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Cardiac stem and progenitor cell identification: Different markers for the same cell?

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1. ABSTRACT

For a long time the heart has been considered a terminally differentiated organ without any regenerative potential. The latter has been classically based on the terminally differentiated nature of cardiomyocytes and the absence of a pool of tissue-specific stem cells. This view has been radically changed due to the discovery of resident cardiac stem and progenitor cells in the adult mammalian heart. However, at minimum, 5 apparently different cardiac stem and/or progenitor cell types have been described so far. Thus, we have changed from a view of the heart as a static tissue to an organ with the highest number of tissue-specific stem cell populations. Most likely, the different putative adult cardiac stem and progenitor cells represent different developmental and/or physiological stages of a unique resident adult cardiac stem cell. Notably, it is not yet known the origin of all these cells. A better understanding of the origin, biology and physiology of the myocardial stem and progenitor cells will impact the development of regenerative medicine as an effective therapy for heart disease and failure.

2. INTRODUCTION

Despite the remarkable progress made in the treatment of cardiovascular diseases during the past half-century, the fact remains that for many the available treatment is at best palliative. Meanwhile the increasing success in treating acute life-threatening ischemic cardiac diseases often results in an extended life for the patient but leaving a chronic condition (1). These chronic sequelae are frequently without effective treatment. Nowadays, there are at least 10 million patients with heart failure in European countries with prevalence in the general European population ranging from 0.4 to 2% (2). The prognosis of heart failure is uniformly poor if the underlying problem cannot be rectified. Half of patients carrying a diagnosis of heart failure will die within 4 years, and in patients with severe heart failure >50% will die within 1 year. Indeed, once congestive heart failure emerges, no current therapies can improve long-term cardiac function (3) and the only available alternative is organ transplantation, with all the logistic, economic and biological limitations associated with this intervention (4). The root problem for all these

patients is a functional deficit arising from a loss of myocardial contractile cells and an inadequate coronary circulation to nurture those remaining, leading to the pathological cardiac remodeling responsible for the development of heart failure (5).

A goal of cardiovascular research for the past decade has been to find a method of replacing the contractile cells (cardiomyocytes) lost through ageing and/or one or more myocardial infarctions, so as to prevent, or reverse, the pathological remodeling of the myocardium. With the explosion of interest for the new exciting field of regenerative medicine, stem cells are widely regarded as "the magic bullet" to repair the damaged myocardium (6). Indeed, stem cell therapy is fast becoming an attractive and highly promising treatment for heart disease and failure. Research into its design and application is currently at the very cutting edge of biomedical research. Current clinical trials are mainly using bone marrow cells of different origins as the therapeutic agent (6,7) and scientifically the race is still on to find the 'best' type and source of cell to reconstitute the myocardium and improve function following myocardial damage.

Until recently, the adult mammalian heart has been considered a post-mitotic terminally-differentiated organ, precluding any possibility for intrinsic regeneration. Thus, a myriad of exogenous cell types have been experimentally evaluated to test their capacity to replace lost cardiomyocytes and recover the myocardial tissue (6-8). Embryonic stem cells, fetal myocytes, skeletal myoblasts, endothelial progenitor cells, bone marrow-derived cells, including mesenchymal and hematopoietic stem cells, amniotic fluid-derived stem cells, and adult testis stem cells have all been transplanted into the post-infarcted myocardium of experimental animal models in order to generate new cardiomyocytes, vascular structures, or both (6). Despite the encouraging results obtained from experiments carried out in small animals, the outcomes from clinical trials have been modest. Recently, a new population of multi-potent progenitor cells (MPCs) present in peripheral blood has been described (9). These cells (s) have proprieties of stem cells and appear to display a primitive phenotype with molecular and functional characteristics similar to human embryonic stem cells (9).

The identification of a few small cardiomyocytes undergoing mitosis in the normal adult heart of rats, mice and humans has challenged the worldwide accepted paradigm that considered the heart a post-mitotic organ, without any intrinsic regenerative potential (5). Undoubtedly, the heart is mainly composed of terminally-differentiated cells, with cardiomyocytes unable to re-enter the cell cycle under any known physiological or pathological stimuli (10). Recently, by monitoring carbon 14 emitted from Cold War-era nuclear bomb tests, Bergmann et al. obtained strong evidence for cardiomyocyte renewal in humans (11). Myocyte turnover and replenishment appears to be the product of the activation and differentiation of a pool of resident cardiac stem and progenitor cells. Indeed, these cells, are spread throughout the myocardial tissue in the four cardiac chambers, can give rise to functional cardiomyocytes *in vitro* and *in vivo* and owing to genetic labeling and transitional tracking of differentiation it is now strongly documented that the newly born cardiomyocytes observed in the adult mammalian heart are the product of resident cardiac stem cell (CSC) differentiation (12, 13).

The first report of mammalian CSCs by Nadal-Ginard, Anversa and colleagues in 2003 (14) was rapidly followed by other works (15-20), which described various surface markers for the identification and the isolation of cells with stem and progenitor properties in the adult mammalian heart, including human. Interestingly, each reporting group has placed emphasis on different markers which made their cell "unique" and different from those previously described. Unfortunately, the use of these markers, each supposedly identifying a specific stem cell, created confusion in the scientific community regarding which cell is the real cardiac stem cell and what is its origin. The most common misunderstanding originates from the uncertain difference between CSCs and progenitor cells. Indeed, although some authors have grouped cardiac cells proven to have stem and/or progenitor features under the same acronym, there is an exact difference between the two cell types. Stem cells, as defined by Potten and Loeffler, are "undifferentiated cells capable of 1) proliferation, 2) self-maintenance, 3) production of large number of differentiated progeny, 4) regeneration of the tissue after injury, and 5) flexibility in the use of these options" (21).

According to this definition, CSCs are clonogenic, self-renewing and multipotent, giving rise to a minimum of three different cardiogenic cell lineages (i.e. myocytes, smooth muscle and endothelial cells) both *in vitro* and *in vivo* and exhibit significant cardiac tissue regenerative capacity when injected into infarcted rat myocardium (14). On the other hand, a cardiac progenitor cell is an immature but already committed myocardial cell that can proliferate and mature into its respective precursor which, in turn, develops into one of the main cardiac cell lineages (22).

Here we will overview the latest advances in the identification and characterization of the different stem and progenitor cell populations reported to be present in the neonatal and adult mammalian heart, including the human, and the possibility that some of these cells are probably phenotypic variants of a "master" CSC.

3. RESIDENT CARDIAC STEM CELLS IN THE NEONATAL AND ADULT MAMMALIAN HEART

Several lines of evidence have been obtained in favor of adult myocardium regeneration. Quaini *et al.* (23) reported an unexpected form of chimerism after the transplantation of hearts from female donors into male recipients. They identified a high number of "primitive" cells of host origin that migrated into the transplanted heart and generated not only new cardiomyocytes but also endothelial and smooth muscle cells. This incontestable evidence provides a conclusive affirmative answer to the

Table 1. Characteristics of resident cardiac stem and progenitor cells identified in the neonatal and adult heart

PHENOTYPE	SPECIES	*In vitro* STEM CELL CHARACTERISTICS	*In vivo* CARDIAC REGENERATIVE POTENTIAL
C-KIT	- Adult rodent[14] - Neonatal and Adult mouse[17, 30, 31] - Post-Natal and Adult human[17, 36, 38] - Adult Porcine[27, 28]	- Yes - Yes - Yes - Yes	- Yes - Yes[17, 31] - Yes - Yes
SCA-1	- Neonatal and Adult mouse[15, 16, 39] - ? Adult human (Sca1-like)[40]	- Self-renewing, colony formation[39], cardiomyocyte[15,16,39] and osteogenic and adipogenic differentiation[16]. - Cardiomyocyte differentiation	- Yes[15, 39] - Yes[41]
SP	- Neonatal and Adult mouse[18-20, 42]	- Cardiosphere formation[20] - Cardiomyocyte[18-20,42], Neuron and Glia differentiation[20]	- Yes[42]
Isl-1	- Post-natal Mice, Rodent and Human[24]	- Cardiomyocyte differentiation	ND
Epicardial progenitors C-KIT	- Adult mouse and human[60]	- Cardiac and vascular precursors	ND, but proliferation and differentiation into cardiac and vascular lineages in the epicardium after AMI.

SP denotes side population; ND denotes Not Determined; AMI denotes acute myocardial infarction.

question of whether or not the heart has regenerative potential. In the same study, cells with similar characteristics were also found in the normal myocardium (23). Based on these data, a variety of studies have established that the heart contains a reservoir of stem and progenitor cells. Indeed, CSCs have been isolated from different animal models by selection based on c-kit, Sca-1, and/or Abcg2 (MDR-1) expression. Because the "stemness" of a cell is not linked to a single specific biological marker, many reporting groups have independently described a "unique" CSC or progenitor cell that has demonstrated to be different from those previously reported, showing a combination of different stem-associated cell surface markers. With the exception of the Islet-1 cells, which decrease dramatically in number into adulthood (24) and seem to be remnants from the cardiac primordia (25), the identification of different cardiac stem progenitor cells by expression of other membrane markers (see Table 1) suggest that these phenotypically different cells are likely to be phenotypic variations of a unique cell type. It is highly unlikely that a tissue which until recently was believed to lack any self-renewal capability is indeed populated by several different types of tissue-specific stem cells.

3.1. c-kit^{pos} cardiac stem cells (CSCs)

A distinct population of resident c-kit positive (c-kit^{pos}) CSCs were first identified and characterized in rodents (14). Importantly, these cells, identified by their negativity for the markers of the hematopoietic lineage (i.e. CD45; Lineage negative (Lin^{neg})) but positive for c-kit, the tyrosine kinase receptor for stem cell factor, are self-renewing, clonogenic and, at least, multi-potent giving rise to the three main cardiogenic cell lineages: myocytes, smooth muscle and endothelial cells. When grown in suspension, CSCs form spherical multi-cellular clusters of hundreds of cells dubbed 'cardiospheres' because of their similarity to the pseudoembryoid bodies yielded by neural stem cells (26). Out-growing cells from these spheres express biochemical markers of myocytes, smooth muscle and endothelial cells (14, 22). c-kit^{pos} CSC-derived cardiospheres, when placed in a specific medium with cardiopoietic factors, differentiate into functional beating cardiomyocytes *in vitro* (Figure 1). Additionally, to determine whether these cells were able of acquiring functional competence *in vivo*, after being BrdU- or genetically-tagged, c-kit^{pos} cells were injected into the border zone of an experimentally produced myocardial infarction (14). These labeled cells formed a band of regenerating myocardium composed of newly-formed cardiomyocytes and vascular structures within the infarcted region, which significantly contributed to improving cardiac function (14). Moreover, this pool of resident c-kit^{pos} CSCs can be recruited and activated by growth factors to regenerating myocardium after ischemia (27, 28) and has pro-survival effects on adult rat cardiomyocytes *in vitro* (29).

Since the first identification of resident CSCs in the adult rodent heart, different groups have proven the existence of cells with similar characteristics and regenerative potential in other species. Interestingly, it has been shown that adult human heart has a population of c-kit^{pos} myocardial cells with analogous characteristics to

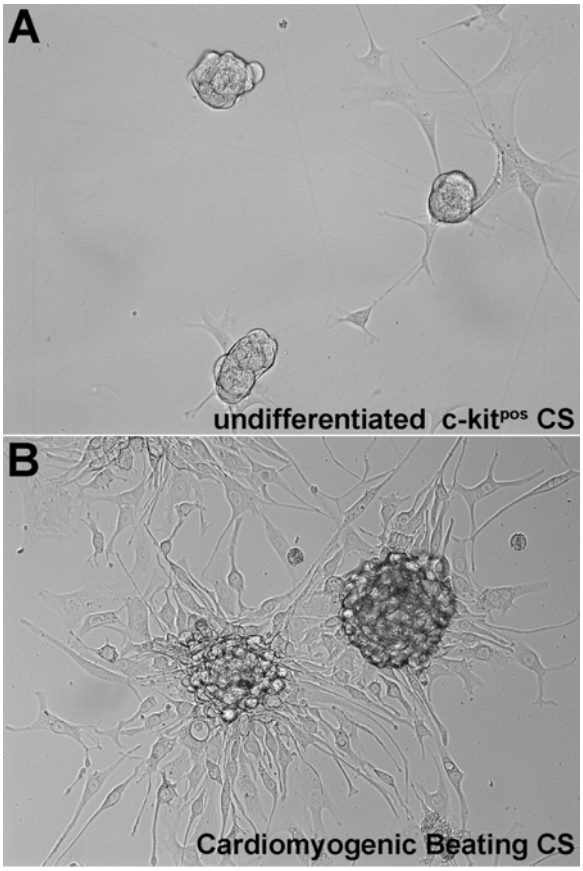

Figure 1. Light microscopy images showing mouse c-kit^{pos} CSC-derived cardiospheres (CS) maintained in undifferentiated conditions (A) and their myogenic differentiation into beating CS when stimulated with specific cardiopoietic factors (B).

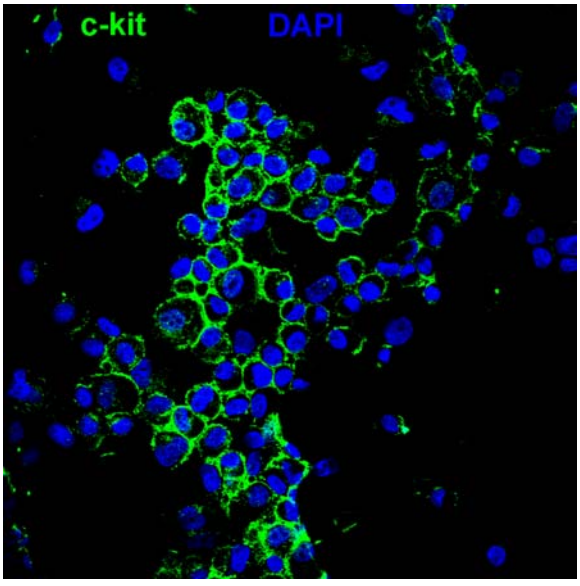

Figure 2. Cytospin preparation and immunofluorescence staining of human c-kit^{pos} CSCs analyzed by confocal microscopy.

CSCs found in rodent and murine hearts. Indeed, Messina *et al.* (17) isolated cardiosphere-forming c-kit^{pos} cells from biopsy samples of human myocardium. These cells were cloned with efficiency similar to rodent CSCs and when injected into immunodeficient animals after myocardial infarction they were able to regenerate functional cardiac tissue (17). In addition, the density of Lin^{neg} c-kit^{pos} CSCs in the adult human and rodent myocardium is similar: 1 cell per approx. 1,000 myocytes or approx. 50,000 CSCs per gram of tissue (32). Furthermore, their existence and activity has been demonstrated in physiological or pathological conditions (33-37).

Recently, we have confirmed and expanded these results. Indeed, we have isolated c-kit^{pos} human CSCs (hCSCs) from myocardial surgical or percutaneous biopsy samples from each of the four cardiac chambers of patients with ischemic and non-ischemic heart disease by explant culture technique and enzymatic digestion (Figure 2) (38). The c-kit^{pos} hCSCs are self-renewing and clonogenic, and their capacity to generate clones from a single cell appears to be similar to their rodent counterparts. Many experiments have demonstrated that all the hCSCs clones express high levels of c-kit and MDR-1 and they score negative for the hematopoietic and endothelial markers CD45, CD34 and CD31. These cells formed cardiospheres and under adequate stimuli differentiated *in vitro* into cardiomyocytes, vascular smooth muscle and endothelial cells. Many of these cloned hCSCs have undergone more than 60 passages so far without evidence of "crisis" or culture senescence. Importantly, when injected into the infarcted heart of *nu/nu* rats, they form histological and functional human myocardium and vascular structures. At the level of analysis performed so far, there are no detectable differences among the isolated cells that can be attributed to the cardiac chamber of origin (38). Thus, c-kit^{pos} hCSCs can be successfully and routinely isolated from small myocardial samples of the four cardiac chambers, expanded to large numbers and maintained undifferentiated and/or differentiated in culture as desired. Different isolation protocols are viable (36, 38) however, from the progeny of a single cell, it is possible to obtain > $1x10^{10}$ differentiation-competent hCSCs (38).

In recent reports, transgenic mice in which EGFP expression is placed under control of the c-kit locus have been used to obtain further data on c-kit^{pos} cardiac cells (30, 31). These data showed that the myocardial c-kit-EGFP^{pos} cell population increases in early postnatal growth as the heart expands in size, but declines rapidly in the first weeks after birth (30, 31). c-kit-EGFP^{pos} cells isolated from neonatal hearts showed evidence of commitment to all three cardiac lineages and several days after plating in specific media, many c-kit-EGFP^{pos} cells began to spontaneously contract (30). After myocardial cryo-injury to adult c-kit^{BAC}-EGFP^{pos} mice, it was reported that myocardial c-kit expression increased significantly at 7 days after injury and declined within a 4 week period to baseline levels. Upon closer examination, c-kit-EGFP^{pos} cells were contributing to the endothelium and smooth muscle layer of blood vessels in the cryo-injured zone and fibrosis in the border zone. Modest c-kit-EGFP^{pos} expression was also observed in striated mature

cardiomyocytes in the border zone (30). Using a different approach, the group of Sussman (31) also studied the response of c-kit^{pos}- cells during normal heart growth and following myocardial infarction induced by permanent coronary artery occlusion in adult transgenic c-kit-EGFP^{pos} mice. This study also reported elevated c-kit expression (approx. 5-fold) in the infarcted and border regions at 10 days after injury, however contrary to Tallini *et al.* (30) they showed c-kit-EGFP^{pos} cell recruitment to the area of injury, with differentiation of c-kit-EGFP^{pos} cells into the 3 main cardiac lineages: cardiomyocytes, smooth muscle and endothelium (31).The reason for the discrepancy between these 2 studies could be due to the choice of myocardial injury model. Unfortunately, as the construct used to develop both the c-kit-EGFP transgenic mice labeled all the c-kit^{pos} cells including bone-marrow derived cells, no firm conclusion can be drawn from these data on the actual genetic status, mechanism of differentiation and commitment, and biological potential of resident c-kit^{pos} CSCs.

3.2. Sca-1^{pos} cardiac progenitor cells

A resident population of progenitor cells has been isolated from the mouse heart based on expression of the stem cell antigen 1 (Sca-1) (15, 16). These adult-heart derived Sca-1^{pos} cells were described as being c-kit^{neg} in one case (15) but c-kit^{pos} in another (16). In the case of the former, Sca-1^{pos} cells also expressed Tie-2, Ang-1, and CD31, which might identify a primitive hemangioblast or its precursors (15). When treated with 5'-azacytidine *in vitro*, these cells expressed Nkx2.5, beta-myosin heavy chain (beta-MHC), alpha-myosin heavy chain (alpha-MHC), cardiac Troponin I and sarcomeric alpha-actin, documenting their cardiomyogenic commitment (15). Furthermore, when injected into the mouse heart following ischemia/reperfusion injury, adult-heart derived Sca-1^{pos} cells contributed to 1.5% new cardiomyocytes in the infarct and border zone (15). A similarly enriched population of adult cardiac Sca-1^{pos} cells isolated from the hearts of 10-12 week old mice formed beating cardiomyocytes with spontaneous calcium transients upon oxytocin treatment (16). Moreover, these Sca-1^{pos} cells also showed osteogenic and adipogenic differentiation *in vitro*. Interestingly, this Sca-1^{pos} population was also positive for c-kit (16). However, multi-potency of the adult Sca-1^{pos} cells remains to be proven in cloned cell assays. More recently, Matsuura and colleagues (39) produced limited dilution clonal colonies of Sca-1^{pos} cardiac progenitors from adult murine hearts (clonal efficiency of 0.1%), which could be expanded for more than 500 population doublings. Over culture time these clonal Sca-1^{pos} cells expressed the cardiac precursor markers, Nkx2.5 and GATA4, and cardiomyocyte differentiation genes and proteins, beta-MHC and sarcomeric alpha-actinin. When clonal Sca-1^{pos} cells were transplanted as monolayered sheets over the infarcted mouse heart, 4 weeks later there was improved cardiac function due to the formation of approximately 0.6 $\times$ 10^5 new cardiomyocytes which amounted to 5% of the entire hearts cardiomyocyte compliment (39).

Investigations have also begun with human myocardium. Smits *et al.* (40) have validated a protocol for the isolation of Sca-1-like cardiac progenitor cells from human cardiac surgical waste pieces (i.e. the auricle: appendix of the atrium). Although the human equivalent of murine Sca-1 is not yet known, it is postulated that the antibody may cross-react with an unknown protein, still leading to a homogenous cell population (40). These human derived Sca-1-like cells give rise to functional cardiomyocytes *in vitro* and generated new cardiac tissue consisting of human cardiomyocytes and blood vessels when injected intra-myocardially after a myocardial infarction in the mouse heart (41). Interestingly, these Sca-1 like cells were characterized as being c-kit low, yet negative for CD45 and CD34 and positive for CD31 and CD105 (40).

3.3. Side population-Abcg2^{pos} cardiac progenitor cells

Recently, the side population (SP) of cardiac cells within the Sca-1^{pos} fraction has been introduced as a reliable marker to identify subpopulations of cells with stem/progenitor cell properties in the developing and adult murine heart (18-20, 42). These cells are best characterized by the ability to efflux Hoechst 33342 dye mediated by a member of the family of ATP-binding cassette (ABC) transporter, Abcg2 (18). Whether the cells present in pre-natal and post-natal life represent the same or different cell populations remains unaddressed. Pfister *et al.* (19) have reported that among cardiac SP cells, the greatest potential for cardiomyogenic differentiation resides in the Sca1^{pos}CD31^{neg} population, which are capable of both biochemical and functional cardiomyogenic differentiation. Tomita *et al.* (20) observed that cardiac SP cell fractions from neonatal and adult mice hearts (heterogeneously positive for c-kit, Sca-1 and CD34) expressed nestin and Musashi-1, markers of undifferentiated neural crest stem cells. These cells formed cardiospheres, which went onto differentiate into neurons, glia of CNS and PNS lineage (*in vitro* and *in vivo*) and beating cardiomyocytes (*in vitro*). Indirect evidence from Cre/lox transgenic mice also supports that these SP- cardiospheres were derived from neural crest–originated cells present within the myocardium (20). Oyama *et al.* (42) documented that SP cells isolated from neonatal rat hearts and treated with oxytocin expressed cardiac specific genes and proteins and showed spontaneous beating. Furthermore, these SP cells significantly migrated and homed to the injured myocardium after intravenous injection and differentiated into cardiomyocytes, endothelial and smooth muscle cells (42). Nevertheless, direct and definitive proof that adult cardiac SP cells contain clonogenic, self-renewing and multi-potent cells is still missing, as is whether they exhibit regenerative potential *in vivo*. However, Martin and colleagues proved that Abcg2 expression regulates the proliferation state of cardiac SP cells in response to murine myocardial cryo-injury (43).

3.4. Homebox gene Islet-1^{pos} cardiac progenitor cells

A population of undifferentiated cardiac cells expressing markers different from those described above has also been identified (24, 25). These cells are remnants of cells present since the embryonic life in the heart fields and anterior pharynx (25). These cells express the homebox gene Islet-1 (Isl-1). They are most commonly located in the outflow tract, in the atria, and throughout the right

ventricle, in agreement with the embryonic contribution of the secondary heart field (SHF) (25). Nonetheless the number of these cells dramatically falls in the first few weeks of post-natal life (24) and a small number of Isl-1 positive cells are present in the fetal and early postnatal heart (25±7 Isl-1 cells in the left ventricle of a 1 day old rat; Ref 24) of rodents and humans. These cells have been proposed to represent residual SHF cells that identify a resident progenitor cell population potentially contributing to growth of the heart (24). Lineage tracing studies and purification of Isl-1^{pos} progenitor cells from the early embryo or post-natal heart have shown their self-renewal capacities and ability to contribute to multiple cardiovascular cells of distinct lineages, including cardiomyocyte, conduction system cells, endothelial and smooth muscle lineages *in vivo* and *in vitro* (24, 44, 45). Recently, Bu *et al.* (46) more closely analyzed the Isl-1^{pos} progenitor cells in the fetal human myocardium. Similar to their fetal rodent counterparts, human Isl1^{pos} progenitor cells, tagged by a Cre/Lox system, are self-renewing, clonogenic and multipotent giving rise to the three major cardiac lineages, i.e. cardiomyocytes, smooth muscle and endothelial cells (46). At the early stage of human cardiogenesis, these cells are Isl-1^{pos}/Nkx2.5^{neg}/KDR^{neg}. During different periods of development, Isl-1 is expressed in a family of partially committed progenitors, that depending on their stage of differentiation, are positive for Nkx2.5, KDR and also Wilms Tumor Gene (WT1); identifying the epicardial lineage (46). The multi-potentiality capacity of these progenitors to engraft in the heart and to regenerate lost myocardium has not been tested (47). Moreover, whether the signaling pathways regulating the Isl-1^{pos} progenitor cells during development and in the early embryo also regulate this population in the adult heart remains to be elucidated. In particular, Wnt/beta-catenin signaling stimulates proliferation of isolated Isl-1^{pos} cells (46, 48, 49), and Notch signaling appears to block differentiation and allows expansion of activated cardiac precursor cells in the failing heart (49). These molecular data should lay the foundations for ascertaining an applicable technique to isolate, maintain and trigger proliferation and differentiation of these cells for clinical application.

4. CARDIOGENESIS AND EPICARDIAL PROGENITOR CELLS

The mammalian heart develops through a series of carefully orchestrated events. Two different mesodermal heart fields contribute to the development of the heart in a temporally and spatially specific manner. The “first” heart field (FHF) derives from cells in the anterior lateral plate mesoderm. These cells form a primitive heart tube and contribute to populate the left ventricle, right ventricle, and inflow region of the heart. More recent studies indicate that the heart tube derived from the FHF may predominantly provide a scaffold upon which cells from the “second” heart field (SHF) migrate and build the requisite cardiac chambers (50). The SHF is marked by the expression of Isl-1, Tbx1 and the growth factors Fgf8 and Fgf10 (25, 51, 52). Isl-1-expressing SHF progenitors contribute to cardiomyocytes that ultimately reside in the outflow tract (OFT), right ventricle, and inflow region. The left ventricle and OFT are therefore mostly exclusive derivatives of the FHF and SHF, respectively. (25, 53, 54). Despite these differences, the FHF and SHF are contiguous in the early embryo, and recent studies suggest that FHF cells transiently express genes that continue to be expressed in the SHF, including Isl-1 (55). Previous studies in avian species have demonstrated that the pro-epicardium (PE) and/or epicardium provide the heart with non-myocardial cells that are necessary for a complete and correct cardiac development. These studies showed that the PE and/or epicardium are a source for coronary vascular progenitors and cardiac fibroblasts (56-58). The epicardium has an extra-cardiac origin. In fact, the primitive epicardial tissue, also known as “epicardial mesothelium”, is formed by a monolayer of epithelial cells that originates from a cluster of cells derived from the septum transversum in mammalians and located close to the liver primordium in other vertebrates. This epicardial mesothelium covers the outer edge of the premature heart and the epicardial cells fill the so-called sub-epicardial space with a dense layer of extracellular matrix. After a process known as epithelial-to-mesenchymal transition of the epicardial mesothelium, a pool of these epithelial cells migrate into the subepicardium space where they generate a population of epicardially-derived cells (EPDCs), which have the features of pluripotent stem cells (59).

Recently, the presence of c-kit^{pos} cells in the fetal and adult human and murine epicardium has been reported (60). These cells are also positive for CD34, Sca-1 and MDR-1, but negative for CD45 and CD31. Based on these findings, the role of c-kit^{pos} epicardial progenitor cells in the process of adult myocardium repair was tested. The number of epicardial c-kit^{pos} cells increases in the epicardial compartment after inducing myocardial infarction in a murine model and the myocardial infarction provides the environment to induce their differentiation into a myocardial, endothelial and smooth muscle phenotype within the epicardial and subepicardial regions (60). Moreover, treatment of adult mouse and human epicardial explants with thymosin beta-4, a G-acting monomer binding protein which is implicated in reorganization of the actin cytoskeleton, stimulated extensive outgrowth of cells that differentiated into fibroblasts, endothelial and smooth muscle cells (61).

It is widely known that the epicardial cells in the embryo that migrate from the PE are characterized by the expression of the product of WT1, which plays a pivotal role in normal heart development. Moreover, it has been demonstrated that WT1 is expressed in PE and epicardium, but not in myocardium (62). Mouse embryos with homozygous WT1 null alleles (WT1 -/-) fail to develop several organs like kidneys, gonads, spleens and adrenal glands, and they exhibit severe defects in mesothelial tissue (63, 64). Importantly, WT1-/- embryos show an impaired developing and extremely thin myocardium, which sometimes consist of no more than a single layer of cells (63, 64). It has been shown that epicardial cells migrate from the PE and spread over the surface of the heart. A subset of epicardial cells turns into a mesenchymal

phenotype, migrates into the subjacent myocardium and differentiates mostly into smooth muscle cells and, a minority into endothelial cells, crucial in the development of coronary vasculature (65). Surprisingly, Pu's team (66) demonstrated that WT1 positive epicardial cells not only give rise to smooth muscle and endothelial cells, but also significantly contribute to cardiomyocyte formation (7-10% ventricular and 18% atrial) during normal cardiogenesis (66). This group analyzed the expression of cardiac genes Nkx2.5 and Isl-1 in the WT1 positive PE/epicardial cells. At the early stage of mouse heart development, Nkx2.5 and WT1 were expressed in adjacent cells, but not co-expressed, suggesting that Nkx2.5 and WT1 could be expressed sequentially, or just transiently co-expressed. In the same way, they demonstrated a robust contribution of Isl-1-expressing precursors to the WT1 positive cells in PE showing that WT1 and Isl-1 were expressed in adjacent regions and a subset of cells were positive for both markers. These data propose that PE/epicardial WT1 positive cells originate from progenitors that express Nkx2.5 and Isl-1 suggesting that they could share a common precursor with the widely known multi-potent cardiovascular progenitors (44, 66). Accordingly, another group led by Sylvia Evans (67), identified a population of Tbx18-expressing epicardial progenitors that contribute to cardiomyocyte formation in the ventricular septum, atria and ventricular wall. Moreover, these cells give rise to cardiac fibroblasts and coronary smooth muscle cells, but not endothelial cells (67). It is clear that there are still many questions regarding the biology of the epicardial progenitors, but the present findings propose these cells could be a possible source for cardiac regeneration and repair.

5. CARDIAC STEM AND PROGENITOR CELLS: THE SAME OR DIFFERENT CELLS?

For a long time the heart has been considered a terminally differentiated organ without any regenerative potential. The latter has been classically based on two lines of evidences: first, the cardiomyocytes, the main cell type of the adult heart, are terminally differentiated cells unable to divide under any physiologic or pathologic stimuli and second, the absence of a pool of resident tissue-specific stem cells. This view has been radically changed by the discovery of resident cardiac stem and progenitor cells throughout the atria and ventricles of the adult mammalian heart. However, as above described, at minimum, 5 apparently different cell types with tissue-specific characteristics of stem and/or progenitor cells have been described in the adult heart so far. Thus, we have changed from a view of the heart as a static tissue to one of an organ with the highest number of tissue-specific stem and progenitor cell populations. As the latter is improbable to be proved correct, aside from Isl-1^{pos} cardiac progenitor cells, it is likely that the different putative adult cardiac stem and progenitor cells reported so far, do not represent different cell types but, instead, different developmental and/or physiological stages of a unique resident adult cardiac stem cell.

One of the main reasons for the apparent confusion surrounding the myocardial stem and progenitor cells is that it is not yet known, at least for the majority of them, the origin of these cells, i.e. whether they are intrinsic cells present in the myocardium from embryonic and fetal life or cells of extra-cardiac origin which have colonized the myocardium in post-natal life, where they acquire tissue-specific properties.

Three papers have described a population of cells resident in the embryonic heart which give rise to all 3 cardiac lineages, suggesting a developmental origin of a common ancestor for the different cardiac progenitor cells (68). However, although pertinent, the phenotype of the multipotent cardiac progenitor cells (Isl^{pos}/Flk1^{pos}) described by Moretti *et al.* (44) and Kattman *et al.* (69) does not include c-kit. Also, both studies describe the location and *in vitro* differentiation of these cells but they have not yet shown the existence of similar multi-potent cells in the adult heart or their ability to reconstitute functional myocardium upon injury. Interestingly, Wu *et al.* (70) describe c-kit^{pos} /Nkx2.5^{pos} bi-potential myogenic precursor cells in the developing mouse embryo which more closely relates to adult c-kit^{pos} CSCs and therefore supports a developmental origin of CSCs. c-kit^{pos}/Nkx2.5^{pos} bi-potent progenitor cells underwent *in vitro* differentiation into both myocardial and smooth muscle cells and demonstrated engraftment and differentiation when transplanted into the chick embryo (70). It is highly tempting to speculate that these cells might represent different developmental stages of the same cell population which acquire different phenotypes and express a particular array of epitopes in response to local cues throughout development and in different regions of the heart. However, this still remains to be demonstrated.

In this regard the many varied phenotypes of cardiac progenitor/stem cells identified in the adult mammalian myocardium brings into question whether they are all exclusively different or actually of the same population of cell yet selected and identified at different physiological states. Recent work from our lab favors a transitional developmental sequence, which involves changes in expression of different receptors and transcription factors before differentiation into one of the 3 cardiac lineages (12). Under this view, it would be fair to argue the existence of one CSC and would predict the existence of a 'true' stem cell in the adult heart which exhibits more primitive characteristics than all the previously described adult "cardiac stem/progenitor cells". Indeed we and others have found a small population of Oct-4^{pos}/c-kit^{neg} cells in the myocardium of adult Oct-4/EGFP transgenic mice (12, 71). Interestingly, the number of Oct4^{pos} cells decreases with age (*Unpublished observations*). A fraction of the Oct-4^{pos} cells were also positive for c-kit suggesting a developmental response of the stem cell as it goes from being perhaps the 'early' quiescent stem cell to an amplifying progenitor. On the other hand, no Oct-4^{pos} cells were positive for the stem cell antigen, Sca-1. Oct-4^{pos} cells were also positive for other embryonic pluripotent markers, i.e. Nanog, Sox-2 and stage-specific embryonic antigen, SSEA-1 (*Unpublished observations*).

Nevertheless, even with the many identified phenotypically distinct cardiac progenitor cells little

evidence exists regarding the functional role of the different cell surface receptors identified on CSCs. Thus, a better understanding of the origin, biology and physiology of the myocardial stem and progenitor cells will impact the development of regenerative medicine as an effective therapy for heart disease and failure.

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Abbreviations: CSCs: Cardiac Stem Cells, AMI: Acute Myocardial Infarction, MPCs: Multi-potent progenitor cells, Sca-1: Stem cell antigen-1, Abcg2: ATP-binding cassette, sub-family G (WHITE), member 2, MDR-1: Multi-Drug Resistance-1, Lin^{neg}: Lineage negative, EGFP: Enhanced Green Fluorescent Protein, hCSCs: human Cardiac Stem Cells, beta-MHC: beta myosin heavy chain, alpha MHC: alpha myosin heavy chain, SP: Side population, Isl-1: Islet-1, OFT: Outflow Tract, FHF: First heart field, SHF: Secondary heart field, PE: Pro-

epicardium, EPDCs: Epicardial-derived cells, WT1: Wilms Tumor Gene

Key Words: c-kit; Sca-1, side population; Isl-1, Cardiac Stem Cells, Cardiac Progenitor Cell, Epicardial Stem Cell, Myocardial Regeneration, Review

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