Cardiogenic Differentiation and Transdifferentiation of Progenitor Cells

Hans Reinecke, Elina Minami, Wei-Zhong Zhu, Michael A. Laflamme

Abstract—In recent years, cell transplantation has drawn tremendous interest as a novel approach to preserving or even restoring contractile function to infarcted hearts. A typical human infarct involves the loss of approximately 1 billion cardiomyocytes, and, therefore, many investigators have sought to identify endogenous or exogenous stem cells with the capacity to differentiate into committed cardiomyocytes and repopulate lost myocardium. As a result of these efforts, dozens of stem cell types have been reported to have cardiac potential. These include pluripotent embryonic stem cells, as well as various adult stem cells resident in compartments including bone marrow, peripheral tissues, and the heart itself. Some of these cardiogenic progenitors have been reported to contribute replacement muscle through endogenous reparative processes or via cell transplantation in preclinical cardiac injury models. However, considerable disagreement exists regarding the efficiency and even the reality of cardiac differentiation by many of these stem cell types, making these issues a continuing source of controversy in the field. In this review, we consider approaches to cell fate mapping and establishing the cardiac phenotype, as well as the present state of the evidence for the cardiogenic and regenerative potential of the major candidate stem cell types. (Circ Res. 2008;103:1058-1071.)

Key Words: stem cell ■ progenitor ■ myogenesis ■ cardiomyocyte ■ myocardial infarct

In L. Frank Baum’s classic novel The Wonderful Wizard of Oz, the title character is only able to provide the Tin Woodman with a placebo heart made of velvet and filled with sawdust. One century later, the emerging field of regenerative medicine aspires to accomplish what Oz could not, that is, harness the potential of stem cells to build (or rebuild) heart tissue out of its component parts. Modern medical management has greatly improved the prospects for heart failure patients, but the only clinically available means of replacing lost myocardium remains whole-organ transplantation. The demand by patients with end-stage heart failure greatly exceeds the supply of suitable donor hearts, and so there has been considerable recent interest in cell-based therapies as an alternate means of “remuscularizing” injured hearts. Valuable proof of principle for cell-based cardiac repair was provided by early preclinical studies in which terminally differentiated cardiomyocytes from fetal and neonatal sources were transplanted into infarcted rodent hearts, resulting in
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The transplanted cells retained their cardiac phenotype, including expression of sarcomeric proteins and formation of intercalated disc structures. Subsequent intravital imaging studies have confirmed the systolic activation of such grafts in both uninjured and infarcted hearts, demonstrating that the nascent myocardium is capable of appropriate electromechanical integration. Thus, although recent work has taught us that cell therapy can also improve ventricular function by indirect (“paracrine”) mechanisms (for a review, see elsewhere), cardiogenic cell therapies offer the potential advantage of adding force-generating units to infarcted hearts that have lost muscle to scar tissue.

The clinical application of fetal and neonatal cardiomyocytes for cardiac repair is precluded by their limited supply and ethical concerns, and so the field has sought to identify other cardiogenic cell sources. We are pleased to report that there has been considerable recent progress, and cell-based cardiac repair has become a mainstream experimental concept with multiple clinical trials underway. At the same time, this field has witnessed a significant degree of controversy, particularly with regard to the capacity of certain unexpected stem cell types to differentiate into cardiomyocytes. In the present review, we begin by discussing techniques for demonstrating the cardiac potential of a candidate stem cell type and potential pitfalls in this phenotyping. We then consider a variety of reportedly cardiogenic stem cell types, both endogenous and exogenous, and evaluate the evidence in support of such claims and the prospects of each in cardiac repair.

Shock Cells and the Cardiac Phenotype: Definitions, Methods, and Potential Artifacts

Stem cells are clonogenic cells capable of both self-renewal and differentiation into more specialized progeny. Traditionally, they have been divided into 2 broad categories: adult stem cells and embryonic stem cells (ESCs). Adult stem cells are derived from postnatal somatic tissues and are generally considered to be multipotent, meaning they can give rise to multiple differentiated cell types. ESCs, derived from the inner cell mass of blastocyst-stage embryos, are pluripotent, meaning they can, in principle, give rise to all the differentiated cell types of the postnatal organism. Differentiated somatic cell types can also be reprogrammed into a pluripotent state similar to ESCs via forced expression of stem cell–related genes, the basis for the recently reported induced pluripotent stem cells (iPSCs).

Cardiac differentiation has been reported for a variety of stem cell types, including both expected and unexpected cell types. Because reports of differentiation into unexpected cell fates (ie, “transdifferentiation” events) continue to be a source of controversy, it is worth considering the experimental techniques for tracing cell fate as well as associated potential pitfalls. In principle, tracing cell fate is straightforward and simply requires that one evaluate 2 parameters, the lineage (or “ancestry”) of any given cell and its phenotype at the time of evaluation. In practice, the challenges to determining each of these parameters and then rigorously demonstrating their colocalization within a single cell are often underestimated. Table 1 lists potential approaches to establishing the cardiac potential of a candidate progenitor cell type, as well as the major pros and cons of each approach. Although not always possible, the use of multiple techniques is the most convincing way to demonstrate cardiac potential.

Tracing the lineage of putative cardiogenic progenitor cells is especially treacherous in experimental designs that involve the culture of admixed cells, coculture with definitive cardiomyocytes, or in vivo transplantation studies. Two artifacts can confound such experiments: transfer of labels to neighboring cells and heterotypic cell fusion. The first of these artifacts is best avoided by the use of indelible (eg, genetic) cell lineage markers whenever possible. Although even transgenes or transgene products have been reported to be exchanged between cultured cells under certain conditions, chemical labels (eg, bromodeoxyuridine [BrdUrd], fluorescent cell tracker dyes, and fluorescent or iron-labeled particles) are known to be readily exchanged between cocultured cells and between graft and host following transplantation. This is a particular concern in the context of myocardial infarction, which involves brisk infiltration by phagocytic cells that are capable of taking up both label and necrotic cardiomyocyte debris. As pointed out by our group and others, such transfer can be difficult or impossible to resolve at the light microscopic level.

Heterotypic cell fusion is a process whereby a donor and host cell merge, resulting in a fused cell with the genetic information of both cells, including any genetic marker (eg, green fluorescent protein [GFP] expression) from the donor cells. Although cell fusion is generally considered to be a rare phenomenon, it can be augmented by inflammation, which predominates in the context of myocardial infarction or allotransplantation. The fusion event can result in a binucleated cell, a mononucleated cell with a single tetraploid synkaryon, or potentially even a karyotypically normal cell (if a fusion event is followed by reductive division in which an entire set of paired chromosomes is lost). Thus, to most convincingly exclude cell fusion in coculture and transplantation experiments, investigators should supplement cytogenetic analysis with the use of separate genetic lineage markers to track donor and graft cells (eg, transplantation of GFP+ cells into a β-galactosidase–expressing recipient or the Cre-lox recombination system, discussed in more detail below). Such precautions are not always possible, but they have been successfully used by multiple groups and should be regarded as the standard, particularly when asserting cardiac potential for unexpected stem cell types.

Demonstrating an unambiguous cardiac phenotype can also be a challenge. The most common approach is to show the expression of 1 or more cardiac “specific” markers by immunocytochemistry or RT-PCR. In reality, there is no single absolutely specific cardiac marker, and so this approach requires the judicious selection of multiple markers and appropriate controls. (Table 2 lists commonly used markers as well as the noncardiac cell types in which they are also reportedly expressed.) Ideally, such phenotyping by cardiac markers is accompanied by functional assays, such as action potential recordings or fluorescent calcium imaging.
Table 1. Approaches to Determining the Cardiac Potential of Progenitor Cells

<table>
<thead>
<tr>
<th>Approach</th>
<th>Advantages</th>
<th>Limitations</th>
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<tr>
<td>Fate mapping using chemical label (eg, BrdUrd, fluorescent dye, iron particles) to track progenitor cells</td>
<td>■ Convenient in many coculture or transplantation studies</td>
<td>■ Susceptible to misinterpretation caused by cell fusion</td>
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<td></td>
<td>■ Often only alternative when genetic labels are unavailable</td>
<td>■ Requires phenotyping by immunocytochemistry or flow cytometry, which are susceptible to artifacts (nonspecific antibody binding, autofluorescence, and errors of colocalization)</td>
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<tr>
<td></td>
<td>■ Iron particles potentially compatible with human studies</td>
<td>■ Susceptible to false positives with transfer of the label to cocultured or host cells</td>
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<td>Fate mapping using in situ hybridization for species or gender-specific DNA sequence (eg, Y-chromosome) to track progenitor cells</td>
<td>■ Convenient in many coculture or transplantation studies</td>
<td>■ Technically challenging</td>
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<td></td>
<td>■ Provides a stable, nontransferable lineage marker</td>
<td>■ Susceptible to misinterpretation caused by cell fusion</td>
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<td></td>
<td>■ Compatible with some human transplantation studies (ie, gender-mismatched transplants)</td>
<td>■ Requires phenotyping by immunocytochemistry or flow cytometry, which are susceptible to artifacts</td>
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<td>Fate mapping using genetic reporter (eg, GFP) to track progenitor cells</td>
<td>■ Less readily transferred than chemical labels</td>
<td>■ Not compatible with all progenitor cell types (eg, difficult to transduce cell types) or hosts (eg, human patients)</td>
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<td>■ Provides additional phenotypic or temporal specificity if transgene includes a genetic reporter driven by a conditional promoter (eg, the cardiac-specific αMHC→GFP transgene)</td>
<td>■ Genetic reporter can potentially interfere with viability or biological function of labeled cells</td>
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<td>Fate mapping using independent genetic reporters to track progenitor cells and neighboring host/cocultured cells</td>
<td>■ Helps distinguish differentiation/transdifferentiation events from cell fusion (this readout is especially straightforward with the Cre-lox recombination system, eg, Cre-expressing graft cells into a “floxed” reporter host)</td>
<td>■ Generally requires phenotyping by immunocytochemistry or flow cytometry, which are susceptible to artifacts</td>
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<tr>
<td></td>
<td>■ Provides additional phenotypic or temporal specificity if transgene involves a genetic reporter driven by a conditional promoter</td>
<td>■ Not compatible with all progenitor cell types (eg, difficult to transduce cell types) or hosts (eg, human patients)</td>
</tr>
<tr>
<td>Species-specific RT-PCR for cardiac transcripts</td>
<td>■ Simple and comparatively high throughput</td>
<td>■ Genetic reporter can potentially interfere with biological function of labeled cells</td>
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<tr>
<td>Direct assessment of in vivo graft function (eg, intravital calcium imaging studies of GFP-tagged grafts)</td>
<td>■ Determines electromechanical integration and functional phenotype of progenitor-derived graft, rather than mere histological outcome(s)</td>
<td>■ Limited to xenotransplantation or coculture experiments involving different species</td>
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<td></td>
<td>■ Requires careful primer design and rigorous control studies</td>
<td>■ Provides limited mechanistic insights</td>
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<tr>
<td>Assessment of cardiac function (eg, echocardiography)</td>
<td>■ Examines the parameters that determine patient outcomes</td>
<td>■ Requires careful primer design and rigorous control studies</td>
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<tr>
<td></td>
<td>■ Available in human studies</td>
<td>■ Generally not feasible in large-animal or human studies</td>
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**Evidence for Cardiomyocyte Repopulation in Postnatal Hearts**

Until quite recently, it was accepted dogma that cardiogenesis was limited to the developing heart in mammalian organisms. This conventional view held that postnatal cardiac expansion under physiological or pathological conditions results from cardiomyocyte hypertrophy rather than via hyperplasia or recruitment of cardiac precursors. The pendulum has now swung the opposite direction, and a new paradigm has been proposed wherein there may be several resident and extracardiac progenitor cell populations that respond to cardiac injury by cell division and subsequent differentiation into functional cardiomyocytes.

The degree to which such progenitors contribute to the renewal of adult myocardium is a source of considerable controversy. In our opinion, one of the most important advances in this area was reported recently by Hsieh et al, and we regard their study as a benchmark to evaluate findings by others in this evolving field of research.22 In their elegant fate-mapping study, these authors addressed 2 critical questions using mouse models of normal aging and cardiac injury. First, is there significant replacement of cardiomyocytes...
switching from expression of (the “pulse”) should result in 100% of cardiomyocytes MerCreMer-ZEG mice with tamoxifen at a given time point (MerCreMer). In theory, treatment of the resulting expression of a tamoxifen-activated form of Cre recombinase, any subsequent re-

Table 2. Commonly Used Cardiomyocyte Markers and Extracardiac Tissues/Cell Types in Which They Can Also Be Expressed

<table>
<thead>
<tr>
<th>Marker</th>
<th>Extracardiac Site(s) of Expression</th>
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<tr>
<td>Nkx2.5</td>
<td>Embryo: pharyngeal endoderm, spleen, stomach, tongue106,111</td>
</tr>
<tr>
<td>GATA4</td>
<td>Adult: ovary, testis, lung, liver, and small intestine112</td>
</tr>
<tr>
<td></td>
<td>Embryo: proximal and distal gut, testis, ovary, liver, visceral endoderm, and parietal endoderm12, 113</td>
</tr>
<tr>
<td>T-box 5 (Tbx5)</td>
<td>Embryo: eye, forelimb, genital papilla, lung, mandible, trachea114</td>
</tr>
<tr>
<td>Myocyte enhancer factor 2C (MEF2c)</td>
<td>Adult: skeletal muscle, brain, lymphocytes115, 116</td>
</tr>
<tr>
<td></td>
<td>Embryo: skeletal muscle, smooth muscle, brain112, 118</td>
</tr>
<tr>
<td>Connexin43</td>
<td>Ovary, testis, smooth muscle, eye, brain, macrophages, fibroblasts118, 120</td>
</tr>
<tr>
<td>Sarcomeric MHC</td>
<td>Skeletal muscle121</td>
</tr>
<tr>
<td>Sarcomeric actin</td>
<td>Skeletal muscle122</td>
</tr>
<tr>
<td>Sarcomeric actinin</td>
<td>Skeletal muscle123</td>
</tr>
<tr>
<td>Cardiac troponin I</td>
<td>Fetal skeletal muscle124</td>
</tr>
<tr>
<td>Cardiac troponin T</td>
<td>Fetal skeletal muscle125</td>
</tr>
<tr>
<td>Atrial natriuretic peptide</td>
<td>Brain126</td>
</tr>
<tr>
<td>Smooth muscle α-actin</td>
<td>Smooth muscle, myofibroblasts127</td>
</tr>
<tr>
<td>Desmin</td>
<td>Smooth muscle, skeletal muscle128</td>
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during normal aging? Second, do endogenous progenitor cells significantly contribute to the replacement of adult mammalian cardiomyocytes after injury?

Figure 1 illustrates the design and readout of the genetic pulse–chase labeling experiments of the authors. In brief, they generated a cardiac-restricted, inducible reporter mouse model by crossing 2 transgenic strains: the Z/EG reporter mouse in which constitutive β-galactosidase expression is replaced by the expression of GFP on the removal of a loxP-flanked stop sequence by Cre recombinase; and a second transgenic mouse in which the cardiomyocyte-specific α-myosin heavy chain (MHC) promoter drives expression of a tamoxifen-activated form of Cre recombinase (MerCreMer). In theory, treatment of the resulting MerCreMer-ZEG mice with tamoxifen at a given time point (the “pulse”) should result in 100% of cardiomyocytes switching from expression of β-galactosidase to GFP (ie, a “blue” to “green” switch). In reality, repeated dosing resulted in ∼80% cardiomyocytes, making the blue-to-green switch, whereas the remaining ∼20% did not (ie, an 80%:20% ratio). The authors also performed appropriate control experiments to ensure the fidelity of their readout (eg, confirming its reliable inducibility in cardiomyocytes and low leakiness in noncardiomyocytes) and to exclude cell fusion.

Their model assumes that cardiomyogenic precursors would not be labeled by the tamoxifen pulse because the αMHC promoter should not be active in these presumably primitive cells. Thus, after a tamoxifen pulse and blue-to-green recombination in cardiomyocytes, any subsequent re-

progenitors would decrease the fraction of GFP-expressing (“green”) cardiomyocytes. In fact, during normal aging, the ratio of green/blue cardiomyocytes remained stable at 80%:20%, indicating that no repopulation had occurred. By contrast, by 3 months following infarction, the mice showed a significant increase in the percentage of blue cardiomyocytes, resulting in a =65%:35% ratio of green-to-blue cardiomyocytes. This finding indicated that newly differentiated cardiomyocytes (blue because they have not undergone Cre-mediated recombination) had been recruited from the progenitor pool after the tamoxifen pulse. In important control experiments, the authors detected a similar frequency of cell death in β-galactosidase–expressing and GFP-expressing cardiomyocytes, ruling out the preferential death of GFP cardiomcyocytes.

In sum, although Hsieh et al did not find significant cardiac repopulation to occur during normal aging, their study is arguably the best evidence supporting cardiomyocyte repopulation, albeit modest, by endogenous progenitors following injury. At present, the phenotype and anatomic location of the cardiomyogenic progenitors implicated in their study remains undetermined. The authors observed that the cardiomyocyte repopulation following injury coincided with an increase in the number of intramyocardial cells expressing c-Kit (reported to be a marker of resident cardiac stem cells23,24 as discussed below), but such data are only correlative. In the following sections, we consider some of the reportedly cardiogenic adult stem cell types that may underlie the endogenous cardiac repopulation demonstrated by Hsieh et al, as well as those that could contribute new muscle as an exogenous cell-based therapy. (Please see Figure 2.) We have organized this discussion by the 3 compartments in which these candidate cell types reside: the bone marrow, the heart itself (so-called resident cardiac stem cells), and peripheral tissues (either circulating or in the stroma).

### Bone Marrow–Derived Stem Cells

Approximately 1 decade ago, a number of studies challenged the long-held view that adult stem cells could give rise to only a restricted set of differentiated cell types. These reports described “transdifferentiation” events, wherein adult stem cells differentiated into unexpected cell types, even across embryonic germ layer boundaries (eg, neural stem cells giving rise to hematopoietic cell types). Although we now understand that many of these apparent transdifferentiation events actually resulted from technical artifacts and/or previously overlooked phenomena, such as cell fusion and “ectopic” stem cells (eg, hematopoietic stem cells resident in skeletal muscle),11,12 the possibility that bone marrow–derived stem cells might contribute to cardiac repair generated tremendous excitement. Importantly, the bone marrow is a very heterogenous compartment and contains multiple stem cell populations with putative cardiac potential (eg, hematopoietic stem cells [HSCs],25 mesenchymal stem cells [MSCs],26–32 very small embryonic-like stem cells,33 multipotent adult progenitor cells,34 etc). We focus here on those marrow-derived progenitors that have drawn the most attention by the field.
Hematopoietic Stem Cells

Interest in the cardiac potential of HSCs was stirred by animal studies in which donor-derived cells were identified in the hearts of bone marrow transplant recipients. In 1 such study, Jackson et al isolated an enriched population of HSCs from the bone marrow of transgenic mice that constitutively express β-galactosidase (such that only cardiomyocytes show LacZ nuclei, thereby providing a convenient means of following both lineage and phenotype). After direct intracardiac injection into infarcted wild-type recipients, Murry et al were unable to find even a single donor-derived cardiomyocyte. In independent studies, both the Robbins and the Jacobsen laboratories were also unable to find bone marrow–derived cardiomyocytes following transplantation in murine infarct models. More recently, Rubart and colleagues used intravital imaging techniques to examine the functionality of bone marrow–derived cells following transplantation into infarcted hearts. These authors found that whereas host cardiomyocytes exhibited the expected cyclic calcium transients, the graft cells did not. This indicated that the graft cells had not achieved a functional cardiac phenotype.

Some (but not all) of these discrepancies regarding the cardiogenic potential of bone marrow–derived stem cells can perhaps be attributed to the previously described phenomenon of cell fusion. Using the Cre-lox recombination system, Alvarez-Dolado et al showed that cell fusion accounted for non of cell fusion. Using the Cre-lox recombination system, Alvarez-Dolado et al showed that cell fusion accounted for non of cell fusion. Using the Cre-lox recombination system, Alvarez-Dolado et al showed that cell fusion accounted for non of cell fusion.

Figure 1. Genetic fate-mapping study indicating the replacement of adult mammalian cardiomyocytes by endogenous stem cells following injury. MerCreMer mice (tamoxifen-dependent Cre recombinase expression from the α-MHC promoter) were crossed with the ZEG reporter strain (ubiquitous LacZ expression, which is replaced by enhanced [E]GFP expression following Cre recombination), resulting in double heterozygous MerCreMer-ZEG mice. Pulsing the latter animals with tamoxifen induces a reporter switch from lacZ to EGFP expression in cardiomyocytes only. No reduction in the ratio of EGFP to lacZ cardiomyocytes was observed during normal aging, but a decrease in this ratio was observed following myocardial infarction, suggesting that new cardiomyocytes had been recruited from progenitor cells.

Summary of the experimental design used by Hsieh et al (22).

A subsequent study by Orlic et al reported a remarkable degree of regeneration following direct injection of HSCs in a mouse infarct model. These authors reported that the transplantation of lineage-null, c-Kit+ bone marrow stem cells from GFP-expressing transgenic mice into infarcted hearts resulted in the formation of nascent GFP+ cardiomyocytes that, on average, occupied more than two-thirds of the infarct zone by 9 days posttransplantation. This impressive myocardial regeneration was accompanied by improved myocardial function and attenuated adverse ventricular remodeling.

A number of subsequent studies have failed to confirm the findings of the preceding authors. Murry et al isolated HSCs using techniques similar to those of Orlic et al and used 2 transgenic mice strains as donors: mice constitutively expressing GFP and those in which the cardiac-specific α-MHC promoter drives expression of a nuclear targeted β-galactosidase (such that only cardiomyocytes show LacZ nuclei, thereby providing a convenient means of following both lineage and phenotype). After direct intracardiac injection into infarcted wild-type recipients, Murry et al were unable to find even a single donor-derived cardiomyocyte. In independent studies, both the Robbins and the Jacobsen laboratories were also unable to find bone marrow–derived cardiomyocytes following transplantation in murine infarct models. More recently, Rubart and colleagues used intravital imaging techniques to examine the functionality of bone marrow–derived cells following transplantation into infarcted hearts. These authors found that whereas host cardiomyocytes exhibited the expected cyclic calcium transients, the graft cells did not. This indicated that the graft cells had not achieved a functional cardiac phenotype.

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Nonetheless, the cardiac potential of HSCs remains controversial. The authors of the original study by Orlic et al
recently revisited this issue and again concluded that c-Kit+ bone marrow cells transdifferentiated following transplantation in a mouse infarct model and formed extensive replacement myocardium. In this study, Rota et al extended their original work to include the use of graft cells with both constitutive and cardiac-specific genetic reporters, Y-chromosome in situ hybridization (to exclude cell fusion, at least in the absence of reductive division), and intravital imaging studies. Although this extensive study strengthens the case for HSC transdifferentiation, challenges to selected aspects of this work immediately followed, indicating the debate is likely to continue.

Mesenchymal Stem Cells

MSCs are another well-characterized multipotent stem cell type that can be isolated from the bone marrow compartment. As typically prepared, MSCs are a fairly heterogenous cell population, but they generally express markers including Sca-1, CD29, CD44, CD81, and CD106 in the mouse and CD29, CD44, CD71, CD90, CD106, CD120a, and CD124 in humans. MSCs differentiate into adipocyte, chondrocyte, and osteogenic phenotypes under specific media conditions, and they can mediate immunomodulatory actions that help them avoid rejection after allotransplantation.

The capacity of MSCs to differentiate into mesodermal lineages generated considerable interest in exploring the potential of these cells for cardiogenic differentiation and infarct repair. Makino et al observed that the exposure of immortalized murine MSCs to 5-aza-deoxycytidine (5-aza-dC), an inhibitor of DNA methylation previously reported to promote the differentiation of pluripotent P19 embryonal carcinoma cells, resulted in the appearance of spontaneously beating foci. The cardiac phenotype of the treated cells was confirmed by a variety of techniques including RT-PCR (for markers including atrial natriuretic peptide [ANP], myosin light chain-2a [MLC2a] and -2v [MLC2v], GATA4, and Nkx2.5), immunocytochemistry (for markers including sarcomeric MHC and α-actinin), electron microscopy, and electrophysiology (cardiac-type action potentials). Although similar findings with 5-aza-dC have been reported by others, some have suggested that this cardiac induction may require “immortalized” MSCs.

Safety concerns regarding 5-aza-dC led to interest in alternative, more targeted approaches to inducing cardiogenic...
differentiation by MSCs. Shim et al\textsuperscript{28} isolated MSCs from the bone marrow of human patients undergoing coronary artery bypass surgery and treated the cells with insulin, dexamethasone, and ascorbic acid. The authors reported that the treated cells immunostained positively for α- and β-MHC and GATA4 but not for skeletal muscle markers such as skeletal MHC and MyoD. However, the efficiency of cardiogenesis by this approach appeared fairly low. The resultant “cardiomyocyte-like” cultures lacked appreciable spontaneous contractile activity, and only a small subset exhibited α-actinin–positive cross-striations. More recently, Shiota et al have reported the cardiac induction of MSC-like progenitors derived via a complex culturing protocol involving the formation of spheres of marrow-derived adherent cells.\textsuperscript{46} After treatment with 5-aza-dC, the spheres showed spontaneous beating activity, as well as immunoreactivity for cardiac markers including Nkx2.5 and MLC2v. The authors tested the capacity of these preparations to mediate cardiac repair in murine infarct model. They reported functional improvements following the transplantation of GFP-tagged, sphere-derived cells, but the degree of remuscularization was extremely low (\textasciitilde7 donor-derived cardiomyocytes per heart.)

The latter study is but 1 of many preclinical studies asserting beneficial effects on contractile function following the transplantation of MSCs in models of cardiac injury. Some\textsuperscript{27,29,32} but not all\textsuperscript{47} of these studies have concluded that MSCs transdifferentiate into cardiomyocytes in vivo. Reports favoring myocardial repopulation by MSCs have generally shown only rare clusters of cells that lack a typical cardiomyocyte morphology but immunostained positively for 1 or more cardiac markers. Moreover, an important caveat with regard to the previously cited studies is that they used transferable chemical markers (such as BrdUrd), rather than a more indelible lineage marker to distinguish graft from host. Indeed, when Fazel et al\textsuperscript{31} transplanted MSCs isolated from a constitutively β-galactosidase–expressing mouse, they observed functional benefits but no LacZ\textsuperscript{+} cardiomyocytes.

Some insights into how MSCs might improve contractile function without directly repopulating the myocardium can be gleaned from an interesting series of studies by Dzau and colleagues.\textsuperscript{20,48–50} Taken together, the latter work suggests that MSCs have limited cardiogenic potential but considerable promise in cell-based therapies via indirect (“paracrine”) effects on cardiac repair. These authors compared the consequences of transplanting MSCs overexpressing the prosurvival gene Akt1 to those obtained with control (ie, β-galactosidase–expressing) MSCs in a rat infarct model. They found that the transplantation of Akt-expressing cells was far more efficacious, inhibiting adverse remodeling, inflammation, fibrosis, and cardiomyocyte hypertrophy, while completely normalizing cardiac function.\textsuperscript{58} Moreover, these effects did not appear mediated by cardiomyocyte repopulation, because studies using the Cre-lox recombination system revealed only rare MSC-derived cardiomyocytes, nearly all of which resulted from cell fusion.\textsuperscript{20} Similar favorable effects were mediated by the injection of either medium conditioned by Akt-overexpressing MSCs\textsuperscript{49} or secreted frizzled-related protein 2 (SFRP-2), a soluble Wnt antagonist released by these cells,\textsuperscript{51} implicating a paracrine mechanism of action.

## Resident Cardiac Stem Cells

### Side Population Cells

Hierlihy et al was the first to isolate side population (SP) cells from heart tissue using Hoechst 33342 dye exclusion, a technique previously developed to enrich resident progenitor cells from bone marrow and other organs.\textsuperscript{52} Hoechst dye exclusion in primitive cells is conferred by the expression of various ATP-binding cassette (ABC) membrane transporters, such as those encoded by the multidrug resistance (MDR) genes. Hierlihy et al found that cells with the SP phenotype comprised \textasciitilde1% of all cells in the mouse heart, but they did not examine their cardiogenic potential. Martin et al\textsuperscript{53} isolated cardiac SP cells from adult GFP\textsuperscript{+} mice and cocultured the latter cells with cardiac main population cells from wild-type mice. After 14 days, an unspecified fraction of the GFP\textsuperscript{+} SP-derived cells immunostained for the striated muscle marker α-actinin; however, it should be noted that the antibody used does not distinguish between cardiac and skeletal forms of α-actinin.

In 2005, Pfister et al\textsuperscript{21} described a subpopulation of murine cardiac SP cells that expresses the stem cell marker Sca-1 but is negative for the endothelial marker CD31. These cells comprised \textasciitilde10% of the total SP cells (\textasciitilde500 to 1000 cells per adult mouse heart). By RT-PCR, it was shown that the CD31\textsuperscript{−}/Sca-1\textsuperscript{+} SP cells expressed Nkx2.5, GATA4, smooth muscle α-actin, and desmin but not α-actinin or α-MHC. After 2 to 3 weeks of coculture with adult rat ventricular cardiomyocytes, it appeared that the SP cells had adopted a more mature cardiac phenotype, as evidenced by positive immunostaining for α-actinin, connexin43, and troponin I. These cells also exhibited contractions and calcium transients in response to field stimulation. Cell fusion was excluded by coculture experiments using 2 separate genetic lineage markers and the Cre-lox recombination system.

Oyama et al reported that they could induce the cardiac differentiation of SP cells from neonatal rat hearts without coculture, by treating the cells with either oxytocin or the histone deacetylase inhibitor trichostatin A.\textsuperscript{54} They also found that GFP-tagged SP cells homed to cryoinjured hearts following intravenous infusion. GFP\textsuperscript{+} cardiomyocytes were observed in the injured hearts (comprising 4.4% of all cells), but cell fusion was not excluded.

Although we look forward to more definitive transplantation studies with cardiac SP cells, the in vivo response of endogenous SP cells to cardiac injury also remains a source of some uncertainty. On the one hand, Martin et al reported that the number of cardiac SP cells increased following cryoinjury in the mouse heart.\textsuperscript{55} By contrast, Mouquet et al observed an acute decrease in the number of cardiac SP cells in a mouse model of permanent coronary occlusion, followed by a gradual increase to baseline levels by 7 days postinfarction (via proliferation of resident cardiac SP cells and homing of bone marrow derived SP cells).\textsuperscript{56} At present, we can only speculate that the apparent discrepancies in SP cell dynamics between the 2 studies result from the differing modes of cardiac injury.

### c-Kit\textsuperscript{+} Cells

In 2003, Beltrami et al reported a population of resident cardiac progenitors in adult rat heart that were identified by
their expression of the surface antigen and stem cell marker, c-Kit. Although rare overall (≈1 c-Kit+ cell per 104 total myocardial cells), histological analysis revealed these cells to be located in small clusters within the interstitium of the ventricular and atrial myocardium with a higher density in the atria and the ventricular apex. Cardiac c-Kit+ cells were found to be self-renewing, clonogenic, and multipotent, differentiating into cardiomyocytes, smooth muscle, and endothelial cells. Whereas their differentiated progeny remained quite immature in vitro (ie, lacking structural organization, identifiable sarcomeres, and contractile activity), this was in stark contrast to their in vivo capabilities. By 20 days following transplantation into the infarct border zone of syngeneic rats, BrdUrd-labeled c-Kit+ cells had colonized the infarcted area and appeared to have matured with expression of sarcomeric proteins (cardiac MHC), cross-striations, and connexin43-positive intercalated discs. Morphometric analyses revealed that the injected cells had given rise to 13×103 new cardiomyocytes, corresponding to a 65-fold increase from the day of injection. This was accompanied by an increase in vascular density with incorporation of the graft cells. Similarly impressive cardiac regeneration and functional improvements by echocardiography were reported after the intracoronary infusion of c-Kit+ cardiac stem cells in both rat and canine infarct models. More recently, these investigators have reported the isolation and expansion of c-Kit+ cells from human surgical specimens.24

The significance of these findings would be far-reaching, but it remains to be seen whether this success can be reproduced in the target population of patients with ischemic heart disease. Other adult stem cell populations are known to be reduced with advanced age and/or disease; indeed, a recent study by Pouly et al failed to demonstrate c-Kit+ stem cells in endomyocardial biopsies from typical human patients.58

Sca-1+ Cells

Oh et al reported the existence of resident cardiac progenitor cells in the adult mouse heart that express stem cell antigen-1 (Sca-1) but not c-Kit. A small percentage of Sca-1+ cells expressed cardiac-specific genes like Nkx2.5 and sarcomeric proteins after treatment with the demethylating agent 5-aza-dC, but no spontaneous beating activity was observed. Following intravenous infusion in a mouse infarct model, Sca-1+ cells homed to injured myocardium, where they appeared to have differentiated into cardiomyocytes in the border zone with expression of sarcomeric α-actin, cardiac troponin I, and connexin 43. These authors used the Cre-lox recombination system to distinguish cell fusion from direct incorporation and concluded that each accounted for ≈50% of the apparently donor-derived cells.

Matsuura et al also reported the isolation of Sca-1 cells from adult murine hearts. Although 5-aza-dC failed to induce cardiac differentiation in their hands, they found that oxytocin resulted in a small fraction (≈1%) of Sca-1+ cells exhibiting spontaneous beating activity and expression of sarcomeric α-actin, cardiac troponin I, Nkx2.5, and MHC. More recently, Wang et al reported that Sca-1+ cells from the mouse heart could be induced to express cardiac markers by treatment with 5-aza-dC, fibroblast growth factor-4, fibroblast growth factor-8, and the Wnt antagonist Dickkopf-1. These authors also transplanted Sca-1+/CD31− cells into the acutely infarcted mouse heart and found that this intervention attenuated the progressive functional decline, while promoting angiogenesis and resulting in “modest” cardiomyocyte repopulation (ie, LacZ+ cardiomyocytes that immunostained with troponin T, α-sarcomeric actin, and N-cadherin). Unfortunately, the authors did not determine the frequency of such cardiomyocytes or perform experiments to exclude cell fusion.

Because humans do not have the Sca-1 gene, it is unclear how an “orthologous” population of progenitors would be isolated from the human heart.

Cardiospheres

In 2004, Messina et al described a novel isolation technique for resident cardiac progenitors from murine hearts, as well as subcultures of human atrial or ventricular specimens. Mild enzymatic digestion of the tissue specimens yielded small, round, phase-bright cells that clustered together in suspension. These sphere-generating cells were then allowed to adhere on poly-l-lysine–coated plates and cultured in a media enriched with cytokines (epidermal growth factor, basic fibroblast growth factor, cardiotoxin-1, thrombin). These “cardiosphere” (or CS)-derived cells were self-renewing, clonogenic, and expressed both endothelial (KDR in human, flk-1 in mouse cells, and CD31) and stem cell markers (CD34, c-Kit, Sca-1). Mouse CS-derived cells showed spontaneous contractile activity, whereas human CS cells did so only after 24 hours of coculture with postnatal rat cardiomyocytes. CS-cells from both human and mouse demonstrated trilineage differentiation into cardiomyocytes and endothelial and smooth muscle cells; however, quantitative data as to the frequency of these events were not reported. CS-derived cardiomyocytes expressed cardiac markers including cardiac troponin I, ANP, and cardiac MHC. In vivo, CS-derived cells were reported to regenerate the infarcted mouse heart.

Subsequently, Smith et al expanded on these findings by isolating CS-forming cells from human biopsy specimens. The human cardiospheres, which were successfully isolated from 69 of 70 biopsies attempted, consistently expressed c-Kit but not the multidrug resistance gene MDR1, indicating that these cells were phenotypically distinct from the resident cardiac progenitors previously identified in situ (c-Kit+, MDR1−). Consistent with findings by Messina et al, human CS-derived cells did not spontaneously contract, but coculture with neonatal rat cardiomyocytes evoked calcium transients in synchrony with neighboring cardiomyocytes, action potentials, and fast inward sodium current. Smith et al also injected lentivirally transduced LacZ+ human CS-derived cells into the border zone of infarcted SCID-beige mice.

Twenty days later, the CS-derived cells were found throughout border regions of the mouse heart, and occasional donor cells immunostained for α-sarcomeric actin and von Willebrand factor. Echocardiography showed improvements in global left ventricular function, but, given the apparently limited cardiomyocyte repopulation by LacZ+ cells, these
functional effects were attributed to a combination of regeneration and paracrine effects.

Based on these studies, explant-derived cardiospheres appear to have cardiogenic potential and considerable promise for cardiac repair. However, a recent study by Shenje et al gives some reason for caution. These authors performed lineage tracing of cardiac explant-derived cells from a transgenic mouse with a highly sensitive cardiac reporter based on the Cre-lox recombination system (i.e., MLC2v-Cre/ZEG mice, in which expression of GFP indicates cardiac differentiation). They observed the emergence of small, round, phase-bright cells as described by others, but they did not place these explant-derived cells in suspension cultures so as to form cardiospheres. Notwithstanding this important technical difference, the explant-derived cells characterized by Shenje et al had a perplexing phenotype in light of the preceding work. Their cells lacked expression of Sca-1, c-Kit, Nkx2.5, and ANP, and the sensitive MLC2v-Cre/ZEG genetic reporter was not activated. GFP+, sarcomeric actinin-expressing explant-derived cells were observed, but these were convincingly shown by ultrastructural studies to result from phagocytosis of GFP-expressing myocytes, rather than activation of the reporter. (The latter finding underscores the challenge of colocalization studies at the light microscopic level when phagocytic cell types are present, a point that we have previously emphasized.) Finally, the authors examined the functionality of these cells following transplantation with intravital imaging studies and found no evidence for calcium cycling by the graft.

Islet-1 (Isl1), a LIM homeodomain transcription factor, is expressed by progenitor cells of the secondary heart field, a structure present during early development that gives rise to the formation of the outflow tract, the atria, and the right ventricle. In 2005, Laugwitz et al showed that a population of Isl1+ cells persists in neonatal mouse hearts and that these cells express the cardiac transcription factors Nkx2.5 and GATA4, but not Sca-1, CD31, or c-kit. Isolated Isl1+ progenitor cells self-renew and maintain the ability to differentiate into functional cardiomyocytes in vitro and in vivo. Rare Isl1+ cells were identified in early postnatal rat, mouse, and human myocardium, but they may not be present in adult human heart.

Stem Cells in the Periphery and/or Circulation

Endothelial Progenitor Cells

Endothelial progenitor cells (EPCs) are more properly viewed as both a circulating and a bone marrow stem cell type, because they are known to reside in both compartments. In 1997, Asahara and colleagues described the phenotype of EPCs, which proliferate in response to tissue ischemia, home to areas of injury, and either incorporate within or otherwise promote neovascularization. EPCs express markers including Flk-1, CD34, and CD133 and can differentiate into definitive endothelial cells. Initial interest in the application of EPCs to cardiac repair was naturally focused on their angiogenic properties.

The capacity of EPCs to transdifferentiate into cardiomyocytes was first reported by Dimmeler and colleagues in 2003. In their study, CD34+ human EPCs were obtained from peripheral blood mononuclear cells of healthy adults or from patients with coronary artery disease. After coculture with neonatal rat cardiomyocytes, EPCs were reported to transdifferentiate into cardiomyocytes based on morphology, α-sarcomeric actinin immunoreactivity by flow cytometry, and expression of other cardiac markers by immunostaining or RT-PCR with species-specific probes. Furthermore, the EPCs showed calcium transients that synchronized with adjacent rat cardiomyocytes, suggesting communication with the host myocardium by gap junctions. Coculture experiments with paraformaldehyde-fixed cardiomyocytes indicated that cell fusion was not required for EPCs to acquire the cardiac phenotype. Still, the efficiency of cardiac induction by EPCs was very low: even after enhancement with inhibition of Notch signaling, <1% of EPCs expressed α-sarcomeric actinin. Ashara and colleagues reported even lower rates of in vitro cardiac transdifferentiation following coculture of EPCs with the rat heart–derived H9C2 cell line. The latter authors also reported the in vivo cardiac differentiation of a related preparation of human circulating cells following transplantation into a rodent infarct model. This conclusion is complicated, however, by the definite demonstration of cell fusion between host myocytes and graft cells, using species-specific fluorescent in situ hybridization probes.

Moreover, Gruh et al were unable to confirm the in vitro cardiac differentiation of EPCs following coculture with primary myocytes. These authors detected no expression of human cardiac transcripts (in contrast to the previously discussed studies), and they concluded that the rare, ostensibly transdifferentiated EPCs observed by FACS or epifluorescence microscopy were artifacts resulting from overlying cells and/or autofluorescence. Thus, although the cardiac potential of EPCs remains a source of controversy, this report by Gruh et al again underscores the challenges inherent to interpreting coculture experiments.

Other Postnatal Stem Cell Types With Reported Cardiogenic Potential

Several other post- (or peri)natal stem cell types have been reported to have cardiogenic potential, among which adipose-derived stem cells (ADSCs) and umbilical cord–derived stem cells (UCBSCs) are just 2 more extensively studied examples. In 2004, Planat-Benard et al reported the differentiation of spontaneously beating cells from ADSCs, albeit at a vanishingly low frequency (0.02 to 0.07%). Several cardiac markers were detected by RT-PCR and immunocytochemistry, and the cells exhibited “pacemaker-like” action potentials, but these action potentials had a strikingly brief duration that would be unusual even for primitive cardiomyocytes. Recently, Yamada et al reported a 10-fold higher yield of ADSCs from brown rather than white fat. Following transplantation in a mouse infarct model, brown fat ADSCs had...
Cell-Based Cardiac Repair With Exogenous Pluripotent Stem Cells

In this section, we consider the cardiogenic potential of pluripotent stem cells—cell types that are uninvolved in endogenous reparative mechanisms but may nonetheless prove useful as an exogenous cell therapy. Although the preclinical development of therapies with pluripotent stem cells certainly lags that with adult stem cells, there has been a recent flurry of encouraging preclinical studies indicating functional efficacy with ESC-based cardiac repair.86–91 It is no coincidence that these encouraging developments are happening now: they reflect a decade “on the learning curve” with human embryonic stem cells (hESCs), as well as the application of recent insights from developmental biology. Given the recent reprogramming of human somatic cells into ESC-like pluripotent cells, it is becoming increasingly plausible to envision a “second generation” of cell-based cardiac therapies that will exploit the tremendous expandability and un questioned cardiac potential of pluripotent stem cells.

Embryonic Stem Cells

ESCs are pluripotent cells derived from the inner cell mass of preimplantation-stage blastocysts. Murine ESCs were first isolated in 198192,93 and their human counterparts in 1998.94 ESCs from both species show similar features in culture. First, they can be propagated indefinitely as a stable, self-renewing population. Second, they have a pluripotent phenotype, meaning they can differentiate into cell types from all 3 primary germ layers both in vitro95 and in vivo.92–94 The latter property represents both an advantage and a disadvantage to the use of these cells: the intracardiac transplantation of undifferentiated ESCs or preparations of insufficiently purified ESC-derived cardiomyocytes results in teratoma formation.88,90,96 Because this would obviously be a disastrous outcome, the development of cell therapies based on pluripotent stem cells will require tight control of their differentiation into useful cell types.

Human ESC-derived cardiomyocytes (hESC-CMs) have an unambiguous cardiac phenotype by transcriptional, immunocytochemical, ultrastructural, and functional end points. They express expected cardiac markers, including cardiac-specific transcription factors (eg, Nkx2.5, GATA4, MEF2c, Tbx-5, and Tbx-20), sarcomeric proteins (eg, cardiac troponins I and T, sarcomeric MHCs and actins), and chamber-specific proteins (eg, ANP, MLC2V, and MLC2A).97–99 They exhibit spontaneous beating activity, characteristic cardiac ionic currents, and nodal-, atrial-, and ventricular-like action potentials.100,101

Historically, the most common method for deriving hESC-CMs in vitro has been via the formation of 3D aggregates, so-called embryoid bodies, and treating the cells with high serum to induce differentiation.97,99 The efficiency of cardiogenesis is generally low in this approach, but the field has applied lessons from molecular and developmental biology to derive preparations of greater cardiac purity. More than a decade ago, the Field and colleagues demonstrated the power of genetic selection to purify cardiomyocytes from pluripotent stem cells by introducing a transgene in which the cardiac specific α-MHC promoter drives expression of neomycin resistance.102 After antibiotic selection of differentiated embryoid bodies, the resultant cultures contained 99.6% α-MHC+ cardiomyocytes versus 0.6% in untreated cultures. Similar approaches have recently been shown to be successful with hESCs.103,104

Our group and others have used insights from developmental biology to devise protocols to more efficiently guide the differentiation of hESCs into cardiomyocytes.86,87,105 For example, we showed that the serial application of 2 TGF superfamily members, activin A and bone morphogenetic protein 4, followed by Percoll gradient purification, resulted in preparations of 80% β-MHC+ hESC-CMs. Following injection into recently infarcted rat hearts, these cells formed stable grafts of human myocardium. Although we do not yet know whether hESC-CMs undergo systolic activation in vivo, echocardiographic and MRI studies indicated beneficial effects on global and regional left ventricular function. Interestingly, these effects appeared to require the presence of engrafted cardiomyocytes, because they were not observed with the transplantation of noncardiac hESC derivatives. Multiple groups have now independently reported partial remuscularization and functional benefits following hESC-CM transplantation in rodent infarct models.86,88,89

Induced Pluripotent Stem Cells

Three independent groups have reported the generation of ESC-like iPSCs by reprogramming human somatic cells with the viral delivery of 4 stem cell–associated genes.106–108 Ethical concerns are avoided because no embryos are harmed, and, in principle, the cells can be used in autologous cell therapies. There are risks associated with the integrating viruses currently used to generate iPSCs, but we anticipate that safer, nonviral approaches will be developed soon.

MacLellan and colleagues demonstrated the cardiac phenotype of murine iPSC progeny in vitro and in vivo following blastocyst injection.109 Murine iPSC-derived cardiomyocytes immunostain for sarcomeric MHC and troponin C, and they express transcripts including Nkx2.5, GATA-4, MEF2c, ANP, MLC2V, and MLC2a. They exhibit calcium transients in response to field stimulation. Of note, Yamanaka and colleagues have reported that human iPSCs can be differentiated into cardiomyocytes using methods developed by our group for hESCs.106 It is too soon to know whether iPSCs will be of value in cell-based cardiac repair, but they are an attractive new candidate.

Conclusions

Remuscularization of injured hearts is an ambitious goal, but recent studies indicating that adult mammalian hearts un-
dmergo limited cardiomyocyte repopulation in response to injury give some reason for hope. In this review, we have considered only 1 aspect of this challenge: the identification of potential sources of replacement cardiomyocytes. Advances in stem cell and developmental biology have resulted in the identification of numerous candidate stem cell types with putative cardiogenic potential. Nonetheless, the ideal cell type remains uncertain, despite all claims to the contrary. In our opinion, the cardiogenic potential of bone marrow–derived and circulating stem cells appears limited, but other candidates including certain resident cardiac stem cell populations and pluripotent stem cells are clearly capable of more efficient cardiogenesis. We are optimistic that research into cell-based cardiac repair will eventually yield effective myogenic therapies, but success will require rigorous attention to cardiac phenotyping, cell fate mapping, and preclinical and clinical testing.

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References
Cardiogenic Differentiation of Progenitor Cells


51. Gneechi M, He H, Liang OD, Melo LG, Morello F, Mu H, Noisieux N, Zhang L, Pratt RE, Ingwall JS, Dzau VJ. Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. Nat Med. 2005;11:367–368.


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Hans Reinecke, Elina Minami, Wei-Zhong Zhu and Michael A. Laflamme

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