Cellular Therapy for Cardiovascular Disease Part 1 – Preclinical Insights

Peter J Psaltis[1], Stan Gronthos[2], Stephen G Worthley[1] and Andrew C.W. Zannettino[2]

[1]Cardiovascular Research Centre, Royal Adelaide Hospital; Department of Medicine, University of Adelaide, South Australia, 5000. [2]Division of Haematology, Institute of Medical and Veterinary Science; Department of Medicine, University of Adelaide, South Australia, 5000.

Introduction

Coronary artery disease (CAD) and congestive heart failure (CHF) are leading causes of morbidity and mortality in Western communities and represent a growing economic burden to most health systems (Cohn et al. 1997). CHF arises from the process of ventricular remodeling, which can occur in response to either ischemic or non-ischemic myocardial insult. Central to this process, is the progressive loss of cardiomyocytes and contractile cardiac tissue. Following severe myocardial infarction (MI), large-scale death of cardiomyocytes (approximately 1.8×10^9 cells), occurs via a process of ischemic "*anoikos*", which is followed by apoptosis of vulnerable cardiomyocytes in peri-infarct territories (Murry et al. 2006). Although more sporadic and insidious, substantial cardiomyocyte loss is also associated with remodeling in other disease states including hypertension, valvular disease and cardiomyopathies.

Current conventional therapy of CHF has evolved from numerous seminal advances over the last two decades (Fonarow, 2000). Improvements in pharmacotherapy addressing the neurohormonal changes associated with the condition have provided the mainstay of treatment. These advances have been accompanied by a concomitant improvement in coronary revascularization techniques, the development of cardiac resynchronization pacemakers and implantable defibrillators, more effective immune modulation for heart transplant recipients and increasing usage of mechanical assist devices. With the obvious exception of cardiac transplantation, these treatment options are limited by their failure to replace lost cardiomyocytes and myocardial scar tissue with new, functioning, contractile tissue. This is compounded by the heart's own incapacity to replace its lost cell mass by self-regeneration. Consequently, in its most advanced stages, CHF is associated with an extremely grave prognosis, with six month mortality rates approaching 50%, rivalling the most aggressive types of malignancy. New approaches to CHF treatment are therefore required.

Cell-based therapy has seen a rapid evolution over the last decade. While much of the ground covered has been at the level of basic science through both *in vitro* and *in vivo* preclinical research, the last six years have also witnessed the application of this novel treatment strategy in clinical trials of MI, CAD and cardiomyopathy The first part of this two-part review will address the biological properties and functional potential of different cell types that have been trialed in preclinical cardiovascular research. Part two will focus on the clinical application of these cells and will discuss the lessons learnt from clinical trial experience so far.

Endogenous Myocardial Repair

For many years, the long-standing dogma has been that adult cardiomyocytes undergo terminal differentiation soon after birth, irreversibly withdrawing from the cell cycle. In the postnatal state, uncoupling of cardiomyocyte karyokinesis and cytokinesis helps to explain the characteristic cell hypertrophy and

Correspondence:

nuclear ploidy, rather than hyperplasia, that occurs following cardiac injury (Kellerman et al. 1992). Furthermore, the inability of cardiomyocytes to progress through the cell cycle contributes to their apoptosis. In recent years, however, this long-held depiction of the adult mammalian heart as a post-mitotic organ has been severely challenged. It is now apparent that not all cardiomyocytes in the adult heart lose their ability to replicate. Initially this was inferred from observations that there is an increase in the number of proliferating cardiac cells after MI, especially in the peri-infarct territories, but also in areas of remote, non-infarcted myocardium (Beltrami et al. 2001). Similar evidence of cell division is also evident in cardiac failure (Kajstura et al. 1998). These findings prompted investigations into the properties and the functional role of these dividing cells under both normal tissue homeostasis and pathological conditions.

Circulating non-cardiac cells

Evidence that extrinsic (non-cardiac) cells may contribute to endogenous cardiac repair processes emerged from transplantation studies in which donor and recipient organs were sex mismatched and small numbers of Y-chromosome containing cells were identified in the female donor hearts of male transplant recipients (Laflamme et al. 2002). This implied the migration of a non-resident cell type(s) to the heart which contributed to resident endothelial and possibly cardiomyocyte cell populations. It is now well established that in response to myocardial insult, a variety of mature and immature cell types are mobilized from non-cardiac organs, such as bone marrow (BM), into peripheral blood to assist with cardiac and vascular repair. These cells include lymphocytes, monocytes/macrophages, granulocytes, mature endothelial cells along with progenitor cells such as endothelial progenitor cells (EPCs), hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) (Mouquet et al. 2005; Wojakowski et al. 2007). Following acute MI, peripheral blood levels of $CD34^+$ EPCs rise in association with plasma levels of vascular endothelial growth factor (VEGF), peak within just a few hours and remain elevated throughout the first week (Shintani et al. 2001; Massa et al. 2005). Different subpopulations of mobilized progenitor cells have also been identified, specifically: $CD34^+/CD117^+$, $CD34^+/CXCR4^+$, $CD34^+/CD38^+$ and $CD34^+/CD45^+$ cells (Wojakowski et al. 2007). Activation of chemokines and chemokine receptors, such as stromal-derived factor-1 (SDF-1; CXCL12) and its receptor CXCR4, play central roles in the mobilization of these cells and their migration to, and engraftment in, the injured myocardium (Askari et al. 2003).

Cardiac resident progenitor cells

In addition to the recruitment of non-cardiac stem cells, there is exciting evidence from several groups, demonstrating the existence of resident cardiac stem/progenitor cells in the hearts of adult mammalian species, including humans (Beltrami et al. 2003; Oh et al. 2003; Laugwitz et al. 2005). Different techniques have been used to identify populations of these cells, including co-expression of stem cell markers (c-kit, SCA-1, Islet-1, MDR1) with cardiac and vascular-associated markers. It is not clear whether these subtypes of cardiac stem cells are actually distinct from each other or merely represent different stages of normal cardiomyocyte development. However, each has shown capacity for cardiomyocyte differentiation both *in vitro* and *in vivo* and in some cases differentiation into endothelial and smooth muscle cell lineages as well (Beltrami et al. 2003; Moretti et al. 2006).

While not fully characterized, cardiac stem cells may occupy niches within atrial tissue, the atrioventricular groove and the apical ventricular myocardium, where they form structural and functional connections to mature cardiomyocytes and cardiac fibroblasts (Messina et al. 2004; Urbanek et al. 2006). Growth factors, such as insulin-derived growth factor (IGF-1) and hepatocyte growth factor (HGF), appear able to stimulate their migration to sites of injury and their proliferation and differentiation into mature cardiac and vascular-specific cells. Unfortunately, these responses are severely attenuated in the hostile environment of acute MI and cardiac remodeling, implying that under these conditions myocardial cell loss goes largely unreplaced, limiting the heart's capacity for self-repair.

The existence of these native stem cell populations does however, provide evidence for an endogenous cardiac repair process that can be targeted by exogenous cells or factors to reduce cardiac remodeling and aid myocardial healing. There is also the exciting potential for the therapeutic application of these autologous cardiac stem cells which

could be harvested by endomyocardial biopsy and then reimplanted into damaged tissue. Indeed preclinical studies investigating this promising strategy are well underway (Oh et al. 2003; Beltrami et al. 2003; Messina et al. 2004; Dawn et al. 2005; Smith et al. 2007).

Exogenous Cell Therapy for Cardiac Repair

Following initial proof-of-concept studies using fetal cardiomyocytes transplanted into fibrotic myocardial scars (Leor et al. 1996), various other cell types have been examined for their capacity to mediate cardiac and vascular repair, in both *in vitro* assays and *in vivo* transplantation studies.

Stem cells are defined by their ability to achieve self-renewal, high replicative potential and the capacity to differentiate into multiple mature cell types. Stem cells can be classified into two broad types: embryonic stem cells (ESCs) and adult-derived stem cells (ASCs). Embryonic stem cells are totipotent with the ability to develop all the tissue-forming cells that constitute an entire organism, while ASCs reside in the postnatal state in tissue niches and function to replenish cell loss as a consequence of tissue damage and death. The differentiation potential of some ASC populations is not only confined to the cells of their specific tissue of origin, but to other cell lineages as well. As detailed below, not all of the cell types applied to cardiac therapy fulfill these criteria for stem cells.

Embryonic stem cells

Embryonic stem cells are derived from the inner cell mass of the blastocyst. They display promising preclinical potential for achieving myocardial regeneration, largely as a result of their enormous proliferative capacity and their toti–differentiation potential. There are numerous reports in the literature describing the properties of both murine ESC (mESC) lines and human ESCs (hESCs), which were first successfully isolated from human blastocysts in 1998 and have consistently shown cardiogenic potential *in vitro* (Thomson et al. 1998; Kehat et al. 2001; He et al. 2003; Kehat et al. 2004). Human ESC-derived cardiomyocytes behave structurally and functionally like early-stage cardiomyocytes, expressing characteristic early cell markers and possessing electrophysiological and ultrastructural features similar to fetal ventricular cardiomyocytes. Cardiac-committed cells can be enriched from hESC culture using Percoll gradient centrifugation. Initial studies, in which these cells were transplanted into animal models of heart disease, demonstrated their durable engraftment, proliferation and differentiation *in vivo*, and importantly, their integration with host cardiomyocytes (Xue et al. 2005; Laflamme et al. 2005).

Unfortunately, current techniques for isolating and generating differentiated hESC-derived CMCs for clinical use are limited by issues of yield and failure to exclude undifferentiated ESCs. The latter issue poses significant risk of teratoma formation at sites of cell transplantation or in other tissues following migration of immature ESCs to secondary sites. Along with tumor risk, immunorejection and ethico-legal considerations are currently major obstacles hindering the clinical application of hESCs (Laflamme and Murry, 2005). While these problems are not insurmountable, the emerging field of nuclear transfer technology provides an appealing future alternative to hESC therapy (Lanza et al. 2004).

Adult cells

Various adult tissues contain resident cell populations that have been tested in cardiac research. Most experience has involved cells from skeletal muscle, bone marrow and blood, however, other tissue sites, such as adipose, dental pulp and periodontal ligament, placental and umbilical cord blood and of course the heart itself, also harbour cell types that are being intensively investigated.

Skeletal myoblasts

Skeletal myoblasts (SkMs) are committed skeletal muscle precursor cells that normally lie in a quiescent state under the basal lamina of skeletal muscle fibers. Their normal role is to regenerate skeletal muscle following injury, both by fusion with the surrounding muscle fiber and by differentiation into contractile mature skeletal myocytes. Autologous SkMs are easily accessible through muscle biopsy and can be rapidly expanded *in vitro* to high cell number for therapeutic application. *In vitro* isolation can be assisted through their expression of the surface marker CD56. Of relevance to cardiac repair, these cells remain committed to a contractile myogenic lineage and are resistant to ischemia.

A number of preclinical studies have demonstrated that these cells can engraft, proliferate and survive in the presence of various forms of myocardial injury, ultimately improving systolic and diastolic cardiac function (Marelli et al. 1992; Taylor et al. 1998; He et al. 2005; Hata et al. 2006). The mechanisms responsible for these functional benefits are unlikely to be related to myoblast transdifferentiation into cardiomyocytes. Studies have documented consistently that these cells fail to adopt a cardiac phenotype after their engraftment into the myocardium (Reinecke et al. 2002). These cells therefore remain committed to their skeletal muscle origin *in vivo*, but may change over time to become more fatigue resistant, by acquiring a greater component of slow-fiber myosin. They also fail to electromechanically couple with host cardiomyocytes through the production of gap junction and the intercalated disc proteins, N-cadherin and connexin-43 (Reinecke et al. 2000). This phenomenon may partly explain the electrical instability, manifested by ventricular tachyarrhythmias, that complicated transplantation of SkMs in early clinical studies (Menasche et al. 2003). In the absence of cardiac transdifferentiaton and electromechanical coupling, SkMs may mediate cardiac repair through various other mechanisms, including autonomous contraction, infarct size reduction and stabilization of scar tissue. They may also influence cardiac remodeling and angiogenesis in a paracrine fashion by the release of cytokines and growth factors such as VEGF and HGF (Chazaud et al. 2003). Numerous clinical studies have addressed the therapeutic potential of skeletal myoblasts in ischemic (post-infarction) cardiomyopathy.

Bone marrow and blood-derived cells

Bone marrow and peripheral blood contain various types of progenitor/stem cells, with the best defined being hematopoietic stem cells (HSCs), angioblasts and endothelial progenitor cells (EPCs) and mesenchymal stem cells (MSCs). Numerous subpopulations of these cell types have been identified through techniques such as flow cytometric analysis, although in many cases they are still incompletely characterized.

Unfractionated bone marrow

A paucity of comparative studies between the different types of BM-derived stem cells has created uncertainty as to which cell-type is optimal for cardiac repair. Many trials, especially those in humans, have avoided the use of specific stem cell fractions, preferring to adopt a "blanket" treatment approach by administering unfractionated bone marrow cells (BMCs) or bone marrow mononuclear cells (BMMNCs). These preparations are heterogeneous in their cellular composition and include monocytes, lymphocytes, nucleated red cells, immature B- and T-cells and a minor proportion of HSCs ($<2.5\%$) and MSCs ($\leq 0.0001\%$) (Gronthos and Simmons, 1995; Gronthos et al. 2003).

Unfractionated bone marrow cells have been transplanted in preclinical models of ischemic and non-ischemic cardiomyopathy, with the most notable benefits relating to improvements in myocardial vascularisation (Fuchs et al. 2001; Agbulut et al. 2003; Silva et al. 2005). In clinical studies, the practicality of using unfractionated BM has been well suited to acute MI, where the time required to prepare autologous stem cells (e.g. MSCs) can preclude cell delivery in the early stages of infarct healing. While the low frequency of clonogenic stem cells in BM may limit its reparative potential, this limitation may be counterbalanced by the pleiotropic properties inherent in using a mixture of immature and mature cell types. Comparative studies between unfractionated BMCs/BMMNCs and fractionated stem cell populations are required to provide scientific rationale to the relative merits of both treatment approaches.

Hematopoietic stem cells

Subtypes of HSCs have been investigated in preclinical models of myocardial injury, with inconsistent results. A seminal study by Orlic et al. directly injected murine $Lin^{-}c\text{-}kit^{pos}$ BM-derived cells into mice post MI (Orlic et al. 2001). These cells were labeled to express enhanced green fluorescent protein and were observed to engraft into the host myocardium within a few days after transplantation. Some engrafted cells exhibited features suggestive of cardiomyocyte differentiation and animals receiving these cell preparations exhibited improvement in invasive hemodynamic measurements of left ventricular function. While similar studies have also supported the *in vivo* engraftment and differentiation of HSC subpopulations into cardiomyocytes and endothelial cells (Jackson et al. 2001), these results have not been consistently

reproducible (Murry et al. 2004; Balsam et al. 2004). Reports have also suggested that apparent changes in HSC phenotype may be due to fusion with surrounding cells in the host tissue, rather than true transdifferentiation (Nygren et al. 2004).

Endothelial progenitor cells

Endothelial precursors capable of transforming into mature, functional endothelial cells can be isolated from the mononuclear population of either BM or peripheral blood. Although bone marrow-derived EPCs are $CD45^-lin^-$, they appear to share common ancestry with HSCs, as indicated by their expression of the immature hematopoietic markers CD34 and CD133, along with vascular endothelial growth factor (VEGF) receptor 2 (VEGFR2) (or fetal liver kinase-1 [flk-1]). Upon maturation to endothelial cells, CD133 expression is lost and replaced by expression of VE-cadherin (CD144), CD31 (PECAM-1) and von Willebrand factor (vWF) (Peichev et al. 2000; Masuda and Asahara, 2003). At least three subpopulations of EPCs, at different stages of maturation and with different profiles of CD34 and CD133 expression, are present in the peripheral blood. These circulating cells are derived from both $CD117^+/VEGFR^+$ BM hemangioblasts and peripheral blood monocytes. In recent years, highly proliferative EPCs have also been isolated from umbilical cord blood (Ingram et al. 2004).

As discussed previously, EPCs are mobilized into the peripheral circulation as part of the normal physiologic response to tissue ischemia and vascular injury, allowing their migration to the site of injury under the influence of growth factors, such as VEGF and SDF-1. There they contribute to neovascularization, by differentiation into mature endothelial cells, incorporation into new vessels (vasculogenesis) and promotion of local vascular cell growth (angiogenesis) (Asahara et al. 1997). The numbers of circulating progenitor cells fluctuate in response to ischemic injury (e.g. MI) and are also influenced by other factors, including the presence of cardiovascular risk factors and non-cardiac diseases, ageing and specific pharmacologic agents, such as HMG-CoA reductase inhibitors (Isner, 2000; Shintani et al. 2001; Llevadot et al. 2001).

As the frequency of EPCs is very low in BM ($CD34^+$ ~2% and $CD133^+$ ~0.5% of total cells) and even more so in peripheral blood, therapeutic application has usually necessitated their isolation by *ex vivo* expansion of MNCs on fibronectin-coated tissue plates. Two distinct subpopulations of EPCs result – early outgrowth and late outgrowth populations – with different capacities for further culture and proliferation (Ingram et al. 2004; Young et al. 2007). Both populations have been successfully trialed in animal models of hindlimb ischemia, achieving viable engraftment and promoting new vessel formation. Combined administration of the early and late outgrowth populations may optimize these pro-vascular effects (Lee et al. 2005).

Preclinical studies of cell transplantation post-MI, have demonstrated pro-angiogenic and anti-apoptotic benefits of angioblasts and EPCs, culminating in improved myocardial function (Schuh et al. 2007). These effects are probably mediated by a combination of improved local tissue perfusion and protective paracrine effects that EPCs exert on dying cardiomyocytes (Kocher et al. 2001; Rehman et al. 2003; Schuster et al. 2004). There is also limited *in vitro* experience indicating that EPCs may be able to undergo cardiomyocyte differentiation (Badorff et al. 2003).

Mesenchymal stem cells

This rare type of stem cell is found both in BM and other tissues, including adipose tissue, dental pulp, periodontal ligament, umbilical cord blood and placenta (Gronthos et al. 2001; Shi and Gronthos, 2003; Rangappa et al. 2003; Kogler et al. 2004; Kim et al. 2005; Miao et al. 2006). Typically in these tissues, MSCs are located in perivascular niches surrounding blood vessels, where they play an important supportive role for other cell types. Their important contribution to the creation and maintenance of stromal and skeletal tissues in BM has led to the use of interchangeable terminology, such as somatic stem cells, stromal stem cells and marrow stromal cells.

Traditionally, BMMSCs are isolated from the MNC fraction based on their adherence to tissue culture plastic and the subsequent formation of colonies of spindle-shaped cells (colony-forming-units-fibroblastic: CFU-F) (Friedenstein et al. 1970). Clonogenic MSCs possess the proliferative potential to enable them to be expanded *ex vivo* to the high cell numbers required for transplantation. These cells have distinct surface molecule expression to other BM-derived stem cells; they

are $CD34^-$ and $CD45^-$ but stain positively for a range of other markers, including SH2 (CD 105), SH4 (CD 73), CD49d,e, CD71, CD106 and CD166 (Pittenger et al. 1999; Bianco et al. 2001).

Plasticity of MSCs isolated from BM and other tissues, is classically demonstrated by differentiation into adipoblasts, chondroblasts and osteoblasts under appropriate culture conditions (Bianco et al. 2001). Of more relevance to potential cardiac application, these cells also have potential to acquire phenotypic characteristics of cardiomyocytes, smooth muscle cells and endothelial cells, prompting expectation that they may be able to provide both myogenic and vasculogenic benefits to injured myocardium (Galmiche et al. 1993; Makino et al. 1999; Toma et al. 2002; Shim et al. 2004; Xu et al. 2004; Iwase et al. 2005; Silva et al. 2005).

There is a large body of preclincial research confirming the pro-myogenic and pro-angiogenic properties of non-induced, *ex vivo* expanded BMMSCs in various animal models of cardiac injury (Toma et al. 2002; Nagaya et al. 2004; Hattan et al. 2005; Amado et al. 2005; Silva et al. 2005; Hou et al. 2006). Studies examining the efficacy of transplantating MSCs post-infarction have consistently reported attenuation of myocardial scarring and infarct size, improved regional and global ventricular function and increased vascular density and in some cases myocardial perfusion (Shake et al. 2002; Amado et al. 2005). Restoration of myocardial mechano-energetics has also been observed and may be an important mechanism by which MSC therapy can avert post-MI heart failure (Amado et al. 2005). Beneficial effects on myocardial vascularization appear to be largely mediated indirectly through cytokine and growth factor release, although potential for MSC engraftment into blood vessels and differentiation into endothelial and smooth muscle cells has also been demonstrated (Davani et al. 2003; Kinnaird et al. 2004a; Silva et al. 2005). While most preclinical studies have not demonstrated significant adverse observations at MSC injection sites, a recent study in a murine model of cryo-infarction reported a high incidence (approximately 50%) of encapsulated areas containing ossifications and/or calcifications in tissue that had received MSC transplantation (Breitbach et al. 2007). This finding highlights the need for all preclinical studies to be thorough in their assessment of adverse histopathological outcomes from cellular therapy.

In addition to their pro-myogenic and pro-angiogenic potential, MSCs are also hypoimmunogenic, enabling them to exist in inflammatory environments without activating host T-lymphocytes (Ruggeri et al. 2001). These immune properties are partly due to a lack of surface expression of MHC Class II molecules and co-stimulatory molecules for T-cell induction (CD40, CD40 ligand, and the B7 molecules CD80 and CD86) (Tse et al. 2003; Le Blanc et al. 2003). Mesenchymal stem cells down-regulate lymphocyte proliferation and suppress the maturation and function of various other immune cells. This occurs both by direct cell contact of MSCs with these target cells and also by the release of immune-modifying soluble factors, such as HGF, interleukin-10 and prostaglandin E (PGE)-2 (Kuroiwa et al. 2001; Rasmusson et al. 2005; Aggarwal and Pittenger, 2005). The culmination of these immune-modifying effects raises the potential for MSCs to be used allogeneically without concurrent immunosuppression. This may have major clinical advantages, as an allogeneic "off-the-shelf" or "ready-to-use" source of MSCs would enable early MSC transplantation after MI. It also promises to avoid the shortcomings of autologous cell therapy, especially in elderly patients with chronic diseases (e.g. CAD, CHF), who characteristically have impaired stem cell recovery, proliferation and function (Heeschen et al. 2004). Clinical application of allo-MSCs awaits careful comparative studies with autologous MSCs.

Mechanisms of Benefit from Cell Therapy

Early *in vitro* and *in vivo* evidence that specific cell types could differentiate into cardiomyocytes gave rise to an expectation that exogenous stem cells might be capable of regenerating injured myocardium, by direct replacement of the cells that are lost during cardiac remodeling. Increasingly, the potential for current cell strategies to achieve actual cardiomyocyte regeneration appears at best limited. Cardiac transdifferentiation has not been observed with SkMs, despite their capacity to improve myocardial function in animal studies. In addition, evidence for *in vivo* cardiac transformation of other cell types, has largely been based on immunofluorescence and immunohistochemical analyses which are prone to tissue artefact and also do not reliably account for the possible confounder

of cell fusion (Terada et al. 2002; Nygren et al. 2004). Cell labeling and imaging techniques have been developed to track the fate of transplanted cells *in vivo*, consistently showing that engraftment of cells is modest, with as few as 0%–6% still present in the recipient heart soon after direct cardiac injection (Hou et al. 2005; Freyman et al. 2006). This reflects poorly on the early retention and longer-term engraftment and survival of cells in disease states such as MI and cardiomyopathy and unfortunately has not yet been overcome by using alternative routes of cell delivery or by administering higher doses of cells. These observations suggest that current approaches to cell therapy fall short of fulfilling the objective of actual myocardial regeneration and instead facilitate cardiac repair through alternative mechanisms.

Recent studies have acknowledged that paracrine actions underlie much of the cardiac reparative effects of transplanted cells, including their ability to increase angiogenesis, reduce infarct size and ventricular wall thinning and improve myocardial contractility (Kamihata et al. 2001; Kinnaird et al. 2004c; Tang et al. 2005b). Mesenchymal stem cells, EPCs, HSCs and SkMs possess the potential to influence other cells in their vicinity, by direct cell-to-cell interaction and by release of a wide array of soluble growth factors and cytokines. These soluble factors are influenced by the developmental status and properties of the transplanted cell populations and by the local milieu in which the cells find themselves (Weimar et al. 1998; Caplan and Dennis, 2006). Growth factors that have been identified as possibly contributing to myocardial and vascular repair include SDF-1, HGF, IGF-1, basic fibroblast growth factor (bFGF), VEGF, angiopoeitin-1 (Ang-1), monocyte chemoattractant protein 1 (MCP-1), interleukins-1 and 6, placental growth factor, plasminogen activator and tumor necrosis factor-alpha (TNF-α) (Kamihata et al. 2001; Kinnaird et al. 2004b).

In the injured myocardium, transplanted cells may exert paracrine actions on a range of resident mature and immature cell types, facilitating endogenous repair processes. This may include protection of stressed cardiomyocytes that are vulnerable to apoptosis in peri-infarct areas, and promitogenic and homing effects on cardiac progenitor cells, endothelial cells and endothelial progenitor cells. The latter contributes to neovascularization, which in turn further supports the viability of donor and host cells, especially in the presence of ischemia. Although such paracrine effects of cell therapy may be only part of the mechanism of benefit in cardiac repair, they may emerge as one of the most important.

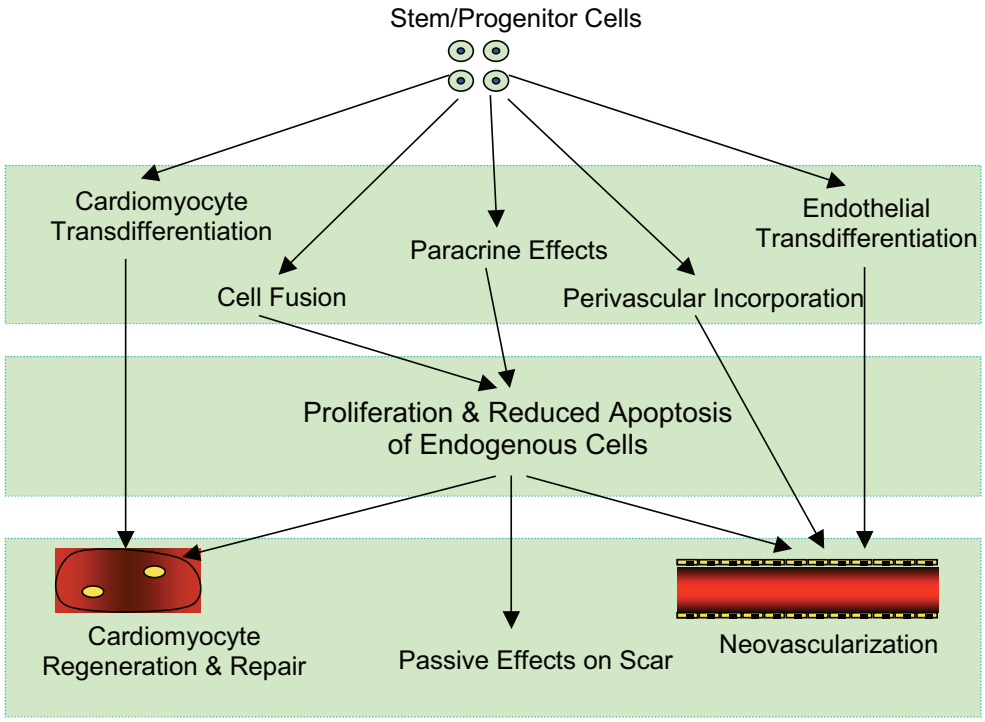

Figure 1. Mechanisms by which cell therapy may achieve cardiovascular repair.

Selecting the Optimal Cell Type

Presently uncertainty exists as to which cell types are best-suited for the treatment of different cardiac pathologies. Cell-based treatment can theoretically benefit the injured heart either by new blood vessel formation or improvement in myocardial systolic and/or diastolic function. Different cell types vary in their ability to influence these two broad mechanisms. In the context of myocardial ischemia, where the objective is reperfusion, the more favorable cells are those that can enhance angiogenesis and neovascularization. Cells derived from bone marrow (BM) or peripheral blood (PB) fall into this category, such as BMMNCs (Stamm et al. 2003), MSCs (Silva et al. 2005), BM angioblasts (Itescu et al. 2002), EPCs (Assmus et al. 2002; Rauscher et al. 2003), or multipotent adult progenitor cells (MAPCs) (Jiang et al. 2002). In situations where cardiac dysfunction predominates, such as in cardiomyopathy or after severe MI, cell types which can induce myogenesis, reduce myocardial wall stress and perhaps stabilize myocardial scar will be of most value. Examples include skeletal myoblasts (SkMs), MSCs and cells from the heart itself, including mature cardiomyocytes, cardiac fibroblasts and cardiac stem cells.

Currently there is a paucity of research experience comparing the effectiveness of these different cell types. In preclinical models of cardiac injury, transplantation of SkMs has been compared with fetal cardiomyocytes (Scorsin et al. 2000), fibroblasts (Hutcheson et al. 2000) and bone marrow-derived cells, including MSCs and $CD133^+$ EPCs (Thompson et al. 2003; Agbulut et al. 2004; Guarita-Souza et al. 2006a). In each of these studies, SkM therapy was shown to improve cardiac systolic performance, in a manner superior to fibroblasts and EPCs and non-inferior when compared to other BM cell preparations

The relative merits of combination versus individual cell therapy are also unresolved. Combination therapy can be achieved by transplanting two or more cell populations together which may be especially attractive in ischemic cardiomyopathy, where cardiac dysfunction is associated with poor tissue perfusion. Examples include the co-transplantation of BMMNCs and SkMs in a rat study of MI (Ott et al. 2004) and the co-administration of SkMs and MSCs in a murine model of Chagas cardiomyopathy (Guarita-Souza et al. 2006b). Alternatively, cell therapy can be combined with coronary revascularization, either by percutaneous intervention (Schachinger et al. 2006) or coronary artery bypass surgery (CABG) (Menasche et al. 2001), in order to enhance the survival of cells being transplanted into ischemic myocardium.

Cellular Biology—Limitations and Future Directions

Despite great optimism, significant challenges remain before cell therapies can be used widely in clinical practice (Chien, 2004; Oettgen et al. 2006). Unresolved clinical issues relate to optimal cell dose, delivery technique, timing of delivery and monitoring of long-term efficacy and safety outcomes, including cell fate. Critical work also remains for basic science to improve our understanding of stem cell biology so that this can be manipulated to ultimately optimize clinical outcomes. Major ongoing areas of preclinical research and development include: (1). cell isolation and purification and (2). *in vitro* manipulation of cell therapy by gene and growth factor regulation or biomaterial scaffolds.

Preparation and purification

Optimization of cell isolation and culture techniques may be an important step towards maximizing both the efficacy and safety of cell therapies. As indicated above, isolation procedures for differentiated hESCs will be critical for minimizing tumor risk with these cells. Traditional techniques to prepare adult cell types are also imperfect. The limited supply of progenitor cells from BM and peripheral blood could potentially be overcome by using alternative tissue sources, such as adipose, which contain a higher number of clonogenic cells from freshly prepared tissue (Zannettino et al. 2007).

Historically, BMMSC culture has involved the plastic adherence of MNCs and *ex vivo* expansion of the cell progeny. Cell purity has been claimed on the grounds that the resulting MSCs express certain surface antigens, despite the fact that these antigens are neither specific nor definitive markers of cells with *bona fide* stem cell properties. Colonies isolated by plastic adherence of MNCs are known to be heterogeneous, containing both immature multipotent MSCs, but also a significant number of contaminating cell types such as more

mature mesenchymal cells (e.g. osteoblasts, osteoprogenitor cells, fat cells and fibroblasts) and non-mesenchymal cells (e.g. macrophages and endothelial cells) (Simmons and Torok-Storb, 1991; Alhadlaq and Mao, 2004). In addition, when e*x vivo* expanded to achieve adequate cell numbers for transplantation, MSC progeny lose their stem cell qualities, including their capacity for proliferation and differentiation.

Techniques have emerged to counter these limitations inherent with MSC preparation by plastic adherence and *ex vivo* expansion. They include prospective immunoselection and clonal expansion of single cells. Immunoselection refers to selection of cells based on their negative or positive expression of specific surface antigens. Antibodies that have been used to enrich for MSCs include STRO-1, VCAM-1(CD106) and STRO-3 (Simmons and Torok-Storb, 1991; Gronthos et al. 2003; Gronthos et al. 2007). Human MSCs expressing high levels of STRO-1, an as yet unidentified antigen, possess many hallmark characteristics of immature stem cells and achieved impressive cardiac protection and neovascularization when applied to a rat model of MI (Kortesidis et al. 2005; Martens et al. 2006). Immunoselection has also been used for the preparation of $CXCR4^+$ blood-derived cells, based on strong evidence that CXCR4 and its ligand SDF-1 play a crucial role in the mobilization and homing of circulating cells to the heart (Petit et al. 2007).

Distinct subpopulations of adult BM progenitor cells, derived from clonal expansion, include multipotent adult progenitor cells ("MAPCs"), "snMSCs" and "hBMSCs". Each of these have been applied to promising effect in preclinical studies of cardiac therapy (Reyes et al. 2001; Jiang et al. 2002). Multipotent adult progenitor cells and hBMSCs appear to possess ESC properties of extensive proliferative capacity and totipotent differentiation into all three germ cell layers. Recently, another group has been able to induce ESC-like stem cells by retroviral transduction of differentiated somatic cells (dermal fibroblasts) with specific transcription factors (Oct3/4, Sox2, c-Myc and Klf4) (Yamanaka and Takahashi, 2006; Takahashi et al. 2007). Although the biological properties of these adult-derived cells appear very favorable for tissue repair, uncertainty remains as to whether these pluripotent cell types are vulnerable to dysregulated mutations and tumorigenesis.

In vitro manipulation

There is considerable interest in combining cellular therapy with other strategies to overcome the current problems of insufficient engraftment and survival of transplanted cells in the heart. Culture and transplantation of cells in carefully designed bio-scaffolds could potentially achieve enhancement of adhesion, growth, and migration signals *in vivo*, while simultaneously providing structural and mechanical support to the damaged cardiac region (Davis et al. 2005). Genetic engineering has also been used to impart cytoprotective effects to newly transplanted cells, so that the efficiency of cell therapy is enhanced. Examples include transfection of cells with genes encoding the anti-apoptotic factor Akt (Mangi et al. 2003), fibroblast growth factor-2 (FGF-2) (Song et al. 2005) and angiopoietin (Jiang et al. 2006). Manipulation of MSCs to overexpress Akt has resulted in further enhancement of the reparative effects of MSC therapy in animal models of infarction, both with respect to global left ventricular ejection fraction and also area of infarct scarring (Mangi et al. 2003; Gnecchi et al. 2005; Lim et al. 2006). This may partly reflect greater resistance of Akt-transfected cells to *in vivo* apoptosis, but in addition has also been shown to relate to stronger paracrine effects that these cells may impart to the host myocardium (Gnecchi et al. 2006).

Mesenchymal stem cells have also been modified using a hypoxia-regulated heme oxygenase-1 (HO-1) plasmid vector which increased short-term cell survival by five-fold in a murine study of MI. This survival advantage was accompanied by benefits in reducing the infarct size and improved cardiac function (Tang et al. 2005a). Another popular candidate gene for pre-transplant cell modification has been VEGF. Transfection of various cell types with plasmids encoding this gene has become an alternative to direct gene therapy which has had some promising results in animal and human studies of coronary ischemia (Rosengart et al. 1999; Vale et al. 2001). The combined cell/gene therapy approach may provide longer-lasting overexpression of VEGF and its angiogenic effects than can be achieved with gene only therapy (Yau et al. 2001; Matsumoto et al. 2005). However, the safety of this approach remains uncertain due to concerns of late vascular tumor formation (Lee et al. 2000).

SDF-1 plays a crucial role in trafficking of progenitor cells from the bone marrow to ischemic

tissue (Ceradini et al. 2004) and consequently some experimental strategies have attempted to upregulate its effects in myocardial ischemia. Pre-transplantation engineering of cardiac fibroblasts (Askari et al. 2003) and mesenchymal stem cells (Zhang et al. 2007) has resulted in enhancement of myocardial repair and cardiomyocyte preservation in rodent studies, while another successful approach has been to precondition MSCs by adding SDF-1 to the cell culture media during *in vitro* expansion (Pasha et al. 2007). Administration of exogenous SDF-1 in the absence of concomitant cell transplantation has resulted in mixed outcomes, with one porcine study showing deleterious effects on left ventricular function following intramyocardial SDF-1 injection two weeks after MI (Koch et al. 2006).

Conclusion

There is now a large body of preclinical evidence supporting the potential for a variety of cell types to facilitate cardiac and vascular repair. Currently, adult-derived cells appear limited in their capacity to achieve true replacement of damaged and/or dead cardiomyocytes and their beneficial effects appear to be predominantly reparative. Much of this cardiac repair seems to be mediated through indirect or paracrine actions of these cells on endogenous tissues and cell populations. Although more capable of proliferating and differentiating to the extent required to replace the cells lost in cardiac disease states than their adult counterparts, embryonic stem cells have a number of inherent challenges before becoming a viable clinical alternative.

Optimizing techniques by which cells used for transplantation are isolated, cultured and manipulated *in vitro* will play a crucial role in improving the engraftment and function of these cells *in vivo*. Equally important in achieving clinical application will be the issues of tailoring specific cell types to specific myocardial diseases and delivering cells to the heart both safely and efficiently. However, the potential of cell-based therapies to provide a paradigm shift in the clinical management of patients with heart failure, demands continued research.

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