Stem cells for myocardial repair

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The adult human heart has limited regenerative capacity and therefore any significant myocardial damage results in progressive deterioration in its function. Cell-based cardiac repair represents an exciting approach for rebuilding the injured heart. Consequently, during the last decade, significant efforts have been made to assess the potential role of skeletal myoblasts, fœtal cardiomyocytes, bone marrow stem cells, endothelial progenitors, resident cardiac progenitor cells, and embryonic stem cells (ESC) to restore the myocardial performance. Clinical translation of these basic studies has progressed rapidly with several Phase I and II clinical trials, utilizing autologous bone marrow cells and skeletal myoblasts, being well underway. In this review, we will describe these basic and clinical efforts. A specific emphasis will be placed on the potential role of human ESC for myocardial repair. These unique pluripotent cell lines can be propagated in the undifferentiated state in culture and coaxed to differentiate into cell derivatives of all three germ layers, including cardiomyocytes. The potential applications of this unique differentiating system in the emerging field of cardiovascular regenerative medicine will be discussed with special emphasis on the steps required to fully harness their unique potential.

Introduction

One of the most exciting areas in basic and applied research today involves the use of stem cells. These unique cells have the capability to transform and replenish the different tissue types that make up the body, and also represent the fundamental building blocks of human development. The recent advances in the areas of stem cell biology and tissue engineering coupled with parallel achievements in molecular and cell biology have paved the way to the development of a new field in biomedicine, regenerative medicine. This approach seeks to develop new biological solutions to replace or modify the function of diseased, absent, or malfunctioning tissue.

The adult heart represents an attractive candidate for these emerging technologies because adult cardiomyocytes have limited regenerative capacity. Hence, any significant loss of heart cells is mostly irreversible and may lead to progressive loss of ventricular function and finally to the development of heart failure. Despite advances in the pharmacological, interventional, and surgical therapeutic measures, the prognosis for heart failure patients remains poor. Non-pharmacological treatments like heart transplantation for end-stage patients are of limited impact as the chronic lack of donors limits the number of patients that could benefit from heart transplantations. Given these circumstances, the development of novel therapeutic strategies for the treatment of heart failure has become imperative.

Although the pathophysiological mechanisms underlying the development of progressive ventricular dysfunction and clinical heart failure are multi-factorial, the initial rationale underlying the cell replacement strategy was simple, to attempt to restore myocardial performance by augmenting the number of functional cardiomyocytes within the diseased hearts.1,2 While these initial experimental strategies mainly focused on repopulating the diseased myocardial areas with a new pool of...
functional myocytes (skeletal myoblasts or foetal cardiomyocytes),\(^3,^4\) it is now clear that cell therapy may lead to improvement of myocardial performance through a variety of mechanisms. Examples of such indirect mechanisms include improving the survival of host cardiomyocytes by induction of angiogenesis, augmenting a possible endogenous repair mechanism, and altering the negative remodelling process that occurs following myocardial infarction by changing the geometry and structural properties of the scar.

Since the initial pioneering cell therapy experiments more than a decade ago, numerous basic studies and several early-stage clinical trials were performed using a variety of cell types with the hope of improving myocardial performance. In the current review, we will briefly describe these efforts (for a more detailed discussion please refer to a number of excellent reviews).\(^1,^2,^5\) We will then focus on one unique cell type, the human embryonic stem cell (hESC), and describe the possible future role of these cells in cardiovascular regenerative medicine as well as the steps required to harness their potential.

**Cell therapy approaches for myocardial repair**

During the last decade, different cell types have been proposed for cell-based cardiac repair. These include foetal cardiomyocytes,\(^4,^6,^7\) skeletal myoblasts,\(^3\) bone marrow-derived haematopoietic\(^8\) and mesenchymal\(^9\) stem cells, mouse\(^10\) and hESCs,\(^10,^11\) and more recently resident cardiac progenitor cells.\(^12\)

Although a variety of cell types have been suggested, the ideal donor cell should probably exhibit the electrophysiological, structural, and contractile properties of cardiomyocytes, and should be able to integrate structurally and functionally with host tissue. In addition, it has to retain an initial high proliferative potential that may enable improved colonization of the scar tissue. The ability to engineer the candidate cell to promote desirable characteristics such as resistance to ischaemia and apoptosis and improved contractile properties may be another advantage of such an ideal cell type. Finally, the optimal candidate cell should be of autologous origin or retain minimal immunogenicity and should be readily available in large quantities for transplantation. Unfortunately, none of the candidate cell sources exhibit all of the aforementioned properties.

**Foetal cardiomyocytes**

Early cell transplantation studies focused on using foetal or neonatal rat cardiomyocytes, as these cells have the inherent electrophysiological, structural, and contractile properties of cardiomyocytes and still retain some proliferative capacity.\(^4,^6,^7,^13\) In these pioneering studies, foetal cardiomyocytes transplanted into healthy mice hearts were demonstrated to survive, align with host cells, and form cell-to-cell contacts with host myocardium.\(^4\) Interestingly, early-stage cardiomyocytes (foetal and neonatal) were demonstrated to be better candidates than more mature cardiac cells due to their superior in vivo survival.\(^13\) More recently, it was demonstrated that these cells could survive and improve cardiac function for up to 6 months in a rat model of chronic myocardial infarction.\(^7\) Cardiomyocyte cell transplantation was associated with smaller infarcts,\(^14\) prevented cardiac dilatation and remodelling following infarction,\(^15\) and also improved the ventricular function in some of these studies.

**Skeletal myoblasts**

Experimental cell therapy approaches for cardiac repair began with the use of skeletal myoblasts derived from skeletal muscle satellite cells.\(^3,^16\) Interestingly, besides their non-cardiac origin, skeletal myoblasts have almost all the properties of the ideal donor cell type. Skeletal myoblast satellite cells can be harvested from autologous sources, they can be rapidly expanded \textit{ex vivo} to clinically applicable numbers of myoblasts without a risk for tumorigenicity, and they have the capabilities to withstand ischaemia better than many other cell types.

Although it was originally hoped that these cells would adapt a cardiac phenotype, it is now clear that myoblasts remain committed to form only mature skeletal muscle in the heart that possess completely different electromechanical properties than those of heart cells. Moreover, given the inability of myoblasts to form electromechanical connections with host cardiomyocytes (due to lack of expression of adhesion and gap junction proteins), it is not surprising that physiological studies failed to demonstrate synchronous beating of the grafted cells within the host tissue.\(^17\) Interestingly, despite these apparent shortcomings, several studies in small and large animal models of infarction demonstrated beneficial effects of grafting of these cells on ventricular performance.\(^3,^18\)

Given their autologous origin, the encouraging preclinical results, and despite the lack of complete understanding of the mechanisms underlying their beneficial effects, skeletal myoblasts were the first cell type to reach the clinical arena.\(^19,^20\) These clinical trials utilized autologous skeletal myoblasts that were harvested by a muscle biopsy, expanded \textit{ex vivo}, and later grafted into the hearts of chronic heart failure patients. The pioneering Phase I clinical trials were performed either using a direct surgical approach [during coronary artery bypass graft surgery (CABG)] or using a percutaneous endocardial catheter-delivery approach and have demonstrated both the feasibility of the procedure and the ability of the cells to engraft in the infarcted myocardium (Table 1).\(^19,^25\)

These trials, however, have also raised a number of issues for concerns.\(^1\) Pathological studies in some of the patients revealed that engrafted cells populated only a small proportion of the infarct. Thus, methods should be developed in the future to improve the efficacy of cell engraftment and survival.\(^2\) An important safety concern was the disturbingly high incidence of ventricular arrhythmias noted in the initial stages of some of
<table>
<thead>
<tr>
<th>Author/study name</th>
<th>Number of patients</th>
<th>Cell delivery</th>
<th>Additional procedures</th>
<th>Cell dose</th>
<th>Endpoint assessment methodology</th>
<th>Control group</th>
<th>Results</th>
<th>Adverse effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herreros et al.</td>
<td>12</td>
<td>Direct-</td>
<td>CABG</td>
<td>2.21 x 10^8</td>
<td>Echocardiography, PET</td>
<td>None</td>
<td>↑LVEF, ↑Regional contractility in transplanted segments</td>
<td>None reported</td>
</tr>
<tr>
<td></td>
<td></td>
<td>intramyocardial</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↑Viability of infarct zone (F-FDG PET)</td>
<td></td>
</tr>
<tr>
<td>Menasche et al.</td>
<td>19</td>
<td>Direct-</td>
<td>None</td>
<td>8.71 x 10^8</td>
<td>Echocardiography</td>
<td>None</td>
<td>↑NYHA functional class, ↑LVEF, ↑Regional contractility in transplanted</td>
<td>Four delayed episodes of sustained VT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>intramyocardial</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>segments</td>
<td></td>
</tr>
<tr>
<td>Pagani et al.</td>
<td>5</td>
<td>Direct-</td>
<td>LVAD</td>
<td>3 x 10^5</td>
<td>Histological analysis</td>
<td>None</td>
<td>Myoblasts survive and form myofibers, ↑Small vessel formation</td>
<td>None reported</td>
</tr>
<tr>
<td>Siminiak et al.</td>
<td>10</td>
<td>Direct-</td>
<td>CABG</td>
<td>1.7 x 10^7</td>
<td>Echocardiography</td>
<td>None</td>
<td>↑LVEF</td>
<td>Four episodes of VT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>intramyocardial</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(no additional episodes following administration of amiodarone prophylaxis)</td>
<td></td>
</tr>
<tr>
<td>Smits et al.</td>
<td>5</td>
<td>Percutaneous-</td>
<td>None</td>
<td>1.96 x 10^8</td>
<td>LV angiography nuclear radiography DSE MRI</td>
<td>None</td>
<td>↑LVEF, LV Wall thickening in target areas (MRI)</td>
<td>One episode of non-sustained VT</td>
</tr>
<tr>
<td>Siminiak et al. -PONZAN</td>
<td>10</td>
<td>Percutaneous-</td>
<td>None</td>
<td>Up to 1 x 10^8</td>
<td>Echocardiography</td>
<td>None</td>
<td>↑LVEF, ↑NYHA functional class</td>
<td>None reported</td>
</tr>
<tr>
<td>Trial</td>
<td></td>
<td>coronary sinus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↑LVEF, ↑Viability of infarct zone (F-FDG PET) Long term survival of</td>
<td>Three episodes of non-sustained VT</td>
</tr>
<tr>
<td>Dib et al.</td>
<td>30</td>
<td>Direct-</td>
<td>LVAD (n = 6) CABG (n = 24)</td>
<td>1-30 x 10^7</td>
<td>PET, echocardiography, MRI</td>
<td>None</td>
<td>Long term survival of engrafted cells (up to 4 years)</td>
<td></td>
</tr>
</tbody>
</table>

F-FDG-PET-F, F-labelled fluoro-deoxyglucose positron emission tomography; VT, ventricular tachycardia; NYHA, New York Heart Association; LVAD, left ventricle assist device; DSE, dobutamine stress echo.
these trials. For example, 10 out of the first 22 patients, reported in the literature, undergoing skeletal myoblast transplantation experienced some form of ventricular arrhythmias.\textsuperscript{19,20} Although direct causal relationship is hard to prove in these non-controlled trials given the expected high incidence of ventricular arrhythmias in this patient population, this potentially life-threaten side effect warrants further considerations. It is postulated to due to their lack of gap junctions, the engrafted myotubes are completely uncoupled to the surrounding ventricular myocytes and may therefore act as anatomical obstacles, increasing tissue inhomogeneity, slowing conduction, and increasing the likelihood for the formation of re-entrant arrhythmias.

Given the fact that these Phase I clinical trials were not randomized, placebo controlled, or blinded, that they used widely different cell transplantation protocols in a relatively small number of patients, and that they were associated with other confounding factors such as concomitant LVAD implantation or revascularization, it is difficult to draw any significant conclusions about their efficacy. Ongoing Phase II clinical trials will hopefully address these concerns and thoroughly evaluate the safety and efficacy of myoblast transplantation.

**Bone marrow-derived stem cells**

Studies in animal models of ischaemia and Phase I & II clinical trials suggest that delivery of haematopoietic stem cells (HSCs) and circulating endothelial progenitor cells (also originating from bone marrow stem cells) may result in improvement in the ventricular function in ischaemic heart disease (IHD) patients. Nevertheless, the initial assumption regarding the capability of bone marrow-derived HSCs to regenerate the heart by transdifferentiation into cardiomyocytes^{8} has been recently challenged by a number of studies demonstrating cell fusion of the progenitor cells with host cells.\textsuperscript{26}

Moreover, recent studies suggest that the HSC continue to differentiate along the haematopoietic lineage\textsuperscript{27,28} and the functional improvement observed may not be related to transdifferentiation into the cardiac lineage, but rather from indirect mechanisms. The main assumption is that the infused bone marrow mononuclear cells (BMMC) stimulate neoangiogenesis and thus prevent late myocardial remodelling, thereby decreasing myocyte apoptosis, collagen dispostion, and scar formation.\textsuperscript{3}

Fueled in part by hopes for cardiac transdifferentiation, as well as by the considerable body of data supporting angiogenic activity,\textsuperscript{4} bone marrow studies moved remarkably quickly from small animals to clinical trials. As expected, from a rapidly developing therapeutic modality, there is a significant heterogeneity between the conducted studies concerning the cell type used [bone marrow is a heterogeneous tissue, containing rare haematopoietic and mesenchymal stem cells (MSC) (0.01%)], the indications, and the clinical endpoints assessed in these studies. In general, the clinical trials using bone marrow-derived stem cells can be grouped into two groups: intracoronary cell infusion to the culprit coronary artery following ST elevation acute myocardial infarction (STEMI) (Table 2) and intramyocardial cell injection for chronic IHD (CIHD) (Table 3).

**Acute myocardial infarction**

Initial Phase I and II clinical studies assured the feasibility and safety of the intracoronary cell infusion in the setting of STEMI.\textsuperscript{29–34} Strauer \textit{et al.}\textsuperscript{29} evaluated in 10 patients the effect of BMMC infusion, 7 days following STEMI, on LV function when compared with control patients who refused to enter the study. Patients who received BMMC infusion displayed improvement in the extent of the hypokinesis/dyskinesis area but did not show any significant change in the left ventricular ejection fraction (LVEF). The following TOPCARE-AMI study compared blood- and bone marrow-derived progenitor cells (delivered 4 days following MI) and demonstrated that both cell types had a similar but mild positive effect on cardiac function when compared to historical controls (improvement of EF from 52 to 60% over 4 months whereas in controls, EF improved from 51 to 54%).\textsuperscript{30}

Wollert \textit{et al.}\textsuperscript{34} (the BOOST trial) conducted a randomized study in 60 patients and reported, using MRI, a substantial improvement in global LVEF in the BMMC infusing group (50.0–56.7% in treated vs. 51.3–52% in controls) following 6 months of follow-up. More recently, however, it was reported that following 18 months, there were no significant differences in global LVEF, although regional contractility in target areas remained significantly improved.\textsuperscript{31} These results were recently reinforced in the first double blinded, randomized, sham controlled, trial conducted by Janssens \textit{et al.}\textsuperscript{33} Following a period of 4 months, patients treated with BMMC had an improved viability in the infarct area and regional systolic function but a similar global LVEF function when compared to sham-infused controls.

**Chronic IHD**

Similar to the skeletal myoblast trials, delivery of bone marrow stem cell to the chronically ischaemic myocardium was performed either using direct intramyocardial injection (as an adjunct to CABG)\textsuperscript{35,36} or using a percutaneous catheter-based endomyocardial injection approach.\textsuperscript{37–39} The hypothesis underlying these studies was that the injected cells may improve the perfusion and survival of the ischaemic tissue. These initial trials included only a small number of patients, lacked a control group, and some also included a confounding revascularization procedure.\textsuperscript{36,37,39} More recently, Perin \textit{et al.}\textsuperscript{38} reported a controlled, non-randomized, trial that included 21 patients with end-stage IHD treated with percutaneous transcatheter injection of BMMC. The treated patients showed improved functional class and myocardial performance with no significant adverse effects. More recently, Patel \textit{et al.}\textsuperscript{35} reported on the first controlled randomized trial evaluating BMMC in IHD. BMMC were implanted in conjunction to CABG surgery in 10 patients. Cell transplantation resulted in a substantial improvement of both symptoms and myocardial performance.
<table>
<thead>
<tr>
<th>Author/study name</th>
<th>Number of patients</th>
<th>Cell type</th>
<th>Cell delivery</th>
<th>Cell dose</th>
<th>Endpoint assessment methodology</th>
<th>Control group</th>
<th>Results</th>
<th>Follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td>Janssens et al.</td>
<td>33 (1 day following AMI)</td>
<td>BMMC</td>
<td>Intracoronary</td>
<td>$3 \times 10^9$ (2.8 $\times 10^6$ CD34+)</td>
<td>MRI, acetate PET</td>
<td>34, sham, randomized, double blinded</td>
<td>No effect on LVEF</td>
<td>4 months</td>
</tr>
<tr>
<td>REPAIR-AMI</td>
<td>94 (3–6 days following AMI)</td>
<td>BMMC</td>
<td>Intracoronary</td>
<td>$2.4 \times 10^8$</td>
<td>MRI, LV angiography</td>
<td>93, sham, randomized</td>
<td>↑LVEF More significant benefit for cell infusion following more than 5 days</td>
<td>4 months</td>
</tr>
<tr>
<td>ASTAMI</td>
<td>50 (5–8 days following AMI)</td>
<td>BMMC</td>
<td>Intracoronary</td>
<td>$2.1 \times 10^8$</td>
<td>MRI, echocardiography, SPECT</td>
<td>50, sham, randomized</td>
<td>↓LVEF</td>
<td>6 months</td>
</tr>
<tr>
<td>Fernandez-Aviles et al.</td>
<td>20 (13 days following AMI)</td>
<td>BMMC</td>
<td>Intracoronary</td>
<td>$7.8 \times 10^7$</td>
<td>MRI, echocardiography, LV angiography</td>
<td>13, no sham, not randomized</td>
<td>↑LVEF LV systolic function in target areas (MRI)</td>
<td>11 months</td>
</tr>
<tr>
<td>Wollert et al.— BOOST Trial</td>
<td>30 (6 days following AMI)</td>
<td>BMMC</td>
<td>Intracoronary</td>
<td>$2.5 \times 10^9$ (9.5 $\times 10^6$ CD34+)</td>
<td>MRI</td>
<td>30, no sham, randomized, blinding not stated</td>
<td>No significant difference between BMMC and CPC</td>
<td>6 months</td>
</tr>
<tr>
<td>Chen et al.</td>
<td>34 (18 days following AMI)</td>
<td>MSC</td>
<td>Intracoronary</td>
<td>$8\times10^9$</td>
<td>PET, echocardiography, electromechanical mapping</td>
<td>35, sham, randomized, blinding not stated</td>
<td>↑LVEF</td>
<td>18 months</td>
</tr>
<tr>
<td>Schachinger et al., TOPCARE-AMI</td>
<td>20 (5 days following AMI)</td>
<td>BMMC (n = 29) CPC (n = 30)</td>
<td>Intracoronary</td>
<td>BMMC-2.1 $\times 10^8$ (5.5 $\times 10^6$ CD34+) CPC (1.6 $\times 10^7$)</td>
<td>MRI, PET, echocardiography, LV angiography</td>
<td>No control group</td>
<td>↑LVEF, ↑LVESV, ↓Infarct size</td>
<td>1 year</td>
</tr>
</tbody>
</table>

AMI, acute myocardial infarction; LVESV, LV end systolic volume; CPC, circulating progenitor cells; PET, positron emission tomography; SPECT, single photon emission computed tomography.

*Studies presented at the American Heart Association Conference 2005, and reported in Cleland et al.*
Table 3  Bone marrow-derived stem cell transplantation in CIHD

<table>
<thead>
<tr>
<th>Author/study name</th>
<th>Number of patients</th>
<th>Cell type</th>
<th>Indication</th>
<th>Cell delivery</th>
<th>Additional procedures</th>
<th>Cell dose</th>
<th>Endpoint assessment methodology</th>
<th>Control group</th>
<th>Results</th>
<th>Follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perin et al.³⁸</td>
<td>14</td>
<td>BMNC</td>
<td>End-stage IHD</td>
<td>Percutaneous trans endocardial + electromechanical mapping</td>
<td>None</td>
<td>$2.5 \times 10^7$ (6 x $10^5$ CD34⁺)</td>
<td>Echocardiography, SPECT, electromechanical mapping, LV angiography</td>
<td>7, no sham, non-randomized, open labelled</td>
<td>↑ LVEF ↓ Total reversible defect ↓ NYHA Class ↓ LVEDV, LVESV ↑ VO₂ Max ↑ CCS score ↑ Stress-induced ischaemia (target areas) No effect on LVEF</td>
<td>2 months (4 months for treated patients)</td>
</tr>
<tr>
<td>Fuchs et al.³⁷</td>
<td>10</td>
<td>BMNC</td>
<td>CCS III-IV with no conventional revascularization treatment option</td>
<td>Percutaneous trans endocardial + electromechanical mapping</td>
<td>None</td>
<td>$7.8 \times 10^7$ (2 x $10^6$ CD34⁺)</td>
<td>Echocardiography, SPECT</td>
<td>None</td>
<td>None</td>
<td>3 months</td>
</tr>
<tr>
<td>Patel et al.³⁵</td>
<td>10</td>
<td>BMNC</td>
<td>IHD and EF &lt; 35%</td>
<td>Direct intracmyocardial</td>
<td>Off pump CABG</td>
<td>–</td>
<td>Echocardiography, SPECT</td>
<td>10, no sham, randomized</td>
<td>↑ LVEF ↑ NYHA class ↑ Perfusion in target areas ↑ LVEF ↓ LVEDV ↑ Target wall thickening ↓ Hypoperfused myocardial areas No effect on LVEF</td>
<td>6 months</td>
</tr>
<tr>
<td>Stamm et al.³⁶</td>
<td>12</td>
<td>CD 133⁺</td>
<td>IHD</td>
<td>Direct intracmyocardial</td>
<td>Direct intracmyocardial</td>
<td>Off pump CABG</td>
<td>1.5 x $10^6$</td>
<td>Echocardiography, SPECT</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Tse et al.³⁹</td>
<td>8</td>
<td>BMNC</td>
<td>IHD</td>
<td>Percutaneous trans endocardial + electromechanical mapping</td>
<td>None</td>
<td>–</td>
<td>Echocardiography MRI</td>
<td>None</td>
<td>None</td>
<td>3 months</td>
</tr>
</tbody>
</table>

LVEDV, LV end diastolic volume; CPC-circulating progenitor cells; SPECT, single photon emission computed tomography; CCS, Canadian cardiovascular society score.
Mesenchymal stem cells

MSC represent another cell type suggested for cardiac repair. This potential multipotent stem cell is derived from the non-haematopoietic, stromal, compartment of the bone marrow. MSC were suggested by some (but not by the majority) studies to differentiate into cardiomyocytes both in vitro and in vivo. Transplantation of MSC into the infarcted myocardium of rats and pigs resulted in improved myocardial performance. One possible advantage of MSC is their ability to be delivered either in autologous procedures or using an allogeneic strategy as some reports suggest that they may be relatively immunoprivileged. These apparent attractive capabilities of the cells have recently led investigators to begin a number of clinical trials.

Cardiac resident progenitor cells

The long-held belief that the adult mammalian heart has no intrinsic capacity for repair has recently been brought into question when several groups have reported evidence that myocardium itself contains a resident progenitor cell population capable of giving rise to new cardiomyocytes. Initially, Beltrami et al. isolated and expanded c-kit positive cells from the adult rat heart. Engraftment of the cells to the acutely ischaemic myocardium resulted in cell differentiation to cardiomyocytes, smooth muscle cells, and vascular endothelium and in significant improvement on the ventricular function. A second progenitor population was isolated on the basis of sca-1 expression from the adult mouse heart. These cells were demonstrated to express cardiac-specific markers after treatment with 5-Azacytidine in vitro and following intravenous injection they homed to the infarcted mouse myocardium. Similar to side population cells reported in the bone marrow, Martin et al. isolated Hoechst dye effluxing cells expressing the ATP-binding cassette transporter, Abcg2. These cells have the ability to express the sarcomeric protein α-actinin after co-culturing with unfractionated cardiac cells. The most recent candidate progenitor population was isolated on the basis of the transcription factor islet-1. Collectively, these results suggested that the limited regenerative capacity of the adult heart, it is clear that the presence of the above mentioned cells within the heart does not translate to a functionally significant cardiac differentiation following myocardial infarction. In addition, the description of four non-overlapping, discrete, progenitor cell populations in a non-regenerating organ may stem from the immature nature of this developing area. Nonetheless, the roles of these cells in tissue maintenance, repair, and possible therapeutic applications will be an exciting area of investigation in the coming years.

Embryonic stem cells

Most of the cell types discussed above are stem cells and therefore share a number of properties. First, they are capable of self renewal, meaning that they can divide and give rise to stem cell progeny with similar properties. Second, the stem cells are clonogenic, meaning that each cell can form a colony in which all the cells are derived from this single cell and have identical genetic constitution. Third, they are capable of differentiation into one or more mature cell types. The different stem cells can be categorized anatomically, functionally, or by different cell surface markers, transcription factors, and the proteins they express. One clear division of the stem cell family is between those present in adult somatic tissue known as adult stem cells and those isolated from the embryo, known as ESC.

Although adult stem cells have been found to be more versatile than originally believed, they typically can differentiate to a relatively limited number of cell types. In contrast, cells in the early pre-implantation mammalian embryo have the potential to contribute to all adult tissues. At the blastocyst stage, a group of cells begins to separate from the outer cells and forms the inner cell mass (ICM). While the outer cells become the trophoectoderm, the ICM cells will ultimately give rise, through specialized progenitor cells, to all the tissues in the body and are therefore truly pluripotent. In 1981, the ICM cells, isolated from mouse blastocysts, were used to generate pluripotent stem cell lines that were termed ESC. The mouse ESC (mESC) were demonstrated to be capable of prolonged in vitro proliferation and self-renewal but also retained the ability to differentiate into derivatives of all three germ layers both in vitro and in vivo. Thus, following cultivation in suspension, the mESC tend to spontaneously create three-dimensional aggregates of differentiating tissue known as embryoid bodies (EBs). Among other cell types, cardiomyocyte tissue appears within this multicellular arrangement as spontaneously contracting areas that can be studied as a cluster or as dispersed cells.

The availability of the mESC system have provided important insights into the mechanisms underlying early-cardiac differentiation, development of excitability, calcium handling, and electromechanical coupling but also demonstrated the potential role of ESC for myocardial regeneration. By using genetically-selected mESC-derived cardiomyocytes, Klug et al. showed that these cells can form stable intracardiac grafts in the mouse heart. Later studies, utilizing the infarcted rat heart model, demonstrated that transplantation of differentiated mESC-derived cardiomyocytes can result in short- and long-term improvement of myocardial performance. Interestingly, whereas studies conducted by the Puceat’s group indicate that transplantation of undifferentiated mESC into the immunocompetent mouse or rat heart survive, integrate, and improve the myocardial function of the infarcted heart, other groups reported on the generation of teratomas and rejection of the cells when undifferentiated mESC were injected into non-syngeneic or immunotolerant animals. More recently, Menard et al. reported that cardiac committed mESC (following incubation with BMP-2), transplanted to the infarcted sheep heart, differentiated to...
mature cardiomyocytes and that cell transplantation resulted in a significant improvement in cardiac function independent of whether the sheep were immunosuppressed or not.

Given the outstanding potential demonstrated by the mESC, it was not surprising that much effort was spent on the development of similar hESC lines. This quest has ended in 1999 when two groups described the generation of hESC lines.27,28 The hESC were demonstrated to fulfill all the criteria defining ESC,27,28 namely: derivation from the pre- or peri- implantation embryo, prolonged undifferentiated proliferation under special conditions, and the capacity to form derivatives of all three germ layers.

More recently, we and other researchers56–58 were able to generate a reproducible cardiomyocyte differentiation system from the hESC. The induction of in vitro differentiation of hESC to cardiomyocytes requires their removal from the mouse embryonic fibroblast (MEF) feeder layer that allows their undifferentiated propagation, and cultivation in suspension where they form EBs.56 The EBs are then cultured in adherent conditions and develop spontaneously contracting activity in ~10% of the clusters.56

Several lines of evidence confirmed the molecular, structural, and functional cardiac phenotype of the generated myocytes.56–58 The generated myocytes expressed cardiac-specific transcription factors and cardiac-specific structural genes. Immunostaining studies confirmed the presence of cardiac-specific proteins and absence of skeletal muscle markers. Electron microscopy revealed varying degrees of myofibrillar organization and the presence of nascent intercalated discs, typical of an early-stage cardiac phenotype. These studies also depicted the progressive maturation from an irregular myofilament distribution to a more mature sarcomeric organization in late-stage EBs.59 Interestingly, in parallel to this ultrastructural maturation process, we could also observe a reproducible temporal pattern of early cardiomyocyte cell proliferation, cell-cycle withdrawal, and progressive ultrastructural maturation.59

The hESC-CMs were also shown to display functional properties, consistent with an early-stage cardiac phenotype.56,58,60,61 These include the presence of typical extracellular electrical activity and intracellular action potential and calcium transients. In addition, the hESC-CMs displayed appropriate chronotropic responses to adrenergic and cholinergic stimuli, demonstrating the properties of functional receptors and signalling pathways. Whole cell patch-clamp recording from the hESC-CMs demonstrated the presence of cardiac-specific action potentials and ionic currents. These studies also revealed important insights to the mechanism of automaticity, excitability, and repolarization in these developing cardiomyocytes.61 More recently, we have demonstrated, using a microelectrode array mapping technique, that this differentiating system is not limited to the generation of isolated cardiomyocytes but rather a functional syncytium is generated with stable spontaneous pacemaking activity and synchronous action-potential propagation.60 Immunostaining studies demonstrated that this functional syncytium results from the formation of gap junctions between the cells.

Optimal functional improvement following cell grafting would require structural, electrophysiological, and mechanical coupling of donor cells to the existing network of host cardiomyocytes. In a recent study, we tested the ability of the hESC-CMs to integrate structurally and functionally with host cardiac tissue both in vitro and in vivo.62 Initially, primary cultures were created from neonatal rat ventricular myocytes and the contracting EBs were added to the co-cultures. Interestingly, within 24 h post-grafting, we could detect synchronous contraction in the co-cultures that persisted for several weeks. Detailed electrophysiological mapping of the hybrid cultures demonstrated tight electrophysiological coupling between the two tissue types. These findings were reinforced by immunostaining data suggesting the development of gap junctions between the human and the rat cells.

To demonstrate the ability of the hESC-CMs to survive, function, and integrate also in the in vivo heart, we assessed their ability to pace the heart and to function as a "biological pacemaker".62 The hESC-CMs were transplanted to the posterolateral region of the left ventricle in a swine model of slow heart rate. Following cell grafting, a new ectopic ventricular rhythm was detected in 11 out of 13 animals studied, in six of which, it was characterized by sustained and long-term activity. Pathological studies validated the presence and integration of the grafted hESC-CM at the site of transplantation. Three-dimensional electrophysiological mapping revealed that this ectopic ventricular rhythm originated from the area of cell transplantation. Recently, our findings were reinforced in a study conducted by Xue et al.63 using a similar model of guinea pig heart block and demonstrating cell integration by optical mapping of the epicardial surface. More recent pathological studies demonstrated the ability of grafted hESC-CMs to survive and proliferate within the uninjured arrhythmic rat heart for as long as 4 weeks.64

From cell culture to bedside—challenges for clinical translation of hESC-CMs

Although hESC-CMs could theoretically have the potential to fulfill most of the properties of the ideal donor cell (they are the only cell source that can provide ex vivo an unlimited number of human cardiomyocytes), a number of critical obstacles are needed to be overcome prior to clinical application.11 (i) Strategies need to be developed for directing and augmenting hESC differentiation into the cardiac lineage. (ii) Purification of the differentiating cardiomyocyte population should be achieved. (iii) Upscaling of the culturing techniques is needed to yield clinically relevant number of cells for transplantation. (iv) Transplantation technique should be developed to enable proper alignment of the graft tissue, high seeding rate of the transplanted cells, and minimal damage to the host tissue. (v) Strategies aimed at preventing immunological rejection of the cells should be developed. (vi) Several regulatory issues should be addressed.
hESC cell line characterization and development of good manufacturing practice

Worldwide it is estimated that there are ~200 hESC cell lines. However, the ability of hESC to differentiate to cardiomyocytes effectively was shown in only a minority of these lines. For effective clinical translation of the enormous potential of hESC, characterization of a wide range of lines representing the diverse human genetic pool is required. Importantly, each cell line should also be evaluated for extended periods, ensuring no modifications occur during prolonged passaging. The essence of uniform characterization criteria is stressed in light of the recent studies reporting karyotype abnormalities in hESC.65

The hESC were derived in medium containing serum and were plated on top of MEF feeder layer. From the perspective of therapeutic applications, a xeno-free and serum-independent culturing system is essential for ensuring high reproducibility and minimizing the risk for xenozoonoses. Recently, supporting systems based on human tissues such as human foetal fibroblast, adult epithelial cells, or foreskin cells were recently suggested as alternatives to the MEF feeder cells.66 More recently, some of the key regulators mediating the ability of ESC to self renew have been described. These studies allowed the generation of a feeder-free cultivation system.67

Cardiomyocyte enrichment, purification, and upscaling strategies

Although cardiomyocytes can be reproducibly generated from the hESC using the EB differentiating system, these cells typically account for only a minority of the total population within the differentiating EBs. Consequentially, developing of strategies to augment cardiomyocyte differentiation and to select the generated cardiomyocytes may be crucial for the ultimate success of these cells in myocardial repair.

Directed differentiation

The currently hESC cardiac differentiation system is essentially spontaneous and thereby characterized by relatively low efficacy. Possible strategies for increasing cardiomyocyte yield may include the use of different growth factors, overexpression of cardiac transcription factors, co-culturing with feeder layers, and mechanical factors.68 The development of a more rational directed differentiation system is hampered, however, by the relative lack of data regarding the inductive cues that lead to commitment and terminal differentiation of human cardiomyocytes. Consequentially, strategies for directed differentiation should follow the elegant developmental studies conducted in a number of model organisms, most notably the chick, amphibians, zebrafish, and the mouse.69

Interestingly, initial studies suggest that the pathways found to play a role in the aforementioned developmental models may also govern the in vitro differentiation of the hESC to cardiomyocytes. Lev et al.,68 from our laboratory, examined the temporal gene expression pattern of signalling proteins and transcription factors governing early hESC cardiogenic differentiation and noted a similar cascade to that noted in vivo with an early expression of cardiac promoting factors (BMP-2, WNT11), followed by the expression of cardiac-specific transcription factors (Nkx2-5, GATA4, and Mef2C), and later the expression of cardiac-specific structural genes. Similarly, the well known cardiogenic inductive role of the primitive visceral endoderm in the developing embryo was also demonstrated to play a role in the cardiomyocytes differentiation of hESC line in an elegant study conducted by Mummery et al.58 Co-culturing of a hESC line (hES2), which does not regularly differentiate spontaneously to cardiomyocytes, with END-2 cells (a visceral endoderm-like cell line) provided the missing trigger for cardiac differentiation.

Selection and purification strategies

Although the development of directed differentiation systems is essential for increasing the cardiomyocyte yield from the hESC, it is unlikely that the degree of purity that will be achieved would be sufficient for clinical purposes. Because the contracting areas derived from hESC are comprised of a mixed population of cells, selection strategies are crucial not only for increasing cardiomyocyte numbers but also for preventing the presence of other cell derivatives as well as ensuring the absence of pluripotent stem cells carrying the risk of teratomas.

An elegant selection scheme to generate pure cardiomyocyte populations was suggested by Klug et al.10 in the mESC model. Undifferentiated mESC were transfected with a fusion gene encoding antibiotic resistance under the regulation of the cardiac-specific α-myosin heavy chain promoter. Differentiation was then induced and purified population of cardiomyocytes were selected based on their resistance to Neomycin. A slightly different approach was reported by Muller et al.70 using green fluorescent protein driven by the cytomegalovirus enhancer and a ventricle-specific promoter (MLC-2V). In conjunction with the use of percoll gradient centrifugation and subsequent FACS, this strategy yielded a relatively pure (97%) population of ventricular cells.

Scaling up

The LV of the human heart contains ~5.8 × 10⁹ cardiomyocytes. Given the fact that 25% of the cells are lost during a typical MI leading to heart failure, cell transplantation strategies aiming to completely regenerate the myocardium would require several hundred millions of cells. Generation of this number of cells can be achieved by increasing the number of hESC used for cardiomyocytes differentiation, by using directed differentiation systems and enrichment strategies, by increasing the ability of the cells to proliferate after cardiomyocyte induction,
and by upscaling the entire process. For example, Zandstra et al., using the mESC system, combined the aforementioned cardiomyocyte selection strategies with the bioreactor technology to generate more than 10^9 vital cardiomyocytes in a single 2 L bioreactor run.

**Achieving immune tolerance**

A major obstacle for the utilization of hESC derivatives in regeneration of different tissue types is the prevention of their immune rejection. Although initial *in vitro* and *in vivo* characterization of the immunogenicity of the hESC demonstrated that they display only minimal immunogenicity, they are still expected to be rejected. Several strategies aimed at achieving immunological tolerance are being explored. These strategies include establishing of ‘banks’ of major histocompatibility complex antigen-typed hESC lines, genetically altering the hESC to suppress the immune response (for example, by knocking-out the major histocompatibility complexes), the concept of haematopoietic chimerism, and possibly also by nuclear transfer techniques (therapeutic cloning).

**Summary and future potential research directions**

The derivation of the hESC lines and the resulting cardiomyocytes differentiation system may bring a unique value to several basic and applied research fields. Research based on the cells may help to elucidate the mechanisms involved in early human cardiac lineage commitment, differentiation, and maturation. Moreover, this research may promote the discovery of novel growth and transcriptional factors using gene trapping techniques, functional genomics, and proteomics as well as providing a novel *in vitro* model for drug development and testing. Finally, the ability to generate, *in vitro* for the first time, human cardiac tissue provides an exciting and promising cell source for the emerging discipline of regenerative medicine and myocardial repair. Yet, despite the enormous potential of these technologies, several hurdles need to be overcome before this strategy can become a clinical reality.

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**References**


