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REVIEW

The role of stem cells for treatment of cardiovascular disease

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Abstract. Cardiovascular disease is a global cause of mortality and morbidity. Current treatments fail to address the underlying scarring and cell loss, which are the causes of ischaemic heart failure. Cellular transplantation can overcome these problems and new impetus has been injected into this field following the isolation of human embryonic and adult stem cells. These cells have shown remarkable ability to produce cardiomyocytes and vascular cells *in vitro* and *in vivo*. Initial transplantation studies have demonstrated functional benefits and it is hoped further randomised clinical trials will concur with initial findings. Much basic science remains to be unearthed, such as the signals for homing, differentiation and engraftment of transplanted cells. Further matters of concern are the role of cell fusion and the mechanisms by which transplanted cells improve cardiac function. In spite of initial progress made in stem cell therapy there is still much to be done and we are some way off from achieving the goal of effective cellular regeneration.

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death in Europe, of which nearly half is attributable to coronary heart disease (CHD) (British Heart Foundation 2000). Globally, CVD accounted for one-third of all deaths in 2001 with the World Health Organization estimating 7.2 million from CHD each year (WHO 2002). This situation is expected to become worse, with CVD becoming the leading cause of death in developing countries by 2010 and a predicted 25 million CVD deaths worldwide by 2020 (Chockalingam & Balaguer-Vintro 1999; WHO 2002). These predictions are made taking into consideration the advances in medical treatment and an overall decline in mortality rate in western countries (Reitsma *et al*. 1999; Tunstall-Pedoe *et al*. 1999; Boersma *et al*. 2003). Whilst mortality may be decreasing, the morbidity associated with CHD is increasing as more people survive and grow old. One of the most devastating sequelae of CHD is the development of heart failure, a condition characterized by the symptoms of shortness of breath, oedema and disability. The outlook for this condition has improved in recent

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years, but mortality remains high, especially compared with other diseases. Patients with heart failure have 1-year survival rates that are comparable, if not worse, than patients suffering with cancer. For example, 1 year from diagnosis of breast cancer 92% of patients are expected to be alive compared with only 62% of patients with a diagnosis of heart failure – even with current treatments (Cowie *et al*. 2000; MacIntyre *et al*. 2000; Quinn *et al*. 2001).

Heart failure in the context of CHD is characterized pathophysiologically by loss of functioning cardiomyocytes secondary to ischaemic injury. As cardiomyocytes have a very low potential for repair and regeneration, the ability of pharmacological agents to improve cardiac function is limited as these agents do not address the fundamental issue of cell loss. This has led to the search for a new approach in the treatment of patients with heart failure.

Currently, there are two therapeutic strategies that overcome the problem of cell loss in heart failure. Heterotropic heart transplantation is able to resolve the problems of heart failure and relieve patients' symptoms, however, this treatment option is severely limited by donor organ availability. Although the technique has been used for approximately 30 years, the long-term outcome for such patients is still complicated by problems of organ rejection on the one hand, and contrasted with both infection and development of lymphoproliferative disorders caused by immunosuppressive regimens designed to modulate the rejection process on the other (Miniati & Robbins 2002).

A second approach to the treatment of heart failure that addresses the issue of cell loss, and consequent decrease in heart function, is the concept of 'cellular transplantation'. Ideally, this process allows the replacement of non-functional cardiomyocytes and scar tissue with new fully functional contracting cells, improving cardiac function, and relieving the symptoms of heart failure. This ideal was initially realized with the use of cells derived from neonatal or embryonic tissue. Although the results showed great promise in ability to recover heart function, the future of this source of cells was limited due to ethical considerations and their short supply (Soonpaa *et al*. 1994; Sakai *et al*. 1999; Muller-Ehmsen *et al*. 2002). Recent advances in the understanding of adult stem cell biology have reinstated the concept of cellular transplantation to replace dysfunctional cells.

Adult as well as embryonic stem cells have been shown to differentiate into cardiomyocytes, hence providing a new and viable source of these cells to treat patients. The full potential of each cell type remains to be seen. Embryonic stem cells appear to be truly totipotent, i.e. have the ability to differentiate into any cell type, whereas stem cells from adults appear to have undergone a process of partial differentiation such that they are committed to certain cell lineages. Provisional results have shown that transplantation of either embryonic or adult-derived stem cells can improve cardiac function to some degree in animal models of myocardial infarction. The precise mechanism for this effect remains unclear and potential explanations will be discussed in the following text.

STEM CELL OVERVIEW

Stem cells are defined by their ability to self renew and to form one or more differentiated cell types. Cells with these characteristics can be usefully categorized in a number of ways; anatomically, functionally or by cell surface markers, transcription factors and proteins they express.

One clear division of the stem cell family is between those isolated from the embryo, and known as embryonic stem (ES) cells, and those found in adult somatic tissue known as adult stem cells. Within these categories, stem cells can be further divided, according to the number

of differentiated cell types they can produce. *Totipotent* stem cells are able to form all fully differentiated cells of the body and trophoblastic cells of the placenta. The embryo, zygote and the descendants of the first two cell divisions are the only cells considered to be *totipotent*.

Pluripotent cells are able to differentiate into almost all cells that arise from the three germ layers, but are unable to give rise to the placenta and supporting structures. At around 5 days following fertilization, ES cells that form the inner cell mass of the blastocyst are considered *pluripotent*.

Multipotential stem cells are capable of producing a limited range of differentiated cell lineages appropriate to their location and are usually found in adult tissues. However, the use of the term 'multipotential' may be somewhat redundant, as it appears now that certain adult stem cells, removed from their usual location, transdifferentiate into cells that reflect their new environment. Stem cells with the least potential for differentiation are termed unipotential, an example of which is the epidermal stem cell found in the basal skin layer that only produces keratinized squames. From this initial introduction, it can be gleaned that ES cells are initially the most attractive option when considering the use of embryonic or adult stem cells for cellular therapies given their totipotential. However, their use is limited by ethical considerations, and thus, practically, adult stem cells are of more use. The remainder of this review will consider in greater detail the characteristics of ES cells and adult stem cells and their use in cellular therapy to treat heart disease.

EMBRYONIC STEM CELLS

ES cells were initially isolated in mice and more recently in humans (Evans & Kaufman 1981; Thomson *et al*. 1998; Reubinoff *et al*. 2000). ES cells are derived from the inner cell mass of the pre-implantation or peri-implantation blastocyst-stage embryo. After mouse derived ES cells have been isolated, they can be cultured for prolonged periods in an undifferentiated state in the presence of leukaemia inhibitory factor (LIF) or mouse embryonic fibroblasts (MEFs). In contrast, human ES (hES) cells remain in an undifferentiated state only when cultured on a feeder layer of mitotically inactivated MEFs.

For the safe use of hES cells in clinical scenarios, an important step in the culture of these cells is the removal of xenoproducts such as MEFs from the process of isolation and culture. To this end, some progress has been made to replace MEFs. Thus far, hES have been shown to remain undifferentiated when grown in the presence of either human fetal fibroblasts, adult epithelial cells, foreskin cells or a matrigel/laminin matrix in media conditioned by MEFs (Xu *et al*. 2001; Richards *et al*. 2002; Amit *et al*. 2003a,b).

Undifferentiated hES can be identified due to characteristic markers. These include stage-specific embryonic antigens, SSEA-3, SSEA-4 and the glycoproteins TRA-1–60, TRA-1–81. Undifferentiated ES cells also express alkaline phosphatase, high telomerase activity and the transcription factor Oct-4.

Human ES cells are pluripotent and can be induced into the differentiation process on removal from feeder layers (see Fig. 1). The multicellular aggregates of differentiated and undifferentiated cells that form as a consequence are termed embryoid bodies (EBs) and resemble early post-implantation embryos. Within the Ebs, cellular derivatives of all three primary germ layers can be found (Itskovitz-Eldor *et al*. 2000).

The technique of feeder layer or LIF withdrawal, and formation of EBs has been successfully used to obtain cells with a cardiomyocytic phenotype from mouse and human ES cells

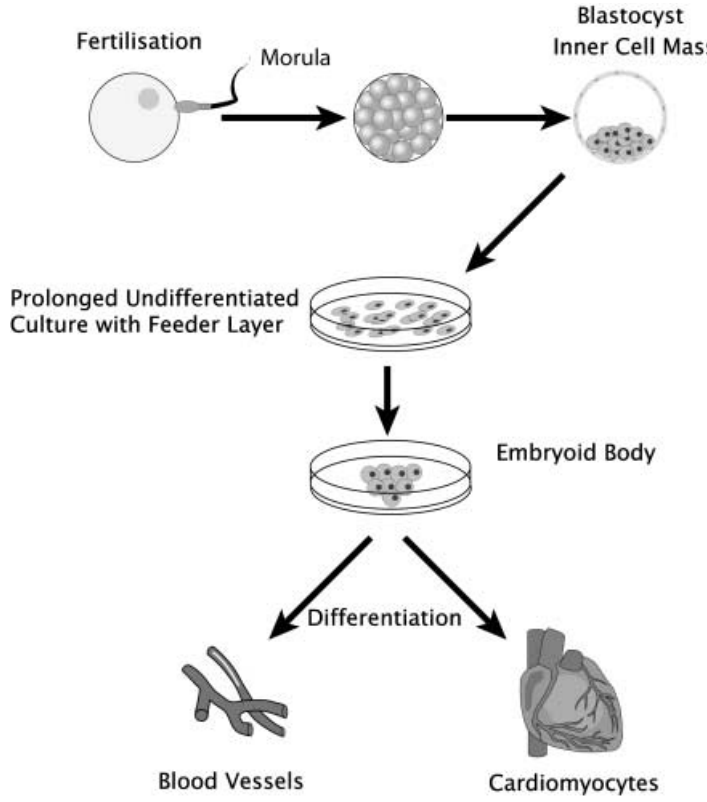

Figure 1. Transdifferentiation of embryonic stem cells into a cardiovascular phenotype. Following fertilised the inner mass from the blastocyst can be cultured *ex vivo* to form multiple cardiovascular cell types.

(Doetschman *et al*. 1985; Kehat *et al*. 2001). After the formation of an EB, most ES cell lines will spontaneously differentiate into a wide variety of cell lineages that include spontaneously beating cells. These beating cells have been extensively characterized and carry a cardiomyocytic phenotype. Morphology and ultrastructure of these cells is organized with sarcomeric structures, formation of intercalated discs, desmosomes and gap junctions characteristic of cardiomyocytes (Westfall *et al*. 1997; Kehat *et al*. 2001). These gap junctions have been demonstrated to function by transference of Lucifer yellow dye between cells and microelectrode array mapping, demonstrating the presence of a functional syncitium with action potential propagation (Westfall *et al*. 1997; Kehat *et al*. 2002). Immunohistochemistry and *in situ* hybridization have demonstrated the presence of appropriate proteins in cardiomyocytes and, furthermore, these are expressed in a time course that is analogous to that seen in cardiomyocytes developing *in vivo* (Guan *et al*. 1999*)*. Normal functioning of the cells is suggested by their spontaneous contraction, but also by their electrophysiological profile and response to pharmacological agents (Wobus *et al*. 1991; Maltsev *et al*. 1999).

Cardiomyocytes formed in EBs are heterogeneous. By examining the electrophysiological profiles of spontaneously contracting cells, three distinct cell lines have been isolated; atrial, ventricular and purkinje cells (He *et al*. 2003). This finding underlines the pluripotential nature of ES cells, however, this attractive property of stem cells is also a hindrance to their potential clinical application. Therapeutic transplantation of cells that retain pluripotential capability can

lead to undesirable effects such as oncogenesis. Equally, transplantation of pre-differentiated cells, such as atrial or non-cardiac lineage cells, into the ventricle could act to destabilize the electrical milieu and lead to arrythymogenesis. Isolation and expansion of specific cells types from EBs is crucial for a therapeutic role to be realized. Two approaches can be applied to this problem, either solely or in combination. One approach, often described as *enrichment*, involves addition of growth factors or other metabolically active agents to the culture medium to preferentially direct differentiation of ES cells to a cardiac lineage. Several factors alone and in combination have been shown to enrich cardiac differentiation – hepatocyte growth factor (HGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), transforming growth factor b1 (TGFb1), platelet-derived growth factor (PDGF), sphingosine-1-phosphate, retinoic acid, 5-azacytidine, vitamin C and over expression of GATA-4 (Grepin *et al*. 1997; Schuldiner *et al*. 2000; Xu *et al*. 2002; Sachinidis *et al*. 2003; Takahashi *et al*. 2003). Besides addition of factors to culture medium, co-culture with visceral-endoderm-like cells helps direct hES cells to differentiate into cardiomyocytes (Mummery *et al*. 2003). Other variables that may also be altered to enhance cardiomyocyte formation from ES cells are the use of different cell lines, culture mediums or feeder layers, timing of EB plating and altering the initial number of cells plated (Wobus *et al*. 2002).

The process of enrichment is yet to yield a collection of cardiomyocytes pure enough to consider for use in clinical therapy. An alternative avenue that promises a purity of cell line necessary for clinical application is to isolate cells after they have differentiated down a cardiac lineage by using a cell marker and separation technique. The key to this process is to use a marker that is specific for the cardiac lineage and ideally able to distinguish ventricular cardiomyocytes from atrial or purkinje cells. One such method involves the transfection of ES cells with a fusion gene of a αMHC promoter linked to a cDNA encoding aminoglycoside phosphotransferase. Following differentiation, selection of cells of cardiac lineage is possible due to expression of the antibiotic resistant gene. Using this protocol, up to a 99% pure cardiomyocyte sample has been obtained (Klug *et al*. 1996). Another method, used to isolate ventricular type cells only, involves the transfection of ES cells with enhanced green fluorescent protein under transcriptional control of ventricular-specific myosin light chain-2v. This reporter gene based approach, in conjunction with fluorescent assisted cell sorting, has allowed isolated collection of ventricular cardiomyocytes (Muller *et al*. 2000). These methods of purification are highly effective, but both insert a foreign epitope into the ES cells that may lead to immunological rejection following transplantation. An alternative strategy that circumvents this problem, and has been applied to hES cells, is the use of a Percoll gradient that separates cells based on their density. This technique has produced a 4-fold enrichment of cardiomyocyte yield, with no undifferentiated hES cells detected following separation (Xu *et al*. 2002). Using these strategies to purify and enrich cardiomyocyte cultures, animal work has been conducted to establish the effect of these ES cell-derived cells.

Transplanted ES cell-derived cardiomyocytes have been shown to engraft in infarcted myocardium and improve cardiac function (see Table 1). Differentiated cardiomyocytes, derived from mES cells, have been transplanted using epicardial injection in myocardial infarction models and in animals with a muscular dystrophy. Results from these studies consistently show a significant improvement in cardiac function using echocardiography or left ventricular pressure transduction. Engraftment of transplanted cardiomyocytes has been confirmed by donor-cell tracking, using transfection with green fluorescent protein, and immunohistochemistry (Klug *et al*. 1996; Behfar *et al*. 2002; Min *et al*. 2002b; Yang *et al*. 2002).

In the failing heart, in addition to the replenishment of cardiomyocytes by ES derived cells, a simultaneous increase in the blood supply is necessary for optimal and prolonged engraftment.

Table 1. Stem cell transplantation experiments in man and animal models grouped by donor cell phenotype

Reference	Host	Injury	Donor cell phenotype	Route of administration	Time after injury	Number of cells	Outcome	Follow-up
Yang *et al*. 2002	Friend leukaemia virus strain B mice	LAD ligation	mES (ES-D3)-derived early differentiated cardiomyocytes, transfected with GFP and ± transfected with VEGF	Epicardial into BZ and infarct 3×10 µl	15 min	3×10^5	Significant increase in function & capillary density. Stem cells transfected with VEGF > increase than stem cells alone > than controls	6 weeks
Behfar *et al*. 2002	Sprague-Dawley/ Wistar	LAD ligation	mES (CGR8)-derived cardiomyocytes	Epicardial into BZ 27G needle	4 weeks	3×10^5	Significant increase in LVEF on echocardiography	5 weeks
Min *et al*. 2000b	Wistar rats	LAD ligation	mES (ES-D3)-derived cardiomyocytes, transfected with GFP	Epicardial into BZ and infarct 3×10 µl	30 min	3×10^4	Significant functional improvement, compared with controls. Engrafted GFP +ve cardiomyocytes seen	6 weeks
Klug *et al*. 1996	Mdx adult mice	None	mES (ES-D3 transfected MHC-neo^r/pGK-transgene) derived cardiomyocytes	Epicardial 30G tuberculin syringe	N/A	1×10^4	Cardiomyocytes engrafted, no measures of function	N/A
Perin *et al*. 2003	Humans	Congestive cardiac failure/IHD	Autologous BM, Mononuclear cells separated, avg 2.4%CD45^{low}CD34$^+$	Percutaneous transendocardial catheter	N/A	$25.5 \pm 6.3 \times 10^6$ cells	NYHA, CCS improved, EF significantly improved 20–29%, SPECT less stress defects	2 & 4 months
Tse *et al*. 2003	Humans	IHD	Autologous BM, Mononuclear cells separated 3.2%CD34$^+$ 27G needle, 16 injections @11 sites	NOGA mapping, Endomyocardial injection via catheter	N/A	1.4×10^7	Less angina attacks, reduced GTN tablet requirement, MRI: improved target wall thickening & motion	3 months
Pak *et al*. 2003	Pigs	LAD distal occlussion	MSC & BM or BM only	Direct epicardial, injection, × 15	1 month	1.5×10^8 MSC, BM N/A	Higher nerve density in MSC group	60 & 96 days
Nishida *et al*. 2003	Dark agouti syngenic rats	LAD ligation permanent	Aspirated BM	Direct epicardial injection, 6×10 µl, 26G needle	Stat	6×10^7	Increase in FS, microvessel density & blood flow & 90 days	7, 30, 60
Strauer *et al*. 2002	Humans	Acute myocardial infarction	Autologus mononuclear cells from BM aspiration, Ficoll gradient, 2.1%CD34$^+$, 0.6% AAC133$^+$	IC catheter, 6–7 injections 2–3 mls per injection, each $1.5–4 \times 10^6$ cells	5–9 days	18×10^6 cells	Repeat cardiac catheter significant reduction in hypo/dys/akinetic segments, improved perfusion on thallium, no significant increase in EF	3 months
Hamano *et al*. 2002	Dogs	LAD ligation, permanent	Autologus BM, density centrifugation	Direct epicardial, 6 injections, 0.1 ml each 27G needle	30 days	12×10^7	Improved wall thickening & density of microvessels in marginal area	30 days some up to 240

Table 1. Continued.

Reference	Host	Injury	Donor cell phenotype	Route of administration	Time after injury	Number of cells	Outcome	Follow-up
Hamano *et al*. 2001	Humans	IHD	Autologus mononulcear cells separated from BM aspiration	Endocardial at CABG, avg 11 injections 0.1 mL each, 26G needle	N/A	5×10^8–1×10^9 cells	60% improved stress tests	1 year
Kobayashi *et al*. 2000	Inbred dark agouti rats	LAD ligation	Unsorted BM	Direct epicardial injection, 6×10 µl PBS	1 h	5×10^6	Siginificant increase in No. of vessels	1, 3 & 7 days
Tomita *et al*. 1999	Sprague-Dawley rats	LV cryoinjury	BM separated with Percoll gradient, fresh, cultured 7 days or 5-azacytidine treated	Direct epicardial injection, 50 µl into centre of scar, tuberculin syringe	3 weeks	10^6	All grps significant increase capillaries, significant increase peak systolic & developed pressure only in BM treated with azacytidine	5 weeks
Orlic *et al*. 2001a	C57BL/6 mice	LAD ligation	Aspirated BM, sorted for $Lin^-c\text{-}kit^+$, from male transgenic eGFP mice	Direct epicardial injection, 2.5 µl into border zones	3–5 h	1.5×10^4–1×10^5	Improved LV haemodynamics, 68% of infarct engrafted with transplanted cells	9 days
Orlic *et al*. 2001b	C57BL/6 mice	LAD ligation	None	G-CSF & SCF 5 days prior & 3 days post injury	N/A	N/A	Increase in survival, EF, & regenerating myocardium seen	27 days
Jackson *et al*. 2001	Irradiated mice	LAD ligation 60 mins	SP cells separated from Rosa C57BL/16 mice, $CD34^{-/low}$, $c\text{-}kit^+$ $Sca\text{-}1^+$	BM transplant	10 weeks prior	2000 for transplant	Engrafted cells of which, 0.02% cardiomyocytes, 3.3% endothelial cells	2 & 4 weeks
Mangi *et al*. 2003	Sprague-Dawley rats	LAD ligation	MSCs $CD117^+$, $CD90^+$, $CD34^-$, transfected with GFP, LacZ or Akt	Direct epicardil injection, × 5 injections into border zone	60 min	2.5 or 5×10^6	MSC with Akt group complete normalization of function, 80–90% regeneration of myocardium	2 weeks
Thompson *et al*. 2003	Pigs	None	Aspirated BM, adherent cells, transfected with GFP	Transendocardial, 15 injections	N/A	N/A	GFP + ve cells found in all animals, no adverse outcome	0–28 days
Gojo *et al*. 2003	C3H/HeJ adult mice	None	BM stromal cells, treated with 5-Azacytidine, $CD34^{low/-}$ $c\text{-}kit^+$,$CD140a^+$	Direct epicardial injection, 31G needle, 10 µl in ventricle or IVC	N/A	10^6	0.25% of cells engrafted	1, 4, 8 & 12 weeks
Shake *et al*. 2002	Pigs	LAD ligation, 60 mins	Aspirated BM, MSC separated	Direct epicardial, 6 injections, 0.5 ml each 30G needle	2 weeks	6×10^7 cells	Significant increase systolic function, Transdifferentiation	2 & 4 weeks
Min *et al*. 2002a	Pigs	LAD ligation	Human MSCs & human Fetal cardiomyocytes	Endocardial injection, BZ	5 min	7×10^6 cells	Transdifferentiation, improved haemodynamics & improved blood flow (microspheres)	6 weeks

Table 1. Continued.

Reference	Host	Injury	Donor cell phenotype	Route of administration	Time after injury	Number of cells	Outcome	Follow-up
Toma *et al*. 2002	CB17 SCID/ Beige mice	None	hMSC, transfected with LacZ	Transdiaphragm epicardial injection, 100 µls 32G needle	N/A	$0.5–1 \times 10^6$	Up to 0.44% engraftment, transdifferentiation	30 mins, 4, 14, 21, 30 & 60 days
Stamm *et al*. 2003	Humans	Myocardial infarction	Autologous, BM aspiration, $AC133^+$ cells separated by MACS	Endocardial at CABG, × 10 injections 0.2 mls, 22G needle	10 days 3 months	1×10^6	No long-term adverse effects, Improved NYHA, Improved LVEF (minimal). Improved perfusion on SPECT	avg 6.5 months
Assmus *et al*. 2002	Humans	Acute myocardial infarction	BM mononuclear cells, 90% endothelial characteristics VEGFR2, CD105, PECAM-1, vWF, VE-Cadherin & CD146	Intracoronary, ballon inflated for 3 ×, 3, 3 mls per patient	4 days	245×10^6, 7×10^6 $CD34^+/CD45^+$	EF improved significant, Reduced WMA, improved CFR, improved viability FDG-PET	4 months
Kocher *et al*. 2001	Athymic nude rats Sprague-Dawley	LAD ligation permnt	BM aspiration $CD34^+$ve separated, Dil labelled	Tail vein injection	48 h	2×10^6 cells	Significant improvement EF	2 & 15 week
Kawamoto *et al*. 2001	Athymic nude rats, Hsd: RH-rnv	LAD ligation, Permnt	Peripheral blood MNCs, Dil labelled	Tail vein injection	3 h	10^6	Significant increase in FS & capillary density, improved regional wall motion, transdifferentiation	28 days
Kamihata *et al*. 2001	ePigs	LAD ligation	Aspirated BM, MNC separated, transfected with GFP	Epicardial injection, BZ & infarct, 25×0.02 mls	1 h	10^8 cells	Significant increase EF, blood flow, vessels on angio & histology, decreased perfusion defects	3 weeks & 12 week
Fuchs *et al*. 2001	Pigs	LCx ameroid implant	Aspirated BM, MNC separated	Transendocardial injection, 10–12 injections 0.2 mls each	4 week	N/A	Improved regional contractility, perfusion & EF	1, 3, 7 & 21 days

BM, bone marrow; BZ, border zone (of infarct); CABG, coronary artery bypass graft; CCS, Canadian cardiovascular score (for angina); EF, ejection fraction; FS, fractional shortening; GFP, green fluorescent protein; IC, intra-coronary; IHD, ischaemic heart disease; LAD, left anterior descending artery; LV, left ventricle; Mdx, muscular dystrophy; mES, murine embryonic stem cell; MNC, mononuclear cell; MRI, magnetic resonance imaging; NYHA, New York Heart Association (score for heart failure); PBS, phosphate-buffered saline; PET, positron emission tomography; SPECT, single photon emission computed tomography; VEGF, vascular endothelial growth factor.

Hence, it is of interest that ES cells differentiate to all cell lines necessary for formation of new blood vessels. Both mES and hES cells spontaneously differentiate to form endothelial and smooth muscle cells *in vitro*, and blood vessel-like structures can be seen within EBs (Vittet *et al*. 1996; Yamashita *et al*. 2000; Levenberg *et al*. 2002). ES cell-derived endothelial cells express endothelial cell markers, including CD31, Flk-1, VE-cadherin, PECAM, Tie-1 and Tie-2. They also take up acetylated-LDL, a functional characteristic of the endothelial cell phenotype. Enrichment and purification strategies, similar to those applied to cardiomyocytes, have been developed for endothelial cells (Yamashita *et al*. 2000; Marchetti *et al*. 2002). A range of transplant studies demonstrate that these cells can form vessels *in vivo* (Yamashita *et al*. 2000; Levenberg *et al*. 2002; Marchetti *et al*. 2002). Encouragingly, human ES cell-derived endothelial cells have been shown to anastomose with host tissue following transplantation in immunodeficient mice and the structures so formed have been found to contain red blood cells (Levenberg *et al*. 2003).

Despite the progress made in transplantation, enrichment, purification, and eradication of xenoproducts in the derivation and culture of human stem cells, several issues still remain. The issue of rejection of donor cells still necessitates the use of immunosuppressive therapy with the accompanying hazards. A way round the issue of immunogenicity of the donor cell may be achieved using techniques such as somatic cell nuclear transfer (SCNT) or parthenogenesis.

SCNT, also known as therapeutic cloning, involves introduction of an adult cell nucleus from the potential recipient, into an enucleated oocyte to generate a cloned embryo (Hochedlinger & Jaenisch 2003). Embryonic stem cells may then be isolated from the resultant blastocyst. ES cells isolated in this way are pluripotent, and differentiated cells such as cardiomyocytes can be obtained (Kawase *et al*. 2000; Munsie *et al*. 2000). Viability of cardiac tissue produced by SCNT has been demonstrated *in vivo* (Lanza *et al*. 2002*)*. The benefit of this process is that cells derived by nuclear transfer are genetically identical to the recipient cells (which would be obtained from the patient requiring treatment), thus eliminating the risk of organ rejection and requirement for immunosuppressive therapy. Alternatively, a similar technique that has the same benefits of SCNT is 'parthenogenesis.' This technique allows formation of an embryo from an egg without sperm. Understanding of this process is not as advanced as SCNT, but pluripotent ES cells have nevertheless been produced. Cardiomoyocytes have been produced *in vitro*, whilst *in vivo* teratomas containing tissue from all three germ layers have formed following implantation in immunodeficient mice (Cibelli *et al*. 2002).

The greatest obstacle to the use of stem cells derived from embryonic tissue relates to the ethical considerations involved. The combined scientific and social barriers preventing hES use in clinical therapy to date have led to consideration of other cell sources for therapeutic transplantation. Recent years have seen adult stem cells take centre stage due to their previously unrealized potential to produce a range of cell types outside of their expected lineage restriction.

ADULT STEM CELLS

Adult stem cells are found in many tissues and organs where they have the capacity to replenish cells that are lost during physiological homeostasis. Unearthing of a hitherto unknown property of some adult stem cells in which they appear to undergo a process of transdifferentiation or exhibit plasticity has led to significant interest in these cells. Plasticity describes a property of adult stem cells whereby they are able to produce specialized cells that are outside of their normal lineage commitment (Poulsom *et al*. 2002). *In vitro* and *in vivo* studies have demonstrated

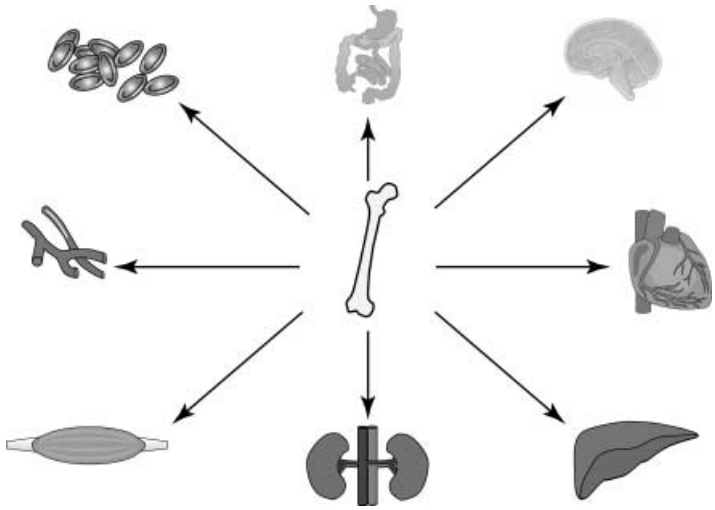

Figure 2. Adult stem cells plasticity, bone marrow derived stem cells have been shown to engraft and transdifferentiate into a variety of cell types other than haematopoietic lineages.

that these cells can transdifferentiate into brain, gut, lung, liver, pancreas, kidney and cardiac cells when placed under specific conditions (Makino *et al*. 1999; Pittenger *et al*. 1999; Alison *et al*. 2000; Mezey *et al*. 2000; Krause *et al*. 2001; Poulsom *et al*. 2001; Deb *et al*. 2003; Ianus *et al*. 2003) (see Fig. 2). Within cardiovascular research, most progress in the use of adult stem cells for cellular transplantation and end organ recovery has been seen with haematopoietic stem cells (HSC), mesenchymal stem cells (MSC) and endothelial progenitor cells (EPC).

HAEMATOPOIETIC STEM CELLS

Haematopoietic stem cells are functionally defined as cells capable of reconstituting and maintaining all blood lineages. For practical purposes, these cells are usually defined with respect to cell surface markers, or ability to efflux Hoechst dye. Cells expressing certain combinations of markers can behave as HSCs. To date, cells expressing $CD34^+$ alone or the combinations Thy-1.1^{lo}Sca-1^{hi}Lineage$^{-/lo}$, Lineage$^{-/lo}$Sca-1$^+$c-kit$^+$, Lineage$^{-/lo}$Sca-1$^+$c-kit$^+$CD34$^-$, and Lineage$^{-/lo}$Sca-1$^+$c-kit$^+$CD38$^+$ have been used successfully in repopulation assays (Spangrude *et al*. 1988; Osawa *et al*. 1996a,b; Randall *et al*. 1996). Using the relevant antibody and fluorescence-activated cell sorting (FACS) or magnetic-assisted cell sorting (MACS) these cells lines can be isolated for research purposes or transplantation (Thomas *et al*. 1999).

So far, *in vitro* studies have been unable to demonstrate the potential for HSC transdifferentiation to a cardiac cell lineage. However, *in vivo* studies have shown transdifferentiation of HSCs to cardiomyocytes and to vascular structures (see Table 1). These studies have also demonstrated improvement in cardiac function. Side population cells, expressing CD34$^{-/LOW}$c-kit$^+$Sca-1$^+$ have been shown to differentiate into cells that bear a cardiomyocytic and endothelial cell phenotype in a mouse model of myocardial infarction (Jackson *et al*. 2001). Furthermore, Lin$^-$c-kit$^+$ HSCs are able to engraft and significantly improve ventricular function following myocardial infarction (Orlic *et al*. 2001a). This potential regenerative capacity has also been

seen with human HSCs in a rat model of myocardial infarction. In this model, intravenous injection of human $CD34^+$ cells lead to a significant reduction in scar tissue formation, improved neovascularization and resulted in an overall improvement in cardiac function (Kocher *et al*. 2001).

An alternative method for HSC delivery to the damaged heart, is to enhance the process of migration and homing from the bone marrow. Mobilization of HSCs using stem cell factor (SCF) and granulocyte colony stimulating factor (G-CSF) before and after myocardial infarction in a mouse model was seen to significantly increase survival and cardiac function. Sections of heart from treated mice revealed newly formed myocytes and blood vessels (Orlic *et al*. 2001b). A second study, in non-human primates, that infused SCF and G-CSF 4 hours post-MI, was able to demonstrate regeneration of vascular structures, with a significant increase in the number of capillaries and arterioles. Correspondingly, a significant increase in blood flow to the infarcted territory was seen *in vivo*, as measured by positron emission topography. In contrast to the former study, using the mouse model, formation of myocytes was not witnessed and there was no benefit to cardiac function (Norol *et al*. 2003). The first human trial of stem cell mobilization to treat CAD used a protocol of intra-coronary granulocyte-macrophage colony-stimulating factor followed by 2 weeks of subcutaneous administration. Treated patients had a significant increase in coronary collateral flow, suggesting new vessel formation (Seiler *et al*. 2001). Further studies of stem cell mobilization are in progress, but results are yet to be published. It is also not clear which of the adult cell lineage is responsible for these changes.

The ability of mobilized stem cells to home to the infarcted heart necessitates a signalling mechanism to attract and retain the cells. Stromal derived factor-1 (SDF-1) has been suggested as a candidate for promoting homing of stem cells. SDF-1 has proven to be essential in HSC homing to bone marrow for reconstitution in irradiated animals (Lapidot & Kollet 2002). Endothelial precursor homing also entails SDF-1 – local injection of SDF-1, in an animal model of limb ischaemia led to significantly increased vasculogenesis and blood flow (Yamaguchi *et al*. 2003). In the heart, SDF-1 is essential to development and SDF-1 expression is significantly up-regulated post-MI (McGrath *et al*. 1999; Pillarisetti & Gupta 2001). More evidence for the role of SDF-1 in stem cell homing has been obtained from an experiment in which SDF-1-expressing cardiac fibroblasts were transplanted into the infarct region of rat hearts 8 weeks after MI. Stem cells were then mobilized with G-CSF. The results showed a significant homing of $CD117^+$ (c-kit) cells, thought to represent endothelial progenitors, to the injured myocardium and a greater improvement in cardiac function when compared with control animals (Askari *et al*. 2003). This suggests that local expression of SDF-1 can be used to enhance adult stem cell homing in animal models of myocardial infarction. The future of this approach in man remains to be seen.

MESENCHYMAL STEM CELLS

Mesenchymal stem cells (MSCs) can be found in bone marrow, muscle, skin and adipose tissue. MSCs are characterized by the potential to differentiate into muscle, fibroblasts, bone, tendon, ligament and adipose tissue (Caplan 1991). MSCs can be obtained from vigorous washing of cells from bone marrow aspirates in culture as MSCs differentially adhere to the plastic culture dish. This technique has been used to isolate MSCs from humans, rats and mice (Krebsbach *et al*. 1997; Colter *et al*. 2000; Barbash *et al*. 2003). Removing cells with HSC or EPC markers by FACS or MACS prior to plating initial cultures further enhances the specificity of this method.

MSCs that have been used in cardiac research are mainly from the heterogeneous population of cells derived from adherent bone marrow cultures as described above. Several studies have demonstrated that these cells can transdifferentiate into cardiomyocytes and vascular-like structures (Makino *et al*. 1999; Min *et al*. 2002a; Shake *et al*. 2002; Toma *et al*. 2002; Gojo *et al*. 2003; Planat-Benard *et al*. 2003; Thompson *et al*. 2003). Unlike ES cells, MSCs do not spontaneously form cardiomyocytes *in vitro*, but require stimulation to proceed along a cardiomyocytic lineage. Thus far, treatment of MSCs with 5-azacytidine, has successfully lead to the development of cells that express a cardiomyocytic phenotype. This phenotype not only displays ultrastructural characteristics of cardiomyocytes, but also the localization of various cardiac-specific markers, including the cytoplasmic proteins troponin, myosin, actin, atrial naturetic factor, as well as transcriptional factors such as GATA4, MEF-2 and Nkx2.5. Spontaneous contraction and action potentials are observed, as are muscarinic, α and β adrenergic receptors. Adaptive functionality is suggested by the appropriate response of these cells to agonists and antagonists with a corresponding rise in intracellular molecules and change in contraction rate (Makino *et al*. 1999; Hakuno *et al*. 2002; Rangappa *et al*. 2002; Planat-Benard *et al*. 2003).

MSCs have been shown to differentiate into cardiomyocytes and endothelial cells *in vivo* when transplanted to the heart in both non-injury and myocardial infarction models. The cells have been strictly characterized by immunohistochemistry and positively stain for cardiac and endothelial specific markers, as well as gap junction proteins. (Wang *et al*. 2000; Rangappa *et al*. 2002; Shake *et al*. 2002; Toma *et al*. 2002; Gojo *et al*. 2003). Myocardial function and capillary formation are significantly increased in experimental groups treated with MSCs when compared with controls (see Table 1) (Mangi & Dzau 2002; Tomita *et al*. 2002; Davani *et al*. 2003). The ability of MSCs to transdifferentiate into specialized cells that improve function of the failing heart makes MSCs a realistic option for cellular transplantation. This is further underlined by the relative ease by which they can be maintained and expanded in culture. The ability to grow MSCs in culture also makes them a promising target for gene transduction. The value of combining gene therapy with cellular transplantation has already been demonstrated in a rat myocardial infarction model. Rat MSCs were isolated, expanded *ex vivo* and retrovirally transduced to over-express Akt1, a mediator of survival signals and glucose metabolism. These cells were then transplanted into the heart of the recipient animal one hour following myocardial infarction. When compared with transplantation with standard MSCs, there was a significant decrease in collagen formation and inflammation – processes that may well be detrimental to recovery of cardiac function. Furthermore, 80–90% of lost myocardium was regenerated and function was completely normalized (Mangi *et al*. 2003). Such combination therapy has yet to be used in a clinical setting.

However, the use of bone marrow stem cells has already been tested in the setting of human heart disease. Unlike animal studies, in which attempts have been made to study specific cell lines, early clinical trials have tended to use a mononuclear cell fraction isolated from bone marrow aspirates. This fraction contains HSCs, MSCs and EPCs, and this should be kept in mind when considering the results of clinical studies. Mononuclear cells have been delivered via several routes, including intra-coronary alone or combined with angioplasty and stenting following acute MI. So far, small numbers of patients have been studied with limited control groups. However, the therapy appears to be safe and functional benefits have been recorded, with reduction in wall motion abnormalities and improvements in left ventricular ejection fraction and myocardial perfusion (Strauer *et al*. 2002). Similar results have been generated by mononuclear cell administration via direct endocardial injection concomitant with coronary artery bypass (Hamano *et al*. 2001). A third technique – transendocardial injection of bone marrow-derived mononuclear cells – has been used to treat patients with chronic severe CAD and heart failure.

This method has also produced encouraging results, with improved cardiac function and patients experiencing less symptoms, as well as being able to reduce their cardiac medications (Perin *et al*. 2003; Tse *et al*. 2003). All trials published to date have been open label and small in size. The promise of these initial findings must be confirmed by larger randomised controlled trials before routine use of adult stem cell therapy can begin.

ENDOTHELIAL PROGENITOR CELLS

Successful cellular therapy for cardiac regeneration will require transplantation of functioning cardiomyocytes and concomitant generation of an adequate blood supply. Endothelial progenitors cells (EPCs) can contribute to tissue revascularization and can be isolated from adult bone marrow or from the peripheral circulation (termed circulating endothelial progenitor cells – CEPs). Adult-derived EPCs and CEPs can be distinguished from mature endothelial cells by a functional *in vitro* assay due to their high proliferation rate. Temporally, they form late-outgrowth colonies in which the cells are mainly EPCs and CEPs, and can form colony-forming unit-endothelial cells (Lin *et al*. 2000; Rafii & Lyden 2003).

Mature endothelial cells, EPCs and CEPs share several endothelial specific markers. However, only EPCs and CEPs express AC133 (CD133) (Miraglia *et al*. 1997; Yin *et al*. 1997). Cells expressing $CD133^{+}VEGFR2^{+}$ can proliferate *in vitro* to form mature endothelial cells (Gehling *et al*. 2000; Peichev *et al*. 2000). Human CEPs have also shown potential to differentiate to cardiomyocytes. When co-cultured with neonatal rat cardiomyocytes, human CEPs formed cells with a cardiomyocytic phenotype, as defined by positive staining for cardiac specific markers such as troponin, atrial naturetic peptide and MEF-2. Functional gap junctions were also demonstrated with transfer of Lucifer yellow dye and calcein between the cells (Badorff *et al*. 2003).

EPCs and CEPs play an important role in neovascularization *in vivo* (Asahara *et al*. 1999). Circulating EPCs are mobilized in response to organ ischemia, trauma and acute myocardial infarction (Takahashi *et al*. 1999; Gill *et al*. 2001; Shintani *et al*. 2001). The increase in CEPs post-MI is mirrored by a rise in the growth and migratory cytokine VEGF-A, and suggests a role for this factor in the mobilization of progenitor cells (Rabbany *et al*. 2003). Also, 3-hydroxy-3-methylglutaryl co-enzyme A (HMG-CoA) reductase inhibitors (statins) have been shown to augment mobilization of EPCs. This important observation may provide an alternative mechanism by which statins decrease morbidity and mortality in patients with ischaemic heart disease (Dimmeler *et al*. 2001).

Transplantation of EPCs and CEPS has been shown to promote neovascularization of the ischaemic heart and improve function (see Table 1). Transdifferentiation to endothelial cells, smooth muscle cells and cardiomyocytes has been characterized by immunohistochemistry (Kawamoto *et al*. 2001; Kocher *et al*. 2001; Yeh *et al*. 2003). In animal models of MI, transplantation of EPCs or CEPs causes a significant increase in capillary density, regional blood flow, and collateral formation in the ischaemic heart. In addition, cardiac function is also significantly improved following transplantation (Kawamoto *et al*. 2001; Kocher *et al*. 2001; Kamihata *et al*. 2002; Kawamoto *et al*. 2003). Encouraging results such as these have led to human clinical studies of EPC transplantation. Two studies have been completed to date; EPCs or CEPs were transplanted either intra-coronary following acute MI or endocardially at the time of coronary bypass surgery. There have been no adverse outcomes as yet, and both studies were able to show an increase in cardiac function with improved myocardial perfusion (Assmus *et al*. 2002; Stamm *et al*. 2003).

WHERE NOW?

Adult and embryonic stem cells are showing great potential for the treatment of cardiovascular disease, in particular ischaemic heart disease and heart failure. Results from animal studies and initial human trials are encouraging and have prompted the development of larger clinical trials. However, important aspects of stem cell biology and the transplantation process remain unresolved.

Currently, one main area of debate is the role of cell fusion in adult stem cell transdifferentiation. The possibility of stem cells acquiring a new phenotype by fusion with a differentiated cell type rather than by transdifferentiation into the new cell type by themselves has been demonstrated *in vitro* (Terada *et al*. 2002; Ying *et al*. 2002). Recently, this phenomenon has been used to explain the development of the hepatocyte phenotype from bone marrow-derived progenitor cells (Vassilopoulos *et al.* 2003; Wang *et al*. 2003). This finding begs the question as to whether cell fusion is the process that explains progenitor-derived cells expressing cardiomyocytic and endothelial cell phenotypes as discussed in this review. The possibility of cell fusion has not been addressed in the majority of stem cell transplant studies and future publications must examine this with rigour if the mechanisms of cellular transplantation are to be understood.

Recent studies have started to try and resolve the issues of cell fusion versus differentiation, using techniques such as a cre/lox recombination system to detect cell fusion. This method has been used in a mouse model, in the presence of ischaemia, where the overall engraftment of transplanted stem cells to the heart was about 3%. Half of the engrafted cells were the result of cell fusion events, the other half were felt to represent transdifferentiation and expressed appropriate cardiomyocytic markers. In the absence of ischaemia in this model, stem cell fusion with cardiomyocytes was seen at a very low level (Alvarez-Dolado *et al*. 2003; Oh *et al*. 2003). However, in contrast to these findings, quantification of DNA failed to reveal cell fusion in a rat myocardial infarction model following stem cell transplantation (Beltrami *et al*. 2003). Human heart sections from female patients who had received male bone marrow transplants were found to contain cells that carried the Y chromosome suggesting donor origin. These donor cells displayed a cardiac phenotype which was shown to result from a process of transdifferentiation rather than cell fusion (Deb *et al*. 2003). Interpretation of these conflicting results is difficult and it remains to be seen what the true extent of cell fusion is and whether it is beneficial or detrimental. It is also of note that several of these studies have postulated the existence of a native adult cardiac stem cell. This was first suggested after demonstration of replicating cells in the adult heart in pathological states (Kajstura *et al*. 1998; Beltrami *et al*. 2001). Two potential sources for native cardiac stem cells have been suggested. Firstly, these cells may have their origins in the bone marrow from which they are released and engraft in the heart, either as a low-level process of ongoing renewal, or in response to injury (Deb *et al*. 2003). Alternatively, it has been suggested that these cells represent a local cardiac stem cell population. The latter theory has been proposed following the isolation of $lin^{-}c\text{-}kit^{+}$ cells and $Sca\text{-}1^{+}$ cells from heart tissue which were found to be self-renewing, clonogenic and multipotent. When transplanted into an animal myocardial infarction model, these cells appeared to recover heart function. Although these 'cardiac stem cells' did not express markers suggesting a haematopoietic or endothelial lineage, there is still the possibility these cells were derived from other sources such as the bone marrow (Beltrami *et al*. 2003; Oh *et al*. 2003). Isolation of a truly local pluripotent cardiac stem cell would change our understanding of the native homeostatic processes in the heart and may provide us with the ultimate cell type for therapeutic cardiac regeneration.

For therapeutic cellular transplantation to occur, many clinical problems remain to be resolved. We must define which patient groups are suitable for this therapy and which stem cell

or cell types are the most effective given the underlying pathology. The optimum timing and method of delivery still remain to be determined and may have a significant bearing on the beneficial outcome of cellular transplantation. Long-term side-effects of treatment are unknown as most of the clinical studies are very recent.

Pressing scientific problems are also unresolved. The mechanism by which transplanted stem cells improve cardiac function is not understood. It was initially assumed that stem cells might improve cardiac function by undergoing differentiation into replacement cardiomyocytes or vascular cells to take on the role of non-functioning tissue. Stem cell-derived cardiomyocytes would therefore improve function by contributing mechanically to contraction, whilst stem cell-derived endothelial cells would improve blood supply by a process of neoangiogenesis. However, direct evidence for these assumptions is poor and provides sufficient doubt to allow alternative theories for the mechanism of functional benefit. These include cell fusion and other mechanisms such as release of local factors modulating cell division, apoptosis, extra cellular matrix homeostasis or metabolic efficiency. The cellular signals and mechanisms involved in homing, engraftment and differentiation of stem cells are partly established for ES cells, but on the whole remain undiscovered for adult stem cells. Understanding of these processes will increase the potential benefits of stem cell therapy. Finally, as mentioned earlier, immunomodulation strategies and methods of removing xenoproducts from the culture and isolation of stem cells are in their infancy, and need more work.

In summary, in spite of the exponential growth of interest and initial progress made in stem cell therapy for treatment of cardiovascular disease, there is much to be done and we are still some way off from achieving the ultimate goal of effective cellular regeneration.

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