

Application of Stem Cells in Cardiology: Where we are and where we are Going

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Abstract: Heart disease including myocardial infarction and ischemia is associated with the irreversible loss of cardiomyocytes and vasculature, both *via* apoptosis or necrosis. However, the native capacity for the renewal and repair of myocardial tissue is inadequate as have been current therapeutic measures to prevent left ventricular remodeling. Cell transplantation has emerged as a potentially viable therapeutic approach to directly repopulate and repair the damaged myocardium. A detailed analysis and a vision for future progress in stem cell applications, both in research and clinical cardiology are presented in this review, highlighting the use of a wide spectrum of stem/progenitor cell types including embryonic or fetal stem cells, myoblasts, and adult bone marrow stem cells. An up-to-date comparison of donor cell-types used, and evaluation of the myocardial disorders that might be most amenable to stem cell therapy are discussed. The roles that myocardial cell fusion and transdifferentiation play in stem cell transplantation, the specific shortcomings of available technologies, and recommendations for practical ways that these concerns might be overcome, are also presented.

Keywords: Stem cells, differentiation, cardiomyocytes, heart disease, myocardial infarct, myocardial ischemia.

1. BACKGROUND

Recent progress in the field of stem cell research has confirmed their potential to be used in tissue regeneration. Heart disease, including myocardial infarction and ischemia is associated with the irreversible loss of cardiomyocytes and vasculature both *via* apoptosis and/or necrosis. The native capacity for renewal and repair of the cardiomyocyte is inadequate as have been the available therapeutic measures to prevent left ventricular remodeling. Cell transplantation, to directly repopulate these tissues represents a viable therapeutic approach for repairing the injured myocardium. However, in spite of remarkable progress in this field, significant problems remain, especially ethical problems and the tumorigenic and arrhythmogenic potential that these techniques present for differentiation into somatic cells. Moreover, uncertainty remains about whether the cells formed new tissue or whether they released compounds that fortified existing cells.

2. INTRODUCTION

Heart disease is an endemic health problem of great magnitude in the world. In spite of considerable clinical and research effort during the last decade and the development of new drugs and surgical modalities of therapy, the mortality and morbidity remain very high. Because the limited potential of the myocardium for self-repair and renewal, a significant proportion of cardiac muscle loses its ability to perform work and this loss may be the most important factor in the heart pump failure occurring in patients with coronary artery disease and dilated cardiomyopathy.

Until recently, reperfusion of the ischemic myocardium was the only intervention available to restore the various

cellular functions affected by myocardial ischemia, including preventing cell death by necrosis or apoptosis. Unfortunately, reperfusion may result in extensive myocardial damage, including myocardial stunning, and the functional recovery of the heart may appear only after a period of cardiac contractile dysfunction that may last for several hours or days. It is evident that the limited capacity of regeneration and proliferation of human cardiomyocytes can prevent neither the scar formation that follows myocardial infarction nor the loss of heart function occurring in patients with cardiomyopathy and heart failure. Replacement and regeneration of functional cardiac muscle is an important goal that could be achieved either by stimulation of autologous resident cardiomyocytes or by the transplantation of allogenic cells (e.g. embryonic stem cells, bone marrow mesenchymal cells or skeletal myoblast). However, a number of impediments to a successful implantation of these cells remain and they will be addressed in this manuscript.

3. EMBRYONIC STEM (ES) CELLS

The most primitive of all stem cells are the ES cells that develop as the inner cell mass in the human blastocyst at day 5 after fertilization. At this early stage, ES cells have vast developmental potential since they can give rise to cells of the three embryonic germ layers. When isolated and grown in the appropriate culture media, the pluripotent mouse and human ES cells can undergo cell proliferation and form embryo-like aggregates (termed embryoid bodies) *in vitro*, some of which can spontaneously contract (Fig. 1). The beating embryoid bodies contain a mixed population of newly differentiated cell types including cardiomyocytes, based on the expression of cardiac-specific genes such as cardiac-myosin heavy chain, cardiac troponin I and T, atrial natriuretic factor, and cardiac transcription factors GATA-4, Nkx2.5, and MEF-2, cellular ultrastructure, and extracellular

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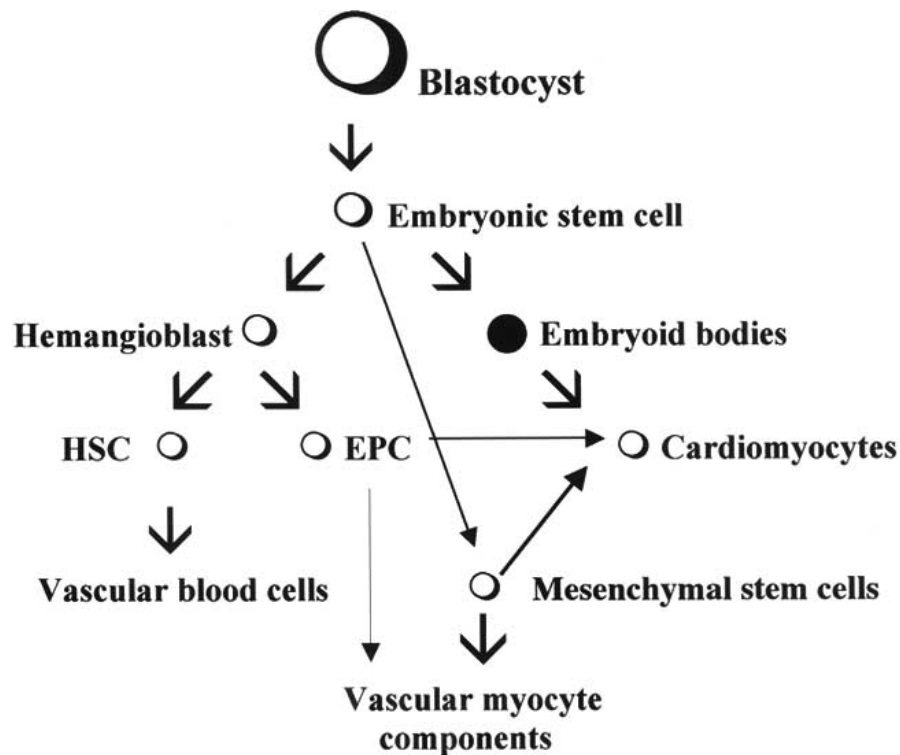


Fig. (1). Pluripotent embryonic stem cells spontaneously differentiate into endothelial progenitor cells (EPC), hemangioblasts, mesenchymal stem cells and embryoid bodies (embryo-like aggregates). Hemangioblasts further differentiate generating both hematopoietic stem cells (HSC) and EPC which give rise to both vascular blood and myocyte components. Under the appropriate conditions (most of which remain to be determined), cardiomyocytes can form from embryoid bodies as well as from EPC and mesenchymal stem cells.

electrical activity [1-3]. These cardiomyocytes can be of the pacemaker-atrium and ventricle-like type and they are distinguishable by their specific patterns of action potential [4-6].

While the precise cellular and molecular events comprising the pathway of ES cell cardiomyocyte-specific differentiation remain largely undetermined, significant progress has been made in identifying the regulatory factors which can enhance or inhibit the process (Fig. 2). Differentiation into a particular cell type is dependent on these factors. For instance, inhibition of bone morphogenetic protein (BMP) signaling by its antagonist Noggin induces cardiomyocyte differentiation from mouse ES cells [7], while retinoic acid specifically induces the formation of ventricular-specific cardiomyocytes [8]. Nitric oxide (NO), generated either by NO synthase activity or exogenous NO exposure has also been implicated in the promotion of cardiomyocyte-specific differentiation from mouse ES cells [9]. Cardiomyocyte differentiation of human ES cells could be enhanced by treatment with 5-aza-2'-deoxycytidine [10]. Also, IGF-1 promotes cardiomyocyte differentiation phenotype and the expression of the cardiomyocyte phenotype in ES cells *in vivo* [11]. Interestingly, increased levels of oxidative stress appear to reduce the cardiotypic development of embryoid bodies [12].

Early experiments with both fetal cardiomyocyte and differentiating ES cell transplantation reported successful formation of stable grafts and nascent intercalated discs between the grafted and the host myocardial cells [13-14]. In

addition, both fetal and embryonic stem cell-generated cardiomyocytes maintain myocardial electromechanical properties. Human ES cell-derived cardiomyocytes are able to effectively form structural and electromechanical connections with cultured rat cardiomyocytes [3]. Similarly, the transplanted human ES cell-derived cardiomyocytes were able to integrate and pace *in vivo* the swine heart with complete atrioventricular block, as demonstrated by detailed electrophysiological mapping and histopathological studies. The similarity in phenotype between the transplanted differentiating-ES cells and harder-to come-by (particularly in humans) fetal cardiomyocytes suggested that ES cells could be a useful surrogate for fetal cardiomyocytes in human cardiac engraftment procedures [14].

When fetal rat cardiomyocytes were transplanted into ischemic damaged hearts, a large percentage of cardiomyocytes die posttransplantation [15]. This, as well as the finding that no increase in graft size occurred while using increasing number of injected cardiomyocytes has prompted a re-consideration of the clinical use of cardiomyocyte transplantation in the treatment of ischemic heart disease. It is apparent that more research is needed to develop a successful strategy that can maximize grafted cardiomyocyte cell survival and accelerate the differentiation process.

3.1. Advantages to ES Cell Transplantation

ES cells can be readily and reproducibly obtained from the inner layer of the blastocyst, and exhibit an excellent

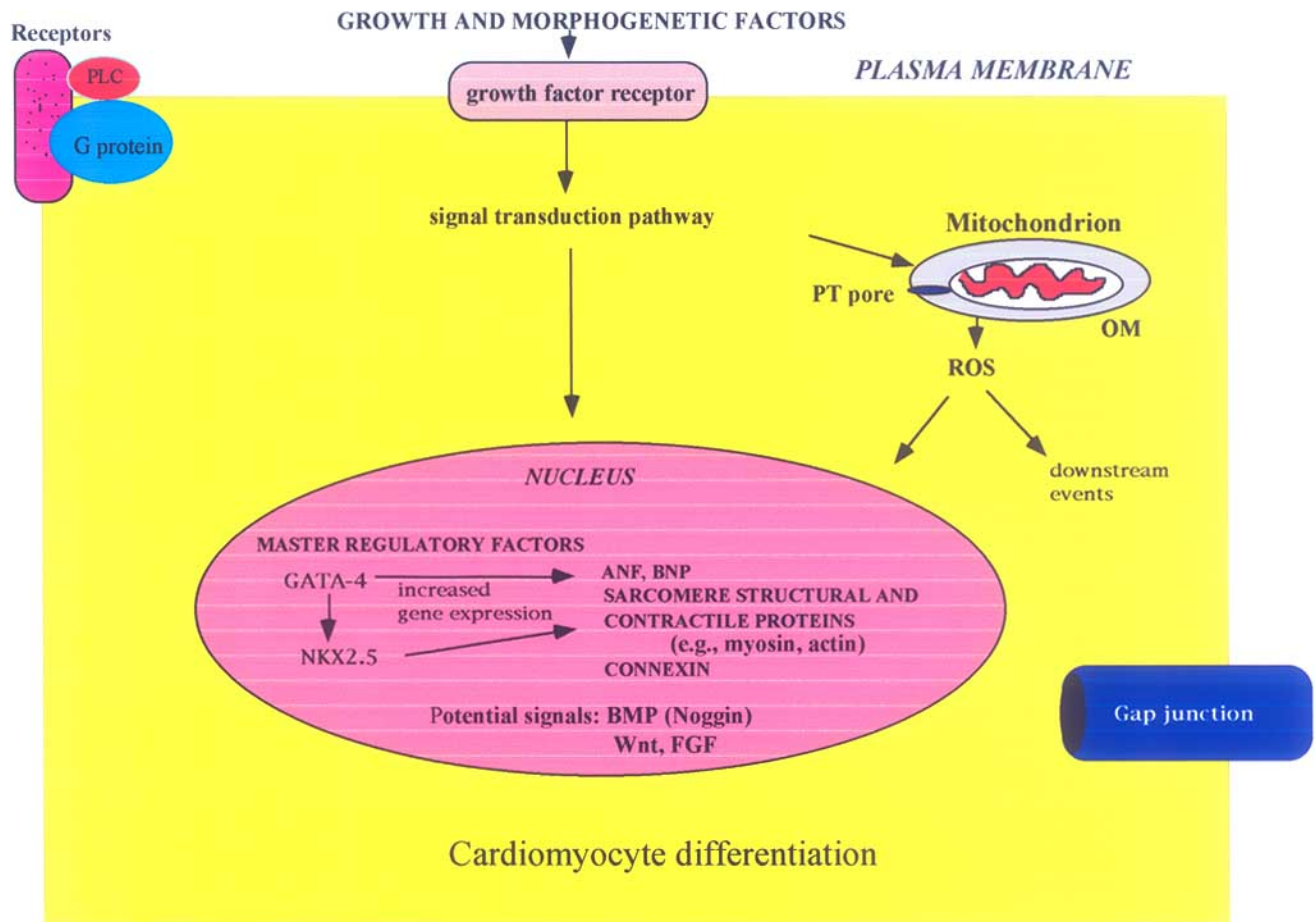


Fig. (2). Signaling pathways potentially involved in cardiomyocyte differentiation.

BMP, bone morphogenetic protein; Wnt, amalgam of wingless (Wg) and int (integration loci); FGF, fibroblast growth factor; OM, outer membrane; permeability transition (PT) pore opening; PLC, phospholipase C.

growth phenotype, both *in vitro* and *in vivo*. The development and application of ES-like cell lines (e.g. P19), which have been highly informative in the identification and characterization of regulatory factors, transcriptional activators and signal transduction events involved in cardiomyocyte differentiation, may also be useful in cell transplant therapy [17-19].

Preliminary data suggest that ES cells may be of a particular value in targeting and modifying congenital heart defect phenotypes [20-21]. Once their safety is confirmed, further clinical studies should address the use of targeted ES cell therapy in infants/children with severe cardiac diseases including cardiomyopathies, congenital heart defects and arrhythmias.

ES cells may also be more amenable to *ex vivo* engineering *via* DNA modifications (e.g. gene therapy, viral transfection, knockouts and over-expressed genes). In fact, the transformation of a normal cardiomyocyte in a pacemaker cell has been successfully achieved in animal models by the injection of plasmid or viral vectors carrying genes encoding specific therapeutic proteins [22-24]. In this manner, ES cells transfected with overexpressed β_2 -adrenergic receptors, or ion channel proteins could be transplanted to restore function in defective myocardial cells [24]. However, the safety and efficacy of these methodologies need to be

absolutely proven prior to their use in humans with cardiac arrhythmias.

3.2. Limitations and Concerns with ESC Transplantation

Considerable ethical and legal concerns about ES cells remain, and these concerns have significantly hampered further research efforts, which could provide needed cell lines as well as answers to many of the questions regarding the efficacy, long-term stability, function and even the extent of the negative effects of ES cell transplantation in cardiovascular disease (as well as in other human diseases).

A concern often raised regarding the use of ES cells relates to their source (i.e. whether they originate from a cell-line or directly from embryo), primarily heterologous versus autologous, posing the potential problem of generating an allogenic response or immunorejection upon transplantation. In addition, pluripotent ES cells which have unlimited growth potential can have tumorigenic side effects, making the screening for teratoma formation well-advised. Moreover, there is evidence that differentiation of a heterogeneous ES cell population is rather inefficient, although several agents (e.g. retinoic acid) appear to be effective in activating a greater extent of ES cell-mediated cardiomyocyte specific

differentiation. The long-term stability of ES cell-differentiated phenotype has also received mixed reviews since several studies have shown a loss of ES cell-differentiated cardiomyocytes over time.

Transplanted ES cell progeny may not always have a normal function since ES cells may promote arrhythmias in the transplanted hearts. On the other hand, the application of ES cells in repairing the damaged aging heart may also be limited; however, while this limitation has been proposed, currently there is not solid data to support it. Nevertheless, cell transplants (either ES or adult stem cells) in hearts of older individuals have frequently proved to be less effective. The inability of the damaged myocardium to provide the appropriate molecular signals for stem cells engraftment seems to limit their capacity for recruitment and integration into the aging myocardium [16].

3.3. Recommendations

- 1) The therapeutic use of ES cell transplantation in cardiac diseases primarily needs a rigorous demonstration that it can work in a stable fashion and with limited adverse effects.
- 2) Despite the limitations on federally-funded research presently imposed, new sources of ES cells and cell-lines for ES cell transplantation studies need to be developed and likely will be, given the strong worldwide, corporate and state-funded interest in this technology and its purported benefits. Investigation into novel ways to isolate and culture autologous ES cells should also prove to be of significance.
- 3) Our overall understanding of the factors that may elicit the homing of ES cells to the heart and stimulate or direct the differentiation of ES cells to functional cardiomyocytes is presently rudimentary (a critique also applicable to adult stem cells). Identification of these factors as well as their mechanism of action will likely optimize both the homing and the differentiation processes as well as contribute to defining the best case scenarios in which ES cell transplantation will be beneficial.

4. ADULT SKELETAL MYOBLAST CELLS

Transplanted satellite stem cells (myoblasts) from skeletal muscle can successfully home and engraft within a damaged myocardium, preventing progressive ventricular dilatation and improving cardiac function [25-27]. These myoblasts can be delivered into the myocardium by either intramural implantation or arterial delivery [28-29] and recently effective deployment of a less invasive catheter approach has been reported [30]. Skeletal muscle satellite cells can proliferate abundantly in culture, and can be easily grown from the patient themselves (self-derived or autologous) thereby avoiding potential immune response. Myoblasts are relatively ischemia-resistant (compared to cardiomyocytes which become injured within 20 minutes) since they can withstand several hours of severe ischemia without becoming irreversibly injured [31]. The functional benefits of intramyocardially transplanted skeletal myoblasts in improving the damaged myocardium secondary to

ischemia have been well documented [32]. Initial clinical trials have shown the efficacy of autologous skeletal myoblast transplantation in patients with left ventricular (LV) dysfunction [27,33]. The use of skeletal myoblasts, delivered by multiple intramyocardial injections, was effective in restoring left ventricular function in the genetically determined Syrian hamster model of dilated cardiomyopathy, demonstrating that the functional benefits of transplanted skeletal myoblast can be extended to non-ischemic cardiomyopathy [34].

4.1. Advantages to Myoblast Transplantation

Since myoblasts can be of autologous origin and can be robustly expanded in culture, a large number of cells can be obtained from only a small skeletal muscle biopsy sample (such as obtained from a patient) in a relatively short period of time. Compared to transplanted cardiomyocytes, myoblast cells appear to be more resistant to ongoing apoptotic damage, which tends to be prevalent at ischemic sites.

4.2. Limitations and Concerns with Myoblast Transplantation

While several reports have suggested that a subpopulation of transplanted skeletal myoblasts were capable of transdifferentiation to a cardiomyocyte-phenotype with increased expression of cardiac genes [35-36], others have been unable to replicate the transdifferentiation of donor myoblasts to cardiomyocytes [37]. The present consensus of the majority of the researchers in this field, is that grafted myoblast cells primarily remain non-cardiomyocytes. On the other hand, there is evidence that when myoblasts are implanted in the heart, their developmental program is influenced in such a way by the cardiac environment that it enables them to improve cardiac performance. Skeletal myoblasts engrafted to an injured myocardium differentiated to a fatigue-resistant, slow twitch phenotype adapted to cardiac workload [38]. Moreover, grafted myoblasts may display incompatible "wiring" or cell-to-cell connections with resident cardiomyocytes and do not respond in the same way to electrical signaling and stimuli [39]. While early preclinical studies did not detect evidence of arrhythmias, recent clinical studies have revealed that a subset of patients receiving skeletal myoblast transplant can experience severe and often life-threatening arrhythmias [40]. The precise reason for these arrhythmias remains unclear but may be related to the heterogeneous electrical properties and interactions between donor and recipient cells. On the other hand, the arrhythmias may be promoted by the medium used to introduce the cells, rather than by the cells themselves [41]. Parenthetically, the functional benefits of myoblast transplantation may be related to the limitation of adverse post-infarction remodeling and/or the paracrine effects of transplanted myoblasts on recipient tissue, rather than to a grafted-myoblast contribution to enhance ventricular systolic function.

4.3. Further Recommendations

While preclinical studies with stem cell and myoblast transplantation have shown similar levels of efficacy [42-43],

there is a need for a detailed evaluation on the relative benefits, adverse effects and efficiency of skeletal myoblast and stem cell transplants in the clinical setting (e.g., heart failure) *vis a vis* the restoration of myocardial function. New methods to better assess and optimize post-transplanted myoblast recruitment and survival, particularly in the long-term, need to be developed and the repertoire of effective, less invasive cell delivery technologies needs to be expanded.

5. ADULT BONE MARROW DERIVED CELLS (BMCS) STEM CELLS

Interest in bone marrow derived stem cells has been mainly motivated by their neovascularization and angiogenesis properties and these effects are enhanced by the presence of specific growth factors and cytokines (e.g. G-CSF). The beneficial effects of these cells to a damaged vascular system was confirmed and subsequently extended to studies on myocardial damage in mice [44] in whom implanted BM cells can differentiate into myocytes and coronary vessels and thereby ameliorate the function of the injured heart. Since implantation of BMCs initially required surgical intervention and the procedure is often accompanied by a high mortality rate, with only a 40% of successful grafting, the development of noninvasive method became imperative. One such approach employed cytokine treatment, stem cell factor (SCF) and granulocyte-colony-stimulating factor (G-CSF), to mobilize endogenous BMCs and direct their integration or homing to the infarcted heart promoting repair. Mice injected with SCF (200 µg/kg/day) and G-CSF (50 µg/kg/day) exhibited a substantial increase in the number of circulating stem cells from 29 in nontreated controls to 7,200 in cytokine-treated mice. The endogenous BMCs were shown to give rise to new cardiac myocytes and coronary vasculature, and the BMC-derived myocardial regeneration resulted in improved cardiac function and survival. Similar findings of cell-mediated repair of myocardial infarction in the mouse were obtained using transplanted BMCs which promoted proliferating myocytes and vascular structure [45].

It is important to point out that bone marrow contains several stem cell populations with overlapping phenotypes, including hematopoietic stem cells (HSCs), endothelial stem/precursor cells (EPCs), mesenchymal stem cells (MSCs), and multipotent adult progenitor cells (MAPCs). When endothelial progenitor cells (EPCs), originating from a common hemangioblast precursor in bone marrow, are delivered to the myocardial target area they may implant, differentiate *in situ* and promote new vessel growth, an approach that has been applied to several animal models of myocardial ischemia [46]. These bone-marrow derived stem/precursor cells also can prevent the progression of cardiomyocyte apoptosis and stem cardiac remodeling [47]. Moreover, there is evidence that adult EPCs can transdifferentiate into active cardiomyocytes [48], although how extensively this occurs is presently unknown. On the other hand, bone marrow-derived mesenchymal stem cells exhibit a high degree of plasticity allowing them to be employed as a self-renewing autologous source of progenitor cells (from adults), with the potential for differentiating into cardiomyocytes and can be used in cellular cardiomyoplasty.

Upon treatment with specific agents (e.g. 5-azacytidine), MSCs can differentiate into synchronously beating cardiomyocytes [49]. The injection of MSCs after their expansion in culture can also be used in the rescue of an abnormal mouse cardiac phenotype [50] and may prove effective in repairing a broader array of cardiac damage including myocardial infarct. In addition, bone marrow-derived HSCs and a subpopulation of HSC cells termed SP cells have been reported upon transplantation to repair infarcted myocardium, promoting new growth of cardiomyocytes, endothelial and smooth muscle cells [51]. While this cell-mediated myocardial repair was initially characterized as resulting from HSC's ability to transdifferentiate to cardiomyocytes, HSC plasticity has been difficult to reproduce and both its significance and basis remain undetermined.

5.1. Advantages of Adult BM Cells Transplantation

There is evidence that treatment with BMCs can ameliorate both myocardial and vascular damage with increasing angiogenesis. The effect of transplanted BMCs (which can include endothelial precursor cells) on vascular growth may significantly impact the recovery of the damaged heart, i.e. by improving oxygen availability, although this may depend on the myocardial setting whether acute myocardial infarction or established heart failure [47]. Moreover, autologously derived cells for transplantation are an attractive alternative, since bone marrow mesenchymal cells can be readily isolated in most cases. In addition, the expansion of BMC number by *in vitro* growth can be readily achieved by vigorous growth of mesenchymal cells in culture. It is significant that this method bypasses much of the ethical and legal maelstrom associated with the use of ESCs.

5.2. Limitations/Concerns with Adult BMC Transplant

The mechanism of BMC-mediated augmentation of cardiomyocyte number and function remains controversial. Some studies have suggested that the effects of adult stem cell transplantation on the recipient heart are not a consequence of transdifferentiation [52], but likely arise as a result of cell fusion with pre-existing cardiomyocytes or occur as a function of paracrine effects of transfected cells [53] while others maintain that there is evidence for a transdifferentiation event [46, 54-57]. Cell fusion has been demonstrated between cardiomyocytes and non-cardiomyocytes *in vivo* and *in vitro* [58-59] and the data in support of transdifferentiation (particularly with HSCs) have not always been replicable. Further research is needed to clarify these issues and reconcile the contradictory claims as well as provide additional information about the extent of cell fusion and when it occurs. Similarly, a careful delineation of transdifferentiation from a well-defined adult stem cell type is warranted. Unfortunately, a critical problem in the replication of these experiments and in determining the effects of BMCs lies in the considerable heterogeneity of the populations of BMCs used.

A limitation of the majority of the clinical studies with adult non-cardiac stem cell transplantation relates to the potential stability of the differentiated phenotype, since these

studies have primarily examined the short-term benefits. However, it is important to underline the absence of adverse events in over 100 patients studied. This is in contrast with the arrhythmia-prone myoblast transplantation [40]. Because the paucity of successful techniques to effectively treat heart failure, there is mounting pressure (mainly from clinicians) to expedite the clinical application of cells transplant even before the mechanisms (as well as the long-term effects) are fully understood.

5.3. How Does the Preceding Information Translate to Human Clinical Studies?

Since most BMC research is presently performed in mice, a critical question is whether this model is truly applicable to humans. Preliminary studies in humans suggest that BMC transplantation and cytokines can home into areas of injury and promote neovascularization in those areas where they are needed. Whether enough BMCs can be transplanted to repair the damaged regions in the human heart which tend to be larger in size than in the mouse heart remains to be seen. Moreover, it is questionable if adult human BM stem cell therapy works against any of the following: apoptotic cell death, ischemic injury, cardiomyopathy, the cardiomyopathy of aging, cardiac conduction/arrhythmia defects and cardiac defects in infant/children.

Preliminary results of human clinical trials have shown a modest improvement in the cardiac function of patients with acute myocardial ischemia and infarct [60-62]. When transplantation was applied to patients with chronic myocardial disease or damage secondary to myocardial infarction the results were less definitive.

6. ADULT CARDIAC STEM (ACS) CELLS

The current information available on various stem cell populations in the adult heart has emerged from research in several laboratories. However, many questions remain concerning the origin, structure, precise location, function and regulation of these cells.

The existence of Lin⁻ c-kit⁺ cells in adult rat myocardium with the properties of stem cells has been reported [63]. These cells are self-renewing and can be propagated for several months, expandable in culture, and multipotent, and can give rise to cardiomyocytes, smooth muscle, and endothelial cells. When injected into an ischemic heart, the Lin⁻ c-kit⁺ cells contribute to the formation of endothelium and vascular smooth muscle and to the regeneration of myocardium in the region of necrosis, improving its pump function and ventricular chamber geometry [64].

The isolation and characterization of a small population of adult heart-derived cardiac progenitor cells (from post-natal mouse myocardium) expressing the surface marker stem cell antigen-1 (Sca-1⁺) and telomerase reverse transcriptase activity, associated with self-renewal potential, have been also recently reported [65-66]. These ACS cells can be selectively isolated by a magnetic cell sorting system and express neither cardiac structural genes nor Nkx2.5. These cells can differentiate *in vitro* forming beating

cardiomyocytes, in response to the DNA demethylating agent 5'-azacytidine. Increased expression of other cardiogenic transcription factors (GATA-4, MEF-2C) was shown by microarray profiling of differentiating ACS cells as previously found in bone marrow stromal cells with cardiogenic potential. Similarly, when treated with oxytocin, the cardiac Sca-1⁺ stem cells expressed genes of cardiac transcription factors and contractile proteins and exhibited sarcomeric structure and spontaneous beating. [67]. After intravenous delivery, the Sca-1⁺ cardiac stem cells can home to myocardium injured by ischemia/reperfusion and can functionally differentiate *in situ*.

Laugwitz and associates [68] recently reported the presence of a population of cardioblasts in both embryonic and postnatal heart (from mouse, rat and human) numbering just a few hundred per heart identified on the basis of their expression of a LIM-homeodomain transcription factor, Isl1. This group of cardiac stem cells was primarily localized in the atria, right ventricle, and outflow tract regions (where Isl1 is most prevalently expressed during cardiac organogenesis). These myocardial derived stem cells can be isolated, transplanted, survive and replicate in the damaged heart with evidence of functional improvement [69].

6.1. Advantages of ACS Cells

While the implantation of skeletal myoblasts and adult BMC transplantation appears promising, ACS cell transplantation might be more effective than adult BMC transplantation, since cardiac stem cells may be better programmed. The further identification, purification and characterization of the ACS cells as well as a detailed knowledge of their interactions with the cardiac milieu or niche are essential if we are to achieve the major goal of regenerating/transplanting the tissue to treat myocardial infarction.

6.2. Limitations of ACS Cells

Until recently, data on the presence of ACS cells has been scarce. This subset of stem cells appears to be extremely limited in number, difficult to identify and expand in culture thereby limiting their characterization and utilization, likely contributing to difficulties in reproducing experiments concerning their isolation and transplantation. In addition, there is presently no consensus in the definition of selective markers specific for this cell-type (see Table 1).

6.3. Recommendations

Considerable work remains to fully delineate the relevant cardiac progenitor cell population and in the optimization of conditions for their efficient transplantation, homing, differentiation and integration into the myocardium. Understanding the factors that are responsible for growth, homing, and differentiation may allow specific ways to improve their production and functional benefits upon transplantation. Moreover, this information may also shed light on the activation of endogenous cardiac stem cells contributing to cardiac repair. Also, to be defined are the kinds of cardiac defects as well as type of injuries that can be

best treated with these cells, including a clear knowledge of the best place in the heart to deliver or direct these cells. For instance, implanting cells within an area of necrosis and/or low oxygen availability may be unsuccessful whereas implanting cells in regions of hibernating myocardium may be successful.

The long-term stability and functionality of transplanted ACS cells wait to be defined. Whether ACS cells can be used as a platform for *ex vivo* gene modification, including the introduction of therapeutic genes, whether robust expression of specific genes can be directed in such cells, and if an increased proliferative response in the cardiac progenitor cells can be modulated by the introduction of cell-cycle progression genes remain to be seen.

7. DELINEATION OF CELL IDENTITY

From the foregoing discussion, it should be evident that a critical element in the identification of the grafted cell in the heart and in a number of cases even prior to the transplant, is the unequivocal assignment of cell-type identity. In Table 1, we provide a list of endogenous molecular markers that have been used to establish a differentiated cardiac phenotype resulting from transplanting different stem cell types, including bone marrow cells, embryonic stem cells and cardiac-derived stem cells. In addition to the endogenous markers available to establish cell identity, GFP has been extensively used as a reporter to define donor cells. Marking cells with the chromosome stain DAPI has been unsuccessful, since the DAPI stain from dead cells can be readily incorporated by non-marked cells [75].

Genotype marking has also been shown to be a powerful tool in assessing cell identity. In several studies of cardiovascular self-repair in which female hearts were allografted into human male recipients, the presence of the Y chromosome was assessed in the coronary vasculature and in cardiomyocytes [76-79] since the Y chromosome can be easily viewed by cytochemical staining or by fluorescence *in*

situ hybridization. However, the assessment of the degree of cardiac chimerism reported in these studies reveals striking variation ranging from very low level of Y-chromosome containing cardiomyocytes (0.02-01%) [77-78] to high levels (30%) [79], underscoring the critical need for establishing rigorous criteria by which chimerism is identified. The identification of a nucleus with a Y-chromosome is in itself not sufficient, but needs to be unequivocally associated with either myocardial vessels or cardiomyocyte structure (i.e. by confocal microscopy). Otherwise, it is possible to attribute the Y chromosome-positive nuclei to host cells involved in immune response and inflammatory infiltration, and not to cardiac regeneration. There is also some indication that the use of chromosomal analysis can result in the underestimation of the transfected cells due to the presence of nuclei that may not be counted when in the histological section [46].

The detection of cell phenotype markers by real-time assays, confocal microscopy and non-invasive detection methodologies employing magnetic resonance imaging (MRI) has just begun to be applied in the assessment of cell transplant. Real-time visualization can provide identification of regions of myocardial infarction and precise MRI-guided delivery of therapeutic agents, with injection sites identified by contrast agents. Novel contrast agents permit MRI visualization of gene expression at a cellular resolution, and can be used as well to detect apoptotic cells [80-81]. The appropriate labeling and detection of stem cells by MRI should enable the tracing of their *in vivo* distribution, and allow a glimpse of their destiny over time [82-83].

8. WHICH STEM CELL-TYPE TO USE FOR CARDIAC DISEASE?

A brief comparison of the advantages and limitations of the cell types presently used in cardiac transplantation is shown in Table 2. While no clear-cut choice has yet emerged as to which cell type is best to transplant in myocardial repair, there are reasons to believe that the development of a

Table 1. Markers of Stem Cell-Derived Cardiomyocyte Differentiation

Cell type	Differentiation Agent	Markers of Differentiated Cardiomyocyte	Refs
ES cells			
Embryonic stem cells	IGF-1, TGF-	-sarcomeric actin, connexin 43, major histocompatibility complex class I, sarcomeric myosin	11,70
P19 embryonal carcinoma line	5'-azacytidine	bone morphogenetic protein-2 (BMP-2), BMP-4, Bmpr 1a, Smad1, GATA-4, Nkx2.5, cardiac troponin I, desmin	71
BMC			
Bone marrow (MSC)	insulin, ascorbic acid, dexamethasone	-skeletal actin, -myosin heavy chain (MHC), MLC-2v, CaV1.2, cardiac troponin I, sarcomeric tropomyosin, cardiac titin	72, 53
Cardiac Stem Cells			
CKIT+Lin-	na	c-kit+	63
Isl1+	na	Csx/Nkx-2.5, GATA4	68
Sca-1+ cKit-	5'-azacytidine oxytocin	High telomerase activity, Sca-1+ Csx/Nkx-2.5, GATA4, MEF-2C, + -MHC, MLC-2, Cardiac- actin	65-67
cardiosphere	CKit+	Cardiac troponin I, myosin heavy chain, atrial natriuretic peptide	69
SP cells	na	ATP-binding cassette transporter (ABCG2)	73-74

na = not available

Table 2. Myocardial Transplants: Advantages and Limitations Associated with Cell-Type

CELL-TYPE	SOURCE	ADVANTAGES	LIMITATIONS
CARDIAC STEM CELLS	Allogenic fetal, neonatal or adult heart	<ol style="list-style-type: none"> 1. Recognition of myocardial growth factors and recruitment to myocardium are likely faster and more efficient than other cell-types 2. <i>In vivo</i> electrical coupling of transplanted cells to existing myocardium has been demonstrated 	<ol style="list-style-type: none"> 1. Poor cell growth <i>in vitro</i> 2. Transplanted cells are very sensitive to ischemic insult and apoptotic cell death 3. Availability from either fetal (F), neonatal (N) or adult sources is low at present; likely immune rejection; F and N cells pose ethical difficulties;
SKELETAL MYOBLAST	Autologous skeletal muscle biopsy	<ol style="list-style-type: none"> 1. Cells proliferate <i>in vitro</i> (allowing for autologous transplant) 2. Ischemia-resistant 3. Transplanted myoblasts can differentiate into slow-twitch myocytes (similar to cardiomyocytes) enabling cellular cardiomyoplasty. 4. Reduces progressive ventricular dilatation and improves cardiac function 5. Can use adult cells 	<ol style="list-style-type: none"> 1. Likely do not develop new cardiomyocytes <i>in vivo</i>. 2. Electrical coupling to surrounding myocardial cells is unclear (may cause arrhythmias) 3. Long-term stability of differentiated phenotype unknown
ADULT BONE-MARROW STEM CELLS	Autologous bone marrow stromal cells (mesenchymal); Bone marrow (endothelial progenitor cells)	<ol style="list-style-type: none"> 1. Pluripotent stem cells can develop into cardiomyocytes 2. Stem cells are easy to isolate and grow well in culture 3. Neovascularization can occur at site of myocardial scar reducing ischemia 4. Transdifferentiation of cells into cardiomyocyte <i>in vivo</i> has been shown 5. Can be derived from autologous source; no immune-suppression treatment 6. Can improve myocardial contractile function 	<ol style="list-style-type: none"> 1. New program of cell differentiation is required 2. Efficiency of the differentiation into adult cardiomyocytes appears limited 3. Signaling, stability and regulation of differentiation unknown
EMBRYONIC STEM CELLS	Allogenic blastocyst (inner mass)	<ol style="list-style-type: none"> 1. Easy propagation and well-defined cardiomyocyte differentiation process 2. <i>In vivo</i> electrical coupling of transplanted cells to existing myocardial cells. 3. Pluripotent cells 	<ol style="list-style-type: none"> 1. Potential for tumor formation and immune rejection (allogenic) 2. Incomplete response to physiological stimuli 3. Legal and ethical issues 4. Donor availability

multiplicity of approaches in the application of cell engineering will be required to develop novel therapies for different cardiac disorders. The approach to treat heart failure may require the transplantation of cell-types (e.g., skeletal myoblasts) that are different than those used in the targeted treatment of cardiac arrhythmias, conduction disorders and congenital defects. It is also possible that the long-term repair of a fully functioning myocardium, may require more than a single cell type—for instance, cardiomyocytes, fibroblasts, and endothelial cells—in the generation and integration of a stable and responsive cardiac graft.

9. OTHER DEVELOPING TECHNOLOGIES IN CELL ENGINEERING

The refinement of nuclear transfer, cybrid and cell fusion techniques may allow further engineering of stem cells to provide cardioprotection, or stimulate antioxidant or antiapoptotic responses in the myocardium. These cell-engineering techniques might also allow the specific targeting of mitochondrial-based cytopathies [84].

To identify aspects of the cardiac milieu that may contribute to the growth and development of transplanted myoblasts *in vivo*, 3-dimensional matrices have been designed to serve as a novel *in vitro* system that mimic some aspects of the electrical and biochemical environment of the native myocardium. These structures may allow a

finer resolution of electrical and biochemical signals that may be involved in myoblast proliferation and plasticity. Myoblasts have been grown on 3-D polyglycolic acid mesh scaffolds under control conditions in the presence of cardiac-like electrical current fluxes, and in the presence of culture medium that had been conditioned by mature cardiomyocytes [85]. Such scaffolds containing either fetal or neonatal aggregates of contracting cardiac cells have been used to generate artificial cardiac grafts transplanted into injured myocardium with recuperation of ventricular function, and formation of functional gap junctions between the grafted cells and the myocardium [86-87].

The combination of gene therapy and stem cell engineering is an attractive approach for treating cardiac disorders. Overexpression (and in some cases the inhibition of expression) of specific proteins can result in striking changes in cardiomyocyte and in cardiac phenotype. Specific cardiomyocyte functions, including ion channel, cardiac conduction, contractility and myocyte proliferation have been shown to be effected by the gene transfer and expression of specific proteins [88-90]. Cell-based therapies for injured or dysfunctional hearts can be enhanced by the use of *ex vivo* genetically modified stem cells to deliver genes and proteins. For instance, transplanted mesenchymal stem cells have been shown to be effective devices to deliver channel proteins involved in pacemaking activity (e.g., channel protein HCN2) resulting in the modification of cardiac rhythm *in vivo* [91]. In an animal model of ischemic

cardiomyopathy, the introduction of the vascular endothelial growth factor (VEGF) and its effect on both angiogenesis and left ventricular function was markedly enhanced in hearts with VEGF-transfected skeletal myoblasts in comparison to hearts directly injected with the adenoviral-VEGF construct [92].

10. CLOSING COMMENTS

The discovery of cardiogenesis in adult animals and human represent one of the most significant advances in cardiology in the last 25 years. Previously, most cardiologists believed that the birth of new cardiomyocytes was only confined to the fetal and neonatal heart. This dogma recently collapsed when researchers discovered that the heart of adult rat, mice and human undergo significant cardiac changes as a function of age. New cardiomyocytes were born/homing-in into myocardial areas relevant to cardiac pathways, and then can integrate structurally so that myocardial function can be restored and new tissue can be produced. These findings have set off a large number of parallel discoveries in rats, mice, and humans, with dramatic implications for how we think about cardiac plasticity and its potential role in rehabilitating individuals with acquired myocardial ischemia/infarct, heart failure and different types of cardiomyopathies, including the cardiomyopathy of aging.

In summary, our increasing capacity to understand the function of different cardiomyocytes/cardiac differentiation pathways in detail will eventually allow the replacement of tissue, the transplant of others, and shift imbalances on the molecular and the biochemistry of the heart. With the beginning and eventually rapid progress in cell engineering, we expect to see the end of cardiac abnormalities that weaken human life and bankrupt the health care system.

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