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The Existence of Myocardial Repair:

Mechanistic Insights and Enhancements

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Abstract

The lack of myocardial repair after myocardial infarction and the heart failure that eventually ensues was thought of as proof that myocardial cell regeneration and myocardial repair mechanisms do not exist. Recently, growing experimental and clinical evidence has proven this concept wrong. Cardiac stem cells and endogenous myocardial repair mechanisms do exist; however, they do not produce significant myocardial repair. Similarly, the preliminary results of stem cell therapy for myocardial repair have shown early promise but modest results. Preclinical studies are the key to understanding stem cell senescence and lack of cellular contact and vasculature in the infarcted region. Additional laboratory studies are sure to unlock the therapeutic mechanisms that will be required for significant myocardial repair.

Keywords

cardiac stem cells; myocardial repair; stem cell therapy

Heart disease is the number 1 cause of mortality in the developed world¹ with postmyocardial infarction (MI) heart failure (HF) being a major contributor. The loss of functional cardiomyocytes leads to infarct expansion and thinning of the infarcted myocardium.² This results in alterations in ventricular geometry, leading to increased wall stress on the remaining myocardium and myocyte hypertrophy.³ Eventually, the progressive hypertrophy results in ventricular dilatation and failure. The most effective way to prevent dilatation and failure is to limit infarct size.⁴ Currently, timely percutaneous interventions are the most effective means for limiting infarct size. Percutaneous transluminal coronary angioplasty within 4 hours of symptom onset is associated with myocardial salvage and recovery of left ventricular (LV) function if thrombolysis in myocardial infarction flow grade 3 is restored.⁵ However, the infarcted tissue will inevitably lead to scar formation. Because no significant regeneration is seen after an MI, the heart has been thought to be a postmitotic, senescent organ with no ability for cell replication. Therefore, HF ensues because of the inability to regenerate myocardium and the resultant strain placed on the remaining functional myocardium.

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Evidence of Endogenous Cardiac Repair

However, there is a large body of evidence now showing that endogenous repair mechanisms do exist in the adult heart.^{5a} Leri et al⁶ discuss 2 patients, a 99-year-old woman who died from pneumonia and a 65-year-old man who died from acromegalic cardiomyopathy. Their hearts at the time of death weighed 140 g and 800 g, respectively. Therefore, one would expect that the cardiomyocytes in the acromegalic heart would have had to hypertrophy to sizes about sixfold larger than the cardiomyocytes in the 99-year-old woman. However, the cardiomyocytes were of similar size, approximately $30,000 \mu\text{m}^3$ in both patients, but there were 240% more myocytes in the younger man.⁶ Several studies have shown the presence of human cardiomyocytes undergoing apoptosis and regeneration in the normal heart,⁶⁻⁸ and this occurs at an increased level in failing hearts^{9,10} and after an MI.¹¹ The very presence of cycling myocytes in the normal human heart indicates that this process helps to maintain homeostasis. Anversa et al¹² therefore propose that myocyte number and cardiac health depend on a balance between myocyte regeneration and myocyte death. Imbalance of these events has been shown to occur in diseased-aged hearts, as compared with age-matched healthy controls.^{12a} In diseased-aged hearts, there is an increase in myocyte formation, which cannot keep pace with the increase in myocyte death.¹³ In fact, in some patients who died after an acute MI, $500 \mu\text{m}^2$ to 5 mm^2 areas of spontaneous regeneration containing proliferating myocytes, smooth muscle cells (SMCs), and endothelial cells (ECs) were observed. In aortic stenosis patients with ventricular hypertrophy undergoing aortic valve replacement, biopsies from the LV outflow tract contained clusters of approximately 6 to 12 undifferentiated and early differentiating cells expressing stem cell antigens. These observations provide definitive evidence of endogenous repair mechanisms.^{14,15} This regeneration, however, was not identified in the hearts of any of the chronic infarct patients, and the number of cycling myocytes was inversely correlated with duration of aortic disease.

Sex-mismatched cardiac transplants, in which a female heart is transplanted into a male recipient, allow for a unique examination of the origin of cells in the human myocardium. All donor cells in the myocardium of the transplanted heart should have a karyotype XX. However, several studies have shown the presence of Y chromosome⁺ myocardial cells in these transplant patients, indicating these cells are of recipient origin.¹⁶⁻¹⁸ Studies by Müller et al¹⁷ and Quaini et al¹⁸ found Y⁺ cardiomyocytes, SMCs, and ECs, whereas another study found only SMCs.¹⁶ The highest levels of cardiac chimerism were seen in those who were transplanted less than 28 days before their death.¹⁸ Furthermore, increased levels of stem cell antigens c-kit, MDR, and Sca-1 were demonstrated in these hearts compared with controls. Of the cells positive for these stem cell antigens, 12% to 16% contained a Y chromosome, thus indicating that these progenitor cells are composed of 2 populations,¹⁸ 1 derived from the recipient and an intrinsic primitive cell in the myocardium.

Mobilization of indicate bone marrow cells (BMCs) has been shown to occur after MI.¹⁹⁻²² These cells include CD34⁺ BM-mononuclear cells, composed of hematopoietic stem cells, endothelial progenitor cells (EPCs), and mesenchymal stem cells (MSCs), bone marrow (BM), side population (SP) cells, and very small embryonic-like stem cells. Circulating progenitor cells have been previously implicated in vascular repair, and neovascularization in the border zone of an infarct has been shown to occur secondary to the circulating EPCs.²³ This seems to be a physiologic maintenance process, as reduced levels of circulating EPCs have been shown to independently predict atherosclerotic disease progression.²⁴ Vascular risk factors, including hypertension, hyperlipidemia, and type II diabetes, have been associated with a lower level and function of circulating EPCs,^{25,26} which may at least partly account for the accelerated atherogenesis in patients with diabetes. Importantly, Leone et al²⁷ demonstrated that higher levels of circulating CD34⁺ cells

correlated with improved LV ejection fraction, LV end-diastolic volume, and wall motion index score 1 year post ST-elevation MI. Furthermore, angiotensin converting enzyme inhibitors, known to decrease ventricular remodeling and improve survival after MI, have been shown to, among other effects, regulate EPCs in a cardiac pressure overload model.²⁶ This regulation results in increased engraftment and differentiation into ECs in the myocardium.²⁶ Studies have shown that BM stem cells are capable of differentiating into cardiomyocytes, SMCs, and ECs in vitro and in vivo.²⁸ However, although BMCs have been shown to circulate and mobilize into the peripheral blood after MI, studies have showed little¹⁹ or no cardiomyocyte differentiation²⁶ after MI without therapeutic application of these cells. Therefore, these cells do not significantly contribute to myocyte regeneration under physiologic or pathologic conditions, if at all.

Primitive cells in the adult myocardium have been identified, isolated, and shown to have the properties of stem cells; namely, they are clonogenic, self-renewing, and multipotent. Cardiac stem cells (CSCs) were first isolated by Beltrami et al,²⁹ express the stem cell antigen c-kit, although being negative for lineage markers, including CD45 and CD34, thus indicating they are primitive, undifferentiated cells not of hematopoietic or endothelial origin. Several other groups have isolated distinct cardiac progenitor cells (CPCs) that express other stem cell markers such as Sca-1³⁰ and MDR1.³¹ Interestingly, CPCs are seen at higher levels in women compared with men, and unlike EPCs, no correlation between medical therapies and CSCs have been found.⁹ These populations of primitive CSCs are composed of a heterogeneous group at different stages of cardiac differentiation. True CSCs express stem cell antigens but lack lineage markers, cardiac transcription factors, and structural proteins, although CPCs exhibit a higher level of differentiation by expressing transcription factors (ie, Nkx2-5, GATA-4), and proliferating/amplifying myocytes also express cardiac structural proteins (ie, α -sarcomeric actin, myosin heavy chain).³² These CPCs have been implicated in cardiac myocyte turnover and proliferate after myocardial injury.³³ Figure 1 is an electromicrograph that illustrates CSCs undergoing cell mitosis and apoptosis.³⁴ CSCs have been isolated from the hearts of patients who died from an acute MI or whose hearts were removed for cardiac transplant, and have been shown to increase in number in the border zone after acute MI; however, these numbers were lower in the chronic ischemic cardiomyopathy patients, indicating an acute proliferation followed by a gradual decline of the CSC population.¹⁵

This article will discuss the evidence for endogenous repair and will review the phenotype and functionality of CSCs, which will elucidate some of the limitations of endogenous repair. It will then discuss current limitations, therapeutic advances, and future prospects in this field.

Cardiac Progenitor Cells

C-kit⁺ Cardiac Stem Cells

After the identification of cycling ventricular cardiomyocytes,¹¹ Beltrami et al²⁹ were the first to identify a unique population of cells in the myocardium that have the phenotypic appearance of primitive cells, express the stem cell marker c-kit, and lack lineage markers that would indicate differentiation (Fig. 2). These c-kit⁺ CSCs consist of 2 subpopulations, namely c-kit⁺ KDR⁻ myocyte progenitor cells (mCSCs) and c-kit⁺ KDR⁺ vascular progenitor cells (vCSCs) both of which can differentiate into cardiomyocytes, ECs, and SMCs; however, mCSCs have greater propensity to differentiate into cardiomyocytes, whereas vCSCs preferentially differentiate into vascular SMCs and ECs.^{35,36} Accordingly, the vCSCs are located in cardiac niches in the coronary vasculature, associated with ECs, SMCs, and fibroblasts, although the mCSCs are found in the myocardial muscle compartment, associated with myocytes and fibroblasts. In the myocardium, 97% are

mCSCs and 3% are vCSCs,³⁷ and overall, these cells occur at a frequency of 1:30,000 myocardial cells.⁶ A small fraction of the c-kit⁺ cells expressed cardiac markers Nkx2.5 and GATA-4, indicating their commitment to cardiac and myogenic differentiation, respectively. Nkx2.5 is a homeobox gene implicated in early cardiogenesis and upregulates expression of other genes required for cardiomyocyte formation and maturation, including GATA-4 and MEF2c.³⁸

These cells are self-renewing, clonogenic, and multipotent.²⁹ In vitro, these cells biochemically differentiate into myocytes, SMCs, and ECs, but they are morphologically and functionally immature²⁹ and require differentiating medium to mature with striations and contractile activity with electrical stimulation.³⁵ In vivo, regeneration of myocardium was shown by intramyocardial injection of these cells at the border of 5-hour-old infarcts followed by the use of green fluorescent protein (GFP) reporter gene labeled cells or bromodeoxyuridine, a DNA-binding agent that incorporates into cells undergoing DNA synthesis. Cardiomyocytes, SMCs, and ECs were observed, localized to the necrotic area. By day 20, these cells had visible striations, and had developed N-cadherin and connexin43, indicating the formation of gap junctions. After this regeneration, there was reappearance of synchronous wall movement and improved myocardial function; these cells were therefore functionally competent and contributed to the myocardial syncytium.²⁹ These results were confirmed using isolated mCSCs.³⁵ The vasogenic ability of the vCSCs was shown by injection of human vascular progenitor cells into an immunodeficient dog with a critically stenotic lesion of the left anterior descending artery (LAD).³⁶ One month later, increased blood flow distal to the lesion associated with the formation of entirely human large conductive (up to 1.5 mm luminal diameter), intermediate, and small human coronary arteries was seen. Subsequent occlusion of the LAD during cardiac catheterization did not result in changes in LV pressure or dP/dt, indicating that complete occlusion of the LAD did not induce ischemia, likely secondary to new collaterals that formed around the LAD.³⁶

Stem Cell Antigen-1⁺ Cardiac Progenitor Cells

Stem cell antigen (Sca)-1 expressing progenitor cells have been previously isolated from skeletal muscle and the BM.^{39,40} In the myocardium, these cells are found in small clusters in the interstitium, accounting for approximately 2% of myocardial cells.^{6,30} This population is heterogeneous, with 40% of these cells expressing CD45 and 10% expressing CD34 and c-kit.^{40,41} The subset of Sca-1 cells with clonogenic properties are characterized by Sca-1⁺, CD29⁺, CD44⁺, CD31⁻, CD45⁻, c-kit⁻, and may or may not express CD34.⁴¹ This is consistent with the observations that Sca-1⁺ CD31⁻ progenitors double in numbers in the myocardium after MI, peaking at day 7 and returning to baseline by day 14,⁴² and differentiation into cardiomyocytes was restricted to the CD45 population.⁴⁰ These cells also expressed GATA-4 and Csx/Nkx2.5 at low levels in culture but did not express any structural cardiac genes, indicating some of these cells are committed myocyte progenitors.^{30,40} These cells underwent increased expression of GATA-4 and Nkx2.5/Csx and began expressing MEF2c and cardiac structural proteins when exposed to 5-azacytine; however, although Oh et al³⁰ demonstrated morphologically differentiating cells, this was not reproduced by Matsuura et al⁴⁰ who demonstrated a fibroblast morphology 4 weeks after exposure to 5-azacytine. Furthermore, these cells did not undergo spontaneous beating. Exposure to oxytocin, however, led to morphological changes, increased expression of cardiac-specific transcription factors and structural proteins (including striations and connexin43), and spontaneous beating 4 weeks after exposure.⁴⁰ In fact, there is evidence of an oxytocin system in the adult myocardium.⁴³ The differentiated cells exhibited a marked increase in beating frequency when exposed to isoproterenol, an effect that was blocked by exposure to a β 1-receptor blocker but not a β 2-receptor blocker, indicating expression of

functional β 1-adrenergic receptors.⁴⁰ Transdifferentiation into endothelial,⁴² osteocytic, and adipocytic⁴⁰ lineages was also demonstrated.

In vivo, these cells homed to the myocardium and regenerated myocardium via both transdifferentiation and cell fusion^{30,41} leading to improvements in left ventricular ejection fraction (LVEF), LV end-diastolic diameter, and LV end-systolic diameter and neovascularization.⁴² Although cell fusion is normally considered a rare process,⁴⁴ these cells undergo an extremely high rate of cell fusion, approximately 50%, which may limit their clinical utility. Interestingly, 0.03% of these cells possess properties of the SP cells in the BM, whereas cardiac SP cells were highly enriched for Sca-1 (93% positive) and are enriched 100-fold in the Sca-1⁺ population.³⁰

Cardiac Side Population Cells

Pfister et al⁴⁵ selectively isolated these cardiac SP cells, found to account for 1.2% to 2% of adult myocardial cells.^{6,46} The phenotype of SP cells, as with those in the BM, is characterized by the efflux of Hoechst 33342 DNA-binding fluorescent dye because of the presence of Abcg2 and MDR1, ATP-binding cassette transporters in the P-glycoprotein superfamily.³¹ Using double knockout mice models (Abcg-1^{-/-} or MDR1^{-/-}), it was shown that, unlike in the BM where Abcg-1 solely regulates the SP phenotype, both proteins regulate the cardiac SP phenotype, and do so in an age-dependent manner.³¹ Furthermore, Abcg-1 expression has been shown to enhance proliferation and survival of these cells, although inhibiting differentiation, therefore playing a role in the regulation of proliferation and differentiation in these cells.³¹ These cells generally expressed Sca-1 and CD31 (73.8 $\pm$ 2.4%), although they had low expression (<1%) of CD45, CD44, CD34, and c-kit, all of which are generally expressed by marrow SP cells.⁴⁵ The minority of cardiac SPs that are CD31 negative have been shown to be the subpopulation of cardiac SP cells with cardiomyocyte potential. The CD31⁻ cells increase expression of GATA-3 and MEF2c, and begin expressing α -actinin and troponin I in a disorganized pattern in culture, and in coculture with adult cardiomyocytes develop organized sarcomeres and exhibit spontaneous contraction (<1 Hz) with electrical stimulation. Gap junction formation was confirmed by expression of connexin43, determination of intracellular calcium gradients, and illustration of synchronized rhythmic contractions with adjacent cardiomyocytes.⁴⁵ This was not seen with BM SP cells or CD31⁺ cardiac SPs. Cell fusion has been shown to be an insignificant mechanism because most differentiated cardiac SPs remained mononuclear. Furthermore, by using reporter genes specific to cardiac SPs and adult cardiomyocytes, they demonstrate no double positive cells.⁴⁵

These cells have been shown to home to the myocardium after administration after cardiac injury, with the majority engrafting in the border zone, and differentiating in vivo into cardiomyocytes, fibroblasts, EC, and SMCs.⁴⁶ However, only 4.4% and 6.7% of these GFP-labeled cardiac SP cells differentiated into cardiomyocytes and ECs, respectively, although 33% transdifferentiated into fibroblasts and 29% into SMCs. This points out that although these cells can differentiate into all cardiac cell types, the low cardiomyogenic potential may limit their clinical utility.

Cardiospheres and Cardiosphere-Derived Cells

A unique isolation and expansion method has been applied to CPCs. The culture of these cells in nonadhesive substrate leads to loosely adherent small, round, phase-bright cells, which proliferate into clonally derived clusters, dubbed cardiospheres.⁴⁷ These cells express CD105 and CD29 at high levels (>99.8%) with lower expression of stem cell antigens c-kit and Sca-1.^{47,48} The coexpression of CD90 by 18% of the cells, with high expression of CD105, likely represents a mesenchymal cell population. Although about 10% of the cells

expressed all of the phenotypes on day 0, by 6 days in culture c-kit is the only conserved marker and is considered to be the marker of the proliferating cells in the cardiospheres.⁴⁷ Furthermore, it has been shown that the Sca-1⁺ fraction in cardiospheres is enriched for cells expressing LIM-homeodomain transcription factor islet-1, known to be important in the prenatal right ventricle and inflow and outflow tract cardiomyogenesis.^{49–51}

Cardiosphere cells therefore do not represent a unique cell population but a heterogeneous group of previously isolated CPCs mixed with other cell types, including mesenchymal cells. The culture of dissociated murine and human CSCs, cardiosphere-derived cells (CDCs), leads to cells with a distinct cardiomyocyte morphology and spontaneous contraction, with human CDCs requiring coculture with adult cardiomyocytes to beat. These cells are clonogenic and multipotent, differentiating into cardiomyocytes, vascular SMCs, and ECs.⁵² Although CSCs have been shown to secrete higher levels of growth factors and cytokines compared with CDCs,⁵³ the large size of the spheres makes them less practical.

Human CDCs have been shown to remain functionally competent after isolation and expansion, capable of differentiating into cardiomyocytes independent of cell fusion⁵⁴ in a rat model. Furthermore, synchronous calcium transients in adjacent differentiated cells and staining for connexin43 indicate these cells are structurally integrated via gap junctions. In vivo experiments have shown myocardial regeneration, improved cardiac function, and increased angiogenesis.^{47,49,54}

The US Food and Drug Administration has approved the plans for a 250 patient study to examine whether “universal donor” cardiosphere-derived cells would have the same effect as the patient's own cardiac stem cells. Universal donor cells would eliminate the need for myocardial biopsy.

Clinical Utility in Humans

For clinical utility, the practitioner must be able to reliably isolate and expand competent cells from patients in a timely manner. As collagen can be detected microscopically in the myocardium and maturation of the scar is complete by 7 and 28 days, respectively, cell therapy would ideally be applied in this period before scar formation.³ Several studies have shown that these cells can be isolated reliably during coronary artery bypass grafting (CABG)³⁷ and endomyocardial biopsy,⁵⁴ with the highest cumulative yield of c-kit⁺, Sca-1⁺, and MDR1⁺ cells from the right atrium, followed by the apex, with almost threefold more cells isolated per tissue weight. However, in a population of end-stage HF patients, with a mean LVEF of $15 \pm 5\%$, the LV contained the highest percentage of c-kit⁺ cells.^{9,55} One study in transplant patients only obtained c-kit⁺ CD45⁺ tryptase⁺ mast cells; however, cells expressing stem cell antigens have been observed in transplanted hearts.^{18,56} Once expanded, these cells retain their growth capability, as determined by preserved telomerase activity and low p16^{INK4a} levels, which permanently prevents entry into the cell cycle and their ability to differentiate. However, using antigenic sorting and classical culture in patients with end-stage HF, it took 28 days and 41 days to obtain a clinically relevant 5×10^6 mCSCs and vCSCs,³⁷ respectively, which precludes the use of these cells in the setting of an acute MI.

Intracoronary administration of stem cells is the most clinically relevant method of application. Given the small size of the CSCs (4–6 μm diameter),¹³ intracoronary injection seems feasible in humans. Studies using a rat model have shown that intracoronary administration of CPCs is safe and efficacious in the settings of both acute⁵⁷ and chronic infarcts.⁵⁸ Although scar formation does interfere with the migration of these cells, they can still permeate into and repair a chronic myocardial scar. Intramyocardial injection of GFP-

labeled CSCs into a chronic infarct in a rat model resulted in a large reduction in infarct size, increased infarct wall thickness, improved LVEF, and attenuated ventricular remodeling.⁵⁸

Clinical Trials Utilizing Cardiac Progenitor Cells

Given the high morbidity and mortality of postinfarction HF and the ability to show, albeit modest, improvement in animal trials, 2 phase 1 clinical trials have been initiated: the Cardiac Stem Cells in Patients with Ischemic Cardiomyopathy (SCIPIO) trial,^{59,60} for which preliminary results are available, and the proof-of-concept Cardiosphere-Derived Autologous Stem Cells to Reverse Ventricular Dysfunction (CADUCEUS) clinical trial which concluded in February 2012.⁶¹

The SCIPIO trial enrolled patients with postinfarction LV dysfunction, defined as LVEF 40%, who were undergoing CABG. Patients were randomly assigned to receive 1×10^6 autologous c-kit⁺ CSCs via intracoronary infusion or control for a mean of 113 days after CABG.⁵⁹ Investigators were able to isolate cells from the atrial appendage and expand CSCs from 80 of 81 patients, the only failure being in a patient with cardiac amyloidosis. Initial results have been published on 16 treatment patients and 7 control patients who were at least 8 months after CABG (4 months after stem cell infusion). At 4 months after CABG and before stem cell treatment, the baseline LVEF of these patients were not significantly different— $31.4 \pm 1.8\%$ and $30.0 \pm 2.3\%$ in the treatment and control groups, respectively. However, 8 months post-CABG (4 months after stem cell treatment), the LVEF in the control group did not improve ($30.2 \pm 2.5\%$), although the LVEF considerably improved to $38.5 \pm 2.8\%$ in the treatment group. Furthermore, in the 8 patients in which 1 year had passed, the LVEF continued to improve to an absolute improvement of $12.3 \pm 2.1\%$ from the baseline. Cardiac magnetic resonance performed in eligible patients ($n = 7$) showed a significant decrease in mean infarct weight by 24% at 4 months and 30% at 1 year. Symptomatic improvement was also evident by decreases in New York Heart Association (NYHA) functional class and Minnesota Living with Heart Failure Questionnaire score in the treatment group but not in the control group. Importantly, no adverse events were noted.⁵⁹

The CADUCEUS trial⁶¹ enrolled patients who recently had an MI (<4 weeks) with successful percutaneous coronary intervention, stent placement, and an LVEF of 25% to 45%. Patients ($n = 31$) were randomly allocated to the treatment group ($n = 23$) or control ($n = 8$). Endomyocardial biopsies were used to isolate the cardiac tissue and were expanded using cardiosphere culture methods. The expanded cells were injected into the infarct-related artery 65 $\pm$ 14 days after MI at low dose (12.5×10^6 cells, $n = 4$) or high dose (25×10^6 cells, $n = 12$). The cells were infused based on attainment of the cell dosages, with a mean expansion time of 35 ± 6 days from endomyocardial biopsy. Baseline LVEF and functional status were similar between the groups, with a mean LVEF of $39 \pm 12\%$ and 71% to 75% of patients in NYHA functional class 1. As seen in preclinical trials,⁶² no adverse events were seen acutely with these cell doses; however, a non-ST-elevated MI in a high-dose patient at 7 months after infusion was possibly related to the treatment. Although an increased number of adverse events occurred in the treatment group, it was not significant compared with the control group. The primary endpoints of this study were sudden unexpected death after infusion, as evidenced by ventricular tachycardia or ventricular fibrillation, or a major adverse cardiac event, as evidenced by MI after infusion or new cardiac tumor formation on magnetic resonance imaging. There were no ischemic or dysrhythmic events related to the cell infusion. In the 12-month follow-up, no patient died. Unlike in the SCIPIO trial, there was no change in LVEF or NYHA functional class. However, there was a trend toward increased peak VO_2 in CDCs treated patients (2.6 mL/kg per min versus -0.5 mL/kg per minute, $P = 0.07$), and on cardiac magnetic resonance, there

was a significant reduction in infarct size at 6 and 12 months in the CDCs group ($-12.3 \pm 5.0\%$ at 12 months) compared with the control group, which showed no change in infarct size ($-2.2 \pm 7.1\%$ change from baseline to 12 months, $P = 0.452$). Increased viable myocardium on cardiac magnetic resonance was interpreted as myocardial regeneration and showed a significant 13.0 ± 11.4 g increase in viable tissue in the treatment group, but not in the control group (0.9 ± 6.2 g).

The results of these trials confirm that intracoronary administration of these cells is safe and that there is potential therapeutic benefit from the administration of autologous CSCs in humans; however, the limited regeneration seen in these patients and the lack of functional myocardial improvement seen in the CADUCEUS trial illustrate the lack of understanding of the properties of these cells. This limits our ability to use them clinically. Furthermore, these studies cannot assess the mechanism of cardiac regeneration in these patients, and functional integration of differentiated CSCs has not been proven in humans thus far. The increase in viable myocardium seen on cardiac magnetic resonance could occur secondary to differentiation of the injected cells; however, other explanations include cardiac hypertrophy or activation of endogenous cardiac progenitors via the indirect paracrine effects of these cells. Although not definitive evidence, the authors of the CADUCEUS trial used human CDCs in a rat model and demonstrated that the increase in viable myocardium was secondary to regeneration and not hypertrophy.⁶¹

Which Cardiac Progenitor is the Best?

Although direct in vivo comparison of the CPC types has not been performed, some conclusions can be drawn from preclinical studies. Comparison of rat model studies showed greater regenerative capabilities for the c-kit⁺ CSCs versus the Sca-1⁺ cells,^{29,30} and given the high rate of cell fusion seen with the Sca-1⁺ cells, their regenerative potential postinfarction may be limited to the border zone secondary to massive myocyte death in the infarct region. On the other hand, studies have shown that the Sca-1⁺ CD31⁻ cardiac side population (CSP) subpopulation has a greater regenerative potential than the unselected Sca-1 population.⁴⁵ Given the small numbers present in the adult heart (500–1000 cells in the rat myocardium) and low rate of cell fusion, studies of the utility of this expanded population may be warranted.

The use of CDCs has shown that selected c-kit⁺ CDCs are inferior to the unselected CDC population, likely because of higher soluble factors secreted by this population and the heterogeneity of cells, including mesenchymal cells, expanded by this culture method.^{48,54} Cardiosphere culturing requires extra steps in tissue processing and culturing. Therefore, Davis et al⁵⁵ compared CDCs with the cellular outgrowth from cardiac samples, which does not require antigenic selection or cardiosphere (CS) formation. Direct in vitro comparison of these 2 groups of cell demonstrates that cardiac outgrowth cells have greater potential to differentiate into cardiomyocytes; however, in vivo studies showed no difference between the 2 treatment groups. Importantly, based on growth kinetics, the authors estimate that 400 mg human atrial appendage tissue could result in 8.0×10^6 cardiac outgrowth cells in 7 days. This is in stark comparison to the mean 28 or 45 days required to obtain 5.0×10^6 mCSCs and 1.7×10^6 CDCs, respectively.³⁷

The argument of which progenitor cell has the greatest regenerative potential is based on studies in mouse and rat models, which demonstrated phenotypically distinct c-kit⁺ cells, cardiac SPs, and Sca-1⁺ cell populations; however, in canines⁶³ and humans,¹⁵ approximately 60% of lineage negative CPCs coexpressed c-kit, MDR1, and Sca-1 antigens, although a smaller number possessed 1 or 2 of those antigens alone. The CPCs expressing multiple antigens or a single antigen (c-kit, Sca-1, or MDR1) were all shown by clonal

analysis to be multipotent and differentiate into myocytes, SMCs, and ECs.⁶³ All generated similar proportions of these cells: approximately 50% myocytes, approximately 40% SMCs, and approximately 10% ECs. However, the c-kit antigen single positive cells had the greatest potential for creating cardiac cells, producing 2.3-, 5.9-, and 7.6-fold more cells than the MDR1⁺, Sca-1, and triple positive CPCs, respectively.⁶³

Direct comparison of the in vivo effects of CDC culturing to the c-kit⁺ cell isolation method using antigenic sorting has not been made, but clinical trials show that the c-kit⁺ cells may have greater regenerative potential than CDCs. This is shown by the 8.3% treatment effect in LVEF at 4 months in the initial results of the SCIPIO trial compared with the approximately 2% nonsignificant increase at 6 months in the CADUCEUS trial. In addition, the reduction in infarct size at 12 months was greater (30% versus 12.3%) using c-kit⁺ CSCs.^{59,61} However, one must be careful when drawing conclusions from the CADUCEUS and SCIPIO trials given that patient populations and cell doses were not identical. The fact that the SCIPIO trial used 12.5 to 25-fold less cells in much older infarcts (mean infarct age 3.7 years versus <8 weeks) and still seem superior to the CDCs used in the CADUCEUS trial, favors the superiority of c-kit⁺ cells. However, the final results of this trial are still pending.^{59,61} Therefore, given these studies, it seems that c-kit⁺ cells have the greatest regenerative potential of the individual CSCs isolated, even in humans where coexpression of other stem cell markers is common. Furthermore, the use of CDCs or cardiac outgrowth methods may be superior to the antigenic selection of c-kit⁺ cells given the heterogeneity of their cell populations. However, direct comparison should be carried out. Although the expansion time currently precludes use of these cells for an acute MI, the cardiac outgrowth method may be the most clinically relevant given the short time estimated to be required to cell expansion.⁵⁵ Ultimately, initial basic studies confirming the growth kinetic estimation are needed.

Current Issues and Mechanisms

Injection of CPCs in a noninjured heart leads to rapid disappearance of the cells, secondary to low engraftment and apoptosis of the nonengrafting cells.^{29,64} After MI, there is increased engraftment of these cells,^{29,41,42,52} and consistent with this, hypoxia has been shown to increase c-kit⁺ CDC migration into the ischemic myocardium.⁵² This results in reduced infarct size and improved heart function;⁵² however, even then engraftment remains low.^{30,41} In addition, these newly formed myocytes have been shown to be small^{29,33,35,57} and resemble fetal–neonatal cardiomyocytes,³⁵ with most being less than 2000 μm^3 in volume, compared with approximately 20,000 μm^3 volume of mature adult cardiomyocytes. Interestingly, in the noninfarcted healthy myocardium, a different outcome is seen. Differentiation of c-kit⁺ CSCs into cardiomyocytes in the uninjured myocardium results in cardiomyocytes that are indistinguishable from adult native cardiomyocytes,⁵⁷ and Y-positive cardiomyocytes in sex-mismatched transplanted hearts seem indistinguishable from native cardiomyocytes as early as 4 days after transplant. These cells were most commonly found singly and therefore retained contact with the resident myocytes. This is contrary to an infarct where there is massive cell death.¹⁸ Consistent with these findings, engraftment and cell apoptosis have been shown to be restricted to cells that lack expression of connexin43 and N-cadherin—both integral molecules in cardiomyocyte cellular connection.⁶⁴

Overall the low engraftment and lack of cell maturation illustrates failures in both endogenous cardiac repair and cell therapy. These findings implicate both the effects of soluble factors and cell-to-cell contact in these failures. Therefore, although the isolation, expansion, and characterization of these cells is the first step toward understanding their regenerative capabilities, it is obvious that better knowledge of their mechanisms of activation, proliferation, and differentiation, and their indirect paracrine effects, is needed.

Notch1, a transmembrane receptor known to control stem cell fate in other tissues,⁶⁵ has been implicated in the proliferation of CPCs. Along with one of its ligands Jagged1, Notch1 has been localized to CSC niches with expression limited to cells not expressing markers of cardiac differentiation, indicating its presence on true c-kit⁺ undifferentiated CSCs. Activation of Notch1 leads to translocation of its intracellular domain, N1ICD, to the nucleus, which then complexes with a binding protein, RBP-Jk, and acts to alter DNA expression. Specifically binding to and activating the promoter site for Nkx2.5, however, required GATA-4 and Smad1, indicating that N1ICD/RBP-Jk alone cannot activate this gene expression.⁶⁶ In vivo studies in a mouse model show that inhibition of the Notch1 system decreases CPC proliferation, differentiation, and myogenic regeneration after an acute infarction. On the other hand, activation of this pathway increases proliferation of CPCs 2.2-fold, commits CPCs to the myocyte lineage, and may increase amplifying cardiomyocytes by preventing terminal differentiation.⁶⁷ In addition, Notch1 has been implicated in the differentiation of EPCs into cardiomyocytes.⁶⁷

Although changes in expression of Notch1 and its ligands in myocardial ischemia are not well-defined, the ischemic myocardium has been shown to upregulate expression of numerous soluble factors that affect CPCs. Overall, these factors reach peak levels at approximately 48 hours after infarction and return to baseline about 7 days later.²⁰ CPCs have been shown to secrete many of these factors and may account for some of the increase seen in the acutely infarcted heart. These factors include: soluble vascular cell adhesion molecule-1 (sVCAM1), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), and stromal cell-derived factor-1 (SDF-1),^{41,64,68} and CPCs are known to express their receptors: VLA-4, vascular endothelial growth factor receptor (VEGFR), c-Met, IGF-1R, and CXCR4, respectively.⁵⁷ The increased engraftment seen with ischemia is largely attenuated using the chemokine receptor-4 (CXCR4) inhibitor AMD3100 and anti-VLA-4 antibodies, therefore, implicating the SDF-1-CXCR4 and sVCAM1-VLA-4 axes.^{41,52,69} The increased production of SDF-1 by ischemia is secondary to the upregulation of hypoxia-inducing factor (HIF)-1 α induced by HGF and IGF-1.⁶⁴ CPCs activated by either ischemia or direct application of SDF-1 showed preferential differentiation into vascular cells, and had a limited myogenic differentiation in a nonreperfused rat model.⁶⁴

HGF has been shown to mobilize CPCs, which is mediated in part by increased actions of matrix metalloprotease-9, known to enhance BM stem cells mobilization to the peripheral blood,⁷⁰ although IGF-1 promotes survival and proliferation of CPCs, at least partially via the Akt/PI3K pathway.^{64,71,72} The importance of IGF-1 in cardiovascular health has been shown in a prospective study of 717 elderly patients without a history of MI or HF. The study showed a decrease in HF risk with logarithmic increase IGF-1 levels. Those patients with IGF-1 values ≥ 140 $\mu\text{g/L}$ (median value) had half the risk for HF over the mean 5.2 year follow-up.⁷³ This may be due to its effects on cardiomyocytes and CPCs, and its vasodilatory effects.⁷⁴ With respect to IGF-1's effects on CSCs, IGF-1⁺c-kit⁺ CSCs represent a primitive subpopulation with the ability to form more mature cardiomyocytes, enhanced vasculogenesis, and improved myocardial function versus unselected c-kit⁺ CSCs.⁷⁵ Furthermore, cells not expressing IGF-1R were shown to have impaired growth reserve and increased apoptosis.⁷⁵ IGF-1's effects have been shown to be mediated, at least in part, by alterations in intracellular calcium oscillations due to release of calcium from the endoplasmic reticulum via IP3-IP3R and sarcoendoplasmic reticulum calcium transport ATPase 2a (SERCA) pump systems.³³ This system can also be activated by adenosine triphosphate (ATP) and histamine via the ATP-specific P2-purinoreceptors and histamine receptor-1. These oscillations have been shown to regulate the activation, growth, and regenerative potential of human CPCs.³³ Application of ATP, histamine, or IGF-1 results in a larger proportion of CPCs exhibiting these calcium oscillations, and at higher frequency,

although blockade of calcium oscillations or the signaling pathways inhibits the growth promoting effects of these molecules. This was shown not to occur via cell contact and not to be associated or altered by intracellular calcium oscillations in coupled cardiomyocytes.³³ Given the increased levels of IGF-1, histamine, and ATP in the myocardium after an infarction,^{20,76} this likely represents a mechanism of activation of endogenous CPCs and modification. In fact treatment with ATP, histamine, or IGF-1 lead to 2.7-fold increase in engraftment, 10- to 12-fold more CPC cycling, and a fourfold increase in differentiation.³³ Enhancement of this system may improve the efficacy of cell therapy.

Therefore, in addition to differentiating into cardiomyocytes and vascular cells, the beneficial effects of these cells are in part because of paracrine effects. These effects include decreased apoptosis, increased neoangiogenesis and recruitment, and activation of endogenous progenitors.⁵² Chimenti et al⁵³ quantified the indirect paracrine effects by estimating that of the new cardiomyocytes and vasculature, an estimated 20% to 50% formed secondary to human CDC differentiation or fusion. These paracrine effects have been shown of c-kit positive cells isolated by other methods.^{57,68,71} However, the ability of CPCs to respond to IGF-1 and HGF is attenuated in aged CPCs. Expression of IGF-1R and c-Met decreases with age in a rat model, and CPCs expressing IGF-1R and c-Met were consistently negative for p16^{INK4A}, which was detected in CPCs positive for angiotensin II (AT-II) receptors.⁷⁷ Furthermore, Ang-II stimulates CPC apoptosis, which implicates the activated renin angiotensin system seen in HF in the increased apoptosis noted in failing hearts. This also indicates a novel mechanism for angiotensin converting enzyme inhibitor attenuation of myocardial remodeling. Thus, although there are increased numbers of c-kit⁺ cells in aged myopathic hearts compared with age-matched healthy controls, a high percentage of these cells are senescent (59% versus 14% in the control) and undergoing apoptosis, which unbalances the equilibrium of CPC apoptosis and cell division.¹³

Despite this, a population of CPCs retaining long telomeres and increased telomerase activity do exist in these hearts and can participate in myocardial regeneration.⁷⁷ Modifying the levels and expression of these soluble factors presents a unique opportunity to increase the efficacy of cell therapy. Using IGF-1 overexpressing transgenic mice or intramyocardial injection of tethered nanofibers of IGF-1 has been shown to lead to prolonged increase of IGF-1 in the myocardium.^{78,79} This results in increased proliferation of CPCs in the myocardium, decreased CPC apoptosis, delayed CPC aging, and some myocardial regeneration compared with control.⁷⁸ Similarly, pretreatment of c-kit⁺ CPCs with IGF-1 and HGF leads to increased engraftment in the myocardium and subsequently decreased apoptosis.⁶⁴ Coadministration of CPCs with tethered IGF-1 increased new myocyte formation and volume by 32% and 48%, respectively, and increased newly formed coronary arterioles by 73% compared with administration of CPCs alone.⁷² There was also greater attenuation of ventricular remodeling and improved cardiac function with the cotherapy compared with CPCs alone.

In addition to the significance of indirect paracrine and autocrine effects on CPCs and myocardial regeneration, the importance of cell-to-cell contact in myocyte maturation has been illustrated.^{45,80} MicroRNA are 20 to 22 nucleotide noncoding RNAs, which exert their effects posttranscriptionally by binding to the 3'-untranslated regions of target mRNAs, leading to cleavage of the target mRNAs and therefore inhibiting protein synthesis and expression of those genes.⁸¹ Hosoda et al⁸⁰ showed that miR-499 is highly expressed in mature cardiomyocytes, lacking in developing myocytes, and junctionally transferred to coupled CSCs, resulting in enhanced cardiomyogenic differentiation in these cells, possibly by repression of Sox6 and Rod1 genes. When miR-499 overexpressing human CSCs were transplanted in a rat model, the resultant cardiomyocytes were 50% larger and had improved fractional shortening, consistent with more mature cardiomyocytes.⁸⁰ The importance of cell

contact was further shown in a study illustrating improved cardiomyogenic differentiation of MSCs in culture with cardiomyocytes versus cardiomyocytes conditioned media.⁸² Therefore, the lack of cell–cell contact in the infarcted region of the myocardium because of the death of almost all cardiomyocytes in that region may inhibit complete maturation of these cells into adult cardiomyocytes. Enhancing this system using hind limb injection of miR-21, -24, and -221 “miRNA cocktail” treated CPCs in immunodeficient mice lead to significantly improved cellular engraftment and improved fractional shortening, at least in part by suppressing proapoptotic genes.⁸³

Comparison of Bone Marrow Cells with Cardiac Progenitor Cells

Numerous studies have shown that BM cells are capable of contributing to myocardial regeneration.^{84,85} However, conflicting results have questioned whether these cells are truly differentiating into myocardial cells, and the competing theories of cell fusion⁸⁶ and paracrine effects^{87–89} have been well-studied. Despite this, an overwhelming amount of evidence is mounting for improved cardiac function after administration of BM stem cells in both animal models^{29,90,91} and in human trials.^{92–94}

In a murine model, the regenerative potential of human CDCs was compared with that of human BM-MSCs, adipocytic-MSCs, and BM-mononuclear cells.⁴⁸ CDCs secreted more angiogenic and antiapoptotic growth factors as compared with the other cell types, and had greater in vitro capacity for cardiomyogenic and endothelial differentiation. These cells demonstrated significantly higher engraftment at 3 weeks than a similar number of BM-mononuclear cells; however, the engraftment only trended toward the CDC group versus the other treatment groups. Importantly, a significantly higher number of human cardiomyocytes were seen with the CDC group than with any other group and only in the CDC treatment group was there a significant increase in LVEF at 3 weeks compared with the control. In addition, infarct size and wall thickness were significantly improved compared with all treatment groups.⁴⁸

When comparing these groups of cells, it is important to point out that the origin of CPCs is unknown. Whether or not these cells are residents of the prenatal myocardium or are of extracardiac origin²⁹ is not well-defined. BM SP cells have been shown to mobilize to the myocardium after infarction.¹⁹ Using lethally irradiated mice with reconstitution of their BM with GFP-expressing BMC showed that under physiologic conditions and 7 days after MI, less than 1% and nearly 25%, respectively, of cardiac SPs are of BM origin.²² These cells engrafted in the infarcted region, and although they initially expressed CD45, by day 7, CD45 expression was decreased by >90% and there was an increase in CD31. This demonstrates phenotype conversion within the myocardium to that consistent with cardiac SPs. Therefore, these cells may be maintained at low levels by BM-SPs, and after the acute depletion of cardiac SPs that occurs with MI, there is both proliferation of the remaining cardiac SPs and contribution from mobilized BMCs. However, this cannot be conclusively stated and the BM-SPs may represent a population of reserve cells able to repopulate tissue-specific SP cells after injury.²²

There is also some experimental evidence to suggest an enhanced effect on LV diastolic and systolic function in a swine post-infarction model by combining BM-MSC infusions with c-kit⁺ CSCs.^{94a,b}

The results of human trials of BMCs have been disappointing thus far. Although studies in acute and chronic infarcts have shown a significant improvement in hemodynamic factors, the improvements are modest at best. Meta-analyses of studies using BMCs in the setting of acute MIs have shown an overall improvement in LVEF of approximately 3% (2.99%–3.66%) compared with controls and with trends toward improved clinical outcomes.^{95–97}

The use of BMCs in chronic infarcts has shown similar outcomes, with improvement in LVEF ranging from +4.1 to 6.3% compared with controls.^{98,99} Although clinical trials using CSCs have been carried out, it is premature to compare the outcomes with the studies utilizing BMCs for several reasons. Although the CADUCEUS trial failed to show improvement in LVEF, there have also been negative trials utilizing BM stem cells, likely related to differences in cell processing and handling and time of administration.^{100,101} Similarly, the improvement in LVEF seen in the SCIPIO trial is from preliminary data, and final analysis should be carried out before drawing conclusions. Although the cardiomyogenic potential of cells of cardiac origin should intuitively be greater than that of BMCs, randomized clinical trial will provide conclusive evidence.^{102–106}

A large-scale trial in 3,000 patients with myocardial infarction is now in progress: Effect of Intracoronary Reinfusion of Bone Marrow-Derived Mononuclear Cells on All-Cause Mortality in Acute Myocardial Infarction (BAMI).^{5a} In addition, adipose tissue-derived regenerative cells are being prospectively studied in the ADVANCE study, a multicenter, randomized, prospective, placebo-controlled phase 2b/3 study in 375 patients with recent acute ST elevation myocardial infarction. Adipose tissue-derived regenerative cells have been touted to improve LV function and myocardial infarction after myocardial infarction, with anti-apoptotic immunomodulatory and pro-angiogenic potential due to paracrine effects.¹⁰⁷

Conclusions

The evidence summarized here provides conclusive evidence that the adult heart is not a postmitotic organ. Rather, myocytes are continuously turning over, and they do so at an increased rate in failing hearts. The lack of significant regeneration via endogenous repair mechanisms and the limited regeneration seen with cellular therapy indicate that these systems fail at some level. In aging myopathy, the failure seems to be because of accelerated myocyte apoptosis, and an increase in aged, senescent progenitor cells, such that myocyte regeneration is overshadowed by myocyte death. This seems to be, at least in part, because of changes in the IGF-1-IGF-1R, HGF-c-Met, and AngII-AngIIIR axes.⁷⁷ In the postinfarcted heart, the failures seem more complex as the changes in cytokine secretion and receptor expression are complicated by the lack of cellular contact and vasculature in the infarcted region.

The mechanisms of CSC activation and proliferation allow insight into the issues of limited engraftment and maturation; however, much work in this field has yet to be done. Manipulation of these cells with cytokines and miRNA can lead to improved engraftment and cell maturation as described above. Further variations in the method of administration may also improve outcomes. Utilizing a cell sheet transplantation method resulted in 40% engraftment at 4 weeks, compared with the 10% survival with intramyocardial injection of a similar cell number of CPCs.⁴¹ However, this procedure would require a thoracotomy. Studies utilizing BM stem cells have shown the efficacy of transendocardial cell application and improved outcomes with repeat cell administration;^{102,103} these administration techniques should be evaluated further using CSCs. Furthermore, although the use of allogeneic transplants with BM-MSCs has been shown to be tolerated without rejection of the cells secondary to secretion of immune modulating factors,¹⁰⁴ this has not been well-defined using CSCs. Using allogeneic rat CDCs, however, resulted in only a slight local immune reaction with no myocyte necrosis, evidence of a systemic reaction, or antidonor antibodies. Despite the lower engraftment seen at 3 weeks, there was significant and similar attenuation in ventricular remodeling and improvement in cardiac function in the syngeneic and allogeneic groups as compared with control groups.¹⁰⁵ Furthermore, CDCs have been shown to retain function after cryopreservation,⁴⁷ and therefore, preexpansion and storage of

allogenic CSCs that can be used in the days after an acute infarction may lead to improved outcomes. More importantly, the possibility of autologous cells being expanded within 7 days of isolation using the cardiac outgrowth method may no longer preclude their use in the acute setting, and further studies should be carried out to confirm the growth kinetic estimations. Overall, CSCs and BM stem cells have thus far failed to result in the presumed and desired clinical outcomes. However, the administration of CPCs have been shown to be safe, and given the grave clinical need to prevent HF, further research into the mechanisms of HF should be carried out that can allow for the enhancement of myocardial regeneration.

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Figure 1.
This electromicrograph illustrates cardiac stem cell mitosis (A, B) and apoptosis (C, D).
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Figure 2.

Clusters of primitive and early committed cells in the heart. A, Cluster of 11 c-kitPOS cells with 3 expressing c-kit only (arrows), 7 expressing Nkx2.5 (arrowheads) in nuclei, and 1 Nkx2.5 and *-sarcomeric actin in the cytoplasm(*); B, Cluster of 15 c-kitPOS cells with 5 c-kitPOS cells only (arrows), 8 expressing MEF2C (arrowheads), and 1 expressing MEF2C and *-sarcomeric actin (*). Bars, 10 μ m