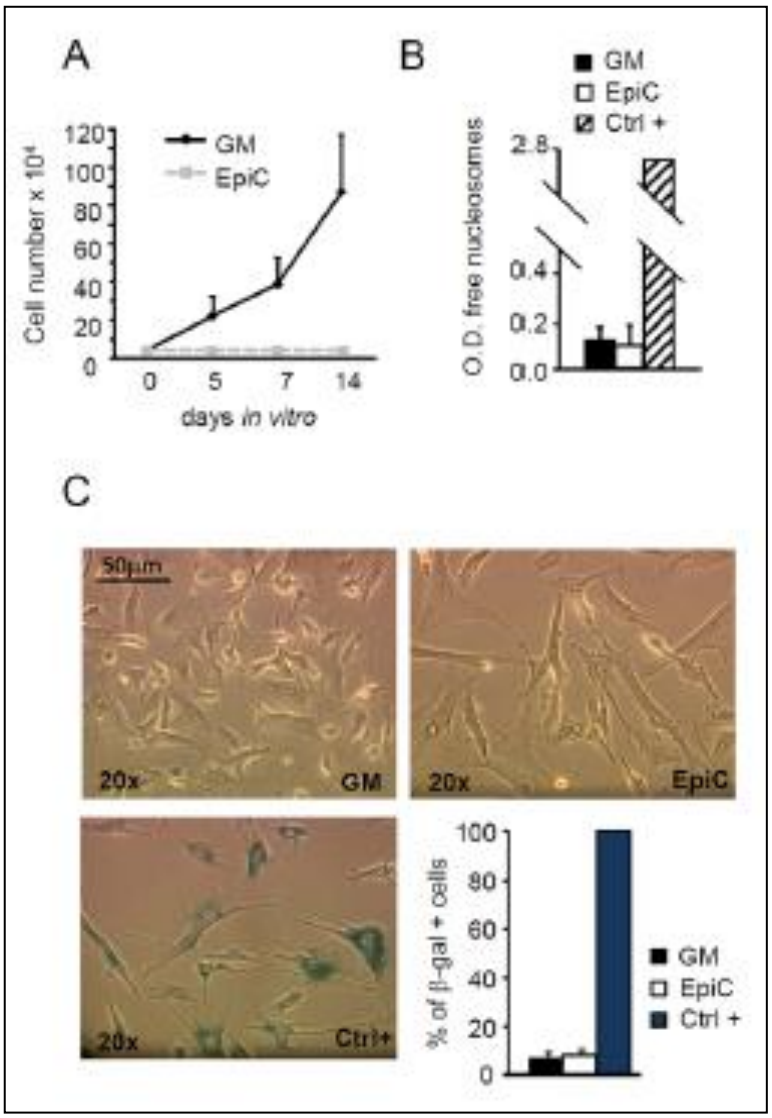


**Figure 1. Effect of EpiC treatment on CStC growth, viability and senescence. (A)** Growth curve of CStC cultured in GM or EpiC for 5, 7, 14 days. **(B)** Quantification of free nucleosomes, used as markers of apoptosis, in CStC in GM or EpiC for 7 days. Ctrl+ = positive control. **(C)** Staining for senescence-related acidic β-galactosidase (β-gal) performed on CStC grown in GM or in EpiC medium. Positivity for β-gal is indicated by the presence of a dark grey stain jn the cytoplasm. Ctrl+ = BMStC at passage 10 (replicative senescence). Bar Graph shows average percentage of β-gal positive cells. Original magnification: 20x.

## 3.2 Effects of EpiC treatment on stem and cardiovascular precursor markers

EpiC treatment of CStC strongly up-regulated markers associated to cardiac resident adult stem cell such as c-Kit and MDR-1 (Figure 2A and Figure S2A). In this condition, we were able to demonstrate that the MDR-1 transporter was functionally active as indicated by the rhodamine extrusion assay (Figure S2B). Other proliferation and differentiation markers including Notch, Jagged-1 and Numb [18,19] were also increased (Figure 2B), while the expression of the pluripotency factors Oct4 and Nanog remained negative (not shown).

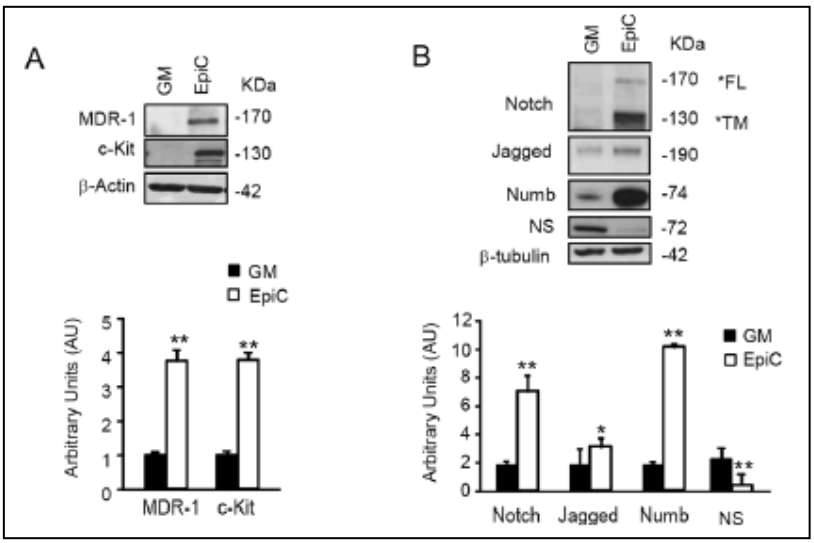


**Figure 2. Effect of EpiC treatment on stem cell marker expression.** Western blot analysis of **(A)** adult cardiac stem cell marker c-Kit and MDR-1 and **(B)** Notch, Jagged-1, Numb and nucleostemin (NS) in CStC, grown in GM or in EpiC. Densitometry is shown in the bar graphs at the bottom of each panel. FL = full length protein; TM = Transmembrane domain.

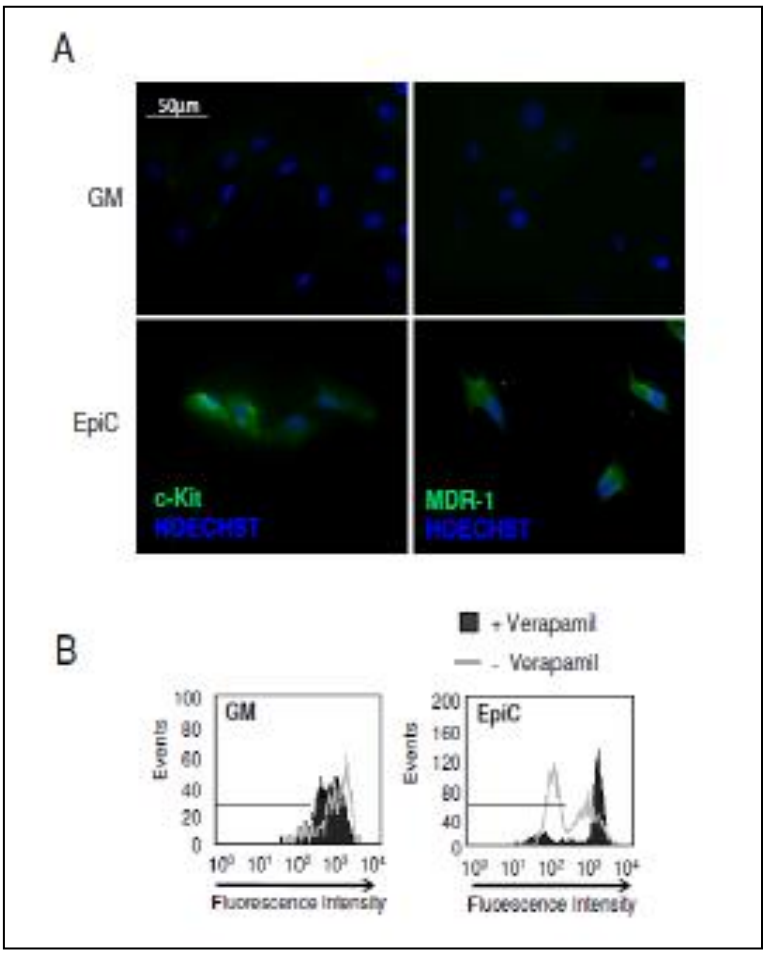


**Figure 2 Supplemetary. Effects of EpiC treatment on c-Kit and MDR-1 expression in CStC. (A)** Representative immunofluorescence images for c-Kit and MDR-1 in GM and EpiC-treated CStC. Original magnification: 20×. **(B)** Rhodamine 123 assay in GM and EpiC treatments (n = 4). Only EpiC-treated CStC were able to extrude Rhodamine through Verapamil sensitive MDR-1 channels.

In this condition, EpiC-treated cells were growth arrested (Figure 1A) and nucleostemin (NS), a nucleolar protein present in proliferating stem cells [20], was down-regulated (Figure 2B). Of note, untreated CStC expressed detectable level of GATA6, α-smooth muscle actin and GATA4, whose expression is associated with processes ongoing during vascular and cardiac commitment [21,22,23]. Interestingly, EpiC treatment increased the expression of markers for both vascular (VEGFR2, GATA6, and α-smooth muscle actin) and cardiomyogenic (GATA4 and Nkx2.5) precursors, while leaving Mef2C expression unaltered (Figure 3A and 3B). However, in spite of the evidence that 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 increased cardiac troponin (TnT-C) expression were observed (Figure 3C).

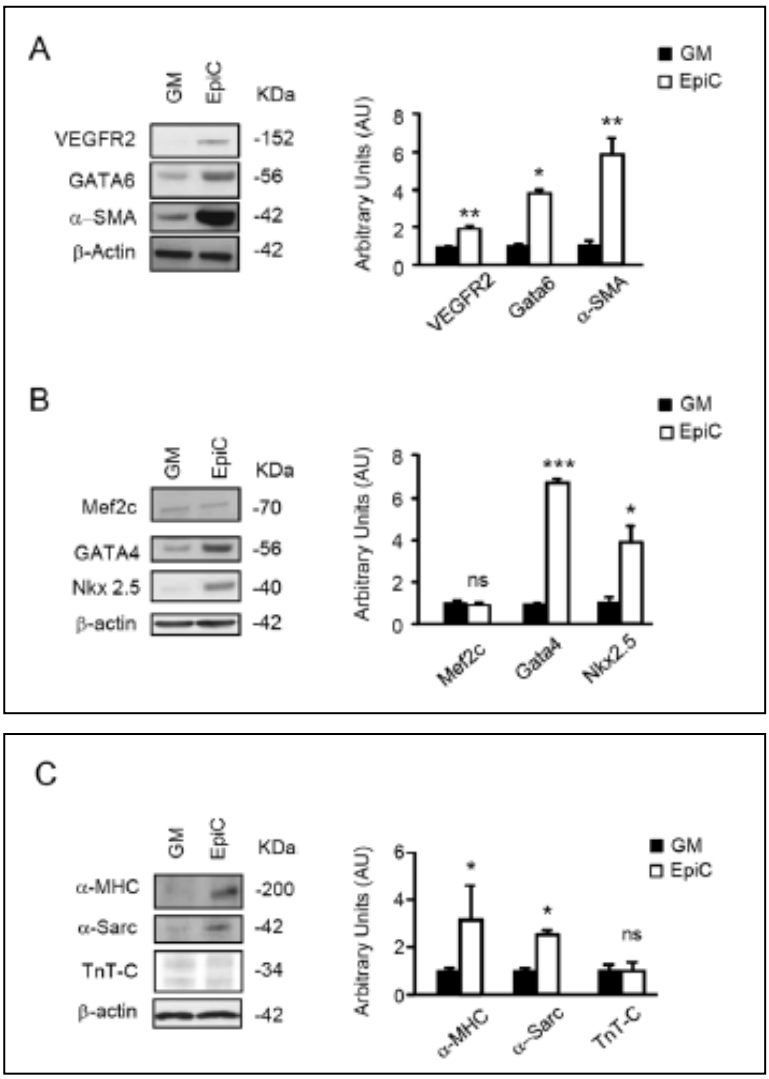


**Figure 3. Effect of EpiC treatment of cardiovascular marker expression.** Immunoblots showing expression analyses of **(A)** vascular markers: VEGFR-2, GATA6, and a-smooth muscle actin (a-SMA); **(B)** early cardiomyogenic markers: Mef2c, GATA4, and Nkx2.5; **(C)** late cardiomyogenic markers: a-MHC, a-Sarc, and Tn-TC in EpiC-treated CStC compared to cells in GM. Bar graphs represent average results, normalized to β-actin, of western blot densitometric analyses.

## 3.3 Effects of EpiC treatment on CStC microRNA expression profile

microRNAs (miR) are 22-23 nucleotide-long, single-stranded ribonucleic molecules usually involved in transcriptional repression and gene silencing [24]. As miR are known to have important roles in cell reprogramming [25] and cell differentiation [26], their expression profile was evaluated in CStC after 7 days of exposure to EpiC. Two hundred and sixty-one microRNAs passed the quality assurance and filtering criteria (Table 1).

| microRNA | GM | EpiC | FC | P-value | FDR |
|---|---|---|---|---|---|
| miR-133a | 0.07 ± 0.07 | 0.86 ± 0.43 | 11.88 | 0.0488 | 0.13 |
| miR-34a | 0.26 ± 0.10 | 1.23 ± 0.13 | 4.78 | 0.0266 | 0.12 |
| miR-664 | 0.30 ± 0.09 | 1.44 ± 0.34 | 4.75 | 0.0085 | 0.06 |
| miR-34a# | 0.31 ± 0.13 | 1.33 ± 0.61 | 4.27 | 0.0209 | 0.10 |
| miR-210 | 0.25 ± 0.10 | 1.04 ± 0.29 | 4.19 | 0.0266 | 0.11 |
| miR-200b | 0.33 ± 0.16 | 1.34 ± 0.63 | 4.06 | 0.0492 | 0.13 |
| miR-146b-5p | 0.29 ± 0.04 | 1.00 ± 0.31 | 3.42 | 0.0442 | 0.14 |
| miR-145 | 0.31 ± 0.23 | 0.95 ± 0.47 | 3.09 | 0.0346 | 0.13 |
| miR-362-3p | 0.48 ± 0.10 | 1.50 ± 0.35 | 3.09 | 0.0002 | 0.02 |
| miR-1300 | 0.31 ± 0.18 | 0.93 ± 0.38 | 3.02 | 0.0126 | 0.09 |
| miR-204 | 0.38 ± 0.08 | 1.14 ± 0.16 | 2.99 | 0.0124 | 0.09 |
| miR-452 | 0.42 ± 0.13 | 1.20 ± 0.17 | 2.87 | 0.0113 | 0.10 |
| miR-30a-5p | 0.43 ± 0.05 | 1.13 ± 0.23 | 2.63 | 0.0056 | 0.08 |
| miR-21 | 0.40 ± 0.19 | 1.02 ± 0.29 | 2.52 | 0.0478 | 0.13 |
| miR-140-5p | 0.52 ± 0.07 | 1.28 ± 0.05 | 2.46 | 0.0114 | 0.09 |
| miR-30e-3p | 0.55 ± 0.12 | 1.31 ± 0.27 | 2.37 | 0.0181 | 0.10 |
| miR-26b# | 0.49 ± 0.07 | 1.09 ± 0.18 | 2.22 | 0.0423 | 0.14 |
| miR-22# | 0.64 ± 0.25 | 1.40 ± 0.31 | 2.19 | 0.0162 | 0.09 |
| miR-30a-3p | 0.56 ± 0.19 | 1.14 ± 0.25 | 2.03 | 0.0140 | 0.09 |
| miR-574-3p | 0.60 ± 0.11 | 1.21 ± 0.16 | 2.02 | 0.0445 | 0.13 |
| miR-663B | 1.32 ± 0.34 | 0.63 ± 0.02 | −2.08 | 0.0397 | 0.14 |
| miR-20b | 1.28 ± 0.34 | 0.58 ± 0.20 | −2.21 | 0.0455 | 0.13 |
| miR-708 | 1.07 ± 0.51 | 0.46 ± 0.19 | −2.34 | 0.0020 | 0.04 |
| miR-92a | 1.58 ± 0.27 | 0.68 ± 0.07 | −2.34 | 0.0303 | 0.12 |
| miR-376a | 1.15 ± 0.70 | 0.43 ± 0.23 | −2.71 | 0.0093 | 0.09 |
| miR-942 | 1.38 ± 0.92 | 0.36 ± 0.22 | −3.82 | 0.0009 | 0.04 |
| miR-18a | 1.16 ± 0.79 | 0.30 ± 0.19 | −3.91 | 0.0072 | 0.08 |
| miR-223 | 1.35 ± 0.38 | 0.32 ± 0.12 | −4.17 | 0.0017 | 0.05 |
| miR-29b-1# | 1.51 ± 0.82 | 0.35 ± 0.09 | −4.38 | 0.0282 | 0.11 |
| miR-155 | 1.83 ± 0.43 | 0.40 ± 0.15 | −4.54 | 0.0206 | 0.10 |
| miR-15b# | 1.53 ± 0.75 | 0.15 ± 0.04 | −10.42 | 0.0429 | 0.14 |

**Table 1: Differentially expressed microRNAs**. microRNA normalized relative expression levels in GM and EpiC-treated cells are expressed as mean ± SD. FC = fold change. FDR = false discovery rate.

Results of the differential analysis showed that 31 microRNAs were significantly modulated by EpiC treatment. In particular, 20 microRNAs were up-regulated > 2-fold (among which miR-133a, miR-34a, and miR-210) and 10were down-regulated (such as miR-155). Unsupervised hierarchical cluster analysis was performed using the whole dataset of 261 microRNAs, revealing that the global expression profile well discriminates between the two groups of treatment. Likewise, unsupervised cluster analysis of the differentially expressed microRNAs correctly discriminated between EpiC and control treated CStC, sorting them into two independent groups (Figure 4). In addition, it showed two clusters of microRNAs with highly correlated expression, the first increased and the latter decreased in EpiC-treated cells, which may be deemed as a specific signature of co-regulated microRNAs.

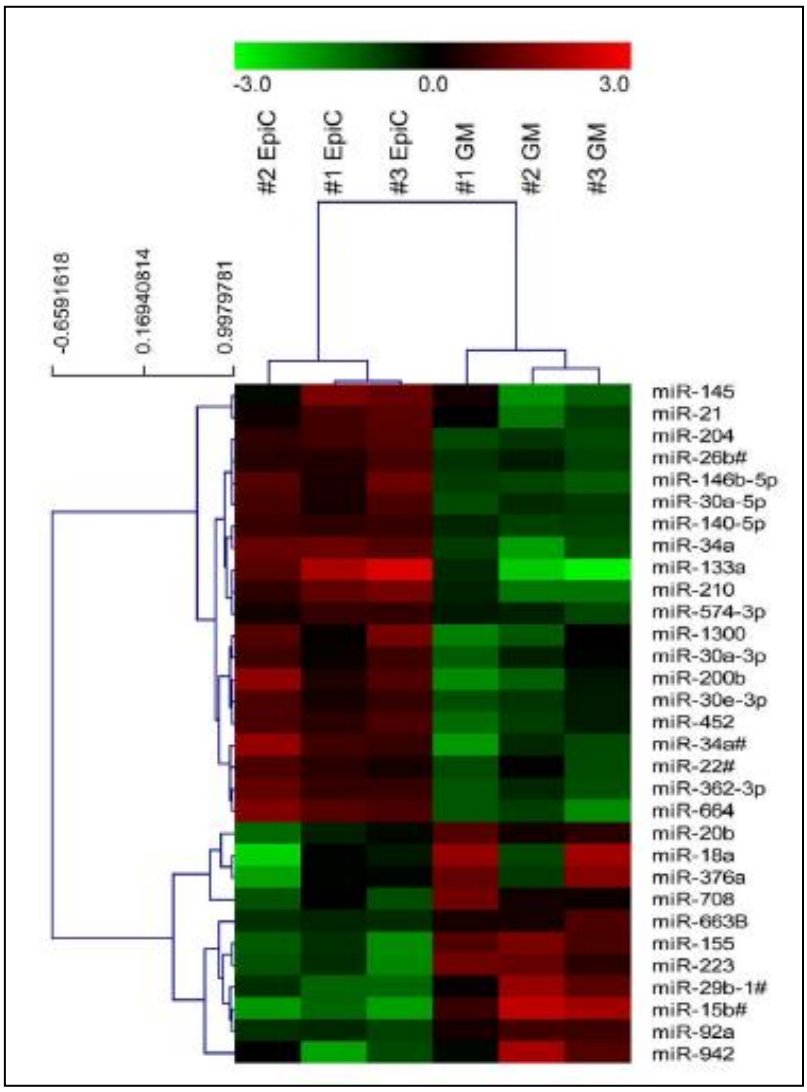


**Figure 4. Hierarchical clustering of differentially expressed microRNAs in GM and EpiC-treated CStC.** Unsupervised cluster analysis was performed using the 31 miRNAs that showed a significant modulation induced by the EpiC treatment. The dendrogram above shows that thedifferential expression profile completely discriminates the two treatment groups. The dendrogram on the left shows two distinct clusters of miRNAs up- and down-regulated by EpiC treatment in CStC (respectively at the top and the bottom of the heatmap).

## 3.4 EpiC treatment changes CStC electrophysiological properties

The exposure to EpiC had a profound impact on CStC function, determining the appearance of electrophysiological 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 (Figure 5A), which activated at voltages more positive than -40 mV, peaked around 0 mV, and was completely blocked by TTX (10 µM, Figure 5B). At a lower concentration (50 nM) TTX had variable effects on sodium current. In fact, in a group of cells, sodium current was not blocked at all (n=3) while in another group the block ranged from 38 to 94% (mean value: 76.3 ± 9.2%, n=3, data not shown). These results suggest that EpiC-treated cells may present both TTX-sensitive and TTX-resistant sodium currents. Accordingly, expression analyses showed that, in the presence of EpiC, the type V (NaV1.5) and type II (NaV1.2) voltage-gated sodium channels (known for having different TTX sensitivity and encoded by the SCN5A and the SCN2A genes, respectively) were significantly up-regulated, both at the mRNA (Figure 5C) and at the protein level (Figure 5D). A pacemaker If current (Figure 5 E and F) has also been recorded in 5 out of 33 EpiC-treated cells (15.1%), with kinetic properties compatible with those of immature cardiomyocytes and of native pacemaker cells (V1/2:

-74.4 ± 5.7 mV, n=3; Figure 5E and F) [27]. Accordingly, EpiC increased the expression of the pacemaker channel subunit HCN4 (Figure S3). Notably, CStC maturation towards the cardiomyocyte lineage was far from being complete, as demonstrated by the absence of the intracellular Ca2+ handling proteins NCX1 and RyR2 (not shown) and the depolarized resting potential characterizing CStC exposed to EpiC (-12.7 ± 2.7 mV, n=7). This last observation is in accordance with the negligible expression of the inward rectifying IK1 current (0.43 ± 0.04 pA/pF at -100mV, n=5; not shown), physiologically important in setting the resting potential of working cardiomyocytes.

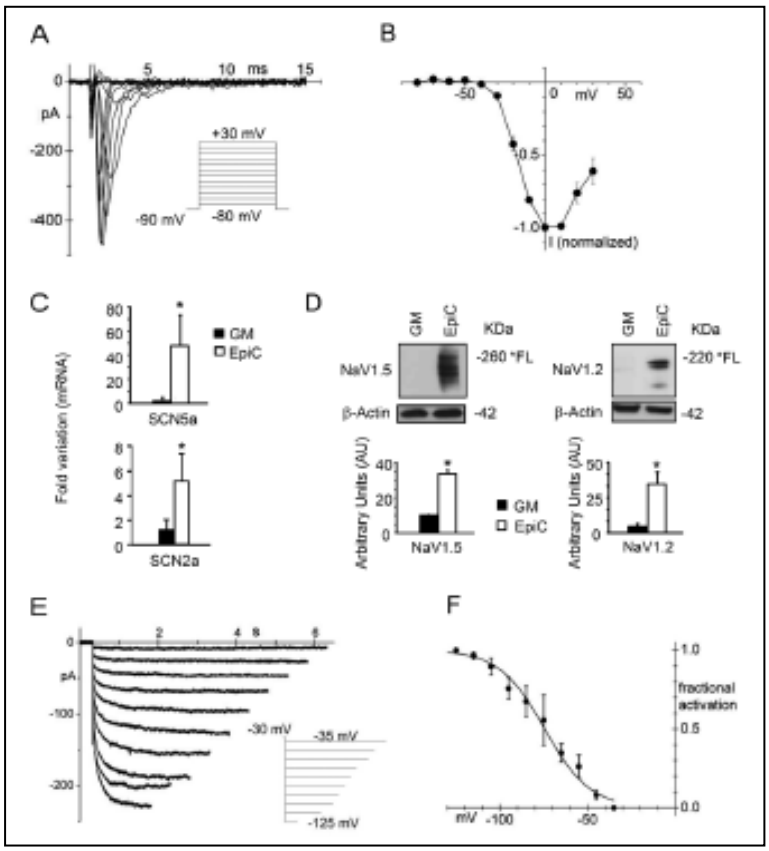


**Figure 5. EpiC-treated CStCs express functionally competent ion channels.** In panel **(A)** a family sodium current recorded from a representative EpiC-treated TTX (10 mM) low -sensitive CStC, following the application of a standard depolarizing protocol (see inset), is shown. **(B)** Mean current-voltage relation of normalized TTX-sensitive currents showing a threshold of activation around 240 mV and a peak around 0 mV. **(C)** SCN5A and SCN2A genes were up-regulated (n = 3,) by real-time PCR analysis**. (D)** Western Blot evidences increase of the type V (NaV1.5) and type II (NaV1.2) voltage-gated sodium channel protein level in CStC cells exposed to EpiC (n = 5). FL = full length protein. **(E)** In few cells, membrane hyperpolarization in the range 235 to 2125 mV (see inset) revealed an inward current with the kinetic features of the native pacemaker If current. **(F)** Plot of the mean activation curve obtained from the analysis of the If currents recorded in a subset of EpiC-treated CStC.

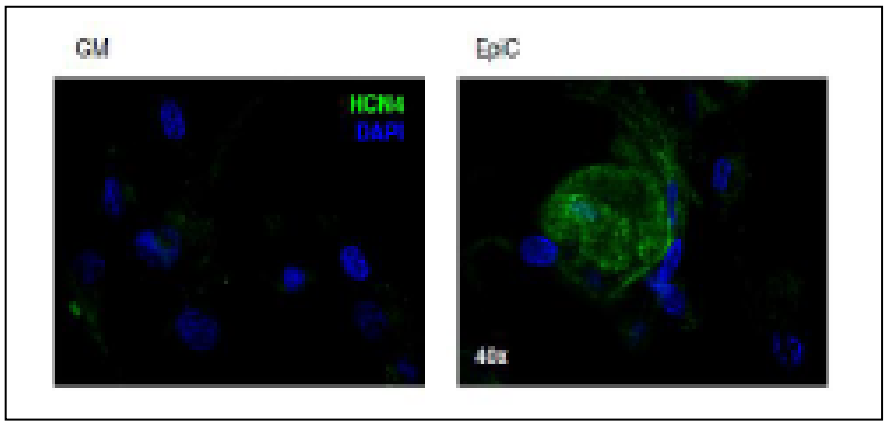


**Figure 3 Supplementary. Effect of EpiC treatment on the expression of the pacemaker channel subunit HCN4 in CStC.** Representative immunofluorescence images for HCN4 in GM and EpiC-treated CStC. Original magnification: 40×.

## 3.5 EpiC treatment modulated CStC epigenetic landscape

As expected by the composition of the EpiC described above, EpiC-treated CStC revealed a significantly lower HDAC Class I activity compared to cells kept in control condition (Figure S4) and changes in a number of genome wide histone modifications could be detected. Specifically, in agreement with its anti-proliferative effect EpiC also induced a significant decrease in Histone H3 phosphorylation at Ser10 (H3S10P, Fig. 6A) [28]. Further, we observed a global decrease in histone H3 lysine 9 trimethylation (H3K9Me3) and H4 lysine 20 trimethylation (H4K20Me3), paralleled by an increase in histone H4K20 monomethylation (H4K20Me, Fig. 6A and B). These modifications are compatible with an open chromatin structure and have been reported in cells undergoing differentiation or cell cycle arrest [29]. On the other hand, an increased density of the permissive marks histone H3K4 trimethylation (H3K4Me3) [30] and histone H4K16 acetylation (H4K16Ac) [31] was observed (Fig. 6 A and B), suggesting the presence of cells with a high developmental potential [32]. Along this line of evidence, repressive markers [33] remained stable (H3K27Me3) or were significantly down-modulated (H3K9Me3 and H4K20Me3), suggesting that EpiC-treated cells underwent a site-selective chromatin remodelling process leading to regulation of gene transcription.

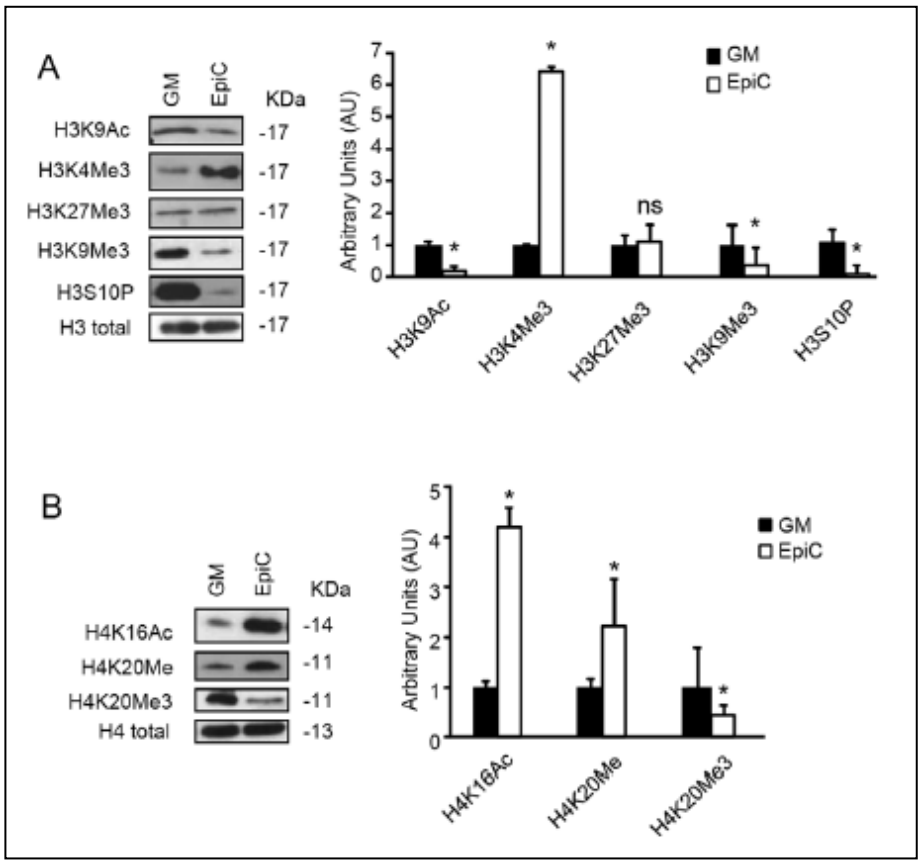


**Figure 6. EpiC treatment modifies CStC epigenetic landscape.** Western Blot analysis showing histone modification changes in EpiC-treated CStC compared to GM. **(A)** H3 modifications: H3K9Ac, H3K4Me3, H3K27Me3, H3K9Me3, and H3S10P. **(B)** H4 modifications: H4K16Ac, H4K20Me, and H4K20Me3. The same filter was probed with anti-total histone H3 or H4, respectively, to control for equal nuclear protein loading. Band densitometric analyses are reported in the bar graphs on the right (n = 3).

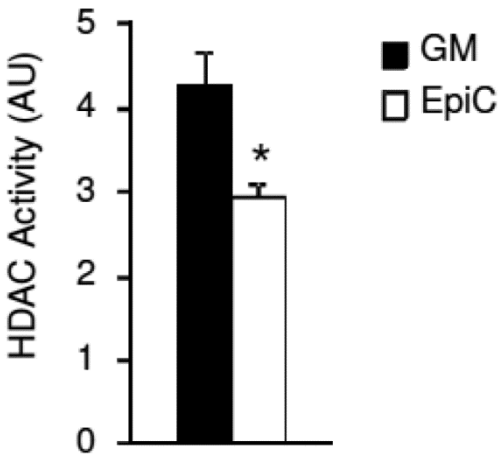


**Figure 4 Supplementary. Effect of EpiC treatment on HDAC activity in CStC.** Bar graphs show Class I HDAC activity in CStC cultured in GM or EpiC for 7 days (n = 4).

## 3.6 EpiC introduces chromatin changes at specific-gene promoters

To validate the hypothesis that Epic may specifically regulate gene expression, a series of chromatin immunoprecipitation (ChIP) experiments were performed in CStC cultured in the presence or the absence of EpiC. Specifically, the highly divergent H3K4Me3 and H3S10P were used to immunoprecipitate chromatin, followed by real-time PCR to detect the relative modulation of these specific histone modifications in the promoter region of c-Kit, MDR-1, Nkx2.5 and nucleostemin. As shown in Figure 7A and B and in Figure

S5A, H3K4Me3 association to c-Kit, MDR-1, and Nkx2.5 promoter was increased by EpiC treatment, suggesting that chromatin conformational modifications may account for the increased expression of these genes (Figure 7C). Accordingly, the presence of H3K4Me3 and H3S10P in the NKX-2.5 and nucleostemin (human GNL3 gene product) promoters was reduced, suggesting local structural changes as the basis of their down-regulation (Figure S5B).

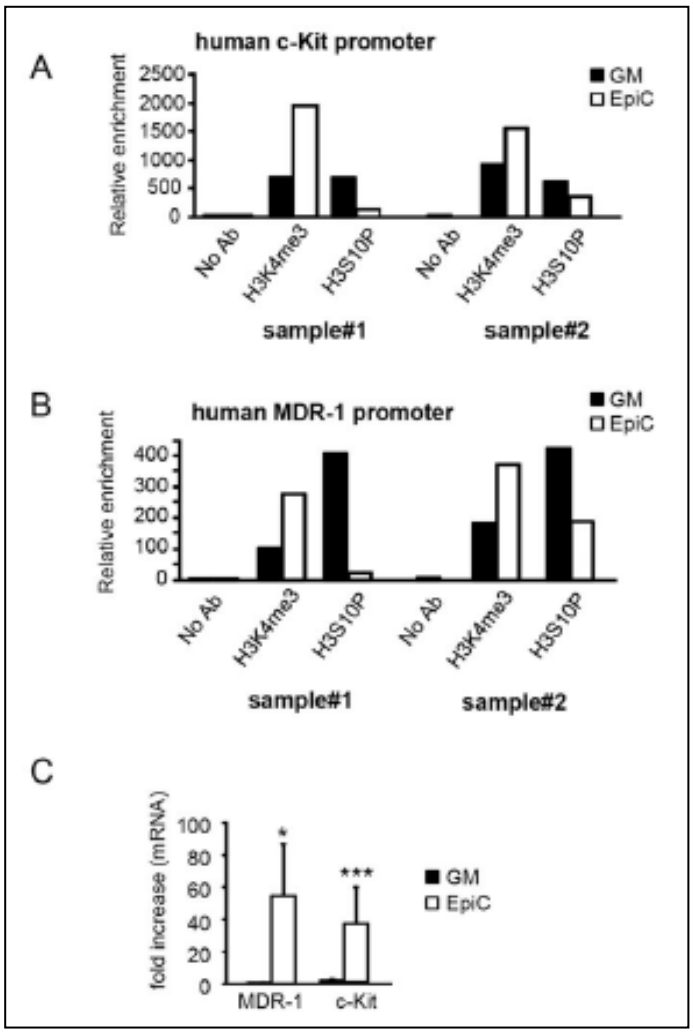


**Figure 7. Effects of EpiC treatment on specific-gene promoters.** ChIP experiments, performed on CStC isolated from 2 different patients, show that H3K4Me3 association to **(A)** c-Kit and **(B)** MDR-1 promoter is enriched after EpiC treatment, while H3S10P is decreased. Data are expressed as relative enrichment of specific histone modifications in EpiC-treated cells compared to GM measured by real-time PCR amplification. **(C)** Real-time RT-PCR analysis of c-Kit and MDR-1 mRNA in EpiC-treated CStC (n = 3).

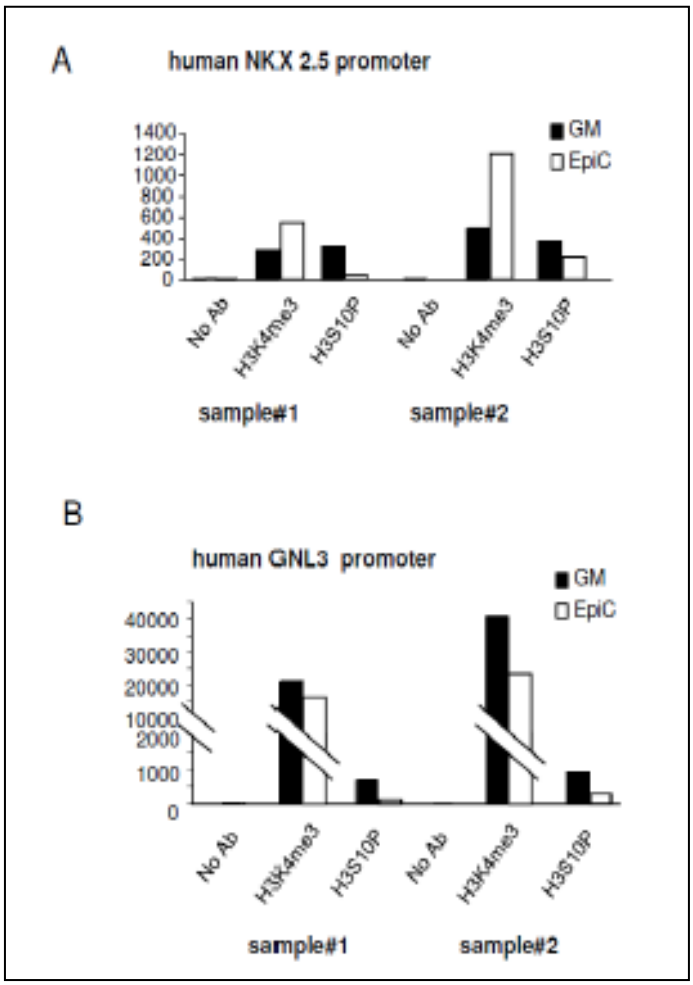


**Figure 5 Supplementary. Effects of EpiC treatment on specific-gene promoters in CStC. (A)** and **(B)** Bar graphs show relative enrichment for H3KMe3 and H3S10P in Nkx2.5 and GNL3 (nucleostemin) promoter.

# 4. Discussion

Resident cardiac stem cells 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 come [17]. Specific markers identify a cardiac stem cell, including the presence of c-Kit and the P-pump (MDR-1) gene products [16,17]. Adult cardiac stem cells are neither easily grown ex-vivo nor effectively differentiated 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 plasticity 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-like stromal cells (CStC) showing in vitro and in vivo cardiovascular plasticity [5]. Although CStC proneness to acquire cardiomyogenic markers was higher than that of syngeneic bone marrow cells, CStC efficiency to differentiate into adult cardiovascular cell types remained low, in spite of the expression of detectable mRNA encoding for early cardiovascular markers including c-Kit, GATA4 and GATA6 [5].

In the present work, CStC differentiation potential was improved by using a novel combination of small epigenetically active molecules, defined here as epigenetic cocktail, or “EpiC”. This cocktail was designed to modify the CStC chromatin landscape and, thus, 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 [34] and whose receptors are known to recruit p300 and CBP acetyl-transferases facilitating their action at the histone level of specific gene loci [35]. Relevant for this study, retinoic acid retains well known morphogenetic properties including a profound effect on heart development and regeneration [36]; (ii) phenyl butyrate (PB), a drug belonging to the family of histone deacetylase inhibitors, which are known to enhance mesoderm maturation [37]; (iii) diethylenetriamine/nitric oxide (DETA/NO), a nitric oxide donor associated to cell survival growth arrest in vascular cells [38] and increased mesendoderm differentiation in mouse ES cells [10]; (iv) a reduced serum content, known to induce spontaneous differentiation in a variety of cell types including C2C12 myoblasts [39] or cardiac mesoangioblasts [40]. 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 decades but, recently, an increasing number of studies are showing the potential role of small molecules to promote cardiogenesis in mouse ES cells

including different BMP inhibitors such as dorsomorphin [41] and Wnt pathway modulators [42]. On the other hand, similar attempts on adult cells were inefficient and the induction of true cardyomyogenesis is still vigorously 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]. Since then, this drug was commonly used to induce cardiomyogenesis in isolated cells [44]. Nevertheless, in some 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 [45]. 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. [46] 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 [46]. In this light, our findings suggest that the EpiC treatment determines global activation-prone changes in chromatin structure, including that at c-Kit, MDR-1 and Nkx2.5 promoters. Importantly, the expression of these genes was induced without apparently altering that of non-cardiac osteogenic and adipogenic differentiation markers. This suggests that CStC response to EpiC may be predominantly cardiovascular oriented [10,47]. In fact, along with the

expression of adult cardiac stem markers, EpiC treatment also induced the up-regulation of specific transcription factors associated with the vascular and cardiomyocyte lineage commitment.

It is widely accepted that microRNAs (miRs), are important for cardiac gene expression regulation and differentiation control [48,49]. In the present study, miR expression has been evaluated by profiling analysis. The evidence that unsupervised cluster analysis correctly separated between EpiC-treated and control CStC, further indicates that the EpiC treatment is potentially able to induce a specific transcriptional programme. Specifically, the expression of miR-133a, associated with cardiomyogenic differentiation [50], was strongly up-regulated, together with the expression of miR-210 and mir-34a, involved in stem cell survival [51] and negative growth control [52], respectively. Of note, miR-155 whose expression is associated with proliferation [53] and to the protection of cardiomyocyte precursors from apoptosis [54], was down-modulated in agreement with the reduced proliferation ability observed in EpiC-treated CStC. Interestingly, a recent paper by Anversa and co-workers shows that microRNA typically associated to adult cardiomyocytes (such as miR-1, mir-499 and mir-133) are expressed in cardiovascular precursors, but at lower levels than in adult cardiomyocytes [49]. In this light Epic-treated CStC expressing miR-133, more closely resemble cardiovascular precursors than control CStC, in which miR-133 expression is very low.

Based on the expression of typical stem and cardiovascular precursor markers and considering previous hypothesis for cardiovascular stem cell differentiation hierarchy in the adult heart [16], it is difficult to establish the differentiation stage to which CStC may belong. The fact that untreated CStC are GATA4, GATA6 and Mef2C positive, but only EpiC-treated CStC are cKit, MDR-1and VEGFR2 positive suggests that our cocktail may induce a cascade of events able to reprogram CStC towards a more immature state, characterized by the expression of cardiovascular stem cell markers [55]. On the other hand, EpiC-treated cells also up-regulated some markers associated with differentiating cardiomyocyte precursors (i.e. Nkx2.5, Gata4, □–sarcomeric actin, □–myosin heavy chain, miR-133a) and exhibited functional, although not yet operational, properties typical of differentiating cardiomyocytes, suggesting that EpiC treatment may induce cardiomyogenic differentiation at least in a fraction of the CStC population. It is thus possible that our approach induced the production of a mixed population composed of cells at different stages of differentiation, or acted on different cell populations present inside the CStC preparations. More experiments are required to elucidate this important point.

Importantly, many cells presented a fast sodium current (normally responsible for the action potential upstroke), which, however, was small in size and with kinetic properties slightly different from those of mature channels. In fact, TTX-sensitivity and expression analyses evidenced that EpiC-treated cells

expressed both the TTX-resistant NaV1.5 isoform, the primary cardiac type, and the TTX-sensitiveNaV1.2 isoform, a typical neuronal type which has been also detected, albeit at low levels, in the mouse and human hearts [56,57].

Moreover, few cells displayed the If current, which, although negligible in the adult ventricle, is present in atria and ventricles during the late embryonic and perinatal stage [27,58].

However, due to the lack of inwardly rectifying potassium currents, such as IK1, EpiC-treated cells exhibited a depolarized resting potential keeping both the Na+ and the HCN4 channels inactive, thus preventing the Epic-treated CStC cells from acquiring induced or spontaneous electrical activity.

In conclusion, it is reported here the first evidence that CStC may be chemically reprogrammed to acquire functionally competent cardiac precursor-like features.

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# Chapter 4: Human Chorionic Villus Mesenchymal Stromal Cells Reveal Strong Endothelial Conversion Properties

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*Differentiation. 2012 Jun;83(5):260-70*

# 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., 2010) and heart (Beltrami et al., 2007; Rossini et al., 2011). 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., 2010). 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., 2010; Insausti et al., 2010), 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

## 2.1 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.

## 2.2 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).

## 2.3 Bone Marrow Stromal Cell Isolation

BMStC were isolated and cultured as previously described (Rossini et al., 2011).

## 2.4 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.

## 2.5 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.

## 2.6 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.

## 2.7 Real-Time Reverse-Transcription-Polymerase Chain Reaction

Real-Time PCR experiments were performed as previously described (Livak and Schmittgen, 2001; Rossini et al., 2011). The sequences of used primers are reported in Table 1.

## 2.8 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).

## 2.9 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.

## 2.10 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., 2011).

## 2.11 *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., 2011). 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., 2011).

## 2.12 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.

## 2.13 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.

## 2.14 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.

## 2.15 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., 2011).

## 2.16 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.

## 2.17 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., 2011).

## 2.18 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

## 3.1 CVStC morphology and proliferation ability

Cells from chorionic villi (CVStC) were obtained from pregnant women undergoing chorionic villus sampling between the 11$^{th}$ and the 13$^{th}$ 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. 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.

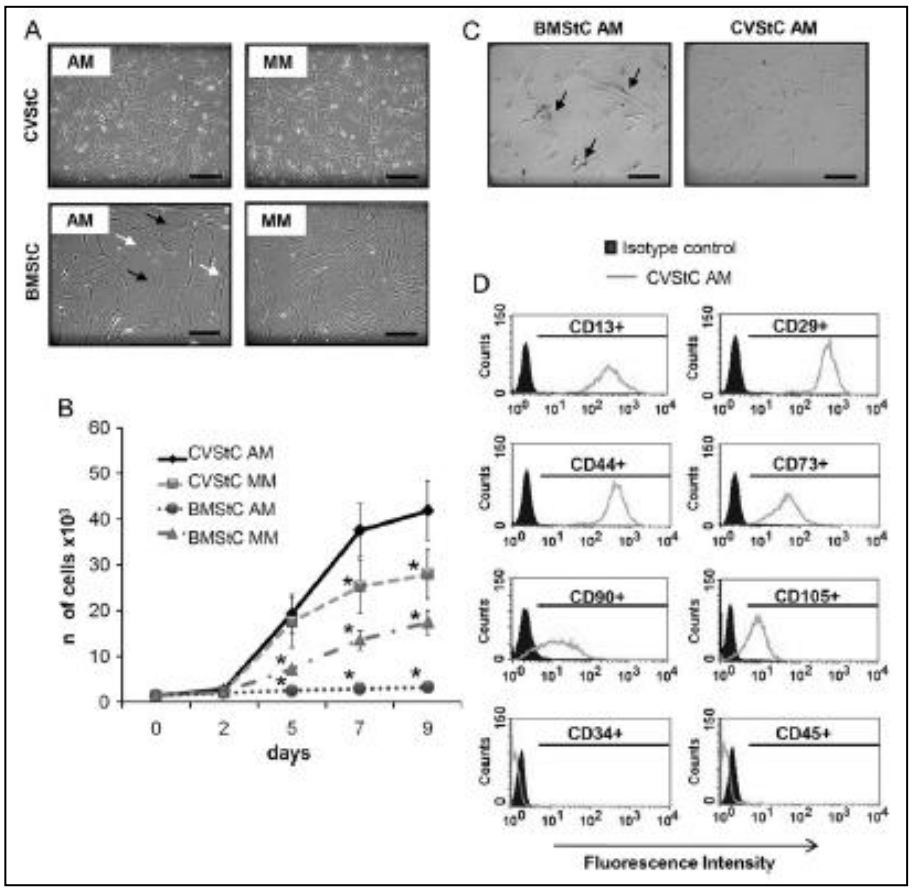


**Figure 1. Morphology, immunophenotyping and proliferation. (A)** CVStC appeared of fibroblast-like shape in both standard mesenchymal medium (MM) and AmniomaxII (AM). They were indistinguishable from bone marrow stromal cells (BMStC) expanded in MM, whereas BMStC developed long cytoplasmic extensions, resembling cellular senescence, when cultured in AM. Original magnification 10x; scale bar 100 µm. **(B)** Growth curves for CVStC and BMStC cultured in MM and in AM. CVStC grew more rapidly than BMStC in both media, however, their proliferation rate was faster in the presence of AM. BMStC cultured in AM did not proliferate and acquired a senescence-like morphology. **(C)** Representative pictures of senescence associated β-galactosidase staining in AM cultured BMStC and CVStC. Positive cells showed dark granules in the cytoplasm (black arrows). Original maginification 10x; scale bar 100 µm. **(D)** Representative FACS cytograms of CVStC surface markers expression.

## 3.2 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.

## 3.3 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).

## 3.4 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).

## 3.5 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., 2010).

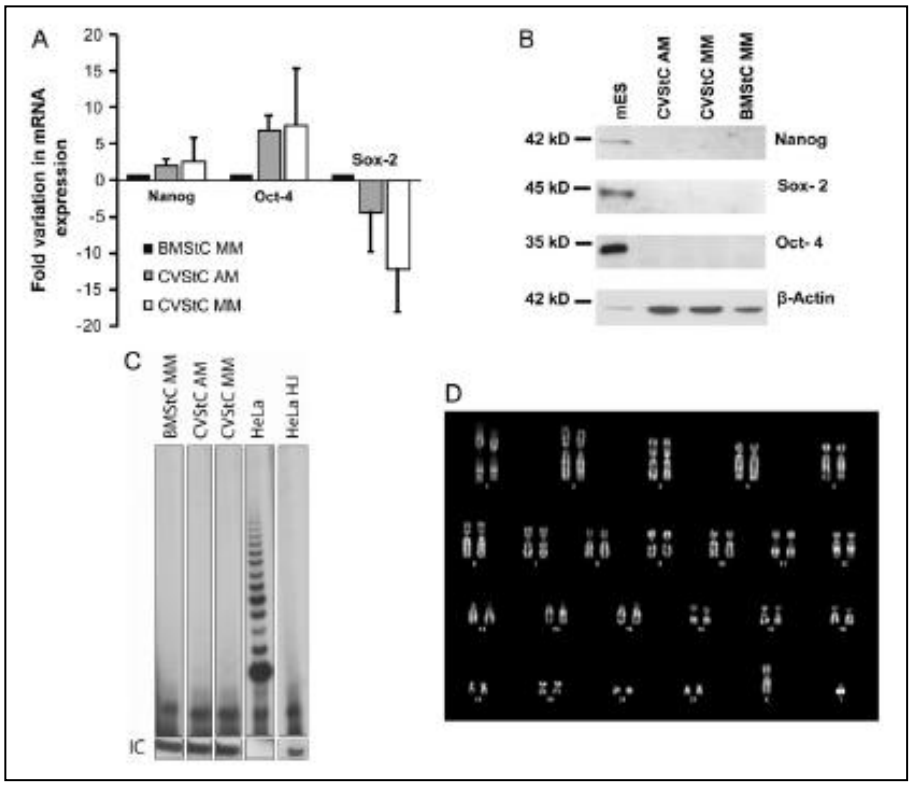


**Figure 2. Pluripotency-related gene expression analysis, telomerase activity and chromosomal stability. (A)** The mRNA level of the pluripotency-related factors Nanog, Oct-4 and Sox-2 was similar in CVStC and BMStC. **(B)** Nanog, Oct-4 and Sox-2 proteins were absent in both cell types. Mouse embryonic stem cells (mES) were used as positive control. **(C)** TRAP assay reveals that CVStC and BMStC did not exhibit hTERT activity. 0.1 mg from HeLa whole cell extract were used before (HeLa) and after (HeLa H.I.) heat inactivation as positive and negative control, respectively. **(D)** Representative CVStC karyogram.

## 3.6 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)

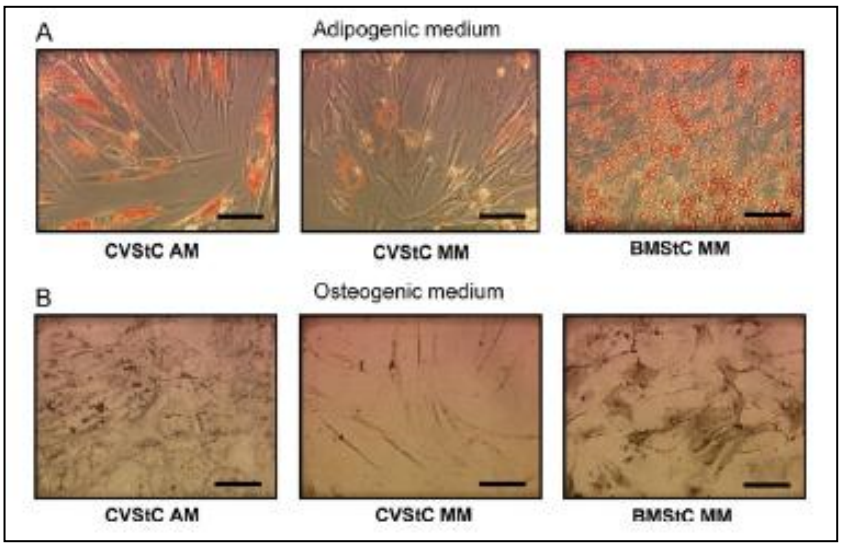


**Figure 3. Response to adipogenic and osteogenic stimuli. (A)** Cells were cultured 3 weeks in adipogenic medium; Oil-Red-O positive red droplets appeared in the cytoplasm of CVStC expanded both in AM and MM. BMStC adipogenic potential was higher than CVStC's, as indicated by large lipid vacuoles formation. Magnification 20x; scale bar 50 µm. **(B)** Osteogenic potential was demonstrated by Von Kossa reaction staining calcium salts (black or brown-black grains). CVStC, pre-cultured in AM, stained more intensely than those grown in MM. The osteogenic response of BMStC was the most pronounced. Original magnification 20x; scale bar 50 µm.

- ***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 $\alpha$-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 ($\alpha$-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).

- ***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., 2011). In this condition CVStC assumed a flattened and elongated morphology, similar to cardiac stromal cells (Rossini et al., 2011) (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., 2010) 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 $\alpha$-smooth muscle actin ($\alpha$-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., 2011). 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.

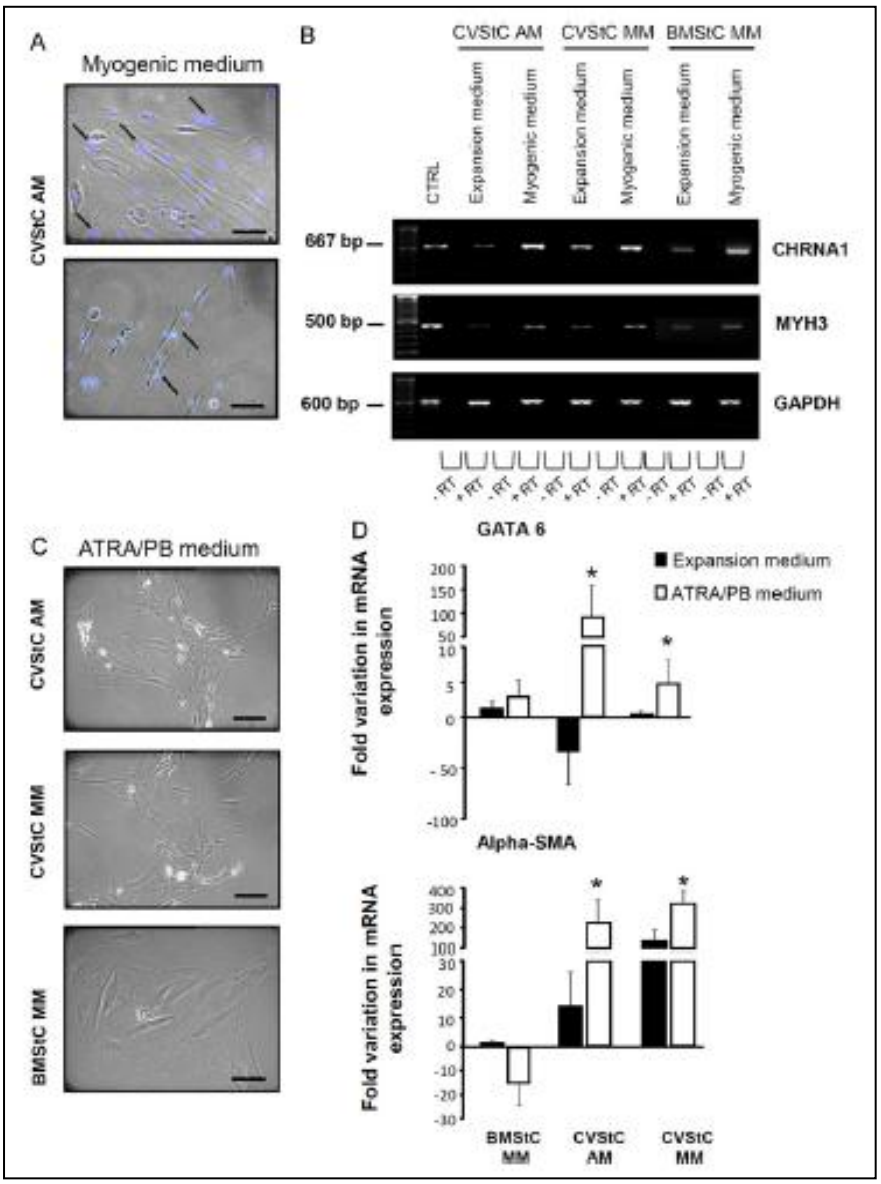


**Figure 4. Skeletal and cardiac muscle differentiation. (A)** Representative pictures of elongated bi- and poly-nucleated myotube-like cells, which appeared in CVStC cultured in low serum. Original magnification 20x; scale bar 50 µm. **(B)** RT-PCR analysis of CHRNA1 and MYH3 myogenic markers in CVStC and BMStC. **(C)** Morphological changes in CVStC and BMStC cultured in ATRA/PB medium. Original magnification 10x; scale bar 100 µm. **(D)** qRT-PCR analysis of CVStC and BMStC exposed to ATRA/PB medium.

- ***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(Rossini et al., 2011), the number of VEGF-receptor 2 (VEGFR2) positive cells (Fig. 5) considerably increased while BMStC remained negative for VEGFR2 before and after exposure to EGM-2 (Fig.5). 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., 2011), 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. Intriguingly, although late endothelial markers such as CD31 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.

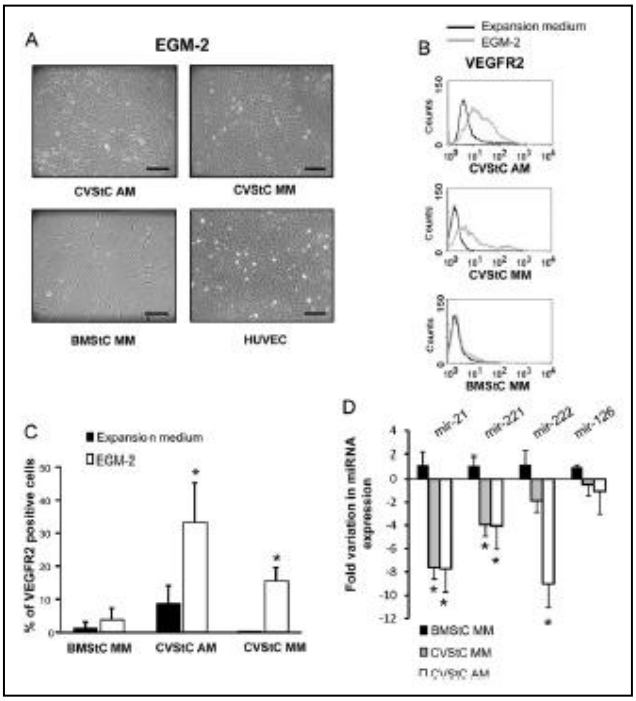


**Figure 5. Endothelial differentiation. (A)** CVStC cultured in EGM-2 showed cobblestone-like shaping; in this condition BMStC exhibited senescence-associated features. Original magnification 10x; scale bar 100 µm. **(B)** Representative cytograms of VEGFR2 expression in CVStC and BMStC before and after 3 weeks of EGM-2 culture; **(C)** Bar graph indicating average results of VEGFR2 positive cells evaluated by FACS analysis; **(D)** qRT-PCR analysis of angiomiR expression in CVStC compared to BMStC.

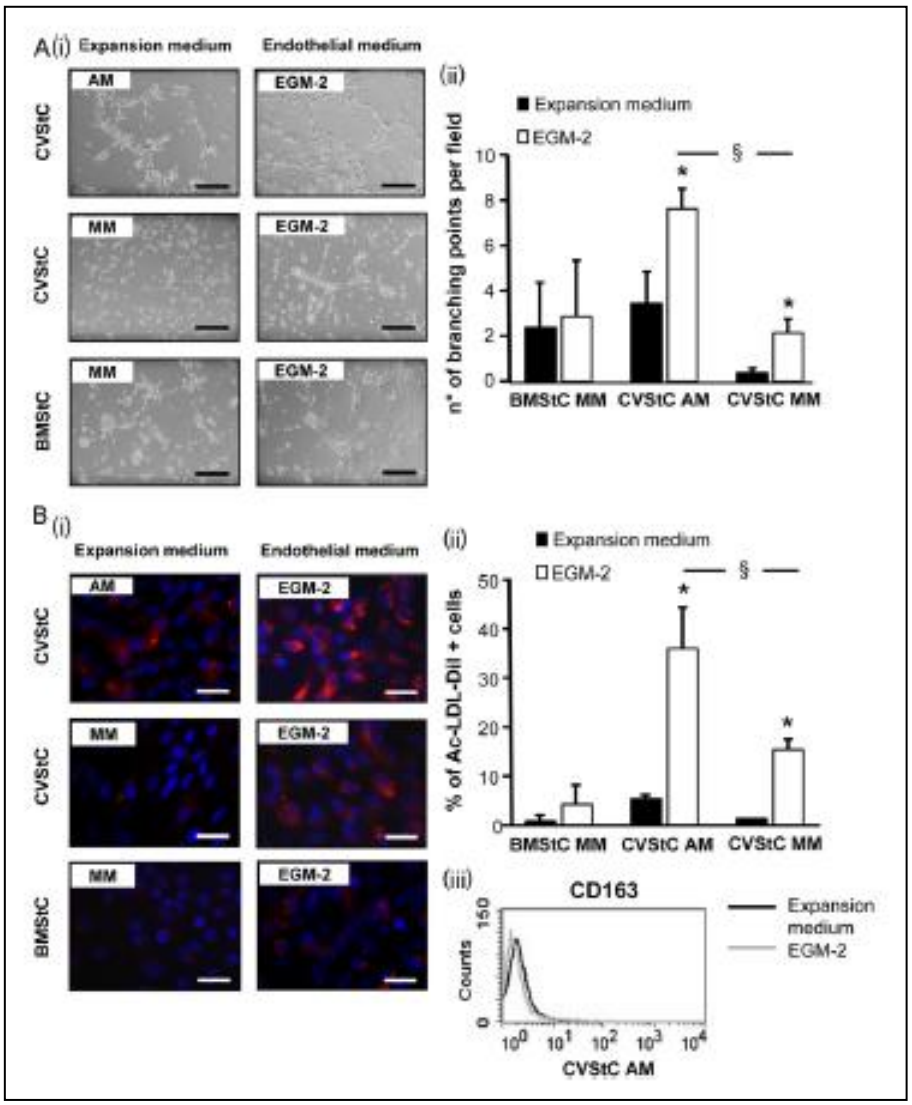


**Figure 6. Functional evaluation of CVStC- and BMStC-derived endothelial cells. (A) (i)** Representative pictures of CVStC- or BMStC-generated capillary-like structures obtained on basement membrane extract (Cultrexs) assessed before and after exposure to EGM-2. Magnification 10x scale bar 100 µm. **(ii)** Bar graph showing average results of Cultrexs assay. **(B) (i)** Analysis of Ac-LDL-DiI uptake in the presence of EGM-2. Original magnification 40x; scale bar 25 µm. **(ii)** Bar graph showing average results for the Ac-LDL-DiI. **(iii)** FACS analysis of CD163 expression in CVStC in AM and exposed to EGM-2.

# 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., 2010; 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., 2011) 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 10th–12th 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., 2010). 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., 2011), 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., 2011). 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., 2010). 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, 2011). Therefore, CVStC appear as a promising biological source suitable for cell therapy applications in vascular diseases.

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# Chapter 5: Summary, Conclusions, and Future Perspectives

Stromal cells can be isolated virtually from all adult [1] and fetal [2] tissues and organs and they have shown a great clinical interest cell therapy. The full characterization of mesenchymal stromal cells obtained from different origin (BMStCs, CStCs and CVStCs), with particular attention on the role of miRNAs, could lead to a better understanding of the molecular mechanisms responsible for the cell identity, differentiation and plasticity and their possible implication in regenerative medicine.

In agreement with the hypothesis that stromal cell plasticity is influenced by the tissue of origin [3], we found that CVStC exhibited restricted plasticity preferentially oriented toward the endothelial lineage, but CVStC ability to acquire osteocyte and adipocyte properties was less efficient than that of BMStC. This finding highlited that differentiative properties of stromal cells obtained from different tissues is at least in part dependent on their origin, but we have also reported that culture conditions and the differentiation strategies significantly influences the output. These observations are of therapeutic relevance, as cell identity and culture conditions are emerging as a major determinant of cell plasticity [4]. It is widely accepted that miRNAs are important for gene expression regulation, and differentiation control and the identification of a tissue signature

of CStCs and BMStCs which remained unmodified after *in vitro* treatments has important consequences, giving confirmatory evidences that miRNA-regulated pathways are involved in cell identity and fate determination. A growing body of evidence indicates that many miRNA play important roles during development [5] and further experiments are required to elucidate this important point. Intriguingly, genes of the Homeobox (Hox) family are among the most abundant targets predicted by our miRNA signature, strongly supporting the role of these miR in specifying the stromal identity of the cells of origin. Also, the presence of specific tissue miRNA signature suggested that their expression may not be dependent only on *in vitro* stimuli but more-likely reflects the epigenetic background of the target cell population. In fact, our study demonstrated that CStCs differentiation potential was improved by using a novel combination of small epigenetically active molecules, defined here as epigenetic cocktail (EpiC), specifically designed to modify the CStC chromatin landscape. The evidence that miRNA unsupervised cluster analysis correctly separated between EpiC-treated and control CStCs, further indicates that the EpiC treatment is potentially able to induce a specific transcriptional programme. Consequently, not only miRNAs 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. The important role of miRNAs in reprogramming and future clinical application is

likely to revolve in part around the nature of their biology. First, miRNA expression does not require protein translation and thus leads to a fast response in protein expression based on inhibition of mRNA translation and stability. Second, miRNAs generally target scores or hundreds of mRNAs that coordinate expression of many different proteins which can rapidly impose a dominant phenotypic change in cell identity, including those that regulate chromatin remodeling and cell proliferation/differentiation. The current focus on using miRNAs for potential clinical translation could lead to a safe methodological approach for induction of cell reprogramming and phenotypic conversion. In fact, miRNA-based approaches are already under direct clinical investigation in a number of other therapeutic contexts [6]. For example, a recent report demonstrated the utility and safety of therapy with miRNA in the treatment of hepatitis C [7].

In conclusion, this work have elucidated the distinctive properties of stromal cells from different origin and the role of miRNA in determining cell identity and plasticity in stromal cells. Our results emphasyzed the fact that the role of miRNA and the identification of the optimal cell for tissue regeneration represent in fact a key issue in cell therapy; these findings could have potentially impact on the development of new reprogramming strategies and great consequences for regenerative medicine.

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# Aknowledgments

*Ed eccomi di nuovo qui, alla fine di un'altra tesi…di un altro percorso…di un'altra avventura!!! Sono passati più di tre anni dall'esame di ammissione alla Scuola di Dottorato…e in questo periodo si sono alternati tanti alti e bassi…momenti di entusiasmo e altri giorni neri in cui mi domandavo: "Ma chi me lo fa fare?"…ma alla fine sono felice di aver resistito per essere arrivata a tagliare anche questo traguardo!!!*

*Ma ora veniamo a ringraziare tutte quelle persone che mi hanno accompagnato e sostenuto in questo percorso…*

*Inanzitutto vorrei ringraziare Giulio Pompilio e Maurizio Capogrossi per avermi dato la possibilità di proseguire la mia esperienza al Monzino, iniziata nel lontano 2009 per la tesi specialistica.*

*Grazie mille a Gualtiero Colombo per il suo impareggiabile e insostituibile aiuto nell'analisi dei dati e nella stesura del paper…ma anche se non sarò più in lab tutti i giorni, non pensare di esserti liberato di me…continuerò ad essere il tuo incubo peggiore finchè non avremo finito di pubblicare i lavori che stiamo finendo!!!*

*Un ringraziamento va anche al mio capo "americano", Vincent Chen per la fantastica opportunità di lavorare nel tuo laboratorio al Sanford Burnham Medical Research Institute…non ti preoccupare Vincent, se mai ti capiterà di leggere questi ringraziamenti, te li tradurrò in inglese!*

*Grazie a Carlo Gaetano in qualità di Mentor, per la disponibilità che hai sempre mostrato nei miei confronti e perché è sempre un piacere ascoltarti parkare di "scienza".*

*Un ringraziamento particolare va ad Alessandra …la supervisor più mitica che chiunque possa desiderare…anzi, scusa ora devo chiamarti Boss!!! Grazie perché è a te che devo la passione e l'entusiasmo per il mondo della ricerca, ma soprattutto grazie per la fiducia che hai sempre dimostrato nei miei confronti e per la meravigliosa opportunità che mi stai regalando venendo a lavorare nel tuo nuovo gruppo. Girls power!!!*

*E parlando della nuova avventura che mi aspetta non posso non ringraziare la mia vecchia e nuova compagna di lavoro…la CRiiiiii!!! E tra pochissimo si riunirà il mitico duo S&S…vai Cri, inizia a contare i giorni!!!*

*Ma pensando a questo percorso che si sta chiudendo non posso non ricordare con nostalgia e con qualche lacrimuccia i miei fantastici e insostituibili amiciiii LPV e non più…in particolare Elena, Yuri, Valerio, Silvia, Ileana…ragazzi, mi mancherete un sacco!!!*

*Ele, cerca di non supplire troppo la mia mancanza con Alzy…se no la prossima volta che ci vediamo non ti ricorderai neanche più chi sono…meno male che verrà Vale nella mia vecchia postazione a tenerti compagnia!!! E soprattutto meno male che hai quella santa donna di Silvia che si ricorda di ricordarti dove metti le tue cose!!!*

*Yuri…sommo sapiente dei microRNA…unico e impareggiabile Controrelatore (anzi, per questo ne parleremo dopo la discussione!!!)…ma prima di tutto amico, sempre pronto ad ascoltare, dare consigli…ma soprattutto sincero, che dice sempre quello che pensa. E forse*

*è proprio per questo che andiamo così d'accordo noi due…un po' str….. quel tanto che basta…eheheheh!!!*

*Vale, invece come faremo adesso con le lezioni di slang romano? Dovremo organizzarci con delle Skype call!!! Anche se l'espressione topica insuperabile me l'hai già insegnata…ma qui forse non è il caso di ripeterla!!!*

*Invece che dire della mitica Ile sempre presente in lab…giorno e notte…weekend e feste…quando facevo notte o dovevo venire il sabato o la domenica, sapevo che almeno avrei trovato una persona amica con cui condividere questi momenti di disperazione, eheheheh!!! Anche se Giulio forse ringrazierà di non sentire più i nostri commenti modalità Scania!!!*

*Grazie alla mia mitica compagna di PhD, Francesca…e soprattutto scusa se in albergo a Dallas russavo…eheheheh!!!*

*E per concludere, ho lasciato i ringraziamenti più importanti…*

*Un "grazie" speciale va a Diego che mi supporta e mi sopporta tutti i giorni con la stessa pazienza e lo stesso amore…grazie per essere sempre al mio fianco!*

*Infine un ringraziamento speciale, il più importante, va ai miei genitori, Marina e Gianluigi, i miei "fan numero uno"…è grazie a voi se sono riuscita ad arrivare fino a questo traguardo…Voi, che mi avete sempre sostenuto e incoraggiato in tutte le mie scelte e decisioni…GRAZIE!!!*