Topical Review

Heart repair and stem cells

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Of the medical conditions currently being discussed in the context of possible treatments based on cell transplantation therapy, few have received more attention than the heart. Much focus has been on the potential application of bone marrow-derived cell preparations, which have already been introduced into double-blind, placebo-controlled clinical trials. The consensus is that bone marrow may have therapeutic benefit but that this is not based on the ability of bone marrow cells to transdifferentiate into cardiac myocytes. Are there potential stem cell sources of cardiac myocytes that may be useful in replacing those lost or dysfunctional after myocardial infarction? Here, this question is addressed with a review of the recent literature.

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Cell transplantation is an area of growing interest in clinical cardiology as a potential means of treating patients with myocardial infarction or cardiac failure. The interest is based on the assumption that left ventricular dysfunction is largely due to the loss of a critical number of cardiomyocytes and that it may be partly reversed by implanting new contractile cells into the postinfarction scars or regions of wall thinning. Four categories of stem cells have been examined for their ability to promote cardiac repair in animal models: bone marrow-derived/circulating progenitor cells (BMPCs) and their subpopulations, skeletal myoblasts (SM), embryonic stem cells (ESCs) and resident cardiac stem (or cardiomyocyte progenitor) cells (CMPCs) (van Laake *et al.* 2005). The origin of these cells is summarized in Table 1. Three of these cell types (BMPCs, SMs and CMPCs) are potentially autologous. Partly for this reason, BMPCs and skeletal muscle cells have been the first to be used in clinical trials. Their use is now considered feasible and for BMPCs safe. In contrast SM fail to integrate electromechanically within the recipient heart and their use is associated with risk of arrhythmias. Efficacy data are now emerging from ongoing randomized double-blind studies. However a note of caution has arisen with BMPCs since early claims that they were able to transdifferentiate in cardiac cells have now been refuted and attributed to fusion with recipient cardiac cells. Their ability to induce neovascularization and rescue ischaemic myocardium when introduced at the correct time post myocardial infarction, is considered a potential mechanism underlying any beneficial effects. Resident CMPCs by contrast have only relatively recently been identified, but are already generating excitement because they appear to differentiate into *bona fide* cardiomyocytes *in vitro* with high efficiency. This is exceptional for any adult stem cell source studied to date. The question being addressed in preclinical experiments is whether *ex vivo* or *in vivo* expansion might be the best approach to increasing their numbers and improving contractile function of the heart. ESCs are at present the major heterologous source of cells being considered and ethically the most sensitive as their derivation requires the destruction of early human embryos. ESCs are developmentally the most versatile of stem cells forming all of several hundreds of cell types in the adult body. They are, however, associated with the risk of tumour formation if not fully differentiated.

While experimental studies and early phase clinical trials tend to support the concept that cell therapy may enhance cardiac repair, several key issues still need to be addressed before introduction into routine clinical practice. These include (1) the optimal type of donor cells in relation to the clinical profile, (2) the mechanism by which cell engraftment improves cardiac function, (3) optimization of cell survival, (4) development of less invasive cell delivery techniques, and (5) the relevance to non-ischaemic heart failure. Here the background and current status of cardiac cell therapy are reviewed and perspectives for improving the prognosis of heart failure discussed.

Pre-clinical studies

Among the most important issues being addressed at present is identifying the most suitable stem cells for replacing muscle mass and finding out which mechanisms

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Table 1. Origin of the cell types

Cell type	Abbreviation	Origin
Bone marrow derived cell	BMPC	Bone marrow
Skeletal myoblast	SM	Adult skeletal muscle
Cardiomyocyte progenitor cell	CMPC	Adult or fetal heart
Cardiac stem cell		
Endothelial progenitor cell/ endothelial precursor cell	EPC	Bone marrow/ peripheral blood
Embryonic stem cell	ESC	Blastocyst stage embryos

might contribute to stem cell-mediated improvement in cardiac function after myocardial infarction (MI) so that they could be used additionally or alternatively to vital muscle replacement. Several studies have described enhanced cardiac function after MI, sometimes sustained, in animals following stem cell transplantation but the transplanted cells were often barely dectable post-operatively (Muller-Ehmsen *et al.* 2002) with no evidence of integration into either the vasculature or muscle. However, in some cases heart remodelling and extracellular matrix deposition appeared altered, so unknown paracrine mechanisms have been proposed as underlying functional improvement (Dowell *et al.* 2003; Laflamme & Murry, 2005; Dai *et al.* 2005). Enhanced blood vessel formation has frequently been observed concomitant with salvage of the myocardial tissue at risk in the infarct region (Kocher *et al.* 2001) and subsequent preservation of left ventricular function by increased recovery of hibernating myocardium (Kloner & Jennings, 2001). This could be mediated by incorporation of transplanted angioblasts (Kocher *et al.* 2001) or endothelial precursor cells (EPCs; Asahara *et al.* 1997; Crosby *et al.* 2000; Ceradini *et al.* 2004) into the neovasculature or be the result of a local inflammation up-regulating signalling pathways associated with angiogenesis (Lebrin *et al.* 2004). MI itself increases the circulating levels of EPCs, which in turn correlates with increased levels of granulocyte-colony stimulating factor (G-CSF). Therapy based on G-CSF injection initially appeared safe in patients with acute MI but did not improve functional recovery (Ripa *et al.* 2006) although later studies show that adverse side-effects may result. Stromal-derived factor-1 is also expressed in the ischaemic myocardium (Askari *et al.* 2003) and plays an important role in recruiting EPCs from bone marrow via chemotaxis to the ischaemic site. Indirect mechanisms for enhancing cardiac function may thus operate independent of possible contributions to muscle mass. Here, we consider the principal candidate cell types for cell therapy in turn for their potential to contribute to growth of new myocardium and/or improve cardiac function. Since BMPCs and SM have recently been the subject of numerous reviews (Laflamme & Murry, 2005; Davani *et al.* 2005; Dimmeler *et al.* 2005; Smits *et al.* 2005; Dimarakis *et al.* 2005; Bartunek *et al.* 2006), we will focus on the only two human stem cell types that have been shown convincingly and robustly to convert to cardiac muscle cells: ESCs and CMPCs.

BMPCs, transdifferentiation and angiogenesis

Orlic *et al.* (2001) first suggested that BMPCs transdifferentiate (i.e. covert to another lineage by differentiation) into cardiomyocytes when injected into infarcted mouse myocardium. The transplanted BMPCs expressed cardiac-specific markers troponin I and cardiac myosin but it was later shown that they could fuse with somatic cells and adopt aspects of the phenotype of the somatic cell (Balsam *et al.* 2004; Murry *et al.* 2004). The interpretation of the data appeared flawed; it is now clear that BMPCs do not transdifferentiate into cardiomyocytes (reviewed in Laflamme & Murry, 2005). Nevertheless, functional improvement post-MI has been described after BMPC transplantation possibly due to an EPC subpopulation enhancing angiogenesis and the local blood supply in ischaemic tissue (Tomita *et al.* 1999; Amado *et al.* 2005; Li *et al.* 2005; Limbourg *et al.* 2005; Piao *et al.* 2005). An alternative hypothesis has centred on proarteriogenic paracrine signalling from mononuclear cells also found in circulating blood (Rehman *et al.* 2003; Kinnaird *et al.* 2004) rather than direct incorporation of an EPC subpopulation into neovasculature (Ziegelhoeffer *et al.* 2004). These 'circulating angiogenic cells' were described as secreting multiple proangiogenic cytokines, including vascular endothelial growth factor, hepatocyte growth factor, granulocyte stimulating factor and granulocyte-macrophage colony stimulating factor, with secretion increasing under hypoxic conditions. Neovascularization in turn may inhibit apoptosis of cardiomyocytes (Kocher *et al.* 2001). Mesenchymal stem cells, present in BMPCs, cord blood and adipose tissue, have also been described as possibly having cardiomyogenic potential (Mangi *et al.* 2003). Overall, their efficiency of cardiomyogenic conversion is low.

Skeletal muscle cells

SM satellite cells (or myoblasts), normally mediate regeneration of skeletal muscle but there were some initial hopes that these cells would transdifferentiate into cardiomyocytes. These cells can be expanded in culture and up to 10^9 cells have been grown from a few grams of muscle tissue, but it appeared later that they remain committed to a skeletal muscle fate (Reinecke *et al.* 2002) although exceptionally rare fusion events between skeletal muscle cells and cardiomyocytes were observed in rat hearts after transplantation (Reinecke *et al.* 2004). SM cells failed to express the adhesion and gap junction proteins that would be necessary to couple electromechanically with each other

or with host myocardium and, as a result, the grafts did not beat in synchrony with host rat myocardium (Leobon *et al.* 2003).

Embryonic stem cells

Differentiation to cardiomyocytes. Human ESCs (HESC) (Thomson *et al.* 1998; Reubinoff *et al.* 2000), like mouse ESCs (mESCs; Evans & Kaufman, 1981; Martin, 1981) are derived from the inner cell mass of blastocyst stage embryos. ESCs grow indefinitely in an undifferentiated state whilst retaining the ability to differentiate to all cell types in the adult body. Among the cells that form in culture, rhythmically contracting cardiomyocytes are particularly striking (reviewed in Passier & Mummery, 2003, 2005). The first report of cardiomyocyte differentiation (Kehat *et al.* 2001) appeared almost 3 years after HESCs were first derived. To induce cardiomyogenesis, this cell line was dispersed into small clumps of 3–20 cells and grown for 7–10 days in suspension to form structures like embryoid bodies (EBs) from mESCs. After plating onto culture dishes, beating areas were observed in ∼8% of the outgrowths 20 days later. This spontaneous differentiation to cardiomyocytes in aggregates was also observed by others using different cell lines (Xu *et al.* 2002) but in this case approximately 25% of the EBs were beating after 8 days of differentiation and 70% after 20 days. Other reports described spontaneous differentiation of cardiomyocytes from HESCs with 1–25% of the embryoid bodies beating after several weeks (He *et al.* 2003; Allegrucci & Young, 2006). The reasons for these differences in efficiency are not clear but counting beating EBs may not accurately reflect the conversion of HESC to cardiomyocytes; variable numbers of cardiac cells and non-cardiomyocytes may be present. An alternative differentiation method is based on coculture of HESCs with a visceral endoderm like cell line (END-2) or growth of HESC EBs in END-2 conditioned medium (Mummery *et al.* 2003; Passier *et al.* 2005). Endoderm (visceral) plays an important role in the differentiation of cardiogenic precursor cells in the adjacent mesoderm *in vivo* during the development of species as diverse as chick, mouse and zebrafish, suggesting that the mechanism is apparently conserved (reviewed in Passier *et al.* 2006).

A cause for concern in relation to clinical applicability is that each HESC line may require a different protocol for optimal maintenance of self-renewal and efficient differentiation (reviewed in Allegrucci & Young, 2006). Significant upscale will also be required before HESC cardiomyocytes (HESC-CMs) can undergo extensive preclinical testing in large animal models like sheep and pigs. This could involve increasing the efficiency of cardiomyocyte differentiation, promoting proliferation of the emerging cardiac precursor cells or cardiomyocytes or developing methods of purification of the required cardiac cell type (Passier *et al.* 2006). Methods for upscaling have been described for mESCs using drug selection in combination with a cardiac specific promoter on EBs grown in a bioreactor: pure populations of up to 10^9 cardiomyocytes have thus been generated (Dang & Zandstra, 2005). Of note, 10^8–10^9 cardiomyocytes may be lost after sublethal MI in humans. The only enrichment method described to date for HESC-CMs used Percoll gradient purification (Xu *et al.* 2002) although others have found this difficult to reproduce. Fluorescence or magnetic sorting based on a cell surface antibody binding would be useful improvements for quantification and selection of HESC-CMs. However, to date few, if any, suitable cell surface protein–antibody combinations have been identified for cardiomyocytes. Genetically marked HESC-CMs, as described in mice (Klug *et al.* 1996; Meyer *et al.* 2000; Muller *et al.* 2000), have also not yet been reported and even if available for experimental use, would be unlikely to be clinically acceptable due to the perceived risk associated with genetic modification.

Characteristics of HESC-CMs. Although functional cardiomyocytes can easily be identified *in vitro* by their beating phenotype, only more detailed interrogation can establish the identity of the specific cardiac cell types generated, their degree of maturity compared with cardiomyocytes developing *in vivo* and whether they possess fully functional excitation–contraction coupling machinery that responds appropriately to pharmacological agents. Primary adult cardiomyocytes do not survive transplantation or beat spontaneously in culture, in contrast to HESC-CMs and primary fetal CMs. On the other hand, beating in culture indicates spontaneous, pacemaker-like activity which may result in arrhythmias if there is electrical 'mismatch' with the recipient heart, as indeed occurred when SMs were transplanted to human hearts. To realize the scientific and therapeutic potential of HESC-CMs, therefore, comprehensive characterization of their phenotype is essential.

Differentiation of human ES cells to the cardiac lineage creates a characteristic gene expression profile (Beqqali *et al.* 2006) reminiscent of both mESC differentiation and the early stages of normal mouse heart development (Fijnvandraat *et al.* 2003). Analysis of HESC-CM RNA and proteins has demonstrated the presence of cardiac transcription factors including GATA-4, myocyte enhancer factor (MEF-2) and Nkx2 transcription factor related locus 5 (Nkx2.5) (Xu *et al.* 2002; Kehat *et al.* 2004; Beqqali *et al.* 2006). Correspondingly, structural components of the myofibres are appropriately expressed. These include α-, β- and sarcomeric-myosin heavy chain (MHC), atrial and ventricular forms of myosin light chain (MLC-2a and -2v), tropomyosin, α-actinin and desmin although in contrast to mouse, heart chamber restricted expression of structural proteins is less well defined in human heart;

MLC-2v, for example, is restricted to the ventricle in mouse and human fetal hearts but MLC-2a is expressed in both atrium and ventricle in humans and not just in atria as in mice (Chuva de Sousa Lopes *et al.* 2006). This implies that using protein or gene expression profiles *alone* to determine the phenotype of HESC-CMs in culture should be done with caution. Antibody reactivity to two members of the tropinin complex, cardiac tropinin T (cTnT), which binds to tropomyosin, and cardiac tropinin I (cTnI), which provides a calcium sensitive molecular switch for the regulation of striated muscle contraction, has been demonstrated. cTnI appears to be truly cardiac specific as antibodies to this protein only react with cells arising from beating and not non-beating regions. In addition, up-regulation of atrial natriuretic factor (ANF), a hormone expressed in both atrial and ventricular cardiomyocytes in the developing heart, has also been observed during cardiac differentiation of HESCs. Moreover, these cells express creatine kinase-MB (CK-MB) and myoglobin (Xu *et al.* 2002). Thus, many of the transcription factors, structural proteins and metabolic regulators of cardiac development are found within HESC-CMs although they also react with antibodies to smooth muscle actin, a protein found in embryonic and fetal, but not adult, cardiomyocytes suggesting a limited degree of maturation (Xu *et al.* 2002). Single HESC cardiomyocytes display various morphologies in culture and may be spindle-shaped, round, or tri- or multiangular, rather than the rod shape of mature cells; sarcomeric immunostaining shows striations in separated bundles, rather than the highly organized parallel bundles, as in human adult cardiomyocytes; and the action potentials determined by patch-clamp electrophysiology show ventricular phenotypes with upstroke velocities ∼10 times lower than those of adult cardiomyocytes (Mummery *et al.* 2003). It is of interest to note that not only are HESC-CMs connected to each other by connexin-43 expressing gap junctions (Mummery *et al.* 2003), they are also capable of forming *de novo* gap junctions with primary human cardiomyocytes. Figure 1 shows HESC-CMs cocultured with primary human fetal cardiomyocytes; injection of the dye Lucifer Yellow into the HESC-CMs results in rapid transfer of the dye via gap junctions, into the underlying primary cardiomyocytes. Counterstaining the cells post-fixation with an antibody recognizing tropomyosin confirms that dye transfer takes place between the cardiomyocytes. This ability to couple with primary cardiomyocytes was not observed in skeletal myoblasts. Despite their immaturity, HESC-CMs may still be useful in understanding the activity of some pharmacological agents in (adult) human CMs, e.g. the L-type Ca^{2+} channel is inhibited by verapramil, indicating that it is already coupled to downstream signalling pathways, as in postnatal CMs. For other purposes, mature human CMs may be required. Possibilities for achieving this in culture range from prolonged cyclic stretch, to forced electrical pacing and biochemical activation of reactive oxygen species (ROSs). ROSs are considered downstream mediators of mechanical stress signals in cells (Hool, 2006). HESCs can provide useful information on the molecular mechanisms controlling early differentiation in the human heart. Analysis of gene expression by microarray

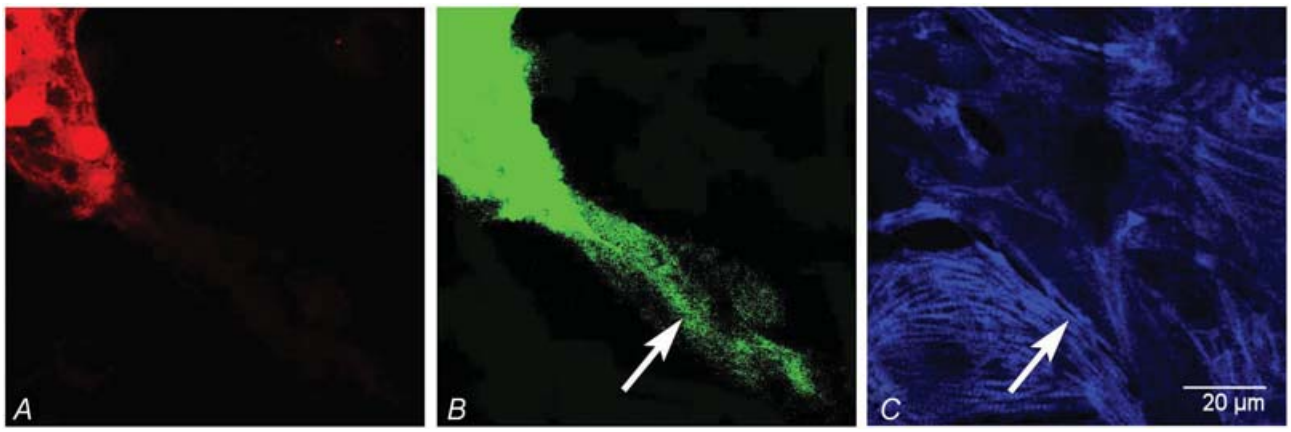

Figure 1. Co-culture of HESC-derived cardiomyocytes with human fetal cardiomyocytes leads to coupling and dye transfer between the two cell types
PKH-26 labelled HESC cardiomyocytes (arrowhead in *A*) in coculture with primary human cardiomyocytes transfer lucifer yellow, which is injected in the HESC-CMs, to the primary human cells (*B*, arrow), indicating the ability to form *de novo* functional gap junctions. The identity of the injected cells, as well as that of the cells to which the Lucifer Yellow transfers, is verified by staining with antitropomyosin antibodies (*C*; arrow indicates same cell as in panel *B*, i.e. positive for both Lucifer Yellow and tropomyosin). This indicates that transfer occurs only between the (tropomyosin-positive) cardiomyocytes. In *A*, the z-section of the CLSM is in the plane of the HESC-CMs, whilst in *B* and *C* it is in the plane of the underlying primary human cells. Magnification ×63.

during HESC-CM differentiation (Beqqali *et al.* 2006) showed that apart from identifying most known cardiac transcription factors and structural protein genes, we observed up-regulation of phospholamban, MEF2C, TBX2 and TBX5 and multiple known genes not previously associated with cardiac development and several unexpected genes enriched or even uniquely expressed in the heart. Some of these are conserved across species and included the synaptopodin like-2 gene, related to myopodin, and SRD5A2L2, a gene primarily known for its function in converting testosterone to its active form, hydroxytestosterone. The restricted expression pattern of this gene in both mouse and human fetal heart is intriguing; further analysis by deletion in the mouse should shed light on its function, not only in mice but also in relation to congenital heart defects in humans. This reflects a more general strategy using HESC for functional analysis and gene mining in human development.

Transplantation of ESC. Whilst promising as a potential new therapeutic strategy, several questions need to be answered before clinical application of HESC-CMs. Assuming they can be produced homogeneously in sufficient numbers, the best way and site to deliver them would still need to be determined. Another important issue is graft rejection. Furthermore, the fate of transplanted ESCs or their derivatives would have to be examined in terms of efficacy and safety. Importantly, several authors report transplantation of mESCs (Klug *et al.* 1996; Behfar *et al.* 2002; Yang *et al.* 2002; Min *et al.* 2002; Himes *et al.* 2004; Hodgson *et al.* 2004; Kofidis *et al.* 2004*a*, *b*; Naito *et al.* 2004; Singla *et al.* 2006), experience with transplantation of HESC-CM is still very limited (Kehat *et al.* 2004; Laflamme *et al.* 2005; Xue *et al.* 2005; Kofidis *et al.* 2006).

Animal models

Rodents have mainly been used for transplantation of ESCs into either uninjured (Klug *et al.* 1996; Behfar *et al.* 2002; Naito *et al.* 2004; Kofidis *et al.* 2004*b*; Laflamme *et al.* 2005) or infarcted (Behfar *et al.* 2002; Yang *et al.* 2002; Himes *et al.* 2004; Hodgson *et al.* 2004; Kofidis *et al.* 2004*a*, *b*; Singla *et al.* 2006; Kofidis *et al.* 2006) hearts. Genetically marked transgenic animals are available and fewer cells are needed for relatively large cardiac grafts. However, larger animals will eventually be indispensable for testing compatibility with human physiology. Studies in mice may not reliably predict generation of arrhythmias by transplanted cells, since at a beating frequency of ∼500 bpm the mouse heart may well override any arrhythmia caused by ectopic pacemaker activity. One groups has specifically created an AV block in a swine model to evaluate the (ar)rhythmogenic potential of HESC-derived cardiomyocytes (Kehat *et al.* 2004).

Methods of cell delivery

Intramyocardial injection of 10 000–500 000 mESCs or 5–150 beating areas from HESCs with a small needle (21–30 gauge, depending on the size of the animal) is the most commonly used technique to deliver ESCs to the heart. Target regions can be either the normal myocardium, the infarcted area or the border zone, or a combination of these. Unfortunately, a variable and often relatively small proportion of the transplanted cells is successfully delivered to and survives in the host myocardium. An alternative approach would be the combination of cells with a (degradable) matrix compound (Kofidis *et al.* 2005*b*) or a cocktail of survival factors that may inhibit apoptosis (Laflamme 2006 personal communication). Such a strategy may have the advantage of both preventing cell loss and forming a temporary support for the thinned infarcted wall. Upscale methods for HESCs are still in development.

Immune rejection

It has been postulated that HESCs, like mESCs (Tian *et al.* 1997), lack MHC protein expression and therefore do not evoke an immune response in the host. However, a recent study showed that HESCs do express MHC class I molecules (Drukker *et al.* 2002) albeit at low levels and expression increased upon differentiation *in vitro,* an effect enhanced by interferon-γ. Transplantation of differentiated ESCs in an *in vivo* model could enhance MHC protein levels in a similar way. On the other hand, the myocardium may be a relatively hospitable environment in terms of immune response (Drukker & Benvenisty, 2004). However, after injury (MI or needle stick manipulation), inflammation occurs which could trigger the recruitment of immunoreactive cells.

Several authors used immune competent, though in some cases syngeneic, wild-type animals and yet reported graft integration. However, direct comparisons of immunodeficient mice with immunocompetent counterparts have supported the view that mESCs do trigger the immune system (Yang *et al.* 2002; Kofidis *et al.* 2005*a*; Swijnenburg *et al.* 2005). In a study with mESCs, cyclosporin was administered to rats in order to prevent immune rejection (Naito *et al.* 2004). HESCs were reported to be less susceptible to immune rejection than adult cells, even when differentiated. Yet, the studies undertaken with HESCs published so far do not reflect confidence in this immune privilege: a combination of cyclosporin and methylprednisolon was administered to pigs (Kehat *et al.* 2004) or immunodeficient rodents were used (Laflamme *et al.* 2005; Kofidis *et al.* 2006). It is not clear whether these were purely preventive measures or previous trials with immune competent hosts had been unsuccessful. Of note is the difference in MHC

protein expression between mESCs and HESCs; their immunogenic potentials are therefore not equivalent. The degree of immunosuppression necessary thus remains to be determined for HESC-CMs. A possible solution for graft rejection is banking of HECSs with a range of HLA profiles or induction of immunotolerance in the recipient (Drukker & Benvinisty, 2004). Nuclear transfer could in principle be used to create patient-specific non-immunogeneic stem cell lines. The nucleus of an adult cell from a recipient patient would be transferred into a donated oocyte. When the oocytes reach the blastocyst stage, HESC lines could be derived that were genetically identical to the donor of the nucleus. Recently discredited reports from Korea have made clear that this has not yet been achieved in humans and is likely to remain highly inefficient and ethically sensitive. A better strategy, also in terms of cost-effectiveness and availability in acute disease, would be to modify ESCs to become universal non-immunogenic cells, for example by knockout of the β-2 microglobulin gene which controls MHC-I presentation (Zijlstra *et al.* 1990).

Functional assessment

The goals of cardiomyocyte cell transplantation are to improve survival rate of patients following MI and to improve cardiac performance. Methods of assessing cardiac function include electrocardiography (ECG) (Kehat *et al.* 2004; Hodgson *et al.* 2004; Xue *et al.* 2005), measurements of cardiac pressure (Yang *et al.* 2002; Min *et al.* 2002), echocardiography (Hodgson *et al.* 2004; Kofidis *et al.* 2004*a*, *b*; Min *et al.* 2002; Yang *et al.* 2002), and electrophysiological mapping (Kehat *et al.* 2004; Xue *et al.* 2005). Magnetic resonance imaging (MRI) has been performed to locate transplanted mESCs (Himes *et al.* 2004). Each of these methods has its own advantages and drawbacks. ECG is inexpensive and widely available, but has no value in assessing dynamic function. Direct pressure measurements provide more information on left ventricular function but are limited unless combined with volume measurements, technically more challenging. However, with the requisite specialized equipment and a skilled investigator, measuring pressure–volume loops with a conductance-micromanometer is an outstanding way to evaluate cardiac performance after ESC transplantation (Lips *et al.* 2004). Echocardiography and MRI are both appealing techniques as they present direct and easily interpretable images of both cardiac kinetics and morphology. Although to date there is significantly more experience with the commonly used echocardiographic visualization, MRI is expected eventually to become the method of first choice because of its higher resolution and accuracy and additional options such as *in vivo* infarct size measurements (Devlin *et al.* 1999).

Tissue engineering

Direct injection of predifferentiated cardiomyocytes may be one approach to a cell-based therapy but the concept of regenerating diseased myocardium by implantation of tissue-engineered heart muscle is also intriguing. The first convincing evidence that heart tissues can be produced at a size and with contractile properties that would lend support to a *failing* heart was recently described by Zimmerman *et al.* (2006). Large, thick rings of force-generating heart tissue were created by seeding fetal rat heart cells with liquid collagen type I and Matrigel in moulds and culturing them at elevated ambient oxygen under autotonic load. The rings were then stacked and stitched onto infarcted rat hearts. After a month, the engineered tissue had survived, coupled to the underlying myocardium and prevented further dilatation of the heart compared to non-contractile control rings without cells or shams. In addition, systolic wall thickening was induced and fractional shortening of the infarcted hearts was improved. The next steps are taking these studies towards using human embryonic stem cells differentiating to cardiomyocytes in tissue engineered structures which may ultimately provide a better strategy for treating heart failure which may be less amenable to direct cell therapy than myocardial infarction.

CMPCs

Increasing evidence indicates that the myocardium harbours several different types of precursor cells (i.e. CPCs and CSCs) that can (re)enter the cell cycle and differentiate to mature cardiomyocytes. Although their name suggests otherwise, the distinction between CPCs and CSCs is not always clear; they may very well represent different stages of the same type of cell or subsets of a more broadly defined cell population found in the heart.

A population of stem cells that possessed the ability to efflux Hoechst was isolated from postnatal mouse hearts. These cells, representing $\sim$1% of the total cell number in the adult heart, were shown to enter the cell cycle when growth of the heart was attenuated and be capable of cell fusion (Hierlihy *et al.* 2002).

In another study, small stem cells with a high nucleus-to-cytoplasm ratio were isolated from hearts of $\sim$2-year-old Fischer rats using fluorescence activated cell sorting and immunomagnetic microbeads (Beltrami *et al.* 2003). These cells were self-renewing, clonogenic, multipotent, and positive for stem cell markers like c-kit, while negative for markers of the blood lineage (Lin), myocytes, endothelial cells and fibroblasts. Notably, 7–10% of the Lin^- $c\text{-}kit^+$ cells showed positive for transcription factors Nkx2.5, GATA-4 and MEF2C, which are expressed early in the myocyte lineage (Lints *et al.* 1993; Lin *et al.* 1997; Watt *et al.* 2004), suggesting that

the population is heterogeneous and contains cells already committed to the cardiomyocyte lineage. *In vitro,* the Lin^- c-kit^+ cells gave rise to immature cardiac myocytes, smooth muscle cells, and endothelial cells. Remarkably, they not only formed new myocardium, but also exhibited improved cardiac function, when injected into the myocardium of infarcted rats (Beltrami *et al.* 2003).

As in the study described above, a cell population expressing stem cell antigen-1 (Sca-1) was isolated from adult mouse hearts (Oh *et al.* 2003). Like the rat Lin^- c-kit^+ cells, these mouse Sca-1^+ cells were negative for cardiac structural genes and blood cell lineage markers as well as haematopoetic stem cell markers. In contrast to the rat cardiac progenitors, however, the mouse cells did not express c-kit but did express high levels of cardiogenic transcription factors like GATA-4, MEF-2C and TEF-1. In response to 5′-azacytidine, the cells differentiated *in vitro* to cardiomyocytes and expressed Nkx2.5, α-myosin heavy chain, β-myosin heavy chain and bone morphogenetic protein (BMP) receptor 1A, which are involved in cardiac development. Sca-1^+ cells delivered intravenously homed to injured myocardium after ischaemia–reperfusion injury, and were found to differentiate as well as fuse with the host cells (Oh *et al.* 2003). Almost simultaneously, an independent study confirmed the presence of a Sca-1^+ stem cell population in the adult mouse heart. This study described the isolation of Sca-1^+ cells that expressed cardiac transcription factors and contractile proteins, and showed sarcomeric structure as well as spontaneous beating when treated with oxytocin (Matsuura *et al.* 2004). This aspect of showing sarcomeric structure is essential in distinguishing reports describing stem cells with a *bona fide* capacity to form cardiomyocytes from those in which structural protein (and gene) expression is found but the cells show no sarcomeric organization. Several reports claiming the ability of mesenchymal cells of various origins (e.g. bone marrow, umbilical cord blood) to differentiate to cardiac myocytes in fact only show that sarcomeric protein is detectable; the cells do not develop sarcomeres and, probably as a consequence, they do not beat spontaneously.

A heterogeneous population of cardiac stem cells was isolated by mild enzymatic digestion of human atrial and ventricular biopsy specimens, and embryo, fetal and postnatal mouse hearts (Messina *et al.* 2004). These cells formed clonal spherical clusters referred to as cardiospheres expressing endothelial as well as stem cell markers, like c-kit, Sca-1 and CD-34. When cultured as single cells on collagen-coated dishes, cardiosphere-derived cells expressed cardiac differentiation markers and, in the case of mouse cells, started spontaneous beating.

Recently, a novel population of cells that are able to proliferate as well as differentiate into cardiac cells has been isolated from rat, mouse and human postnatal hearts. These cells, marked by the expression of isl1 and the absence of both Sca-1 and c-kit, are also abundantly present in the embryonic heart (Laugwitz *et al.* 2005). Whereas isl1^+ cells express early cardiac differentiation markers like Nkx2.5 and GATA-4, they lack transcripts of mature myocytes. When cocultured *in vitro* with differentiated myocytes, they spontaneously acquired myocyte characteristics, like expression of cardiac specific proteins, contractile activity and electromechanical coupling (Laugwitz *et al.* 2005).

Clinical trials

Clinical trials of stem cells for cardiac repair have so far used two types of cell: SMs and BMPCs. BMPCs also include a subpopulation of $CD34^+$, $CD133^+$ cells with haematopoietic and angiogenic potential, referred to as circulating or BMPC-derived EPCs earlier.

Skeletal myoblasts

SMs represent an autologous source of cells that demonstrate a contractile phenotype. As such, they represent a logical target when attempting to repair damaged myocardium. To date, SM cells have only been used in trials of heart failure, and not for acute MI owing to the method of preparation and route of delivery. The use of SMs in humans was first reported by Hagege *et al.* (2003) in a single patient with recalcitrant heart failure who showed symptomatic and echocardiographic improvement following the epicardial injection (i.e. at the time of bypass surgery) of these autologous cells. Subsequently, a Phase I non-randomized study of transepicardial myoblast transplantation during coronary artery bypass grafting showed an improvement in symptoms (e.g. breathlessness) and left ventricular ejection fraction (LVEF), as measured by echocardiography. Unfortunately, four out of the 10 patients treated experienced ventricular arrhythmia. These patients received internal cardioverter defibrillators (Menasche *et al.* 2003). By contrast, no significant ventricular arrhythmias were observed in another Phase I study that recruited 12 patients and again used the transepicardial approach to deliver autologous SMs. This study demonstrated a significant increase in LVEF, as well as improved cardiac viability on positron emission tomography (PET) at 3 months, suggesting that the recovery of myocardial function was associated with an increase in functional cell mass (Herreros *et al.* 2003). A one-year follow-up showed maintenance of the global improvement in cardiac function with no adverse events, including absence of arrythmias (Gavira *et al.* 2006). Nevertheless, preoperative use of antiarrhythmic therapy or simultaneous implantation of internal defibrillators has been used to address these possible safety issues. Menasche, (2004) has recently provided a comprehensive overview

of both the controlled trials and case reports studies. As a result of the inconclusive early studies, a larger scale clinical trial was undertaken but unfortunately the incidence of arrhythmias was high and sufficient cause for concern that the trial was terminated prematurely. The future of this approach to therapy is at present unclear and awaits careful comparison with the outcome (safety and efficacy) of trials with alternative autologous sources.

BMPCs

Variable results have been reported in the first clinical trials, four of which are at present complete. Probably the most important trial is the BOOST trial, which used MRI to determine LVEF and infarct size. In this study 30 patients were treated with BMSC intracoronarily within 1 week of the MI and 30 patients received standard treatment. The early results at 6 months showed a significant improvement of LVEF in the stem cell treated group compared to the controls (Wollert *et al.* 2004). This beneficial effect was in part due to an increased end-diastolic volume in the stem cell group. More importantly, however, by 18 months the control group had undergone a gradual recovery of LVEF and there was no longer a significant difference between the stem cell treated and control groups (Meyer *et al.* 2006). The early modest positive effect that had been observed (6.7%) was therefore apparently not sustained. In the much larger REPAIR AMI trial, which included 95 patients in the stem cell group, a much smaller early beneficial effect was reported (Cleland *et al.* 2006). Although unfortunately, the LVEF was determined by angiography, a technique not ideal for the assessment of LVEF or infarct size (Schachinger *et al.* 2006). A third trial with a neutral outcome was conducted in Norway. In this ASTAMI trial 49 patients received stem cells with a similar number as controls. The treatment was safe and, as in the previous studies, no adverse events were reported. However no beneficial effects of the additional treatment on heart function were observed by nuclear imaging or MRI. Interestingly, a group in Leuven reported on a fourth, smaller, trial ($n = 33$) where no benefit on LVEF was measured by MRI in the stem cell treated group *versus* controls (Janssens *et al.* 2006). However a reduction of infarct size was observed, together with local improvement in of cardiac wall motion. This last study was in fact the only one of the four with a correct placebo control group. Although all four of these trials had shown no adverse effects of stem cell treatment on patients and the consensus was that they appeared safe, enthusiasm was tempered somewhat by a recent report which showed that the application of selected BMSC enhanced atherosclerotic lesion formation in vessels treated with stem cells (Mansour *et al.* 2006). Overall, however, it seems that intracoronary BMSC injections are safe and if they have a beneficial effect it is small and not sustained. For the future, larger trials with proper placebo controls will be necessary for a definitive conclusion. Guidelines for such trials were recently published and some of these trials are ongoing (HEBE in the Netherlands, and BOOST II in Germany) (Bartunek *et al.* 2006).

Selected populations of EPCs, usually derived from peripheral blood of the patient following G-CSF mobilization, have also been included in recent clinical trials. An early study showed no significant difference between using these selected cells and unselected BMCs (Dobert *et al.* 2004), both showing improvement in myocardial viability and perfusion in combination with coronary stenting although no control group was included. Two more recent studies, however, did include a control group. In combination with (drug eluting) stents, significant increases in LVEF (Bartunek *et al.* 2005; Numaguchi *et al.* 2006) were observed following intracoronary infusion of cells in patients with acute MI although there were no differences with controls in patients with 'old' MI. However, in one of the studies (Bartunek *et al.* 2005), cell infusion was associated with increased incidence of intracoronary events. Most of the trials to date assessed cardiac function at 4–6 months after treatment and the long-term outcome has not yet been described.

Conclusions

This review illustrates the complexity of mechanisms underlying recovery from myocardial infarction and the different ways in which transplanted cells might be of benefit. The crucial questions requiring an answer are whether the nature of cells used for transplantation is important, whether their long-term survival in the infarct is essential for sustaining functional recovery or is only necessary transiently to rescue ischaemic tissue, or, indeed, whether effects seen can be attributed to the presence of cells at all. One experiment to test this in animals would be the incorporation of a 'suicide gene' into the genome of the cells to be transplanted, transplanting the cells post-myocardial infarction and at various times thereafter determining what effect removal of the cells by activation of the suicide pathway has on cardiac function. Observation of reduction towards controls would suggest the presence of cells was necessary at that time point; lack of an effect would suggest the requirement for cells had been lost.

The results of several clinical studies have shown that several different approaches relating to cell-type and delivery appear safe and in some cases there is a statistically significant improvement in parameters of cardiac function. This appears to be related to the size of the initial infarct and the time after infarct at which the cells are delivered to the heart. The questions remain of whether this results in a significant biological improvement, and

of which approach to cell therapy – muscle replacement, vascular generation or both – is most likely to improve the prognosis of cardiac patients. It is clear that to date HESCs and CMPCs are the only independently validated sources of human cardiomyocytes but the question is still open on whether they will 'perform' better in the clinic than the probably safer option of autologous bone marrow.

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