

Stem cell therapy for cardiac disease

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Congenital heart disease occurs in 1% of liveborn infants, making it the most common birth defect worldwide. Many of these children develop heart failure. In addition, both genetic and acquired forms of dilated cardiomyopathy are a significant source of heart failure in the pediatric population. Heart failure occurs when the myocardium is unable to meet the body's metabolic demands. Unlike some organs, the heart has limited, if any, capacity for repair after injury. Heart transplantation remains the ultimate approach to treating heart failure, but this is costly and excludes patients who are poor candidates for transplantation given their comorbidities, or for whom a donor organ is unavailable. Stem cell therapy represents the first realistic strategy for reversing the effects of what has until now been considered terminal heart damage. We will discuss potential sources of cardiac-specific stem cells, including mesenchymal, resident cardiac, embryonic, and induced pluripotent stem cells. We will consider efforts to enhance cardiac stem cell engraftment and survival in damaged myocardium, the incorporation of cardiac stem cells into tissue patches, and techniques for creating bioartificial myocardial tissue as well as whole organs. Finally, we will review progress being made in assessing functional improvement in animals and humans after cellular transplant.

More than 19 million children and adults in the United States and Europe suffer with heart failure, resulting in ~230,000 deaths annually at a cost of >\$140 billion/y (1,2). This includes the 2.5 million children born each year worldwide with congenital heart disease (3), many of whom eventually develop heart failure. Heart failure occurs when damaged myocardium becomes unable to meet the body's metabolic demands. Unlike some organs, the heart has a severely limited, if any, capacity for repair after injury.

Currently, heart transplantation remains the ultimate approach to treating end-stage heart failure, but this therapy is invasive, costly, and excludes patients who are not candidates for transplantation given their comorbidities. Most importantly, there are not enough organs for transplanting the increasing number of patients with end-stage heart failure,

including children with complex congenital heart disease who are surviving well into adulthood. New, accessible therapies are needed to treat the millions of patients with debilitating heart failure worldwide. Myocardial engineering, including stem cell transplantation, may represent the first realistic strategy for reversing the effects of what has until now been considered terminal damage to the heart. We review the potential sources of cardiac-specific stem cells and their incorporation into tissue patches, as well as the progress made in assessing functional improvement in transplanted animals and human patients.

CELL SOURCES

Mesenchymal Stem/Stromal Cells

Mesenchymal stem/stromal cells (MSCs) reside in the bone marrow stroma and can differentiate into osteoblasts, chondrocytes, and adipocytes (4,5). In addition, MSCs differentiate *in vitro* into spontaneously beating cardiomyocytes (CMs) after exposure to the demethylating agent 5-azacytidine (6). Because of their cardiomyogenic potential, MSCs have been transplanted in animal models of myocardial infarction (MI). Collectively, these studies have demonstrated improved left-ventricular function, reduced infarct scar size, attenuated left-ventricular remodeling, greater vascular density, and increased survival following transplantation (7). Besides transdifferentiating into CMs, MSCs are relatively easy to grow and can be expanded in culture to cell numbers required for transplantation (8). Another advantage of MSCs for the repair of damaged myocardium is their ability to suppress immune rejection and curb the inflammatory response. Despite expressing major histocompatibility complex class I and low levels of class II antigens as well as Fas ligand, MSCs fail to elicit an alloreactive lymphocyte response when added to mixed lymphocyte cultures (9).

MSC transplantation has been tested in the clinic. In a randomized, placebo-controlled study, patients with acute MI received either intracoronary injections of autologous MSCs or saline (10). Evaluation of treated patients as compared with a placebo group and with that of pretransplantation after injury, the MSC-treated group showed increased wall movement velocity at the infarct site, left-ventricular ejection fraction, and end-systolic pressure to end-systolic volume ratio at 3-mo post-therapy. Cardiac mechanical and electrical properties were also significantly improved. Osiris Therapeutics completed a phase I clinical trial to assess the safety and preliminary efficacy of

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intravenous allogeneic adult human MSCs (11). Although MSC infusion proved safe in patients, with no ectopic tissue formation, the trial results also showed improved outcomes for MSC-treated patients with respect to left-ventricular function, cardiac arrhythmias, pulmonary function, and symptomatic global assessment. Whether MSCs transdifferentiate into new CMs remains controversial (12), and so the mechanisms for MSC-mediated functional benefit remain to be fully elucidated. However, several companies are now conducting phase II clinical trials to evaluate “off-the-shelf” MSC therapy in patients with acute MI and congestive heart failure.

Resident Cardiac Stem Cells

Since the 1960s, studies of CM proliferation in rodents had indicated that the adult mammalian heart was a terminally differentiated, postmitotic organ without the capacity for cellular regeneration (13). Over the past 10 y, however, several findings have challenged this view. Observation of cell division, telomerase activity, telomere shortening, and CM apoptosis have provided evidence of CM turnover in adult human hearts (14–17). In addition, pockets of mitotically active cells in hypertrophic myocardium and hearts of patients with end-stage heart failure have been described (18). Using a genetic fate-mapping approach, Hsieh *et al.* showed that whereas adult CMs are not replaced in the uninjured heart during normal aging up to 1 y in the mouse, they are refreshed after MI or pressure overload (19). Recently, Porrello *et al.* showed that the hearts of 1-d-old neonatal mice can regenerate after partial surgical resection, but this capacity is lost by 7 d of age (20). The most compelling evidence for CM renewal in maintaining cardiac homeostasis comes from a study in which the amount of ¹⁴C (generated from above-ground nuclear testing between 1955 and 1963, before the implementation of the Limited Nuclear Test Ban Treaty) integrated into the DNA of human myocardial cells was used to date the birth of myocardial cells (21). Carbon dating indicated postnatal cell turnover that declines with age: about 1% per year at age 25 to 0.45% at 75. This provided strong evidence not only for low-level turnover of CMs in the adult heart but for the idea that adult myocardial tissue is capable of incorporating new muscle cells to preserve tissue mass and function.

c-Kit and stem cell antigen-1 as markers of cardiac progenitors.

Detection of the Y chromosome in undifferentiated cells and differentiated CMs found in female donor hearts transplanted into male patients not only supports the concept of cardiac chimerism in humans but also suggests the existence of cardiac progenitor cells that give rise to new CMs (22). This putative stem cell population was positive for surface antigens *c-Kit*/CD117 and/or multidrug resistance-like protein 1, or stem cell antigen-1 (*Sca-1*). Cardiac stem cells have been identified and isolated by others based on the expression of these markers (17,23). In the presence of 5-azacytidine or oxytocin, *Sca-1*⁺ cells are reported to exhibit spontaneous beating, express cardiac-specific genes, and, when injected intravenously into mice following MI, home to the infarct site, differentiate, and

fuse with host myocardium (23). Likewise, multipotent *c-Kit*⁺ cells reconstitute the injured myocardium by forming new blood vessels and myocytes, comprising as much as 70% of the ventricle in some reports (24,25). The structural and functional regeneration of infarcted myocardium occurred independently of cell fusion. Remarkably, Bearzi *et al.* obtained similar results after isolating human *c-Kit*⁺ cells from myocardial tissue of patients who underwent cardiac surgery, expanding them in culture, and injecting them into the infarcted myocardium of immunodeficient mice and immunosuppressed rats (26).

Side population cells. Side population (SP) cells, known for their ability to efflux vital dyes such as Hoechst 33342, were initially discovered as hematopoietic stem cells (27). ATP-dependent transporters, including multidrug resistance-like protein 1 and ATP-binding cassette subfamily G member 2 (ABCG2), are believed to mediate dye exclusion, and both ABCG2 and multidrug resistance-like protein 1 have been cited as molecular determinants of cardiac SP cells in the adult myocardium (28,29). Further characterization shows that cardiac SP cells widely express *Sca-1* and CD31 but are largely negative for the hematopoietic markers CD45 and CD34, cellular adhesion marker CD44, and *c-Kit* (30). Moreover, functional differentiation and maturation of cardiac SP cells are also restricted to the *Sca-1*⁺/CD31⁻ subpopulation. Direct injection of heart-derived *Sca-1*⁺/CD31⁻ cells into the peri-infarct region immediately following MI in mice limits left-ventricular remodeling, attenuates contractile dysfunction, and improves myocardial energy metabolism (31). The transplanted *Sca-1*⁺/CD31⁻ cells promoted neo-angiogenesis and underwent *in vivo* differentiation into CMs and endothelial cells.

Cardiospheres. Cardiospheres present another potential source of endogenous cardiac stem cells. Cardiosphere cells are derived from cultured explants of mouse hearts and human atrial or ventricular biopsy samples following gentle enzymatic digestion (32). They are able to migrate over the adherent portion of the explanted tissues. When collected and cultured, they form clonal, multicellular clusters capable of self-renewal as well as differentiation into CMs and vascular cells. Immunophenotypic characterization shows that newly formed cardiospheres express stem cell markers CD34, *c-Kit*, and *Sca-1*, and endothelial markers CD31 and kinase insertion domain receptor/vascular endothelial growth factor receptor 2/Flk-1. Recently, Ye *et al.* have shown that the *Sca-1*⁺ subpopulation of cardiosphere cells preferentially include *Isl-1*-expressing precursors that give rise to second heart field structures (33). Since their description, alternative approaches and more efficient methods to generate cardiospheres have been developed (34,35). Of particular note, the yield of cardiosphere cells from injured hearts is greater than from uninjured hearts, and cardiospheres are easily isolated and expanded from “middle aged” hearts (33), supporting their feasibility in autologous cell transplantation. Clinical studies in animal models of MI have shown that cardiosphere injection into infarcted mouse and pig hearts preserves ventricular function, improves hemodynamic

indices, produces less adverse remodeling, and reduces infarct size (33,34,36–38).

Despite displaying partial overlap in marker expression, the origins and exact lineage relationships among these various adult cardiac stem cells remain unknown. In fact, the validity of these cell populations as resident cardiac stem or progenitor cells has been questioned. Pouly *et al.* reported that c-Kit⁺ cells harvested from either human right-ventricular endomyocardium or right-atrial appendage lack other stem cell markers such as multidrug resistance-like protein 1, and coexpress CD45, suggesting they are of hematopoietic origin (39). In addition, c-Kit⁺ cells stained positive for the mast cell lineage marker tryptase, implying that these cells may not be CM precursors but rather mast cells. Studies in which Sca-1 was used to identify adult stem cells from human hearts were based on immunoreactivity to nonhuman Sca-1 antibodies. To date, a human homologue of Sca-1 has not been found (40), although the preponderance of studies that have identified cells based on the expression of “human Sca-1” make an immunoreactive analogue likely. There have also been some discrepant findings with regard to cardiospheres. Studies by several groups have demonstrated that some explant migrating cells do not differentiate into functional CMs or yield any significant physiological benefit in the infarcted mouse heart (41–43). Notwithstanding, the clinical utility of cardiospheres is currently being tested in phase I clinical trials in patients with MI (44).

Human Embryonic Stem Cells

Human embryonic stem cells (hESCs) grow and divide indefinitely while maintaining the potential to develop into derivatives of all three embryonic germ layers. Under appropriate culture conditions, a small fraction of hESCs (5–15%) spontaneously differentiate into CMs with structural and functional properties characteristic of endogenous CMs (45). To improve yield of hESC-derived CMs for cell therapy, current efforts are focused on directing the differentiation of hESCs into the cardiac lineage (46). Innovative enrichment, purification, and selection strategies have been developed to guide cardiac differentiation to relatively pure homogeneity (Figure 1).

Defined culture media have been developed to direct CM differentiation from hESCs. Coculture studies with mouse END2 cells have identified prostaglandin I₂ as an inducer of CM differentiation (47). Similarly, 5-azacytidine treatment during hESC differentiation has been shown to enhance CM differentiation, suggesting that DNA demethylation is a key factor in directing tissue-specific differentiation (48). Exposure to SB203580, a small-molecule inhibitor of p38^{MAPK}, has also been observed to improve the efficiency of CM differentiation from hESCs (49,50) and has implicated p38^{MAPK} in the regulation of the ectoderm–mesoendoderm switch during early ESC differentiation (50). By mimicking the signaling environment of the early mouse embryo, Yang *et al.* established a three-stage protocol that supports cardiac development at high frequency in differentiating hESC cultures (51). This protocol exposes human embryoid bodies (hEBs) to a combination of activin A, bone morphogenetic protein 4, and basic fibroblast growth

factor (bFGF) during the first 4 d of differentiation to induce primitive-streak formation, representing the onset of gastrulation. Between days 4 and 8, the differentiating hEBs are treated with vascular endothelial growth factor and Dickkopf homolog 1 to induce the development of cardiac mesoderm and further maturation.

Advances in understanding the genetic and epigenetic regulation of CM differentiation have also suggested potential new approaches to producing stem cell–derived CMs for therapy. The small, regulatory RNAs, microRNA (miR)-1 and miR-133, are specifically expressed in the mouse heart, and their targeted deletion or knockdown results in dysregulation of cardiac morphogenesis, electrical conduction, cell cycle, and cardiac hypertrophy (52,53). Recently, Ivey *et al.* showed that miR-1 and miR-133 regulate the differentiation of mouse embryonic stem cells (mESCs) and hESCs into the cardiac lineage (54). Lentiviral induction of either miR-1 or miR-133 expression in mESCs enhanced early mesoderm differentiation and repressed development of endoderm and neuroectoderm. However, further differentiation revealed a predominant role for miR-1 in promoting the differentiation of mESC- or hESC-derived mesoderm into cardiac and skeletal muscle cells. More recently, Wong *et al.* have shown that miR-125b similarly controls early CM specification through its effects on the pluripotency factor LIN28 (55). Whereas miRNAs direct cell lineage

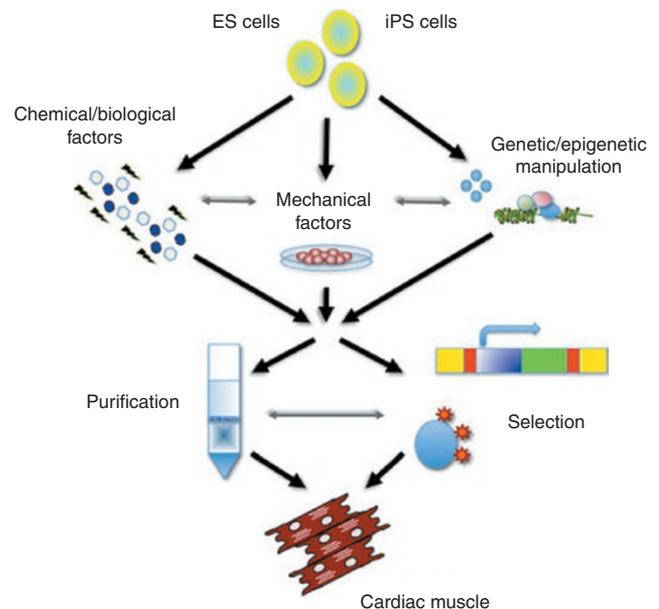


Figure 1. Approaches to preparing embryonic stem (ES) cell- and induced pluripotent stem (iPS) cell-derived cardiomyocytes (CMs) for tissue repair. Methods for directing differentiation of pluripotent stem cells to CMs have focused on chemical (e.g., 5-azacytidine, p38^{MAPK} inhibitors, PGI₂) and biological (e.g., activin A, bone morphogenetic protein, basic fibroblast growth factor, vascular endothelial growth factor, Dickkopf homolog 1) factors, genetic (e.g., miRNAs) and epigenetic (e.g., miRNAs, chromatin remodeling) manipulation, and mechanical factors (e.g., hydrodynamics, surface tension). In transplantation experiments, these approaches have been complemented by purification methods that take advantage of the biochemical properties of CMs (e.g., Percoll density centrifugation, mitochondrial content), and selection strategies that rely on the expression of cardiac-specific genes (e.g., reporter lines, molecular beacons) and surface markers.

determination by controlling protein dosage, epigenetic regulation through chromatin remodeling has been shown to control cell fate as well. Takeuchi *et al.* identified a minimal set of factors necessary to execute the cardiac transcriptional program (56). Baf60c, a cardiac-enriched subunit of the Swi/Snf-like barrier-to-autointegration (BAF) chromatin remodeling complex, in combination with cardiac transcription factors guanine-adenine-thymine-adenine (GATA) motif binding zinc finger transcription factor-4 (Gata4) and T-box transcription factor-5 (Tbx5), was able to induce cardiac differentiation in mouse embryos when ectopically expressed (56).

In combination with directed differentiation strategies, the use of genetic selection has the potential to address issues of CM homogeneity and isolation of cardiac progenitors. Several laboratories have developed transgenic reporter hESC lines to derive pure CM populations. This relies on a cardiac-restricted promoter to drive the expression of a reporter gene or selectable marker. Huber *et al.* used lentiviral vectors to produce stable hESC lines in which enhanced green fluorescent protein (eGFP) was expressed under control of the cardiac-specific human MLC2v promoter (57). Xu *et al.* generated stable hESC lines using a reporter consisting of the cardiac-specific mouse α -myosin heavy chain promoter driving expression of the neomycin resistance gene (58). Kita-Matsuo *et al.* designed a set of lentiviral vectors to generate multiple stable hESC lines with eGFP and mCherry reporters or with puromycin resistance downstream of the mouse α -myosin heavy chain promoter (59). Recently, Ritner *et al.* generated a cardiac-specific hESC reporter line using a lentiviral construct consisting of a fragment of the mouse α -myosin heavy chain promoter upstream of eGFP. The specific promoter fragment allowed the identification and analysis of early cardiac progenitors expressing NKX2-5 before the onset of cardiac troponin T or chamber-specific myosin light chain (MLC) expression (60).

Collectively, fluorescence-activated cell sorting or antibiotic selection of these lines has yielded 85–99% pure CMs or cardiac progenitors. eGFP-expressing cells derived from the ventricular MLC2 (MLC2v) transgenic line formed stable intracardiac cell grafts following transplantation (57). Injection of neomycin resistance-selected hEBs into the hind limb muscles of SCID mice resulted in no teratoma formation after 23 wk (58). Contractile forces in puromycin resistance-selected CMs were similar to those generated by rat neonatal ventricular CMs (59). Whereas isolation of hESC-derived CMs from these lines was based on positive selection, Anderson *et al.* implemented a negative selection strategy to deplete undifferentiated, proliferating hESCs from cultures of hESC-derived CMs (61). Their transgenic hESC line utilized a Herpes thymidine kinase/ganciclovir suicide gene system under the control of a constitutive phosphoglycerate kinase promoter. An important caveat of this approach, however, is that other nonproliferating cell types remained in the culture while proliferating CMs were depleted. CMs produced with this line still required purification, and the excluded proliferating progenitors may yet be proven to be of benefit for transplantation.

As an alternative to genetically modified hESC lines for tracking and isolating CMs and progenitors, King *et al.*

adapted dual-fluorescence resonance energy transfer “molecular beacon” technology for transient, real-time detection of gene expression during hESC differentiation (62). Molecular beacons are single-stranded oligonucleotide probes that have been employed to assay gene expression *in vitro*, as in real-time PCR, and *in vivo* using microscopy. These investigators showed that appropriately designed, dual-fluorescence resonance energy transfer molecular beacon pairs can identify the expression of specific mRNAs and facilitate the collection of specific hESC populations by fluorescence-activated cell sorting, while leaving the hESC genome intact (62).

Transplantation with hESC-derived CMs in animal models of MI has yielded promising, albeit modest, results. Although the methods for generating CMs and monitoring engraftment, as well as the number of transplanted CMs, varied between studies, hESC-derived CM transplantation has led to measurable benefit (63–65). Overall, CM transplantation preserved left-ventricular function, attenuated post-MI wall thinning, and even partially remuscularized the infarct region. Despite these encouraging results, the cardiac-specific benefits determined from *in vivo* engraftment studies appear transient. Van Laake *et al.* reported that although cardiac function was improved 4 wk after MI, the functional benefit was no longer significant at 12 wk, even after tripling the number of transplanted CMs in a repeat study (63). These results question the utility of fully differentiated CMs for cardiac repair. The integration of hESC-derived CMs into existing muscle may be hampered by their limited developmental plasticity, whereas partially differentiated cardiac progenitors may retain the plasticity needed for extensive engraftment (60). In addition, the electrophysiological differences between human and mouse CMs, for example, the significant difference in baseline beating rates, might affect integration of human cells into mouse myocardium, limiting the conclusions that might be drawn from these studies.

Another limitation of hESC-derived CMs is that the allogeneic transplanted cells may be subject to immune rejection. hESCs appear to have a lower immunostimulatory potential as compared with adult cells. DNA microarray data of undifferentiated and differentiated hESCs indicate that almost half of the upregulated immunoregulatory genes in hematopoietic cells, lymphoid organs, and other tissues are not similarly expressed in hESCs, implying that hESCs are immunologically immature (66). This observation suggests that immunosuppressive regimens for hESC-based therapeutics may not need to be as rigorous as those for conventional organ transplantation (66). Nevertheless, transplanted hESC-derived CMs may be susceptible to immune rejection to some degree.

Induced Pluripotent Stem Cells

The advent of induced pluripotent stem cell (iPSC) technology (67,68) offers a possible solution to immune rejection. This technology entails reprogramming terminally differentiated adult human fibroblasts to pluripotent stem cells through ectopic expression of four pluripotency factors: OCT4, SOX2, c-MYC, and KLF4. Reprogrammed cells exhibit many features characteristic of hESCs, including morphology, cell surface

antigens, gene expression, telomerase activity, and proliferation rates, and have the capacity to differentiate into cells of all three germ layers, including CMs (68,69). Because adult fibroblasts can be obtained directly from the patient, it has been assumed that patient-specific, iPSC-derived CMs could be transplanted without immune rejection. However, a recent study showed that isogenic iPSCs may still elicit an immune response (70). Another obstacle to iPSC-based therapy is the time needed to derive patient-specific cells. Using currently available protocols, iPSCs cannot be used under urgent circumstances, such as in the setting of acute MI or rapidly progressive heart failure. Although the prospect of iPSC banking to provide “off-the-shelf” therapy may be useful in genetically homogeneous populations (71,72), this approach may not be practical in more diverse communities (73).

It is also important to recognize that iPSCs have typically been generated using viral vectors, which carry the risk of potentially harmful genomic integration. Indeed, some established iPSC lines are genetically unstable, exhibiting large-scale genomic rearrangements, copy-number variations, and abnormal karyotype even at early passage (74,75). Although most alternative methods for reprogramming are significantly less efficient as compared with viral integration (76,77), several more efficient, nonintegrating technologies have recently been described (78,79). Furthermore, in some iPSC lines reprogrammed using nonintegrating viral methods, high levels of mutational changes were still observed (74). Recent studies have also revealed that iPSCs contain epigenetic features that indicate either incomplete or aberrant reprogramming. In particular, iPSC DNA methylation patterns are frequently reminiscent of the somatic cell of origin (80), suggesting that iPSCs may be incompletely reprogrammed to the naive pluripotent state seen in hESCs. It is unclear whether the observed genetic instability and epigenetic imprinting accrues during reprogramming or was present in the somatic cell of origin. Nevertheless, patient-specific iPSCs derived from somatic cells of older patients may be more likely to contain genomic mutations and disadvantageous epigenetic programs. Thus, the safety and efficacy of therapeutic iPSCs as currently derived remain to be tested.

Induced CMs

A recent study provides another promising approach to bypassing immune rejection. Ieda *et al.* identified a minimal set of transcription factors to reprogram postnatal cardiac fibroblasts into functional CMs (81). They showed that Gata4, myocyte enhancer factor-2c (Mef2c), and Tbx5 were sufficient for CM induction. Induced CMs expressed cardiac genes, exhibited calcium oscillations, and possessed action potentials resembling those of adult ventricular CMs. Some induced CMs reprogrammed fully to display spontaneous contractile activity. *In vivo*, cardiac fibroblasts transduced with Gata4, Mef2c, and Tbx5 transdifferentiated into CMs within 2 wk after injection into immunosuppressed mouse hearts. This suggests that endogenous cardiac fibroblasts provide a pool of cells that could be transdifferentiated *in vivo* to regenerate heart muscle.

TISSUE PATCHES

To improve the survival and engraftment of transplanted cells, tissue engineering efforts have focused on *in vitro* cultivation systems in which cells are grown on preformed scaffolds. These afford matrix and culture parameters suitable for generating three-dimensional contractile and vascularized cardiac patches that resemble native cardiac muscle. Zimmermann *et al.* developed a technique to create engineered heart tissue whereby neonatal rat CMs were combined with liquid collagen I and Matrigel in circular molds subjected to mechanical strain (82). Grafts implanted post-MI in rats demonstrated structural and electrical integration with the host myocardium as well as improved diastolic and systolic ventricular function as compared with controls. Caspi *et al.* constructed highly porous, multicellular scaffolds composed of poly-L-lactic acid and polylactic-glycolic acid in which hESC-derived CMs, human endothelial cells, and embryonic fibroblasts were cultured (83). The three cell-based culture strategy generated human cardiac tissue that was vascularized and contracted spontaneously and synchronously with gap junctions mediating impulse conduction. Transplantation of the engineered cardiac tissue to healthy rat hearts resulted in the formation of stable tissue grafts and functional vascularization (84).

Bioreactors provide a way to control environmental conditions for constructing functional cardiac tissue and are designed to recapitulate the microenvironmental conditions within native cardiac muscle. Neonatal CMs within cardiac tissue patches cultivated under pulsatile fluid flow exhibited highly ordered sarcomeres, intercalated discs, and better contractile properties than those cultivated in nonpulsatile medium flow (85,86). Application of electrical stimulation during cultivation of neonatal rat cardiac constructs enhanced ultrastructural differentiation and augmented contractile behavior as compared with nonstimulated constructs (87,88). Using the rat omentum as a natural bioreactor to prevascularize neonatal rat cardiac patches, Dvir *et al.* showed that omentum-generated patches coupled structurally and electrically with the host myocardium 4 wk after implantation and attenuated ventricular remodeling and dysfunction (89).

Decellularization of tissues to create native scaffolds is another approach that has been taken to reconstitute whole tissues and organs. Using detergent-based coronary perfusion to decellularize cadaveric rat hearts, Ott *et al.* produced a whole-heart scaffold with intact geometry, vasculature, and extracellular matrix (90). They then repopulated the decellularized heart with cardiac and endothelial cells and mounted the recellularized heart in a bioreactor. By supplying oxygen and electrical stimulation within the bioreactor, the organ displayed pumping capacity within several days. This decellularization/recellularization technique holds tremendous promise for the generation of bioartificial myocardial tissue, as well as whole organs for transplantation.

Beyond the use of conventional biodegradable scaffolds, Shimizu *et al.* engineered cell sheets using thermoresponsive cell culture dishes to detach intact sheets and then stacked them three-dimensionally to create thick cardiac constructs (91). The temperature-sensitive culture surfaces were covalently grafted with poly(N-isopropylacrylamide) upon which

neonatal rat CMs were grown. On reducing the temperature below 32°C, confluent cells spontaneously lifted up as a single contiguous sheet with cell–cell junctions and extracellular matrix–deposited adhesive proteins preserved. *In vitro*, layered cardiac sheets pulsed simultaneously, and when implanted subcutaneously into nude rats, displayed spontaneous macroscopic beating, synchronized electrical potentials, neovascularization, and cardiac structures including sarcomeres, desmosomes, and gap junctions. Transplantation of three-dimensional cardiac sheets onto the epicardial surface of injured rat hearts resulted in functional integration of electrical signals between the cardiac graft and host heart without any observed arrhythmias (92).

FUNCTIONAL ASSESSMENT IN ANIMAL MODELS

Hemodynamic Assessment

Although some tissues and organs require that transplanted cells perform specific metabolic tasks, it is essential that cells transplanted to the heart coordinate both mechanically and electrically with native tissue. Testing the efficacy of cell-based therapies in various animal models of MI has created a need for accurate assessment of cardiovascular variables in the intact animal. In addition to tissue measurements of infarct size, vascularity, cellular proliferation, and CM apoptosis post-mortem, changes in left-ventricular structure and function can be measured *in vivo* by a variety of techniques, of which serial echocardiography has been employed in the majority of studies. Left-ventricular end-systolic volume, end-diastolic volume, end-systolic diameter, and end-diastolic diameter, as well as anterior and posterior wall thickness during systole and diastole can be obtained with two-dimensional and M-mode echocardiography (65). These measurements allow for calculation of left-ventricular ejection volume, fractional shortening, and mass. Three-dimensional imaging using cardiac magnetic resonance or multidetector computerized tomography permits similar measurements but with a higher degree of precision and accuracy due to improved temporal and spatial resolution that also offers detailed examination of changes in tissue morphology (42,93).

Other indices for quantifying global and regional left-ventricular function can be obtained with Doppler tissue imaging, which measures blood flow velocities and strain rates, both of which have been shown to correlate with the maximum rate of rise of left-ventricular pressure, or dP/dt_{max} , an indicator of myocardial contractility (94). Some studies have used more invasive procedures such as pressure–volume loop analysis to directly measure hemodynamic parameters (38,95). Left-ventricular volume and pressure are recorded from a specially designed catheter with a pressure sensor and conductance electrodes for simultaneous pressure–volume measurements. End-systolic and end-diastolic volumes, heart rate, maximum pressure, and contractile and relaxation parameters can be obtained. Sonomicrometry has also been utilized to determine wall thickness, wall motion, segment shortening, end-systolic and end-diastolic pressure–volume relationships, stroke work, stroke volume, and ejection fraction (96). This technique

involves implanting small piezo-electric transducer crystals in the infarct region of the heart, allowing the distances between the transducers to be measured continuously through ultrasound waves transmitted and received by the crystals.

Electrophysiological Assessment

Ventricular tachyarrhythmias and sudden cardiac death caused the suspension of clinical trials using autologous skeletal myoblasts transplanted into the myocardium of MI patients (97). This underscored the importance of electrical integration of transplanted cells into the recipient myocardium. Thus, a fundamental operating principle for cell transplantation is the requirement for electrical coupling with the host myocardium. *In vitro* and *ex vivo*, electrophysiological assessment has included patch clamp, multielectrode array, and optical mapping analyses. Patch clamp technique has been used to characterize action potentials from CMs derived from hESCs *in vitro*. Electrical recordings of dissociated hESC-derived CMs have revealed fetal ventricular, atrial, and pacemaker action potentials (98). hESC-derived cardiac action potentials were shown to be sensitive to the L-type calcium channel blocker verapamil, implicating the presence of L-type calcium channels; moreover, the L-type calcium channels were responsive to adrenergic stimuli (98). More recently, as much as one-third of hESC-derived CMs have been shown to possess a mature electrical phenotype, as determined by patch clamp analysis (99).

Multielectrode array mapping has been employed to acquire electrophysiological data. This technique consists of an array of embedded, substrate-integrated contact electrodes that allow simultaneous recording of extracellular field potentials from all electrodes over extended periods of time. The detected field potentials from the attached cells can be correlated to the shape and duration of the underlying action potential. Using multielectrode arrays to assess electrical conduction of hESC-derived CMs, Satin *et al.* detected functional sodium channels that were sensitive to the sodium channel blocker tetrodotoxin (100). Ritner *et al.* used multielectrode arrays to show that hESCs give rise to a heterogeneous combination of chamber-specific cell types (60).

Optical mapping uses voltage-sensitive dyes to record action potentials *ex vivo*. Voltage-sensitive dyes bind with high affinity to the cell membrane and, when excited, emit light in direct proportion to the transmembrane voltage, thereby producing an optical signal that mimics an action potential. Optical mapping not only maps membrane depolarization but also the repolarization process. Verheule and coworkers utilized high-resolution optical mapping to study atrial conduction in canine models of chronic atrial dilatation and chronic rapid atrial pacing to determine whether the underlying mechanisms of atrial fibrillation inducibility are similar (101). Based on the conduction profiles obtained, they found that the underlying substrate for atrial fibrillation in the chronic atrial dilatation model is distinctly different from that of the chronic rapid atrial pacing model. The same group used optical mapping to evaluate the electrophysiological effects of transplanted hESC-derived CMs on injured rat myocardium (102). High-resolution

analysis demonstrated a slight conduction delay at the site of CM grafting.

The capacity of transplanted CMs to electrocouple with the host myocardium is often examined by postmortem immunohistochemical analysis, usually through connexin 43 immunohistochemistry to demonstrate that the transplanted cells form gap junctions within the infarcted region. Besides identification of proteins involved in establishing electrocellular connections, additional evidence is clearly warranted to demonstrate electromechanical stabilization between donor cells and host myocardium. One study used pigs as a large animal model of atrioventricular heart block to show functional electrical integration. Injection of hESC-derived CMs into the left ventricle of pig hearts with atrioventricular block resulted in successful pacing of the heart, manifested by the presence of a new ectopic ventricular rhythm measured by body surface electrocardiography and catheter-based electroanatomical mapping (103). In another study, hESC-derived CMs were injected subepicardially into the left-ventricular anterior wall of guinea pigs. After atrioventricular nodal cryoablation to suppress the intrinsic heart rhythm, *ex vivo* optical mapping of control cryoablated hearts exhibited complete electrical silence, whereas transplanted hearts propagated spontaneous action potentials from the site of injection (104).

FUTURE PERSPECTIVES

The past decade has improved our knowledge of stem cell biology and the development of the cardiovascular system. However, a more profound understanding of cardiac myogenesis will be required for the development of advanced stem cell therapeutics to repair or regenerate damaged myocardium. The future will likely include (i) further investigation to delineate the human CM lineage tree; (ii) methods to isolate specific cardiac progenitor pools or specialized CM subtypes; (iii) strategies to ensure survival of transplanted cells, their functional integration with the host myocardium, and circumvention of immune rejection; (iv) development of technologies to accurately assess integration; (v) determination of parameters that optimize engraftment, such as delivery method, timing of transplantation post-MI, and cell preparations; and (vi) large-animal models of heart failure that closely resemble human cardiovascular physiology and disease for assessing cell engraftment, host immune response, and myocardial function.

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