Introduction 3
The adult heart as a terminally differentiated organ with limited proliferation capacity ... 3
The heart as a self-renewing organ 6
Cardiac Stem/Progenitor cells 8
C-kit^{+} cardiac stem/progenitor cells 8
Sca-1 + cardiac progenitor cells 10
Cardiac Isl1+ progenitor cells in embryonic and postnatal 11
Cardiac side population cells 12
Homeostasis of CSP cells under physiologic and injured conditions and the role of the bone marrow 18
A hierarchy model of stem cell biology 21
The Side Population phenotype as a stem cell marker 22
ABC transporters are the molecular determinants of the SP phenotype 24
ABC transporters as protective mechanisms of stem/progenitor cells 26
The goal of this thesis 28
Materials and methods 31
Materials 31
Methods 32
Molecular Biology Techniques 32
Isolation of total eukaryotic RNA 32
DNAse treatment 33
Reverse transcription reaction 33
Polymerase Chain Reaction (PCR) and Real-Time PCR 33
Agarose gel electrophoresis 34
Animal experimental protocol 35
Cell culture techniques 36
Isolation and immunostaining of Cardiac Side Population cells 36
Immunocytochemistry 37
FACS analysis 38
Propidium Iodide (PI) staining 38

Maintenance of cell cultures of CSP cells 38
Cardiac Side Population Co-Cultures with adult rat cardiomyocytes 39
Duplication assay 39
Cell counting 40
Heat inactivation of FBS 40
Maintenance of expanded Cardiac Side Population cells in liquid nitrogen 40
List of antibodies 41
Results 42
In vitro expansion of CSP cells 42
Determination of the duplication rate 43
Gene expression analysis of in vitro expanded CSP cells 45
In vitro expanded CSP cells retain the ability to efflux the Hoechst dye 52
Cardiomyogenic differentiation potential of in vitro expanded CSPs 53
Spontaneous differentiation of CSP cells 57
Cell fusion studies 58
Study of the effects of myocardial injury on the proliferation status of the CSP cells 59
Discussion 62
Characterization of expanded cardiac side population cells 62
Expansion of csp cells 65
Cardiomyogenic differentiation capacity of expanded cardiac side population cells 66
Effects of MI on CSP cells proliferation in vivo 67
Bibliography 70

Introduction

The adult heart as a terminally differentiated organ with limited proliferation capacity

During the last 60 years the prevailing theory in the field of cardiology was that the proliferation of cardiomyocytes ceases after birth. According to this theory the cardiomyocytes shortly after birth exit the cell cycle and remain arrested in a non-proliferative situation. The only widely acceptable way of cardiac growth in an adult human was the enlargement of the cardiomyocyte size, hypertrophy, but not an enlargement of cell number, hyperplasia. So it became a general belief that the cardiomyocytes of an adult heart are terminally differentiated and that the adult heart, similarly with the brain, does not possess any self-renewal ability. So according to the above generally accepted idea the age of the cardiomyocytes in a human individual is the same with the age of the individual at any time point of its life.

This theory was based mainly on studies of DNA synthesis of fetal and adult cardiomyocytes. BrdU labeling and 3[H] thymidine incorporation were used as indications of newly formed DNA in cardiomyocytes and subsequently as indications of proliferation. Soonpaa *et al* [1]demonstrated, by 3[H] thymidine incorporation assays

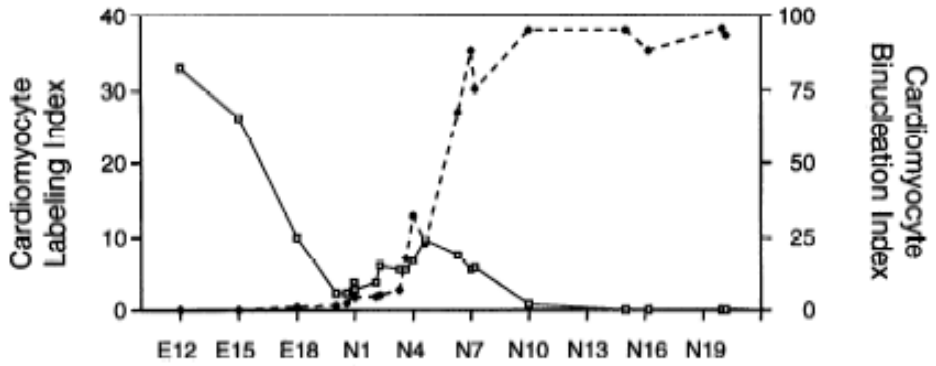

Figure 1: Cardiomyocyte proliferation and binucleation indexes during late fetal and early post-natal life. Data are obtained by measuring [H] ^{3} incorporation in the cardiomyocyte nucleus and by counting the number of nuclei in each cardiomyocyte. Adapted from [1].

that there are two different periods in the life of a mouse cardiomyocyte where elevated DNA synthesis is detected (Figure 1). The first period is during the fetal life before embryonic day 12 and that synthesis of DNA was resulted in myocyte cell proliferation. From embryonic day 12 until birth and until further post-natal day 4 the DNA synthesis index is decreases eventually to almost undetectable levels. At post-natal day 4 a second round of DNA synthesis is taking place but the outcome this time is only binucleation of the existing cardiomyocytes and not cell proliferation. Finally at post-neonatal day 10 there were no detectable levels of 3[H] thymidine positive myocytes. So during the adult life of mice there is no detected evidence of cardiomyocyte proliferation [1].

The above conclusion was in accordance with another study of the same group that evaluated the percentages of cardiomyocytes undergoing DNA synthesis under homeostatic and post-injury conditions. In that study were used transgenic mice overexpressing nuclear-localized p-galactosidase (P-Gal) reporter gene exclusively in the myocardium (myosin heavy chain, MHC, driven P-Gal expression). The expression of MHC-P-Gal in combination with the incorporation of [H^3] thymidine were the indications for the presence of myocytes that synthesize DNA. In the non-injured hearts the detected index of proliferation was one cardiomyocyte in 180.000 total analyzed cells or approximately 0.0006%. The synthesis of DNA in injured heart was assessed at various time points using a cautery myocardial damage model. At days 1 and 3 post-injury there no cardiomyocytes positive for incorporation of [H^3] thymidine were detected neither at the border nor at the distal area from the injury. One week post-injury in the peri-injury area the cardiomyocyte labeling index was 0.008%, in the distal area was $\leq$0.0006 and in the atrium was 0.005%. In contrast with these inappreciable differences of positively stained cardiomyocytes there was observed a 20% elevation in mean ventricular cardiomyocyte surface area in cells prepared from injured animals. The above results strongly suggest that the prevalent response mechanism of the adult heart after injury is hypertrophy and not hyperplasia. The possibility that some DNA synthesis takes place in adult cardiomyocyte can not be excluded but it is rather linked with multinucleation (karyokinesis) and not with proliferation (no cytokinesis) [2].

However, there are several reports of BrdU labeling assays in other species, as rats, that show significant differences in the fraction of adult cardiomyocytes that synthesize DNA. Interestingly, and in contrast with what happens in mice, adult rat ventricular myocytes demonstrate elevated levels of BrdU staining. Different groups report that 0.2% [3], 0.45% [4], 0.9% [5] and 2% [6] of adult rat cardiomyocytes are stained positively for BrdU. Additionally to the above reports there are others about myocytes undergoing mitosis in adult rats [7]. Therefore it is reasonable to support that in the heart of adult rats there is a low level of DNA synthesis and this phenomenon is not related exclusively to myocyte binucleation but also to myocyte proliferation (Figure 2).

The observed differences between mice and rats in terms of adult cardiomyocyte DNA synthesis could not be explained based on the myocyte cell volume and myocyte size as they both remain relatively constant in these species [8-11]. So the myocyte cell number makes the critical difference between the two species and it is reasonable to assume that in rats the DNA synthesis and myocyte proliferation is taking place even in adult animals [11].

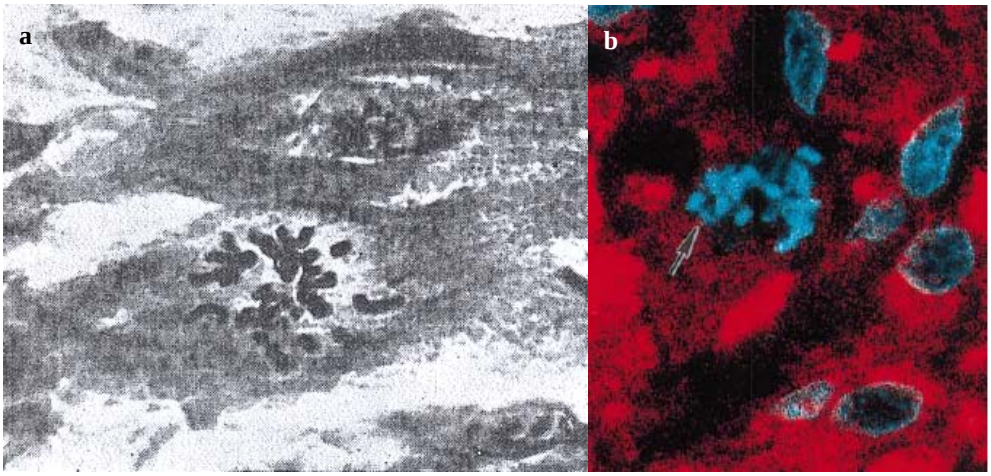

Figure 2: Mitotic images of adult cardiomyocytes. 2a: Representation of rabbit chromosomes in metaphase. (Adapted from [12] 2b: Representation of nuclear mitotic division in adult rat myocytes located in a distant region of the infarct area. Adapted from [11].

The heart as a self-renewing organ

The presence of cycling myocytes in the adult normal and failing heart is a very strong argument against the view of the cardiac tissue as a post-mitotic organ comprised only by terminally differentiated cardiomyocytes. The accumulation of data against the above theory continued with several studies of sex-mismatched heart transplantation reporting the detection of (non-cardiac) host-derived newly formed cardiomyocytes.

In one of these reports Muller *et al* , using in-situ hybridization with specific probes for X and Y chromosomes, studied 21 biopsies from 13 male heart transplant recipients who received organs from female donors [13]. Totally 31.787 cardiomyocytes were examined and 0.16±0.04% (mean ± SEM, median value 0.09%) were of recipient origin. The host derived cardiomyocytes were an active part of the transplanted myocardium as they were connected with gap junction with the adjacent cardiomyocytes. Host-derived endothelial and smooth muscle cells were identified in addition to myocytes. These results of cardiac chimerism were confirmed by other groups [14-16] implying that in the adult humans there is a non-cardiac source of cells with ability to differentiate *in vivo* to three main cardiac cell types. However there is a degree of uncertainty about the level of myocyte chimerism in cardiac transplants. Glaser et al [17] reported the existence of only vascular smooth muscle cells of host origin in human cardiac sex-mismatched transplants but no evidence of host-derived cardiomyocytes. Additional studies are required to determine the degree of cardiac smooth muscle cell, endothelial and myocyte chimerism and to identify the factors that modulate this process.

The detection of mitotic figures of cardiomyocytes in the adult heart and the host-origin derived cardiomyocytes raised questions about the origin and the identity of these newly formed cells. It is unlikely that mature cardiomyocytes are going backwards the differentiation pathway and disassemble their well-organized sarcomeric structures to reenter the cell cycle. This biologically too complex process would be disadvantageous to the heart function [18]. Additionally it is well documented that the adult myocytes are withdrawn from the cell cycle in an irreversible way. So the proliferating cardiomyocytes in the adult heart could be originated from a non cardiac endogenous cell

population as the heart transplantation studies revealed [13]. An alternative explanation is an existing endogenous stem/progenitor cell population in the adult heart with a cardiomyogenic differentiation capacity. The neural tissue in adult mice and humans was another example of a solid organ that was believed to lack any regenerative ability up to the identification of the neural stem/progenitor cells [19].

Cardiac Stem/Progenitor cells

C-kit$^+$ cardiac stem/progenitor cells

In parallel with the sex-mismatched transplantation studies an extensive effort was started to discover the presence of a previously faceless special type of cell, the endogenous cardiac stem/progenitor cell. in the adult myocardium of several animal models (rat, mouse, dog, pig and human) expression of c-kit$^+$, MDR$^+$ and Sca1$^+$ was detected by immunohistochemistry (Figure 3) [20, 21]. Subsequently these cells were isolated using the c-kit antigen by either immuno-magnetic micro beads or by FACS sorting. C-kit+ cells were negative for the expression of any marker of mature cardiomyocytes, smooth muscle cells, endothelial cells or fibroblasts.

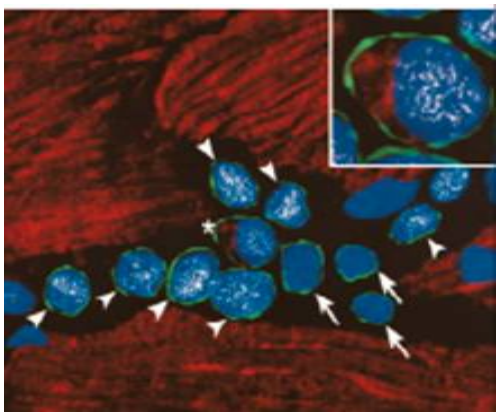

Figure 3: Cardiac stem cells and early committed progenitor cells. The arrows indicate cells expressing c-kit (green). The arrowheads indicate progenitor cells expressing c-kit and Nkx2.5 (white). The asterisk indicates an early committed cell expressing c-kit and a-actinin. The nuclei are shown in blue. Adapted from [20].

The freshly isolated c-kit$^+$ cells were also negative for the expression of any hematopoietic marker, as CD45 and CD34, suggesting their non-hematopoietic phenotype. 7-10% of the c-kit$^+$ cells were expressing GATA-4, Nkx2.5 and MEF2, which are important transcription factors for the myocyte lineage. Finally no marker

specific for the skeletal muscle lineage was expressed in c-kit$^+$ cells. Quantification studies demonstrated that there is approximately 1 cell per ~30000-40000 myocardial cells expressing any of c-kit$^+$, mdr1$^+$ and Sca1$^+$ markers. 65% of the cardiac stem cells express all the three antigens, ~20% express only two and ~15% express only one. Finally 5% of all cardiac stem cells express exclusively one of the c-kit$^+$, mdr1$^+$ and Sca1$^+$ stem cell markers [18].

Cardiac stem cells expressing c-kit$^+$, mdr1$^+$ and Sca1$^+$ were expanded in a modified media that was specific for neural stem cells. The doubling time of the c-kit$^+$/mdr1$^+$/Sca1$^+$ cells was approximately 40 hours and almost 80% of the population was cycling, as suggested by Ki67 staining. The c-kit$^+$/mdr1$^+$/Sca1$^+$ cells were examined for their differentiation potential towards the main cardiac cell lineages. Under appropriate culture conditions the c-kit$^+$/mdr1$^+$/Sca1$^+$ cells differentiated to endothelial cells, smooth muscle cells, cardiomyocytes and fibroblasts. In contrast there was no evidence of differentiation towards to any type of skeletal muscle, blood and neural cell type [20].

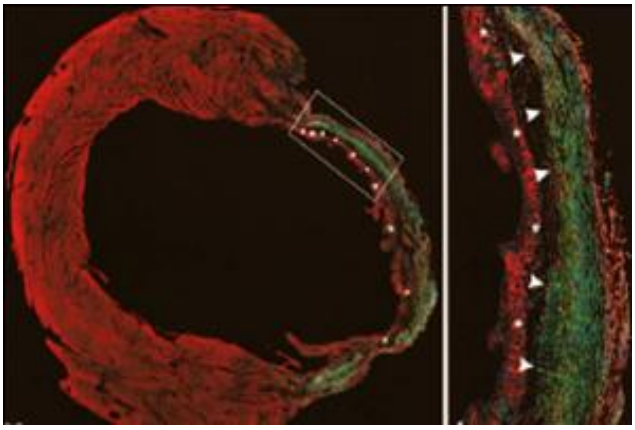

Figure 4: Regenerating myocardium (green, arrowheads) of adult rat at 10 days after injection of EGFP-clonogenic c-kit$^+$ cells. Adapted from [20].

Subsequently their ability to home and differentiate into functional myocardium was evaluated *in vivo* (Figure 4). For this study the model of myocardial infarction was used (described in the section of materials and methods) and approximately 200000 c-

kit$^+$/mdr1$^+$/Sca1$^+$ cells, labeled with BrdU, were injected into two opposite regions bordering the infarct. At 10 and 20 days post-MI the animals were sacrificed and a band of BrdU-labeled regenerating myocardium it was detected in nine of twelve treated infarcts at day 10 and in all the treated infarcts at day 20 post-MI. The regenerated tissue was composed by both BrdU positive myocytes and endothelial cells as revealed by immunohistochemistry. The newly formed myocardium significantly improved the performance of the heart. The infarcted area was contracting, the infarct size was reduced and all the hemodynamic parameters were improved in comparison with the control animals [20]. Hence the c-kit$^+$/mdr1$^+$/Sca1$^+$ cells represent an endogenous multipotent cardiac stem cell population capable to differentiate to all cardiac cell lineages *in vitro* and *in vivo*. More importantly administration of c-kit$^+$/mdr1$^+$/Sca1$^+$ cells after severe cardiac injury is improving the heart performance by regenerating the myocardium.

Sca-1 + cardiac progenitor cells

Another very interesting putative cardiac progenitor cell population was identified based on of the expression of the marker Sca1$^+$. This newly discovered population represents approximately 14-17% of the total cardiomyocyte depleted heart population [22]. The cardiac Sca1$^+$ cell population is negative for the expression of any hematopoietic markers. They also do not express the c-kit stem cell antigen and the transcription factor Nkx2.5 but they do express the factors GATA-4 and MEF2C. Most cardiac Sca1$^+$ cells are positive for the expression of another endothelial marker, CD31 or its receptor CD38, but they are also negative for the expression of other endothelial markers as the von willebrand factor (vWF) [22]. The expression of different types of markers by the Sca1$^+$ cells suggests that there is a degree of heterogeneity.

The cardiomyogenic capacity of the Sca1$^+$ cells was tested using the cytosine analog 5-azacytidine, which is a demethylation factor. The cells were treated with 3 μM of 5 azacytidine for 3 days and were analyzed for the expression of cardiomyocyte

markers at four weeks. 4.6±1.2% and 2.8±0.9% of the cells were expressing sarcomeric a-actinin and troponin-I respectively, as it was revealed by immunostaining [22].

Finally the homing and the *in vivo* differentiation capacity of this putative cardiac progenitor cell population was evaluated. Approximately 10^6 cardiac $Sca1^+$ cells, labeled with PKH2-GL, were injected via the jugular vein in mice that underwent occlusion of the coronary artery for one hour. At 1 day post-injection the administered cells (0.8± 0.05%) were detected in the myocardium and especially in the non-infarcted region. At 2 weeks post-injection the percentage of the delivered cells had been increased to 5.1±1.1%. Approximately 18.1±4.4% of PKH2-GL- $Sca1^+$-labeled cells were positive for sarcomeric a-actinin, as it was revealed by immunohistochemistry, suggesting clearly their differentiation to mature cardiomyocytes. [22].

By isolating $Sca1^+$ cells from a-MHC-Cre mice and using Lox-LacZ-Lox mice as recipients the option of fusion of the injected cells with endogenous cardiomyocytes was examined. The expression of LacZ can only be detected in the case where the injected cells, expressing Cre, fuse with cardiac cells containing the Lox-LacZ-Lox locus. Roughly 50% of the injected differentiated cells expressed LacZ suggesting that they were fused with cardiomyocytes. The fact that a significant (50%) fraction of the injected cells demonstrated differentiation capacity in an autonomous way strongly indicate that the $Sca1^+$ cells represent another putative cardiac stem/progenitor cell population. It would be very interesting to study the effects of that partially regenerated myocardium on the cardiac performance after the injury [22].

Cardiac Isl1+ progenitor cells in embryonic and postnatal

Recently another population of endogenous cardiac stem/progenitor cells was identified. The LIM-homeodomain transcription factor islet-1 (isl1) stains a cell population with considerable contribution in the formation of the embryonic heart and especially the right parts of the myocardium [23]. The isl1 cardioblasts are progressively decreased and after birth there are only relatively few still detectable (500-600 in the

heart of a 1-5-day old rat). These newly discovered cardioblasts can be expanded *in vitro* when co-cultured with a mesenchymal feeder layer promoting their self-renewal ability. Co-culture of the isl1 cardioblasts with neonatal myocytes demonstrated that the isl1 cells are capable of differentiating to mature cardiomyocytes. The differentiated isl1 cells express cardiomyocyte specific markers like sarcomeric a-actinin and exhibit normal cycling of Ca^{2+} in comparison with cardiomyocytes, indicating active contraction. The isl1 cardiac cardioblasts are also capable to differentiate towards the other cardiac cell lineages as endothelial cells and smooth muscle cells. Thus, they represent another putative cardiac stem/progenitor cell population that resides in the myocardium. However, the isl1 cells are abundant in embryonic life but later on at the early postnatal life their number in the myocardium decreases significantly. It is still undefined if the isl1 cells are detectable in adult myocardium and in what amount. Despite the above restriction, the isl1 cells are embryonic-like cardiac stem cells and it is possible that they hold great potential to differentiate towards all the cardiac cell types under appropriate stimulation. [23].

Cardiac side population cells

The first attempt to identify a multipotent cell population in the adult heart was carried out by Hierlihy *et al* [24] and consisted in studying whether the adult heart, possesses a cell population capable of exporting the DNA dye Hoechst 33342, the so called side population (SP).

The side population of cells was originally described in the bone marrow and was demonstrated that this small fraction of cells (approximately 0.05% in bone marrow) was enriched in long-term repopulating hematopoietic stem cells [25]. Since then the side population cells have been found in several other organ and tissues like skeletal muscle, mammary gland, liver, lung, brain, retina and skin [26] and in most cases they represent a cluster of cells with stem or progenitor cell characteristics. Side population cells can export the Hoechst dye 33342 (Hoechst negative or Hoechst low) in contrast to main

population (MP) cells that lack that efflux capacity (Hoechst positive or Hoechst high). The ability to export the Hoechst dye in cardiac side population cells is attributed mainly to two ABC-transporters, termed MDR-1 (P-glycoprotein) and BCRP-1 (ABCG2).

Therefore according to Hierlihy *et al* the cardiac side population (CSP) represents approximately 1% of the total cardiomyocyte-depleted heart population in an eight weeks old mouse. CSP cells do not express CD34, c-kit, Sca1, Flk-2 and Thy1.1 suggesting that they do not belong to the hematopoietic or the skeletal muscle lineage. They also possess the ability to form colonies in a methylcellulose media and when co-cultured with primary cardiomyocytes they demonstrate their cardiomyogenic potential by expressing connexin 43, a well established cardiomyocyte marker. Although, there is no information about the level of functional maturation (sarcomeric organization and contraction ability) of these CSP-derived cardiomyocytes [24], the CSPs cells represent a putative stem/progenitor cell population on the adult murine heart.

The existence of a CSP population in the adult myocardium was also reported by another group [27]. In agreement with the work by Hierlihy *et al*, CSP cells were negative for the expression of the markers c-kit, CD34 and CD45. In contrast, the expression of Sca1 was high and approximately 10% of CSP cells were positive for the expression of the endothelial marker Tie-2 [27]. Interestingly the presence of a specific inhibitor of the ABCG2 (BCRP1) ABC-transporter abolished the efflux capacity of the SP cells was abolished. This result suggested that the ABCG2 transporter is mainly responsible for the SP phenotype in the heart [27].

Martin *et al* tested the cardiomyogenic differentiation ability of the cells using a different model from Hierlihy *et al.* By co-culturing green fluorescence protein (GFP)-CSP cells with non GFP-MP cells for a period of two weeks, a-actinin positive GFP cells were detected by immunocytochemistry. The hematopoietic differentiation and proliferation capacity of the CSP was tested using a methylcellulose media colonies forming assay. Bone marrow SP cells were used as a positive control. The CSP cells proliferated and formed hematopoietic colonies [27]. The hematopoietic potential of CSP cells was also tested by Asakura *et al,* showing that they lack the capacity to give rise to

blood cells. Finally, the formation of characteristic structures of mature cardiomyocytes, as sarcomeres, was not determined in this report, similarly to the Hierlihy *et al* report.

Pfister *et al* [28] confirmed the existence of cardiac side population cells (Figure 5) in adult mice as a multipotent stem/progenitor cell population competent to differentiate into endothelial cells, smooth muscle cells and cardiomyocytes. CSP cells express 84±2 Sca1 and are negative for the expression of the hematopoietic markers CD34 and CD45 [28].

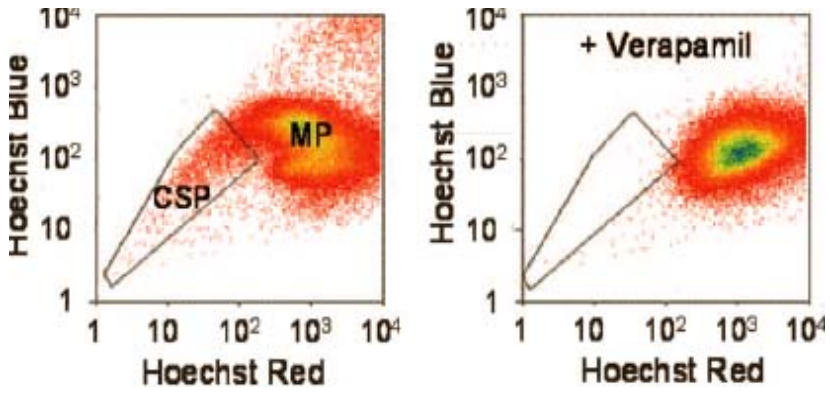

Figure 5: Representative figure of CSP cells. A: Following heart enzymatic digestion a single cell suspension is stained with Hoechst 33342 dye and is analyzed by FACS. The CSP cells are exporting the Hoechst 33342 dye so they appear as "Hoechst low" cells on the "side" of the main population, so-called "Hoechst high" cells. B: Incubation in the presence of the ABC-transporter inhibitor verapamil blocks the efflux process. Adapted from [28].

This result is in accordance with the previous reports about CSP cells, establishing their non-hematopoietic phenotype. The cell adhesion molecule CD44 and the c-kit stem cell marker were not detected in CSP cells. Several transcription factors, important for the cardiomyogenic differentiation [29, 30], as Nkx2-5 and GATA-4 were found to be expressed using RT-PCR. A-actinin and a-myosin heavy chain (a-MHC) were not detectable confirming the undifferentiated status of freshly isolated CSP cells. Interestingly enough CSP cells express only in the mRNA level the well established stem cell marker c-kit (unpublished data). The absence of c-kit expression in the protein level (c-kit was not detected by FACS analysis) could be the result of proteolytic cleavage of the extra-cellular part of the protein during the enzymatic digestion of the heart tissue.

CSP cells also express, in the mRNA level, smooth muscle actinin (SMA) and desmin as well as the endothelial cell marker Tie-2 [28].

The cell adhesion and endothelial marker CD31 (PECAM-1, platelet/endothelial cell adhesion molecule-1) was used in an effort to characterize further the cardiac SP cells. The expression of the CD31 antigen divides the CSP cells in two sub-populations. The cardiac SP population is comprised by approximately 25 % of $CD31^-/Sca1^+$ cells and 75% of $CD31^+/Sca1^+$ cells [28]. In contrast with previous reports [27] the portion of the SP band with endothelial-like characteristics is approximately 7-fold higher.

The next experiment was to test whether the CSP cells represent a multipotent cell population in the adult myocardium of mice. The cell culture models of long-term mono-culture and of co-culture with adult rat cardiomyocytes were used to assess the cardiomyogenic differentiation capacity of the CSP cells. The two CSP sub-populations, depending on the presence of the antigen CD31, CSP/ $CD31^+/Sca1^+$ cells and CSP/$CD31^-$ /$Sca1^+$ cells were cultured in comparison to each other. In the mono-culture model CSP cells were seeded on laminin-coated culture dishes. The CSP/ CD31+/Sca1+ cells failed to adhere on the culture plates and to express any marker of differentiation in both the differentiation assays [28].

In contrast, in the mono-culture model more than 30% of the CSP/$CD31^-/Sca1^+$ cells expressed both the muscle-specific transcription factors GATA-4 and MEF2C. At a latter time point CSP/CD31-/Sca1+ cells expressed the cardiomyocyte specific contractile proteins troponin-I and a-actinin. However the expression of these markers was not well organized in sarcomeric structures as in mature cardiomyocytes [28].

In the co-culture model the GFP^+/CSP/$CD31^+/Sca1^+$ cells and GFP^+/CSP/$CD31^-$ /$Sca1^+$ cells were plated onto rat cardiomyocytes for a period of 14-21 days. As in the previous model the GFP^+/CSP/$CD31^+/Sca1^+$ cells did not express any marker of differentiation. In agreement with the results from the mono-culture model the GFP^+/CSP/$CD31^-/Sca1^+$ cells express the muscle-specific transcription factors GATA-4 (64%) and MEF2C (35%). More than 10% of the GFP^+/CSP/$CD31^-/Sca1^+$ cells expressed a-actinin in a well-organized sarcomeric pattern (Figure 6). As a further evidence of the functional differentiation the GFP^+/CSP/$CD31^-/Sca1^+$ cells demonstrated

active contraction in the same manner as the co-cultured adult rat myocytes, as revealed by cell contractility and intracellular calcium transient assays. The differentiated $GFP^+/CSP/CD31^-/Sca1^+$ cells were expressing connexin 43 as an indication of functional coupling with the adult rat cardiomyocytes. The option of cell fusion was examined by two independent experiments using GFP-CSP cells co-cultured wit rat cardiomyocytes expressing red fluorescence protein (RFP) and using the Cre-Lox-P system. In both assays less than 2% of the differentiated CSP cells were attributed to fusion with cardiomyocytes. So although cell fusion can not be excluded the CSP cells possess an intrinsic ability for autonomous cardiomyogenic differentiation [28].

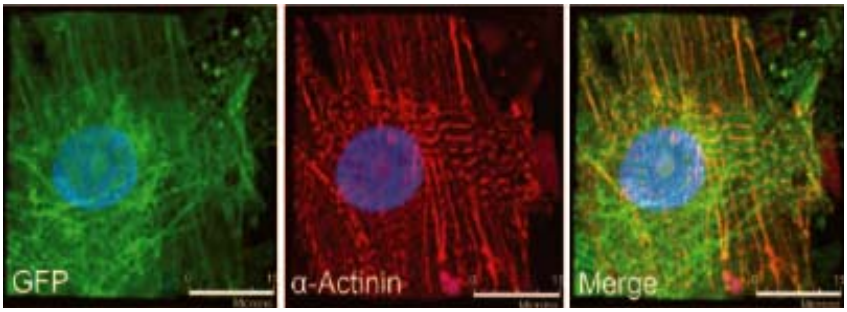

Figure 6: $GFP^+/CSP/CD31^-/Sca1^+$ cells co-cultured with adult rat cardiomyocytes differentiated in functional mature contracting cardiomyocytes exhibiting clear sarcomeric organization of a-actinin. Adapted from [28].

In parallel with the cardiomyogenic differentiation capacity of the CSP cells their capacity to differentiate towards other cell types was evaluated. In response to appropriate stimulation the CSP/CD31+/Sca1+ cells expressed vWF, a well established endothelial marker (unpublished data). Additionally they expressed tropomyosine, an indication of their capacity to form smooth muscle cells (unpublished data)[31]. Finally in methylcellulose colony forming assays $Sca1^+$ CSP cells demonstrated higher colony forming capacity in comparison with $Sca1^+$ CMP cells[28].

Among the different reports about cardiac side population cells there are some differences about their immunophenotypic profile and their abundance in the adult heart. The observed differences could be attributed to the variable parameters that can affect the isolation and sorting processes. It is noteworthy that the formation of fully differentiated

cardiomyocytes from CSP cells was only reported when CSP cells were co-cultured with adult cardiomyocytes [28]. The differentiation process of the CSP cells is dependent on the presence and probably the physical coupling between the CSP cells and the adult cardiomyocytes. Despite those differences the CSP cells represent a novel stem/progenitor cell population in the adult heart that *in vitro* is potent to give rise to several cardiac cell types and more importantly to mature, contracting, fully differentiated cardiomyocytes.

Homeostasis of CSP cells under physiologic and injured conditions and the role of the bone marrow

The presence of CSP cells in the adult myocardium raises several questions about the origin, the properties and the role of CSP cells in the heart physiology and pathophysiology. The model of the permanent coronary occlusion in mice was used by Mouquet *et al* [32]. in an *in vivo study* of the effects of severe cardiac injury on the CSP homeostasis. Myocardial infarction (MI) caused an acute (1 day post-MI) depletion (60%) of the CSP cell pool. The CSP cells remained significantly reduced at 3 days post-MI and their level returned to normal only at 7 days post-MI. The depletion of the CSP cells was significant in both the infarcted and non-infarcted areas of myocardium at days 1 and 3 post-MI.

The reconstitution of the CSP cells resulted from two different mechanisms, the proliferation of the endogenous CSP pool and the mobilization of bone marrow cells, after the injury, into the heart. Under homeostatic conditions (sham operated animals) approximately 10% of the CSP cells were expressing the cell cycling marker Ki67, suggesting that only a small portion of the CSP cells is proliferating under homeostatic conditions. Within the infarcted area at 1 day post-MI there was a reduction of the proliferating ($Ki67^+$) CSP cells in comparison with sham operated animals. However at 3 and 7 days post-MI approximately 20% and 25% of the CSP cells were cycling ($Ki67^+$). In the non-infarcted area the level of proliferating CSP cells remained 10% at 1 and 3 days post-MI. Only at 1 week post-MI was detected an elevated level (approximately 30%) of proliferating CSP cells ($Ki67^+$ cells). So following severe cardiac injury (MI) the proliferation of the endogenous CSP pool is increased and the number of CSP cells in the injured myocardium return to baseline levels approximately 1 week post-MI [32]. However the mechanisms and the signaling molecules mediating this response remain unknown.

The role of the bone marrow in the maintenance of the CSP pool under homeostatic conditions and its contribution to the reconstitution of the CSP pool to base-line levels post-MI was evaluated using a mouse transplantation model. Male C57BL/6 mice underwent transplantation with GFP-expressing bone marrow cells following lethal irradiation. The transplanted mice were sacrificed at the age of 12 weeks old and their CSP cells were analyzed for the expression of CD45 hematopoietic marker.

In sham-operated and reconstituted with GFP-BM mice the GFP-expressing CSP cells were less than 1% suggesting that in the normal heart the number of bone marrow derived CSP cells is not significant. Therefore under homeostatic conditions the contribution of bone marrow derived cells in the CSP pool is minimal and the proliferation of the residential CSP pool is the only driving force of its maintenance.

In contrast, the input of bone marrow cells to the CSP pool is elevated post-MI. Within the infarcted area the bone marrow derived (GFP+) CSP cells at 1, 3 and 7 days post-MI increase gradually to 5%, 20% and 25% respectively. On the contrary in the non-infarcted area the contribution of bone marrow cells to the CSP pool was insignificant with less than 5% of the total CSP population remaining at the same time point post-MI. In an effort to enlighten the characteristics of bone marrow derived CSP cells, the expression of the hematopoietic marker CD45 was used. At day1 approximately 50% of the CSP cells were expressing CD45 revealing their hematopoietic identity. At day 3 and day 7 post-MI the cluster of the $CD45^{+}$ cells decreased eventually to less than 10% regardless of the increase of bone marrow derived GFP+ CSP cells [32].

Taken together the above results suggest that proliferation of endogenous CSP cells and mobilization of bone cells to the heart are the responsible mechanisms for the replenishment of the CSP pool. The bone marrow derived CSP cells home into the myocardium and undergo an immunophenotypic conversion adopting a non-hematopoietic profile. Some information about the identity of the mobilized bone marrow cells is indicated by the increase of the circulating SP cells and a decline of the bone marrow side population cells (supplementary information in [32]). Although the functional characteristics of the bone marrow derived CSP cells remain undefined the mobilization of bone marrow cells to the heart after injury is in accordance with the

reported contribution of bone marrow cells in the healing process of the injured myocardium [13, 33].

A hierarchy model of stem cell biology

A generally accepted definition of stem cells describes them as a special cell type that possess the abilities of self-renewal and differentiation towards more committed cell lineages that eventually give rise to terminally differentiated cells [34]. Based on these two fundamental abilities a prevailing theory has been developed that classifies the stem cells into a hierarchy model. Although data accumulated in recent years challenge the above model, the purpose of this report is not compromised by accepting the hierarchical model as a basis for stem cell classification [35].

On top of the hierarchy model we find the zygote and the descendants from two first two divisions. These very limited in number cells are called totipotent because they are able to form both the embryo and the trophoblasts of the placenta. These totipotent cells begin to differentiate into the second class of stem cells that is composed by cells forming the blastocyst and a cluster of cells forming an inner cell mass (ICM). The ICM cells are called pluripotent because they are able to differentiate into cells from all the three germ layers. The first and the second class of stem cells represent the so-called embryonic stem cells (ESCs)[34].

In contrast with the embryonic tissues the adult tissues possess stem cells that demonstrate mainly multipotent differentiation capacities, meaning that they can give rise to only a limited range of cell types depending on their tissue of origin. For example, the neural stem cells have a tri-lineage differentiation capacity generating precursors of neurons, oligodendrocytes and astrocytes [36]. However up to date there is enough evidence to support the existence of pluripotent stem cells, similar with ESCs, in the adult organism. Bone marrow mesenchymal stem cells (MSCs) are competent to differentiate into cell lineages that arise from the three germ layers [37]. At the lower level of this stem cell "hierarchy model" reside all the cell types that demonstrate unipotential differentiation ability, being able to form only one type of cells. In other words the last class of unipotent stem cells is composed by the so-called committed progenitors cells which are committed towards a specific cell type [38].

The Side Population phenotype as a stem cell marker

In the field of stem cell biology there is a major obstacle that in the case of adult stem cells from solid tissues becomes more apparent. The identification and isolation of stem/progenitor cell populations from solid tissues relies on the existence of unique cytoplasmic markers. In several cases in order to isolate pure multipotent stem cells it is necessary to apply a combination of markers. The CD34 antigen and the recently identified peanut agglutinin ^{low}/heat stable antigen ^{low} profile are representative examples of unique stem cell antigens in the hematopoietic and neural stem cells respectively [39]. Despite the recent progress many solid tissues as the lung and the pancreas still lack a definitive stem cell marker.

Goodell *et al* in a revolutionary report described the existence of a, Hoechst 33342 dye low, cell population in bone marrow preparations that was enriched for long-term repopulating hematopoietic stem cells [25]. The so-called side population (SP) was identified based on the property of these cells to efflux the dye at a greater rate than other cells (main population cells, MP) in those bone marrow preparations (Figure 7).

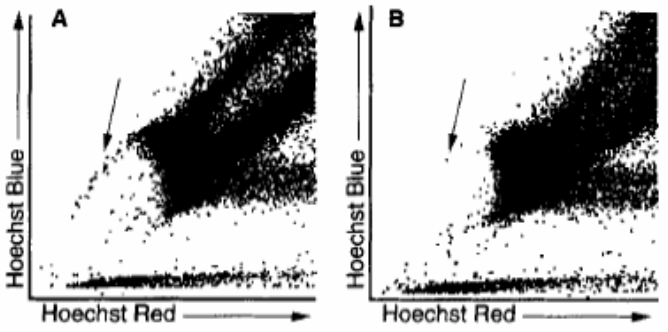

Figure 7: Figure a: Identification of BMSP cells A: Whole BM stained with Hoechst 33342. B: Whole BM stained with Hoechst 33342+verapamil. Adapted from [25].

Since then side population cells have been isolated in several tissues as the adult heart, the liver, the skeletal muscle, the skin, the brain, the mammary gland, the kidney, the lung, the testis, the retina, the aorta and the pituitary gland [26], [40-42]. In most of

the cases the isolated SP cells demonstrate stem/progenitor cell activity being able to differentiate in various cell types according to the organ of origin [26]. Consequently the SP phenotype represents a novel putative marker of stemness and it can be used for the identification of putative stem cell populations in solid organs in combination with the expression of additional markers.

ABC transporters are the molecular determinants of the SP phenotype

The Hoechst 33342 dye is a nuclear counterstain that binds preferentially AT-rich regions of the minor groove of DNA and emits blue and red light when bounded to DNA (www.Cambrex.com). Hence the intensity of Hoechst 33342 fluorescence depends on the cell-cycle status (DNA content of the cell) and the structure of chromatin of the analyzed cell population. The reliability of the Hoechst staining assay as a stem cell identification protocol relies exclusively on the ability of the dye to be exported by the assumed stem/progenitor cells.

There are two major membrane pumps, which belong in the ABC-binding cassette (ABC) transporter superfamily, involved in the generation of the SP phenotype. The first is the multidrug resistance 1 transporter (MDR1a/1b, ABCB1, P-glycoprotein) and the second is the breast cancer resistance protein 1 (BCRP1, ATP-binding cassette, subfamily G, ABCG2) [43, 44]. The basic functional domains of an ABC transporter consist of a transmembrane spanning region linked by a flexible linker to an intracellular ATP-binding cassette domain. The transporter ABCG2 (BCRP1) is also known as a half-transporter compared to with the transporter MDR1a/1b which is a full-transporter. The ABCG2/BCRP1 pump is composed by only two domains, a six-spanning transmembrane domain and an ATP binding cassette whereas the MDR1a/1b pump is composed by the same motif repeated twice, as shown in the figure 8.

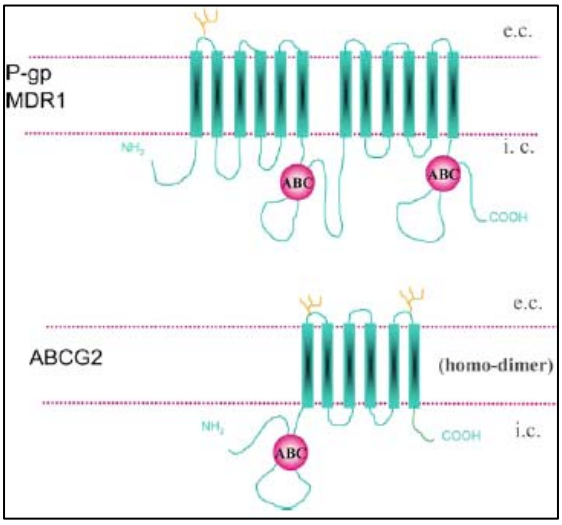

Figure 8: Structure and molecular topology of ABC transporters ABCG2 and MDR1

Studies using knock-out mice for these transporters revealed significant differences in the impact of each transporter on the generation of the SP phenotype in bone marrow and skeletal muscle. The Mdr1a/1b$^{-/-}$ mice demonstrated physiological levels of bone marrow SP cells [44]. However, the BCRP1$^{-/-}$ mice demonstrated significant deficiencies in the amount of the SP cells and their repopulating capacity [45]. The importance of the transporters ABCG2 and MDR1 was also examined through retroviral mediated-enforced overexpression in murine bone marrow cells. Enforced expression of the MDR1 pump resulted in a 3.6% increase of the SP fraction. In contrast overexpression of the BCRP1 pump produced a 62.5% increase in the SP fraction. However, in BCRP1$^{-/-}$ mice there were no developmental defects or any other deficiencies in the process of hematopoiesis suggesting that the HSC cells in these mice were maintained unaffected by the absence of the transporter.

Overall, the above data strongly suggest that the SP phenotype in the bone marrow is depended exclusively on the function of the BCRP1/ABCG2 transporter. The inability of the MDR1a/1b transporter to compensate for the loss of the SP phenotype in the bone marrow of the BCRP$^{-/-}$ mice also suggests that there is not functional overlapping and that maybe there is a tissue-dependent specificity of the function of these pumps. Finally, the absence of any hematopoietic defects in the BCRP1$^{-/-}$ mice in parallel with the absence of the SP fraction indicates the lack of a direct correlation between the SP cells and the HSC cells. In conclusion, the SP phenotype should not be considered as a stem cell marker without additional evidence as the expression of established stem cell markers and evidence from a functional assay.

ABC transporters as protective mechanisms of stem/progenitor cells

As mentioned previously, by definition, every cell that demonstrates the properties of self-renewal and multipotency is considered stem cell. Besides these two fundamental properties the stem/progenitor cells exhibit additional characteristics that make them more sophisticated. The capacity to export cytotoxic compounds and DNA-binding dyes as Hoechst 33342 is likely to provide a mechanism responsible for the survival of the stem/progenitor cells. It is well established that the ABC transporters are capable of transferring a wide variety of substrates as metabolic products, drugs, toxins, endogenous lipids, peptides, nucleotides and sterols [46]By this way they could protect the stem/progenitor cells from the presence of cytotoxic products of the metabolism or from toxins produced due to hypoxic conditions.

In vivo studies of bone marrow SP cells derived from wild type and BCRP knock out mice showed that the $BCRP^{-/-}$-SP cells were more sensitive to the effects of mitoxantrone, a known chemotherapeutic drug, clearly suggesting a protective effect of the BCRP1 pump [45]. Another recent *in vivo* study revealed that BCRP1 confers protective action to bone marrow progenitor cells under hypoxic conditions. The ABC-transporter BCRP1 binds specifically heme and bone marrow progenitor cells lacking this transporter are vulnerable to cytotoxic actions of the accumulated porphyrins. [47]. The physiological significance of the MDR1 transporter under hypoxic conditions was also emphasized by *in vivo* studies in ischemic pig hearts. The expression of the P-gp transporter in not detected in normal cardiomyocytes but it is up regulated under chronic hypoxic conditions [48].

Additional evidence, supporting the proposed protective role of the ABC-transporters, accumulated by studies showing an anti-apoptotic role of the MDR1a/1b pump. In one of these studies the human tumor cell lines CEM and K562, which express high levels of the P-gp pump, were more protected to several forms (chemotherapeutic

drugs, UV irradiation, Fas cross linking and TNF) of caspase-dependent cell death. Further insight in the anti-apoptotic action of MDR1a/1b resulted from studies revealing that the MDR1a/1b inhibits, upon Fas-mediated apoptotic stimuli, the activation of caspases 3 and 8. The anti-apoptotic effect mediated by the MDR1a/1b was reversible by the addition of anti-MDR1a/1b antibodies or the pharmacological inhibitor of P-gp, verapamil. [49] [50]. Thus there are several lines of evidence supporting that the expression of the ABCG2 and the MDR1 ABC-transporters provide a strong protective effect on the SP cells. These transporters provide to the SP cells a defensive mechanism for their long-term survival under different cytotoxic stimuli produced by apoptosis and/or hypoxia.

The goal of this thesis

During the last decade several "different" cardiac stem/progenitor cell populations have been discovered in the adult heart of mice, rats, dogs, pigs and humans [18]. Beside the endogenous cardiac stem/progenitor cells there are several reports about non-cardiac progenitor cell populations that exhibit cardiomyogenic differentiation [33, 51-54]. Additionally, great focus has been given on the role of bone marrow derived in the physiology of the heart under normal and pathologic conditions. So far more than a few ongoing clinical trials demonstrate the beneficial effects of administration of bone marrow cells in patients with cardiac failure [55]. However, there are reports that challenge the findings of sex-mismatched transplantation studies and subsequently the regeneration ability of the heart [56]. Despite, the contradictory reports, the prevailing theory that the heart is composed mainly by terminally differentiated cardiomyocytes and lacks any self-renewal ability is not as strong as it used to be.

The discovery of the endogenous and non-cardiac cells population was a breakthrough in the cardiovascular field. There are still many obstacles to be overcome in order to exploit the cardiac stem/progenitor cells in a therapeutic way. There are several unresolved questions concerning stem cell niche of these populations in the myocardium, the factors that promote their self renewal capacity, differentiation, proliferation, homing and migration. Another unsolved issue is the degree of overlapping between the different cardiac cell populations and the identification of the set of cell markers for the most potent cardiac stem/progenitor cell. There are several biological pathways, as Wnt signaling pathway, involved in the regulation of the properties and functions of the stem cells but little is known about the significance of these pathways in the diverse cardiac stem cells. So far the c-kit$^+$/mdr1$^+$/Sca1$^+$ cardiac cells are the most well studied cells among the other cardiac progenitor cell types, Sca1$^+$ cardiac cells and CSP cells.

There are several explanations about the amount of the remaining questions in the field of cardiac stem/progenitor cells and especially about the Sca1$^+$ and the CSP cells.

Firstly it should be mentioned that the absolute number of each type of cell in the adult heart is very low and very difficult to be studied and subsequently to be exploitable in any therapeutic way. This obstacle becomes more evident in the case of CSP cells where 3000-5000 CSP cells can be sorted per mouse heart. Another important problem is the lack of well established *in vivo* functional assay to prove the multipotency of the cardiac stem/progenitor cells, similar to the transplantation model for the hematopoietic stem cells. It is also of great importance to mention that for the CSP cells there is not any unique identified marker, as in the case of c-kit^{+} cells, which would allow marking them and tracking them in functional *in vivo* assays. Finally, it is also noteworthy that there is no established *in vitro* culture protocol that would permit the extensive study of all the biological properties of the cardiac stem/progenitor cells.

Recently, as described earlier, our lab demonstrated that freshly isolated CSP cells hold the ability to differentiate towards three of the main cardiac cell lineages. Moreover in another report it was shown that the CSP cells are acutely depleted in a severe cardiac injury and that by self-proliferation and contribution from bone marrow cells the CSP pool is replenished to base-line levels at 1 week post-MI. The last finding suggests in an indirect way the necessity of the CSP population presence in the heart physiology even though their exact role is not clear. The documented multipotency of freshly isolated CSP cells proposes that they could participate in the normal cell turnover of the adult heart but this remains to be proved.

The primary focus of this work was to establish and evaluate an *in vitro* model for the expansion and the detailed study of the CSP cells. Before any therapeutic application, it is very important to understand in great detail the basic biological principles and the unique characteristics of these cells. The most important prerequisite for this model will be the ability to promote the self-renewal capacity of the CSP cells by maintaining the cells in a proliferative but at the same time undifferentiated state. The characteristics of the expanded cells will be compared with those of freshly isolated cells in order to draw a conclusion about the maintenance of their properties. Their cardiomyogenic capacity will be evaluated using the same co-culture system as Pfister *et al [28]*.

The expansion of the CSP cells will allow us to work with a several-fold higher number of cells than in the case of freshly isolated cells. Applying the accumulated knowledge from the other well studied stem cell models we will examine in greater detail the nature of the CSP cells as stem/progenitor cells. The expansion of the CSP cells will permit us to study further the factors that promote their self renewal ability and to identify the existence or not of common regulatory mechanisms with other types of progenitor cells. It is of particular importance to study the pathways that govern the proliferation of the CSP cells initially *in vitro* and subsequently *in vivo.* An *ex vivo* system of expanding CSP cells provides an appropriate way to evaluate the putative impact of various parameters on the actual proliferation of the cells.

The physical presence of the CSP cells in the adult myocardium and particularly their response after a severe cardiac injury are not sufficient to adequate the loss of functional myocardial tissue. Thus expanding *ex vivo* the CSP pool allows to deliver an increased number of CSP cells in the injured myocardium in an effort to compensate the harmful consequences of a severe cardiac injury. Additionally, it is would be interesting to attempt to augment the cardiomyogenic potential of the CSP cells by manipulating the expression of some genes involved in that process.

Moreover, one part of this study focuses on the delineation of the *in vivo* effects of a severe cardiac injury on the proliferation of the CSP cells. At different time points post-MI (1, 3 and 7 days) CSP cells are sorted and stained with propidium iodide. The numbers of CSP cells in the various cell cycle phases at the given time points are determined by FACS analysis. The study of the response of the CSP cells in the acute cardiac failure is very interesting and informative about the homeostatic mechanisms of the CSP cells in the adult heart.

Materials and methods

Materials

- Antibodies: the antibodies used for this study are described later in this section.
- The animals used this study (Mice C57BL/6J and C57BL/6-Tg (ACTbEGFP) 1Osb/J, rats male Wister) were purchased by Jackson laboratory or they were in bred colonies.
- Cell culture media used in this study were DMEM (Invitrogen) and α-MEM (Cambrex).
- PCR Materials (Taq-DNA polymerase, dNTPS, $MgCl_2$, 10X polymerase buffer) used in this study were purchased by Promega.
- RNeasy Mini Kit (Qiagen).
- iScript cDNA synthesis kit (Bio-Rad).
- SYBR Green Supermix (Bio-Rad).
- iCycler Single color Real-Time PCR detection system (Bio-Rad).
- PCR system-2700 Gene Amp (Applied Biosystems).
- DAPI mounting media for fluorescence Vector Laboratories Inc. H-1200.
- Collagenase B and Dispase were purchased by Roche.
- All the other chemicals used in this study were purchased by Sigma.

Methods

Molecular Biology Techniques

Isolation of total eukaryotic RNA

Total eukaryotic RNA was collected from freshly isolated CSP cells and from *in vitro* expanded CSP cells using either TRIzol reagent or an RNeasy Mini Kit from Qiagen. Expanded CSP cells were resuspended in TRIzol using a ratio of 1 ml TRIzol reagent per 10 cm^2 of the culture dish. The CSP cells into the TRIzol reagent were passed several times through a 5 ml syringe using an 18G needle. Then the samples were incubated at room temperature for 5 minutes and subsequently it was added chloroform in a ratio of 0.2 ml chloroform per 1 ml TRIzol. Following centrifugation at 4^0C for 15 minutes at 12000-13000 rpm the supernatant was transferred into a new tube and isopropanol was added in a ratio of 0.5 ml isopropanol per 1 ml TRIzol. The new solution was incubated in room temperature for 20 minutes and then it was centrifuged at the same conditions for 15-20 minutes. The precipitated RNA, in the form of pellet at the bottom of the tube, was cleaned twice using 70% ethanol in a ratio of 0.5 ml/1 ml TRIzol. The pellet was centrifuged at the same conditions for 3 min and then the supernatant was removed by aspiration. Finally the pellet was left to dry at room temperature and it was dissolved in 50-100 uL of RNAse/DNAse free-water and stored at -80^0C.

The RNeasy Mini Kit from Qiagen was used to isolate total RNA from 25000-40000 freshly isolated CSP cells according to the manufacturer protocol. Total RNA samples were resuspended in 20-30 μl DNAse-RNAse-free water. The CSP cells were sorted using a MoFlow2 cytometer/sorter.

DNAse treatment

Total RNA isolated from CSP cells was incubated with DNAse in order to eliminate contaminating genomic DNA. The DNA-free kit from Ambion was used according to the manufacturer protocol. Briefly, samples were incubated with 1-3 Units of DNAse at 37^0C for 1 hour. The DNAse was added in two separate doses. The DNAse was removed from the solution by adding a special DNAse inactivation reagent, supplied in the kit. Samples were incubated with the inactivation reagent at room temperature for 2 minutes. Finally, the solution was centrifuged at 12000 rpm for 1.5 minute at room temperature and the supernatant (containing the DNA-free total RNA) was collected in a new DNAse-RNAse-free eppendorf tube.

Reverse transcription reaction

cDNA was generated from DNA-free total RNA using iScript cDNA synthesis kit from Bio-Rad according to the manufacturer protocol.

Polymerase Chain Reaction (PCR) and Real-Time PCR

cDNA generated either from freshly sorted CSP cells or cultured CSP cells was used to assess the expression of various gene markers in expanded CSP cells and freshly isolated cardiomyocytes. The primers used are listed in Table 1. The protocol of the PCR was:

Step I:	1 cycle at	95^0C for 8 min
Step II:	35-45 cycles at	95^0C for 30 sec
		52-60 ^{0}C for 40-60 sec
		72^0C for 30-60 sec
Step III:	1 cycle at	72^0C for 7 min

The annealing temperatures and the incubation period of the various steps varied according to the characteristics of each particular set of primers and PCR product.

The expression of the house-keeping gene GAPDH used for normalization of the results. RT-PCR performed using the iQ SYBR Green Supermix according to the following protocol:

Step I:	1 cycle at	95^0C for 3 min
Step II:	45 cycles at	95^0C for 30 sec
		60 ^{0}C for 40-60 sec
		72^0C for 30-60 sec
Step III:	1 cycle at	95^0C for 30 sec
Step IV	1 cycle (80 repeats)	55^0C (set point) Increased 0.5/10sec.

The analysis of the results was performed based on the

Agarose gel electrophoresis

The PCR products were analyzed with agarose gel electrophoresis and they were visualized under UV light. TAE (40mM Tris-Acetate, 1mM EDTA, pH 8.0) and TBE (Tris-base 54.0 g, Boric Acid 27.5 g and EDTA 2.92 g) were used as running buffers. The concentration of the agarose used in the gels was between 1-1.5% according to the size of the PCR products.

Animal experimental protocol

All surgical operations were done in a surgery room under sterile conditions. C57BL/6J mice were anesthetized using 65 mg/kg pentobarbital. During the operation the mice were ventilated using a standard rodent ventilator. The hair was removed from the animal's chest using a depilatory cream. Skin region was disinfected using aseptic techniques (70% ethylic alcohol) and betadine scrub. An incision was performed in the chest between the fourth and the fifth rib. After the pericardium was cut the left anterior coronary descending coronary artery was permanently ligated with a 7-0 silk suture. Animals were closely monitored until full recovery from anesthesia and then they were received appropriate treatment to prevent any discomfort effects of the surgery. They received buprenorphine, as an analgetic, in a concentration of 0.03-0.06 mg/kg for a period of 48 hours post-operationally. Sham operated animals will undergo an identical surgical operation procedure without tightening of the coronary artery.

Cell culture techniques

Isolation and immunostaining of Cardiac Side Population cells

Animals were anesthetized before being sacrificed receiving pentobarbital in a concentration of. The hearts were excised and washed thoroughly with PBS. Subsequently they were minced, under sterile conditions, initially with a pair of scissors and then with a razor blade. Subsequently was added 5 ml of digestion solution per heart (Dispase II from Roche Diagnostics (2.4 U/ml), 0.1% Collagenase B from Roche Diagnostics and 2.5 mmol/L $CaCl_2$). The heart tissue was incubated at 37^{o}C for 30 minutes and during that incubation period the digestion solution was titrated once. The digestion reaction was terminated by adding equal volume of ice cold HANK'S solution from Gibco [(2% FBS, 10 mmol/L HEPES) (FW 238 from Sigma)] and the cardiomyocyte-depleted single-cell suspension was filtered twice initially through a 70 μm filter and subsequently through. a 40 μm filter. The number of the cells was calculated using a standard hemocytometer. The cell suspension was incubated with Hoechst 33342 (5 μg/ml) at 37^{o}C for 90 minutes in Duldecco's modified Eagle's medium (DMEM) (2% FCS, 10 mmol/L HEPES). During the incubation period the staining solution was mixed several times. After the completion of the incubation period the cell suspension was washed twice with ice cold HANK'S solution and the cells were resuspended in an appropriate volume of ice cold HANK'S solution and were analyzed and/or sorted with a MoFlow2 cytometer. Propidium Iodide (PI), at a concentration of 2μg/ml, was used to distinguish between dead and alive cells.

Immunocytochemistry

Immunocytochemistry for cell-surface antigens

CSP cells (*in vitro* expanded or freshly isolated) were resuspended in 50-100 μl of staining buffer (HANK'S solution or PBS). The CSP cells were stained with antibodies that were either directly conjugated with fluorochrome labels (FITC and PE) or unconjugated. For the purpose of this study were used antibodies reactive to Sca1, CD44, β1-integrin, V-CAM, CD73, α4-integrin, CD31, CD34, CD45. Table 2 showing detailed information of the antibodies used in this study. The staining reactions were incubated in the dark at 4^{o}C for 30 minutes and subsequently were washed twice in 1 ml of staining buffer. During this incubation period the suspension was mixed once or twice. When the primary antibodies were unconjugated appropriate secondary antibodies were used and the staining reactions were performed in the same conditions. Subsequently the cell suspension was washed twice in 1 mL of staining buffer. The cell pellet was resuspended in 50-100 μl of staining buffer and was analyzed through a MoFlow2 or a BD FACScan cytometer.

Immunocytochemistry for intracellular antigens

CSP cells (*in vitro* expanded or freshly isolated) were fixed and permeabilized at 4^{o}C for 20 minutes using the BD Cytofix/Cytoperm solution from BD Biosciences or a solution of 4% paraformaldehyde. During this incubation period the suspension was mixed once or twice. Subsequently the cells were washed twice with a BD Perm/Wash solution from BD Biosciences. The pellet was resuspended in 50-100 μl of the same solution containing appropriate concentration of antibody solution. The staining reactions for the intracellular antigen were performed at 4^{o}C for 30 minutes. Subsequently were used proper secondary antibodies at the same staining conditions with the primary antibodies. Before analysis the cells were washed twice in HANK'S solution and were analyzed through a MoFlow2 or a BD FACScan cytometer.

FACS analysis

FACS (Fluorescence Activated Cell Sorting) was performed using a MoFLow2 and a FACScan cytometer. The FACScan cytometer is a three-color cell analyzer. The fluorochromes are excited by a blue laser at 488nm. The MoFlow2 cytometer was equipped with three lasers. An argon multi-line UV (333-363 nm) laser was used for the excitement of the Hoechst 33342 dye and the emitted fluorescence was collected with a 405-430 nm band pass filter (Hoechst blue) and 660 nm (Hoechst red). Analysis of acquired data was performed using the SUMMIT software from Cytomation Incorporation.

Propidium Iodide (PI) staining

Cell cycle analysis was performed on CSP cells sorted from operated animals (sham and MI). CSP cells were fixed and permeabilized at 4^{o}C for 20 minutes using the BD Cytofix/Cytoperm solution according to manufacturer protocol. The cells were washed twice using the BD Perm/Wash solution and were incubated for 20 min with 5 μl of Propidium Iodide (PI) at a concentration of 10 μg/ml supplied from Sigma. Then the cell suspension was washed twice in BD Perm/Wash solution and the stained cells were resuspended in 50-100 μl of HANK'S solution and were analyzed with the MoFlow2 cytometer.

Maintenance of cell cultures of CSP cells

Freshly isolated CSP cells were plated initially in 60 mm non-coated plastic culture dishes at a density of approximately 2000 cells per 10 mm of culture area. The CSP cells were grown in a mesenchymal-type cell culture media (α-MEM culture medium supplemented with 20% FBS, 2 mM L-Glutamin and 1% penicillin/streptomycin). CSP cells were maintained until sub confluence conditions and then were plated again in a lower density. The CSP cells were trypsinised using 0.05%

Trypsin/EDTA from Gibco at 37^0C for 4-5 minutes. Trypsin was inactivated by adding equal amount of culture medium (α-MEM, 20% FBS, 2 mM L-Glutamin and 1% penicillin/streptomycin) and the cells were centrifuged at 4^o C for 5 minutes at 1500 rpm. Culture medium was changed every three-four days. The CSP cells were expanded for numerous passages (up to P15).

Cardiac Side Population Co-Cultures with adult rat cardiomyocytes

Adult rat cardiomyocytes were isolated from male Wister rats using a collagenase digestion method, as previously described [28]. The cardiomyocytes were seeded at a low density (~75%) into 60 mm culture dishes on top of laminin-coated coverslips. The rat cardiomyocytes were maintained in DMEM culture medium supplemented with 5 mM/L creatine, 2mmol/L L-carnitine, 5 mmol/L taurine, 1 % penicillin/streptomycin, 7% FBS and 100 μM bromodeoxyuridine (BrdU). CSP cells used in co-culture experiments were sorted from GFP transgenic mice and were cultured according to the above described protocol. GFP-CSP cells were co-cultured with adult rat cardiomyocytes, at a density of 10,000-15,000 cells per dish. The co-cultures were maintained for three-four days in DMEM culture medium supplemented with 7% FBS and 1 % penicillin/streptomycin. Culture media was changed every three four days.

Duplication assay

CSP cells, cultured for 7 passages, were seeded in duplicates in 60 mm culture dishes in a density of 20000 cells per dish. CSP cells were cultured using the α-MEM culture medium (supplemented with 20% FBS, 2 mM L-Glutamin and 1% penicillin/streptomycin). The number of cells was calculated every three days, twice for each culture dish. CSP cells were trypsinised as described above and were centrifuged with the same conditions as above. The cell pellet was resuspended in 1 ml of culture medium and the number of the cells was calculated using a standard hematocytometer.

The CSP cells were initially sorted from wild type mice. The culture medium was changed every three days.

Cell counting

Cell counting was performed with standard hematocytometer (LEVY hematocytometer, 0.1 MM deep, HAUSSER SCIENTIFIC) using light microscopy. The final number of cells was calculated based on the formula:

$N_o = \alpha * 10^3 * 10$

α = the number of the cells that is counted as an average from the four quarters of the hematocytometer.

10 = dilution factor of the cell suspension

10^3 = internal factor of the hematocytometer

Heat inactivation of FBS

FBS was stored in -20^0C. Initially FBS was thawed in 37^0C water-bath and then was transferred in a 56^0C water bath for 40 min.

Maintenance of expanded Cardiac Side Population cells in liquid nitrogen

Cell suspension was centrifuged at 4^oC for 5 min at 1500 rpm and the cell pellet was resuspended in 1 ml solution of heat inactivated FBS/DMSO in an analogy 9:1 respectively. Subsequently the cell solution is stored at -80^0C for two-three days and then is stored permanently in liquid nitrogen at -150^oC.

List of antibodies

Name	Vendor	Species Reactivity	Type	Isotype	Fluorochrom
Sca1 (Ly-6A/E)	Pharmingen	Rat anti-mouse	Monoclonal	Rat IgG2a,k	FITC-conjugated
CD44 (Pgp-1, Ly-24)	Pharmingen	Rat anti-mouse	Monoclonal	Rat IgG2b,k	APC-conjugated
CD31 (PECAM-1)	Pharmingen	Rat anti-mouse	Monoclonal	Rat IgG2b,k	FITC and PE conjugated
CD49d (α4 integrin chain)	Pharmingen	Rat anti-mouse	Monoclonal	Rat IgG2a,k	PE-conjugated
CD73 Ecto-5'-nucleotidase	Pharmingen	Rat anti-mouse	Monoclonal	Rat IgG2a,k	PE-conjugated
a-actinin (sarcomeric)	Sigma	Mouse anti-mouse	Monoclonal	Mouse IgG1	TRITC-conjugated
CD34	Pharmingen	Rat anti-mouse	Monoclonal	Rat IgG2a,k	PE-conjugated
CD45 (Leukocyte common antigen, Ly-5)	Pharmingen	Rat anti-mouse	Monoclonal	Rat IgG2b,k	PE-conjugated
V-CAM	Pharmingen	Rat anti-mouse	Monoclonal	Rat IgG2b,k	FITC-conjugated
CD29 Integrin- β1 chain	Pharmingen	Rat anti-mouse	Monoclonal	Rat IgG2a,k	Un-conjugated

Results

In vitro expansion of CSP cells

The CSP cells were cultured *in vitro* in a culture medium that is used in mesenchymal stem cell cultures (www.cambrex.com). Various numbers of CSP cells, ranging from 5000-15000, were seeded initially in a 60 mm non-coated and fibronectin-coated culture dishes. Most of the CSP cells remained floating during the first two-three days and few cells were attached to the culture dish as shown in figure 9. At day 5 the CSP cells were attached and some of them had double nuclei, indicating active proliferation. At day 9 the number of the cultured CSP cells had grown significantly and colonies of CSP cells were detected in various regions of the culture dishes. In addition to the CSP cells, MP cells were also cultured following the same protocol. The MP cells were also attached to the surface of the culture dish. The figure 9 depicts the adaptation process of the CSP cells in the culture dishes. The morphology of the cultured cells resembles to that of fibroblastic-like cells. The CSP cells grow only in a monolayer and not in multiple layers.

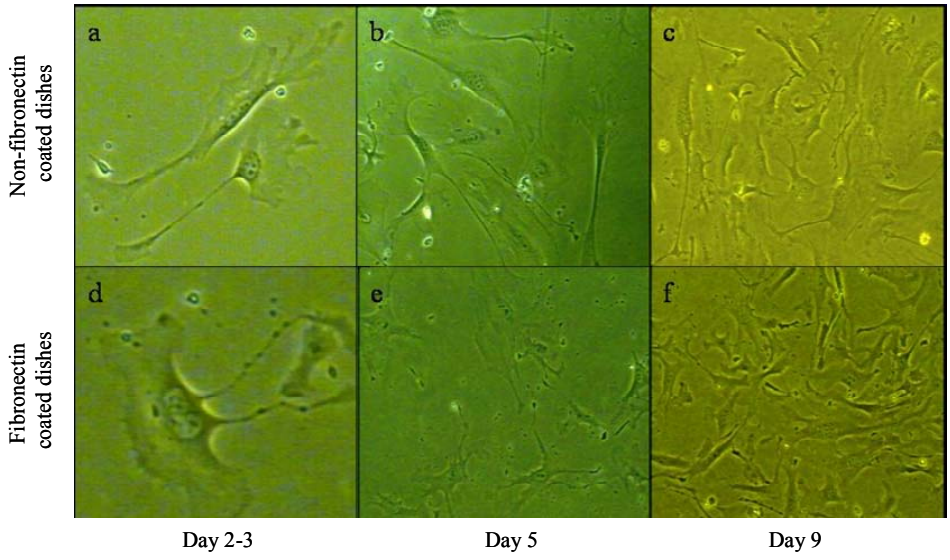

Figure 9: Expansion of CSP cells in fibronectin and non-coated culture dishes

Determination of the duplication rate

The next step was to define the growth potential and the proliferation rate of the CSP and the MP cells. For this purpose a modified proliferation protocol was used from Boland et al [57]. Approximately 20,000 CSP and CMP cells (from passage 4-5) were plated into 60 mm non-coated culture dishes and their proliferation was monitored every three days by manual counting of the cells. According to the diagram below the duplication period of the CSP cells is 2.5-3 days. In contrast the CMP cells demonstrated minimal growth potential with a duplication period longer than 1 week. The proliferation rate of both cell types decreases as cells reach confluence.

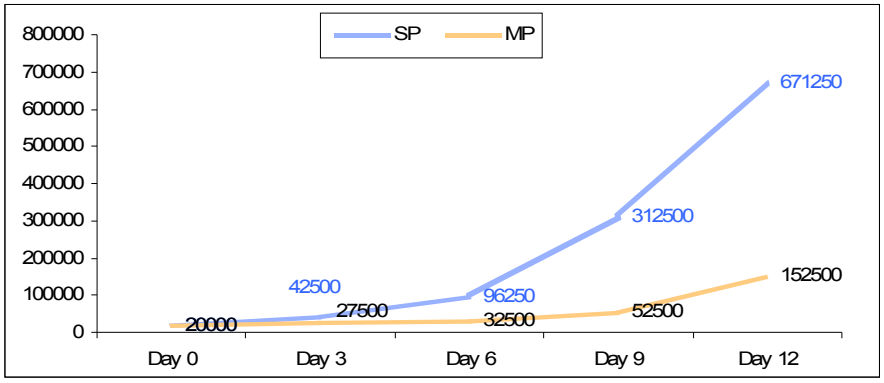

Figure 10: Comparison of the growth rate of cultured CSP and CMP cells.

Subsequently the differences in the proliferation rate between CSP cells from passage 5 and from passage 16 were assessed. 20,000 CSP cells were seeded in 60 mm culture dishes and the cell number was calculated at two different time points. As presented in figure 3 the older cells (~3 months old) demonstrate a significantly higher growth potential in comparison with the younger cells.

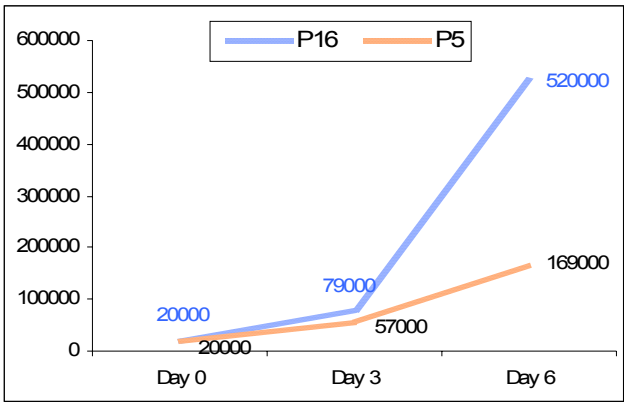

Figure 11: Comparison of the growth rate between CSP cells from different passages

Gene expression analysis of in vitro expanded CSP cells

The effects on the phenotype, the nature and the functional properties of the CSP cells under the culture conditions are unknown. A major issue in stem/progenitor cell cultures is the maintenance of the self-renewal ability and the multipotency. In order to examine these essential properties in this relative "new" CSP cell population the expression of various markers was tested using FACS analysis, conventional PCR and real-time PCR.

Conventional PCR was used to analyze the expression of the ABC-transporters MDR1 and BCRP1, the transcription factors GATA-4 and Nkx2.5, the receptor of the growth factor IGF1, the regulatory molecule TGF-β, the well established stem cell factor c-kit, the smooth muscle actinin (SMA), the desmin and the primary mediator of the non-canonical Wnt signaling pathway Wnt11.

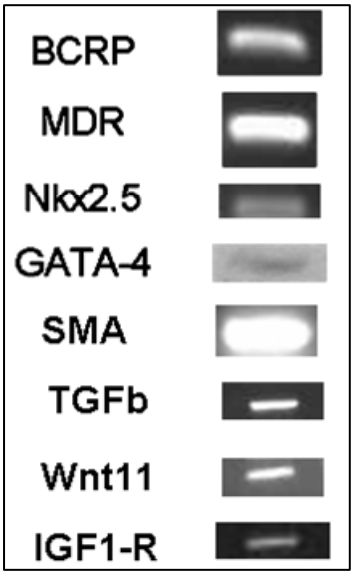

Figure 12: Detection of the expression of various gene markers in expanded CSP cells by conventional PCR.

The expanded CSP cells express in the mRNA level several important genes involved in various processes. Firstly of all both the ABC-transporters MDR1 and BCRP1 are expressed indicating that the culture conditions do not affect the nature of the

cells as side population cells. Interestingly the CSP cells express the transcription factors Nkx2.5 and GATA-4 suggesting possible cardiomyogenic potential. Wnt11 is a member of the non-canonical Wnt pathway which is also involved in differentiation towards cardiomyogenic lineages. TGF-b is another factor involved in cell differentiation and transformation. Finally the expression of IGF1-R is implicated in the biology of other stem cell populations as c-kit^{+} cardiac stem cells. The expression of desmin and c-kit were not detected.

By immunostaining technique and FACS analysis several other markers were tested in an effort to characterize further the cultured CSP cells. The markers under investigation were the Sca1^{+}, CD31, CD44, CD45, CD34, CD73, a4- & b1- integrin chains and V-CAM (CD106). Figures 13-19 present the results of the FACS analysis for the detection of the above markers.

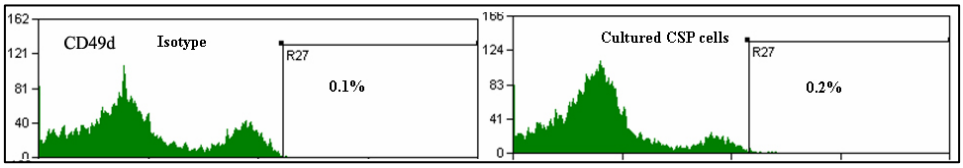

Figure 13: Cultured CSP cells are negative for the expression of the marker CD49d (a4-integrin).

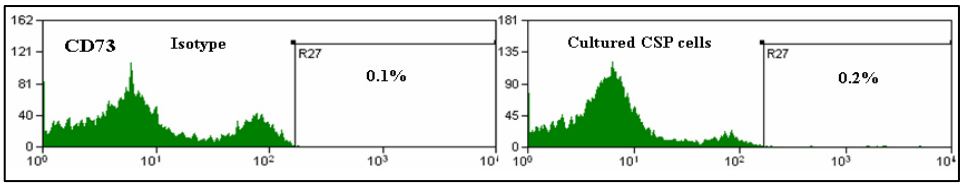

Figure 14: Cultured CSP cells are negative for the expression of the stromal stem cell marker CD73.

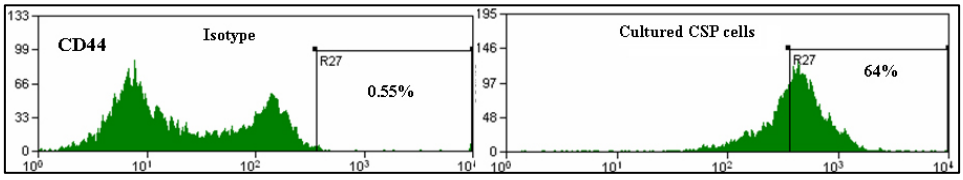

Figure 15: 65% of the cultured CSP cells are negative for the expression of the marker CD44.

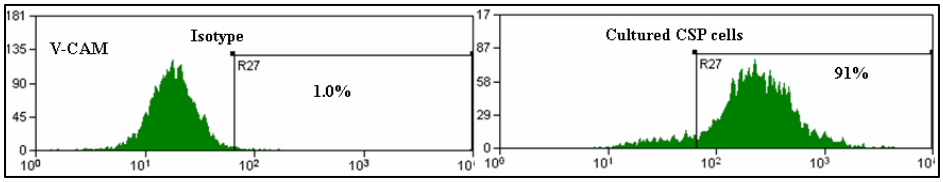

Figure 16: 90% of the cultured CSP cells are negative for the expression of the marker V-CAM (CD106).

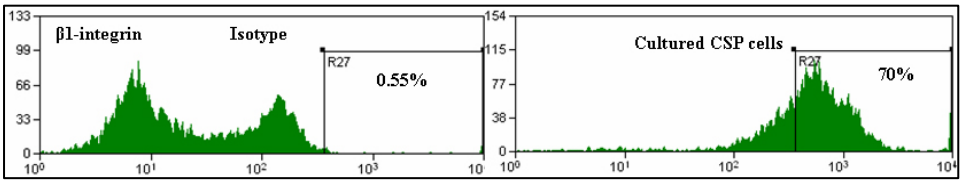

Figure 17: 70% of the cultured CSP cells are positive for the expression of the marker CD29 (β1-integrin).

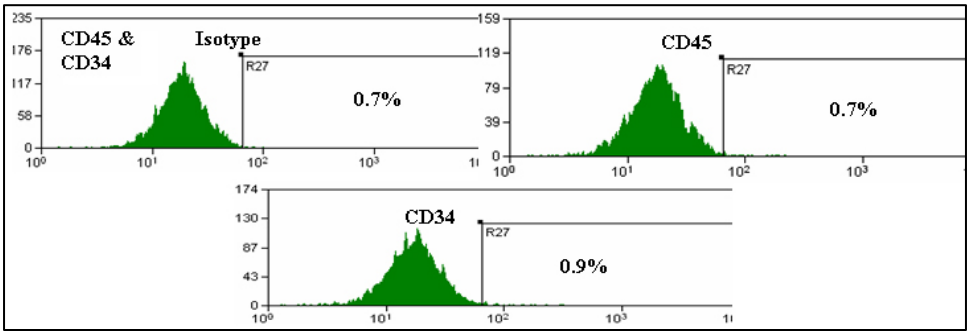

Figure 18: Cultured CSP cells are negative for the expression of the hematopoietic markers CD45 and CD34.

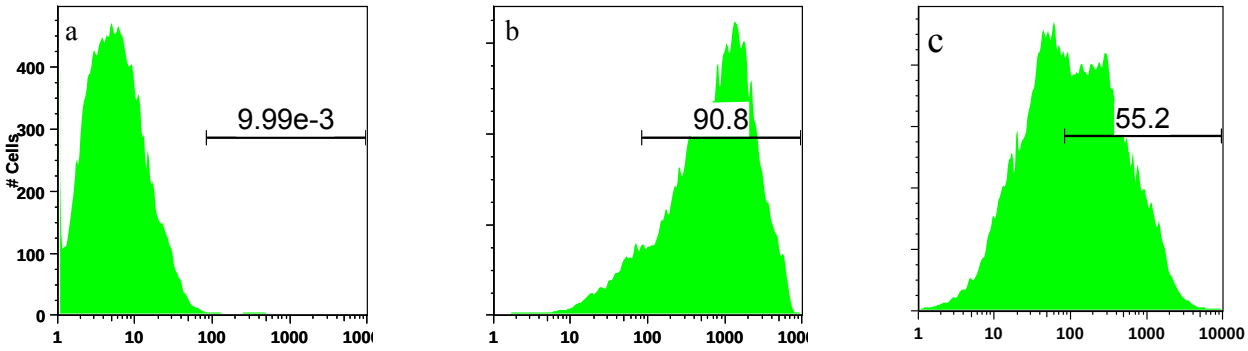

Figure 19: Cultured CSP cells are positive for the expression markers (b) CD90 and (c) Sca1. a is the Isotype control

In the following figure the overall expression profile of the *in vitro* expanded CSP cells is presented.

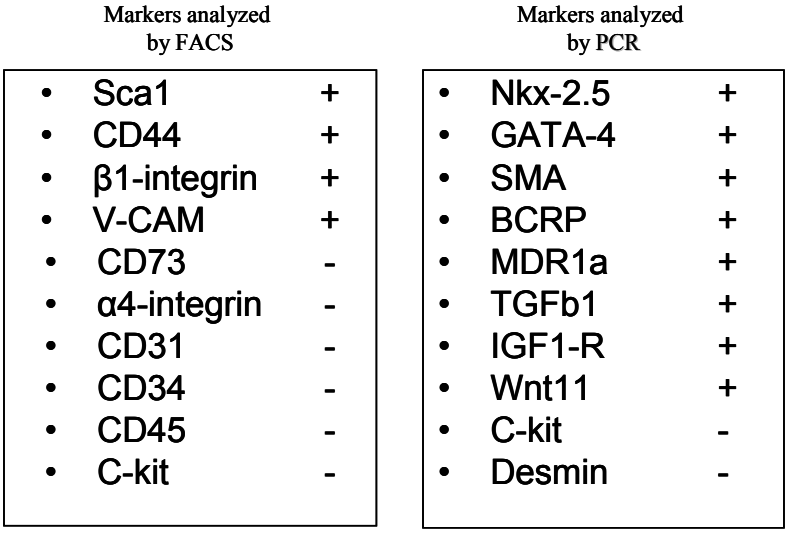

Figure 20: Gene markers expressed in expanded CSP cells.

The next step was to evaluate the expression of several gene markers with particular importance for the CSP population. This evaluation was performed in comparison with the expression in freshly isolated mouse cardiomyocytes. The adult cardiomyocyte comprise a well defined terminally differentiated cell type. Given the cardiomyogenic differentiation capacity of the freshly isolated CSP cells, the cardiomyocytes represent a final destination in the differentiation potential pathway. Based on that assumption it is interesting to compare the differences between these two populations in terms of gene expression.

Real-time PCR was used to compare the expression of the ABC-transporters BCRP1 and MDR1a, the self-renewal factor Bmi-1, the neural stem cell marker nestin and the myocyte sarcoplasmic reticulum calcium handling protein SERCA [58]. The figures 21-25 show the expression of each analyzed gene in the two cell populations. The red bars represent the gene expression index, which corresponds to the quotient of the expression of the gene of interest and the expression of the GAPDH. The black bars represent the normalized value of the expression between the two populations. For the normalization the expression value in the cardiomyocytes was used as denominator for

both cell populations. The expression of SERCA was also used as a control of the fidelity of the assay and of the templates used for cDNA synthesis.

The transporter BCRP1 (ABCG2) is expressed in both populations and is slightly higher in the CSP cells. The expression indexes of BCRP1 mRNA are 0.79 and 0.64 for the CSP cells and the cardiomyocytes respectively. There is approximately 20% increased expression of this ABC-transporter in the CSP cells.

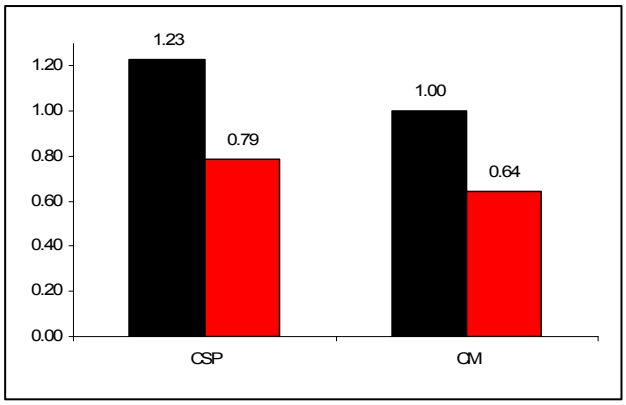

FIGURE 21: Comparison of BCRP1 mRNA expression between adult mouse CM & expanded CSPs.

In contrast the expression of the transporter MDR1a is significantly higher in the CSP cells than in the cardiomyocytes. The expression indexes were 1.27 and 0.12 for the CSP cells and the cardiomyocytes respectively. Overall there is almost an 11-fold difference in the expression of the MDR1a transporter between these two populations.

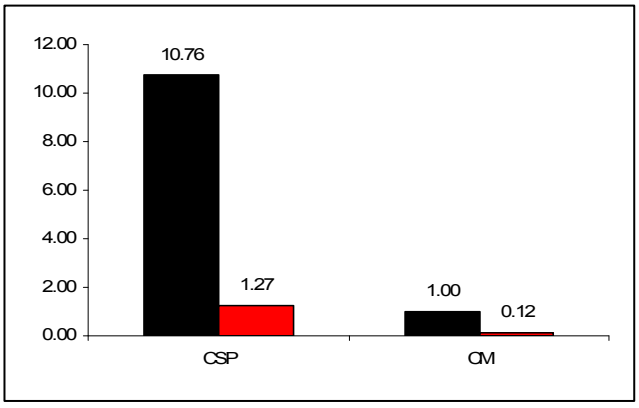

FIGURE 22: Comparison of MDR1a mRNA expression between adult mouse CM & expanded CSPs.

Subsequently the expression of Bmi-1, which is a well established self-renewal factor in HSC cells, was examined. The expression indexes were 9.71 and 1.86 for the CSP cells and the cardiomyocytes respectively. The CSP cells express more than 5-fold higher amount of Bmi-1 mRNA than the cardiomyocytes.

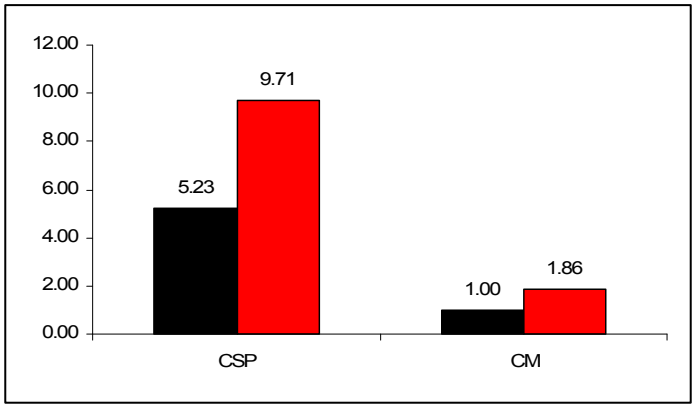

FIGURE 23: Comparison of Bmi-1 mRNA expression between adult mouse CM and expanded CSPs

Another gene marker that was evaluated is the neural stem cell marker nestin. The expression indexes were 4.91 and 0.34 for the CSP cells and the cardiomyocytes respectively. There is approximately a 14.5-fold higher expression of nestin in the CSP cells than in the cardiomyocytes.

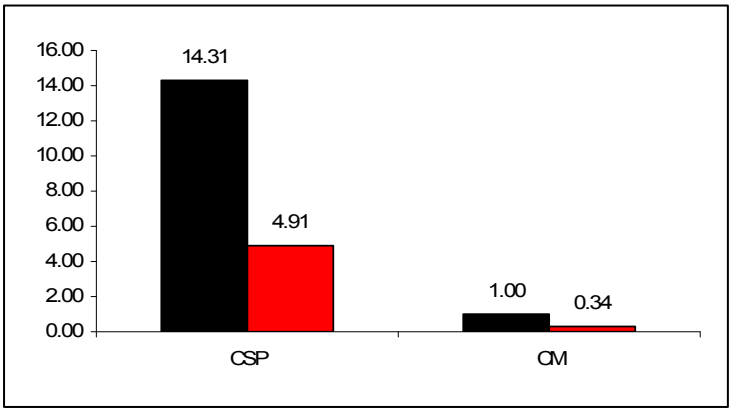

FIGURE 24: Comparison of Nestin mRNA expression between adult mouse CM and expanded CSPs

Finally the expression of SERCA as a marker of mature myocytes was examined. The expression indexes were 1 and 1713 for the CSP cells and the cardiomyocytes respectively. There is a 2141-fold higher expression of SERCA in the cardiomyocytes cells than in the CSP.

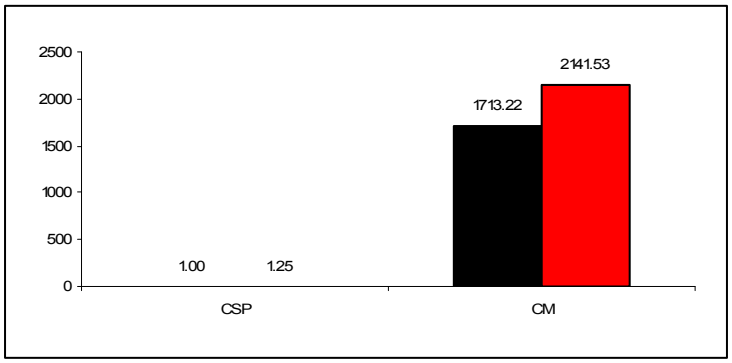

FIGURE 25: Comparison of SERCA mRNA expression between adult mouse CM & expanded CSPs.

In vitro expanded CSP cells retain the ability to efflux the Hoechst dye

According to the gene expression profile, as presented above, the *in vitro* expanded CSP cells express both ABC-transporters, MDR1 and BCRP1. The functional competence of these ABC-transporters was evaluated by testing their capacity to efflux the Hoechst dye. About half million of expanded CSP cells was stained accordingly with Hoechst 33342 dye and the results are presented in figure 26. Approximately 85% of the expanded CSP cells are capable of exporting the Hoechst dye ("Hoechst low" cells). The same experiment was repeated with the application of verapamil as negative control. Where, only around 30% of the analyzed CSP cells were "gated" as "Hoechst low" cells.

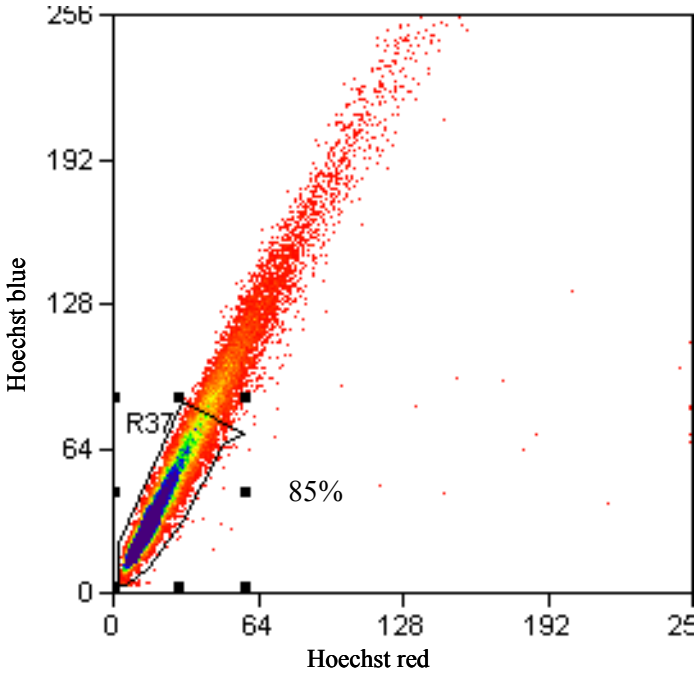

Figure 26: Control experiment for the ability of expanded CSP cells to efflux the Hoechst dye. The Hoechst dye is excited by the UV laser at a wavelength around 350 nm and emits blue and red light. The scale in the diagram is linear so the amount of the dye inside the cell is correlated with its position in the diagram (high or low).

Cardiomyogenic differentiation potential of in vitro expanded CSPs

The cardiomyogenic potential of the *in vitro* expanded CSP cells was tested using a co-culture model with adult rat cardiomyocytes. CSP cells (of passage 4), sorted from GFP mice, were seeded in laminin-coated coverslips with wild type adult rat cardiomyocytes. The differentiation experiments were performed using twice CSP cells of passage 4, once of passage 7 and once of passage 14, which had been frozen at passage 9. The co-culture experiments lasted three or four weeks. The differentiation of the CSP cells to functional cardiomyocytes was evaluated with several experimental ways. Initially the cardiomyogenic potential of the CSP cells was tested by the expression of cardiomyogenic transcription factors GATA4 and MEF2C. As revealed by immunocytochemistry CSP cells express both of these transcription factors.

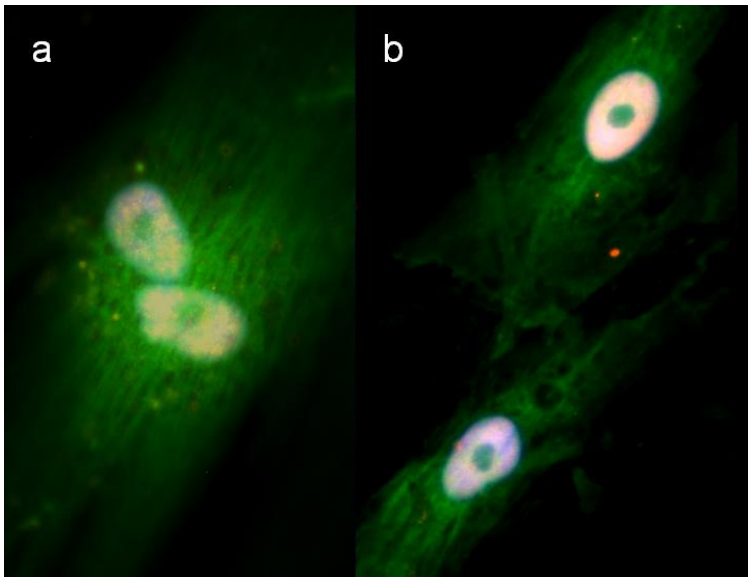

FIGURE 27: CSP cells (expressing GFP, green area) in co-culture with adult cardiomyocytes (not shown) express MEF2C (a) and GATA-4 (b). The expression of these transcription factors is located into the nucleus (light blue) and is indicated by the reddish area.

Subsequently the differentiation process of the CSP cells was estimated by the expression and the structure of the sarcomeric a-actinin. The expression of the sarcomeric a-actinin was demonstrated by immunocytochemistry. The functional maturation of the CSP cell-derived cardiomyocytes is indicated by the presence of sarcomere structures, comparable with those found in rat cardiomyocytes, and the observation of contraction in the same pace as in the rat cardiomyocytes. Figure 12 depicts an example of a differentiated CSP cell with fully developed sarcomeric organization.

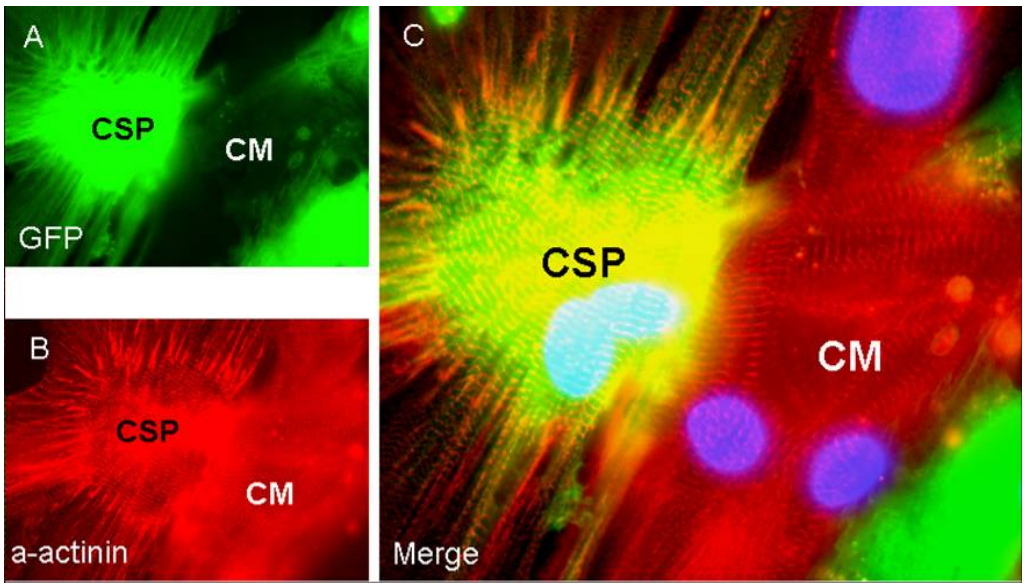

FIGURE 28: In vitro cultured CSP cells of passage 4 differentiate in functional cardiomyocytes. A: CSP cell expressing GFP in contrast with the cardiomyocyte (CM). B: Both the CSP and the CM cells express sarcomeric a-actinin. C: Merged figure depicts the co-localization of the GFP expression with the sarcomeric a-actinin in the differentiated CSP cell. On the right side of the merged picture there is an un-differentiated CSP (GFP+) cell.

In figures 29 and 30 are shown additional examples of GFP-CSP cell-derived cardiomyocytes.

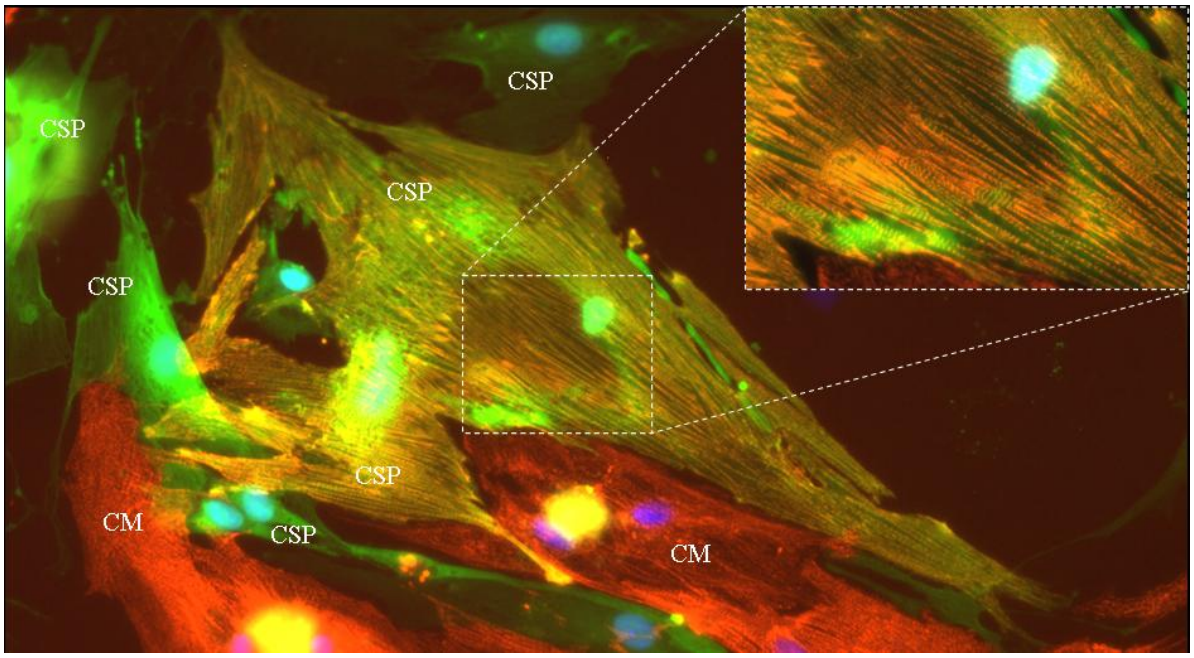

Figure 29: GFP-CSP cells (orange area) differentiate in a-actinin (red area) expressing cardiomyocytes. The detailed image (40X) in upper right site presents in magnification the sarcomeric organization of the a-actinin. The nuclei are depicted in blue due to staining with DAPI.

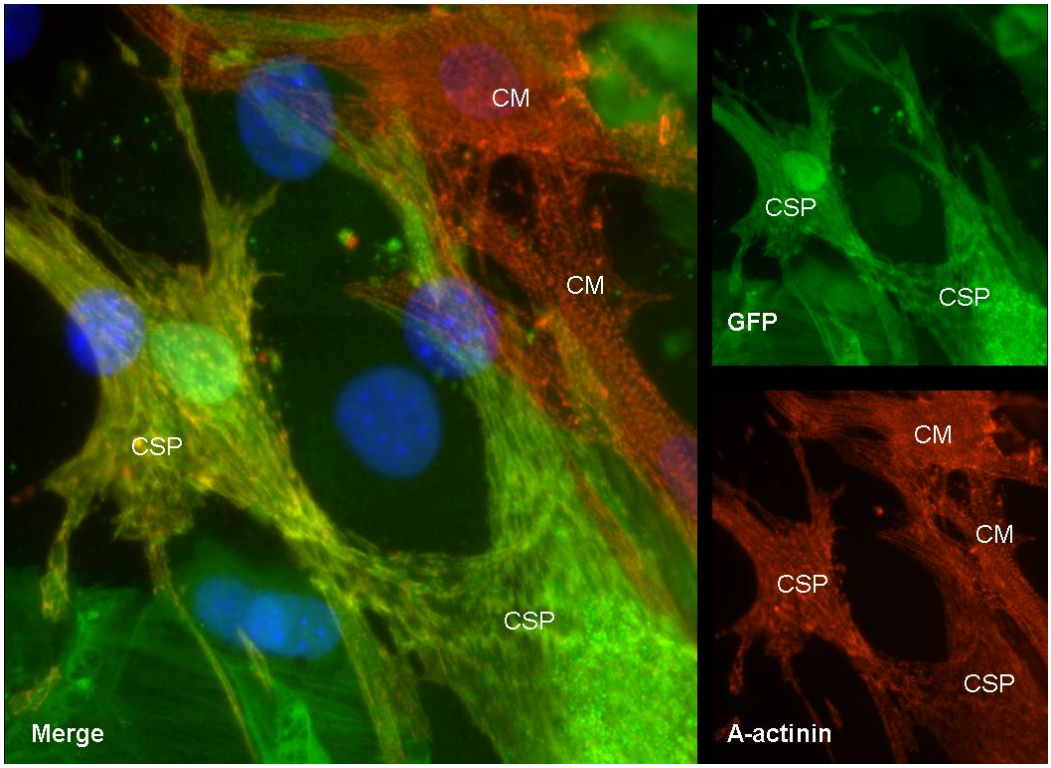

Figure 30: The CSP cells used in this differentiation experiment had been frozen at passage 9. When defrosted they were transduced with lentivirus expressing GFP. At passage 14 they were co-cultured with adult rat cardiomyocytes and the incubation time lasted four weeks.

Spontaneous differentiation of CSP cells

The process of cardiomyogenic differentiation of CSP cells to mature cardiomyocytes depends on the presence and especially on the physical coupling of the CSP cells with the adult cardiomyocytes, as it was shown by Pfister *et al* [28]. However, the direct contact of CSP cells with the cardiomyocytes is not absolutely necessary for the differentiation, as indicated in figures 31 and 32.

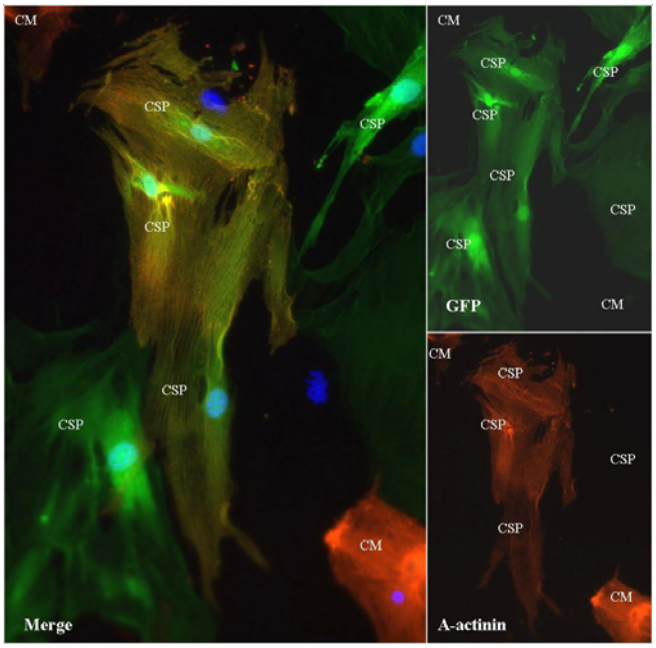

Figure 31: GFP-CSP cells were co-cultured with adult rat cardiomyocytes for a period of two weeks. The differentiated CSP cells (orange in merged picture) were not in direct contact with the adjacent cardiomyocytes suggesting that a cluster of CSP cells possess intrinsic cardiomyogenic potential.

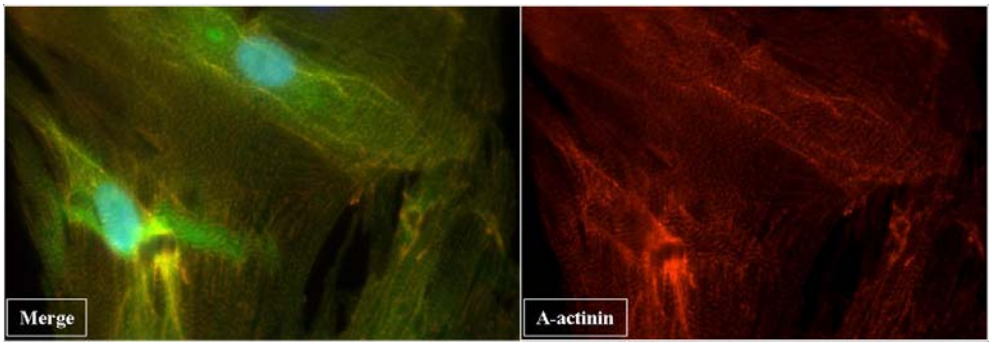

Figure 32: The a-actinin expressed by the differentiated cells from figure 12 is well organized in sarcomeres structures.

Cell fusion studies

So far it has been shown that that the expanded CSP cells can differentiate into functional beating cardiomyocytes when co-cultured with adult cardiomyocytes. The differentiation of the CSP cells could occur due to cell fusion between the two co-cultured populations. In an effort to examine the effect of cell fusion in the process of differentiation, GFP-CSP cells were co-cultured with adult cardiomyocytes, transduced with red fluorescence virus (RFP). The differentiated CSP cells due to fusion would have an orange color in contrast with the autonomously differentiated CSP cells that should be green. The results of this experiment demonstrated that none of the differentiated CSP cells was fused with a cardiomyocyte, since no orange color was observed. A representative example is shown in figure 33.

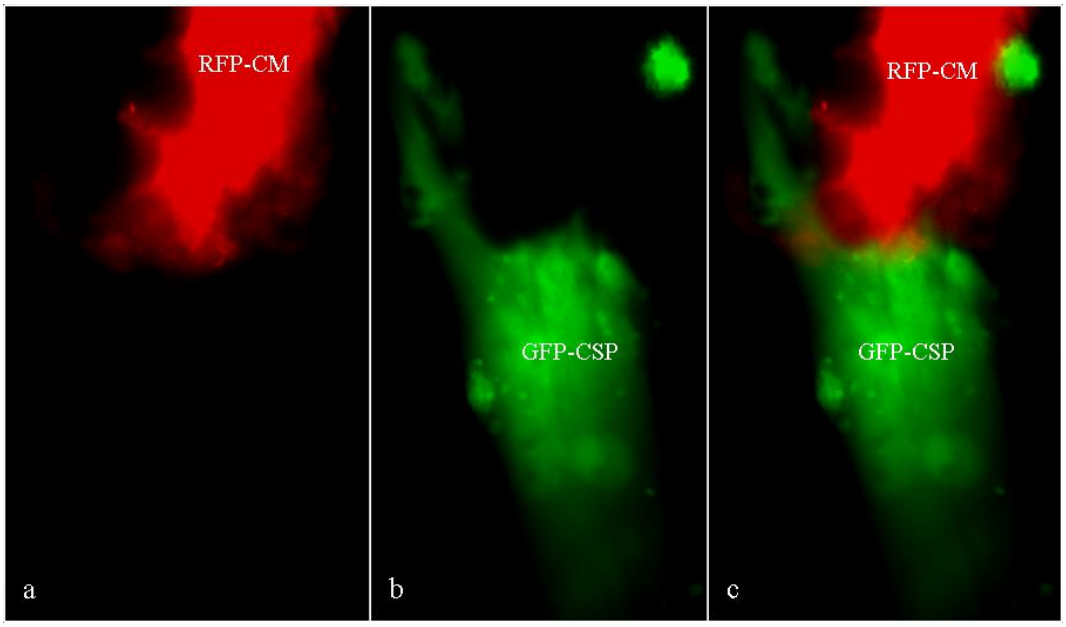

Figure 33: Cell fusion study of expanded CSP cells differentiating towards cardiomyocytes independent of cell fusion. a: RFP-transduced adult rat cardiomyocyte. b: GFP-CSP-derived contracting cardiomyocyte c: merge image showing that there is no orange color.

Study of the effects of myocardial injury on the proliferation status of the CSP cells

The consequences of a severe cardiac injury, as an infarction of the left ventricle of the heart, on the CSP cells were evaluated using a myocardial infarction model. At various time points (1, 3 and 7 days post-MI) CSP cells were sorted from sham and MI operated C57/Bl6 mice and they were stained with PI. The MI injury model and the PI staining method are both described in the materials and methods section. The PI dye stains double stranded nucleic acids so the cells are stained relatively to the amount of DNA and double stranded RNA.

In sham operated animals at day 1, approximately 37% of the CSP cells are in the S/G2/M cell-cycle phase and 63% are arrested in the G1 phase. In MI operated mice at 1 day post-MI the CSP cells that are in the S/G2/M and G1 cell-cycle phases are 45% and 55% respectively. The reported difference between the sham animals and the MI-operated animals is not significant despite the slight increase of CSP cycling cells 1 day post-MI.

Cell cycle Analysis of total CSP cells			
Animal type	**Analyzed cells**	**G0/G1**	**S/M**
Sh 1st 1d	1300	64.6	35.4
Sh 2nd 1d	1300	61	39
Average	1300	62.8	37.2
MI 1st 1d	1300	45.4	54.6
MI 2nd 1d	1300	64	36
MI 3rd 1d	1300	55	45
Average	1300	54.8	45.2
T-test	0.3396		
ratio S/M sham/MI	1.22		

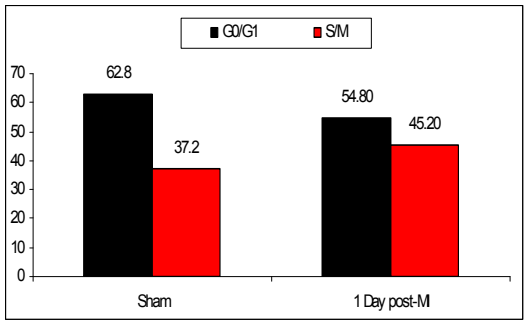

Table 1 &Figure 34: PI analysis of CSP proliferation at day 1 post-MI

In sham operated animals at day 3, approximately 35% of the CSP cells are in the S/G2/M cell-cycle phases and 65% are "located" in the G1 phase. In MI operated mice at day 3 post-MI the CSP cells that are in the S/G2/M and G1 cell-cycle phases are 62% and 38% respectively. The ratio of CSP cells being in the S/G2/M phases among sham-operated and MI-operated mice was 1.63. The reported difference between the sham animals and the MI-operated animals is significant (t-test value= 0.0053).

Cell cycle Analysis of total CSP cells			
Animal type	Cell #	G0/G1	S/M
Sham 1st 1day	1300	61.5	38.5
Sham 2nd 1day	1300	68.5	31.5
Average	1300	65	35
MI 1st 1day	1300	39.6	60.4
MI 2nd 1day	1300	34	66
MI 3rd 1day	1300	40.5	59.5
Average	1300	38.03	61.97
T-test	0.0053		
ratio S/G2/M sham/MI	1.63		

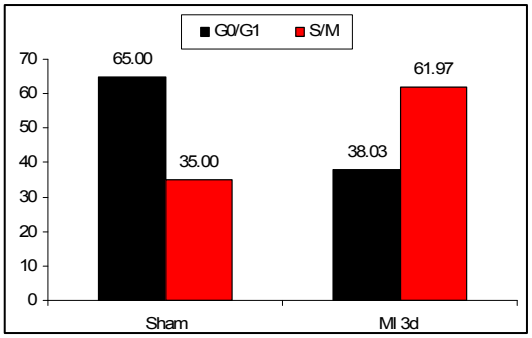

Table 2 &Figure 35: PI analysis of CSP proliferation at day 3 post-MI.

Similarly with day 3, 1 week post-MI in sham-operated animals 35% of the CSP cells were in the S/G2/M cell-cycle phases and 65% were in the G1 phase. In contrast in MI operated mice at 1week post-MI the CSP cells that are in the S/G2/M had increased to 62% and the CSP cells "located" in the G1 cell-cycle phase were 38%. The ratio of CSP cells being in the S/G2/M phases among sham-operated and MI-operated mice was 1.67.

Cell cycle Analysis of total CSP cells			
Animal type	**Cell #**	**G0/G1**	**S/M**
Sham 1wk	350	64	36
MI 1st	1500	44	56
MI 2nd	1500	36	64
Average	1500	40	60

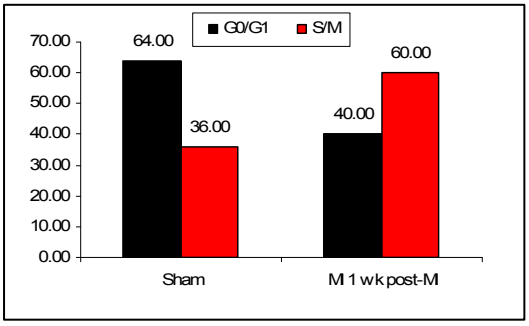

Table 2 & Figure 36: PI analysis of CSP proliferation 1 week post-MI.

Overall the above data suggest that at 1 day post-MI there are no significant differences in the number of CSP cells that are undergo proliferation between sham- and MI-operated animals. In contrast at day 3 and 7 the number of CSP in the S/G2/M cells cycle phases were significantly higher than in the sham animals.

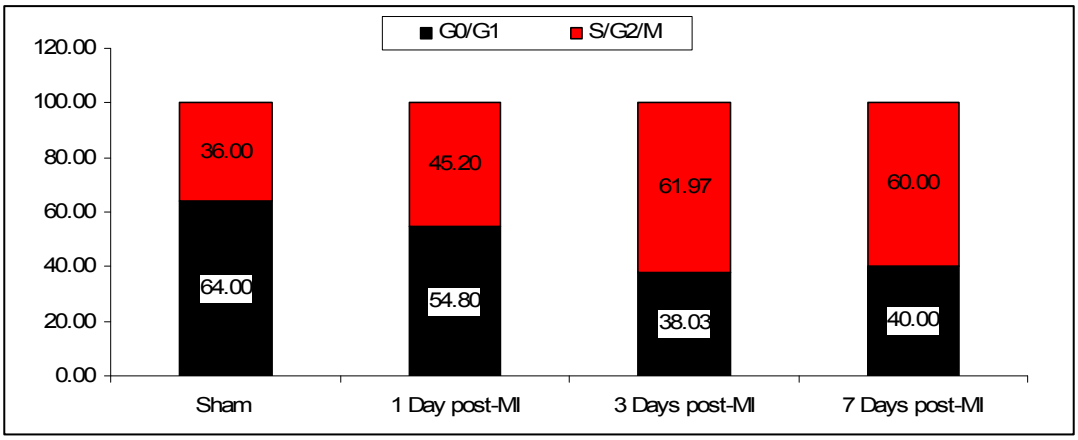

Figure 37: The above diagram depicts the total differences in CSP proliferation at different time points after myocardial infarction in comparison with a non-injured (sham) model.

Discussion

Characterization of expanded cardiac side population cells

The goal of this study was to establish an *in vitro model* for the expansion of the cardiac side population cells. So far, previous attempts to maintain the CSP cells *in vitro* were not successful [59]. This is the first example of expansion of CSP cell in a media commonly used for the isolation and the proliferation of mesenchymal stem cells [60] and lung side population cells (unpublished data from the laboratory of Fine A. and Summer R. from Boston University). There are additional similarities among the expanded CSP cells and the mesenchymal stem cells besides the use of common culture media. The expanded CSP cells are selected in an almost identical way with the mesenchymal stem cells. Both of these cell types are seeded, as primary cell populations, in a culture dish and after a certain amount of time (3-4 days) the non-adherent cells are removed while the attached cells start to grow. An additional common feature of the two populations is the cellular morphology. The CSP cells and the mesenchymal stem cells are both very similar to fibroblasts, as it is shown in figure 38.

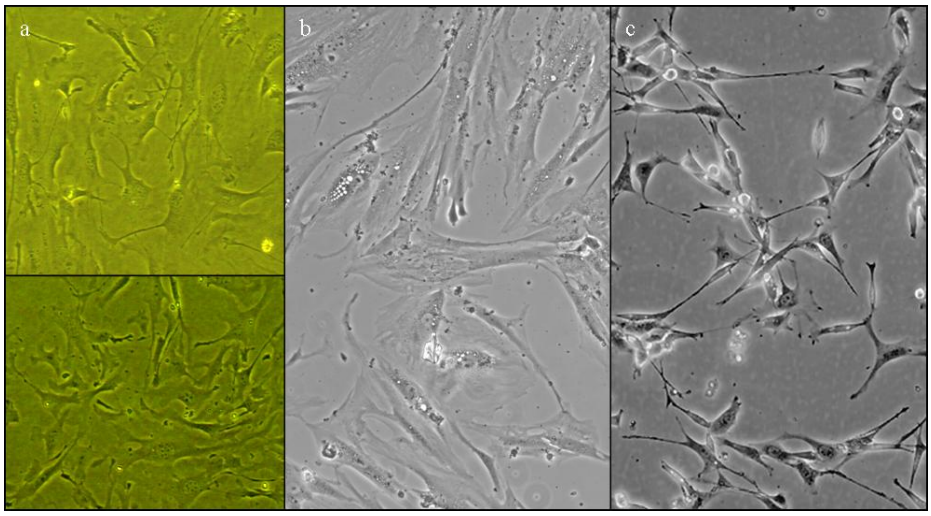

Figure 38: Mouse CSP cells (a) and human mesenchymal stem cells (b) demonstrate a fibroblastic morphological phenotype that resembles closely the phenotype of mouse fibroblasts (c).

The above characteristics suggest that the CSP cells resemble to mesenchymal stem cells. In order to address further this hypothesis the CSP cells were analyzed for the expression of several markers (CD29, CD44, CD73, CD90 and CD117) that comprise a defining set of markers for the mesenchymal stem cells [54]. The mesenchymal stem cells are expressing all the above mentioned antigens except the CD117, which is the cell-surface marker c-kit. The CSP cells are expressing the antigens CD29 (70%), CD90 (92%) and CD44 (60-70%) and they are negative for the expression of the antigens CD117 and CD73.

Despite the above similarities, there are important differences between the two cell types as it concerns their differentiation potential. The mesenchymal stem cells are considered pluripotent giving rise to cell types from the three germ lineages. In contrast, the freshly isolated CSP cells can differentiate to endothelial cells and mesenchymal type cells (cardiomyocytes and smooth muscle cells). The expanded CSP cells can differentiate to cardiomyocytes but not to adipocytes and chondrocytes. In conclusion, the mesenchymal stem cells do not share the same differentiation capabilities with CSP cells but they have several similarities in terms of morphology and immunophenotypic profile. It is possible that the CSP cells could represent a special type of tissue specific mesenchymal progenitor cell.

The expression of several additional markers was examined in order to characterize further the expanded CSP cells. The endothelial marker CD31 was not expressed in more than 3-5% of the cultured CSP cells at any passage. Approximately 60%±10 of the cultured CSP cells express the stem/progenitor cell antigen $Sca1^+$. Both the CSP/$Sca1^+$ and CSP/$Sca1^-$ cells attach on the culture dish but it is unclear whether there is any difference in the functional properties (proliferation rate and differentiation potential) of these two CSP sub-populations. The expression of the antigen $Sca1^+$ of the cultured CSP cells is an indication of their self renewal ability, even though the freshly isolated CSP cells are exhibiting a higher expression level (85%±2) [28].

Another similarity among freshly isolated CSP cells and expanded CSP cells is that they both express the transcription factors Nkx2.5 and GATA-4. Although it is not determined whether these transcription factors are expressed in the protein level the

expression in the gene level is suggesting that the expanded CSP cells hold some cardiomyogenic differentiation potential. An interesting point would be to determine the exact percentage of the expanded CSP cells that express GATA-4 and Nkx2.5 at any time point by immunocytochemistry.

The expanded CSP cells express both the ABC-transporters MDR1 and BCRP1 in the mRNA level. The MDR1 transporter is more abundant than the expression of BCRP1 in expanded CSP cells, as it was revealed by real time PCR analysis. The MDR1 transporter expression was approximately 2-fold higher than of BCRP1 in the expanded CSP cells. More importantly the transporters MDR1 and BCRP1 remain functional under the *in vitro* conditions, as it was shown in figure 26. The mentioned variation in the number of Hoechst low cells among the expanded CSP cells could be due to problems of the staining process. The concentration of the Hoechst dye and the precise calculation of the analyzed cells could affect the percentage and the number of the SP cells. Although, the persistence of the expression and the function of the MDR1 and the BCRP1 in the expanded cells is strong additional evidence that expanded CSP cells maintain their identity as side population cells.

The expanded CSP cells express several genes with particular importance in the regulation of the processes of cardiomyogenic differentiation and self-renewal. By conventional PCR it was shown that the CSP cells express Wnt-11 and TGF-b. Wnt-11 is a member of the non-canonical Wnt signaling pathway and is the ligand of the Frizzled transmembrane receptor [61]. The Wnt signaling pathway is involved in the regulation of the properties of various stem cell populations and there are several reports showing that it is also involved in the regulation of the side population cells of the mammary and pituitary glands [62, 63], [42, 64, 65]. Additionally the non-canonical Wnt signaling is important for the process of cardiomyogenic differentiation [66]. Thus the expression of Wnt-11 by the expanded CSP cells, given their cardiomyogenic potential, suggests that the non-canonical Wnt signaling pathway may represent an putative regulatory mechanism of their differentiation. The TGF-b factor is involved in the process of cardiomyogenic differentiation of embryonic stem cells, similarly with the Wnt-11 [67].

Hence, the CSP cells express several regulatory molecules that are associated with the cardiomyogenic potential of progenitor cells.

The expanded CSP cells express also molecules that are important for their self-renewal capacity. These molecules are the receptor of the growth factor IGF-1, the regulatory molecule Bmi-1 and the neural stem cell marker nestin. The IGFR-1 is involved in the regulation of proliferation and survival of cardiac stem cells expressing c-kit$^+$/mdr1$^+$/Sca1$^+$ [21] [68]There are several reports linking the Polycomb group (PcG) gene Bmi-1 with the regulation of the self-renewal and proliferation processes in hematopoietic stem cells [69-71]. The expression of the Bmi-1 gene is 5-fold higher in the expanded CSP cells in comparison with freshly isolated cardiomyocytes. The expression of an important regulatory gene in the expanded CSP cells strengthens the hypothesis that the expanded CSP cells maintain a high level of self-renewal ability.

Similarly with the Bmi-1 the expression of another stem cell marker, indicative of the stemness of the expanded CSP cells, is the gene nestin. Nestin is a neural stem cell marker [72] but it is also expressed in a significantly high level in side population cells isolated from the adult pituitary gland [42]. Taken together the above results suggest that the expanded CSP cells express several regulatory molecules which are important for the self-renewal property of stem cells. It is very interesting to examine to exact fraction of the expanded CSP cells that express the above markers.

Expansion of csp cells

Subsequently the growth rate of the CSP cells it was determined in comparison with cardiac main population cells. The main population cells, as mentioned in the introduction, lack the ability to efflux the Hoechst dye but besides that property they also grow in the same expansion media as the CSP cells. The CSP cells proved to hold significantly higher proliferation capacity than the cardiac main population cells. The duplication rate of the CSP cells is almost 3-fold higher than that of the main population cells, 2.5-3 days in comparison with 8-9 days respectively. Thus, the main population

cells do not exhibit similar proliferative capacity with the CSP cells, clearly indicating the different nature of the two cell populations.

Another interesting characteristic related to the growth potential of the CSP cells is that the older cells (passage 16) grow significantly faster than the younger (passage 5) CSP cells. However, the reasons for this difference are not known. A possible cause is genomic instability, aneuploidy, which characterizes almost all the solid tumors and occurs early during neoplastic development [73]. During the elongated culture period of the CSP cells, some changes in the chromosome stability could progressively occur, accumulate and finally affect the properties of the CSP cells.

Cardiomyogenic differentiation capacity of expanded cardiac side population cells

The main functional characteristic of the freshly isolated CSP cells is their potential to differentiate into mature contracting cardiomyocytes. Using a co-culture system the expanded CSP cells were cultured with adult rat cardiomyocytes in order to examine their cardiomyogenic potential. It was shown that the expanded CSP cells express a-actinin in well organized sarcomeric structures and also they contract in the same pace as the adult cardiomyocytes. They also express GATA-4 and MEF2C, which are important transcription factors for the cardiomyogenic process [28]. The above confirm that the expanded CSP cells retain the ability to form mature cardiomyocytes.

The differentiation of the freshly isolated CSP cells was shown by Pfister *et al* to be dependent on the physical coupling of the CSP cells with cardiomyocytes in the culture dish. However, in this report there is some evidence of spontaneous cardiomyogenic differentiation of some CSP cells independent from the physical contact with cardiomyocytes. As shown in figure 31 the differentiated CSP cells express sarcomeric a-actinin and they are not in direct contact with any cardiomyocyte but they are directly connected with each other. However, the differentiated expanded CSP cells

in most of the cases were in physical contact with the adult cardiomyocyte, as shown in figures 28, 29, 30 and 33.

Finally the option that the differentiated CSP cells were false positives due to cell fusion among GFP-CSP cells and adult cardiomyocytes was examined. For this reason GFP-CSP cells and adult cardiomyocytes transduced with an RFP virus were used. In the case of cell fusion the false positive differentiated CSP cells should have an orange color as opposed to green if they were not fused. After three weeks in co-culture no fused orange-colored cells were detected. Thus, the differentiation of the expanded CSP cells to cardiomyocytes is not attributed to cell fusion, similarly with the freshly isolated CSP cells. Additional evidence that the cardiomyogenic capacity of the CSP cells is autonomous of cell fusion is presented in figure 33. These differentiated CSP cells are not fused with a cardiomyocyte because there are not any cardiomyocytes surrounding them. In conclusion, the *in vitro* conditions do not increase or create any tendency of the expanded CSP cells to fuse with cardiomyocytes.

Taken together, the above data suggest that the CSP cells can be expanded *in vitro* without compromising their cardiomyogenic potential. The expanded CSP cells express several gene markers in a very similar way with the freshly isolated CSP cells and retain the ability to differentiate in mature cardiomyocytes, exhibiting contraction and clear sarcomeric organization. Moreover they express several important regulatory molecules of stem cell properties. Therefore the model of the expansion of the CSP cells in combination with the co-culture model of their differentiation represent powerful tools for further studying this endogenous cardiac progenitor cell population.

Effects of MI on CSP cells proliferation in vivo

In order to evaluate the effects of a severe cardiac injury on CSP homeostasis adult mice were subjected to myocardial infarction. As control mice subjected to sham operation were used. At different time points (1, 3 and 7 days post-MI) the CSP cells

were sorted from the operated mice (sham and MI) and were stained with propidium iodide (PI).

At day 1, in sham operated animals, approximately 63% of the freshly isolated CSP cells were arrested in the G1/G0 cell cycle phase. At the same time point, in MI operated animals 55% of the CSP cells were detected to be arrested in the same cell cycle phases. The difference between the cycling cells in the two types of animals was not significant at that time point. At day 3 and day 7 post-MI in sham operated animals were detected approximately 65% of the CSP cells in the G1/G0 cell cycle phases. In contrast at the same time points in MI-operated animals the number of arrested CSP cells had decreased to approximately 40%. Thus at day 3 and day 7 the number of cycling cells in MI-operated animals was 62% and 60% respectively. The differences in the numbers of cycling cells at day 3 and 7 were significant.

The above data suggest that after MI in the myocardium the CSP cells start to proliferate in response to unknown signals caused by the injury. In MI operated animals the number of proliferating CSP cells becomes significantly higher from that in sham operated mice only three days after injury and remains in the same significant level for at least 1 week post-MI.

As it was shown by Mouquet *et al* at day 1 post-MI there is an acute depletion (40%) of the total CSP pool and this depletion is more severe (80%) in the infarcted area compared to the non-infarcted area (20%). The CSP cells start to recover at day 3 and day 7 post-MI by two independent mechanisms. The proliferation of the endogenous CSP cells and the mobilization of bone marrow derived cells (GFP^+) to the myocardium, as indicated by the presence of GFP^+ cells in the CSP pool. In parallel with the increase of the proliferation of the CSP cells and the mobilization of bone marrow cells there is an increase in the numbers of the side population cells in the peripheral blood and a decrease in the side population cells of the bone marrow.

The proliferation of the CSP cells after injury was also measured using the cell cycling marker Ki-67 [32]. According to the results from the Ki-67 staining in sham-operated animals only 10% of the CSP cells are cycling. In MI-operated animals the fraction of the cycling cells in the infarcted area at day 1 was 5%, at day 3 was 20% and

at day 7 was 25%. In the non-infarcted area the portion of the cycling cells at day 1 and day 3 was 10% and only at day 7 increased to 30%. Thus this method also suggests that the proliferation of the CSP cells becomes significant at day 3 and remains higher than in the sham animals up to day 7. The differences in the detected amount of proliferating CSP cells between the two methods are caused probably due to technical issues. The amount of the analyzed CSP cells from the different animals at the different time points was approximately 1500. The PI staining method is commonly used in proliferation assays when the number of cells is much higher, approximately 50000. So it is possible that the quantity of analyzed cells could create significant variability. Although the differences in the results from the two methods are profound, the main message is exactly the same. The proliferation of the endogenous CSP cells is increased and becomes significant only at 3 days post-MI and remains higher than the sham-operated animals even 7 days post-MI. Thus both methods strongly suggest that the proliferation of the endogenous CSP cells is a homeostatic mechanism for the replenishment of the CSP progenitor cell pool.

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