Cell, Vol. 120, 295–298, February 11, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.cell.2005.01.025

Pursuing Cardiac Progenitors: Regeneration Redux

Minireview

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Recent studies have questioned the accepted dogma that the regenerative capacity of the heart following injury is limited. Several apparently distinct populations of resident cardiac progenitor cells may have the potential to regenerate functional heart muscle. Despite this progress, the physiologic role and therapeutic potential of cardiac resident progenitor cells remain unclear.

Cardiac myocyte cell loss related to myocardial infarction is the most common cause of congestive heart failure in the United States (Braunwald, 1997). Heart failure is the most common diagnosis of hospitalized patients in the United States, and the prevalence is increasing as the population ages. While advances in the prevention and treatment of atherosclerotic heart disease have reduced cardiovascular morbidity, once damage to the heart has occurred present therapies rarely result in long-term improvement of cardiac function (Jessup and Brozena, 2003). Despite the application of new medical therapies, the prognosis of patients diagnosed with heart failure is comparable to patients diagnosed with many forms of cancer. Usually, inexorable decline ensues from the time of diagnosis. Amidst the present era of regenerative medicine and excitement regarding stem cell therapies, attention has focused on the possibility of re-growing heart muscle in order to treat cardiac failure (Chien, 2004).

The task of inducing tissue repair of the myocardium is fraught with obstacles. Conventional teaching suggests that cardiac myocytes exit the cell cycle after a terminal round of cell division shortly after birth. The postnatal heart is thought to grow and adapt primarily via cardiac myocyte hypertrophy (growth of individual cells) as opposed to myocyte hyperplasia (an increase in cell number). In skeletal muscle, specific myogenic transcription factors have been identified with the capacity to drive nonmuscle cells to become skeletal muscle myocytes (for review see Buckingham, 2001) and resident myoblast progenitors serve as a repository for skeletal muscle regeneration in response to tissue injury. By contrast, the cardiomyogenic program has not been clearly elucidated. For this reason it has been difficult to unambiguously identify or isolate cardiac progenitors during development or in the intact heart. Prior to recent reports (discussed below) it was not believed that cardiac progenitors, or cardioblasts, existed in the postnatal heart.

Several approaches have been investigated to regenerate or repair damaged hearts. Embryonic stem cells possess the capacity to differentiate into cardiac myocytes (in addition to multiple other cell lineages) that resemble fetal or embryonic cardiac myocytes with poorly organized sarcomeric structures. ES cell-derived cardiac myocytes flux calcium, have spontaneous action potentials, and spontaneously contract and will engraft and electrically and mechanically couple to host cardiac myocytes when transplanted into the heart (Kehat et al., 2004). However, attempts to utilize embryonic stem cells to regenerate functioning heart muscle in vivo have been complicated by ethical concerns and the disturbing possibilities of teratoma formation and potentially fatal cardiac arrhythmias (Couzin and Vogel, 2004).

Nevertheless, the possibility that the heart can be repaired is not so far fetched. Zebrafish hearts will regenerate functioning myocardium when injured (Poss et al., 2002). Like the amphibian limb, the zebrafish heart appears to regenerate by forming a blastema at the site of injury where de-differentiation of cardiac myocytes produces proliferative myoblasts. However, no similar response to injury has been observed in mammalian hearts. Perhaps the ability to regenerate tissue in several organs, including the heart, has been lost or diminished through evolution in parallel with the emergence of increased complexity of patterning and function. If so, defining the molecular basis of cardiac regeneration in amphibian species may provide fundamental insights into cardiac myocyte regeneration.

But is the adult mammalian heart completely unable to regenerate cardiac muscle? Several recent reports raise hope that cardiac progenitor cells exist within the adult heart. Recent work from the laboratory of Kenneth Chien takes advantage of a new paradigm emerging from the study of developmental cardiology to identify a population of undifferentiated cells that reside within the mature heart (Laugwitz et al., 2005). If these observations bear fruit, they will serve as an important example of how a detailed understanding of organogenesis can inform regenerative medicine.

Until recently, the heart was believed to arise from bilateral clusters of mesoderm that include cardiac progenitors identified by coexpression of several early cardiogenic transcription factors including Nkx2-5 and GATA4. Within the past few years, several lines of evidence have led to a surprising shift in the established paradigm regarding the origin of cardiac myocytes. A second population of cardiac progenitors is now recognized that migrates into the outflow region of the looped heart and provides mature cardiomyocytes that contribute to the right ventricle and probably to the atria and left ventricle as well (Cai et al., 2003; Kelly et al., 2001; Kelly and Buckingham, 2002; Waldo et al., 2001). This previously unrecognized population of cells is called the secondary or anterior heart field. Precursors of the secondary heart field probably arise in or close to the cardiac crescent, where primary heart field cells also arise and subsequently migrate to the anterior pharynx before later contributing to the heart (see Fig-

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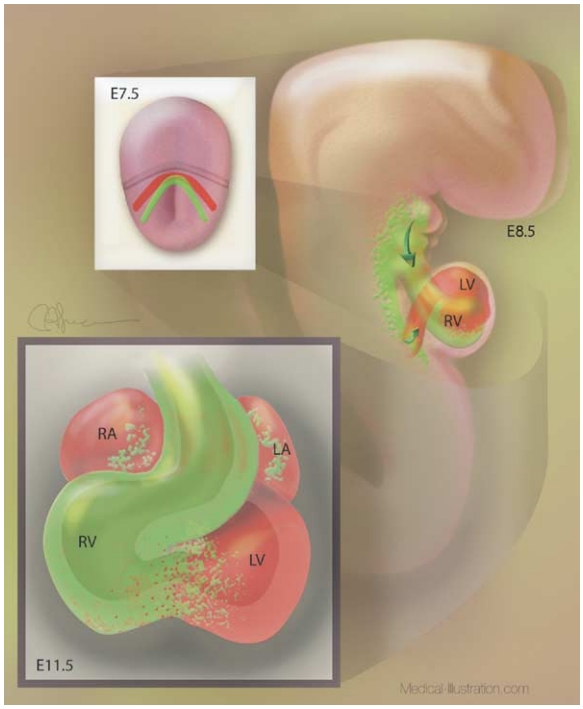

Figure 1. The Secondary Heart Field

Islet-1-expressing myocardial resident precursor cells are thought to arise from the secondary heart field (green) that originates near the cardiac crescent (upper left, E7.5, red). Secondary heart field cells then migrate to the anterior pharynx (right) and infiltrate the rostral and caudal poles of the heart to give rise to myocardial cells of the right ventricle (RV, bottom left) with contributions to the right atrium (RA), left atrium (LA), and left ventricle (LV). Myoblasts and myocardial cells of the primary heart field are shown in red.

ure 1). Secondary heart field myoblasts express many of the same cardiac markers as those previously analyzed in the primary heart field, including Nkx2-5 and GATA4, but they remain undifferentiated until later in development and they express some additional markers.

Sylvia Evans and coworkers showed that a subpopulation of cells in the anterior pharynx expresses the homeobox gene *islet-1* (*isl1*) (Cai et al., 2003). Expression of *isl1* is lost when these cells differentiate into cardiac myocytes. Interestingly, some *isl1*$^+$ cells can be identified in the mature hearts of newborn rodents and humans where they remain undifferentiated (Laugwitz et al., 2005). They are found most commonly in the outflow tract, the atria, and the right ventricle, in agreement with the embryonic contribution of the secondary heart field. Unlike some other putative resident cardiac progenitor populations (see below), these cells fail to express stem cell antigen 1 (Sca-1), CD31, or c-kit, though they do express Nkx2-5 and GATA4. Importantly, Chien and coworkers have shown that these cells can differentiate into cardiac myocytes both in vivo, using an inducible cre-lox system, and in vitro. *Isl1*$^+$ cells from hearts can be expanded in culture. Co-culture with cardiac myocytes leads to expression of terminal differentiation markers and electrophysiologic characteristics of fully differentiated cardiac myocytes including responsiveness to β-adrenergic agonists.

However, a number of questions remain to be addressed before the therapeutic potential of this population can be assessed. *Isl1*$^+$ cells have been isolated only from very young animal and human specimens, and the number of progenitor cells falls rapidly over the first few weeks of life. Do any resident progenitors persist into adulthood? Most of the ex vivo studies were performed on cells taken from 1- to 5-day-old animals, where only 500–600 *isl1*$^+$ cells were identified per rat heart. The rare *isl1*$^+$ cells identified at later times were not evaluated for their ability to expand or differentiate. Importantly, *isl1*$^+$ cells were identified in multiple organisms including humans, but the single human sample examined beyond 8 days of age (at 148 days) failed to reveal any *isl1* progenitors.

The factors that influence the ability to expand and differentiate these resident progenitors need to be elucidated. Are these cells multipotential? Although a high percentage (25%) of cultured cells expressed troponin T under differentiation conditions, only 2.3% displayed calcium transients characteristic of cardiac myocytes. A clearer understanding of the cell surface and secreted factors provided by co-cultured myocytes will allow for enhanced efficiency of cardiomyocyte differentiation. Can these factors be identified and/or enriched obviating the need for co-culture? Questions of multipotentiality, and the degree to which significant and meaningful expansion is possible, including expansion from a single isolated cell, will need to be addressed, especially given the potential concerns regarding tumor and teratoma formation that will accompany clinical trials.

Isl1$^+$ cells are not the only resident cardiac progenitors that may reside within the mammalian heart. A population of cells with stem cell-like properties has been identified in bone marrow, muscle, and skin by the ability to exclude Hoechst dye, resulting in a characteristic appearance after fluorescence-activated cell sorting (FACS) that led to the name "side population" or SP to describe this pool of cells (Gussoni et al., 1999). SP cells are multipotent with limited capacity to differentiate into striated skeletal myoblasts. Cells with the ability to efflux Hoechst dye have been identified in the heart (Martin et al., 2004). These cells are rare, and their ability to differentiate into contracting cardiac myocytes or to contribute to functional repair of damaged heart muscle has not yet been extensively evaluated.

Piero Anversa and colleagues reported the discovery of a distinct resident population of cardiac stem cells (Beltrami et al., 2003). These cells are negative for blood lineage markers CD34, CD45, CD20, CD45RO, and CD8 (Lin$^-$) and positive for c-kit (c-kit^{POS}), the receptor for stem cell factor (SCF). In the adult rat myocardium, Lin$^-$ c-kit^{POS} cells are relatively rare (~1 per 10^4 myocytes) but more prevalent than the *isl1*$^+$ progenitors described above. The cells are roughly one-tenth the size of cardiac myocytes. Cardiac c-kit^{POS} cells isolated by FACS sorting are heterogeneous with rare (7%–10%) cells expressing Nkx2-5, GATA4, and Mef2, and fewer still (0.5%) expressing genes encoding sarcomeric proteins. These cells retain these characteris-

Table 1. Properties of Resident Populations of Cardiac Progenitors and Stem Cells

Cells	Markers	n	Differentiation Method	Differentiated Phenotype	Clonogenic	Self-Renewal	Multi-Potent	Regenerative Capacity
Islet1$^+$ Progenitors	(+) isl1, Nkx2.5, GATA4 (–) Sca-1, c-kit (–) sarcomeric proteins	500–600[a]	Co-culture with neonatal cardiocytes	+ GATA4, Nkx2.5 + sarcomeric proteins + striations/contraction + E-M coupling + Ca^H transients + action potential	ND	Yes	No?	TBD
Lin$^-$ c-kit$^+$ stem cells	(+) c-kit (±) GATA4, Nkx2.5, MEF2 (–) CD34, CD45, Lin skeletal markers, smooth muscle markers low-level sarcomeric proteins detected	~1 per 1×10^4 myocytes	Differentiation medium (MEM + 10% FCS + 10^{-8} M dexamethasone)	+ GATA4, Nkx2.5, MEF2[b] + sarcomeric proteins smooth muscle proteins endothelial proteins – striations/contraction – sarcomeres	Yes	Yes	Yes (cardiocytes smooth muscle, endothelial cells)	Yes[e]
Sca-I$^+$ stem cells	(+) Sca-I, SP[c], GATA4, MEF2 (–) c-kit, CD34, CD45, Lin, Nkx2.5 (–) sarcomeric proteins	ND	5-aza-cytidine	+ GATA4, Nkx2.5, MEF2 + sarcomeric proteins[d] – striations/contraction – sarcomeres	ND	Yes?	ND	TBD[f]

[a] 500 – 600 isl1$^+$ cells were isolated from postnatal day 5 rat heart (Laugwitz et al., 2005).
[b] These markers were observed in 7%–10% of cells (Beltrami et al., 2003).
[c] A small percentage of Sca-I$^+$ cells effluxed Hoechst dye (Oh et al., 2003).
[d] <5% of cells expressed cTnI (Oh et al., 2003).
[e] Bands of regenerating myocardium were observed in injured hearts following intracardiac injection (Beltrami et al., 2003).
[f] Engraftment of Sca-1$^+$ cells was observed in injured hearts following i.v. injection (Oh et al., 2003).
ND, not determined. TBD, to be determined.

tics with passage in culture and can be expanded. When placed in differentiation medium, c-kit^{POS} clones differentiate into cells that biochemically (but not phenotypically) resemble cardiac myocytes. Sarcomeres or striations and/or contractile activity are not observed. Moreover, c-kit^{POS} clones also differentiate into smooth muscle cells and endothelial cells, strongly suggesting that they are multipotent. Of note, a common precursor or stem cell giving rise to these three cell lineages has not been identified previously.

Clinically relevant definitions of stem cells include the capacity to functionally repopulate appropriate tissues of a damaged recipient (Lakshmipathy and Verfaillie, 2005). Anversa and colleagues injected labeled c-kit^{POS} cells into the border zone of hearts of syngeneic rats after experimental myocardial infarction (Beltrami et al., 2003). A band of labeled regenerating myocardium was observed in 19 of 20 treated infarcts. The labeled cells expressing sarcomeric proteins were small relative to mature cardiac myocytes, but these cells exhibited visible striations and expressed connexin-43, a component of the fascia adherens of intercalated discs. An increase in capillary and arteriole density, and contribution of labeled cells to blood vessels, was also observed, as was a marked improvement in multiple indices of cardiac performance. Cardiac remodeling with improvement in systolic and diastolic indices may occur via multiple mechanisms, some of which are not dependent on replenishing cardiac myocyte reserve. Alteration of scar formation and remodeling, induction of angiogenesis, and inhibition of myocyte apoptosis are other potential mechanisms. In humans, significant myocardial regeneration is not observed following myocardial infarction, suggesting that if c-kit^{POS} cardiac stem cells exist they are either nonresponsive or inhibited from migrating and differentiating in response to acute myocardial infarction. Alternatively, perhaps the resident population of cardiac stem cells becomes senescent over time as most myocardial infarctions occur in older patients.

Schneider and colleagues reported a resident population of cardiac progenitor cells that copurifies with the nonmyocyte fraction and is characterized by expression of stem cell antigen 1 (Sca-1$^+$) (Oh et al., 2003). These cells express telomerase reverse transcriptase that has been associated with self-renewal potential and appear to be distinct from the *isl1*$^+$ and c-kit^{POS} cells described above. While they do express the early cardiac markers GATA4, Mef2, and Tef1, they do not express Nkx2-5 or genes encoding cardiac sarcomeric proteins. Though these cells do not spontaneously differentiate in vitro, when exposed to the cytosine analog 5-azacytidine, a small subpopulation of cells (<5%) demonstrate biochemical evidence of cardiac myocyte differentiation expressing sarcomeric α-actin, cardiac troponin I (cTnI) Nkx2-5, α- and β-myosin heavy chain, and type 1A receptor for bone morphogenic proteins. It remains unclear whether the cells that adopt some characteristics of cardiac differentiation represent a homogenous or (more likely) heterogeneous population of cells. Clearly, the majority of cells do not differentiate into cardiac progenitors under the conditions tested, raising the question of what othercell fates, if any, were induced following prolonged exposure to 5-azacytidine.

Nevertheless, in a proof-of-concept experiment, the capacity of cardiac Sca-1$^+$ cells to home to the heart and engraft following intravenous injection was tested in mice subjected to ischemia-reperfusion injury (Oh et

al., 2003). Remarkably, labeled Sca-1$^+$ cells were identified in the infarct border zone two weeks following injury. These cells expressed sarcomeric α-actin, cTnI, and connexin-43, suggesting that they underwent differentiation into cardiac myocytes in vivo. However, control experiments revealed that there was evidence of fusion between the Sca-1$^+$ cells and host cardiac myocytes (in up to 50% of the cells), a finding relevant to the interpretation of the experiments performed with c-kit^{POS} cells (Beltrami et al., 2003). Further studies will be required to determine whether a subpopulation of cardiac Sca-1$^+$ cells exists with restricted developmental potential to differentiate (at high frequency) into cardiac progenitors or cardiac myocytes.

As described in Table 1, the resident population of *isl1*$^+$ cardiac progenitors described by Chien and colleagues differs fundamentally from SP cells, from the c-kit^{POS} cardiac stem cells, and from the cardiac Sca-1$^+$ progenitors. The resident population of *isl1*$^+$ cells in the heart probably represents specified cardiac progenitors or "cardioblasts." By contrast, GATA4 and Nkx2-5 are expressed by only 7%–10% of the resident population of Lin$^-$ c-kit^{POS} cells, suggesting that this is a mixed population that includes both stem cells and determined cardiac progenitors. Similarly, the resident population of Sca-1$^+$, Lin$^-$ cells described by Schneider and colleagues most likely represents a heterogeneous population including a resident stem cell population.

It remains to be determined whether *isl1*$^+$ cells exist in the adult heart beyond the early postnatal period. In contrast, small clusters of Lin$^-$ c-kit^{POS} cells are observed in the adult in multiple species including humans. Similarly, in murine species a resident population of Sca-1$^+$ cells is easily identifiable in the myocardium. Though each of these cell populations has the potential for self-renewal ex vivo, only the *isl1*$^+$ cardiac progenitors have thus far been induced to differentiate at high frequency and to assume the phenotypic characteristics and physiological profile of cardiac myocytes as evidenced by (1) expression of early and late markers of the cardiac muscle cell lineage (and absence of markers for other cell lineages), (2) formation of organized sarcomeres, (3) spontaneous contraction including chronotropic responsiveness to adrenergic stimulation, and (4) spontaneous calcium flux and cardiac action potentials. However, the capacity of *isl1*$^+$ cardiac progenitors to engraft in the heart and to regenerate myocardium, to electrically couple, and to contribute to cardiac work has not been tested.

These are early days in the field of cardiac regeneration. Excitement regarding the possibility that bone marrow-derived stem cells or mesenchymal stem cells could differentiate into cardiac myocytes has rapidly led to clinical trials (Couzin and Vogel, 2004). More recently, the ability of hematopoetic and mesenchymal stem cells to differentiate into cardiac myocytes has been challenged. Before putative resident cardiac stem cells are tested in clinical trials, it would be prudent to define basic mechanisms in animal models. The initial analysis of *isl1*$^+$ cells has been relatively extensive, and the demonstration of calcium transients and cardiac action potentials is encouraging, but a recent report indicates that a population of small cells resident in skeletal muscle can also be expanded in culture and differentiated into cardiac myocytes with mature sarcomeres, calcium transients, cardiac action potentials, and β-adrenergic responsiveness (Winitsky et al., 2005). When delivered by tail vein injection after acute myocardial infarction, these cells, called SPOC cells, home to the damaged myocardium and differentiate into myocytes. The relationship between these cells and the apparently distinct resident stem cell populations needs to be explored. Suggestions of multipotentiality require more strict purification criteria and clonal assays. Resident stem cells in other tissues are dependent upon appropriate local environments, or niches, to maintain viability (Fuchs et al., 2004), but detailed information regarding a cardiac resident stem cell niche is lacking. Despite the enticing therapeutic potential of cardiac regeneration, a host of important questions remain to be answered before rational approaches will allow for optimal clinical utility.

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