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Engineering cardiac healing using embryonic stem cell-derived cardiac cell seeded constructs

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1. ABSTRACT

Myocardial infarction is one of the leading causes of death in industrialized nations. Recent advances in tissue engineering and cell biology have changed our understanding of regenerative activities in the infarcted heart and have raised considerable hopes for novel therapeutic approaches to treat patients. Studies have shown that cell transplantation results in small improvements in the infarct area and while these therapies hold promise, significant challenges remain in order to increase both cellular engraftment efficiencies and transplanted cell function. Robust cardiac healing will require appropriate revascularization of the infarct site, mechanical recovery of damaged tissue and electrophysiological coupling with native tissue. Embryonic stem cells, uniquely, have the potential to generate bonafide cardiomyocytes and other derivatives which should contribute toward these multifaceted requirements. Guiding embryonic stem derived cells to support healing and regeneration of heart tissue using tissue engineered constructs may provide advantages over direct cell transplantation, including replacement of damaged infrastructure, temporary support for transplanted cells, and control of size, shape, strength and composition of the graft.

2. INTRODUCTION

Heart disease is the leading cause of death in industrialized nations (1) and hospitalization rates for ischemic heart disease, acute myocardial infarction (MI), and congestive heart failure (CHF) continue to increase. In the United States in 2003, 7,200,000 people experienced acute MI and 5,000,000 were diagnosed with CHF (2). CHF is the major cardiovascular disorder with increasing mortality rates (3, 4) and accounts for 6% of deaths from cardiovascular disease in the United States (2). Both CHF and the damage associated with MI are characterized by non-contractile scar formation attributed to cardiomyocyte necrosis. After an ischemic episode, the human heart cannot undergo significant regeneration since adult cardiomyocytes are terminally differentiated and do not typically undergo mitosis. While it is generally accepted that adult cardiomyocytes can synthesize new DNA there is considerable debate surrounding this phenomena and the frequency of these events is small at best (5). As a result, cell loss in the adult heart is essentially irreversible and leads to reduced cardiac function.

Traditional therapies to treat patients who survive an extensive myocardial infarction and develop advanced heart failure are limited. While some patients can benefit

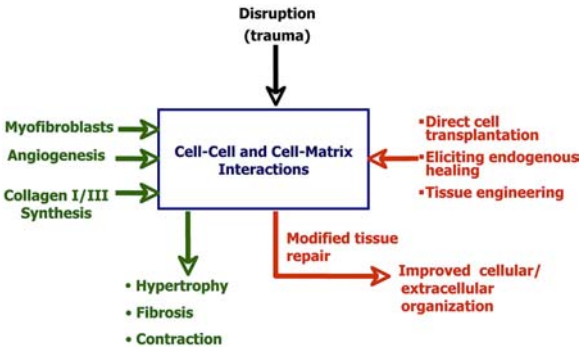

Figure 1. Representative schematic of modified tissue repair after an ischemic episode. Following trauma (black), a normal healing response (green) involves the migration of myofibroblasts to the site of injury. These cells are responsible for the deposition of collagens I and III. This resulting scar tissue does not possess the appropriate mechanical properties and thus the result is tissue contraction and cardiomyocyte hypertrophy. Altering the normal healing mechanisms by introducing either a tissue engineered construct or an injection of dissociated cells can result in a process of modified tissue repair thus helping the organ to heal.

from left ventricular assist devices (LVADs), these devices serve only as a bridge to transplantation and do not provide a long-term solution. Currently, the only effective treatment for patients suffering from severe heart failure is organ transplantation. However, organs are in great demand and donor supply continues to decline, widening the gap between need and availability. In combination with an aging population and an increase in associated vascular disease, the need for alternative therapies is reaching an all time high.

Tissue engineering is a field that aims to create, repair or replace tissues or full organs by integrating the concepts of cell biology, biomaterials and medicine (6). Tissue engineering strategies could revolutionize the way physicians treat patients who are reaching the end stages of congestive heart disease by providing alternatives to full organ transplantation, thus improving the quality of life for millions of people. The purpose of this review is to highlight some of the recent advances in the field of cardiac tissue engineering with an emphasis on embryonic stem cells as a possible source of therapeutic cells.

3. MYOCARDIAL INFARCTION AND REPAIR

3.1. Myocardial infarction and heart failure

In order to maintain the structural integrity of the heart following a myocardial infarction (MI), a reparative process of fibrosis takes place in the area of ischemic damage. The normal healing response involves an inflammation phase, followed by the formation of granulation tissue, and concludes with scar tissue formation (7, 8). White blood cells, predominantly neutrophils, are recruited to the site of injury by cytokines and growth factors. Monocyte derived macrophages locate to the wound site where they are responsible for clearing the infarct zone of necrotic tissue. They also recruit cells such as fibroblasts, endothelial cells, and other cells to create the granulation tissue. Subsequently this tissue is replaced by collagens type I and III. Post infarct healing is usually considered complete 6-8 weeks following MI (7). The scar tissue is decellular cross-linked collagen that resists deformation and rupture. The increased deposition of ECM proteins appears to lead to the separation of myocytes which results in the loss of directed and coordinated beating at the site of the infarct (9).

3.2. Approaches to repair damaged heart tissue

Replacement and regeneration of functional cardiac muscle after an ischemic insult to the heart could be achieved by implanting exogenous donor-derived or allogeneic cells, such as: fetal or embryonic cardiomyocyte precursors, bone marrow-derived stem cells, or skeletal myoblasts. To become a feasible and effective therapy, cell transplantation into the heart requires survival of engrafted cells, integration with the host cardiac cells, as well as electrical and mechanical coupling between host and graft cells (3, 10-12). Simultaneous myocardial revascularization is also required to ensure viability of the repaired region and to prevent further scar formation (13). Numerous studies have supported the concept that cell transplantation can, at least transiently, improve aspects of cardiac function (14); however, direct cell transplantation suffers from several limitations including poor cell survival, appropriate cellular function and cell sourcing. Tissue engineered constructs, in combination with cellular components or growth factors could facilitate cell transplantation through temporary support for transplanted cells. Moreover, a construct provides the option to control parameters such as size, shape, strength and composition of the graft.

A major challenge of extending the goal of cardiac tissue engineering to a full restoration of heart function is that this requires interventions into the fundamental mechanisms of tissue repair and regeneration and any therapy, whether cell based or not, should modulate cellular fate responses at a molecular level (Figure 1). Current strategies for achieving this goal can generally be divided into three broad categories: 1) direct transplantation of cells into the damaged tissue, 2) therapies targeted at eliciting endogenous healing (e.g. growth factor introduction), and 3) tissue engineered constructs in combination with either of 1 or 2. Clearly, for robust tissue regeneration the appropriate milieu must be provided for both implanted and resident cells. A guided healing process incorporating an appropriate construct and *bonafide* cardiomyocytes or their progenitors is particularly promising. Embryonic stem cells (ESC) are an exciting candidate cell source for cardiomyocytes and the other cell types required for cardiac regeneration. This review will focus on progress in cardiac tissue engineering strategies that incorporate (or could incorporate) embryonic stem cells as a source of differentiated progeny. This cell source currently meets the following criteria; easy to isolate and proliferate (thus able to generate cell the cell mass required for tissue replacement); able to integrate appropriately into host tissue; able to differentiate towards the adult phenotype in the host or be fully differentiated at the time

Table 1. Comparative mRNA expression and immunoreactivity of fetal, adult and ES cell derived cardiomyocytes

		Fetal	Adult	ESC-derived	ES cell line	Ref
Contractile protein expression	Actin	Skeletal > Cardiac	Cardiac	Skeletal > Cardiac	Mouse R1, mRNA expression	174
	MHC	beta > alpha	Alpha	Beta > alpha	Mouse R1, mRNA expression	174
	MLC	2v	2v	2v	Mouse R1, mRNA expression	174
	Desmin	+	+	+	Mouse D3, immunoreactivity	59
	Nebulin	-	-	-	Mouse D3, immunoreactivity	59
	ANF	+	±	±	Mouse D3, immunoreactivity	59
Alpha 1c L-type calcium channel $I_{,ca}$		N/A	+	±	Mouse P19, mRNA expression	175
Scn5a heart specific calcium channel $I_{,ca}$		N/A	+	±	Mouse P19, mRNA expression	175
ERG delayed rectifier potassium channel, I_{Na}		N/A	+	±	Mouse P19, mRNA expression	175

Table 2. Comparison of cell types for cellular cardiomyoplasty

Cell type	Availability	Robustness/ survival post implantation	Cell integration with host	Fully differentiated after implantation	Mechanical and electrical coupling	Cell contribution towards active contractile performance
Skeletal myoblasts	Excellent	Good	Formation of myotubules in some cases	No	No	Yes to some degree
Cardiomyocytes	Poor	Cells sensitive to ischemia	Debated	Fetal phenotype remains after implantation	Yes to some degree	Yes to some degree
Adult progenitors	Poor	Not applicable	Poor, limited numbers found after infarct	Whether it is cell fusion or *de novo* differentiation is debated	Not yet assessed	Not yet assessed
Bone Marrow	Poor [1]	Good	Good	Debated	Yes to some degree	Yes to some degree
ESCDC	Excellent	Good	Good	Yes	Yes	Yes

[1] While bone marrow is relatively easy to obtain, the percentages of cells which are thought to contribute to improved cardiac function is extremely low HSC 1-2% and MSC 0.5%. ESCDC: embryonic stem cell-derived cardiomyocytes

of implantation; able to achieve mechanical and electrical coupling with the host tissue; able to contribute to active contractile and structural cardiac performance.

4. CARDIOMYOCYTES

The current goal for patients who are suffering from impaired cardiac function is to improve left ventricular function following an MI. A more aggressive goal for the future is full restoration of heart function through regeneration. Cardiomyocytes (or their progenitors) are an ideal cell type for transplantation and ultimately will be one of the cell types required for restoration of full heart function due to their inherent electrophysiological characteristics (15).

Critical to the use of cardiomyocytes for clinical therapy will be the ability to produce appropriate numbers of transplantable cells. The properties of cardiomyocytes have been extensively studied. They have been isolated and cultured at various developmental stages from many species including humans (16, 17). It has been observed that cultured cardiomyocytes adapt to *in vitro* culture and therefore this environment may not reflect *in vivo* capacities (18-22). Some investigators have expressed the concern that once in culture cardiomyocytes may "de-differentiate" and reverse to a fetal phenotype (23-26) while others are less convinced that this phenomena occurs (27, 28) and the concept remains controversial. Table 1 summarizes some of the properties of adult, fetal and embryonic stem cell derived cardiomyocytes and Table 2 summarizes some of the key properties of various cell types used for cellular cardiomyoplasty.

Cellular cardiomyoplasty has been the focus of intensive research over the last decade. Li *et al.* were the first to show that fetal cardiomyocyte transplantation improved cardiac function and minimized scar tissue formation in an injury model where a metal rod cooled with liquid nitrogen was applied to the left ventricular wall for 1 minute (29). Other studies have demonstrated the feasibility of cellular cardiomyoplasty in larger animal models (30, 31). Leor *et al* have also demonstrated the benefits of this therapy with human fetal cells. They injected fragments of human or rat fetal ventricles into the scar of 7-24 day-old reperfused myocardial infarcts in rats and showed survival of grafted tissue up to 65 days post transplantation. Even so, staining for alpha-actin revealed that the majority of cells were positive for this marker indicating a persistent fetal phenotype and lack of differentiation into an adult-like cell in this model. These studies indicate that the maturation of fetal and neonatal cardiomyocytes after transplantation remains incomplete with respect to morphological characteristics and expression of contractile proteins, which could lead to long-term engraftment problems and lack of electro-mechanical coupling. However, other investigators have shown that engrafted cardiomyocytes were highly differentiated. In one of the earliest studies Soonpa *et al* grafted fetal cardiomyocytes isolated from transgenic mice. Two months following the procedure grafted cells were indistinguishable from host myocytes (32). Vascularization and angiogenesis of new tissue has also been reported (33). However, it is still unknown whether grafted cells will repair the damaged tissue to the extent necessary during periods of increased mechanical and oxygen demand. As well, studies with longer follow up times have shown substantial separation between host and graft. The reader is directed to

Reffelmann *et al* for an excellent review of cardiomyocyte transplantation studies(3).

While all of these studies show promise for this type of therapy and some benefits to cellular transplantation post infarction have been shown, their effect might not be enough to improve heart function to the desired extent mostly due to the death of transplanted cells by ischemia (34-36). Moreover, most studies have examined the properties of ventricular myocytes although there are clear differences between these cells and atrial myocytes. Ultimately, the type of myocyte required for therapy may depend on whether the injury was sustained in the atria or the ventricles.

4.1. Adult progenitors

Until recently it was generally accepted that the heart did not have a population of resident progenitors. Several groups have challenged this dogma and the isolation of resident cardiomyocyte progenitor populations from the adult heart has been reported. Beltrami *et al* have reported cells expressing c-kit (37, 38) that, upon injection into the infarcted myocardium may be capable of differentiating into cardiomyocytes, smooth muscle cells and endothelial cells. Oh *et al* have isolated a similar progenitor population that expressed Sca-1 (39, 40). Two weeks post injection into the infarcted myocardium, these cells expressed several cardiomyocyte markers such as actin and cardiac troponin I. Interestingly, their study found that approximately half of transplanted cells appeared to have fused with native myocytes while the other half had differentiated without fusion. The concept of cell fusion vs. differentiation is highly debated. Other groups have isolated cells by Hoeschst dye efflux or by their expression of LIM homeodomain transcription factor islet-1 (isl1) (41-43).

4.2. Embryonic stem cell derived cardiomyocytes

In the last decade embryonic stem cells have emerged as one of the most promising cell sources for cardiac tissue engineering and regenerative medicine. *In vivo*, the epiblast forms three primary germ layers, the ectoderm, the mesoderm and the definitive endoderm. ESC are typically derived from the inner cell mass of the preimplantation blastocyst (44, 45). At this stage of development the inner cell mass (ICM) consists of a small group of undifferentiated cells (46). ES-like cells have been derived from several species, including mouse, rats, chickens, rabbits, primates and human embryos (23, 47-50).

4.2.1. Mouse embryonic stem cells

The most extensively studied ESC are those of mouse origin. These cells are isolated and inhibited from differentiation by the addition of leukemia inhibitory factor (LIF) (51), and factors in serum or BMP-4. Mouse ESC (mESC) can be encouraged to differentiate by the removal of LIF and by allowing cultures to overgrow, by plating cells in suspension on non-adhesive substrates (such as bacteriological grade culture dishes) in "hanging drop"systems (to control cell number and for cell-cell contact) (52-54) or in low substrate adhesion conditions such as in methylcellulose (55, 56). After a few days *in vitro*, suspension-grown cells aggregate into irregular clumps termed embryoid bodies (EB).

Mouse ESC-derived embryoid bodies have been used to study tissue-specific development, including cardiomyogenesis. Patterns and timing of gene expression during EB differentiation are similar to the pattern in the early mouse embryo. The patterns and efficiency of differentiation can be controlled by altering parameters such as ESC density, media components (i.e. high or low glucose concentration) and amino acids, the addition of growth factors and extracellular (ECM) proteins, pH and osmolality (57). Cardiomyocytes readily differentiate from aggregates composed of 400-800 starting cells that form in the presence of high concentrations of serum. These cells display properties similar to those observed in cardiomyocytes isolated from primary cultures or *in vivo*. They have been shown to express cardiac specific genes and characteristic sarcomeric structures (58). Murine ESC-derived cardiomyocytes express alpha- and beta- cardiac myosin heavy chain, alpha-tropomyosin, myosin light chain 2v (MLC-2v) and atrial natriuretic factor, phospholamban and type B natriuretic factor (59). They demonstrate cardiac specific ion currents and the expression of membrane-bound ion channels. These cells develop spontaneously or could be induced to differentiate by factors such as di-methyl sulfoxide (DMSO) (52), oxytocin (60), retinoic acid (RA) (52), dynorphin B (61), cardiogenol derivatives (57) , hepatocyte growth factor (HGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) (62), transforming growth factor b1 (TGFb1), platelet derived growth factor (PDGF), sphingosine-1-phosphate, 5-azacytidine, vitamin C and over expression of GATA-4 (63, 64). Low frequency magnetic fields have also been shown to induce Nkx2.5 and GATA-4 mRNA expression, two factors linked to the onset of cardiogenesis (65). Although these properties of ESC-derived cardiomyocytes appear uniform, we and others have shown that the cardiac differentiation potential can varies with different parent cell lines (66). While D3 cells result in higher yields of MF20+ (a cardiomyocyte marker) cells, the overall number of cells produced in the culture is significantly lower than R1 cells (67).

Mouse ESC-derived cardiomyocytes show electrophysiological properties that are characteristic of primary myocardium and subsequently differentiate into atrial, ventricular, Purkinje and pacemaker-like cells. Strategies to control the emergence of these different cell types may enable studies to determine the most appropriate cell for transplantation as well as the depletion of inappropriate cell types. This is of particular concern because the transplantation of differentiated cells, such as atrial or non-cardiac lineage cells into the ventricle could lead to destabilization of the electrical milieu and lead to arrhythmias (64).

The use of cardiac-restricted promoters to select cardiomyocytes from differentiating ESC has been shown by various groups (59, 67). In the one of the earliest studies, Field's group injected highly purified mESC-

derived cardiomyocytes into the ventricular myocardium of adult dystrophy mice. These cells were selected using the alpha-MHC gene promoter combined with the neomycin resistance gene. Following differentiation, a neomycin analogue was added to the cultures to select for cardiomyocytes. These cells were found to be present in the graft for at least 7 weeks post transplantation. No tumor formation or significant immune responses were observed. Xiao's group has reported improved left ventricular function in post-infarcted rats after transplantation of "beating cells" isolated from mESC (68). Expression of sarcomeric alpha-actin, alpha-myosin heavy chain, and troponin I was observed, and cells were rod-shaped with typical striation, suggesting the differentiation into a mature cardiomyocyte phenotype. Cells were able to form "normal coupling units" with native cardiomyocytes. Other studies where mESC-derived cardiomyocytes transplanted into the retroperitoneal cavity of mice were shown to form spontaneously contracting grafts 7 and 30 days post transplantation (69). Cells were positive for cardiac troponin I, cadherin, connexin 43 and proliferating cell antigen. Large animal model studies have also shown promising results. Menard *et al* implanted $30x10^6$ cardiac committed mESC into infarcted sheep myocardium at 25 different sites and were able to show that cells engrafted into the tissue and expressed connexins (70). Colonization of the scar tissue was also accompanied by a marked improvement in ventricular ejection fraction.

The production of large quantities of ESC derived cardiomyocytes, along with other cell types needed to repopulate the myocardium, will be required in order for this therapy to become clinically relevant. Screening processes will also have to be implemented in order to ensure that cardiomyocytes produced are of the highest purity. Recently, our group and others have been working on scaling the production of mESC-derived cardiomyocytes to bioreactor systems. Bauwens *et al* have used a perfusion based bioreactor system that has been able to produce as many as 3.5×10^6 functional cardiomyocytes (71). Recently, Schoerder *et al* have used a 2L bioreactor to produce 1.28×10^9 cardiomyocytes (72). Their system allowed for the inoculation of a single cell suspension into the reactor, therefore bypassing the EB formation step. This is a key finding, as EB induction would have proven to be extremely difficult in an industrial setting. By adjusting parameters such as impeller size and speed, they were able to induce EB formation within the reactor and achieve a cardiomyocyte yield that was almost a 10-fold increase over previous studies. Ultimately, it would be useful to define universal culture parameters such as impeller tip speed, Reynolds number, and power input per unit volume in order to better translate the optimization parameters found in their study to a larger scale process.

Lastly, it is extremely important to understand the fundamental mechanisms regulating ESC development and differentiation *in vivo* in order to better manipulate these conditions *in vitro* to generate large numbers of functional ESC derived cardiomyocytes. An important study by Gordon Keller's group details the targeting of green fluorescent protein (GFP) to the brachyury (Bry) locus(73). This transcription factor is expressed in all mesoderm precursors but downregulated as these cells differentiate into their specific derivatives. Their study identified two GFP+ populations which were separated based on their expression of Flk 1. The cardiac potential of both populations was tested and data suggested that only GFP+ Flk 1- cells developed into functional cardiomyocytes. These cells expressed GATA-4, GATA-5, Fog-1, Tbx5, Nkx2.5, mef2a, hand1, and hand2 in a time dependent manner indicating differentiation into cardiac precursors when cultured in appropriate conditions(73).

4.2.2. Human embryonic stem cells

The successful derivation of human ESC lines has opened up the possibility of ESC truly being renewable human cell source for therapeutic purposes. Although many important lessons in the culture of human ESC (hESC) have been learned from mouse ESC, there are fundamental differences between the two. EBs formed from human ESC have been shown to give rise to various types of cells such as neurons, cardiomyocytes and cells from the pancreatic lineage (74-80). Cardiomyocytes derived from hESC possess similar characteristics to cardiomyocytes derived from mESC. Cells isolated from the spontaneously contracting EBs exhibit the structural, molecular and functional properties of fetal cardiomyocytes (81). Human ESC-derived cardiomyocytes express transcripts for GATA-4, Nkx2.5 and contain structural proteins for alpha and beta-myosin heavy chain, atrial and ventricular myosin light chain, tropomyosin, alpha-actin, desmin and atrial natriuretic peptide (82-84). These hESC-derived cardiomyocytes undergo a maturation process that is similar to what has been reported *in vivo* and in the murine model. Small mononuclear cells are round or rod shaped and mature to form highly organized sarcomeric structures. Contractile proteins accumulated over this period formed well-defined sarcomeres with recognizable A, I and Z bands (82).

Intensive research is currently underway in order to define serum and feeder layer free culture conditions for optimal human cardiomyocyte differentiation. Activin A has been identified as a factor that supports this phenomenon in a feeder free system (85, 86). Feeder free hESC-derived cardiomyocyte proliferation has also been reported in the presence of transforming growth factor beta-1, LIF, basic fibroblast growth factor, and fibronectin matrix (87). Other studies have shown similar findings by employing specific combinations of bFGF and the suppression of bone morphogenetic proteins (88).

Unfortunately, the differentiation protocols have not been optimized for human ESC and therefore the cell yields limit in-depth experimental analysis. A recent study from Yoon *et al* has shown that treatment with 5-azacytidine can significantly increase the percentages of beating cells within EBs and simultaneously increase the upregulation of various cardiac specific markers (89). Data from the Mummery group suggests an inverse relationship between the percentage of cardiac differentiation and the concentration of fetal calf serum (90). Their findings suggest that medium supplemented with selenium, insulin,

transferring and heat inactivated bovine serum albumin can increase the percentage of actinin positive cells (90).

The clinical application of hESC currently represents a promising approach for tissue regeneration in the treatment of cardiac disease. Reproducible differentiation systems for human cardiomyocytes are quickly becoming established (58, 91, 92) and insights will be gained into the mechanisms involved in cardiomyogenesis which will in turn aid in the development of novel treatment options. Furthermore, systems have been established where multiple cell lineages can be generated from hESC or their derivatives, an aspect that would allow targeting multiple aspects of cardiac healing (vascularization and appropriate electrical coupling, for example). Ideally, a small number of hESC could be expanded into a large population, differentiated into the desired cell type and used for therapies such as tissue engineering, regenerative medicine, or gene therapy. A recent study has also examined hESC-derived cardiomyocytes in a mouse infarct model (93). Cellular engraftment was detected post sacrifice and MRI detected an improvement in heart function, especially when allopurinol/uricase and ibuprofen were administered before and after cell implantation. Work by Laflamme *et al* has demonstrated that enriched human ESC progenitors injected into the ventricular walls of athymic rats formed stable cardiac grafts (94). Angiogenesis was observed, both graft and donor derived. Kehat *et al* have also shown that human ES cell derived cardiomyocytes can substitute for pacemaker cells in a porcine model of atrioventricular blockage thereby increasing the chances of survival (95).

As with mESC, significant efforts to produce large numbers of hES cardiomyocytes are currently underway. Recently Cameron *et al* have compared the difference in EBs cultured in both static and stirred differentiation systems. They found that EBs cultured in stirred suspension were more homogenous in shape and size(96). Moreover, a 15-fold expansion in EB numbers was observed in the stirred system as opposed to a 4-fold expansion in the static cultures.

4.2.3. Embryonic stem cell purification strategies

Successful used of ES-derived donor cells would require the production of highly purified cardiomyocyte cultures. Currently, two different strategies have been developed in order to isolate undifferentiated progenitor cells from differentiating ESC: 1) selection of differentiated progeny by the use of surface markers combined with flow cytometric fluorescence activated (FACS) or magnetic-activated cell sorting (MACS) and 2) through the manipulation of genes in order to introduce selectable markers.

FACS has been investigated as a method for cell purification in mouse cells with potential applications in a human cell system. FACS provides several advantages over gene manipulation, the most important one being the avoidance of antibiotics. Recently Muller *et al* has combined the two approaches by deriving a transgenic ES cell line by expressing an enhanced version of the EGFP under the transcriptional control of the ventricular specific 2.1 kb myosin light chain 2v (MLC-2v) promoter and the 0.5 kb enhancer element of the cytomegalovirus (97). Post differentiation, FACS sorting along with a Percoll gradient purification step allowed them to produce a 97% pure cardiomyocyte population.

Gene manipulation offers a simpler approach over sorting as ideally, post differentiation all remaining cells should be cardiogenic in karyotype. Klug *et al* have developed a highly successful approach used to enrich mESC cardiomyocyte cultures by transfecting ESC using a fusion gene of an alpha-MHC promoter linked to a cDNA encoding aminoglycosilyde phosphotransferase. Following the differentiation of these cells, selection is carried out due to the expression of the antibiotic resistance gene and a relatively pure population of cells of cardiac lineage is obtained (59). Using this protocol, up to a 99% of cells isolated are cardiomyocytes. Using these types of gene transfection cells of ventricular lineage have been isolated by the introduction of an enhanced green fluorescent protein a transcriptional control of ventricular-specific myosin light chain-2v. This reporter gene based approach has allowed researchers to specifically isolate ventricular myocytes (97). While these purification methods are extremely effective, there is a concern that the insertion of a foreign epitope into the ESC might lead to rejection following transplantation. This strategy is unlikely to be utilized for human cells because of safety and regulatory constraints.

Ensuring that the resulting cell population is of highest purity is crucial. In addition to possible teratomas formation by remaining undifferentiated cells, there are concerns over the fact that cardiomyocytes formed from EBs are heterogeneous. This can be problematic as when spontaneously contracting cells are examined, three distinct cell types can be identified: atrial, ventricular and Purkinje cells. Transplantation of atrial or Purjinke-like cells into an infarcted ventricle could further destabilize the already fragile ischemic environment and could lead to arrythymogenesis. Table 3 highlights some of the most recent cellular cardiomyoplasty studies with an emphasis on studies performed using both mouse and human ESC.

5. CARDIAC TISSUE ENGINEERING

While all of the cellular transplantation studies summarized above have shown some level of improvement in cardiac function post infarction, issues still need to be resolved in order to increase cellular engraftment percentages, and viability of transplanted cells. Cells are also lost through the normal blood circulation. A more efficient delivery vehicle will be needed in order to contain the transplanted cells at the site of injury. Precise cytokine delivery could also be used to improve healing post infarct yet this will prove to be difficult in the direct transplantation model. Tissue engineered constructs may have a significant role to play in facilitating regeneration of heart tissue. The use of cardiac tissue engineered constructs has significant potential advantages over direct cell transplantation, including; replacement of damaged

Table 3. Cellular transplantation experiments grouped by donor cell type

Cell source	Host	Delivery method	Injury type	Cell number transplanted	Outcome	Percentage of engrafted cells	Ref
Autologous SM	H	IM injection	CABG	150 $x10^6$	Improved LVEF, improved cardiac contraction	Not quantified	176
Autologous SM	H	IM injection at time of LVAD implant	None	300 $x10^6$	Myotubule development in scarred myocardium	Total cell survival was estimated to be < 1%	177
CM	R	Injection	Cryoinjury	4 $x10^6$	Transplanted cardiomyocytes formed sarcomeres and were linked by junctions	21% cells were b-galactosidase positive	33
CM embryonic	R	Injection into BZ	Left coronary artery occlusion	1.5 $x10^6$	Less scar tissue formation in implant group. Cells were immature	Not quantified	178
CM	R	Injection into midmyocardium	None	2 $x10^6$	Increased cell death over time 57% at 24 hr, 15% at 12 wk. Transplanted cells became elongated over time.	15% cell survival at 12 weeks	179
CM	R	Injection into midmyocardium	LAD ligation	3-5$x10^6$	Increased LVEF and decreased infarct zone dyskinesis	62% cell survival at 6 months	180
Autologous BM	H	Endocardial at CABG	IHD	5 $x10^8$ -1 $x10^9$	60% improvement in stress test	Not quantified	181
Autologous BM	H	PTCA	MI involving left coronary	1.5-4 $x10^6$	Significant decrease in infarct region	Not quantified directly	182
mESCDC	M	Epicardial syringe	None	1 x 10^4	Engrafted cardiomyocytes	Not quantified	59
mESCDC	R	Epicardial into BZ	LAD ligation	3 x 10^4	Improvement	7.3% cells were GFP+, evidence of cardiogenesis found	68
mESCDC	M	Epicardial	LAD ligation	3 $X10^5$	Increase in function and capillary density	Not quantified directly	138
mESCDC	S	Injection into BZ	Intracoronary embolization	30 $X10^6$	LVEF improved by 6.6%	Scar tissue was composed of 22% ESCDC	70
hESCDC	GP	Injection into the anterior epicardium	None	5 MD EBs	Membrane polarization spread from graft to surrounding myocardium.	Not quantified directly	183
hESCDC	R	Injection into left ventricular wall	None	0.5-10 x 10^6	Formation of grafts. Dose dependent response depending on amount of cardiomyocytes implanted	Not quantified directly. Grafts showed large percentages of cardiac cells	94
hESCDC	P	Injection into the ventricular wall	Atrioventricular block + pacemaker implant	40-150 EBs	Implanted EBs were able to pace the blocked heart wall	Not quantified directly. Grafts showed large percentages of engrafted cells	95

BM: bone marrow; BZ: border zone; CABG: coronary artery bypass grafting, EB: embryoid bodies, GFP: green fluorescent protein, GP: guinea pig, H: human, hESCDC: human embryonic stem cell derived cardiomyocytes, IHD: ischemic heart disease, LAD: left anterior descending artery, LVEF: left ventricular ejection fraction, M: mouse, mESCDC: mouse embryonic stem cell derived cardiomyocytes, , mEDC: mouse early derived cells, MD: microdissected MI: myocardial infarction, P: pig, PTCA: percutaneous transluminal coronary angioplasty, R: rat, S: sheep, SM: skeletal myoblasts

infrastructure, temporary support for transplanted cells, and control of size, shape, strength and composition of the graft. Another principal advantage of tissue engineering approaches is that the strongest ischemia tolerant cells can be selected *in vitro* before implantation whereas injection of isolated cells leads to the death of up to 95% of cells during or shortly after the procedure (14). One tissue engineering strategy is to isolate ESC-derived cells, grow them on a three-dimensional scaffold under controlled culture conditions and to deliver the construct into the patient's body. Another would be to simply use the tissue engineered construct as a cell deliver method with seeding occurring immediately before implantation. In either strategy, ideally, regeneration of cardiac tissue would occur and the new tissue formation would induce construct degradation thereby avoiding a second surgery.

The biomaterial is one of the key components in the design of the tissue engineered construct. The material should promote tissue ingrowth from the host surroundings and guide the formation of tissue from the implanted cells. The ideal scaffold must be biocompatible and must also be able to withstand the stress and strain exerted by the heart without cracking. Moreover, the degradation rate of the material must be timed with new tissue formation and degradation products must be non-toxic. Macroscopic properties are also important, as pores must be interconnected and at least 50 microns in size to allow for cellular infiltration and subsequent vascularization (98).

Biomaterials are generally placed into two different categories: synthetic and natural. The main advantages of materials based on synthetic polymers are that they can be synthesized with direct control of chemical and macroscopic properties. As well, there is great potential for the integration of time-control release of bioactive compounds from the matrix. Other advantages of synthetic materials are their relatively inexpensive cost, the

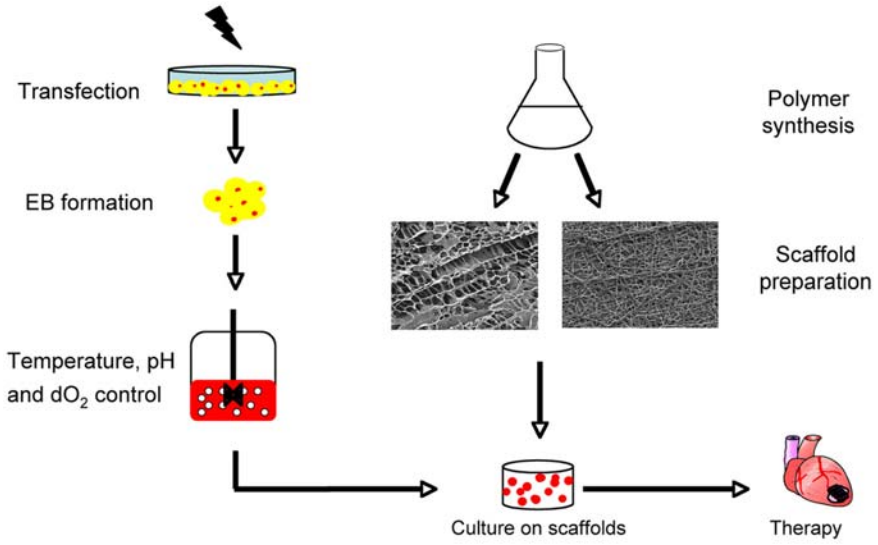

Figure 2. Scheme of the proposed strategy for cardiac tissue engineering using both embryonic stem cell derived cardiomyocytes and porous polyurethane scaffolds. The incorporation of a polymer based biomaterial may have a crucial impact in supporting cellular engraftment, infiltration, and survival.

flexibility in fine-tuning the chemistry and their ease of processing. However, there are concerns that the release of degradation products from these materials can elicit a strong immune response from the host. Naturally occurring biomaterials can offer advantages over synthetic ones. Foremost, the material can be chosen specifically for each application based on the extracellular matrix of the implant site, representing "the right matrix at the right place" (14). In addition, the use of recombinant proteins can minimize the polydispersity of the polymer produced, a problem with many synthetic systems. However, depending on the type of naturally based material, batch to batch variability can be of a greater concern than with synthetic materials, depending on the method of preparation of the material. For example our laboratory has found that there is a great batch-to-batch variability in decellularized constructs. The content of ECM and the vascular structure can vary from specimen to specimen, affecting decellularization times and effectiveness. Cardiac constructs have been engineered *in vitro* by seeding cardiomyocytes onto collagen gels (99), collagen membranes (100), polyglycolic meshes (101), electrospun poly(lactide) and poly(glycolide) meshes (102), polyurethane films (103, 104), synthetic PCLA (105), and alginate scaffolds (106). Researchers have also tried to mimic native cardiac architecture by stacking pulsatile cardiomyocyte sheets (107). Table 4 summarizes some of the advantages and disadvantages of materials used for cardiac tissue engineering.

Since human fetal cardiomyocytes would be impossible to obtain in large enough quantities for therapeutic purposes, ESC derived cardiomyocytes represent the best option for tissue engineering therapies. To date investigations with embryonic stem cells in the presence of scaffolds have focused on the embryonic stem cell differentiation kinetics on different scaffolds materials (108-110) but work in culturing ES cell-derived cardiomyocytes on scaffolds is still in its infancy. For example, Wu *et al.* have used endothelial progenitor cells cultured on PGA-PLLA scaffolds in order to make functional microvessels and work with mesenchymal stem cells has been primarily on cartilage and bone (111-115). There are a few studies that have used ES cell derived cardiomyocytes in tissue engineering constructs. Work from our lab has focused on culturing ES cell-derived cardiomyocytes on biodegradable protein coated polyurethane scaffolds with a view to evaluating the impact of scaffold architecture and protein type on the response of the cardiomyocytes (Figure 2) (103). More recently we have moved to using thermally induced phase separation to fabricate a three dimensional porous structure. Architectural differences cause significant changes in cell morphology with the cells on the porous structure becoming round in shape (Figure 3). Interestingly, cellular viability assessed using various cytoplasmic stains was high and contraction of the constructs was observed, despite the rounded morphology. A recent study by Ke *et al* investigated the effectiveness of grafting an ESC-derived cardiomyocyte seeded cardiac patch on an infarcted heart. Cells were seeded onto scaffold patches of polyglycolic-acid mesh. Hemodynamics, cardiac function, and survival rates were measured 8 weeks after infarction. Mortality rates were significantly higher among mice that had experienced MI alone or MI plus cell-free patches. ESC-derived cardiomyocyte seeded patches also significantly improved left ventricular function following infarction(116). Kofidis *et al* investigated the use of a supporting scaffold, collagen, to deliver mouse embryonic stem cell-derived cardiomyocytes to the infarcted myocardium. The constructs were implanted following MI by left arterial ligation and explanted after 2 weeks. Cells formed stable intramyocardial grafts and were incorporated into the infarct area. The collagen scaffold was incorporated into the surrounding scar tissue (117).

Table 4. Material properties of various scaffold materials used in cardiac tissue engineering applications.

Material	Synthetic/ Natural	Mechanical properties	Degradation rate	Disadvantages	Cells cultured	Constructs created	Ref
Collagen membranes/ gels	Natural	Poor	Degrade fast if not chemically crosslinked	Batch to batch variability. Potential contamination	Neonatal CM	Contracting constructs	162
Alginate	Natural	Poor Sheer modulus 0.75 kPa	Fast. Decrease in construct stiffness when cells are cultured		Neonatal CM	Clusters of cells contracting	184
Polyglycolic acid	Synthetic	Tensile strength 0.08-1 GPa		Degradation products could be released too soon and exceed clearance capacity of tissue	Neonatal CM	Cell molecular and physiological characteristics were similar to CM cultured on TCPS	101, 185, 186
					ESCDC	Not analyzed, implanted	116
Polylactic acid	Synthetic	Poor Tensile strength 0.028-0.88 GPa depending on MW	Scaffolds tended to crumble and degrade fast	Degradation products could be released too soon and exceed clearance capacity of tissue	None		185, 186
PLGA	Synthetic	Poor Depends on ratio of PLA/PGA	Scaffolds tended to crumble and degrade fast	Relatively rigid. Not practical for elastomeric tissues	Neonatal CM	CM cultured on printed laminin lanes formed elongated, rod-like cells with myofibrils aligned in direction of patterns	187
Polye-caprolactone	Synthetic	Tensile strength 0.01-0.8 GPa	Slower than PLA, can be used in applications where delivery device must be in place for over a year	Degradation rate too slow	Neonatal CM	Constructs contracted after 3 days	185, 188
nNIPAAM	Synthetic	N/A	N/A	N/A	Neonatal CM	Layered sheets	107
Polyaniline	Synthetic	N/A	N/A		Cardiac myoblasts	Adhesion and proliferation of cells	189
Polyurethanes	Synthetic	Varying, depending on components	Can be customized to specific applications	Can be customized to specific applications	ESCDC	Constructs contracted w/o electrical stimulation	103, 104, 190-196

An improvement in the large-scale culture of scaffolds will have to be made in order to be able to translate this therapy from the bench scale to bedside. Work to date has focused mostly on proof of principle studies where small numbers of constructs are cultured. In fact, most studies have been hampered by the lack of cells. In the studies mentioned above the number of cultured cells ranged from 50,000 to 5 million cells per scaffold. Constructs developed by researchers have been for the most part small in size and in number due, in part, to the difficulty in isolating large numbers of cardiomyocytes. This is a significant challenge as mammalian tissues have proven to be the most difficult to grown under bioreactor conditions due to their high nutrient and oxygen demands (118). Constructs are highly sensitive to nitrogenated wasted and shear stress (118). Mass transfer to and from tissues is a critical issue in bioreactor design. In the body, cells are always in close proximity to capillaries (usually no further than 100 microns), which are able to fill the tissue's mass transfer requirements. Cardiac constructs have been cultured in a variety of bioreactor configurations (119-126). For the most part, constructs cultured under bioreactor conditions contain more cells and metabolic activity is greater than in constructs cultured in static conditions. In addition, collagen and elastin deposition is increased in constructs cultured under pulsatile conditions, along with an increase in contractile proteins such as myosin heavy chain and connexin 43.

6. CHALLENGES OF SCAFFOLD-ES CELL CONSTRUCTS

6.1. Vascularization

Post implantation a vascular network will be required to provide oxygen to the engrafted cells. After the infarct, transplanted cells need to survive long enough for the vasculature to penetrate the graft in order to restore normal function. Cardiomyocytes are extremely sensitive to changes in oxygen concentration and undergo necrosis or apoptosis under ischemic conditions. Therefore, vascularization or angiogenesis needs to take place relatively soon after implantation in order to ensure graft

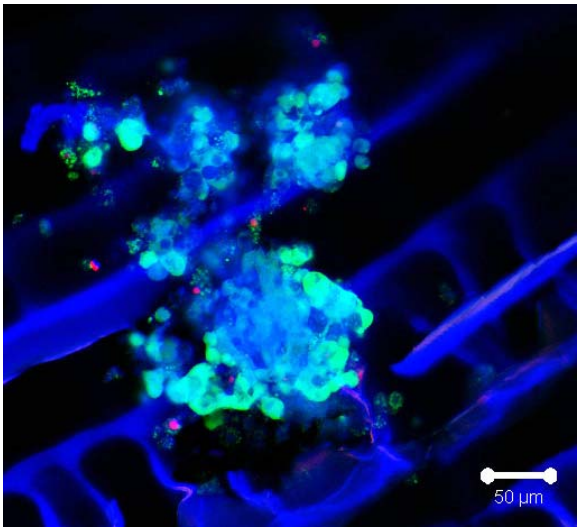

Figure 3. Confocal microscopy image of mESC-derived cardiomyocytes cultured on polyurethane scaffolds. A Live/Dead assay revealed a large percentage of viable cells (green) and few dead cells (red). The underlying polymer structure is highlighted by the use of a UV laser (blue). Bar represents 100 µm.

survival. To date, two distinct cell populations are being examined as candidates to promote vascularization: first, the coculture of primary endothelial cells in order to enhance the endogenous growth factor post infarct angiogenic response and second, promoting angiogenesis with endothelial progenitor cell therapy(127-131).

The role of cardiac endothelial cells (both from capillaries and endocardium) in cardiac development is of fundamental importance. Thus, the interaction between these two cell types will be of utmost significance in therapeutic applications. Evidence exists that the presence of cardiac endothelial cells plays an essential role in cardiomyocytes maintaining their adult phenotype(132, 133). When cardiomyocytes were cultured in the presence of vascular endothelial cells from aorta or fibroblasts, cells underwent dedifferentiation and reexpression of fetal proteins(134, 135). Furthermore, the addition of endothelial cells also stimulated the secretion of atrial natriuretic peptide from atrial cardiomyocytes(136, 137). These findings are not at all unexpected as the heart develops from the double-walled primary heart tube. This tube consists of two types of cells: endothelial cells and cardiomyocytes. Endocardial endothelial cells subsequently invade the cardiac jelly and migrate towards the myocardial tube.

Endothelial cells have been demonstrated to play a regenerative role post implantation. Implanted early-differentiated cells (derived from ESCs) overexpressing VEGF survived and differentiated into cardiomyocytes thus significantly improving cardiac function in a MI model(138). In addition, the presence of these cells was found to be correlated to the ability to stimulate the growth of new vessels in injured myocardium(138). *Ex vivo* approaches to vascularization have involved the coculture of endothelial cells within scaffolds in order to better mimic the cardiac environment (132). Addition of endothelial cells onto the construct promotes cardiomyocyte survival and enhances the spatial organization within the constructs (133). In addition, connexin 43 distribution patterns have been shown to be strikingly different between cardiomyocyte-endothelial cell and cardiomyocyte cell culture alone. A recent study by Pimentel *et al* showed enhanced connexin 43 expression in cardiomyocytes when VEGF was added to the cultures further clarifying the role of this protein in cardiomyocyte-endothelial cell signaling (139).

Primary endothelial cell sources are not widely available. There are several commercially available transformed cell lines, but these have been developed mostly for research purposes. Several groups have suggested the idea of a human circulating endothelial progenitor cell population(140-142). A side population of CD34 positive cells isolated from human peripheral blood displayed endothelial gene expression patterns *in vitro* and incorporated into neovascularization of ischemic hind-limb models in mice and rabbits(143). Further investigation has since shown that these cells, when mobilized with growth factors and/or cytokines, such as granulocyte colony stimulating factor, will home in to areas of injury and will often integrate into growing vessels(144, 145). This phenomenon can be explained by several different theories. First; that CD34 positive cells facilitate angiogenesis thus increasing perfusion and preventing ischemic cells from detaching from the infarct border and second, that CD34 positive cells could be secreting paracrine factors that induce angiogenesis.

Endothelial cells have been isolated from gastrulating mouse embryos (146) suggesting that *in vitro* ESC differentiation could be a powerful tool for recapitulating embryonic development and therapeutic purposes. Furthermore, endothelial cells have also been isolated from differentiating mouse ESCs (147-150). One of the most commonly used murine embryonic stem cell lines, the D3 line, has been demonstrated to differentiate into endothelial cells through three cell expansion stages and two cellular purification stages (149-151). The endothelial markers Flk-1, Flt-1, vascular endothelial cadherin (VECad), platelet endothelial cell adhesion molecule 1 (PECAM-1), CD105, and CD34 were detected *in vitro* following isolation (149-151). These findings are significant as it could be beneficial to be able to generate both cardiomyocytes and endothelial cells from the same ESC source. Recently, the isolation of endothelial cells from hEBs has also been documented (152, 153). Thus, the isolation of human embryonic endothelial cells has potential therapeutic applications, including cell transplantation in conjunction with cardiomyocytes (ESC derived or not) for repair of ischemic tissues and tissue engineering of vascular grafts. Future work should include a more detailed analysis of the molecular pathways controlling endothelial cell differentiation and vasculogenesis during hEB differentiation and the role of growth factors in this process. In addition, efforts to

identify and isolate early embryonic progenitors will aid in understanding the regulatory elements in vasculogenesis. A recent study from Furuta *et al* has challenged the traditional vasculogenesis lineage and proposed a different developmental pathway in which the angioblast lineage directly diverges from the mesoderm before and most importantly independently of hemangioblast development (154). In an elegant system using the coculture of OP-9 stromal cells with mouse embryos, the authors were able to detect a significant number of angioblasts one day before the earliest hematopoetic cells. Both types of cells expressed Flk1 and Tie2 receptor tyrosine kinases, in addition to Oct 3/4 (154). Studies of this kind will bring forth a new set of parameters by which to examine vasculogenesis in the developing embryo and thus aid in the development of useful culture parameters for ESC differentiation.

Integrating these concepts into scaffold-based therapeutic approaches will aid creating a complex, multifaceted approach to *in vitro* tissue engineering of a cardiac tissue construct. In particular, ES cell-material interactions and their role in promoting normal cell function and in some cases differentiation towards the appropriate phenotype must be investigated in order to develop a scaffold that can provide the appropriate physical and mechanical cues. In addition, second generation biomaterials that incorporate growth factors such as VEGF or bFGF into scaffolds to be released in a time-dependent fashion will aid in recapitulating *in vivo*-like events. Alginate scaffolds where PGA microspheres released bFGF in a time controlled manner were developed by Perets *et al* (155). In an *in vivo* model the released bFGF accelerated scaffold vascularization in the mesenteric membrane of rat peritoneum. Furthermore, when the scaffold was implanted into the infarcted rats, a significantly larger number of blood vessels were observed. The release of growth factors need not be confined to the addition of a sole cytokine. New polymeric devices have been created were VEGF and platelet derived growth factor (PDGF) were both released at different rates from a single scaffold. The combination of these two factors in a precisely controlled dose dependent manner resulted in the formation of a highly structured and mature vascular network(156). As with other approaches though, a fine balance needs to be achieved in order to deliver the appropriate amount of growth factors into the system as the excessive delivery of angiogenic growth factors can lead to aberrant vasculature and hemangiomas (132).

6.2. Graft integration

While ES cell-derived cardiomyocytes isolated through different protocols appear to be similar to native cells, it is unclear whether these cells will be able to integrate into the host tissue and perform the functions of the lost cells. This appears to be possible to some degree, as studies have shown limited improvement in organ specific function following transplantation. Field's group were the first to show that direct implantation of mESC-derived cardiomyocytes could form stable intracardiac grafts (59). Since then, other groups have also implanted mESC-derived cardiomyocytes into other rodent models of cardiac injury and have been able to show an improvement in cardiac output through echocardiography (8, 68, 70, 93, 157). Interestingly, one such study showed that undifferentiated ESC injected into mice myocardium differentiated into cardiomyocytes and did not form teratomas as expected (158). However, these results are highly controversial and as yet have not been repeated by any other researchers. A recent study has also highlighted the role that post infarct signaling has on the engraftment of ESC-derived cardiomyocytes. In the absence of MI, very few cells were engrafted in the following 2 weeks post surgery. Moreover, in the MI model very few cells were found in the normal myocardium while the injury site was extensively repopulated with ES derived cells (159).

The alternative to injection of isolated cells into the cardiac environment is to develop an artificial heart muscle construct *in vitro* and implant it *in vivo* when the need arises. This approach could be more cumbersome since a surgery would have to be performed in order to implant the construct. Preliminary studies have shown that engineered heart muscle constructs can be successfully implanted *in vivo* (107, 160-162). The degree of engraftment depends on a number of factors including the size of the construct, the implantation surgery and the material from the construct is made. Zimmerman's group was able to develop cardiac constructs by mixing cardiomyocytes from neonatal rats with liquid collagen I, matrigel, and culture medium. Implantation of these constructs onto uninjured hearts resulted in a heavy vascularized construct which retained an organized heart muscle structure (163). Interestingly, in the absence of immunosuppressants the constructs were almost completely absorbed and the loss of cardiac sarcomeric structures was observed. A recent study by Miyawaga *et al* transplanted cardiomyocyte sheets into the injured myocardium and demonstrated significant attachment, along with vascularization and increased staining for connexin 43 (164). Fibroblast sheets implanted in a similar fashion failed to improve systolic performance in the heart, supporting the idea that cellular contraction prior to implant would be beneficial as opposed to relying on cell coupling post implantation. Leor *et al* used porous alginate constructs as matrices for fetal cardiac cells (165). Scaffolds implanted into myocardial scar tissue revealed intensive vascularization from the coronary network. Scaffolds were almost indistinguishable from the host heart and reductions in LV dilation alongside no change in LV contractility were observed. Longer follow up times are required for these all of these studies as 9 weeks was the longest time point examined in order to better characterize these events. Recently, Levenberg *et al* seeded early differentiating hESC onto biodegradable PGA/PLA scaffolds and implanted the constructs onto the dorsal region of SCID mice (109). Their findings indicated that scaffold-supported hESC remained viable for at least two weeks. In addition, the differentiation patterns observed *in vitro* remained intact *in vivo* and in some cases continued to progress. However, explanted constructs were completely encapsulated by fibrogranulomatomus tissue, although host blood vessels had permeated the capsule.

6.3. Immunosupression and transplanting

Clinically, there are several therapies that are available to physicians to improve the prognosis of heart failure. While at the present time a transplant is the most effective, new tissue engineering therapies are emerging as possible alternatives. However, as with a heart transplant, cellular transplantation therapies will also have to overcome issues of immunosuppression. Transplantation of exogenous cells into injured myocardium has been established as an emerging alternative in both animal and human models. Cells transplanted have included atrial cardiomyocyte tumor cells, neonatal porcine myocyte and human fetal cardiomyocytes into various animal models (69, 157, 159, 166-169).

Several researchers have examined the effects of xenotransplantation of mESC-derived cardiomyocytes in infarcted animal models. A study from Ming's group showed improved function in rats six weeks post cell ESC-derived cardiomyocyte transplantation. Hemodynamic and ecocardiograph data showed significant improvement of cardiac function in the cell transplanted MI group. Similar findings were also found by Naito *et al* (170). When the injected cells dissected from beating regions of EBs into the border zone between the infarcted myocardium and normal myocardium, cells displayed typical cardiac phenotype and gap junctions. Interestingly, in all of these studies, the recipients of xenotransplantation of mESC did not receive any immunosuppressant drugs. The tolerance of the xenograft is presumed to result from the nature of the cells since ESC are believed to express less of the major histocompatibility complex (MHC) (171, 172). It could also be because the myocardium lacks a lymphatic drainage system. It has been reported that ESC do not express many membrane surface antigens and share immune-privileged features that are important for tolerance inductions (171, 172). It is also been shown that embryonic tissue possesses a range of proteins that counteract maternal T cell responses. Therefore the tolerance of cellular xenografts probably correlates with the degree of differentiation of the donor cells. ESC are believed to be the most plastic cells and presumably adapt to a new environment, even in a xenograft situation.

So far, there is no clear answer as to why the recipients do not reject the xenografted ES cells after their differentiation, even if the stem cells are immune-privileged. Chimerism may be a possible explanation as it may occur at an early stage after cell transplantation. Cross talks may happen between engrafted cells and host cells, modulating the expression of antigens of donor cells and also the immune response of a host. It has been shown that following cell transplantation, stem cells can down regulate the host immune response and induced mixed host immune response and mixed immune chimerism favoring long-term graft acceptance. In addition ESC contain a smaller number of T cells, which results in greater transplant tolerance (169, 173).

7. CONCLUSIONS AND FUTURE DIRECTIONS

The use of ESC-derived cardiomyocytes in combination with the appropriate scaffold holds invaluable promise for cardiac tissue engineering. While researchers have made significant headway in the last couple of years, numerous challenges need to be addressed before a successful ES cardiac construct is made. However, to meet this challenge the mechanisms of ES cell differentiation in a three-dimensional scaffolds must be elucidated. In particular, ES cell-material interactions and their role in cell function and differentiation must be investigated in order to develop a scaffold that can provide the appropriate physical and mechanical cues. Most of these parameters have been examined individually and the knowledge gained from each study needs to be pooled in order to create a suitable cardiac tissue replacement. Despite the parameters that need to be taken into account there is reason to be optimistic that ES cell-construct based therapies will find their place in the regenerative treatment of cardiovascular disease. In the next years we will see tremendous advances in the field of scaffold design to create scaffolds that have degradation rates in tune to the healing properties of the heart and release bioactive molecules to aid in the regeneration of diseased tissues. Progress is also forging ahead in the mass production of ESC-derived cardiomyocytes in order to produce an unlimited source of viable cells. These advances will aid in the generation of contractile cardiac constructs to replace or substitute diseased tissue. An interdisciplinary approach between doctors, basic scientists and engineers will be necessary to achieve this goal.

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