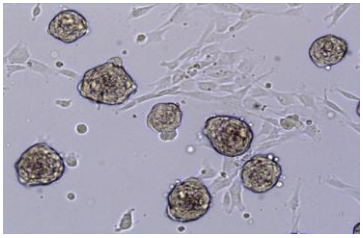
Università degli Studi di Roma "La Sapienza"

"Potential biological meaning and origin of cardiac progenitor cells isolated as Cardiospheres".



Tutore
Prof. Alessandro Giacomello

Docente guida
Prof. Alessandro Giacomello

Coordinatore
Prof. Marco Tripodi

Dottoranda
Elvira Forte

Dottorato di Ricerca in Scienze Pasteuriane
XXIII CICLO

Contents

ABSTRACT

Several populations of progenitor cells have been identified in the adult heart, based on the expression of specific surface markers (c-kit, Sca1, SSEA1, Isl-1), or on functional properties such as the ability to expel a vital dye (Side Population) or to spontaneously migrate from tissue explants and grow in three dimensional structures called cardiospheres (CSps), representing a niche-like microtissue with a cardiogenic gradient of differentiation. It is not clear which is the origin of these endogenous progenitor populations and how they function *in vivo*, given the limited regenerative capacity of the heart. Several possibilities can be envisioned. These cells could be remnants of the embryonic development, or derive from the de-differentiation of cardiomyocytes. A better understanding of their biological meaning is important not only for the extension of our knowledge in the field, but also to gain insight on the regenerative potential and differentiative fate of the cells for the clinical translation of cell therapy. To deeply investigate these issues, we approached the question from two different sides. Firstly to test the de-differentiation hypothesis, we used a double transgenic mouse expressing the recombinase MerCreMer under the α- Myosin-Heavy-Chain promoter, specific of differentiated cardiomyocytes, thus a pulse with 4-OH-tamoxifen (TAM) would activate Cre , allowing the expression of the reporter gene LacZ, in a spatial and temporal regulated manner. With this system, ideally all the differentiated cardiomyocytes should be irreversibly labeled after TAM treatment, even if they subsequently undergo de-differentiation. B-gal positive cells resulted very rare in culture, as shown by x-gal staining and real time PCR, suggesting that

cardiomyocytes de-differentiation is not the main mechanism underlying the presence of immature cells within the CSps, nor their formation, even though, given the limited sensibility of the method, (the efficiency of recombination in the cardiomyocytes is 80% at maximum) the occurrence of this phenomenon cannot be conclusively excluded. Then, we explored the potential of the epicardial-derived cells as contributors/founders of our *in vitro*-derived regenerative system that is the CSps. The epicardium, in fact, is crucial for a normal development of the heart, given its double role as source of multipotent mesenchymal progenitors and of instructive soluble signals that stimulate coronary vessel development, as well as cardiomyocyte proliferation and differentiation. Using another transgenic mouse strain, in which epicardial and epicardial-derived cells are labeled, we observed a large number of positive cells at all culture stages. Our hypothesis, supported by RT-PCR data, is that epicardial-derived cells undergo mesenchyma-to-epithelium (MET) transition during CSps formation, reactivating their embryonic developmental program. This is in line with a recent report presenting MET as a crucial mechanism in cell reprogramming. The information acquired in this work of thesis, although in need to be supported by future *in vitro* and *in vivo* experiments, represents a starting point for a better evaluation not only of the biological meaning of the heart-tissue-derived cardiac regenerative cells in the form of CSps, but also for the prevision of their *in vivo* fate once translated in clinic as therapeutic tools.

INTRODUCTION

The adult heart as a terminally differentiated organ

The mammalian adult heart has long been considered as a terminally differentiated organ, a view supported by clinical observations: primary myocardial tumors are rarely observed in adults and significant myocardial injury results in a permanent reduction in cardiac performance.

Cellular loss in adult myocardium (caused by ischemia, genetic mutations, viral infections, drug abuse, chemotherapy, etc.) does not result in an efficient regeneration, but in deposition of non contractile fibrotic tissue. The reparative response can be divided in three overlapping phases [1] (fig.1.1). During the inflammation phase, cells in and around the affected areas upregulate and secrete cytokines and chemokines such as tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1),interleukin (Il)-1β, Il-6 or Il-8, which trigger an immediate and massive infiltration of circulating leukocytes into the ischemic core [2]. Adjacent endothelial cells bolster the recruitment of pro-inflammatory immune cells by upregulating their expression of cell adhesion molecules such as E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). The initial wave of infiltrating cells is followed by monocytes that home to the damaged area and mature into macrophages. The immune cells gradually clear out cellular debris and matrix degradation products in the injury site, leaving behind sparse tissue with enlarged capillaries. As the acute inflammatory response is suppressed, the gap left behind is filled by granulation tissue. Mesenchymal cells infiltrate the infarct marking the transition to the proliferative phase[2]. Myofibroblasts deposit extracellular matrix while a rich capillary network is formed.

During the maturation phase the cellularity of the granulation tissue (mainly composed of blood vessels, macrophages and myofibroblasts) decreases and the matrix is cross-linked forming a dense collagen-based scar. The ischemic area is rich in inflammatory cytokines and protease activity, which harms surrounding healthy cells [3]. The extra-mechanical burden on the remaining ventricular cells further compromises the integrity of the cardiac tissue. Thus, the initial localized injury creates a ripple effect that spreads slowly to larger areas of the heart. The loss of functional tissue and subsequent remodeling eventually causes ventricular dysfunction and electrical instability, leading to heart failure and malignant arrhythmias [4]. Current treatments provide some survival benefits and amelioration of symptoms, but they do not recover function of the damaged tissue. The only routinely used therapeutic approach to replace the injured tissue is heart transplantation, which is a last chance treatment given the limited donors' pool.

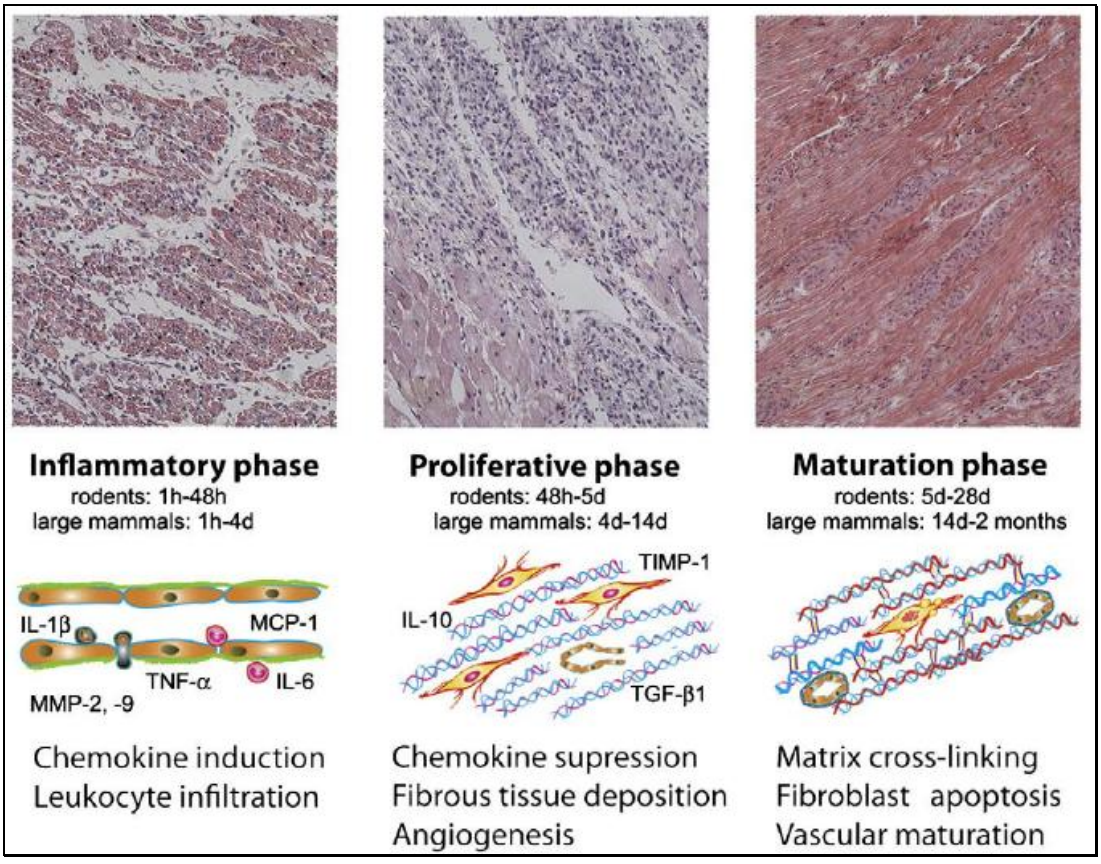


Figure 1.1. Phases of infarct healing [1]

Evidences of cardiac renewal in post natal hearts

In the past 10 years, evidences have shown that the mammalian heart is endowed with a limited regenerative potential and turnover capacity, starting from the observation of cycling myocytes in the adult heart, both in physiological and pathological conditions [5, 6]. In an elegant genetic fate mapping experiment [7], using transgenic mice in which differentiated cardiomyocytes would permanently express the GFP reporter, it has been shown that, in response to an injury, the cardiomyocyte pool (GFP+ cells) in the adult heart is significantly replenished by cells that were undifferentiated (GFP-) at the time of the injury, while the percentage of GFP-labeled cardiomyocytes is nearly unchanged during physiological ageing.

Another recent study [8], analyzing cell cycle markers on FACS sorted nuclei from cTnT positive cells, support the evidence of cardiomyocytes cell cycle withdrawal soon after birth in mice. By day 14 proliferating cardiomyocytes could not be detected and almost 98% of them were bi-nucleated.

However the regenerative capacity seems to differ among different species, in fact in humans the adult myocardium displays proliferating cardiomyocytes and less bi-nucleation (30-65%) compared to the adult mouse, suggesting a prolonged coupling of karyokinesis with cytokinesis, which leads to cell renewal.

Indeed, through the use of radiocarbon dating of human postmortem cardiac tissue [9], it has been confirmed that cardiomyocytes undergo turnover in the adult human heart with an annual rate of about 1% in young adults, declining below 0.45% by the age of 75. It is not clear yet, which is the mechanism involved. Therefore cardiomyocyte turnover may occur through multiple not exclusive mechanisms, including cell-cycle re-entry and dedifferentiation of cardiomyocytes,

differentiation of endogenous cardiac progenitor cells (CPCs), or circulating progenitors recruited from the bone marrow (BM) [10].

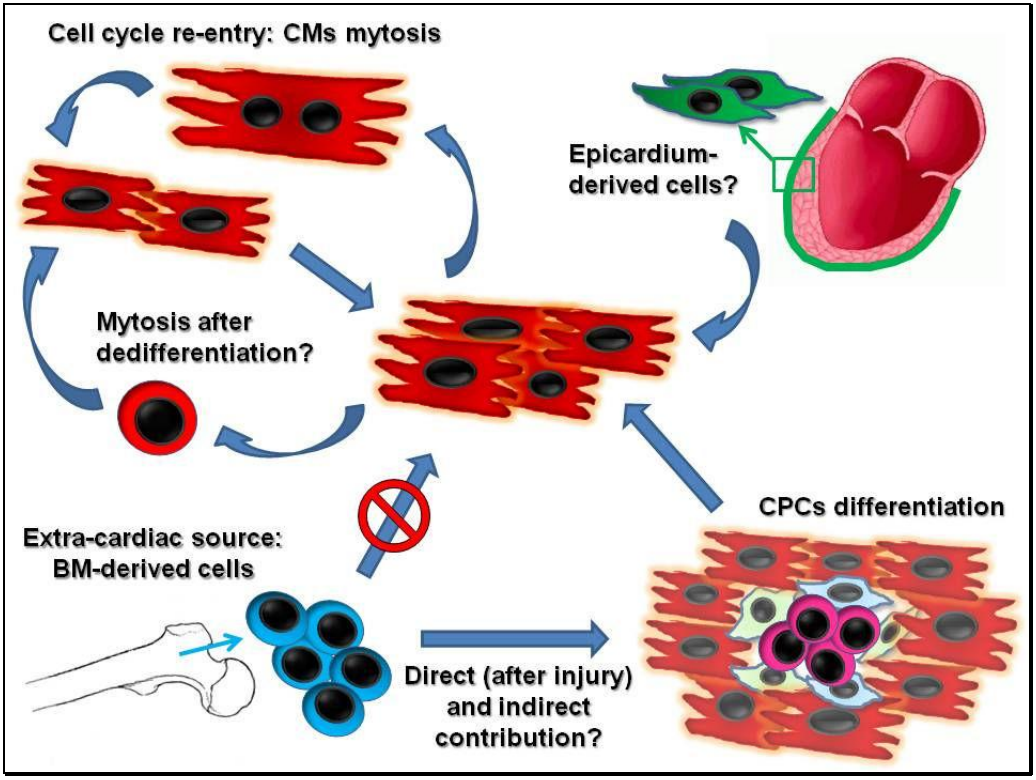


Figure 1.2. Possible cell sources and mechanisms for cardiomyocytes turnover and regeneration in physiological and pathological conditions [10]

Potential sources of cardiac regeneration

Cardiomyocytes de-differentiation

Some vertebrates, like urodeles and zebrafish, are able to regenerate significant portions of the heart and similar mechanisms are involved in blastema's formation. After amputation a blood clot seals the injury site, then the clot matures into fibrin and fibroblasts and inflammatory cells are attracted to the site. Starting from the edges of the wound new cardiomyocytes are formed, regenerating the myocardial tissue [11, 12]. In newt, it has been demonstrated that cardiomyocytes are the main responsibles for myocardial regeneration. In fact a subset of cardiomyocytes [13] can partially dedifferentiate into a progenitor-like state, expand and then re-differentiate in cardiac cells.

Two recent reports [14, 15], based on lineage tracing systems, have shown that even in zebrafish the regeneration mostly relies on a subset of pre-existing differentiated cardiomyocytes, rather than on progenitor cells as previously suggested [16]. Cardiac damage induces a widespread activation of the epicardium which produces important factors for neovascularization and reactivation of a subset of subepicardial GATA4-positive cardiomyocytes within the ventricular wall. As in newt, proliferating cardiomyocytes undergo limited dedifferentiation: they start to detach from one another and to disassemble their sarcomeric structure with a concomitant down-regulation of sarcomeric genes and reactivation of cell cycle regulatory genes, such as plk1 and mps1.

In mammalian hearts during fetal development, cardiomyocytes are able to go through mitosis by continuous disassembling and reassembling of their contractile apparatus [17, 18]. However cell cycle withdrawal is gradually achieved while

reaching the end of gestation and this corresponds to the maturation of the cardiovascular system, achieving higher blood pressure levels. After birth, the increase in heart mass (about 30-50 fold from birth to adulthood [19]) is due to hypertrophy rather than cell division [20]. In the first ten years of human life cardiomyocytes are reported to undergo karyokinesis in the absence of cytokinesis resulting in bi-nucleation and polyploidy [8].

It is interesting, however, that even in humans, after an injury, hibernating cardiomyocytes show similar structural changes: depletion of sarcomeric structure and expression of structural proteins closely resembling that of fetal heart cells, as if cardiomyocytes could initiate the first step toward proliferation, but subsequently face a block in the cell cycle. A recent study [21], showed that muscle cells from the adult mammalian heart are able to de-differentiate *in vitro* and acquire antigenic and morphologic features of CPCs. It is not clear however how frequently this phenomenon occurs *in vivo* and which functional properties these cardiomyocyte-derived cells have.

Several groups have reported that a reactivation of the cell cycle is feasible by either removing cell cycle inhibitors (e.g., p27) or by over-expressing cell cycle activators [22], such as cyclinD1, cdk1, members of the E2F family, CDC5 and p38. Growth factors, such as neuregulin1 [23] and periostin [24], promote proliferation of fully differentiated cardiomyocytes even if their action seems to be limited to mononucleated cells (10-30% of total cardiomyocytes) and only a small percentage of these cells can actually complete karyochinesis.

Controlled layer or lineage transition (CLT)

Controlled layer or lineage transitions (CLT), such as the epithelial-to-mesenchymal transition (EMT), are important biological processes through which polarized epithelial cells assume a mesenchymal phenotype, with enhanced migratory capacity, invasiveness, elevated resistance to apoptosis and a greatly increased production of extracellular matrix components [25]. Upon reaching their destination, migrating mesenchymal cells usually undergo reverse EMT [26]. The development of the mouse heart involves four distinct CLT episodes [27], each generating a distinct set of cardiovascular progenitor cells that differentiate into the cellular components of the mature heart. The first EMT that gives rise to cardiovascular progenitor cells takes place during gastrulation, when epithelial cells of the epiblast begin to delaminate, adopt mesenchymal characteristics and migrate to form the mesoderm layer that occupies the space between the two outer epithelia of endoderm and ectoderm [28]. The timing and location of epithelial cell delamination within the primitive streak determines the destination of the migrating mesodermal/mesenchymal cells in the developing embryo and this specifies their subsequent fate [29]. Mesodermal cells in the lateral plate adjacent to the foregut differentiate into progenitor cells that express early cardiac regulatory genes, such as Nkx2.5, Mef2c and Gata4 [30, 31]. Early cardioblasts are divided into two types representing the primary and secondary cardiac fields [32]. The cardiac stem cells of the primary field form the original heart tube that surrounds the endocardial vascular layer, contributing mainly to the future left ventricle; the secondary field cells then migrate and wrap around the primitive heart and contribute to the formation of the atria, the right ventricle and part or most, of the left ventricle [32, 33].

The second mesenchymal transformation that shapes the heart takes place when a subpopulation of endocardial cells (the endothelial cells of the original inner cardiac tube) in the atrioventricular canal area undergo endothelial-to-mesenchymal transition (EndMT), migrate into the adjacent cardiac jelly and build the endocardial cushions that develop into the heart valves [34, 35]. There is evidence that EndMT continues in the adult valves, supplying cells to maintain and repair the valvular leaflets [36].

The third EMT occurs in the epithelium formed by the epicardial cells at the outer surface of the heart. Pre-epicardial tissue, which appears as a cauliflower structure from splachnopleuric mesoderm during development, attaches to the exterior surface of the heart and spreads out over the entire organ in a single epithelial cell layer called the epicardium [37]. Shortly thereafter, the epicardial epithelium undergoes EMT, generating a mesenchymal stem cell population named epicardial-derived progenitor cells or EPDCs. EPDCs invade the cardiac tissue and differentiate into interstitial fibroblasts, perivascular fibroblasts and smooth muscle cells (SMCs) of the developing coronary blood vessels [11, 38]. Cell-lineage tracing experiments using Cre-recombinase technology show the potential contribution of epicardium to a small fraction of ventricular myocytes, suggesting a more substantial role of EPDCs in heart tissue formation than was thought previously [39, 40]. Transplantation of embryonic EPDCs improved cardiac function after myocardial infarction (MI) in mice, but the cells did not differentiate into cardiovascular cells [41].

The fourth EMT takes place in the neural tube and generates the cardiac neural crest (NC) progenitor cells, between the cranial and trunk neural crest, that migrate to the heart and take part in the remodeling of the aortic arch arteries and the

septation of the common outflow tract into the aortic and pulmonary arteries [42, 43]. Lineage-tracing experiments, using transgenic mice with gene markers that are under the control of NC-specific promoter elements or Cre-recombinase technology, suggest that NC cells also contribute to the conduction system and epicardium, but this remains controversial [44-47]. Recent reports show that nestin^{+} stem cells of NC origin reside in the adult heart and take part in *de novo* blood vessel formation and reparative fibrosis after ischemic injury [48].

While the significance of EMT and EndMT in organogenesis is well supported, little is known about the contributions of these processes to repair and regeneration in adults [25]. Terminally differentiated epithelia have recently been shown to modify their phenotype in response to repair-associated or pathological stress, [49] and EMT seems to yield adult cells with stem cell characteristics [50, 51].Therefore, one could predict that, in line with their role during heart development, EMT and EndMT contribute to the pool of cardiovascular progenitor cells to maintain cardiac homeostasis.

Besides the connection of mesenchymal transition to the emergence of stem cells, both EMT and EndMT are recognized as important mechanisms in the generation of the myofibroblasts that take part in fibrosis [52, 53]. Therefore, it is possible that EMT and EndMT contribute to both cardiac regeneration and fibrosis after injury. In the last few years a number of studies have provided supportive evidences for this notion. For example, EndMT in the adult mouse heart gives rise to myofibroblasts that migrate and produce scar tissue in mouse models of pressure overload and chronic allograft rejection, recapitulating pathways that take place during formation of the atrioventricular cushions in the embryonic heart [54]. Moreover, EMT is induced after injury in activated epicardial cells expressing stem cell

markers, which migrate to the infarct and contribute to both cardiac and vascular cell types [55]. These experiments suggest a function of epicardial cells that echoes their role in the regeneration of the adult zebrafish heart after partial resection [16]. Therefore, both endothelial and epicardial cells become activated after injury in response to specific signals (such as EGF, FGF-2, TGF-β and PDGF) giving rise to cardiac, vascular and myofibroblast/SMCs, similarly to their capacity during development.

Recruitment of exogenous progenitors or stem cells

Almost ten years ago, different groups reported the presence of stem cell-like cells in the adult myocardium that bear sex chromosomes or genetic markers of recipient origin in the case of heart transplants (sex-mismatched transplants)[56], or of the donor in the case of BM transplants [57]. These extra-cardiac CPCs may arise from sources that continuously feed the heart with undifferentiated cells and acquire their tissue-specific properties once in the cardiac niche. The extent of this phenomenon is debated. Quaini et al. report that within 4-28 days after allotransplantation, up to 30% of transplanted myocardium is regenerated by stem cells originating from the recipient [56], which were able to differentiate in new myocytes, as well as endothelial and SMCs [58]. On the other hand, reported negative results include the detection of infiltrating host cells comprising up to 5.6% of the vascular SMCs, but none of the over 6000 cardiomyocytes surveyed in the donor heart [59] and low levels (0.02% to 1%) of cardiomyocyte chimerism within allografted human hearts [60]. Further complicating the picture, the validity of stem-cell plasticity as a mechanism for generating non-lymphohaematopoietic tissue has also been questioned [61] and some authors have suggested that the

apparent cardiac chimerism might be explained by previously overlooked cell fusion events [62].

It is conceivable that circulating mononuclear cells responsible for generating solid-organ–specific cells are actually a group of non-lymphohaematopoietic organ-specific stem cells which move between their own solid tissue and the peripheral blood [63]. Numerous reports show that in the heart, at least for endothelium [64] and myofibroblasts [53], this can in fact be the case.

Endogenous reservoirs of progenitor or stem cells in the heart

Surprisingly several populations of autologous cardiac stem cells have been identified in the past decade. It is not clear if these populations are related and which is their origin. It is possible to speculate that they may be remnants of embryonic precursors, or they can derive from extra-cardiac sources as the BM. Experiments of conditional lineage tracing are undoubtedly required to understand the origin and identity of these CPCs populations and their behavior in physiological and pathological conditions[65]. In this way it would be possible to assess if these cells are actually recruited to the site of injury, if they can undergo proliferation and convert into cardiomyocytes.

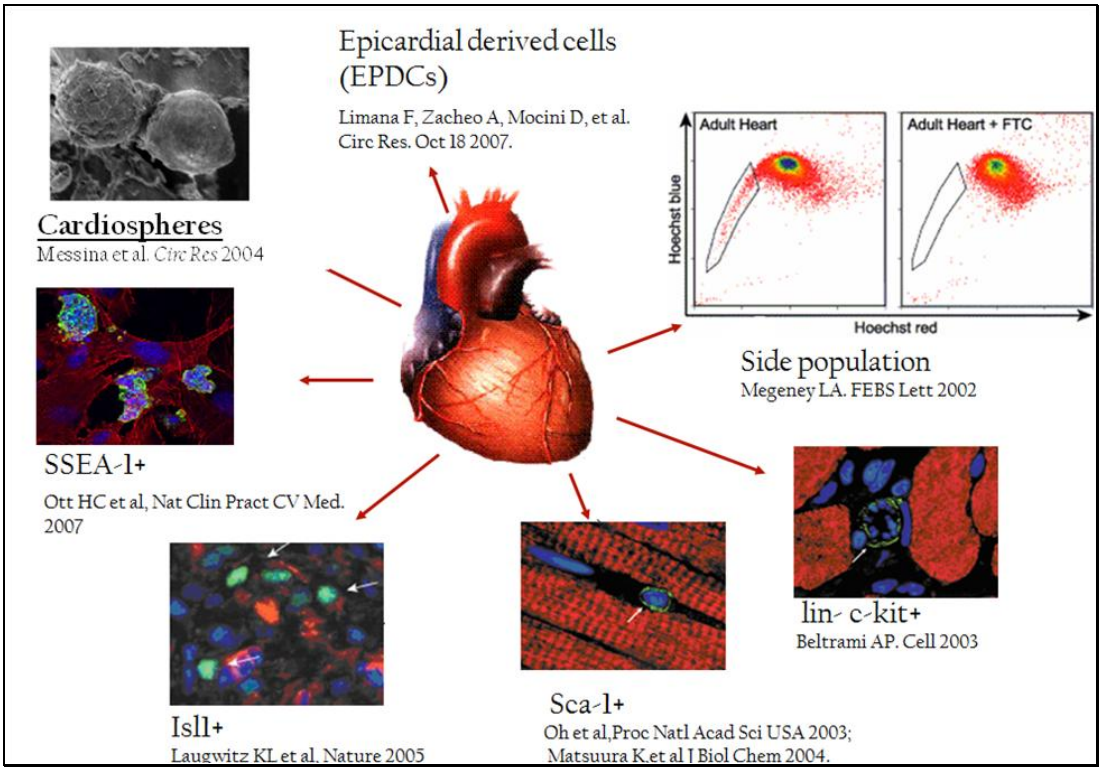


Figure 1.3. Different populations of resident cardiac stem and progenitor cells isolated since 2002.

So far autologous CPCs have been isolated with different protocols, based either on functional properties or surface markers expression:

- ✓ *Expression of cell-surface stemness markers (c-kit or Sca-1).*

Based on the expression of the stem cell factor receptor, Anversa et al [66] identified a small population of c-Kit^{+}/lineage negative cells, located in small

clusters within atria and ventricles. These cells were described as clonogenic, multipotent, capable of self renewal and they were isolated from different species, including human hearts. Although the phenotype of c-kit$^+$ differentiating cells remains immature *in vitro*, they appeared to regenerate cardiac tissue when injected in rat hearts after MI, giving rise to cardiomyocytes, smooth muscle and endothelial cells, with a significant functional improvement after 20 days. The initial enthusiasm aroused by these studies was partially reduced by the identification of c-kit$^+$ cells in tissues of other solid organs. These cells supposedly leave the BM in small numbers to scavenge pathogenic molecules as part of the mechanisms to activate local innate immune responses [67]. Furthermore, by means of *in situ* detection, c-kit$^+$ cells from bioptic samples of human heart have been recently reported to co-express markers of mast cells and to lack cardiac markers, such as Nkx2.5 and Isl1 [68]. A possible change in cellular phenotype during the culture time course needs to be taken under careful consideration to explain these conflicting results [69]. Using a transgenic mice expressing eGFP under the c-kit promoter, Tallini et al. [70] observed c-kit cells at different stages of differentiation in the embryonic heart, peaking at post-natal (PN) day 2; thereafter EGFP$^+$ cells declined and where rarely detected in adult hearts. C-kit-eGFP$^+$ cells isolated from PN 0-5 were able to differentiate in endothelial cells, SMCs and beating myocytes. Cryoablation in the adult heart resulted in increased expression of c-kit–eGFP, peaking after 7 days. C-kit expression occurred in endothelial and SMCs in the re-vascularizing infarct area and in terminally differentiated cardiomyocytes in the border zone surrounding the infarct. Thus they suggested that *in vivo* c-kit expression is associated with neo-vascularization, but not with *de novo* myogenesis. They conclude that the increased c-kit expression in differentiated cardiomyocytes,

in the injury border zone, could rather be the result of c-kit re-expression in committed myocytes[71].

One year later Schneider and al. [72] reported the presence of a population of cells expressing the Stem Cell Antigen (Sca-1) in the non-myocyte fraction of the heart. These cells co-localize with vasculature and express CD31, but have a distinct phenotype from endothelial and hematopoietic stem cells: they are negative for CD45, CD34, flk1, ckit, VE-cadherin, vWF and express early cardiac markers, such as GATA4 and MEF2c, suggesting a possible commitment towards cardiac and endothelial lineages. The exposure to 5-azacytidine induces expression of Nkx2.5 and cardiac sarcomeric proteins. The capability to adopt a cardiac muscle fate in embryogenesis was substantiated by blastocyst injection. When injected intravenously in infarcted mice they were able to home and differentiate in the peri-infarct area, despite a significant percentage of fusion with resident myocytes (50%).

Matsuura et al [73] isolated similar Sca1+ cells from hearts of 12-week old mice. When treated with oxytocin, these cells expressed genes of cardiac transcription factors and contractile proteins and showed sarcomeric structure and spontaneous beating. They may be able to differentiate in different cell types when exposed to different environments; however Sca1+ cells multipotency has not been proven on the progeny of single cells. Recently [74] they demonstrated that the transplantation of sheets of clonally expanded Sca1+ cells ameliorates cardiac function after MI in mice. Clonal Sca1+ cells efficiently differentiated into cardiomyocytes and secreted cytokines, including soluble VCAM-1 (sVCAM-1), which induced migration of endothelial cells and CPCs and prevented

cardiomyocyte death from oxidative stress through activation of Akt, ERK and p38 MAPK.

The group of Doevendans [75] identified cardiomyocyte progenitor cells (CMPCs) in fetal and adult human hearts using an antibody against mouse Sca1. Human CMPCs are localized within the atria, atrioventricular region and epicardial layer and they can be induced to differentiate *in vitro* into cardiomyocytes and form spontaneously beating aggregates, after stimulation with 5-azacytidine. Recently [76] this group investigated the effect of intra-myocardial injection of human CMPCs or pre-differentiated CMPCs-derived cardiomyocytes (CM-CMPCs) in immunodeficient infarcted mice. The results were higher ejection fraction and reduced left ventricular remodeling up to 3 months after MI, when compared to controls. Both CMPCs and CM-CMPCs were able to generate new cardiac tissue consisting of human cardiomyocytes and blood vessels, excluding the need for *in vitro* pre-differentiation.

- ✓ *Expression of the islet-1 gene (isl1+ cells).*

Laugwitz et al. [77] reported that early after birth the mammalian heart harbors a rare subset of cells positive for the LIM homeodomain transcription factor islet-1 (isl1), that disappears soon after the neonatal period. These cells are mostly found in the outflow tract, atria and right ventricle, suggesting that they could be remnants of the embryonic secondary heart field. Postnatal isl1+ murine cells can be expanded *in vitro* on mesenchymal feeder layers and they undergo terminal differentiation when co-cultured with neonatal cardiac cardiomyocytes, acquiring electromechanical properties similar to those of adult cardiomyocytes, thereby fulfilling the criteria for endogenous cardioblasts. Moreover their *bona fide* identity

as true CPCs comes from extensive and detailed genetic fate mapping studies *in vitro* and *in vivo*, providing strong evidences of their cardiac-specific multipotency [78, 79]. However these cells were isolated only from very young animals, including human neonatal specimens and the number of Isl1+ cells dramatically decreases over the first weeks of life. Furthermore it remains to be addressed if these cells are multipotent and able to engraft and regenerate the myocardium.

- ✓ *Expression of the stage-specific embryonic antigen (SSEA-1+ cells).*

Recently uncommitted cardiac precursor cells (UPCs) have been identified in the heart of adult rats through a typical embryonic antigen, SSEA-1, that is expressed early in heart development[80]. SSEA-1+ cells isolated from adult rats differ from neonatal cells because they do not express cardiac specific transcription factors (Nkx2.5, GATA4). This suggests that only uncommitted stem cells persist in the adult heart. Adult UPCs in co-culture with cardiac-derived mesenchymal cells can differentiate in mature cardiomyocytes, endothelial cells and SMCs through multiple stages, in which the cells co-express markers such as Oct4, Flk-1 or Sca-1 together with SSEA-1. Then, once committed, they finally express cardiac transcription factors such as Nkx2.5, GATA4 and Isl-1. Beating colonies are obtained by culturing UPCs in differentiating media or in co-culture with neonatal cardiomyocytes. UPCs improved ventricular function when injected in infarcted hearts and SSEA-1+ cells are capable of forming new cardiomyocytes and endothelial cells in the infarct area [80].

- ✓ *Ability to efflux Hoescht dye (Side Population).*

As other solid organs, the heart presents a Side Population (SP) of cells characterized by the ability to efflux vital dyes, due to the expression of ATP-binding cassette transporters, such as ABCG2 and MDR1. Abcg2-positive cells were identified during embryogenesis and they persist as a small pool in multiple organs [39]. Cardiac SP cells express stem cell markers, such as Sca1 and cKit and are $CD34^+$, $CD31^-$, $CD45^-$. They are capable of proliferation and differentiation towards mesodermal derivatives: isolated SP cells can be induced to express cardiac specific genes *in vitro* when treated with agents such as oxitocyn or tricostatin A and they are able to proliferate and form hematopoietic colonies when plated into metylcellulose media. The cardiogenic potential is higher in cells expressing the Sca1+ and negative for the endothelial marker CD31. However the actual regenerative contribution of these cells *in vivo* is not clear [81].

- ✓ *Cells deriving from the epicardium*

Limana et al. [55] first demonstrated the presence of CPCs outside of the previously described "niche", in the adult epicardium of both mouse and human. Authors identified two different populations of c-kit^+ and $CD34^+$ cells, which can express early and late cardiac transcription factors (Nkx2.5, GATA4) and acquire an endothelial phenotype *in vitro*. Following MI, there is an increase in the absolute number and proliferation of epicardial c-kit^+, which undergo EMT [82] and migrate in the subepicardium [82]. EMT represents the first step for the acquisition of mesenchymal phenotype and consequent migration from the subepicardial space to the myocardial wall [82] where they generate myocardial precursors and vascular cells. They also demonstrated that in presence of an intact pericardium, these c-kit+ cells reactivate the expression of three embryonic epicardial related

genes Tbx18, WT1, Raldh2, probably due to the release of important trophic factors in the pericardial cavity [83].

EPDCs can also be isolated from human adult atrial tissue. In culture they spontaneously undergo EMT and differentiate in SMCs thus retaining characteristics similar to their embryonic counterpart [84, 85]. Further these cells have the potential to positively modify the phenotype of co-cultured adult cardiomyocytes increasing their contractility [86]. Transplantation of human adult EPDCs in the infarcted mouse heart preserves LV function and attenuates ventricular remodeling at 6 weeks after injury, even if only few cells engrafted in the host tissue, mainly acquiring a smooth muscle phenotype [41]. Noteworthy co-transplantation of adult EPDCs with CPCs does not result in differentiation of these latter in functional cardiomyocytes. The synergistic improvement of cardiac function can be explained by the activation of distinct paracrine pathways by the two different populations [87]. Thus the beneficial effect of these cells relies mostly on an indirect paracrine protection of the surviving tissue. Therefore transplanted EPDCs recapitulate only in part their role during embryonic development, i.e. the modulatory effect, but not their physical contribution [87].

Cardiospheres and CSps-derived cells

In our lab it has been demonstrated for the first time that CPCs can be isolated directly from cardiac tissue through selection of spontaneous spherical clusters, termed CSps [88]. After few weeks of culture, a heterogeneous population of cells shed spontaneously from tissue fragments (fig2). Explant-derived-cells (EDCs) are collected by mild enzymatic digestion, plated on poly-D-lysine in presence of

growth factors and low serum and they generate 3D-clusters called Cardiospheres (CSps).

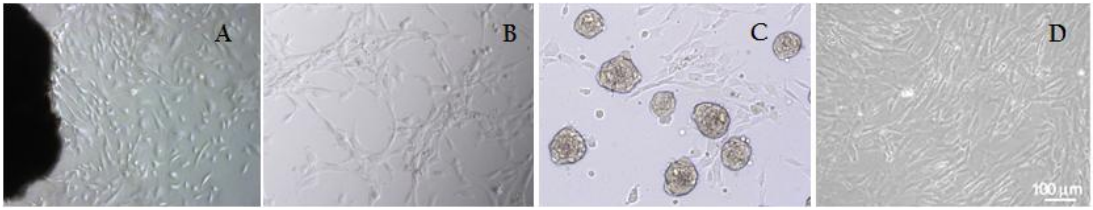


Figure 1.4. Stages of Cardiospheres culture. A- Cells migrating out of human explants; B- EDCs after 4 days of culture; C-floating CSps; D- CSps-derived cells (CDCs)

CSps are characterized by a core of undifferentiated c-kit^{+} cells and differentiating cells towards the periphery, expressing markers of mesenchymal, endothelial, smooth muscle and cardiac cells, such as CD105, myosin heavy chain (MHC), troponin I (TnI), connexin-43 (Cx43), smooth muscle actin (SMA) and Von WIllebrand factor (vWF) (fig.1.6) [88, 89]. Recent experiments on explants obtained after retrograde perfusion in the absence of hematological cells (CD45^{-}, lin^{-}) disprove the notion that blood-derived elements figure prominently in the generation of CPCs from cardiac biopsies; furthermore c-kit+ cells appear to be negative for the mast cell marker AA4 [89] (fig.1.5). However in defined pathological conditions [90], as in mice depleted of the endogenous stem cell reservoir by irradiation and transplanted with BM before inducing MI, it has been shown that BM-derived cells can contribute to replenish the cardiac stem cell niches and participate to CSps formation.

In order to increase cellular yield, CSps can be expanded in monolayer on a fibronectin-coating as CSp-derived cells (CDCs), which retain the capability to form secondary CSps when re-plated in the previous conditions [91]. The vast majority of this heterogeneous population is CD105+ and significant subpopulations are $CD90^+$, $cKit^+$, $CD34^+$ and $CD31^+$. CDCs are also $MDR1^-$, $CD133^-$ and $CD45^-$ and negative for a wide cocktail of blood lineage markers, resembling overall a mixture of CPCs and mesenchymal supporting cells (fig. 1.7).

Both CSps and CDCs have been proved to significantly improve cardiac function in treated animals [91]. In a porcine pre-clinical model of post-infarct left ventricular dysfunction intracoronary delivery of CDCs has been shown to result in formation of new cardiac tissue, to reduce relative infarct size, to attenuate adverse remodeling and to improve hemodynamics [92]. The evidence of efficacy of this study, without obvious safety concerns at 8 weeks of follow-up, offered the fundamental background for human studies in patients after MI and in chronic ischemic cardiomyopathy. At present CDCs are under evaluation in the ongoing CADUCEUS clinical trial for autologous cell therapy (see www.clinicaltrials.gov for details).

However CSps seem to have an enhanced functional potency for myocardial regeneration, compared to CDCs grown in monolayers, probably because the 3D structure closely resembles the tissue “niche”, in terms of interaction among different cell types and the matrix. Recently a study has been conducted comparing CSps, CDCs and cells deriving from CSps dissociation [93] (fig. 1.8-1.10). When implanted in the infarct border zone in a mouse model of acute MI, CSps exerted greater benefits compared to monolayer-cultured CDCs or dissociated CSps. All cell-treated mice exhibited a reduced LV remodeling, but those treated with CSps

showed a net increase in LVEF which corresponded to thicker LV walls and smaller infarcts (fig.1.9). Analysis by PCR array, qPCR and immunostaining showed that culturing cells as CSps augments the expression of stemness markers as c-kit, SOX2, Nanog and stem cell-relevant factors as IGF-1 and Tert, while proliferation is inhibited compared to CDCs (fig 1.8). This may be related to the recapitulation of a stem cell niche microenvironment and HDAC-mediated epigenetic modifications. Many extracellular matrix (ECM) components and adhesion molecules, including lamin-β, integrin α2, E-selectin were also up-regulated and CSps resulted much more resistant to oxidative stress than monolayer-cultured cells (fig 1.8-1.9). These features are expected to favor the engraftment of stem cells after implantation in the damaged heart, explaining at least in part the greater functional benefit deriving from CSps. Therefore while CDCs can be easily expanded to significant number for clinical application, the 3D structure provides a protective microenviroment, improving cell engraftment and regeneration.

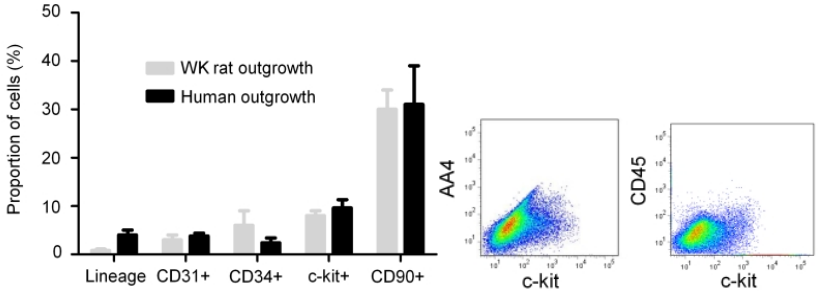


Figure.1.5 Flow cytometry analysis of the first outgrowth collection from human and rat cardiac tissue [89].

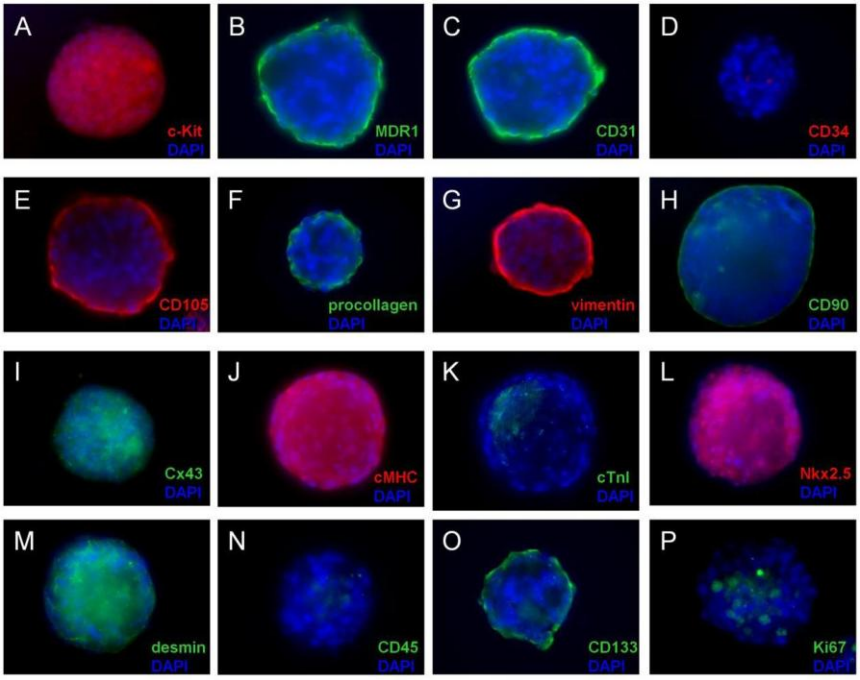


Figure 1.6 Confocal images of immunofluorescence characterization of human CSps [89].

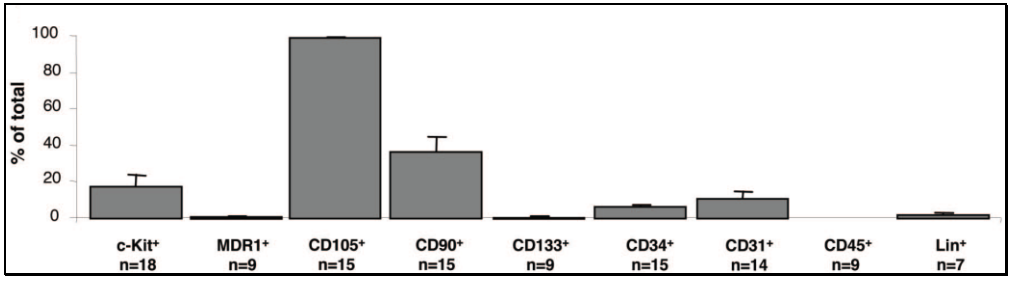


Figure 1.7 CDCs phenotypic characterization by FACS. [91]

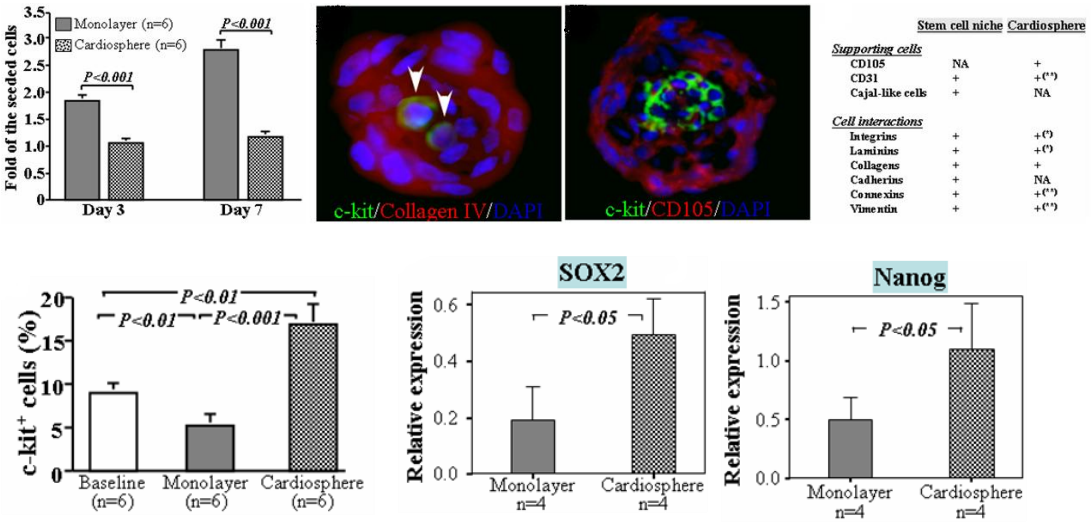


Figure 1.8. Growth, proliferation and recapitulation of stem cell niche-like microenvironment of cells under CSp culture condition [93]

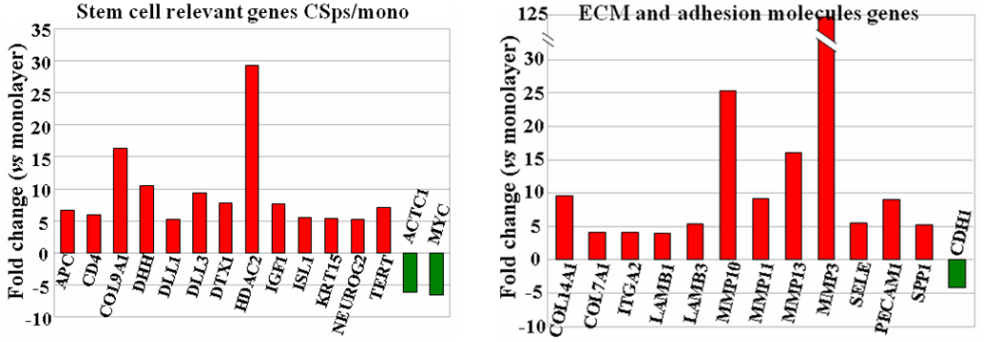


Figure 1.9 PCR array analysis of the gene expression in CSps versus cells grown in mononalyer: stem cells relevant genes; ECM relevant genes [93]

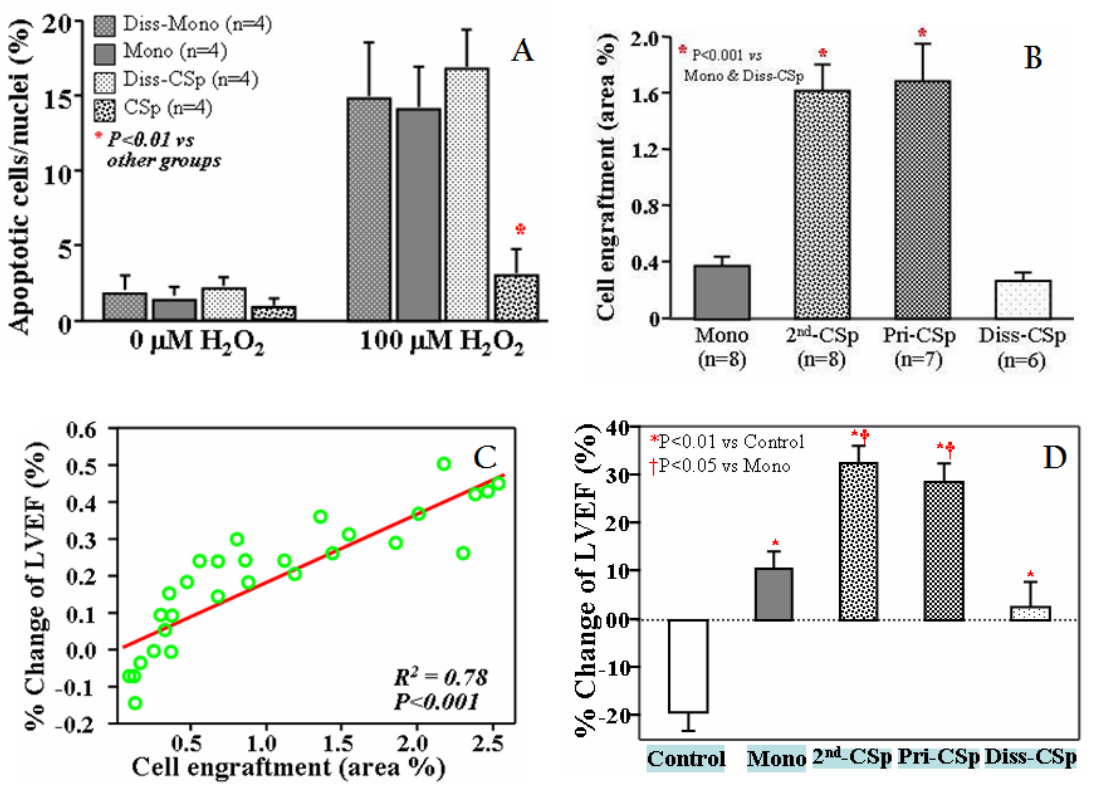


Figure 1.10 A: Higher resistance to apoptosis of CSps vs cells in monolayer; B-D: higher rate of cell engraftment of CSps compared to cells in monolayer results in a greater functional benefit *in vivo*.

Opposed from predicted single-marker selection criteria, the main advantage of this isolation method is the exploitation of the intrinsic functional property of the cells to spread out of the tissue and grow in three-dimensional structures, representing a more physiological culture condition compared to monolayers, where natural spatial cellular connections cannot be promoted.

The single-marker and the function-based selection approaches have been combined by several groups and used to obtain regenerating cells from primary cultures of human cardiac or muscle biopsies, subsequently sorted for specific antigens. The sca-1 positive cardiomyocyte progenitor cells (CMPCs) obtained by Doevendans et al. [76] are an example of this combined procedure, as well as CSp derived Lin^{-}/c-kit^{+} progenitor cells [94].

Interestingly, in our experience, [95] when CDCs were sorted for c-kit and injected in a mouse model of acute MI, the functional improvement was significantly lower than that of the whole population, suggesting that cooperative and synergistic effects among multiple cell types in the CDC pool play an important role on the overall beneficial outcome. Apart from the c-kit positive stem-like cells, other cell types expressing mesenchymal markers might also have a feeder-layer role, allowing the activation of multiple mechanisms for cardiac repair beyond myocyte formation (paracrine pro-survival effects, angiogenesis, mobilization of endogenous CPCs).

Scope of the dissertation

Despite several populations of progenitor cells being identified in adult heart, these cells are clearly not sufficient to provide a significant regeneration of the myocardial tissue. Transplantation in infarcted hearts of progenitor cells expanded *ex vivo* has shown limited and conflicting results. Beneficial effects observed so far are mainly due to the release of paracrine factors. Just a small percentage of cells are able to survive and to engraft long-term in the host tissue. Furthermore the hostile inflammatory and ischemic environment may promote the differentiation toward a fibrogenic/vasculogenic lineage more than a cardiogenic one.

In this regard, a better understanding of the origin of the employed progenitor cells population is required to control their differentiative fate *in vivo* and enhance their therapeutic effect.

The main aim of my work was to gain insight on the origin and biological meaning of CSp-forming cells. First, using transgenic mice we evaluated the eventual contribution of pre-existing differentiated or de-differentiating cardiomyocytes to CSps formation.

Secondly, after observing the significant role of CSp-forming cells in supporting proliferation and cardiogenesis in mouse embryonic stem cells (ESCs), we investigated the potential contribution of epicardial/epicardial-derived cells by means of lineage tracing and gene expression analysis.

Project I: Contribution of de-differentiating cardiomyocytes to Cardiospheres formation

Introduction

As mentioned above, cardiomyocyte de-differentiation and proliferation has been proven to be one of the main mechanisms accounting for the regenerative capacity of lower vertebrates such as zebrafish and newts [14, 15]. Several groups have reported that in particular conditions mammalian adult cardiomyocytes can be induced to re-enter the cell cycle and proliferate [22, 24]; furthermore a recent study [21] showed that muscle cells from the adult mammalian heart are able to de-differentiate *in vitro* and acquire antigenic and morphologic features of CPCs.

In order to assess the role of cardiomyocytes in cardiac explants culture, we used three different transgenic mice strains expressing the recombinase Cre under cardiac specific promoters (MLC2v, TnT, Myh6), with a reporter locus included between two LoxP sites (Rosa26 flox or RBPj flox).

Cre is a tyrosine recombinase from the bacteriophage P1 [96], which mediates recombinations of DNA molecules between two specific sites called LoxP (locus of X-over P1). LoxP sites consists of just 34bp. Cre specifically recognize and binds the Recombinase Binding Elements (RBE) , two 13bp sequences arranged as inverted repeats surrounding a central 8bp crossover region. The central 8bp are asymmetric with respect to sequence and define the directionality of the site [97].

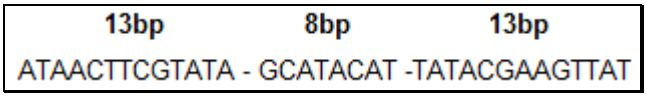


Figure 2.1. LoxP sequence.

Cre can recombine essentially any DNA substrate which contains LoxP sites, with no requirements for accessory proteins or specific substrate topology [98]. The simplicity of the Cre-loxP system has lead to its widespread use for both *in vitro* and *in vivo* genetic manipulation. This system, in fact, allows the creation of conditional mutant animal models where the inactivation of the target gene or the activation of the reporter locus is limited to a specific cell type or tissue. Conditional mutant mice are obtained by crossing a "floxed" strain, in which the locus of interest is modified by the insertion of two loxP sites, with a transgenic line expressing the recombinase Cre under a specific promoter or inserted into a particular locus in order to achieve a tissue-specific expression [24].

In our case the expression of Cre under cardiac specific promoters would lead to the removal of a stop codon in the Rosa26 flox locus, with selective expression of β-galactosidase in cardiomyocytes. The Rosa26 lacZ-loxP reporter strain contains a Cre-dependent, loxP-inactivated lacZ cDNA cassette targeted within the ubiquitously expressed Rosa26 locus [99, 100] (Figure 2A). Cre-mediated recombination of this allele deletes neomycin and a series of polyadenylation sequences, resulting in the juxtaposition of a splice acceptor site and the lacZ cDNA [101]. One of the strain we used presented also another "floxed" locus, RBPj flox [102] in heterozygosis, mainly to have a different reporter of recombination (detectable by PCR) than for its biological meaning.

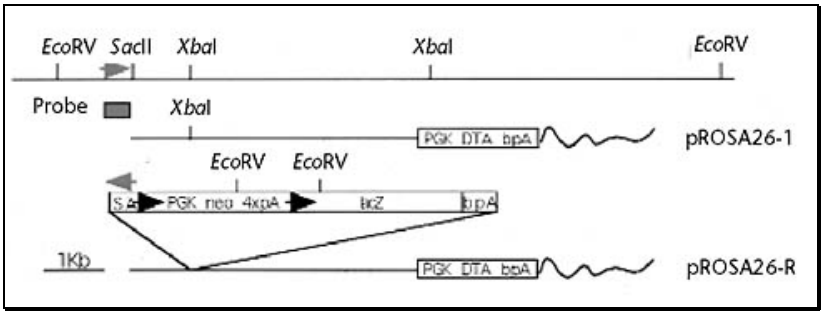


Figure 2.2 Targeting Rosa26 locus. [101]

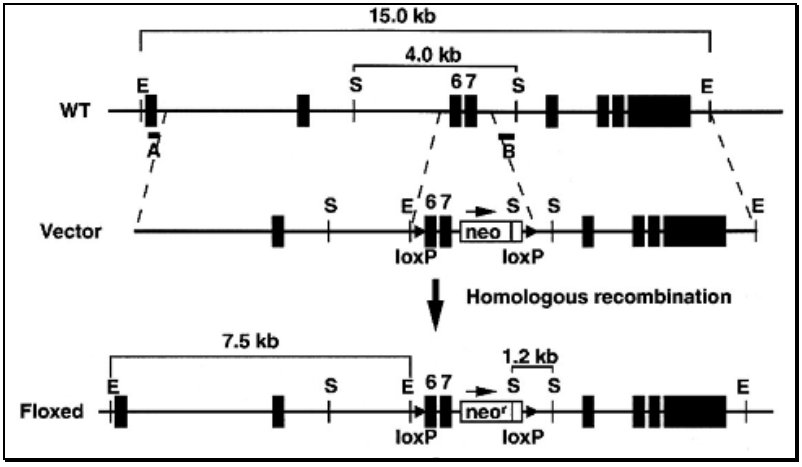


Figure 2.3 Targeting RBPj locus. [102].

Since it was our interest to be able to distinguish between pre-existing cardiomyocytes and *de novo* differentiating one, we took advantage of another

strain of transgenic mice Myh6-MerCreMer/Rosa26flox [103], in which the recombinase Cre fused with two Mer sequences, is subcloned downstream of α-MHC 5.5-kb cardiac-specific promoter.

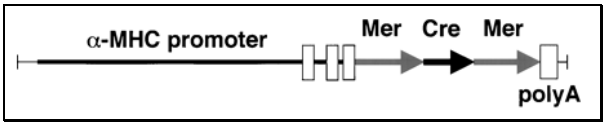


Figure 2.4 cDNA encoding the mutant estrogen receptor ligand-binding domain (Mer) flanking Cre recombinase (Cre) subcloned downstream of the αMHC 5.5-kb cardiac-specific promoter [103].

Mer is a mutant form of the estrogen receptor ligand-binding domain. The estrogen receptor is a hormone-activated transcription factor that is usually sequestered by heat-shock proteins in absence of the ligand. In presence of 17β-estradiol, the estrogen receptor is released, allowing participation in transcriptional regulatory complexes. Since its initial description, the ligand-binding domain of the estrogen receptor (amino acids 281 to 599) has been extensively used as a strategy for fusion-proteins sequestration within mammalian cells [103]. More recently, a mutation in the estrogen receptor at amino acid 525 (glycine to arginine) was described that rendered it insensitive to the endogenous 17β-estradiol, but sensitive to the estrogen antagonist tamoxifen [93]. The use of this mutant estrogen receptor (Mer) domain in fusion-protein strategies permits estrogen-insensitive but tamoxifen-inducible activity within mammalian cells. In particular the Cre recombinase fused to two Mer sequences is inactive and sequestered in the cytosol. After a pulse with Tamoxifen activated Cre can translocate into the nucleus and recombine target floxed sites, activating the expression of the reporter LacZ in

all the αMHC-expressing cells in a time and space specific manner. In this way, only the cells that were differentiated at the time of tamoxifen treatment will be irreversibly labeled by LacZ expression and we could distinguish in culture between three different conditions:

αMHC+ ßgal+	Pre-existing differentiated cells
αMHC+ ßgal-	de novo differentiation
αMHC- ßgal+	de-differentiation? transdifferentiation

Material and Methods

Mice

All experiments were conducted in accordance with the *Guide for the Use and Care of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee.

C57bl wild type and three strains of transgenic mice were used: TnT-Cre x Rosa26 flox/+; MLC2v-Cre x Rosa26 flox/flox(+) x RBPjk flox/+; Myh6-MerCreMer x Rosa (flox/+) x RBPj (flox/+). All mice were provided by Doc. Mark Mercola Burnham Institute for Medical Research San Diego (CA).

Genotyping

For the genotyping of adult mice we used genomic DNA extracted from the tail tip. For newborns, we stained the upper part of the heart in X-galactosidase solution in order to know the genotype before starting the tissue culture and we confirmed the results by PCR on the genomic DNA extracted from the same cardiac tissue or from toes. Genomic DNA extracted from other tissues (skin, brain, lung and skeletal muscles) was used as a control to detect aspecific recombination.

Genotyping primers		
Cre fw	GCC TGC ATT ACC GGT CGA TGC AAC GA	650bp
Cre rv	GTG GCA GAT GGC GCG GCA ACA CCA TT	
CreER fw	ATA CCG GAG ATC ATG CAA GC	300bp

CreER rv	AGG TGG ACC TGA TCA TGG AG	
Rosa 523	GGA GCG GGA GAA ATG GAT ATG	Tan 55°C wt 600bp Rosa flox 325bp
Rosa 26F2	AAAGTCGCTCTGAGTTGTTAT	
Rosa 1295	GCGAAGAGTTTGTCCTCAACC	
RBPj F	ATA ATT TGC CAA GCC AAA GC	wt 110bp flox 250bp
RBPj R	TAA CTG TCT GGG ACC GAA GG	

Primers to detect deletion		
RBPj7s	CCA AGC CAA AGC CCC TTT CT	Tan 64°C wt 110bp flox 250bp del 480bp
RBPj8as	CCG AAG GCG ATT GAA CAG TG	
vRBPjre2a	GCA GGC AAC AAT TGA GTG TG	
RosaLacz Fw	TGC CCC CTC TTC CCC TCG TG	350bp
RosaLacz Rv	ACG CCA GGG TTT TCC CAG TCA	

DNA extraction:

Genomic DNA from tissue has been isolated as previously described (http://web.mit.edu/jacks-lab/protocols/DNA_Isolation_tables.html). Briefly samples were incubated overnight at 56 °C in digestion buffer (10mM Tris pH 8.0, 100mM NaCl, 10mM EDTA pH 8.0, 0.5% SDS in distilled water), to which we added Proteinase K (Sigma-Aldrich) at a final concentration of 0.4mg/ml. Genomic DNA was then purified and precipitated through a series of centrifugation steps with 6M NaCl and Isopropanol and finally resuspended in sterile water. For cells and embryos we used the Quick" Embryo Genotyping Protocol from Pfaff laboratory http://pingu.salk.edu/~pfaff/protocols%20and%20solns/geno.html. In this case the lysis buffer was made of 50 mM KCl, 10 mM Tris pH 8.3, 2 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% NP-40, 0.45% Tween-20, 0.1 mg/ml Proteinase K. Samples were incubated one hour or overnight (depending on the amount of material) in 500ul of buffer, then they were boiled for 10 minutes and 1ul was used in PCR.

X-gal staining:

X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside Invitrogen), was resupended in N,N-Dimethylformamide to obtain a 50x stock. X-Gal stock was diluted to a final concentration of 1mg/ml in staining solution made of: $K_3Fe(CN)_6$ 20mM, $K_4Fe(CN)_6*3H_2O$ 20mM, $MgCl_2*3H_2O$ 2mM, 0.01% DOC (Na salt), 0.02% NP40 in PBS.

Staining of intact tissue (whole mounts). Hearts were fixed in 4% paraformaldehyde on ice for 20 minutes, rinsed twice in PBS, once in staining solution and left in X-Gal solution at 37°C for at least one hour.

Staining on cells and explants. For fixation we used 2% paraformaldehyde 0.2% gluteraldehyde for 5 minutes at 4°C.

As a positive control for X-gal staining human embryonic fibroblasts were transfected with an Adenovirus expressing LacZ.

Tamoxifen treatments:

On cells and explants: 4-OH Tamoxifen (Sigma) resuspended in ethanol was added to the culture. Three different concentrations were tested 500nM, 1uM and 2uM for 2 to 4 days. The LacZ expression was analyzed after 1, 2 or 14 days from the treatment. We used a beta-pancreatic cell line MerCreMer- Rosa flox, kindly provided by Doc. Fred Levine's lab, as a positive control.

In vivo administration: 50mg Tamoxifen free base (Sigma)[103] or 4OH-tamoxifen (Sigma) [7]were dissolved in 500ul EtOH 100%, then 9.5ml of Corn Oil (Sigma) were added and the emulsion was sonicated to have a homogeneous solution. 100ul (0.5mg tam) were injected daily intraperitoneally in adult mice (20mg/kgBW/day) for 15 days [7], instead of 5 days as previously described [103], because it was shown to be insufficient[104] for a significant recombination in adult hearts.

Other mice were treated with 80mg/kg BW of TAM administrated by oral gavage for 7 days. This treatment has been previously shown to be well tolerated and effective, even though causing a marked dilated cardiomyopathy, with a peak 3 days after the treatment, but completely reversible after 21 days [104].

The control group consisted of Myh6-MerCreMer x Rosa26flox/+ mice not treated with tamoxifen and Myh6-MerCreMer x Rosa26+/+.

Evaluation of the efficiency of recombination

The upper part of the hearts was fixed with a solution of 2% paraformaldehyde, 0.2% gluteraldheyde, 0.02% NP-40, 0.01% NaDC in PBS, for 3 to 4 hours at 4°C.

Afterwards the tissue was transferred in a 30% sucrose solution overnight, embedded in gelatin 7.5%-sucrose 15% on dry ice and we made 25µm cryosections. Those sections were fixed with PFA 4% for 15 minutes, covered in x-gal staining solution and left overnight at 37°C. After washing repeatedly in PBS, nuclei were counter-stained with "Nuclear fast Red" (Vector Laboratories), according to the manufacturer's instructions. After dehydration, slides were mounted with Permount (Fisher Scientific).

The percentage of recombinant lacZ-positive cells was quantified using the NIH Image Progessing and Analysis Software ImageJ.

The deletion of RBPj locus was evaluated by PCR. PCR products were obtained by loading the same amount of genomic DNA on a 1.5% agarose gel; the intensity of the band was quantified with ImageJ software.

Explants and Cell culture

Isolated adult mouse hearts (excluding atria and the upper part of the ventricles) were diced into small tissue pieces 1 to 2 mm^3 in size, washed with Ca^{2+}-Mg^{2+}-free phosphate-buffered solution (PBS) (Gibco) and digested for 5 minutes at 37°C with 0.05% trypsin-EDTA solution (Gibco). The remaining tissue fragments were washed with complete explants medium (CEM) (Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 20% fetal calf serum, 100U/mL penicillin G, 100µg/mL streptomycin, 2mmol/L L-glutamine and 0.1mmol/L 2-mercaptoethanol), transferred onto petri dishes coated with fibronectin (25µg/mL, BD Biosciences or Gibco) and cultured as explants in CEM at 37°C and 5% CO_2.

Part of the explants was cultured on coverglass or in 4-well chamber-slides, in order to have samples to stain before each cell-harvesting (passage). Explant-derived cells (EDCs) were harvested three times at intervals of one week, starting after 7days

(newborn mice) or 10 to 14 days (adult mice) from the beginning of the culture. At each passage, cells were collected by pooling two washes with Ca^{2+}-Mg^{2+}-free PBS, one wash with 0.53mmol/L EDTA (Versene, Invitrogen) (1 to 2 minutes) and one wash with 0.05g/L trypsin and 0.53mmol/L EDTA (Invitrogen) (2 to 3 minutes) at room temperature under visual control.

The obtained cells were seeded on poly-D-lysine (BD-Biosciences) coated multiwell plates (BD-Falcon or Corning) in CSps-medium (CSM): 35% IMDM and 65% DMEM/F-12 Mix, 3.5% FBS (Hyclone), 1% penicillin-streptomycin, 1% L-glutamine, 0.1mM 2-mercaptoethanol, 1 unit/mL thrombin (Sigma), 2% B-27 (Invitrogen), 40ng/mL bFGF, 15ng/mL EGF and 1ng/mL cardiotrophin-1 (Peprotech). The media was partially changed every second day. Part of the cells were plated on poly-D-lysine coated coverglass or in 4 well chamber-slides, fixed and stained after 4 days. Cardiospheres were obtained after one week/ten days. They were collected by gently pipetting, pre-plated on fibronectin-coated coverglass or chamber slides for 6-12 hours and then stained.

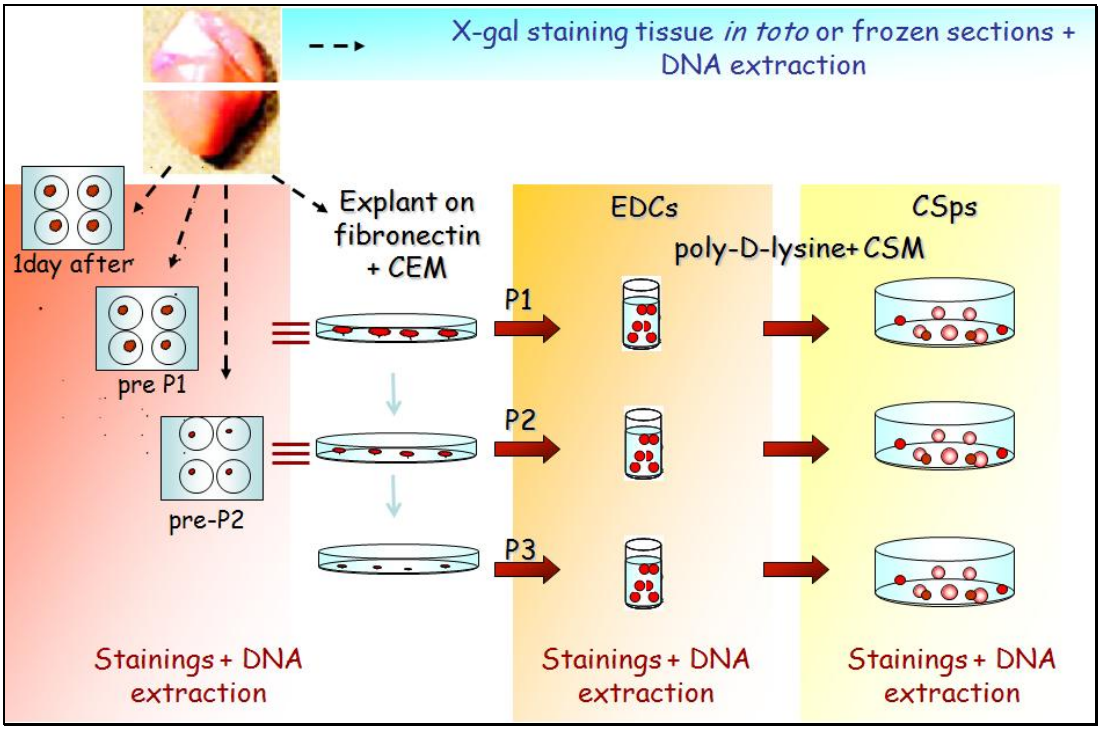


Figure 2.5 Experimental design. Explants, EDCs, CSps were used for DNA or RNA extraction and fixed at each step of the culture for stainings**.**

Immunostainings

Explants, CSps-forming cells and CSps were fixed with 4% paraformaldehyde (4°C, 10 minutes), rinsed twice with Ca^{2+}-Mg^{2+}-free PBS and permeabilized (0.2% Triton X-100 and 1% BSA). Non-specific antibody-binding sites were blocked with 10% goat or horse serum (according to host species in which the secondary antibody was generated), prior to incubation with primary antibodies. To detect cardiac

differentiation we used a mouse anti human and mouse α-actinin antibody (Sigma Aldrich) and mouse anti-mouse light meromyosin antibody (MF20 - Developmental Studies Hybridoma Bank), both tested on beating mouse embryoid bodies (EBs) as positive control and non beating Cripto-/- EBs as negative control. As endothelial markers anti-Tie2 (Santa Cruz) and anti-von Willebrand Factor vWF (Chemicon) antibodies were used. To detect cell proliferation we used rabbit anti-ki67 (Novus) and anti-phospho histone 3 H3P (Upstate). After washing in PBS containing 0.2% Triton X-100 and 1% BSA, cells were incubated with secondary antibodies: donkey anti-mouse Cy3 antibody (Jackson ImmunoResearch) or Alexa goat anti-rabbit 546, Alexa goat anti-mouse or goat anti-rabbit 488 (Invitrogen). To detect ki67 on cells previously stained with x-gal, we used a secondary antibody, goat anti-rabbit HRP-conjugated (Promega), thus we quenched endogenous peroxidase activity by treating our samples with 0.5% peroxidase for 5 minutes and color reaction was developed using diaminobenzidine chromogen (DAB) solution (Vector Lab). Cells were than counterstained with hematoxylin. Vectashield (Vector Lab) or Ultramount (DAKO) were used as mounting media.

Microscopy

Images of whole mount stainings and x-gal stained explants were taken with the Stereoscopic microscope NIKON SMZ 1500. Images of cells and explants in culture were acquired with an Olympus Ix7l inverted microscope. For slides we used the Epifluorescent Zeiss Axioplan. Confocal microscopy was performed with a Leica TCS DMIRE 2 (LCS lite software Leica). No significant fluorescent signal was detectable with any of the secondary antibodies alone.

Real-Time quantitative RT-PCR analysis

Total RNA was extracted using TRIZOL® Reagent (Invitrogen) according to the manufacturer's protocol. One microgram of total RNA was used to synthesize first-strand cDNA with random primers, using the the QuantiTect Reverse Transcription kit (Qiagen). Quantification of gene transcripts was carried out by real-time quantitative RT-PCR. Primers were tested and we obtained calibrations curves on serial dilutions of the positive control sample. Gene expression for each sample was normalized to the housekeeping gene GAPDH and normalized to the control (heart tissue for αMHC). Real-time PCR was performed with Sybr Green I Mastermix (Roche), using a LightCycler ® 2.0 Real-Time PCR System (Roche). Each reaction was run in triplicate and contained 2 µl of cDNA template along with 500nM primers in a final reaction volume of 20µl. Cycling parameters were: 95°C for 8' (to activate DNA polymerase), then 40-45 cycles at 95°C for 3', 60°C for 5' and 72°C for 10'. Melting curves were performed with LightCycler software version 4.05 (Roche) to ensure only a single product was amplified. As negative controls, reactions were prepared without any template.

RT-PCR Primers		
mGAPDH fw	AAT GGA TAC GGC TAC AGC	Tan 60
mGAPDH rw	GTG CAG CGA ACT TTA TTG	
mαMHC fw	CAT GCC AAT GAC GAC CT	Tan 60
mαMHC rw	CCT ACA CTC CTG TAC TGC C	

Results

TnT-Cre x Rosa 26 flox/+

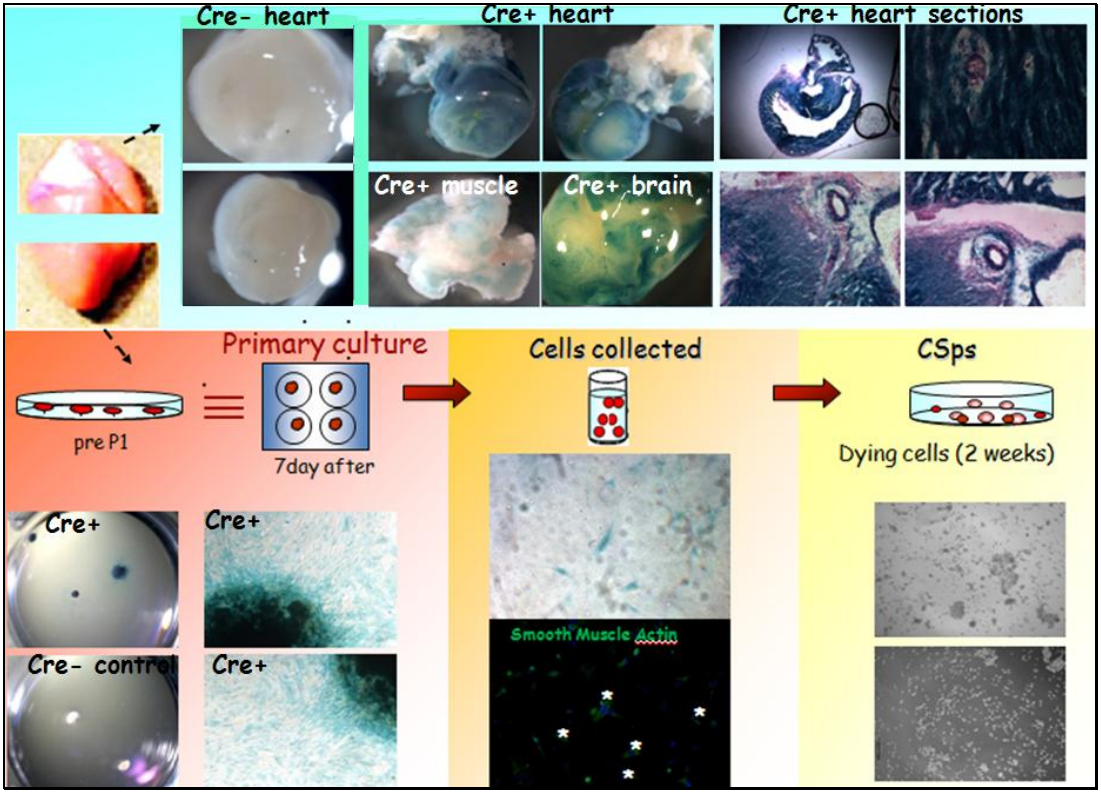


Figure 2.6. Expression of the Cre recombinase in TnT-Cre mice is not specific for cardiomyocytes.

One of the mouse strain we used to trace cardiomyocytes *in vitro*, expressed the Cre recombinase under the TnT promoter, activating the reporter locus Rosa26flox

in positive cells. The upper part of the hearts was used for x-gal staining *in toto*. Most of the EDCs were positive but cells from this strain did not form CSps. Furthermore staining on brain, on tibial muscles, on cardiac cryosections and co-staining on EDCs showed that this reporter was not specific for cardiomyocytes, but it was also expressed in SMCs and skeletal muscle.

MLC2v-Cre x Rosa 26 flox/+ xRBPj flox/+

Mice expressing the Cre recombinase under the myosin light chain 2v promoter [105] were bred with a reporter strain presenting two floxed loci, Rosa26 and RBPj in heterozygosis. MLC2v expression is restricted to ventricles at the earliest stages of ventricular chamber specification. Mice that present the knock-in with Cre in just one allele display no morphogenic defects and express normal levels of MLC2v. In a previous work [105] it has been shown that almost 80% of ventricular cardiomyocytes in postnatal heart had undergone recombination. Previous observations in our lab confirmed that the recombination occurs specifically in ventricular cardiomyocytes. In fact real time PCR for RBPj on cardiomyocytes and fibroblasts deriving from mice heterozygous for RBPj flox, showed complete absence of the PCR product just in cardiomyocytes. From whole mount x-gal staining it was observed almost 100% of recombination in adult heart, but a lower percentage in newborns (fig.2.7).

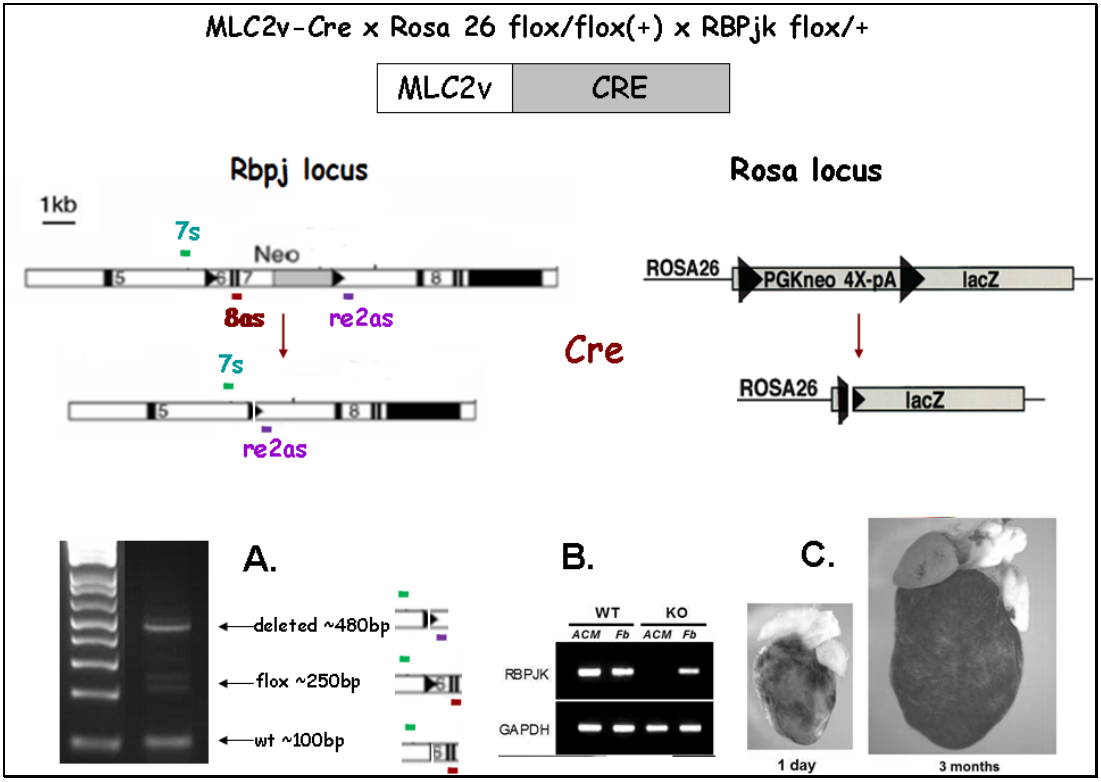


Figure 2.7 Characterization of the MLC2v-Cre x Rosa 26 flox(+) x RBPj flox(+) strain. A- Primers to detect the RBPj flox locus deletion. B- qPCR showing that the deletion of RBPj is specific for cardiomyocytes in KO mice (ACM: dispersed adult cardiomyocytes, Fb: fibroblasts). C- x-gal staining to detect deletion of the Rosa26 flox: recombination was almost 100% in adult heart, but incomplete in newborns.

For our experiments, we used litters of 6-day-old mice. X-gal staining showed that the efficiency of recombination was low and variable among litters. Regarding the other reporter locus, we observed aspecific deletion in some litters probably due to random activation of Cre during gametogenesis. Since MLC2v and RBPj are on the same chromosome 5, Cre and deleted RBPj are inherited together. To exclude aspecific activation, we tested by PCR different tissues, including brain, lungs, skin and tail.

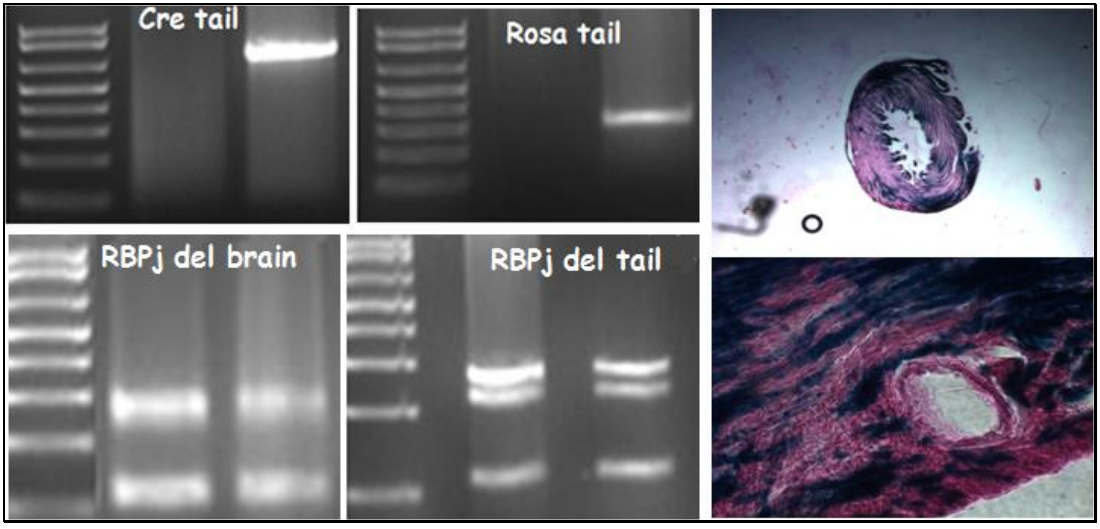


Figure 2.8. Example of genotyping and x-gal staining on sections of 6-days old MLC2v-Cre x Rosa 26 flox/+ x RBPj flox/+ mice.

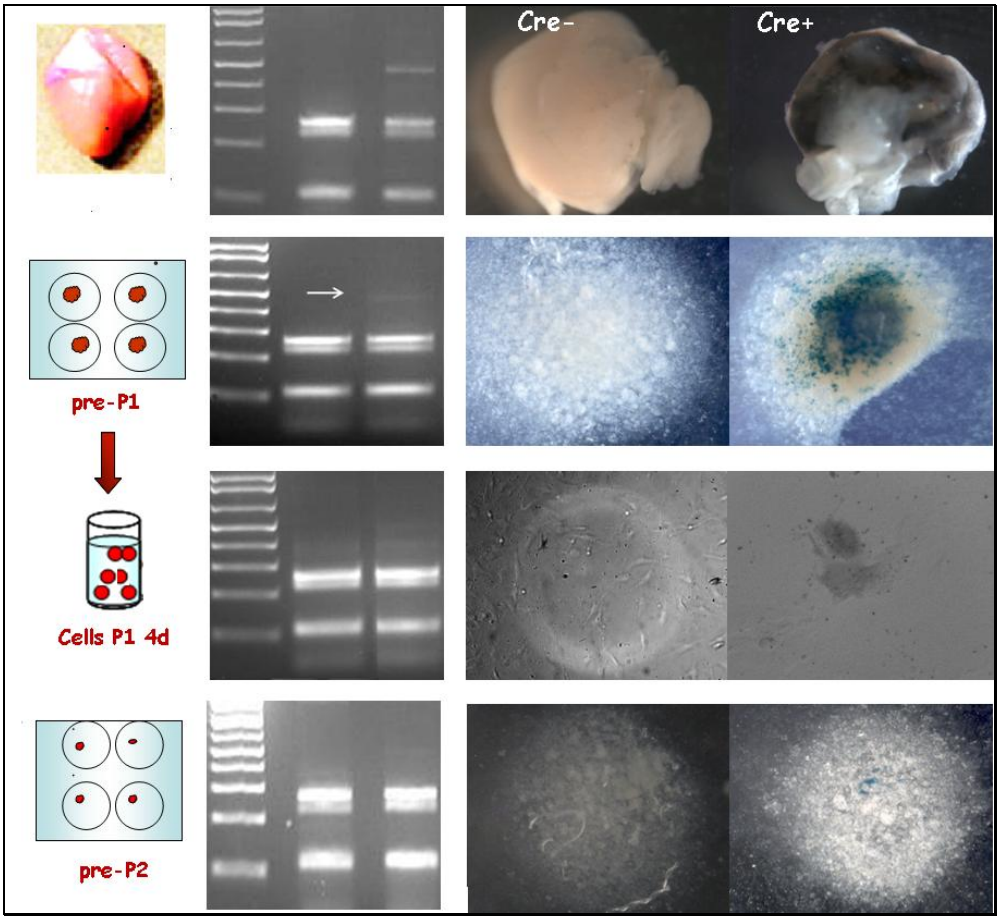


Figure 2.9. Example of an experiment with 6-day-old MLC2v-Cre x Rosa 26 flox/+ x RBPj flox/+ mice. PCR to detect RBPj deletion and x-gal staining on pre-plating tissue, explants pre-P1 (7 days of culture), EDCs 4 days after being harvested, explants pre-P2 (14 days of culture). From a Cre^{+} and a Cre^{-} mouse.

On average 30% of the RBPj flox allele resulted deleted in the cardiac tissue pre-plating, in accordance with the expected percentage of cardiomyocytes in the adult heart [106].The percentage dropped to 17% in the explants after seven days in culture and no deletion band was detectable by PCR on genomic DNA extracted from EDCs, CSps and even from explants after 2 weeks (fig. 2.9). Very rare x-gal positive cells were detected among CSp-forming cells and they resulted negative to the proliferation markers ki67 after co-staining (fig.2.10).

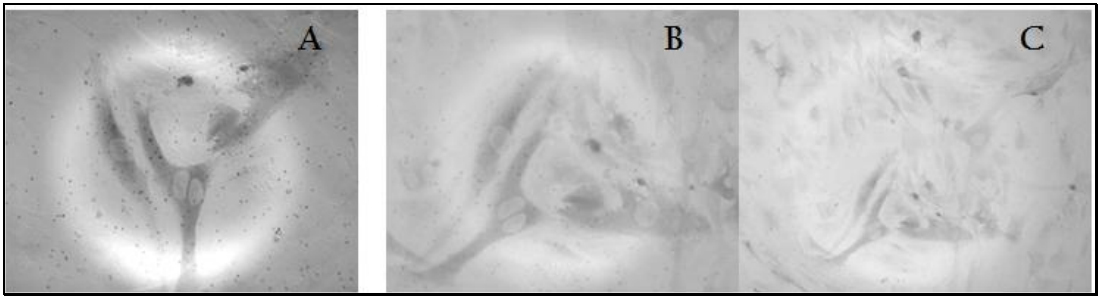


Figure 2.10. x-gal positive EDCs are not proliferating cells. A- LacZ positive EDCs. B-C co-staining for ki67 revealed with a HRP-conjugated secondary antibody. Counter-stain with hematoxylin. Magnification 20x and 10x.

Thus, despite the limitations of this model, these preliminary observations suggest that pre-existing differentiated cardiomyocytes tend to die with time in culture and very rare contaminating cardiomyocytes can be detected in EDCs.

Myh6-MerCreMer x Rosa 26 flox/+

As previously discussed, this mice strain consent a temporal regulation of the Cre recombinase expression. Only the differentiated cardiomyocytes present at the time of Tamoxifen pulse will be irreversibly marked. We tried the treatment on the

explants *in vitro* using three different concentrations. While efficient recombination was observed already at the lowest concentration on the pancreatic β-cell line we used as positive control, we could not detect LacZ-positive cells among CSp-forming cells and the treatment resulted toxic, as shown by the lower number of CSps obtained compared to controls. Hence we decided to treat adult 12-week-old mice *in vivo*.

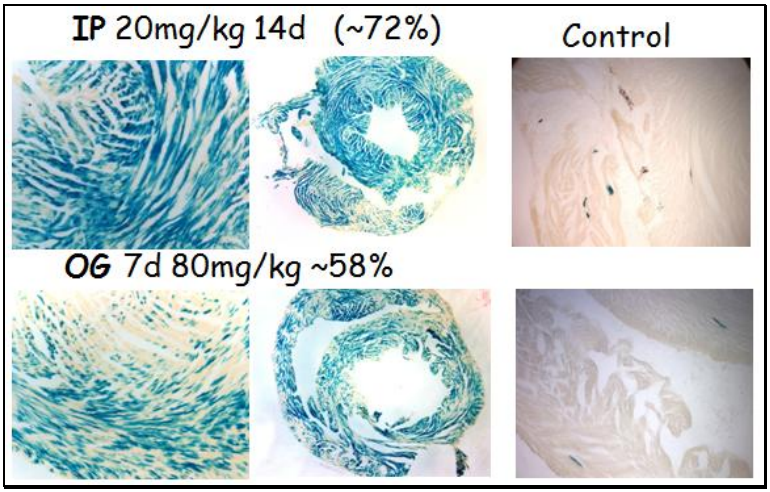


Figure 2.11 Efficiency of recombination in Myh6-MerCreMer x Rosa 26 flox/+ mice. X-gal staining on 25µm cryosections from mice treated with 20mg/kg TAM intraperitoneally for 14 days or with 80 mg/kg TAM by oral gavage for 7 days. Syngeneic mice not treated with TAM were used as control.

Among the different protocols adopted, the most efficient one resulted to be the administration of 20mg/kg BW of 4-OH-tamoxifen for 15 days and mice were

sacrificed after 2 weeks in order to give time for the accumulation of a significant amount of the reporter enzyme β-galactosidase.

In these conditions we obtained an average recombination efficiency of 72% on three mice (fig.2.11). X-gal staining was dramatically reduced in the explants already after one week of culture, before the first passage.

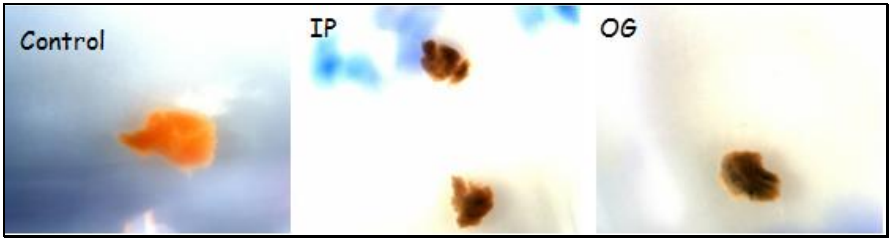


Figure 2.12 Myh6-MerCreMer x Rosa 26 flox/+ mice. X-gal staining in explants 1 day of culture

No blue cells were detected among the CSps-forming cells, fixed and stained 4 days after being harvested. Single blue cell were rarely detected at the periphery of larger CSps (fig.2.13).

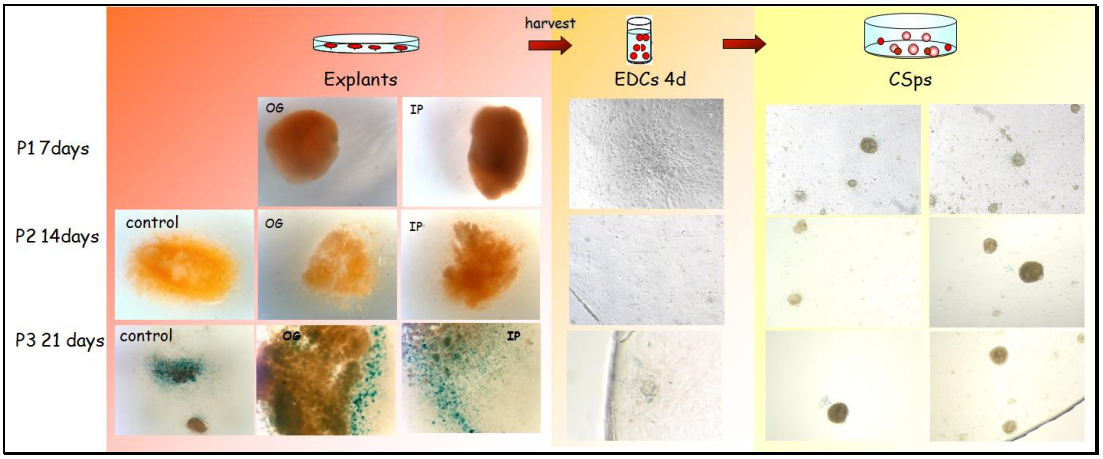


Figure 2.13 Myh6-MerCreMer x Rosa 26 flox/+ mice. X-gal stainings on explants, EDCs, CSps from different passages. Drastic reduction of x-gal expression in explants already after one week of culture; aspecific reactivation in some samples after 21days.

In some samples, after 3 weeks in culture, there seemed to be a re-activation of LacZ expression even in control explants (i.e. from mice not treated with tamoxifen). Most likely it was an artifact due to senescence-associated beta-galactosidase activity [107]. In fact, using specific primers to detect the deletion of the Rosa locus, we failed to detect bands in samples other than the explants after one day of culture, suggesting that pre-existing differentiating cardiomyocytes die with time in culture and that there is no significant contamination among EDCs, first collected after a week. Furthermore real time PCR for Myh6 showed that in the explants after 7days of culture (before the first passage) the expression is reduced by 10^{-4} folds, compared to the tissue and almost 10^{-5} folds after 2 weeks. In cells collected from the explants, after 4 days of culture in CSp-growth medium, the

expression of Myh6 is further reduced 10^{-2} folds relatively to the explants from which they derived and 10^{-6} folds from myocardial tissue, while it was not detectable in newly generated CSps (7days after plating the EDCs).

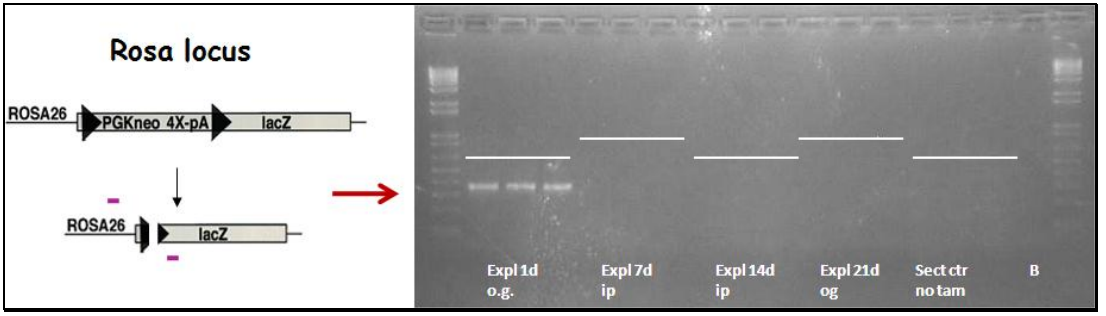


Figure 2.14 PCR to detect the deletion of Rosa26 locus.

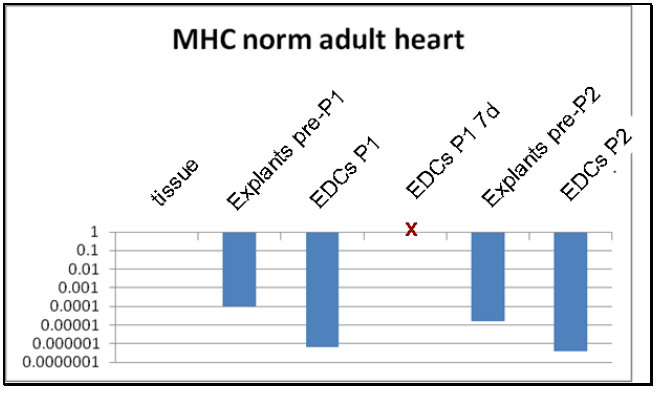


Figure 2.15 qPCR for αMHC on cardiac tissue, explants, EDCs, CSps

Immunostainings for cardiac markers, such as αActinin and light meromyosin MF20, showed that CSps obtained from adult mice did not contain terminally differentiated cardiomyocytes with a fully differentiated sarcomeric structure (fig.2.17); and staining of explants after 6 days and 10 days of culture confirm that cardiomyomyocytes in the plated tissue die with the time in culture (fig.2.16).

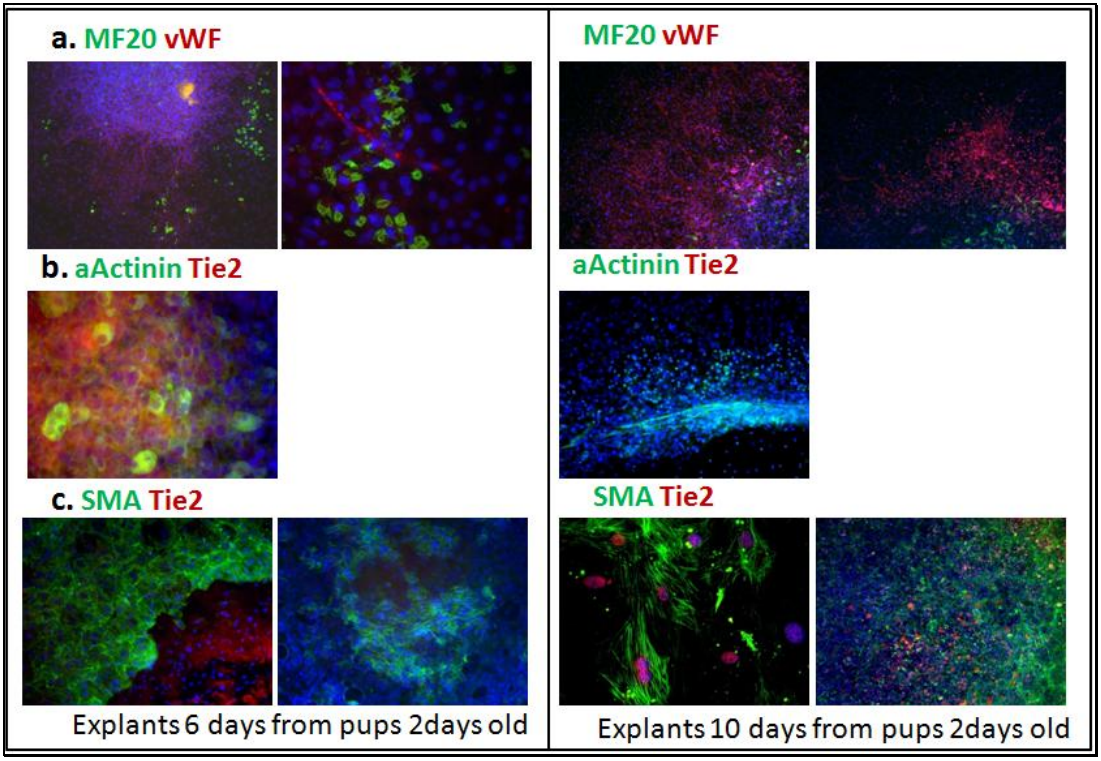


Figure 2.16. Immunostainings on murine explants after 6 days and 10 days of culture.

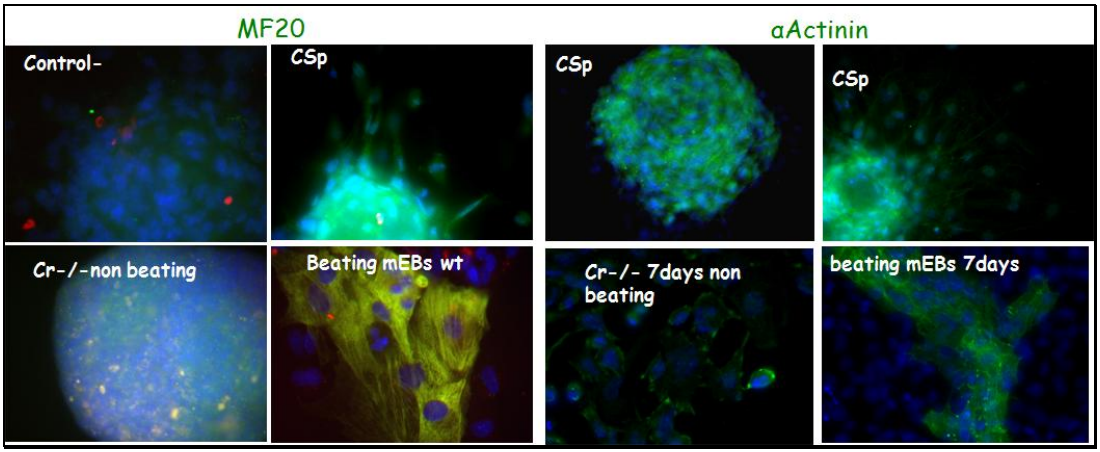


Figure 2.17. Immunostainings for cardiac markers Mf20 and αActinin on CSps, not-beating-Cripto^{-} mEBs, beating Myh6_mCherry mEBs (positive control), CSps with no primary antibody added (negative control).

Discussion

The first part of our results, obtained using this experimental strategy, lead us to the conclusion that most likely mature cardiomyocytes do not directly (as CSp-forming EDCs) or indirectly (as de-differentiating cells) contribute to CSps generation. In fact, in 6-day-old MLC2v-Cre x Rosa 26 flox/+ xRBPj flox/+ mice we observed deletion of the RBPj reporter locus only in explants after 1 day in culture and after 7 days (despite a drastic reduction), while it was not detectable in EDCs or in explants after 2 weeks. Recombination of the Rosa locus resulted low and variable. Rare blue positive cells were observed among the EDCs which resulted negative to ki67 staining.

With Myh6-MerCreMer x Rosa 26 flox/+ 3-month-old mice we obtained a higher efficiency of recombination of the Rosa locus, corresponding to approximately 72%. No positive cells were detected among EDCs and rare blue spots were observed in larger CSps. Aspecific LacZ activity was detected in some samples after 21 days in culture, but by PCR delection of the Rosa locus was observed only in explants after one day of culture. Besides, the expression of αMHC was dramatically reduced in EDCs and not detectable in CSps and staining on explants at different time points confirmed that cardiomyocytes tend to die with time in culture.

The main limitation to the use of a conditional transgenic mouse model for lineage tracing experiments is that the efficiency of recombination is not 100%, so the results may be under-estimated. This is because, while Cre is expressed in temporally and spatially graded patterns, activation of a Cre-dependent reporter is a binary readout in which only cells surpassing a Cre exposure-threshold become activated. The specific threshold depends on the Cre-dependent reporter and the cellular context. Thus lack of the Cre reporter activation must be interpreted carefully, because this does not exclude Cre expression at levels below the threshold required for reporter recombination [108].

However the combination of different techniques allowed us to exclude a significant contamination of pre-existing differentiated cardiomyocytes in EDCs and CSps, suggesting that, at least in our culture conditions, cardiomyocytes de-differentiation is not a crucial factor in CSps formation.

Similar results have been obtained by two other groups, on different animal models [89, 109].

Project II: Role of epicardial/epicardial derived cells in CSps formation

Introduction

A potential source for endogenous CPCs has been recently identified in the epicardium, an epithelial layer lining the cardiac surface. For many years the epicardium was believed to be a derivative of the myocardium, an inert layer of cells with the basic role to protect the myocardial from external factors. Starting from 1969 [110], embryological studies showed a non-myocardial origin for the epicardial tissue. It mostly derives from the proepicardium, a transient organ located in the pericardial cavity which in mammalian arises from the mesothelium of the septum transversus and consists of finger-like protrusions with an outer lining of epithelial cells and a core of undifferentiated mesenchymal cells. Recent lineage tracing experiments have shown that the proepicardium derives from a common cardiac precursor pool of cells $Nkx2.5^{+}/Isl1^{+}$, that generates most of the cell types in the heart [111]. During the process of cardiac looping, proepicardial cells migrate, either as individual cells (in mammalian) or as sheets (in birds) and envelope the developing myocardium. This process is probably spatiotemporally regulated by the expression of adhesion molecules and ECM components on the myocardial surface [38]. Integrin α4β1, a cell adhesion receptor, is expressed on proepicardial cells as they migrate onto the surface of the heart. It binds to fibronectin a major component of the extracellular matrix and to VCAM-1 a member of the immunoglobulin family. It has been shown that in knockout mice ablation of the α4 subunit of integrin α4β1 [112], of VCAM-1 [113] or of Podoplain

(a mucin-like transmembrane glycoprotein) [114], impairs epicardial outgrowth and spreading on the myocardium, resulting in several abnormalities such as hypoplasia, defective atrio-ventricular (AV) valve development, incomplete intra-ventricular septation and alteration of the coronary vasculature. Thus the correct formation of the epicardial layer is crucial for the normal development of the heart. This is because the epicardium is both a source of multipotent mesenchymal progenitors and of instructive soluble signals that stimulate coronary vessel development, as well as cardiomyocyte proliferation and differentiation. During development, some epicardial cells undergo an epithelial-to-mesenchymal transition (EMT) and delaminate from the epicardium to invade the subepicardial space, which consist of hydrated extracellular-matrix rich in proteins. Some of these epicardial-derived cells (EPDCs) remain as mesenchymal cells or form blood islands; others migrate further into the myocardium and endocardial cushions, where they differentiate into different cell types. A recent study showed that Wilms tumor-1 (WT1) has a crucial role in regulating this process[115]. Wt1 is a zinc-finger protein involved in the normal development of several organs, such as kidney, gonads, spleen and heart. Wt1-mutant mice present coronary vascular defects, so in order to determine WT1 function in epicardial cells, conditional knockout Wt1-loxP/GFP x Gata5-Cre mice were used, in which the expression of Wt1 is selectively lost in epicardial cells. These mice died between E16.5-E18.5 for cardiovascular failure: Cre^+ E16.5 embryos presented edema and accumulation of blood in the systemic veins; pericardial hemorrhage; coronary artheries did not form; the right ventricles of some mutant embryos had thinner free walls compared to control embryos. Despite all this, the epicardial layer was integer and, in fact, Cre^+ hearts were lined with a GFP^+ epithelium. These epicardial cells presented a significantly reduced expression

of Snail (activator of EMT) and of Vimentin, while epithelial markers E-cadherin and cytokeratin were upregulated. Using a tamoxifen-inducible Wt1-knockout immortalized epicardial cell line (Cre+ CoMEECs), they observed that TAM treatment led to loss of Wt1 expression with a robust dose-dependent increase in E-cadherin expression, down-regulation of N-cadherin, α-SMA and Snai1 and reduced mobility. Similar changes were observed in ESCs obtained from Wt1 KO mice. While wild type ESCs expressed high levels of vimentin, α-SMA and Snail, but not E-cadherin, (indicating that these cells had undergone EMT), Wt1-KO ESCs presented high levels of E-cadherin, did not express mesenchymal markers, were unable to undergo EMT, their mobility was impaired and the generation of mesoderm precursors (hematopoietic, endothelial, cardiac) was compromised, but could be rescued by the expression of Snai1. Thus Wt1 controls EMT processes through direct regulation of the Snail transcription factor and E-cadherin. This function appears to be crucial for the generation of mesenchymal cardiovascular progenitor cells from the epicardium and during ESCs differentiation. Analysis of quail-chick chimeras and retroviral labelling experiments suggest a primitive epicardial origin for the mesenchymal cells of the subepicardial layer, the endothelium and the SMCs (SMCs) of the coronary vasculature, the perivascular and intermyocardial fibroblasts and some cells in the atrioventricular valves[38, 116]. More recent studies in mouse, using the Cre-lox technique to examine the fate of $Wt1^{+}$ [40] and $Tbx18^{+}$ [39] epicardial cells, showed that Wt1/Tbx18 descendants contribute to the coronary vessel SMCs and the fibroblasts of the myocardial interstitium. Unlike reports in chick, they found that in mice only a small population of coronary vascular endothelial cells is derived from the epicardium. Furthermore both papers suggest a contribution of epicardial cells to

cardiomyocyte formation; however, this suggestion is questionable, since expression of Tbx18 is not confined to the epicardium, but it is also found in cardiomyocytes of the interventricular septum and the left ventricle [117]. As mentioned before, there is evidence that epicardium-derived secreted molecules signal to the underlying myocardium, modulating both EPDC-derived coronary vascularization and ventricular development. In particular both retinoic acid (RA) and Erytropoietin (EPO) act in parallel to induce cardiac mitogen release from epicardial cells [118]. RA signaling in the epicardium up-regulates myocardial FGF2, thereby leading to cardiomyocyte proliferation. FGF9, FGF16 and FGF20 are also released from the epicardium and specific inactivation of their receptors FGFR-1 and FGF-R2c in the myocardium leads to defects in cardiomyocytes development [119]. Even EPDCs are a source of paracrine signals for cardiomyocytes and intermyocardial fibroblasts, which are at least partly epicardially derived, promote cardiomyocyte proliferation during development [120]. On the other hand, myocardial tissue releases important factors to modulate epicardial functions. For example FGF1, FGF2 and 7 released from the myocardium increase the expression of FGFR-1 in the epicardium, triggering the activation of Hedgehog (HH) signaling which controls the expression of multiple angiogenic factors such as VEGFA, VEGFB, VEGFC and Angiopoietin-1, crucial for coronary vasculature development. Furthermore FGFR-1 overexpression promotes EMT from epicardium and its knowckdown impairs the ability of epicardial cells to invade the myocardium and contribute to intramural vasculature. Secretion of the Platelet Derived Growth Factor-B (PDGFB) by myocardium is important to stimulate EMT and promote expression of SMCs markers. In addition embryonic hearts express high level of angiopoietin-1 which binds the receptor Tie2 expressed in the epicardium and

endocardium and this interaction resulted essential for vasculature development and maintanance. Thymosin ß4 (Tmsb4), a 43-aminoacid G-actin-sequestering peptide, is expressed throughout development in myocardium and epicardium [121, 122]. This peptide is known to regulate actin cytoskeletal organization and it is required for several biological cell functions, including cell motility and organogenesis [123]. It has been shown that Tmsb4 stimulates cardiomyocyte migration *in vitro* and increases cardiac function while promoting the survival of cardiomyocytes *in vivo* [122]. Notably, coronary vasculature formation is impaired in cardiac specific Tmsb4-knockout mice, suggesting that the loss of secreted Tmsb4 in these mice may result in altered paracrine signaling to the epicardium [121]. Finally, the addition of Tmsb4 to embryonic epicardial explants significantly increases the number of endothelial and SMCs and, under these culture conditions, endothelial cell differentiation can be further enhanced in the presence of FGF7 and VEGF [121]. The synergistic action of Tmsb4 and VEGF is consistent with the VEGF downregulation observed in Tmsb4-knockout mouse heart [121]. In conclusion, a finely regulated interplay between epicardium and myocardium, mediated by release of paracrine factors, is essential for embryonic heart development.

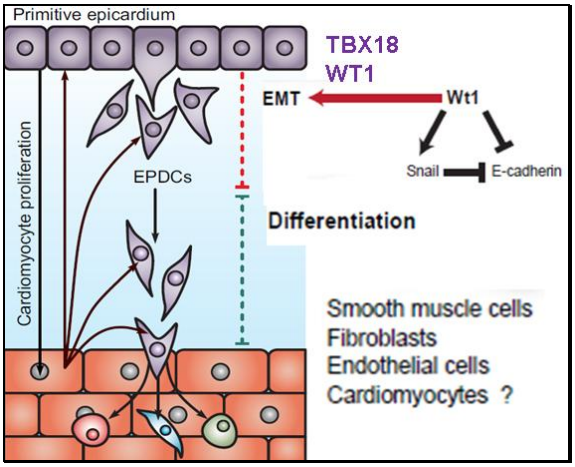


Figura 3.1 Role of the epicardium during embryonic development (modified from [116])

Recent studies have shown that in certain conditions the adult epicardium can reactivate the embryonic gene expression program, suggesting that, apart from the mechanical function, it can contribute to repair processes as observed in zebrafish.
For example, exposure to specific soluble factors such as Prokineticins and thymosin bβ4 has been clearly shown to modulate adult epicardial cells. Prokineticins are small secreted bioactive peptides, comprising two classes: prokineticin-1 and prokineticin-2. They exert their biological functions through activation of two G-protein-coupled receptors, PKR1 and PKR2, which have two opposite effects in the heart: PKR2 displays detrimental actions, leading to hypertrophic cardiomyopathy and vascular leakage [124], whereas PKR1 confers survival signaling in cardiac myocytes and reduces apoptotic cell death following hypoxia [125]. Mice overexpressing PKR1 in cardiomyocytes display an increased number of proliferating EPDCs, of coronary arterioles and a higher capillary density.

Notably, cardioblasts overexpressing PKR1, up-regulate prokineticin-2 which in turn enhanced*, in vitro*, both neonatal and adult EPDC differentiation into SMCs and endothelial cells. Prokineticin-2 actions are mediated by PKR1 expressed in EPDCs since prokineticin-2-induced proliferation and differentiation are reduced in the PKR1-null mutant EPDCs [126].

As we mentioned above Tmsb4 has an important role during cardiogenesis, particularly for coronary vasculature formation. It has been shown that systemic administration of Tmsb4 in infarcted mice preserves cardiac functions and decreases scar volume by two different mechanisms. On one side acting on Akt, Tmsb4 improves early cardiomyocytes survival, cardiomyocytes and endothelial cells migration [122]. On the other hand, [127] it induces an organ-wide epicardial thickening and the reactivation of the embryonic developmental program in adult epicardial cells, as indicated by: increase of blood vessel/epicardial substance (Bves) expressing cells (marker of epicardial cells or cells of epicardial origin in adult and embryonic tissues); elevated VEGF, Flk-1, TGF-β, FGFR-2, FGFR-4, FGF-17 and β-Catenin expression; increase of Tbx-18 and Wt-1 positive myocardial progenitors also migrating in the myocardium and subepicardial space. The result is a significant increase in capillary outgrowth from the epicardium within the border zone and infarcted area [121, 127].

Besides, MI induces embryonic reprogramming of a subset of c-kit^{+} epicardial cells with reactivation of Raldh2, Tbx18 and Wt1, especially in the presence of an intact pericardium, suggesting an important role of soluble factors in the pericardial fluid [83]. Whether these mediators are secreted by epicardial or myocardial cells is not known. Most likely both compartments release important trophic factors. In particular insulin-like growth factor 1 (IGF-1), hepatocyte growth factor (HGF) and

high mobility group box 1 protein (HMGB1), were significantly increased in the pericardial fluid post MI (PFMI), compared to pericardial fluid of non ischemic patients.

Previous studies in our laboratory [128] have shown that CSps and CDCs exert an important paracrine effect both *in vitro* and *in vivo*, releasing different cytokines and growth factors, in particular VEGF, HGF and IGF1. Furthermore direct co-culture experiments conducted in collaboration with Doc. Mercola's lab have shown that CSps forming cells are able to significantly increase cardiogenesis in mouse ESCs. For these experiments we used a line of CGR8 Myh6-eGFP cells. We seeded cells CSp-forming cells in dark mw384 and the following day, we plated on top of them single cells deriving from dispersion of EBs at day 4 of the differentiation protocol (Mesendoderm committed cells). We changed media after 2 days adding FGF and serum 0.6% and after 4 days with serum free medium. After 6days of co-cultures (day 10 of EBs differentiation) we could observe beating GFP positive EBs and the amount of cardiogenesis was quantified measuring the fluorescence intensity with IN Cell 1000 Analyzer™ (GE Healthcare).

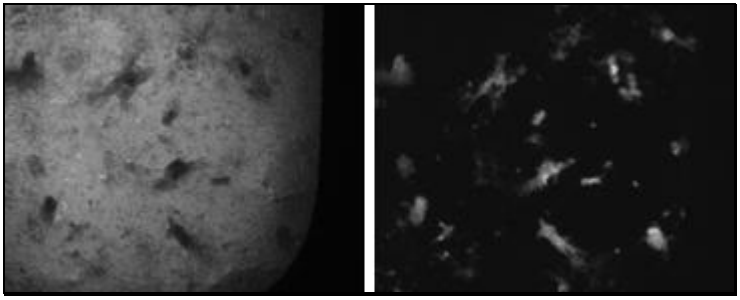


Figura 3.1 Images of co-culture of CGR8 Myh6-eGFP mESCs and CSp-forming cells. Left panel: bright field, Right panel: green channel (eGFP).

Thus given the trophic role of CSps and CDCs, which are able to substain cardiomyocytes proliferation and differentiation, we decided to evaluate the potential contribution of epicardium or epicardial derived cells in CSps formation. The eventual presence of this contribution would give an added value to CSps biological meaning, owing to the potential of epicardial cells to undergo epithelial-to-mesenchymal transition (EMT), with important functional and clinical implication for the fate of transplanted cells.

To this propose, we used transgenic cGATA5-Cre Rosa26flox mice. In this animal model the Cre recombinase is expressed under the control of a specific portion of chicken GATA5 promoter [129], which is selectively activated in proepicardium starting from E9.5; thereby all epicardial and epicardial derived cells will be LacZ positive in the adult heart [130]. Then we proceeded with a gene expression analysis on murine and human samples of epicardial markers (TBX18, WT1), epithelial/endothelial markers (E-cadherin, Ve-caherin, Tie2), mesenchymal markers (Vimentin, SMA) and a marker of EMT (Snail).

Material and Methods

Mice

All experiments were conducted in accordance with the *Guide for the Use and Care of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee. Two-month-old GATA5-Cre Rosa26flox mice were kindly provided by Dr Pilar Lozano's lab. Gene expression analyses were conducted on CD1 wild type mice 7-day-, 3-month-old or embryos E10.5.

Explants and Cell culture

Mouse hearts and human biopsies from auricola of two patients (male 63 years old, female 62 years old) were cultivated as explants as described above(pag. 42). Briefly the myocardial tissue was triturated and enzymatically digested with 0.05% trypsin-EDTA solution (Gibco) before being plated on fibronectin coated petri dishes in CEM. After 7 days for mice, or about 20 days for human we harvested explant derived cells (EDCs) for the first time. EDCs were collected 2 or 3 more times from each explant culture, at intervals of a week. From each harvest 100000-200000 cells were seeded on poli-D-lysine in CSps-medium (CSM) at a density of 9000 cells/cm^2 to obtain CSps. The remaining cells were washed in PBS without Ca^{+2}/Mg^{+2}, spinned down, resuspended in RNA later (Invitrogen) and stored at -20°C before RNA extraction. CSps were obtained after 5 to 10 days. They were collected by gentle pipetting, leaving adherent cells behind, and centifugated at low speed to eliminate contaminating cells. Approximately 25% of the obtained CSps were expanded on fibronectin coated wells, while the rest were washed in PBS, spinned down and resuspended in RNA later. CDCs were collected at confluence after 4 days. Part of the cells (approximately 70000) were re-plated in CSM on poli-D-lysine to obtain seconday CSps; the remaining ones were preserved in RNA later and stored at -20°C before RNA extraction.

X-gal staining:

X-gal stainings to detect epicardial or epicardial derived cells in cultures of hearts deriving from cGATA5-Cre Rosa26flox mice were conducted as described before (pag.40). Explants or CSps were fixed with 2% paraformaldehyde 0.2% gluteraldehyde for 5 minutes at 4°C. Afterwards the samples were rinsed twice in PBS and once in staining solution made of $K_3Fe(CN)_6$ 20mM, $K_4Fe(CN)_6*3H_2O$ 20mM, $MgCl_2*3H_2O$ 2mM, 0.01% DOC (Na salt), 0.02% NP40 in PBS. Finally we

added X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Invitrogen), diluted to a final concentration of 1mg/ml in staining solution and we incubated the samples for at least one hour or overnight at 37°C for the staining to be developed.

RNA extraction and cDNA synthesis

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen) which includes a guanidine-isothiocyanate lysis buffer and silica-membrane columns for purification. To extract total RNA from adult cardiac tissue, embryonic hearts and pieces of explants we used TRIZOL® Reagent (Invitrogen) according to the manufacturer's protocol. Tissue samples were homogenized with Turrex, when necessary, and an additional centrifugation step was performed in order to remove the excessive extracellular material. One microgram of total RNA was used to synthesize first-strand cDNA with random primers, employing the QuantiTect Reverse Transcription kit (Qiagen). Quantification of gene transcripts was carried out by real-time quantitative RT-PCR.

Real-Time quantitative RT-PCR analysis

Real-time PCR was performed with SYBR Green I detection chemistry, utilizing the iQ SYBR Green Supermix (Biorad) on a MiniOpticon™ Two-Color Real-Time PCR Detection System with CFX Manager software (Biorad). Each primer pairs was tested on serial dilutions of a positive control sample with a gradient of temperature in order to obtain calibration curves and select the appropriate annealing temperature, according to efficiency and r^2 values. For endothelial and mesenchymal markers we used murine or human heart tissue as positive control. For murine epithelial and epicardial markers we used cDNA deriving from 10.5E CD1 mice embryos. Human Cdh1 primers were tested on cDNA from MCF-7 cells, an E-cadherin-positive weakly invasive human breast carcinoma cell line (kindly

provided by Prof. Marchese, Dep. of Experimental Medicine, “Sapienza” University of Rome). Human Wt1 primers were tested on cDNA from the human erythromyeloblastoid leukemia cell line k562 [131] and primers for human TMSBX4 were tested on cDNA from myeloma cells (kindly provided by Prof. Santoni, Dep. of Molecular Medicine, “Sapienza” University of Rome). Efficiency values in a range of 100±10%, with r^2>0.990, were accepted.

Each reaction contained 1 µl of cDNA template along with 200nM primers in a final reaction volume of 25µl. The sequences and annealing temperatures of the primers used are shown in Table 1 and 2. Cycling parameters were: 95°C for 3 minutes (to activate DNA polymerase), then 40 cycles at 95°C for 10 seconds, 10 seconds at the chosen annealing temperature and 30 seconds at 72°C. Melting curves were performed using Bio-Rad CFX Manager software to ensure only a single product was amplified. As negative controls, reactions were prepared in which no cDNA template was included.

Gene expression for each sample (e^{-Ct}) was normalized to the expression level of the housekeeping gene GAPDH ($e^{-Ct\ gene}/e^{-Ct\ GAPDH}$). Data were plotted as: ratio between CSps expression/EDCs expression; average expression in EDCs, CSps, CDCs, IICSps; average expression in EDCs and CSps from different harvesting (P1 and P2).

All results are presented as mean value ± standard deviation, unless specified. Significance of difference between any two groups was determined by two-sided Student t test. A final value of $P<0.05$ was considered significant.

Table 1. Murine PCR Primers

Gene	Sequences	Length	Tan	Efficiency	R2
GAPDH NM_008084.2	5'-CGTCCCGTAGACAAAATGGT-3' 5'-TTGATGGCAACAATCTCCAC- 3'	110	56	98.7	0.996
Cdh5/VeCad NM_009868.3	5'-CGTGGTGGAAACACAAGATG-3' 5'-AGACGGGGAAGTTGTCATTG-3'	98	56.5	101.9	0.999
Chd1 PB NM_009864.2	5'-CAAGGACAGCCTTCTTTTCG-3' 5'-TGGACTTCAGCGTCACTTTG-3'	165	55	91.8	0.995
Wt1[115] NM_144783.2	5'-GCCTTCACCTTGCACTTCTC-3' 5'-GACCGTGCTGTATCCTTGGT-3'	186	59	100.8	0.997
Tbx18 PB NM_023814.4	5'-GTACCTGGCTTGGCACGAC-3' 5'-GCATTGCTGGAAACATGCG-3'	151	55	100.8	0.992
Snai1[115] NM_011427.2	5'-CGTGTGTGGAGTTCACCTTC-3' 5'-GGAGAGAGTCCCAGATGAGG-3'	120	56	96	0.997

SMA NM_007392.2	5’-CTGACAGAGGCACCACTGAAC-3’ 5’-AGAGGCATAGAGGGACAGCA-3’	123	59	96.2	0.991
TMSBX NM_021278.2	5’-ATGTCTGACAAACCCGATATGGC-3’ 5’-CCAGCTTGCTTCTCTTGTTCA-3’	124	60	105.2	0.993

Table 2. Human PCR primers

Gene	Sequences	Length	Tan	Efficiency	R2
GAPDH NM_002046.3	5’-ACAGTCAGCCGCATCTTC-3’ 5’-GCCCAATACGACCAAATCC- 3’	110	57	96	0.999
Tie2 NM_000495.3	5’-GTCTCTGCTCTCCAGGATGG-3’ 5’-TGGCAAATCCACTATCTTTGG-3’	83	57	95.5	0.991
Chd5 NM_001795.3	5’-CAACGGAACAGAAACATCCC-3’ 5’-CTGCTGCTGCCACTGCT-3’	134	56.5	92.6	0.99
Cdh1 NM_0004360.3	5’- CAGGAGTCATCAGTGTGGTCA -3’ 5’- TGTGCTTAACCCCTCACCTT -3’	104	58	94.9	0.995

Wt1 NM_024425	5’-GCCAGGATGTTTCCTAACGC-3’ 5’-CGAAGGTGACCGTGCTGTAA-3’	91	59	101.6	0.991
Tbx18 NM_001080508.1	5’-GCCCCTGCTGACTATTCTGC-3’ 5’-CTGCATGGATAAGCTGGTCTG-3’	228	59	101.6	0.991
Snai1 NM_005985.2	5’-AATCGGAAGCCTAACTACAGCG-3’ 5’-GTCCCAGATGAGCATTGGC-3’	147	60.4	97.7	0.995
SMA NM_001613.2	5’-ATGAAGATCCTGACTGAGCG-3’ 5’-GCAGTGGCCATCTCATTTTC-3’	123	58	93	0.998
Vimentin NM_003380.3	5’-ACCCACTCAAAAAGGACACTTC-3’ 5’-GGTCATCGTGATGCTGAGAA-3’	88	56	92	0.997

Results

Staining of explants and CSps deriving from cGATA5-Cre x Rosa26flox adult mice revealed a large amount of LacZ-positive cells, suggesting a possible contribution of epicardial/epicardial-derived cells in CSps formation. The main limitation of this model is that the recombination cannot be modulated in a time-specific manner: Cre is expressed when the cGATA5 promoter becomes active in proepicardial cells at E9.5, therefore all cells deriving from those proepicardial precursors will be irreversibly labeled by LacZ expression, including epicardial cells, EPDCs, a subset of inter-myocardial fibroblasts and the SMCs of the coronary arteries. Previous studies have shown that in particular conditions adult epicardial cells can re-activate genes that are normally expressed during development such as WT1, Tbx18, Raldh2, with consequent EMT, which led to the generation of multipotent mesenchymal progenitor cells. To verify if this could happen even in our culture system, we analyzed by RT-PCR the expression of epicardial markers (TBX18, WT1), epithelial/endothelial markers (E-cadherin, Ve-cadherin, Tie2), mesenchymal markers (Vimentin, SMA) and a marker of EMT (Snail).

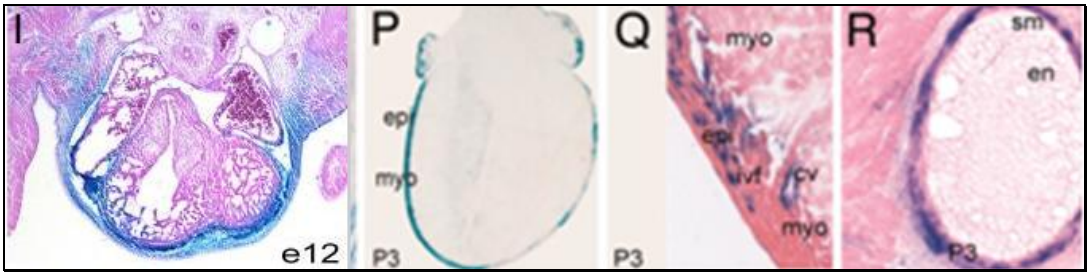


Figure 3.3 Characterization of cGATA5-Cre x Rosa26flox mice (modified from[130]). I) Whole-mount staining and histological section of an E12 embryo showing Cre activity in the epicardium, pericardium and body wall. P) In postnatal hearts (postnatal day 3), LacZ activity is detected in the epicardium and subepicardial layers and in a subset of intermyocardial fibroblasts (Q) and in the smooth muscle of the coronary arteries (R). Note the absence of Cre activity in the coronary endothelium (R). Epi, epicardium; myo, myocardium; ivf, interventricular fibroblasts; cv, coronary vessel; sm, smooth muscle; en, endocardium.

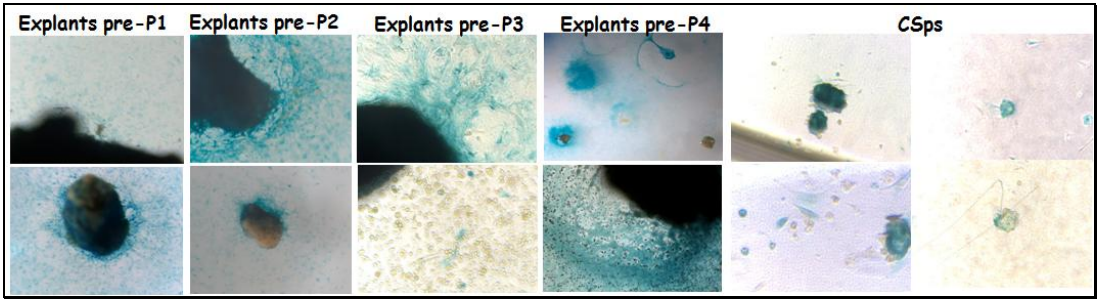


Figure 3.4 cGATA5-Cre x Rosa26flox mice. X-gal staining on explants pre-P1 (7 days of culture), pre-P2 (14 days), pre-P3 (21 days), pre-P4 (28days) and CSps.

Preliminary data on cultures from 7-day-old CD1 mice showed a significant reactivation of TBX18 and WT1, increased expression of Cdh1 and Snail in CSps

compared to adult heart, explants and EDCs, while SMA appears to be reduced in CSps. Vimentin expression was not significantly different in CSps compared to EDCs, while Tmsb4 was more expressed with the time in culture (fig. 3.5-3.6).

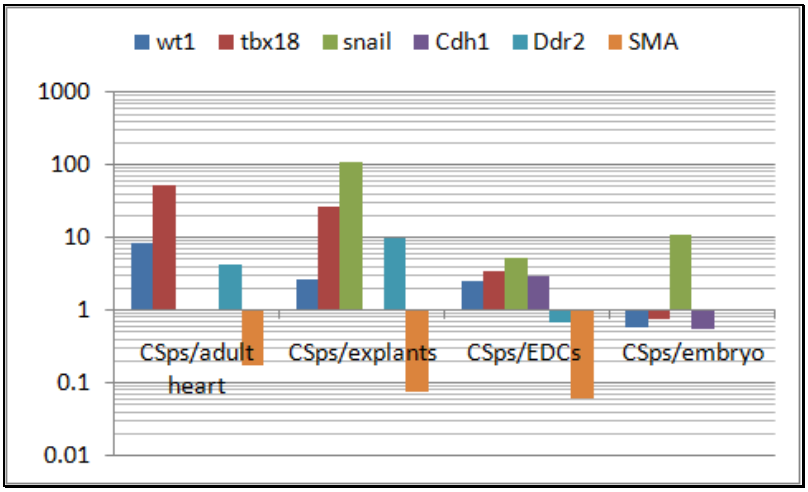


Figure 3.5 Gene expression analyses on murine samples. Expression of Wt-1, Tbx18, Snai1, Cdh1 and SMA in: CSps versus adult heart; CSps vs explants; CSps vs EDCs; CSps versus E10.5 embryonic heart.

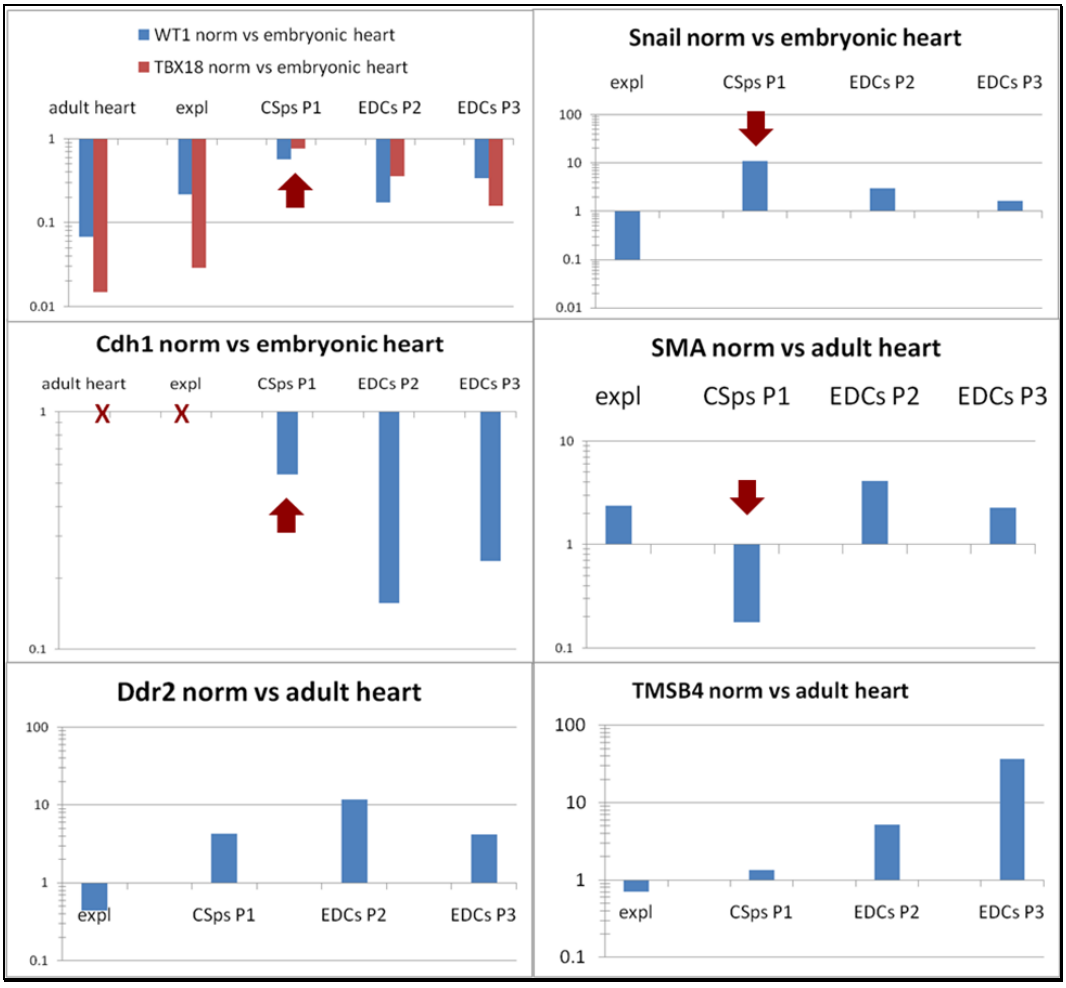


Figure 3.6 Real time PCR analyses on explants, EDCs and CSps from 7-day-old CD1 mice.

Subsequently, we analyzed gene expression in samples deriving from two human biopsies. In fig. 3.7 we plotted the average ratio of the expression in CSps compared to the EDCs from which they derived, calculated on 8-10 samples for each examined gene. Seven genes appear to be expressed in a significantly different manner in CSps compared to EDCs: endothelial markers (Tie2, Cdh5), Wt1 and Snail are upregulated, while Cdh1 and SMA are significantly downregulated. In fig. 3.8-3.11 we represented for each gene the average values of expression in EDCs, CSps, CDCs, IICSps and the levels of expression in EDCs and CSps from different cellular harvests. From these graphs it is possible to infer that the expression of hWt1 is significantly higher in three dimensional structures (CSps and IICSps) compared to monolayer cells (EDCs and CDCs). In the same conditions SMA and Cdh1 expression is significantly reduced. The expression of Cdh5, Tie2 and Snail is increased in CSps, CDCs, IICSps compared to EDCs, especially in the 3D structures. Instead levels of Vimentin expression are not significantly different among the four cell populations examined. The main discrepancy with the results observed in mice seems to be the Cdh1 expression, upregulated in murine CSps and significantly downregulated in human CSps and IICSps. However, in a recent work [132], PCR array analysis of the gene expression of ECM and adhesion molecules in human cells cultured as CSps or monolayers, has shown that 12 out of 84 ECM and adhesion genes examined were up-regulated more than 4-fold in CSps relative to monolayer cells, including *COL14A1*, *COL7A1*, *ITGA2*, *LAMB1*, *LAMB3*, *MMP3*, *MMP10*, *MMP11*, *MMP13*, *SELE*, *PECAM1* and *SPP1*, while Cadherin type 1 (*CDH1*) was the sole gene up-regulated in cells cultured as monolayers.

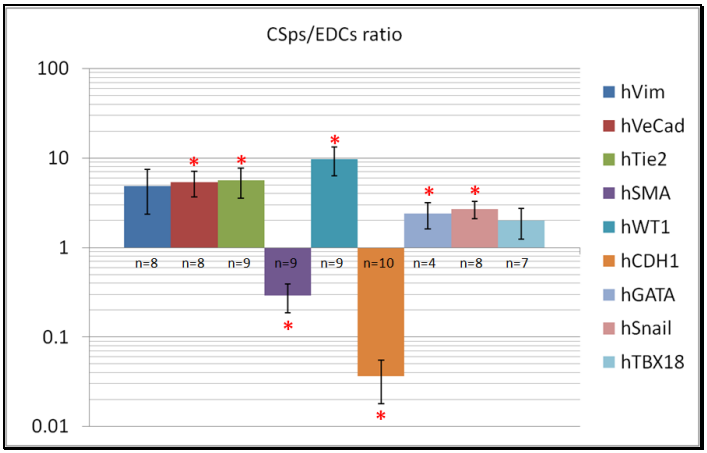


Figure 3.7. Expression levels of endothelial (Tie2, Cdh5), epithelial (Cdh1), epicardial (Wt1, Tbx18) and mesenchymal (SMA, Vimentin) markers, in human CSps versus EDCs.

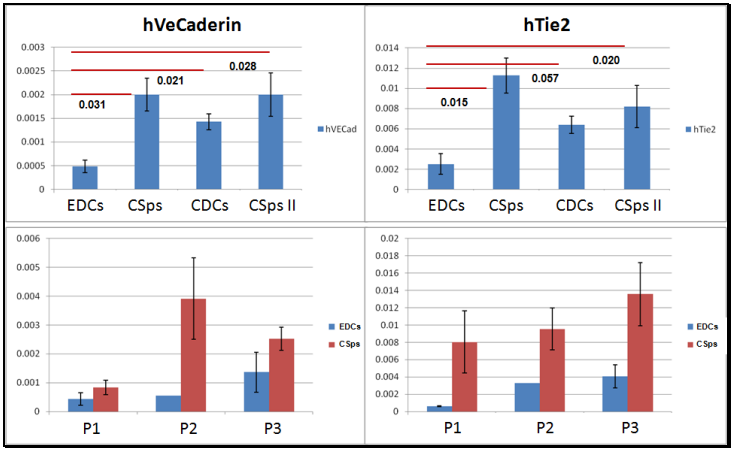


Figure 3.8. RT-PCR analyses of endothelial markers (Cdh5, Tie2) in EDCs, CSps, CDCs and IICSps. Bottom panels: average expression values in EDCs and CSps from different harvests (P1, P2, P3)

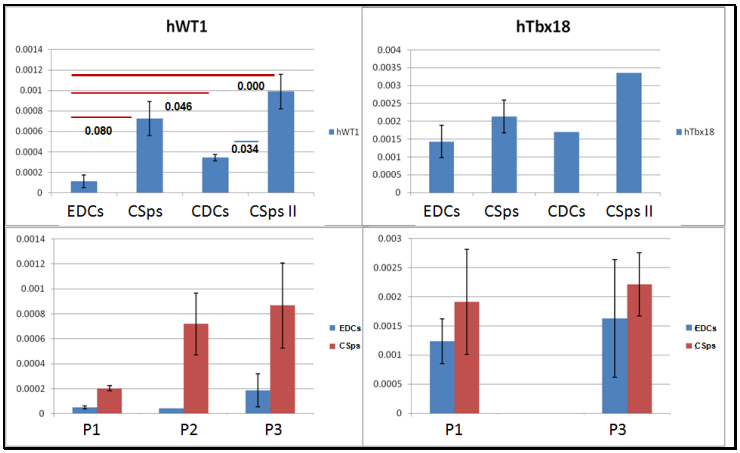


Figure 3.9. RT-PCR analyses of embryonic epicardial markers (Wt1, Tbx18) in EDCs, CSps, CDCs and IICSps. Bottom panels: average expression values in EDCs and CSps from different harvests (P1, P2, P3)

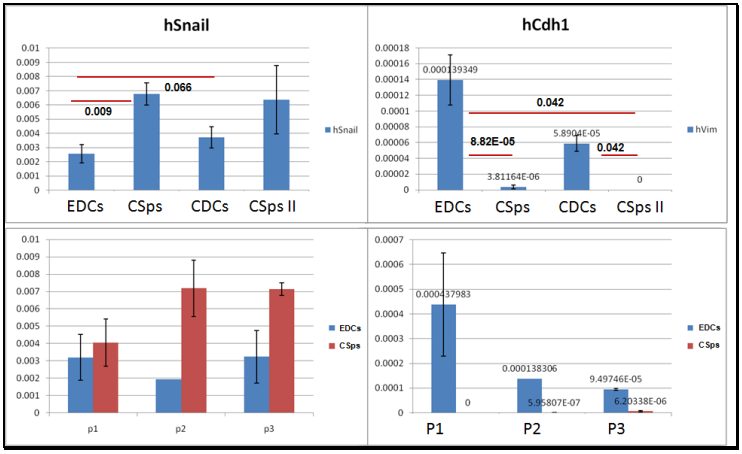


Figure 3.10. RT-PCR analyses of Snai1 (EMT marker) and Cdh1 (epithelial marker) in EDCs, CSps, CDCs and IICSps. Bottom panels: average expression values in EDCs and CSps from different harvests (P1, P2, P3)

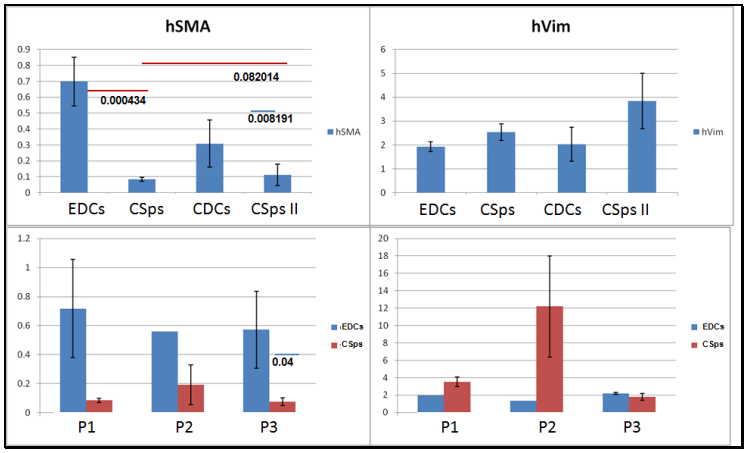


Figure 3.11. RT-PCR analyses of mesenchymal markers (SMA, Vimentin) in EDCs, CSps, CDCs and IICSps. Bottom panels: average expression values in EDCs and CSps from different harvests (P1, P2, P3)

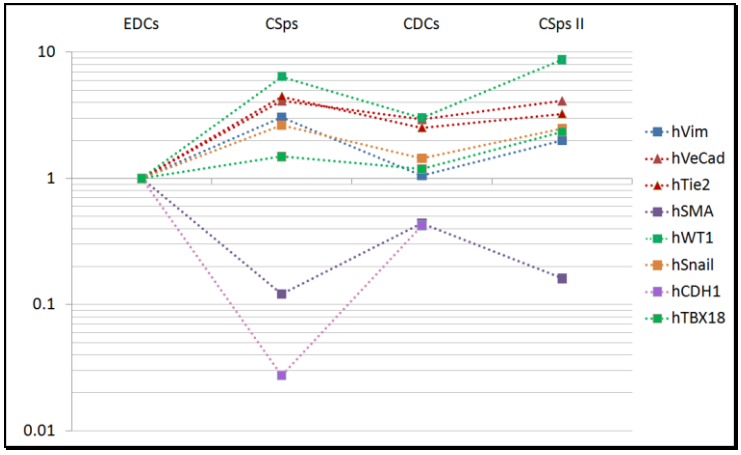


Figure 3.12. Summarizing graph to show the gene expression trends in the four different cell populations: EDCs, CSps, CDCs, IICSps.

Discussion and future directions

Based on these data, the reduced expression of SMA and the increased levels of adhesion molecules in CSps, including Tie2 and Cdh5, may suggest that SMA^+ cells (embriologically derived from the epicardium) take part in CSps formation through a MEndT or MET process, with consequent reactivation of markers of the embryonic epicardium, such as Tbx18 and Wt1. Recent works, indeed, have demonstrated that MET is a fundamental step to initiate cellular reprogramming [133, 134]. Reactivation of Wt1 in the CSp may in turn, induce EMT as suggested by the increased expression of Snail. In fact Wt1 is known to be a central mediator of EMT that leads to the generation of mesenchymal cardiovascular progenitor cells in the epicardium and during ESCs differentiation, through direct reactivation of the Snail transcription factor and inhibition of E-cadherin [115]. Intriguingly EMT has been proven to be important to induce a stem-cell phenotype even in other tissues. For example, when exposed to TGF-β1 for 12 days or by over-expressing the EMT-inducing transcription factors Snail or Twist, normal mammary epithelial cells could be induced to adopt the $CD44^{high}/CD24^{low}$ expression profile, typical of stem-like cells, with an enhanced ability to form "mammospheres" and the potential to differentiate into cells expressing lineage-specific markers of myoepithelial or luminal epithelial cells [50, 51]. Moreover chronic exposure to TGF-β [135] induced EMT, accompanied by a de-differentiation process in both a non-transformed model, such as human fetal hepatocytes and in human hepatic cancer cells, Hep3B, as shown by: the loss of expression of hepatic proteins; the diminished expression of liver enriched transcription factors typical of mature hepatocytes, such as HNF4α or HNF1α; the increase in the expression of liver transcription factors characteristic

of early embryonic stages, such as HNF3β and of proteins described as markers of hepatic stem cells, such as CK7, Thy1, c-Kit and/or EpCAM.

Furthermore, as we mentioned before, EMT/EndMT processes occurring during embryonic development, create cells that act as progenitors of many different tissues from the mesoderm or neural crest. In the heart four different controlled transition layer occur during organogenesis, including that involving the epicardium [27], each generating a distinct set of cardiovascular progenitor cells that differentiate into the cellular components of the mature heart. These processes have been already shown to be re-activated in response to cardiac injury, mainly contributing to vasculogenesis and fibrosis.

Therefore it is plausible that these same transitions occur in CSps, induced by the culture conditions or by the ischemic gradient present in these 3D structures. In this view, a model/hypothes of spontaneous *in vitro* reprogramming process could be proposed (fig.3.13) as a starting point for the future steps. In fact, a better understanding of the relevance of these processes in CSps formation will be essential to improve the knowledge of their biological regulatory pathways and potentially their therapeutic effect, since it will give us insights on how to modulate their differentiation potential, in order to promote and/ot maintain a cardiogenic destiny rather than a fibrogenic one, which could be physiologically favored in the hostile inflammatory environment of the infarcted heart.

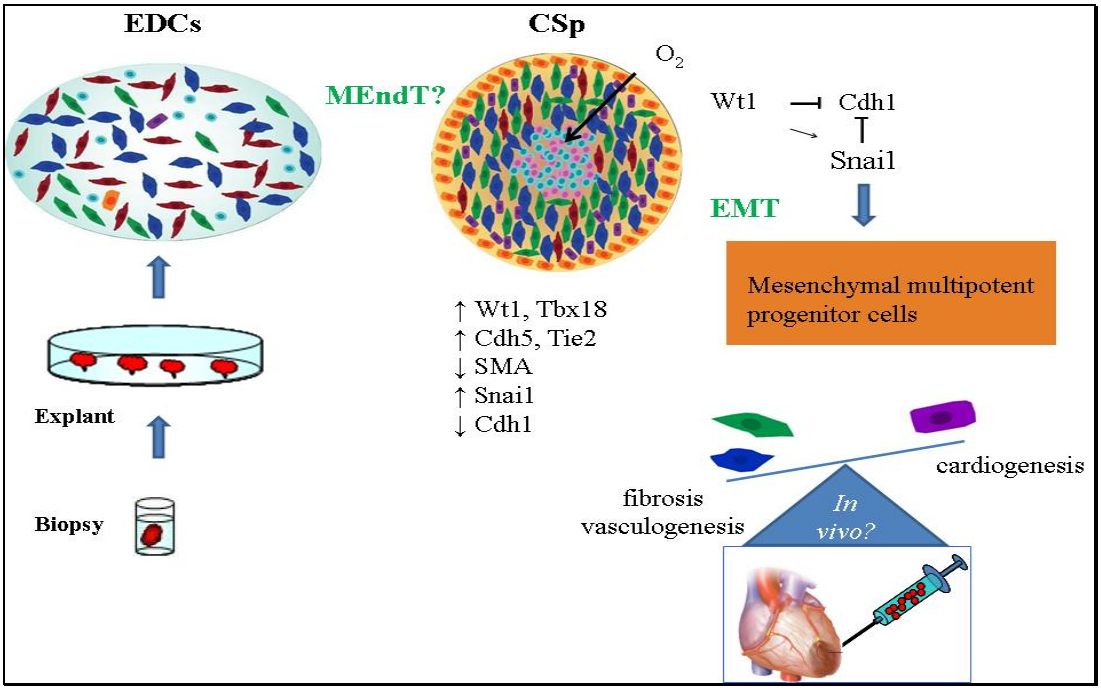


Figura 3.13. Proposed model. The reduced expression of SMA and the increased expression of Tie2 and Cdh5 in CSps compared to EDCs, may suggest the occurrence of End/EMT, probably in SMA^+ cells (embryologically derived from epicardium), with re-activation of embryonic epicardial genes (Tbx18, Wt1) during CSps formation. Wt1 is known to be an active mediator of the EMT that leads to the generation of mesenchymal multipotent progenitor cells from epicardial cells, by Snai1 activation and inhibition of Cdh1. In CSps, Snai1 is upregulated and Cdh1 is downregulated compared to monolayer cells, suggesting the possible occurrence of EMT (probably due also to the hypoxic gradient inside the CSps) that may generate progenitor cells. A better understanding of the relevance of these processes in CSps formation will be essential to improve their therapeutic effect, since it will give insights on how to modulate their differentiation potential in order to promote a cardiogenic outcome rather than a fibrogenic one.

For this propose, many questions remain to be answered. It is interesting to see how the expression of the examined genes varies consistently between 3D structures and monolayer cells, confirming the importance of the niche-like structure of CSps, but CSps consist of different cell types, thus we cannot yet univocally determine which cells are involved in these transitions. The use of conditional $Wt1^{CreERT2}$ Z/EG transgenic mice [40] and of a reporter construct to transfect human explants will help us understand which cells actually reactivate Wt1, how they are involved in CSps formation and it will allow us to trace their fate both *in vitro* and *in vivo*, after CSps or CDCs injection into the infarct border zone of syngeneic mice subjected to LAD ligation. *In vitro* treatments with molecules known to be important regulators of EMT/MET, such as TGFβ, Metmorfin, BMP7, miR200-205, will give us information on how these transitions are relevant in our cellular model. A PCR array will be performed to analyze a larger number of genes known to be involved in EMT and reprogramming and the production of relevant proteins will be confirmed by ELISA and Western Blot. Analysis by transmission electron microscopy will give us important hints on the morphology and phenotype of single cells inside the CSps and the laser capture microdissection technique, combined to gene expression analysis, will allow highlighting differences between the CSp's core and periphery, that may be due to the hypoxic gradient present in the 3D structure, which, as already mentioned, is a well known EMT-inducing factor itself.

In conclusion, CSps represent a valuable method to isolate CPCs, since the 3D structure ensures a niche-like microenvironment which promotes the maintenance of a stemness phenotype in the core, while conferring higher engraftment *in vivo* and resistance to ischemic stress. As mentioned above, de-differentiation of cardiomyocytes, recruitment of circulating cells deriving from the bone marrow,

controlled layer/lineage transitions or the presence of remnant cells from embryonic development are considered to be potential sources of CPCs.

Our work ruled out a significant contribution of cardiomyocyte to CSps formation, while evidencing the involvement of epicardial-deriving cells, reactivating their embryonic developmental program in the 3D niche-like microenvironment of the CSp. Thus the information acquired represents a starting point for a better evaluation not only of the biological meaning of heart-tissue-derived cardiac regenerative cells in the form of CSps, but also for the prevision of their fate *in vivo* once translated into clinic as therapeutic mediators in cardiac cell therapy.

REFERENCES

1. Dobaczewski M., Gonzalez-Quesada C., Frangogiannis N. G. The extracellular matrix as a modulator of the inflammatory and reparative response following myocardial infarction. *J Mol Cell Cardiol*, *48*, 504-511.
2. Frangogiannis N. G. (2008). The immune system and cardiac repair. *Pharmacol Res*, *58*, 88-111.
3. Vanhoutte D., Schellings M., Pinto Y., Heymans S. (2006). Relevance of matrix metalloproteinases and their inhibitors after myocardial infarction: a temporal and spatial window. *Cardiovasc Res*, *69*, 604-613.
4. Pfeffer M. A., Braunwald E. (1990). Ventricular remodeling after myocardial infarction. Experimental observations and clinical implications. *Circulation*, *81*, 1161-1172.
5. Kajstura J., Leri A., Finato N., Di Loreto C., Beltrami C. A., Anversa P. (1998). Myocyte proliferation in end-stage cardiac failure in humans. *Proc Natl Acad Sci U S A*, *95*, 8801-8805.
6. Beltrami A. P., Urbanek K., Kajstura J., et al. (2001). Evidence that human cardiac myocytes divide after myocardial infarction. *N Engl J Med*, *344*, 1750-1757.

7. Hsieh P. C., Segers V. F., Davis M. E., et al. (2007). Evidence from a genetic fate-mapping study that stem cells refresh adult mammalian cardiomyocytes after injury. *Nat Med*, *13*, 970-974.
8. Walsh S., Ponten A., Fleischmann B. K., Jovinge S. Cardiomyocyte cell cycle control and growth estimation in vivo--an analysis based on cardiomyocyte nuclei. *Cardiovasc Res*, *86*, 365-373.
9. Bergmann O., Bhardwaj R. D., Bernard S., et al. (2009). Evidence for cardiomyocyte renewal in humans. *Science*, *324*, 98-102.
10. Chimenti I G. R., Barile L, Forte E., Ionta V, Angelini F, Messina E and Giacomello A (2010). Evidence for the existence of resident cardiac stem cells. *Chapter from the book: Regenerating the heart Sprinter Science &Business Media*.
11. Reese D. E., Mikawa T., Bader D. M. (2002). Development of the coronary vessel system. *Circ Res*, *91*, 761-768.
12. Oberpriller J. O., Oberpriller J. C. (1974). Response of the adult newt ventricle to injury. *J Exp Zool*, *187*, 249-253.
13. Bettencourt-Dias M., Mittnacht S., Brockes J. P. (2003). Heterogeneous proliferative potential in regenerative adult newt cardiomyocytes. *J Cell Sci*, *116*, 4001-4009.
14. Jopling C., Sleep E., Raya M., Marti M., Raya A., Belmonte J. C. Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. *Nature*, *464*, 606-609.
15. Kikuchi K., Holdway J. E., Werdich A. A., et al. Primary contribution to zebrafish heart regeneration by gata4(+) cardiomyocytes. *Nature*, *464*, 601-605.
16. Lepilina A., Coon A. N., Kikuchi K., et al. (2006). A dynamic epicardial injury response supports progenitor cell activity during zebrafish heart regeneration. *Cell*, *127*, 607-619.
17. Engel F. B. (2005). Cardiomyocyte proliferation: a platform for mammalian cardiac repair. *Cell Cycle*, *4*, 1360-1363.
18. Buja L. M., Vela D. (2008). Cardiomyocyte death and renewal in the normal and diseased heart. *Cardiovasc Pathol*, *17*, 349-374.

19. Gruenwald P., Minh H. N. (1961). Evaluation of body and organ weights in perinatal pathology. II. Weight of body and placenta of surviving and of autopsied infants. *Am J Obstet Gynecol*, *82*, 312-319.
20. Adler C. P., Friedburg H. (1986). Myocardial DNA content, ploidy level and cell number in geriatric hearts: post-mortem examinations of human myocardium in old age. *J Mol Cell Cardiol*, *18*, 39-53.
21. Zhang Y., Li T. S., Lee S. T., et al. Dedifferentiation and proliferation of mammalian cardiomyocytes. *PLoS One*, *5*, e12559.
22. Tamamori-Adachi M., Goto I., Yamada K., Kitajima S. (2008). Differential regulation of cyclin D1 and D2 in protecting against cardiomyocyte proliferation. *Cell Cycle*, *7*, 3768-3774.
23. Bersell K., Arab S., Haring B., Kuhn B. (2009). Neuregulin1/ErbB4 signaling induces cardiomyocyte proliferation and repair of heart injury. *Cell*, *138*, 257-270.
24. Roland H. Friedel W. W., Benedikt Wefers and Ralf Kühn (2011). Generating Conditional Knockout Mice *Methods in Molecular Biology*, *693*, 205-231.
25. Antonio Díez-Juan A. I., Iñigo Valiente, Isabel Moscoso, David Lara and Antonio Bernad (2010). New and old cell homeostasis concepts in the healthy and diseased adult heart. *NATURE REVIEWS | CARDIOLOGY (CNIC EDITION)*, *VOLUME 7* 1-6.
26. Kalluri R., Weinberg R. A. (2009). The basics of epithelial-mesenchymal transition. *J Clin Invest*, *119*, 1420-1428.
27. Acloque H., Adams M. S., Fishwick K., Bronner-Fraser M., Nieto M. A. (2009). Epithelial-mesenchymal transitions: the importance of changing cell state in development and disease. *J Clin Invest*, *119*, 1438-1449.
28. Nakaya Y., Sheng G. (2008). Epithelial to mesenchymal transition during gastrulation: an embryological view. *Dev Growth Differ*, *50*, 755-766.
29. Abu-Issa R., Kirby M. L. (2007). Heart field: from mesoderm to heart tube. *Annu Rev Cell Dev Biol*, *23*, 45-68.
30. Brand T. (2003). Heart development: molecular insights into cardiac specification and early morphogenesis. *Dev Biol*, *258*, 1-19.
31. Moorman A. F., Christoffels V. M., Anderson R. H., van den Hoff M. J. (2007). The heart-forming fields: one or multiple? *Philos Trans R Soc Lond B Biol Sci*, *362*, 1257-1265.

32. Buckingham M., Meilhac S., Zaffran S. (2005). Building the mammalian heart from two sources of myocardial cells. *Nat Rev Genet*, *6*, 826-835.
33. Srivastava D. (2006). Making or breaking the heart: from lineage determination to morphogenesis. *Cell*, *126*, 1037-1048.
34. Person A. D., Klewer S. E., Runyan R. B. (2005). Cell biology of cardiac cushion development. *Int Rev Cytol*, *243*, 287-335.
35. Norris R. A., Moreno-Rodriguez R. A., Sugi Y., et al. (2008). Periostin regulates atrioventricular valve maturation. *Dev Biol*, *316*, 200-213.
36. Yang J. H., Wylie-Sears J., Bischoff J. (2008). Opposing actions of Notch1 and VEGF in post-natal cardiac valve endothelial cells. *Biochem Biophys Res Commun*, *374*, 512-516.
37. Winter E. M., Gittenberger-de Groot A. C. (2007). Epicardium-derived cells in cardiogenesis and cardiac regeneration. *Cell Mol Life Sci*, *64*, 692-703.
38. Wessels A., Perez-Pomares J. M. (2004). The epicardium and epicardially derived cells (EPDCs) as cardiac stem cells. *Anat Rec A Discov Mol Cell Evol Biol*, *276*, 43-57.
39. Cai C. L., Martin J. C., Sun Y., et al. (2008). A myocardial lineage derives from Tbx18 epicardial cells. *Nature*, *454*, 104-108.
40. Zhou B., Ma Q., Rajagopal S., et al. (2008). Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart. *Nature*, *454*, 109-113.
41. Winter E. M., Grauss R. W., Hogers B., et al. (2007). Preservation of left ventricular function and attenuation of remodeling after transplantation of human epicardium-derived cells into the infarcted mouse heart. *Circulation*, *116*, 917-927.
42. Hutson M. R., Kirby M. L. (2007). Model systems for the study of heart development and disease. Cardiac neural crest and conotruncal malformations. *Semin Cell Dev Biol*, *18*, 101-110.
43. Snider P., Olaopa M., Firulli A. B., Conway S. J. (2007). Cardiovascular development and the colonizing cardiac neural crest lineage. *ScientificWorldJournal*, *7*, 1090-1113.
44. Stottmann R. W., Choi M., Mishina Y., Meyers E. N., Klingensmith J. (2004). BMP receptor IA is required in mammalian neural crest cells for development of the cardiac outflow tract and ventricular myocardium. *Development*, *131*, 2205-2218.
45. Stoller J. Z., Epstein J. A. (2005). Cardiac neural crest. *Semin Cell Dev Biol*, *16*, 704-715.

46. Kappetein A. P., Gittenberger-de Groot A. C., Zwinderman A. H., Rohmer J., Poelmann R. E., Huysmans H. A. (1991). The neural crest as a possible pathogenetic factor in coarctation of the aorta and bicuspid aortic valve. *J Thorac Cardiovasc Surg*, *102*, 830-836.
47. Nakamura T., Colbert M. C., Robbins J. (2006). Neural crest cells retain multipotential characteristics in the developing valves and label the cardiac conduction system. *Circ Res*, *98*, 1547-1554.
48. El-Helou V., Beguin P. C., Assimakopoulos J., et al. (2008). The rat heart contains a neural stem cell population; role in sympathetic sprouting and angiogenesis. *J Mol Cell Cardiol*, *45*, 694-702.
49. Wu Y., Deng J., Rychahou P. G., Qiu S., Evers B. M., Zhou B. P. (2009). Stabilization of snail by NF-kappaB is required for inflammation-induced cell migration and invasion. *Cancer Cell*, *15*, 416-428.
50. Radisky D. C., LaBarge M. A. (2008). Epithelial-mesenchymal transition and the stem cell phenotype. *Cell Stem Cell*, *2*, 511-512.
51. Mani S. A., Guo W., Liao M. J., et al. (2008). The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell*, *133*, 704-715.
52. Kalluri R., Neilson E. G. (2003). Epithelial-mesenchymal transition and its implications for fibrosis. *J Clin Invest*, *112*, 1776-1784.
53. Wynn T. A. (2008). Cellular and molecular mechanisms of fibrosis. *J Pathol*, *214*, 199-210.
54. Zeisberg E. M., Tarnavski O., Zeisberg M., et al. (2007). Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. *Nat Med*, *13*, 952-961.
55. Limana F., Zacheo A., Mocini D., et al. (2007). Identification of myocardial and vascular precursor cells in human and mouse epicardium. *Circ Res*, *101*, 1255-1265.
56. Quaini F., Urbanek K., Beltrami A. P., et al. (2002). Chimerism of the transplanted heart. *N Engl J Med*, *346*, 5-15.
57. Bayes-Genis A., Muniz-Diaz E., Catasus L., et al. (2004). Cardiac chimerism in recipients of peripheral-blood and bone marrow stem cells. *Eur J Heart Fail*, *6*, 399-402.

58. Minami E., Laflamme M. A., Saffitz J. E., Murry C. E. (2005). Extracardiac progenitor cells repopulate most major cell types in the transplanted human heart. *Circulation*, *112*, 2951-2958.
59. Glaser R., Lu M. M., Narula N., Epstein J. A. (2002). Smooth muscle cells, but not myocytes, of host origin in transplanted human hearts. *Circulation*, *106*, 17-19.
60. Laflamme M. A., Myerson D., Saffitz J. E., Murry C. E. (2002). Evidence for cardiomyocyte repopulation by extracardiac progenitors in transplanted human hearts. *Circ Res*, *90*, 634-640.
61. Wagers A. J., Sherwood R. I., Christensen J. L., Weissman I. L. (2002). Little evidence for developmental plasticity of adult hematopoietic stem cells. *Science*, *297*, 2256-2259.
62. Alvarez-Dolado M., Pardal R., Garcia-Verdugo J. M., et al. (2003). Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. *Nature*, *425*, 968-973.
63. Frisen J. (2002). Stem cell plasticity? *Neuron*, *35*, 415-418.
64. Zampetaki A., Kirton J. P., Xu Q. (2008). Vascular repair by endothelial progenitor cells. *Cardiovasc Res*, *78*, 413-421.
65. Hansson E. M., Lindsay M. E., Chien K. R. (2009). Regeneration next: toward heart stem cell therapeutics. *Cell Stem Cell*, *5*, 364-377.
66. Beltrami A. P., Barlucchi L., Torella D., et al. (2003). Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell*, *114*, 763-776.
67. Massberg S., Schaerli P., Knezevic-Maramica I., et al. (2007). Immunosurveillance by hematopoietic progenitor cells trafficking through blood, lymph, and peripheral tissues. *Cell*, *131*, 994-1008.
68. Pouly J., Bruneval P., Mandet C., et al. (2008). Cardiac stem cells in the real world. *J Thorac Cardiovasc Surg*, *135*, 673-678.
69. Koninckx R., Hensen K., Rummens J. L., Hendrikx M. (2008). Cardiac stem cells in the real world. *J Thorac Cardiovasc Surg*, *136*, 797-798; author reply 798.
70. Tallini Y. N., Greene K. S., Craven M., et al. (2009). c-kit expression identifies cardiovascular precursors in the neonatal heart. *Proc Natl Acad Sci U S A*, *106*, 1808-1813.

71. Chimenti I., Gaetani R., Barile L., Frati G., Messina E., Giacomello A. (2009). c-kit cardiac progenitor cells: what is their potential? *Proc Natl Acad Sci U S A*, *106*, E78; author reply E79.
72. Oh H., Bradfute S. B., Gallardo T. D., et al. (2003). Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. *Proc Natl Acad Sci U S A*, *100*, 12313-12318.
73. Matsuura K., Nagai T., Nishigaki N., et al. (2004). Adult cardiac Sca-1-positive cells differentiate into beating cardiomyocytes. *J Biol Chem*, *279*, 11384-11391.
74. Matsuura K., Honda A., Nagai T., et al. (2009). Transplantation of cardiac progenitor cells ameliorates cardiac dysfunction after myocardial infarction in mice. *J Clin Invest*, *119*, 2204-2217.
75. van Vliet P., Roccio M., Smits A. M., et al. (2008). Progenitor cells isolated from the human heart: a potential cell source for regenerative therapy. *Neth Heart J*, *16*, 163-169.
76. Smits A. M., van Laake L. W., den Ouden K., et al. (2009). Human cardiomyocyte progenitor cell transplantation preserves long-term function of the infarcted mouse myocardium. *Cardiovasc Res*, *83*, 527-535.
77. Laugwitz K. L., Moretti A., Lam J., et al. (2005). Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. *Nature*, *433*, 647-653.
78. Chien K. R., Domian I. J., Parker K. K. (2008). Cardiogenesis and the complex biology of regenerative cardiovascular medicine. *Science*, *322*, 1494-1497.
79. Wu S. M., Chien K. R., Mummery C. (2008). Origins and fates of cardiovascular progenitor cells. *Cell*, *132*, 537-543.
80. Ott H. C., Matthiesen T. S., Brechtken J., et al. (2007). The adult human heart as a source for stem cells: repair strategies with embryonic-like progenitor cells. *Nat Clin Pract Cardiovasc Med*, *4 Suppl 1*, S27-39.
81. Hierlihy A. M., Seale P., Lobe C. G., Rudnicki M. A., Megeney L. A. (2002). The post-natal heart contains a myocardial stem cell population. *FEBS Lett*, *530*, 239-243.
82. Castaldo C., Di Meglio F., Nurzynska D., et al. (2008). CD117-positive cells in adult human heart are localized in the subepicardium, and their activation is associated with laminin-1 and alpha6 integrin expression. *Stem Cells*, *26*, 1723-1731.

83. Limana F., Bertolami C., Mangoni A., et al. Myocardial infarction induces embryonic reprogramming of epicardial c-kit(+) cells: role of the pericardial fluid. *J Mol Cell Cardiol*, *48*, 609-618.
84. Wada A. M., Smith T. K., Osler M. E., Reese D. E., Bader D. M. (2003). Epicardial/Mesothelial cell line retains vasculogenic potential of embryonic epicardium. *Circ Res*, *92*, 525-531.
85. van Tuyn J., Atsma D. E., Winter E. M., et al. (2007). Epicardial cells of human adults can undergo an epithelial-to-mesenchymal transition and obtain characteristics of smooth muscle cells in vitro. *Stem Cells*, *25*, 271-278.
86. Eid H., Larson D. M., Springhorn J. P., et al. (1992). Role of epicardial mesothelial cells in the modification of phenotype and function of adult rat ventricular myocytes in primary coculture. *Circ Res*, *71*, 40-50.
87. Winter E. M., van Oorschot A. A., Hogers B., et al. (2009). A new direction for cardiac regeneration therapy: application of synergistically acting epicardium-derived cells and cardiomyocyte progenitor cells. *Circ Heart Fail*, *2*, 643-653.
88. Messina E., De Angelis L., Frati G., et al. (2004). Isolation and expansion of adult cardiac stem cells from human and murine heart. *Circ Res*, *95*, 911-921.
89. Davis D. R., Zhang Y., Smith R. R., et al. (2009). Validation of the cardiosphere method to culture cardiac progenitor cells from myocardial tissue. *PLoS One*, *4*, e7195.
90. Barile L., Cerisoli F., Frati G., et al. (2009). Bone marrow-derived cells can acquire cardiac stem cells properties in damaged heart. *J Cell Mol Med*.
91. Smith R. R., Barile L., Cho H. C., et al. (2007). Regenerative potential of cardiosphere-derived cells expanded from percutaneous endomyocardial biopsy specimens. *Circulation*, *115*, 896-908.
92. Johnston P. V., Sasano T., Mills K., et al. (2009). Engraftment, differentiation, and functional benefits of autologous cardiosphere-derived cells in porcine ischemic cardiomyopathy. *Circulation*, *120*, 1075-1083, 1077 p following 1083.
93. Littlewood T. D., Hancock D. C., Danielian P. S., Parker M. G., Evan G. I. (1995). A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. *Nucleic Acids Res*, *23*, 1686-1690.

94. Bearzi C., Rota M., Hosoda T., et al. (2007). Human cardiac stem cells. *Proc Natl Acad Sci U S A*, *104*, 14068-14073.
95. Smith R. R., Chimenti I., Marban E. (2008). Unselected Human Cardiosphere-derived Cells are Functionally Superior to c-Kit- or CD90-Purified Cardiosphere-derived Cells. *in AHA*
Scientific Session 2008 2008 Orlando, FL, USA: published in Circulation 118:p S_420.
96. Abremski K., Hoess R. (1984). Bacteriophage P1 site-specific recombination. Purification and properties of the Cre recombinase protein. *J Biol Chem*, *259*, 1509-1514.
97. Ghosh K., Van Duyne G. D. (2002). Cre-loxP biochemistry. *Methods*, *28*, 374-383.
98. Abremski K., Wierzbicki A., Frommer B., Hoess R. H. (1986). Bacteriophage P1 Cre-loxP site-specific recombination. Site-specific DNA topoisomerase activity of the Cre recombination protein. *J Biol Chem*, *261*, 391-396.
99. Friedrich G., Soriano P. (1991). Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice. *Genes Dev*, *5*, 1513-1523.
100. Zambrowicz B. P., Imamoto A., Fiering S., Herzenberg L. A., Kerr W. G., Soriano P. (1997). Disruption of overlapping transcripts in the ROSA beta geo 26 gene trap strain leads to widespread expression of beta-galactosidase in mouse embryos and hematopoietic cells. *Proc Natl Acad Sci U S A*, *94*, 3789-3794.
101. Soriano P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet*, *21*, 70-71.
102. Han H., Tanigaki K., Yamamoto N., et al. (2002). Inducible gene knockout of transcription factor recombination signal binding protein-J reveals its essential role in T versus B lineage decision. *Int Immunol*, *14*, 637-645.
103. Sohal D. S., Nghiem M., Crackower M. A., et al. (2001). Temporally regulated and tissue-specific gene manipulations in the adult and embryonic heart using a tamoxifen-inducible Cre protein. *Circ Res*, *89*, 20-25.
104. Koitabashi N., Bedja D., Zaiman A. L., et al. (2009). Avoidance of transient cardiomyopathy in cardiomyocyte-targeted tamoxifen-induced MerCreMer gene deletion models. *Circ Res*, *105*, 12-15.

105. Chen J., Kubalak S. W., Chien K. R. (1998). Ventricular muscle-restricted targeting of the RXRalpha gene reveals a non-cell-autonomous requirement in cardiac chamber morphogenesis. *Development*, *125*, 1943-1949.
106. Banerjee I., Fuseler J. W., Price R. L., Borg T. K., Baudino T. A. (2007). Determination of cell types and numbers during cardiac development in the neonatal and adult rat and mouse. *Am J Physiol Heart Circ Physiol*, *293*, H1883-1891.
107. Lee B. Y., Han J. A., Im J. S., et al. (2006). Senescence-associated beta-galactosidase is lysosomal beta-galactosidase. *Aging Cell*, *5*, 187-195.
108. Ma Q., Zhou B., Pu W. T. (2008). Reassessment of Isl1 and Nkx2-5 cardiac fate maps using a Gata4-based reporter of Cre activity. *Dev Biol*, *323*, 98-104.
109. Shenje L. T., Field L. J., Pritchard C. A., et al. (2008). Lineage tracing of cardiac explant derived cells. *PLoS One*, *3*, e1929.
110. Manasek F. J. (1969). Embryonic development of the heart. II. Formation of the epicardium. *J Embryol Exp Morphol*, *22*, 333-348.
111. Zhou B., von Gise A., Ma Q., Rivera-Feliciano J., Pu W. T. (2008). Nkx2-5- and Isl1-expressing cardiac progenitors contribute to proepicardium. *Biochem Biophys Res Commun*, *375*, 450-453.
112. Yang J. T., Rayburn H., Hynes R. O. (1995). Cell adhesion events mediated by alpha 4 integrins are essential in placental and cardiac development. *Development*, *121*, 549-560.
113. Kwee L., Baldwin H. S., Shen H. M., et al. (1995). Defective development of the embryonic and extraembryonic circulatory systems in vascular cell adhesion molecule (VCAM-1) deficient mice. *Development*, *121*, 489-503.
114. Mahtab E. A., Wijffels M. C., Van Den Akker N. M., et al. (2008). Cardiac malformations and myocardial abnormalities in podoplanin knockout mouse embryos: Correlation with abnormal epicardial development. *Dev Dyn*, *237*, 847-857.
115. Martinez-Estrada O. M., Lettice L. A., Essafi A., et al. Wt1 is required for cardiovascular progenitor cell formation through transcriptional control of Snail and E-cadherin. *Nat Genet*, *42*, 89-93.
116. Mercader J. M. G.-R. a. N. (2009). The epicardium: development, differentiation and its role during heart regeneration. *NATURE REVIEWS | CARDIOLOGY (CNIC EDITION)*, *6*, 67-72.

117. Christoffels V. M., Grieskamp T., Norden J., Mommersteeg M. T., Rudat C., Kispert A. (2009). Tbx18 and the fate of epicardial progenitors. *Nature*, *458*, E8-9; discussion E9-10.
118. Stuckmann I., Evans S., Lassar A. B. (2003). Erythropoietin and retinoic acid, secreted from the epicardium, are required for cardiac myocyte proliferation. *Dev Biol*, *255*, 334-349.
119. Lavine K. J., Yu K., White A. C., et al. (2005). Endocardial and epicardial derived FGF signals regulate myocardial proliferation and differentiation in vivo. *Dev Cell*, *8*, 85-95.
120. Ieda M., Tsuchihashi T., Ivey K. N., et al. (2009). Cardiac fibroblasts regulate myocardial proliferation through beta1 integrin signaling. *Dev Cell*, *16*, 233-244.
121. Smart N., Risebro C. A., Melville A. A., et al. (2007). Thymosin beta4 induces adult epicardial progenitor mobilization and neovascularization. *Nature*, *445*, 177-182.
122. Bock-Marquette I., Saxena A., White M. D., Dimaio J. M., Srivastava D. (2004). Thymosin beta4 activates integrin-linked kinase and promotes cardiac cell migration, survival and cardiac repair. *Nature*, *432*, 466-472.
123. Mannherz H. G., Hannappel E. (2009). The beta-thymosins: intracellular and extracellular activities of a versatile actin binding protein family. *Cell Motil Cytoskeleton*, *66*, 839-851.
124. Urayama K., Dedeoglu D. B., Guilini C., et al. (2009). Transgenic myocardial overexpression of prokineticin receptor-2 (GPR73b) induces hypertrophy and capillary vessel leakage. *Cardiovasc Res*, *81*, 28-37.
125. Urayama K., Guilini C., Messaddeq N., et al. (2007). The prokineticin receptor-1 (GPR73) promotes cardiomyocyte survival and angiogenesis. *Faseb J*, *21*, 2980-2993.
126. Urayama K., Guilini C., Turkeri G., et al. (2008). Prokineticin receptor-1 induces neovascularization and epicardial-derived progenitor cell differentiation. *Arterioscler Thromb Vasc Biol*, *28*, 841-849.
127. Bock-Marquette I., Shrivastava S., Pipes G. C., et al. (2009). Thymosin beta4 mediated PKC activation is essential to initiate the embryonic coronary developmental program and epicardial progenitor cell activation in adult mice in vivo. *J Mol Cell Cardiol*, *46*, 728-738.

128. Chimenti I., Smith R. R., Li T. S., et al. Relative roles of direct regeneration versus paracrine effects of human cardiosphere-derived cells transplanted into infarcted mice. *Circ Res*, *106*, 971-980.
129. MacNeill C., French R., Evans T., Wessels A., Burch J. B. (2000). Modular regulation of cGATA-5 gene expression in the developing heart and gut. *Dev Biol*, *217*, 62-76.
130. Merki E., Zamora M., Raya A., et al. (2005). Epicardial retinoid X receptor alpha is required for myocardial growth and coronary artery formation. *Proc Natl Acad Sci U S A*, *102*, 18455-18460.
131. Miwa H., Beran M., Saunders G. F. (1992). Expression of the Wilms' tumor gene (WT1) in human leukemias. *Leukemia*, *6*, 405-409.
132. Li T. S., Cheng K., Lee S. T., et al. Cardiospheres recapitulate a niche-like microenvironment rich in stemness and cell-matrix interactions, rationalizing their enhanced functional potency for myocardial repair. *Stem Cells*, *28*, 2088-2098.
133. Li R., Liang J., Ni S., et al. A mesenchymal-to-epithelial transition initiates and is required for the nuclear reprogramming of mouse fibroblasts. *Cell Stem Cell*, *7*, 51-63.
134. Li D., Zhou J., Wang L., et al. Integrated biochemical and mechanical signals regulate multifaceted human embryonic stem cell functions. *J Cell Biol*, *191*, 631-644.
135. Caja L., Bertran E., Campbell J., Fausto N., Fabregat I. The transforming growth factor-beta (TGF-beta) mediates acquisition of a mesenchymal stem cell-like phenotype in human liver cells. *J Cell Physiol*.

NON STANDARD ABBREVIATION AND ACRONYMS

ACM: adult cardiomyocyte

AV: atrioventricular

BM: bone marrow

CDC: CSp-derived cell

CEM: complete explants medium

CMPC: cardiomyocytes progenitor cell

CPC: cardiac progenitor cell

Cr: Cripto

CSC: cardiac stem cell

CSM: cardiosphere medium

CSp: cardiosphere

DAB: 3,3'-Diaminobenzidine chromogen

EB: embryoid body

EDC: explant derived cell

EPDC: epicardial derived progenitor cell

ESC: embryonic stem cell

EMT: Epithelial-to- mesenchymal transition

EndMT: endothelial-to-mesenchymal transition

Fb: fibroblast

H3P: histone 3 phosphorylated

IICSp: secondary CSp

MET: mesenchymal-to-epithelial transition

MF20: meromyosin

MHC: myosin heavy chain

MI: myocardial infarction

MLC2v: myosin light chain 2v

NC: neural crest

SMA: smooth muscle actin

SMC: smooth muscle cell

TAM: Tamoxifen

TnT: troponin I

Tmsb4: Thymosin β4

Wt1: Wilm's tumor-1

vWF: von Willembrand factor

ACKNOWLEDGEMENTS

This work has been conducted in part at the Department of Molecular Medicine, "Sapienza" University of Rome, with funding provided by: "Istituto Pasteur – Fondazione Cenci Bolognetti" to Prof. Alessandro Giacomello; and in part in collaboration with Doc. Mark Mercola at the Del E. Webb Neuroscience, Aging and Stem Cell Research Center of the Burnham Institute for Medical Research of La Jolla, San Diego (CA).

I would like to thank my tutor, Prof. Alessandro Giacomello, for his continuous support and Dr Elisa Messina for her help and critical discussions. I started my training in their lab over six years ago and they introduced me to science, giving me the opportunity to work and collaborate also with other prestigious labs. I want to thank all the lab members starting from Isotta Chimenti for her endless support and precious advises, Lucio Barile, Roberto Gaetani, and also Vittoria, Francesco, Silvia e Simone, because all of them, in a way or the other gave me something; all the Professors who organized our PhD course, especially Prof. Paola Vittorioso and Marco Tripodi for their guidance and suggestions.

I'm very thankful to Doc. Mark Mercola for hosting me in his lab as a visiting graduate student, giving me the chance to experience an extraordinarily active scientific environment, attending seminars and lectures on the latest scientific findings. I'm grateful to all members of Mercola's lab, in particular to my mentor Dr. Ramon Diaz Trelles who thought me how to work with transgenic mice and all the related techniques; helped me in the critical interpretation of the results, in designing experiments and spent so much time patiently answering all my questions. Dr. Erik Willems helped me with the co-culture experiments (mES and CSps forming cells), thought me how to work with mESCs and helped me with the FACS analysis for another project I didn't have time to finish. I want to thank also Serena, Paola, Fabio, Christian, Masa, Wequing, Ke, Agustin, Christine, Natalia, Karen, Alex, Paul, Ruchika, Fred, Denise, Sean, Brandon, Cecilia, all extraordinary lab mates, and all the special friends I've met in San Diego which have been as a family to me, while abroad. Last but not least I would like to thank my parents and my sister Giovanna, because without their loving support all of this wouldn't have been possible; all my family especially my grandfather because I know he was proud of me, even though he couldn't wait to see me back.