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Regenerating functional heart tissue for myocardial repair

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Abstract

Heart disease is one of the leading causes of death worldwide and the number of patients with the disease is likely to grow with the continual decline in health for most of the developed world. Heart transplantation is one of the only treatment options for heart failure due to an acute myocardial infarction, but limited donor supply and organ rejection limit its widespread use. Cellular cardiomyoplasty, or cellular implantation, combined with various tissue-engineering methods aims to regenerate functional heart tissue. This review highlights the numerous cell sources that have been used to regenerate the heart as well as cover the wide range of tissue-engineering strategies that have been devised to optimize the delivery of these cells. It will probably be a long time before an effective regenerative therapy can make a serious impact at the bedside.

Keywords

myocardial repair; tissue engineering; tissue regeneration; heart tissue; cardiac tissue; heart repair; heart regeneration

Introduction

Heart disease is one of the leading causes of death worldwide [1], often manifesting as an acute myocardial infarct (MI), an ischemic event in the myocardium that results in cell death and inflammation [2]. Scar tissue forms in the infarct site to maintain the heart's architecture while the remaining cardiomyocytes slowly hypertrophy in an attempt to compensate for those that were lost [2]. The myocardial wall subsequently thins and a downward spiral ensues as inadequate ventricular contractions lead to further dilatation, eventually resulting in organ failure and death [3]. Various pharmacologic and surgical therapies can help limit myocardial remodeling and alleviate some of the symptoms of heart failure. β -blockers, ACE inhibitors, and a variety of other drugs can reduce the stress on the failing heart to slow the progression of heart failure. Meanwhile, surgeons can revascularize myocardial tissue downstream from a coronary artery occlusion using a coronary artery bypass graft (CABG). This procedure limits the amount of ventricular tissue that is lost during ischemia and significantly improves patient survival. Unfortunately, even with the best drugs and CABG procedures, severe myocardial infarctions inevitably result in ventricular dysfunction, characterized by a reduction in ejection fraction and cardiac output. In these circumstances, left ventricular assist device (LVAD) or a heart transplant are the only remaining treatment options. LVADs prolong survival, but problems such as increased risk of thrombosis,

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infection, gastrointestinal bleeding, and end organ failure limit its effectiveness [5]. Consequently, LVAD are commonly used as a “bridge to transplantation” device rather than a long-term therapy [5]. Thus, the number of patients with heart failure has been steadily increasing. Although we can treat the symptoms and delay its progression, heart transplantation is the only therapy that can cure heart failure. However, limited donor supplies and eventual organ rejection precludes the use of heart transplantation for the majority of patients with heart failure.

In contrast, heart tissue engineering works to regenerate functional heart tissue that can be implanted in the scar tissue of a failing heart to restore ventricular function. Cellular cardiomyoplasty, or cellular injection directly into the myocardium, has been one of the more conventional approaches [4]. A wide array of cell types has been studied, from skeletal myoblasts to hematopoietic stem cells and embryonic stem cells [6–8]. Newer sources such as induced pluripotent stem cells (iPSC) take advantage of recent reprogramming technologies to circumvent many of the problems associated with other cell sources such as the requirement for immunosuppression or limited transdifferentiation potential [9]. In addition, work from several laboratories has identified novel cardiac progenitors that could exist in small numbers in the adult heart [10–13]. Early attempts at reactivating these endogenous progenitors after an acute myocardial infarction have been met with some success [11]. Meanwhile, tissue-engineering strategies aim to optimize cell delivery by providing mechanical support to the heart and a substrate for cell growth and maturation [4]. Newer, scaffold-less technologies utilize temperature-responsive culture plates to create cell sheets held together by the cells’ own extracellular matrix [14]. Finally, cutting edge self-assembling injectable scaffolds are being designed [15–18] that combine a less invasive delivery approach with the mechanical advantages of traditional scaffolds.

The American Heart Association estimated that heart disease cost the US roughly \$316 billion in 2010, which includes health care services, medications, and productivity lost [1]. These figures are likely to grow as the baby boomer generation continues to age and our ability to treat heart failure improves. Clearly, a cost-effective therapy for treating acute myocardial infarction is needed to meet these demands. This review highlights the various cell types that have been used to develop such a therapy as well as cover several tissue-engineering strategies that have been devised for the delivery of these cells.

1. Cell Sources

Several requirements must be met in order for a cell type to effectively regenerate the myocardium. Most notably, the cell type should exhibit contractility that can couple electromechanically with the host tissue. Uncoordinated myocardial contractions are counterproductive and may result in arrhythmias. Moreover, the additive effects of coordinated cellular contractions must be strong enough to propel large volumes of blood throughout the systemic vasculature. The ideal cell type must also be able to survive the harsh, often ischemic environment that follows an acute myocardial infarction. To make matters more challenging, an enormous number of cells must replace the roughly 1 billion cardiomyocytes lost during a myocardial infarction; therefore, the ideal cell type should be self-renewing to enable the large-scale growth of billions of cells [19]. Finally, the cell source should be autologous. Allogeneic implants require long-term immunosuppressive therapy, which has been associated with increased cancer rates and reactivation of bacterial and/or viral infections [20].

Various types of cell sources have been tried with these goals in mind. Some satisfy more requirements than others (Outlined in Table 1 with references). While contractile, skeletal muscle cells are limited in their ability to contract synchronously with the host myocardium

[21]. Instead, many others have searched for endogenous cardiac stem cells. Bone marrow derived stem cells are autologous and easy to obtain; however, it is still debated whether these stem cells truly have the potential to differentiate into cardiomyocytes *in vivo* [22, 23]. Endogenous epicardial cells, which are a more definitive cardiac precursor than bone marrow derived cells, can be primed to migrate to the infarct following ischemic injury and help repair the damaged myocardial tissue [11]. Embryological studies of the heart have revealed a novel cardiac progenitor identified by the expression of the LIM-homeodomain transcription factor Islet-1 (Isl-1) [24, 25]. These Isl-1 cardiac progenitors can differentiate into all three cardiovascular lineages [24], but it is still not known whether these cells survive late into adulthood. Although ESCs have not lived up to their publicity, they have been utilized to regenerate the heart [8]. They are pluripotent and self-renewing [26], which is ideal for generating large numbers of functional cardiomyocytes. Similarly, iPSCs can produce functional cardiomyocytes or cardiac progenitors [27, 28], but with the added advantage of being autologous. Controversy still exists over the safety of iPSC technologies [29], but they nevertheless offer a new approach to regenerating the heart. Each cell type satisfies a unique set of requirements. It will be a challenge to find a cell type that exhibits all of the necessary characteristics for effective heart regeneration. Thus, clinicians and scientists must select the cell type that is best suited to their goals.

1.1 Skeletal Muscle Precursors

Skeletal myoblasts are skeletal muscle precursor cells that reside within the skeletal muscle tissue where they can replace injured and dead cells [30]. Myoblasts self-renew in response to mitogens of the fibroblast growth factor family [30], an important characteristic for translating skeletal muscle cells into the clinic where millions of cells will be needed for engraftment. Theoretically, the contractions produced by mature skeletal muscle cells could restore normal ventricular function. Additionally, unlike the heart, skeletal muscle contains an abundant source of autologous myoblasts that are easily obtainable through a muscle biopsy [31–33]. Perhaps the greatest advantage of skeletal myoblasts over cardiomyocytes is their ability to survive in ischemic conditions [30]. While rat skeletal muscle cells form large grafts and proliferate within the myocardium in a rat cryoinjury model [34], a large proportion of cardiomyocytes die in the initial days following implantation [35].

Despite these advantages, skeletal myoblasts have shown only modest improvements in restoring heart function [33]. Initially, some proposed that myoblasts transdifferentiated into cardiomyocytes after transplantation in the heart [36, 37]. More detailed analysis; however, showed this to be false [23]. While myoblast implants maintained the expression of skeletal muscle cell markers like fast skeletal muscle myosin heavy chain up to 12 weeks after implantation, they failed to express cardiac troponin I or atrial natriuretic peptide [23]. Furthermore, they did not detect the expression of N-cadherin or connexin-43, two proteins that are critical for electromechanical coupling between neighboring cardiomyocytes [23]. Surprisingly, these same proteins were expressed in a small population of skeletal muscle cells co-cultured *in vitro* with a high density of neonatal cardiomyocytes [21]. More importantly, this small population of skeletal muscle cells used gap junctions to couple electromechanically with neighboring neonatal cardiomyocytes [21]. Many have suggested that as myoblasts mature, they lose their ability to express N-Cadherin and Connexin-43, which might explain why gap junction formation does not occur in maturing skeletal muscle cells *in vivo* [21]. In addition to the absence of gap junction proteins, nearly every histological study of skeletal muscle cell implants reported a layer of scar tissue separating the graft from the host myocardium [30]. Such a separation precludes the formation of gap junctions between host and donor cells. One study reported that implanted autologous rat skeletal muscle cells replaced the scar tissue in a rat cryoinjury heart model [34]. However, only a small cohort of animals was examined just 4 days after injury. Taken together, the

evidence in animal models strongly suggests that skeletal myoblasts do not transdifferentiate into cardiomyocytes and cannot couple electromechanically with the host myocardium, which will limit its use as regenerative therapy.

Interestingly, myoblast implants have been shown to slow ventricular dilatation and improve left ventricular ejection fraction (LVEF) better than baseline, control treatments in sheep and rat infarct models [38, 39]. One group measured the stroke work performed by skeletal muscle cell grafts in rabbit infarct models to assess the contractility of the graft [37]. Positive stroke work was detected in myoblast implant hearts as well as in control, a kinetic infarct areas that did not receive muscle cell transplantation [37]. Thus, the positive stroke work may be a measurement of neighboring cardiomyocytes rather than the muscle cell graft itself. Other mechanisms may explain the improvements in geometry and LVEF. Studies involving other cell types have shown beneficial effects due to the secretion of paracrine cytokines and growth factors that guide myocardial remodeling [19]. Skeletal muscle cells may be functioning in a similar manner; however, there is no direct evidence that proves this to be the case. Alternatively, the stiffness or elasticity provided by the skeletal muscle cells themselves may provide some mechanical support to the heart, enough to affect tissue remodeling and attenuate ventricular dilatation.

Phase-I clinical trials have shown skeletal muscle cell implantation in patients diagnosed with heart failure to be safe and beneficial [31, 32, 40]. However, patients that received skeletal muscle cell grafts also received a CABG operation; therefore, it is impossible to conclusively attribute the improvements to the skeletal myoblast implantation [31, 32, 40]. The results could be due to revascularization of the tissue rather than any regenerative effects produced by the skeletal muscle cells [32]. One study gave patients only autologous myoblast injections into the infarct area and noted increased LVEF up to 12 months later, but similar analysis with echocardiography and magnetic resonance imaging yielded contradictory results [41]. A Phase-II, double blind, randomized clinical trial found no significant differences in regional wall motion score index or LVEF 6 months after myocardial injections of autologous skeletal muscle cells [33]. However, patients that received a high dose of skeletal muscle cells displayed a significant reversal of myocardial tissue remodeling, consistent with earlier animal and human studies [33]. The authors also noted that the magnitude of the effects were similar to those produced in other studies that employed anti-tissue remodeling drugs [33]. The mechanism by which skeletal muscle cells slow or prevent myocardial remodeling is still unknown. They may provide mechanical support that prevents ventricular dilatation and/or secrete paracrine factors that attenuate myocardial remodeling. The results from the Phase-II clinical trials may not necessarily spell the end for skeletal myoblasts as a cell source to engineer functional heart tissue. Skeletal myoblasts are easy to obtain and have been shown to improve myocardial remodeling despite some of its obvious disadvantages.

1.2 Bone Marrow Stem Cells

Like skeletal myoblasts, autologous bone marrow derived stem cells are relatively easy to obtain by bone marrow biopsies or aspirations [7, 42]. Early studies in mice suggested that bone marrow cells injected into the infarct site had the capacity to transdifferentiate into cardiomyocytes, smooth muscle cells, and endothelial cells [7, 42]. Since then, reports have emerged that challenge the claim that bone marrow cells can transdifferentiate into cardiomyocytes [22, 43]. Balsam et al. found that the small population of bone marrow derived stem cells that survived direct myocardial implantation had differentiated into hematopoietic cell lineages rather than cardiomyocytes [43]. Notably, the authors also report a modest improvement in heart geometry 6 weeks later compared to control mice [43]. The discrepancies in bone marrow transdifferentiation have been hypothesized to be the result of cell fusion events that are thought to occur between existing cardiomyocytes and injected

bone marrow cells [44–46]. Differences in cell isolation and experimental protocol may also explain the contradictory results. Nevertheless, the improvements in cardiac function observed in the various animal model studies have led to a series of clinical trials to investigate the efficacy of bone marrow cell implantation after an acute myocardial infarction [47, 48].

Improvements in LVEF and wall motion score index, another measure of ventricular function, were observed 4 months after implantation of bone marrow cells in patients that had experienced a myocardial infarction [48]. The regional blood flow around the infarct was also elevated in patients that received the bone marrow cell therapy [48]. Taken together, the short-term improvement in cardiac function could be the result of revascularization of the infarct site, which could reduce apoptosis of hypertrophied cardiomyocytes and myofibroblasts. Notably, cardiac function was maintained 5 years later in patients that had received the bone marrow cell implants and no late adverse events related to the therapy were reported [49]. A meta-analysis of 8 clinical trials lasting longer than one year and up to five years involving 725 patients also showed modest, long-term improvements in LVEF and heart geometry [50]. Patients who had received the bone marrow cell transplantations experienced a lower risk of death than those who had not [50].

Overall, the results from using bone marrow stem cells have been varied and the improvements modest [7, 48, 50]. The changes in heart geometry and function resemble those produced with skeletal myoblasts, suggesting that bone marrow cells and skeletal myoblasts might be functioning through a similar mechanism. In fact, bone marrow cells injected into injured skeletal muscle have been found to produce vascular endothelial growth factor to increase muscular perfusion [51]. Similar results have been found in the heart [52, 53]. Other paracrine signaling candidates include fibroblast growth factor [54], insulin-like growth factor, and stromal derived growth factor [55]. There is also evidence that bone marrow cells improve vascularization of the infarct, which can have a number of beneficial effects on the tissue. A clinical phase-II, double blind trial is currently underway to determine the ideal cell number and delivery method for bone marrow transplantation [56]. Like skeletal myoblasts, autologous bone marrow cells are relatively easy to acquire and have shown success in small clinical trials.

1.3 Cardiac Stem Cells

1.3.1 c-Kit⁺ and Sca-1⁺ Stem Cells—Much of the controversy over bone marrow cells and skeletal myoblasts comes from their apparent inability to transdifferentiate into cardiomyocytes [21, 22, 46]. For a long time, the heart was believed to be a terminally differentiated, static organ. Several reports, however, have challenged this notion [57–59]. One such study relied upon the rapid increase in ¹⁴C that arose from nuclear weapons tests to measure cardiomyocyte turnover following the nuclear weapons test ban in 1963 [57]. Using mathematical models, the authors estimated cardiomyocyte turnover to be roughly 1% at age 20, which declines to about 0.4% at age 75 [57]. Another postmortem analysis estimated human cardiomyocyte turnover to be as high as 46% [58], over 20-fold higher than the previous study by Bergmann and colleagues [57]. The explanations for these discrepancies are beyond the scope of this review, but these studies illustrate the heart's regenerative potential, which could be harnessed in the future to repair damaged myocardial tissue.

The first attempts at isolating cardiac stem cells utilized well-characterized stem cell antigens such as the stem cell factor protein receptor, c-Kit [12], and stem cell antigen 1 (Sca-1) [13], markers commonly identified in other, well-known stem cell populations in the body. Beltrami et al. identified a novel population of c-Kit⁺ resident cardiac stem cells in the adult rat heart that are clonogenic, multipotent, and self-renewing with the ability to

differentiate into smooth muscle cells, endothelial cells, and cardiomyocytes *in vitro* [12]. However, these *in vitro* cardiomyocytes displayed an immature phenotype and lacked the ability to spontaneously contract even in response to adrenergic stimulation [12]. Nevertheless, injecting c-Kit⁺ cells into the infarct site improved heart function 20 days later [12]. Another group found that c-Kit⁺ stem cells activated *in vitro* with hepatocyte growth factor and insulin-like growth factor and injected into the myocardium differentiated into smooth muscle cells and endothelial cells that coalesced to form capillaries and arterioles that were connected with the host coronary vasculature [60]. Cardiomyocyte differentiation was rarely observed [60]. A similar study by Dawn et al. found that intravascular injection of c-Kit positive stem cells delayed cavity dilatation and restored heart function over time [61]. Intravascular catheter delivery is less invasive than surgical procedures, which is a significant advantage for patients.

Sca-1 is another common stem cell antigen used to isolate cardiac stem cells [13, 62]. Oh et al. isolated Sca-1⁺ cardiac stem cells from the adult heart that have the potential to differentiate into cardiomyocytes after culturing the cells for 4 weeks with the histone demethylating agent 5-azacytidine [62]. Sca-1⁺ stem cells treated with 5-azacytidine migrate specifically to the infarct site when they are injected intravenously following a myocardial infarction; however, their differentiation into cardiomyocytes was shown to be the result of cell fusion events at least 50% of the time [62].

Other populations of cardiac stem cells expressing well-characterized stem cell antigens have been identified and imparted only modest benefits to the infarcted heart following implantation [63–65]. The expression of these stem cell antigens across many different cell types in the body has called into question the notion that these cells are truly cardiac in origin [19]. Hematopoietic stem cells (HSC) circulate between the bone marrow and peripheral organs in mice; however, very few of these circulating stem cells are found in the heart [66]. This does not preclude the possibility that HSC migrate to the heart following a myocardial infarction, especially given the hypoxic nature of the infarcted tissue. A study by Pouly et al. showed that bone marrow stem cells migrating to the infarct not only expressed the hematopoietic marker CD45, but they also failed to mature into cardiomyocytes as indicated by the absence of GATA4 and Nkx2-5 expression, markers of cardiac origin [67]. On the other hand, Quaini et al. conducted a postmortem analysis of male transplant recipients who had received female donor hearts and found infiltration of host male cells into the donor myocardium [59]. Most of the cells harboring a Y-chromosome also stained positive for the cardiomyocyte markers GATA-4 and Mef2c along with Sca-1 and c-Kit [59]. Thus, it has not been conclusively determined if Sca-1 and/or c-Kit positive stem cells represent true cardiac stem cells. They could be circulating bone marrow cells that happen to reside within the heart during isolation; however, the lack of CD45 expression shown by many of these studies seems to suggest otherwise. On the other hand, they could represent a small population of cardiac progenitors in the adult myocardium that can migrate to the infarct site following an acute myocardial infarction, providing a potential population of regenerative cells already within the myocardium. There is currently no consensus on the phenotypic definition of cardiac stem cells that express Sca-1 or c-Kit.

1.3.2 Isl-1 Progenitor Cells—Work in cardiac embryology has identified a more restricted cardiac progenitor cell type that has the ability to differentiate into smooth muscle cells, endothelial cells, and cardiomyocytes [10, 24, 68, 69]. Early in heart development, the cardiac mesoderm folds to form the cardiac crescent [70]. Two fields of progenitors define the cardiac crescent and give rise to most of the myocardium. The first heart field contributes to some of the atria and most of the left ventricle [70]. The second heart field, which forms after the first, yields the atria, right ventricle and the outflow tract [70]. Cells in the secondary heart field are defined by the expression of the LIM homeodomain

transcription factor Isl-1, which promotes their progenitor-like state and delays differentiation [25]. Isl-1 is also expressed in motor neuron and interneuron development in the spinal cord [71] and plays a role in pancreatic islet development [72]. Importantly, Isl-1 progenitors have been identified in adult myocardial tissue [10, 73]. Lineage tracing studies have also identified isl-1 derived cells in the sinoatrial and atrioventricular nodes of the heart that are separate from neural crest cells that migrate to the myocardium during embryogenesis [73, 74]. Notably, they do not express Sca-1 or c-Kit [10], suggesting that Isl-1 progenitor cells are distinct from the cardiac stem cell populations reported above.

Isl-1 progenitor cells were first purified from embryonic and post-natal hearts and subsequently cultured *in vitro* on cardiac mesenchymal cell layers [10]. The feeder layer secretes soluble factors that activate the Wnt/ β -catenin pathway to promote self-renewal and expansion while simultaneously inhibiting differentiation [69]. The ability to expand Isl-1 progenitors, but maintain their undifferentiated state is a huge advantage for tissue engineering. Furthermore, their more restricted phenotype reduces the risk of teratoma formation that can result from implanting pluripotent stem cells [75].

Isl-1 progenitors can also be purified from embryonic stem cells cultured *in vitro* as embryoid bodies [68, 69], which mimic the embryonic environment *in vitro* [69]. After purification, these progenitors can be expanded *in vitro* on a Wnt3a-secreting feeder layer [68, 69]. The feeder layer secretes soluble factors that activate the Wnt/ β -catenin signaling pathway [69], which has a primary role in cardiogenesis [76–78]. β -catenin directly activates Isl-1 expression to promote self-renewal and expansion while simultaneously inhibiting differentiation [69, 79]. Additionally, β -catenin has been shown to activate fibroblast growth factor (FGF) signaling, which further promotes cardiac progenitor proliferation [80]. The ability to expand Isl-1 progenitors while maintaining their undifferentiated state could theoretically provide the vast quantity of cells that would be required to repair damaged myocardial tissue. Furthermore, the more restricted phenotype of Isl-1 cardiac progenitors reduces the risk of teratoma formation that can result from transplantation of pluripotent stem cells [75]. In the future, human embryonic stem cells or induced pluripotent stem cells can be cultured *in vitro* as embryoid bodies and the Isl-1 progenitors subsequently purified. Large numbers of Isl-1 progenitor cells can then be expanded *in vitro* before being implanted in the heart where they can differentiate into all three cardiac lineages. Isl-1-derived cardiomyocytes have been shown to couple electromechanically with neonatal cardiomyocytes *in vitro* [10]. Furthermore, the differentiation of Isl-1 progenitors into smooth muscle cells and endothelial cells may help to revascularize the tissue. In fact, overexpression of Isl-1 in endothelial cells significantly improved angiogenesis in *in vitro* and *in vivo* rodent models [81]. Alternatively, Isl-1 progenitor cells can be expanded and guided towards cardiomyocyte differentiation prior to implantation. The Isl-1 derived cardiomyocytes can then be isolated for use with tissue-engineered scaffolds before implantation into the patient.

Although Isl-1-derived cardiomyocytes couple electromechanically *in vitro*, their phenotype will have to match that of adult cardiomyocytes. Isl-1 derived cardiomyocytes cultured *in vitro* most likely exhibit a more fetal phenotype due to the absence of mechanical load and shear stress [82]; however, it has not been directly examined. Isl-1 progenitors must also survive the hypoxic, inflammatory environment of the infarct site. The ability of Isl-1 progenitor cells to differentiate into endothelial and smooth muscle cells may enhance revascularization of the tissue and thus promote cell survival. Currently, there are no studies that utilize Isl-1 progenitors to repair the heart due to the difficulty in purifying human Isl-1 progenitor cells and maintaining homogenous Isl-1 populations *in vitro*. Rat and mouse Isl-1 progenitor cells have traditionally been isolated from transgenic mice expressing an Isl-1-dependent reporter [24]. Such transgenic approaches, however, are not conducive to human

embryonic stem cell lines. Isl-1 progenitor cells have been identified in adult rodent and human hearts, albeit at very low numbers [10, 73, 83]. Ideally, resident Isl-1 progenitors could be activated with small molecules to recapitulate the embryonic myogenesis programming. Proliferation and maturation of these endogenous Isl-1 progenitors would thereby offer a non-invasive, regenerative therapy to repair myocardial tissue. This approach may be limited by the small populations of Isl-1 progenitors that exist in the adult heart [10, 73, 83]. Although most evidence has shown Isl-1 progenitors to contribute to the right ventricle, atria and outflow tract, more sensitive lineage tracing experiments have revealed Isl-1-derived cells in the left ventricle and proepicardium [25, 84]. This is supported further by detection of Isl-1 in the first heart field [85]. Consequently, Isl-1 progenitors offer several advantages over other cell types, but have remained largely untested as a treatment for heart failure due to technical limitations.

1.3.3 Epicardial Progenitor Cells—Endogenous cardiac progenitors that can differentiate into all three cardiac lineages have also been found in epicardium [86]. During embryogenesis, epicardial progenitor cells migrate into the myocardium where they differentiate into smooth muscle cells, cardiac fibroblasts, and endothelial cells to form the coronary vasculature [70]. The ability to also differentiate into cardiomyocytes offers a novel cell source for regenerating the heart [86, 87]. Two populations of epicardial progenitors have been identified based on the expression of two transcription factors—Wilm's tumor 1 (Wt1) and T-box transcription factor 18 (Tbx18) [86, 87]. Both populations have been shown to contribute cardiomyocytes to the atria, ventricular septum, and ventricles themselves during embryonic development [86, 87]. It is not known whether Wt1⁺ and Tbx18⁺ progenitor cells represent the same population.

The migration of Wt1-expressing epicardial progenitor cells appears to be driven in part by the expression of thymosin- β 4 (TB4), a G-actin monomer-binding protein that influences the actin cytoskeleton and cell migration [88]. TB4 has also been shown to promote cardiomyocyte survival and migration [88, 89]. Remarkably, systemic or intracardiac injection of TB4 following a myocardial infarction improved cardiac function in adult mice [89]. Exogenous TB4 is thought to reactivate embryonic myogenesis pathways and increase angiogenesis within the myocardium [90]. An interesting study by Smart et al. found that priming mice with several doses of TB4 several days before and after left anterior descending (LAD) ligation improved myocardial regeneration [11]. *De novo*, electrically coupled cardiomyocytes were found along the border zone of the infarct [11]. Importantly, these cardiomyocytes were derived from Wt1 expressing epicardial progenitor cells [11]. Similar to earlier studies, cardiac geometry and function were improved one month after LAD ligation [11].

These studies show TB4 and epicardial progenitors to be a potential therapy for acute myocardial infarctions [11, 88–90]. Systemic delivery of a regenerative compound to repair the heart endogenously is a gold standard for regenerative therapies. There are no invasive procedures and the patient's own progenitor cells are activated so that there is no need for immunosuppressive therapy. Before this can happen, long-term studies are needed to determine if the effects seen with TB4 are transient or permanent. Moreover, the generation of *de novo* cardiomyocytes occurred only if the mouse was primed with TB4 prior to LAD ligation [11]. This may limit the use of TB4 in humans because acute myocardial infarctions occur randomly. Preventative doses of TB4 can be envisioned for patients at risk for myocardial infarctions, but the long-term side effects of taking TB4 have not been studied. Nevertheless, reconstitution of the embryonic heart development program is an enormously attractive therapeutic option and one that deserves further investigation.

1.4 Human Embryonic Stem Cells

Human ESCs offer enormous potential because they can differentiate into any cell type in the body, including cardiomyocytes [91]. They can be grown *in vitro* and guided to differentiate into hESC-derived cardiomyocytes (hESC-CM) [8, 92–97]. Interestingly, hESC-CM grafts can survive up to 12 weeks in the heart following a myocardial infarction [92–94]. Transplanted hESC-CM also couple electromechanically with each other, but less so with the host tissue [95]. Some studies have noted the presence of scar tissue largely separating the grafts from the host tissue, which may explain the limited electromechanical coupling [92, 94, 96]. Notably, none of the studies involving hESC-CM implantation into rats or mice noted cardiac arrhythmias [92–96], but the slow contractility of human cardiomyocytes compared to the rapid beating of rodent hearts may have hidden any arrhythmogenicity. In fact, transplanted hESC-CM were able to control the electrical pacing of pig hearts, which are more similar in heart rate to those of humans, cautioning the future use of hESC-CM in the clinic [97]. Functionally, hESC-CM produced only short-term improvements [8, 92–94]. Assessments 3 months after implantation did not show a significant difference in heart function between transplant and control groups [8, 93]. The short-term benefit of hESC-CM mirrors the results of other cell types described above, implying a similar mechanism of action. Laflamme et al. noticed angiogenesis in and around the graft, suggesting that hESC-CM are secreting angiogenic factors [96].

Different hESC lines have different cardiogenic potentials, but none of the hESC lines examined can generate the large numbers of cardiomyocytes that are required for clinical therapies [26]. Consequently, efforts have been made to identify small molecules and culture techniques that increase the yield of hESC-CM [94, 98]. Bone morphogenic proteins and members of the transforming growth factor- β family have been shown to promote embryonic cardiomyogenesis [99]. Activation of these two pathways in hESC also increases the yield of cardiomyocytes *in vitro* [94]. Treatment with 5-azacytidine has also been shown to increase cardiomyocyte yield *in vitro* [98]; however, the full effects of such an agent have not been elucidated.

Once the hESC differentiate into cardiomyocytes, they must be purified from a mixed population of differentiated and undifferentiated cells because injections of pluripotent stem cells are likely to result in teratoma formation [75]. There are currently few techniques to efficiently enrich human cardiomyocytes. The most common method is to sort cells by size using a Percoll gradient; however, the purity is limited [98]. Some studies have identified the surface protein, Flk-1, that specifically labels cardiac stem cells for FACS purification [100]. These Flk-1 positive cells also differentiate into smooth muscle cells and endothelial cells; therefore, the use of Flk-1 does not yield an entirely pure population of cardiomyocytes [98]. Recently, a mitochondrial dye has been developed by Hattori et al. that can yield cell fractions with greater than 99% viable cardiomyocytes [101]. The dye, tetramethylrhodamine methyl ester perchlorate (TMRM), is non-toxic and can be washed out within 24 hours [101]. FACS is used to distinguish between three populations of cells that show varying degrees of TMRM labeling [101]. Cardiomyocytes specifically display the greatest TMRM, allowing for their isolation [101]. The other fractions contain only a small percentage of cardiomyocytes, indicating that almost all of the viable cardiomyocytes are collected in the fraction with the greatest TMRM fluorescence [101]. TMRM can be used to label cardiomyocytes from fetal and neonatal hearts as well as ESC-derived and induced pluripotent stem-derived cardiomyocytes [101]. Thus, TMRM is a widely applicable dye that can significantly improve cardiomyocyte yields.

Unfortunately, although it is possible to obtain >99% hESC-CM populations, the phenotype of these cells resemble fetal or neonatal cardiomyocytes [26], a problem that is shared amongst most other cell types. hESC-CM express cardiomyocyte markers like cardiac

troponin T and cardiac transcription factors, but their sarcomeres are unorganized and their overall morphology reminiscent of fetal cardiomyocytes [93, 94, 102]. It is not fully understood why cultured cardiomyocytes develop a fetal phenotype. It seems likely that the absence of mechanical load may be involved [103].

Several obstacles stand in the way of using hESC-CM to regenerate the heart, from differentiation to purification and implantation. Advances have been made to improve the yield of cardiomyocytes, but the greatest challenge may be the ethical debate surrounding the general clinical use of human embryonic stem cells. Other problems include life-long immunosuppression that would be needed to prevent graft rejection. Immune suppression has been shown to improve ESC and iPSC grafts in a mouse model of allogeneic and xenogeneic implantation models [104]. However, long-term immunosuppression has been associated with increased cancer rates [20].

1.5 Induced Pluripotent Stem Cells

Autologous fibroblasts taken from a skin sample can be reprogrammed genetically into a stem cell-like phenotype using a combination of four genes: c-Myc, Oct3/4, SOX-2, Klf4, Lin28, or NANOG [105, 106]. There are two major advantages to using iPSC over embryonic stem cells. In contrast to hESC, iPSC can be produced from a skin sample rather than destroying an embryo, thereby avoiding the ethical issues of hESC while also providing an easily accessible source of cells. Secondly, autologous iPSC may eventually reduce the need for immunosuppressive drugs; however, recent evidence suggests that even syngeneic iPSC are subject to immune rejection [107]. Importantly, iPSC-derived cardiomyocytes behave in much the same way as hESC-CM [108, 109]. The cardiomyocyte yield is lower and differentiation appears later than ESC-derived cardiomyocytes, but the cells still display all of the normal physiological characteristics of hESC-CM, including defined sarcomeres, electromechanical coupling, and hormone sensitivity [108, 109]. More recently, early treatment of iPSC with bone morphogenic protein-4 followed by administration of a Wnt inhibitor enhanced cardiomyocyte differentiation in human iPSCs [110]. Mouse and human Isl-1 cardiac progenitors have also been created from iPSC [28].

Very little work has been done to examine the functional effects of implanting iPSC-CM into the infarct [9]. However, iPSC implanted in a mouse heart after an acute myocardial infarction differentiated into cardiomyocytes that also expressed connexin-43, suggesting electromechanical coupling with the host myocardium [9]. Moreover, iPSC implants reduced apoptosis and fibrosis two weeks later, ultimately resulting in better functional characteristics two weeks after implantation [9]. This study is encouraging, but is limited by its short duration. Longer studies are needed to determine if these effects are transient.

There are some caveats that deserve consideration before using iPSC technology in humans. Most notably, new methods that do not rely on genomic integration are needed. Genomic insertion of stemness factors could activate oncogenes, resulting in tumorigenesis [111]. Consequently, new protocols are being developed that utilize adenoviruses, plasmid vectors, removable transposons, direct delivery of recombinant proteins, and synthetic modified mRNA to induce pluripotency [111–117]. In addition to the problems associated with genomic manipulation, murine iPSC have been reported to display epigenetic similarities to the cell type from which they were derived, a phenomenon known as “epigenetic imprinting” [118, 119]. It is still unclear whether this occurs in human iPSC; however, a comparable study examining a single human iPSC clone revealed similar epigenetic patterns. Remarkably, gene expression profiles for iPSC and ESC vary only slightly [120, 121]. Expression patterns and differentiation potential vary even amongst individual hESC lines [122]; therefore, it is difficult to conclude if these genetic and epigenetic variations have any significant effect on the overall function and phenotype of iPSC-derived

cardiomyocytes. Finally, autologous iPSC will retain any genetic abnormalities that might exist, such as channelopathies. These abnormalities will have to be repaired before implantation in patients with such disorders, which reintroduces the risks associated with gene therapy. The field of induced pluripotency is growing fiercely and has become one of the most intensely debated topics in science today. Even if iPSC can produce the ideal cell source, other obstacles remain that may hinder their integration with host tissue and ultimately the regeneration of a functional myocardium.

2. Tissue Engineering

A large proportion of regenerative studies in the heart have been performed by injecting cells directly into the damaged myocardium or along the border zone of the infarct [9, 22, 30, 92]. The procedure is relatively easy and non-invasive. Some have used even less invasive transvascular catheters to deliver cell suspensions to the heart [41, 61]; however, excessive cell death, poor retention, and inconsistent graft sizes are often reported [34, 35, 43]. When Matrigel was added to the suspension, cell survival and retention was improved [35], suggesting that anoikis is a contributing factor to poor survival. Anchoring cells to a substrate can provide pro-survival cues to the cells and prevent the implants from being washed out of the beating myocardium [19]. The limitations of cellular injections have led to the development of a wide array of heart tissue engineering approaches.

Tissue engineering combines cell biology and engineering to generate living, three-dimensional structures *in vitro* that anchor the cells prior to engraftment into the host [82]. The goal is to develop a tissue that is contractile, non-immunogenic, vascularized, displays mechanical properties similar to the myocardium, non-toxic, and electromechanically coupled to the host myocardium. Conventional methods involve seeding biological or synthetic scaffolds with cells *in vitro* prior to engraftment [123–126]. Ideally, these cells colonize the scaffold, coupling with one another along the way. After several days in culture, the cellular graft is ready for surgical implantation. An alternative approach utilizes temperature-responsive culture dishes to generate scaffold-less, two-dimensional cell sheets that can be stacked on top of one another to create three-dimensional grafts [127–129]. Cell sheets maximize cell density and electromechanical coupling by maintaining intercellular adhesions; however, their fragile nature makes them difficult to transplant surgically [130]. Additionally, a new and innovative strategy collectively known as “tissue engineering *in situ*” utilizes a soluble scaffold material that polymerizes in the myocardium after injection [17, 131–133]. After polymerization in the myocardium, the scaffold behaves like more conventional scaffolds, providing mechanical support to the heart and a substrate to anchor the injected cells [3]. An enormous number of different scaffolds with different cell types have been examined in regards to regenerating functional myocardium, many of them proof of principle experiments [17, 131–133]. This review sets out to highlight the major obstacles and achievements in tissue engineering as well as compare the three general approaches described above (Outlined in Table 2 with references).

2.1 Biological Polymers

Biological polymers such as collagen were some of the first compounds used to construct three-dimensional tissues. Li et al. found that neonatal rat cardiomyocytes grown on gelatin scaffolds exhibited spontaneous contractility *in vitro* and *in vivo* when implanted subcutaneously [124]. Although the neonatal cardiomyocytes survived engraftment in a rat infarct model, no significant improvements in left ventricular function or dimensions were observed five weeks later [124]. When alginate scaffolds were seeded with fetal rat cardiomyocytes and implanted in the infarct site of the heart, neovascularization and myofibril organization improved nine weeks later [134]. Echocardiography revealed an attenuation of left ventricular hypertrophy [134]. Alternatively, alginate grafts have been

“pre-vascularized” by implanting seeded alginate grafts along the omentum for several days [135]. After pre-vascularization, the graft is excised from the omentum and implanted along the epicardium [135]. Alginate grafts that had been prevascularized on the omentum preserved ventricular function and geometry better than grafts grown *in vitro* [135]. Pre-vascularization in the peritoneum has produced similar effects [136]. The effects on tissue remodeling could be due to a variety of factors. Undoubtedly, mechanical support provided by the scaffold material would delay ventricular hypertrophy. Furthermore, neovascularization, likely the result of paracrine factors secreted by the neonatal cardiomyocytes, might also be contributing to the delay in ventricular hypertrophy. Ideally, the improvements in left ventricular remodeling are due to electromechanical coupling between the implanted neonatal cardiomyocytes and surviving cardiomyocytes in the host. There is some evidence of connexin-43 expression between neonatal and host cardiomyocytes [134, 135], but further evaluation is needed to verify electromechanical coupling.

Collagen is one of the most ubiquitous extracellular proteins in the body, providing a natural microenvironment for the seeded cells. The first collagen scaffolds seeded with embryonic chick cardiomyocytes exhibited spontaneous contractility and responded to normal physiological stimuli [137]. Alternatively, Zimmermann et al. molded soluble collagen I, Matrigel, and neonatal rat cardiomyocytes into contractile rings [138]. Interestingly, increasing the matrigel content or subjecting the rings to cyclic stretching or electrical stimulation promoted cardiomyocyte maturation and myofibril organization [139–141]. Multiple contractile rings were then cultured together under cyclic stretching and subsequently implanted into the infarcted heart [142]. Four weeks later, histological analysis revealed mature cardiac tissue that had coupled with the host myocardium [142]. Surprisingly, new blood vessels that connected with the host circulation had penetrated the full thickness of the graft [142]. The contractile rings maintained ventricular function up to four weeks later, although it should be noted that no improvement over baseline function was observed [142]. Several speculations exist as to why the contractile rings did not fully regenerate the myocardium. The contractile ring construct was placed along the epicardium of the heart [142] may not be the appropriate geometry to significantly enhance the pumping efficiency of the heart. Alternatively, the cardiomyocytes may not be able to generate enough force to produce a detectable increase in ventricular contractility even though they appear mature morphologically. Optimized contractile rings generate around 4mN/mm² of force compared to 56mN/mm² for mature adult heart tissue of the same size [82].

Contractile collagen-matrigel rings have also been adapted for clinical use [143]. The effects of contractile ring implantation were observed only after immunosuppressives were administered to the mice [142]. This is likely due to culturing cells in xenobiotic compounds like fetal bovine serum prior to implantation, which can be highly immunogenic [82]. Matrigel can also induce inflammation; therefore, Natio et al. replaced fetal bovine serum with a slew of growth factors while insulin and triiodothyronine improved contractility better than matrigel [143]. Following on the heels of this study, Chacques et al. seeded collagen I matrices with patient-derived bone marrow cells and implanted the grafts in ten infarct patients [144]. No improvements in left ventricular function were apparent ten months later [144]. Improvements in left ventricular end-diastolic volume (LVEDV), however, were observed, but all ten patients also received a CABG operation at the time of engraftment [144]. Thus, the effects on LVEDV cannot be conclusively attributed to the collagen-bone marrow cell implants.

Biological scaffolds help promote cell adhesion, survival and differentiation; however, there are several disadvantages. Most notably, collagen and other biological molecules are known to be immunogenic [134, 142]. As noted above, the contractile collagen-matrigel rings

survived in the heart only after immunosuppressives were given several days prior to implantation [142]. Additionally, the mechanical properties of biological scaffolds do not mirror those of the native myocardium while rapid degradation kinetics and inconsistencies from batch to batch make it difficult to control the quality and size of each scaffold [145]. Clearly, more work is needed to resolve some of the mechanical and immunogenic problems surrounding biological scaffolds. Additional studies examining how scar tissue affects graft integration may help explain why collagen-matrigel rings did not restore heart function even though they coupled electromechanically with the host and developed highly organized myofibers. The results of a study like this can also be applied to other tissue engineering strategies.

Rather than synthesizing biological scaffolds that mimic native myocardial tissue, some groups developed de-cellularized porcine and human heart tissue that leaves behind a preformed scaffold with the native architecture largely in tact. The idea has its roots in the de-cellularization of heart valves and other tissues where removing the resident cells from the tissue eliminates any allogeneic immune reaction and provides natural pores and channels that are suitable for cellular engraftment. Growth factors and adhesion proteins are retained in the matrix and larger structures like blood vessel basement membranes provide a template for angiogenesis after the scaffold has been reseeded with cells [146]. In fact, gross images of de-cellularized whole hearts clearly show vestiges of the epicardial vasculature [146]. A large amount of publicity was generated in 2008 when Ott et al. grew beating hearts from de-cellularized cadaveric rat hearts that had been repopulated with neonatal cardiomyocytes and other stromal cells of the heart [146]. The cardiomyocytes coupled electromechanically, forming a beating heart that could rhythmically pump blood at a rate controlled by external electrodes. Moreover, a patent coronary vasculature was clearly visible within 8 days [146]. Large-scale re-cellularization of whole hearts may not be possible in humans due to the enormous number of cells that would be required. Additionally, further work is needed to dissect the contractility, rhythmicity, and efficiency of the re-cellularized cadaveric hearts. There was no assessment of the electrical conduction system in the cadaveric hearts. Failure to form a functional conduction system would most likely result in life threatening arrhythmias, which might be controllable with external cardiac pacemakers and implantable cardioverter-defibrillators. Furthermore, the use of recellularized cadaveric hearts does not address the current donor shortage. De-cellularization of porcine hearts or those from other large animals might be able to make up the difference. Alternatively, using smaller tissue sections to form epicardial patches could improve cardiac function like traditional scaffolds. In fact, de-cellularized heart tissue patches and injections improved angiogenesis and attenuated ventricular dilatation in rodent infarct models [147, 148]. The results were modest and mirrored the effects of other tissue engineering approaches; however, they have not been directly compared.

Not all de-cellularization protocols are the same and there is a delicate balance between complete removal of cells and cellular debris and the preservation of growth factors and other proteins within the extracellular matrix (ECM). Thorough de-cellularization often results in the loss of ECM proteins [149], which can remove growth factors or alter the structural integrity of the graft. On the other hand, careful preservation of the ECM fails to completely remove cellular debris [149], thereby increasing the risk of an allogeneic immune response in the host after implantation. An analysis of four different de-cellularization protocols revealed highly variable effects on the ECM yet, all four protocols generated a scaffold that promoted cell engraftment and the formation of organized myofibers [149]. "Tissue re-engineering" or reseeding de-cellularized matrices with new cells has been successful in other fields of medicine, notably the creation of bioprosthetic heart valves. Comparative studies like the one above that pit de-cellularized matrices against more conventional approaches could be useful in the future. Some studies have shown that a

mixture of a variety of synthetic and/or biological compounds is superior to scaffolds and injections that utilize only a single polymer. Further studies will show whether nature's mix of ECM proteins are superior to human engineering or not.

2.2 Synthetic Polymers

Unlike biological scaffolds, the size, shape, hydrophilicity, and surface of synthetic scaffolds can be engineered to optimize specific parameters such as cell adhesion or vasculogenesis [145]. Consequently, a wide range of synthetic polymers has been used to construct three-dimensional tissues [125, 126, 150, 151]. Polyglycolic acid (PGA) and polylactic acid (PLA) are a common choice because of their non-toxic degradation products glycolic acid and lactic acid [125, 151, 152]. Ke et al. seeded PGA scaffolds with ESCs and implanted the grafts in the murine heart following LAD coronary artery ligation [153]. The ESCs survived up to eight weeks later while the mice that had received the ESC-PGA implants exhibited higher survival rates and displayed improved hemodynamics [153]. A brief histological analysis revealed some α -myosin heavy chain staining, but the phenotype was not investigated further [153]. PGA scaffolds have also been seeded with bone marrow cells and mitogenic compounds to induce angiogenesis within the graft [125]; however, the lack of a baseline control and sham operated mice makes it difficult to assess the true regenerative effects of this therapy. One study compared various characteristics of gelatin, PGA, and PLA scaffolds that were seeded with autologous rat smooth muscle cells [154]. Cells were more evenly distributed in the PLA scaffolds and displayed more suitable degradation kinetics compared to the gelatin and PGA scaffolds [154]. PGA and PLA have been used for a number of tissue engineering applications because of their non-toxic degradation products and non-immunogenic properties; however, they display relatively rapid degradation kinetics and are not as elastic as native heart tissue thereby limiting the amount of mechanical support they can provide to the heart [145]. Moreover, the degradation products can increase the acidity of the local microenvironment, possibly leading to further tissue damage [145].

Polyurethane offers a more flexible polymer than either PGA or PLA, resulting in better mechanical support [155]. McDevitt et al. cultured cardiomyocytes *in vitro* on polyurethane films coated with laminin lanes to guide cardiomyocyte growth and organization into longitudinal, electromechanically coupled myofibers [155]. The cardiomyocytes displayed a more mature, organized phenotype, but there was no *in vivo* analysis to assess polyurethane's effect on the myocardium [155]. Alternatively, cell-free polyurethane urea films implanted around the infarct site of a rat heart attenuated ventricular remodeling up to eight weeks later, providing evidence for the profound effect mechanical support can have on myocardial remodeling [156]. Another comparative study coated polyurethane films with gelatin, fibronectin, or collagen and seeded them with ESC-derived cardiomyocytes [126]. The study found that polyurethane films coated with collagen were the best at generating contractile grafts [126]. Little histological analysis and no *in vivo* data comparisons were provided [126]. Although polyurethane possesses more suitable mechanical properties than many other synthetic polymers, it is limited by the toxic degradation product diisocyanate [145]. Recently, a biocompatible poly(glycerol sebacate) scaffold has been developed that can be tuned to match the stiffness of the heart [157, 158]. It will be interesting to test these polymers *in vivo* given adaptability.

A long list of other synthetic polymers has been tested for their use in cardiac tissue engineering. Briefly, neonatal cardiomyocytes seeded on poly ϵ -caprolactone (PCL) scaffolds display spontaneous contractility [159]. PCL and gelatin have been electrospun to create longitudinally aligned nanofibers [160]. Cardiomyocytes attached better and displayed a more elongated morphology indicative of a more mature phenotype [160]. Iyer et al. used poly(ethylene glycol) acrylate molds to create matrigel-coated microchannels to

help align cardiomyocytes longitudinally and create more organized myofibrils [161]. The study found that pre-culturing the microchannels with fibroblasts and endothelial cells prior to seeding with cardiomyocytes led to the creation of contractile organoid structures [161]. Seeding the microchannels with all three cell types simultaneously or killing the fibroblasts and endothelial cells prior to the addition of cardiomyocytes was not sufficient to produce contractility [161]. This co-culture study suggests that the extracellular matrix may not be sufficient for cardiomyocyte maturation and that seeding scaffolds with mixed populations of cells appears to be optimal for cardiac tissue engineering [161]. This is not surprising considering the diversity of cells that exist within the myocardium and the amount of cell-to-cell communication that occurs within it. Finally, Kellar et al. seeded a vicryl mesh with Dermagraft, a clinically approved, cryopreserved fibroblast graft commonly used to heal diabetic ulcers, in an attempt to revascularize the heart [162]. Clinically approved Dermagraft is an attractive option as a translational therapy, but it lacks any intrinsic ability to regenerate the heart because of its lack of cardiomyocytes.

In contrast to biological polymers, synthetic scaffolds can be molded to fit a particular application. They offer better mechanical support and are non-immunogenic [145]. Toxicity and irregular degradation kinetics, however, still hinder its use clinically. In addition, biological and synthetic scaffold technologies do not achieve the necessary cell densities to provide the heart with enough contractile force to restore ventricular function. This is partly due to the intrinsic properties of the scaffolds, which can promote the aggregation of cells rather than an even distribution. Nevertheless, interesting new avenues of research have emerged from studying synthetic and biological scaffolds, some of which can be applied to other tissue engineering strategies.

2.3 Cell Sheets

Cell sheets are an alternative, scaffold-less approach to regenerating myocardial tissue that utilizes the cells own deposited extracellular matrix to bind the cells together and maintain intercellular connections [130]. The cell sheets are fashioned by culturing cells on temperature-responsive poly(N-isopropylacrylamide) (PIPAAm)-coated plates [14]. At normal cell culture conditions, PIPAAm exists in a hydrophobic, globular form, exposing most of the culture surface beneath it [14]. In these conditions, the cells can attach to the surface of the plate and deposit an extracellular matrix [14]. When the temperature of the dish is reduced to 20 degrees, the PIPAAm changes to a hydrophilic state and spreads out over the surface of the culture dish [14]. As it does so, the PIPAAm disrupts any attachments the monolayer had with the culture surface and the sheet lifts up from the culture dish, ready for transplantation [14]. Cell sheets maintain their intercellular connections, providing electromechanically coupled, cell-dense grafts that retain the natural pro-survival and maturation environmental cues provided by the extracellular matrix [14].

The first contractile cardiomyocyte sheets were constructed from chick embryonic cardiomyocytes [14]; however, individual sheets do not provide nearly enough cells to restore ventricular function. Hence, the ability to stack multiple cell sheets on top of one another is an essential attribute that enables it to be developed further as regenerative therapy. Individual cardiomyocyte sheets can quickly adhere to one another, forming gap junctions and intercellular adhesions within minutes [163, 164]. Two-layer and four-layer thick cardiomyocyte sheets survived up to one year subcutaneously, exhibiting coordinated contractions that could be observed macroscopically through the skin [129, 163]. Interestingly, two-layer and four-layer thick cell sheets implanted in eight week-old nude rats showed lower cellularity and increased fibrosis compared to those implanted in three week-old nude rats [129]. This is of particular concern because similar problems might arise when cardiomyocyte sheets are implanted in adult myocardial infarct patients.

Oxygen and nutrient diffusion is a major limiting factor for large engineered tissues [130]. Shimizu et al. found that a maximum of three cardiomyocyte sheets can be stacked on top of one another before necrosis starts to appear, which is still too thin to provide an adequate number of cells to significantly improve human ventricular function [165]. Consequently, Shimizu et al. took a multi-step transplantation approach where three-layer thick cardiomyocyte sheets were implanted subcutaneously at one, two, and three day intervals [165]. Each step allowed for neovascularization to occur within the graft in a stepwise fashion so that the entire graft was penetrated with new blood vessels [165]. A series of ten transplantation procedures occurring at one-day intervals generated contractile cardiac tissue 1 mm thick concomitant with blood vessels throughout the entire thickness of the graft [165]. The same procedure was performed over resectable arteries or veins to produce a thick, contractile tissue that could be surgically removed and anastomosed with the host circulation elsewhere in the body [165]. Subcutaneously tissue engrafted over resectable arteries and veins survived the re-transplantation procedure and reperfusion occurred following anastomosis with the carotid artery and jugular vein in the neck [165]. A similar approach can be envisioned in humans. ESC-CM sheets can be engrafted subcutaneously over a resectable vein, such as the saphenous vein in the leg. Multiple engraftments over several days will generate thick, contractile tissue that can be surgically removed and reconnected to the host coronary circulation. Unfortunately, this approach is more invasive than many others, a disadvantage that needs to be considered.

Cardiomyocyte sheets have been shown to survive and couple electromechanically with the host tissue following implantation in a rat myocardial infarct model [127, 128, 166]. Bilayered cardiac sheets preserved left ventricular dimensions and heart function better than control and fibroblast sheets eight weeks after transplantation [128]. Although there was some immunohistochemical evidence of gap junction formation between the graft and host tissue, gross histological sections showed fibrotic tissue largely separating the cell sheets from the host tissue [128]. Additional electrical conduction studies and small molecule dyes, however, have verified electromechanical coupling with the host can occur [127, 166].

Other cell sources have been studied using cell sheet technology, namely skeletal myoblasts [167, 168], mesenchymal stem cells [169], and endothelial cell-fibroblast mixtures [170]. In brief, skeletal myoblast sheets implanted in a porcine infarct model showed functional improvements and higher survival rates compared to controls 6 months later [168]. Mesenchymal stem cell monolayers showed similar improvements one month after engraftment [169]. Similar to the other approaches described above, the improvements in heart function may be the result of paracrine effects as all three cell sources were found to secrete significant angiogenic and paracrine signaling molecules *in vitro* [167, 169, 171]. Most of the current heart tissue engineering studies using cell sheets employ embryonic or neonatal cardiomyocytes, which cannot be translated to the clinic. Thus, studies involving more clinically relevant cell types are more encouraging. Future work involving ESC-derived or iPSC-derived cardiomyocytes will add even more clinical relevance to cardiomyocyte sheet technologies.

2.4 Tissue Engineering In Situ

2.4.1 Biological Compounds—A new field has emerged in tissue engineering that employs self-assembling, soluble scaffolds that can be injected into the infarct site of the heart [17, 132, 133]. The soluble scaffold polymerizes after injection, taking the native shape of the heart while providing mechanical support [3]. At the same time, the scaffold also supplies adhesion and pro-survival signals to improve cell retention [3]. Fibrin is a natural, self-assembling peptide found in the body that is used to form clots along damaged endothelium. Simultaneous injection of fibrinogen and thrombin into the infarct provides

time for the fibrinogen to spread out within the scar tissue before cleavage to fibrin and subsequent polymerization [172]. Skeletal myoblasts injected with self-assembling fibrin glue increased cell survival and attenuated left ventricle dilatation and dysfunction [172]. Fibrin glue has also been injected with endothelial cells [173] and bone marrow mononuclear cells [16]. Both cell types increased vasculogenesis within the scar compared to control, fibrin glue alone, or cell injections alone [16, 173]. Notably, endothelial cells embedded in fibrin glue improved left ventricle function after two months in a sheep chronic ischemia cardiomyopathy model [173]. Martenset al. studied fibrin polymerization kinetics in order to optimize fibrin and thrombin concentrations for transcatheter delivery [174]. An appropriate delivery window of 10 minutes could be achieved without clogging the catheter [174]. Furthermore, cells that are injected in fibrin glue are generally retained within the heart tissue and do not migrate to the kidneys or the spleen where they can cause serious damage [174].

More familiar natural scaffolding materials such as alginate, collagen, and matrigel have also been developed as self-assembling scaffolds [131, 132, 175]. These materials polymerize at 37 degrees after injection into the tissue rather than relying on a proteolytic enzyme like thrombin [175]. Human cardiac progenitor cells isolated from peripheral blood injected with a soluble collagen matrix enhanced cell survival and vasculogenesis in an athymic rat ischemic hind limb model [15, 133]. Soluble alginate injections alone preserved ventricular dimensions and function better than controls up to two months after implantation [132, 176]. Self-assembling alginate has also been safely tested for intracoronary catheter delivery [177]. Similar to alginate and collagen, Matrigel injections increased anterior wall thickness [131, 178] and enhanced embryonic stem cell survival. However, the cells grew in small clusters and did not display typical cardiomyocyte phenotypes [131]. More importantly, injections of ESC and Matrigel did not restore ventricular contractility [131]. A comparative study between all three self-assembling materials found no significant difference angiogenic potential [179]. There were no functional studies to assess any differences that might have existed. Finally, Zhang et al. mixed collagen and matrigel to form a “hydrogel” that was subsequently mixed with cardiomyocytes [180]. Improvements in anterior wall thickness and fractional shortening were observed one month later; however, the cardiomyocytes did not display any detectable levels of connexin-43 [180], indicating that electrical coupling had not occurred.

Chitosan is a novel biological scaffolding material that is beginning to be applied to various tissue-engineering applications [181]. It is a biodegradable polymer derived from chitin that consists of glucosamine and N-acetyl-glucosamine subunits [181]. Three reactive functional groups exist on the chitosan subunits, allowing its properties to be extensively manipulated [181]. Consequently, a temperature-sensitive, injectable chitosan scaffold has been engineered [181]. Murine ESCs survived better and took on an elongated, cardiomyocyte phenotype when they were injected directly into the infarct with a temperature-sensitive chitosan scaffold [17]. Notably, the chitosan scaffolds maintained ventricular dimensions and function one month later in mouse and rat infarct models [17, 182]. Only a few heart tissue-engineering studies have been conducted using chitosan [17, 182]; however, its unique qualities offer a variety of new opportunities. The cationic properties of chitosan attract anionic glycosaminoglycans and proteoglycans of the extracellular matrix, which anchor cytokines and growth factors that are important for wound repair [181]. Thus, it is not surprising that chitosan has been shown to improve all of the stages of wound healing [181]. Chitosan is biocompatible, biodegradable, antimicrobial, and highly adaptable, making it well suited for heart tissue engineering applications.

2.4.2 Designer Synthetic Peptides—The injectable scaffolds described above are natural polymers whose properties happen to be suitable for tissue engineering. However,

some groups have taken an entirely engineered approach by designing self-assembling, modular, “designer” peptides from scratch [18, 183–185]. These peptides can be modified to possess RGD integrin binding domains and other cell adhesion motifs or they can be used to tether specific drugs or compounds [18, 183]. Davis et al. designed a self-assembling scaffold modified to carry insulin-like growth factor 1 (IGF-1) to promote cardiomyocyte survival and maturation [184]. The anchored IGF-1 remained in the tissue up to one month later in a healthy heart [184]. Inflammation will likely shorten IGF-1 retention due to the release of metalloproteinases, but the IGF-1 scaffold nonetheless improved cardiomyocyte viability and maturation *in vivo* while improving fractional shortening and ventricular dimensions for up to three weeks in a rat infarct model [184]. The same scaffold injected with Lin-/cKit+ progenitor cells in a rat infarct model improved LVEF and ventricular geometry one month later [185]. Some connexin-43 and N-cadherin staining was evident, but further examination is needed to determine if these *de novo* cardiomyocytes truly couple electromechanically [185]. A similar study that anchored RGD motifs to the scaffold instead of IGF-1 found that bone marrow stem cells survived better and expressed cardiac troponin T and connexin-43 [18].

Synthetic peptides that can self-assemble offer a modular approach to design a scaffold that best-suits the heart microenvironment. However, it lacks natural adhesion motifs that are recognized by most cells [3]. Adding these adhesion motifs may alter the peptide’s ability to self-assemble or change its mechanical properties. Additionally, no study has examined the potential toxic side effects of injecting designer peptides into the infarcted myocardium. Like most of the other scaffolds and cell therapies that have been studied, long-term assessments of the effects of designer peptides are needed. Smart, designer peptides are a burgeoning field. Its adaptability offers innumerable possibilities for regenerating functional heart tissue, offering an exciting, innovative approach to engineering heart tissue.

Concluding Remarks

Over the past decade, a plethora of different cell types and engineering approaches have tried to regenerate functional heart tissue; yet, a clinical regenerative therapy is still years away. Modest short-term improvements have been made, but current strategies appear to delay cardiac dysfunction rather than restore normal function. Several themes emerge from examining the literature on cardiac tissue repair. Scar tissue that forms after a myocardial infarction is a recurring problem because it prevents electromechanical coupling and alters the heart’s geometry. Consequently, grafts implanted along the exterior of the heart may not be in the best position to significantly improve ventricular function. One study compared direct myocardial injections or epicardial deposition of skeletal myoblasts using a gelfoam or cell sheet [186]. All three delivery strategies improved LVEF better than controls; however, the gelfoam and cell sheets proved to be most effective [186]. The study used skeletal myoblasts that are incapable of coupling electromechanically with the host; therefore, the results may be due to better mechanical support provided by the gelfoam and cell sheets rather than the graft’s position within the heart. Advances in reducing or eliminating scar tissue formation will likely prove invaluable to the field of heart tissue engineering, not to mention post-MI patient care in general.

Clearly, cardiomyocytes will be the preferred cell type for any tissue engineering application. However, the source and the quantity to use are still unclear. Endogenous cardiac progenitors are ideal because they already reside in the heart, but their low numbers may be insufficient to replace the enormous number of cardiomyocytes lost during a myocardial infarction. hESC and iPSC lines can theoretically generate a limitless supply of cardiomyocytes, but nutrient diffusion limitations and poor survival severely reduce the number of cardiomyocytes that can be implanted in the heart. These limitations aside, hESC

and iPSC-derived cardiomyocytes tend to exhibit neonatal or fetal phenotypes that are less contractile than adult myocytes. Advances in tissue-engineering such as cyclic electrical or mechanical stimulation may help to solve some of these problems.

Several challenges remain and, undoubtedly, more will arise as technologies develop. Although regenerative therapies may be years away, heart tissue engineering can be used for alternative purposes, most notably drug screening. The use of thymosin- β 4 is an ideal non-invasive regenerative approach. Further long-term studies are required and limitations still exist, but the results are nonetheless encouraging. An aging baby boomer population in the US and the increased rates of obesity and heart disease demand a regenerative therapy that is cost-effective. Moreover, increasingly effective cardiovascular treatment regimens have led to a growing population of patients with heart failure. Disease progression can be slowed, but eventually, the heart succumbs and the only option left is heart transplantation. Regenerative cell therapies can replace heart transplantation and restore ventricular function in this growing population of patients. The current global fiscal crisis will make this goal even more difficult as funding for research is reduced and countries look to cut health care costs wherever it is possible. Hopefully, these challenges will drive more innovation and creativity to turn a non-invasive, long-term, regenerative therapy into a clinical reality.

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Abbreviations

CABG	Coronary artery bypass grafts
ESC	Embryonic stem cells
HSC	Hematopoietic stem cells
hESC-CM	Human embryonic stem cell derived cardiomyocytes
iPSC	Induced pluripotent stem cells
Isl-1	Islet 1 transcription factor
LAD	Left anterior descending
LVAD	Left ventricular assist device
LVEDV	Left ventricular end-diastolic volume
LVEF	Left ventricular ejection fraction
MI	Myocardial infarct
PCL	Poly ϵ -caprolactone
PGA	Polyglycolic acid
PIPAAm	Poly(N-isopropylacrylamide)
PLA	Polylactic acid
Sca-1	Stem cell antigen 1
Tbx18	T-box transcription factor 18
TB4	Thymosin- β 4

TMRM Tetramethylrhodamine methyl ester perchlorate
Wt1 Wilm's tumor 1

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Table 1

Advantages and disadvantages of cell sources and clinical results obtained from trials

Cell Source	Advantages	Disadvantages	Clinical Results
Skeletal Myoblasts	Capable of surviving hypoxic conditions [30, 34] Autologous [31–34] Easy to obtain through biopsy [31–33]	Cannot transdifferentiate into cardiomyocytes [23] Poor electromechanical coupling with the host [21, 23, 30]	Phase I trial-safe and feasible. Short term benefits [31, 32, 40, 41] Phase II trial showed no significant difference between treated and untreated individuals
Bone Marrow Stem Cells	Endogenous progenitors that are easy to acquire [47–50] Possible differentiation to cardiac lineages [7, 42]	Still not known whether these cells can differentiate into cardiac cells [7, 22, 42–46]	Meta-analysis of 8 long- term trials show modest improvements in heart function [50]
Endogenous c-Kit ⁺ or Sca-1 ⁺ stem cells	Endogenous stem cells in the heart [12, 62] Possible differentiation into cardiomyocytes [12, 62]	Could be circulating hematopoietic stem cells rather than cardiac stem cells [66, 67]	N/A
Isl-1 Progenitors	Self-renewing, clonogenic, and migratory [10, 25, 69] Bona fide cardiac precursor that differentiates into all three cardiac lineages [10, 68, 69]	Still not known if these cells survive late into adulthood [10]	N/A
Epicardial Wt1 and Tbx18 progenitors	Endogenous progenitors [86] Differentiate into all three cardiac lineages [86]	Must be primed with TB4 [11, 88–90] Small population in the heart [86, 87]	N/A
hESC-derived Cardiomyocytes	Self-renewing with the ability to differentiate into cardiomyocytes [92–94] Electromechanical coupling [92, 95, 97]	Ethical issues Immature cardiomyocyte phenotype [26, 93, 94, 102] Low cardiomyocyte yield [26, 98] Risk of teratoma formation Allogeneic	N/A
iPSC-derived Cardiomyocytes	Self-renewing with the ability to differentiate into cardiomyocyte [108, 109] Electromechanical coupling [9, 108, 109] Autologous [105, 106] Easy to acquire [108, 109]	Immature cardiomyocyte phenotype [9, 108, 109] Lower cardiomyocyte yield than hESC [108, 109] Epigenetic imprinting [118, 119] Possible tumorigenic capabilities due to genomic manipulation	N/A

Table 2

Comparison of the advantages and disadvantages of various delivery methods and materials for cardiac repair.

Delivery Technique	Advantages	Disadvantages	References
Cell Injections	Less invasive	Cannot provide support to cells or the heart Inconsistent graft size Poor retention Poor cell survival Poor cell density	[34, 35, 43]
Scaffolds			
Biological Polymers	Provide survival and maturation cues Mechanical support to the heart Non-toxic degradation products	Rapid degradation kinetics Immunogenic Inconsistent quality control Low cell density	[82, 124, 134, 137–144]
Synthetic Polymers	Better degradation kinetics than biological polymers Consistent size and quality Better stiffness characteristics	Some toxic degradation products Lacks natural adhesion and survival motifs Low cell density	[125, 126, 151–160, 162]
Cell Sheets	Non-immunogenic extracellular matrix Cell dense, electromechanically coupled sheets	Difficult to manipulate Immature phenotype	[14, 127–129, 163–171, 187]
Injectable Scaffolds	Less invasive Takes the native shape of the heart Can be specifically engineered to carry beneficial compounds or moieties	Relatively new technology that needs further characterization	[15–18, 131, 133, 172–174, 176–182, 184, 185]