Cardiovasc Toxicol (2008) 8:1–13
DOI 10.1007/s12012-007-9008-5

Adhesion Proteins, Stem Cells, and Arrhythmogenesis

Nikki Gillum · Narine Sarvazyan

Published online: 5 January 2008

Abstract Cell-transplantation therapy is a promising treatment option that is being actively explored as a way to repair cardiac muscle. The ultimate goal is to reconstitute the architecture of the cardiac muscle and to reestablish electrical propagation, while avoiding hypertrophy and scar formation. In this review, we focus on recent advances in the field as well as the difficulties encountered when the engraftment of cells into the host tissue is to be confirmed and functionally characterized. This is critical since incomplete or partial engraftment of transplanted cells within the host cardiac network exacerbates the heterogeneity already present in the injured myocardium and increases its propensity to arrhythmia. We conclude with a brief discussion of how the modulation of cell adhesion via modification of coupling proteins within transplanted cells may facilitate engraftment and alleviate the arrhythmogenic potential of cardiac grafts.

Keywords Adhesion proteins · Stem cells · Arrhythmogenesis · N-cadherin · Intercalated disc

Introduction

To date toxicology studies which involve stem cells fall into two general categories. The first category includes studies that consider stem cells as future sources of cells which otherwise have limited availability (e.g., human cardiomyocytes). Upon successful differentiation these cells can be used for in vitro toxicological testing. The second category includes studies of stem cells aimed to identify genetic or toxic elements which can be inadvertently introduced into the host tissue. There is, however, a third, largely overlooked category of studies which are the subject of this review. Indeed, according to the updated and approved definition by the Society of Toxicology: Toxicology is the study of the adverse effects of chemical, physical, or biological agents on living organisms and the ecosystem, including the prevention and amelioration of such adverse effects. The subject of this review, i.e., adverse arrhythmogenic effects of allogeneic stem cells engrafted into hearts of living animals or human patients, directly falls into this description. By publishing this review in Cardiovascular Toxicology we hope to expand and emphasize additional crosslinks that exist between the centuries-old field of toxicology and a new, rapidly evolving field of regenerative medicine.

N. Gillum · N. Sarvazyan (✉)
Pharmacology and Physiology Department, The George Washington University, 2300 Eye Street, Washington, DC 20037, USA
e-mail: phynas@gwumc.edu

The Need for Cardiac Repair

Myocardial ischemia results in a substantial loss of cardiomyocytes. Although many recent studies suggest that the heart is capable of some degree of regeneration/proliferation [1, 2], this repair may not be significant to maintain efficient cardiac output. As a result, the heart undergoes a remodeling process. Since cardiomyocytes are mitotically limited, the surviving cells hypertrophy as a means to compensate for the additional workload. At the same time, fibroblasts release collagen and proliferate, replacing the necrotic tissue with a fibrotic scar. Even though fibroblasts are capable of electrically coupling cardiomyocytes [3], propagation delays ensue, creating an arrhythmogenic substrate [4, 5]. Additionally, the fibrotic

tissue changes myocardial compliance thereby reducing the heart's ability to pump oxygenated blood to the rest of the body. To compensate for the reduced cardiac output, the heart continues to enlarge, eventually leading to irreversible heart failure.

Treatment options for heart failure patients are limited to lifestyle changes, diuretics, and drugs that increase contractility. As a last resort, there are left ventricular assist devices and organ transplantation. However, only a few thousand heart transplants are available and the number of Americans who are living with terminal heart failure exceeds this amount by more than a 100-fold [6]. In an effort to solve a long-standing problem of cardiac muscle repair, there is a surge of alternative methods designed to promote its regeneration. These efforts aim to reconstitute the heart tissue and to reestablish proper electrical propagation throughout the cardiac muscle while avoiding hypertrophy and scar formation. One of the most promising and exciting approaches is cell transplantation.

Cell Sources for Cardiac Transplantation

Cardiac Stem Cells (CSC)

Within the past few years, a number of independent lines of evidence suggested that even within a mature heart, there are cells that are capable of producing new cardiac myocytes [7–9]. These cells are being called cardiac stem cells or cardiac progenitors. The putative role of CSC in the heart's ability to withstand imposed wear and tear is exciting but actively debated. This is because a myocardial infarction results in the loss of tens of millions of cells, and this might be too great to be repaired through the cell division of the few cardiac progenitors [10, 11]. Notably, even the proponents of CSC find that these cells give rise only to smaller mononucleated myocytes that phenotypically resemble both neonatal cardiomyocytes or embryonic stem cells during the early stages of their differentiation into cardiac lineage [12]. Proof of cardiac replication was also found in the sections of cardiac tissue from human biopsies [13]. In these samples, the areas of CSC, called "niches," were identified by positive staining for c-kit, an antigen associated with stemness [14]. The transplantation of these human CSC into an infarcted rat myocardium was associated with increased ejection fraction and attenuated ventricular dilation, the latter linked to CSC-derived endothelial cells and myocytes [13]. While these findings might appear too good to be true, if confirmed by subsequent studies, they offer an enormous promise because they suggest that the adult heart possesses true multipotent CSC. On a cautious side, it is important to note that the validity of the markers, such as Sca1, c-kit, isl+, to adequately identify CSC is questionable [11]. There is also a concern regarding the minute amount and mitotic capacity of these cells, which may limit the ability of endogenous CSC to make a difference in cardiac muscle repair [10, 15].

Mixed Cell Populations

Another potential source of cells for cardiac transplantation is the so-called "cardiospheres" [16]. Similar to CSC described above, cardiospheres contain proliferative cells that express stem-cell-related antigens [17]. However, cardiospheres contain a more heterogeneous cell population, which includes cardiomyocytes, endothelial cells, and smooth muscle cells [16, 18]. To date, it is still unknown as to whether a pure preparation of cardiomyocytes or a heterogeneous cell population might be optimal for cardiac transplantation. Indeed, the injection of a mixed population may provide some additional advantages. First, the formation of new vessels can aid in the survival of transplanted cells or endogenous ischemic cells [19, 20]. Second, the presence of diverse differentiation stages may promote engraftment between CSC and the host myocardium. Lastly, the injected mixed population can provide additional growth and paracrinal factors that might assist in muscle regeneration.

Skeletal Myoblasts

The first clinical trials aimed at the transplantation of autologous cells used skeletal myoblasts (SkM) as a cell source for myocardial repair [21]. These myogenic precursors were believed to be a good choice, since SkM are fairly resistant to ischemia and have a high-growth potential. Indeed, prior animal studies demonstrated enhanced left-ventricular function following SkM grafting to infarct scars [22, 23] and similar benefits, such as an increase in ejection fraction and systolic thickening, have also been seen in SkM-treated patients [21]. However, soon after transplantation it became evident that skeletal myoblasts do not fully integrate with the host myocardium, and as a result, patients suffered from ventricular arrhythmias that necessitated implantable defibrillators [24]. The main reason of SkM arrhythmogenicity is the near absence of the electrical coupling with the host, an effect clearly confirmed by intracellular recordings from the GFP-labeled SkM [25]. As a result, the injection of unmodified SkM increases electrical heterogeneity of the tissue, begetting ventricular arrhythmia. Interestingly though, cell-cell coupling can be improved by genetically engineering SkMs to express connexin-43, a gap junction protein found in the

heart [26]. This, potentially, can lead to the development of electrically coupled grafts from genetically modified SkM.

Hematopoietic Stem Cells (HSC)

A lot of excitement was generated by an earlier report in which transplantation of c-kit$^+$ lin$^-$ GFP-labeled hematopoietic stem cells into an infarcted myocardium led to regeneration of 68% of the ventricle nine days after transplantation [27]. The newly developed tissue was vascularized and comprised of proliferating myocytes by the progeny of the injected stem cells. Unfortunately, subsequent efforts by other laboratories were not able to repeat these results, generating a heated debate about the findings and the protocols employed [28–30]. It was argued, for example, that the presence of GFP-expressing cells is only a transient event and that those cells do not stain positive for cardiac-specific markers [29]. Another study compared transplantation of unfractionated bone marrow (which contains both HSC and mesenchymal SC, more below) and purified HSC into the myocardium. The result again was only a transient presence of engrafted cells, which appeared to be nonmyocytes, because they stained negatively for α-actinin, troponin-T, and connexin-43 [30]. It was also argued that what appeared to be "hematopoietic-derived cardiomyocytes" was a result of cell fusion with existing host myocytes, and that any positive effect of HSC grafting was due to paracrine factors [28]. All in all, not enough evidence exists today to suggest that HSC can transdifferentiate into cardiomyocytes.

Mesenchymal Stem Cells (MSC)

Engraftment of unfractionated bone marrow cells as well as pure MSC do appear to have positive effects on cardiac function and these benefits were confirmed in studies by different laboratories [31–33]. Due to that and the fact that MSC can be obtained from the patient's own bone marrow (which significantly reduces ethical concerns and immunogenicity issue), the MSC-based therapy appears to be the most feasible clinical option, at least from today's standpoint. The exact mechanisms behind MSC-based benefits are actively debated. Among the suggested possibilities are the following four:

First, there are studies that argue for transdifferentiation of MSC into cardiac muscle cells [31, 34, 35]. As an example, one can cite a recent study in which GFP-labeled MSC were isolated from a male mouse and injected into a female heart, and the progeny of the transplanted cells was identified by fluorescence, Y-chromosome markers, and microspheres [31]. Ten days after injection the infarct size was decreased by 17% and a population of new myocytes was noted. Based on the cell size and genotypic markers analysis it was concluded that these new myocytes were not a result of cell fusion, but were the result of MSC transdifferentiation. Another group [35] reported cardiac troponin-T and alpha-actinin immunostaining within MSC transplanting cells, suggesting again that a small amount of MSC can differentiate into cardiomyocytes.

Second, many argue that it is an increase in tissue revascularization [36–39]. Clusters of MSC have been shown to express key vessel markers, such as von Willebrand factor, while cardiac markers were absent [36]. Angiogenic factors, such as VEGF, FGF, and stem cell homing factor, have been shown to increase in MSC-treated hearts [40, 41]. Similarly, ischemic limb studies have also detected enhanced angiogenic factor expression at the site of injury following MSC injection [38]. The increase in capillary density one month after MSC injection into infarcted hearts has also been shown [37, 40, 42]. The fact that these improvements persist while only a negligible amount of MSC remains in the myocardium, can also be used to support neovascularization as a main beneficiary mechanism.

The third alternative is decreased remodeling, which includes reduced fibroblast proliferation and collagen deposition. This possibility is supported by the previously mentioned ischemic limb study [38], where the degree of fibrosis was substantially decreased in the MSC-treated group. A direct upregulation of anti-proliferative and a downregulation of collagen I and III genes was also detected when MSC-conditioned media was added to cardiac fibroblasts [43].

The fourth alternative is an increase in a pro-survival milieu surrounding damaged cells [44, 45]. This mechanism seems to be the case in a study where the positive effects of MSC engraftment were noted as early as 72 h post injection [45]. Since, it is clear that the transplanted cells themselves cannot account for these benefits; the paracrinal effect is the most likely explanation for the observed improvement. The effect was further amplified when the MSC were genetically modified to overexpress a pro-survival protein, Akt [44]. The MSC-Akt-treated animals had reduced cardiac remodeling, as seen thru decreased intramyocardial inflammation, lesser collagen deposition, and a diminished degree of cardiomyocyte hypertrophy.

Embryonic Stem Cells (ESC)

Pluripotent stem cells are found within the inner cell mass of the blastocyst of a developing embryo. Unlike adult stem cells, ESC retain the ability to differentiate into

specialized cells from all three germ layers. Moreover, it is possible to guide ESC differentiation thru the mesodermal lineage to generate cardiomyocytes [46, 47]. This can be accomplished by utilizing the hanging-drop method which creates pseudo-embryos (Fig. 1a), termed embryoid bodies. The three-dimensional environment created within the embryoid body allows stem cells to physically interact. This interaction leads a sub-population of ESC to differentiate into spontaneously contracting cardiomyocytes [50, 51]. These ESC-derived cardiomyocytes exhibit a temporal pattern of protein expression that follows early myocardium development (i.e., GATA-4 and Nkx2.5 transcription factors are expressed prior to alpha/beta-myosin heavy chain, cardiac troponin-T, and the Na^+/Ca^{2+} exchanger [52]).

Existing evidence suggests that undifferentiated ESC are not guided into the cardiac phenotype simply by transplanting them into an injured heart [53, 54]. In fact, injection of ESC leads to teratoma formation and does not appear to improve cardiac function [53]. Therefore, studies that transplant ESC usually differentiate the ESC first, and then enrich them for the cardiomyocyte-like cell population. Often, this is achieved by transfecting ESC with an antibiotic resistance gene that is under the control of a cardiac-specific promoter (Fig. 1b), so that only cells of the cardiac lineage survive the selection process [55, 56]. Additionally, a fluorescence gene might be linked to a cardiac promoter to facilitate the selection of cardiac lineage cells via fluorescent-activated cell sorting. The latter approach has been shown to yield a 95% pure cardiomyocyte population [57]. Generally, population purity is then confirmed by identifying the expression of cardiac-specific markers and/or by visualizing contractile activity [57–59].

The scalable expansion of ESC into cardiac-like cells can also be accomplished by exposing undifferentiated ESC to a number of specific proteins or low-molecular weight compounds [60–62]. For example, the TGF-beta family of secreted factors has been shown to induce mesodermal differentiation of ESC [63, 64]. Therefore, sequential exposure of ESC to actinin A and BMP4 was shown to enhance cardiac differentiation 50 times more than serum induction alone [65]. Transplantation of these cells resulted in the "re-muscularization" of the ischemic myocardium, with both echocardiograph and MRI studies showing improved diastolic and systolic function. In another study by the same group [49], when transplanted cells were heat-shocked and then injected into the heart along with a cocktail of pro-survival factors, the graft size was noticeably increased, as well as the graft survival. These studies are very encouraging as they demonstrate the feasibility of expanding ESC-derived cardiac cells, thus bringing us closer to the future use of ESC-derived cells for cardiac transplantation.

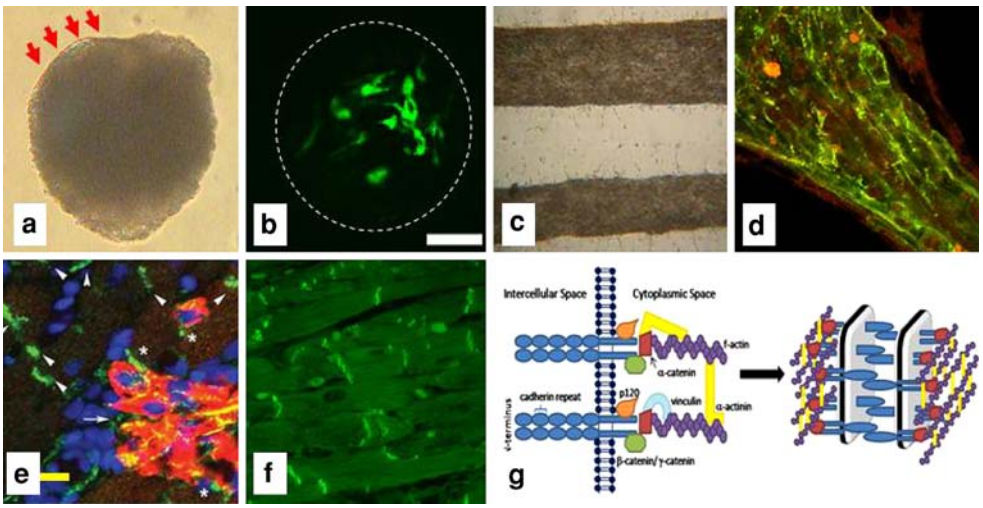

Fig. 1 (**a**) Phase contrast image of 10-day-old contracting embryoid body; arrows indicate contracting area. (**b**) Areas of embryoid body differentiating into cardiac phenotype can be seen by expressing GFP, which is linked to alpha-myosin heavy chain promoter. (**c**) In vitro cardiac fibers developed using Matrigel technique (details in [48]). (**d**) Immunohistochemical confirmation of connexin-43 (red) and N-cadherin (green) epitope colocalization within the in vitro cardiac fiber. (**e**) Cardiomyocytes derived from human embryonic stem cells are shown four weeks after transplanting into rat myocardium. Beta-myosin heavy chain (red) is used to label the human graft. Pan-Cadherin (green) is detected between adjacent myocytes (arrowheads), and infrequently, found between the host and graft (asterisks). Scale bar = 10 μM. With permission from the publisher [49]. (**f**) Intercalated disc appearance within mature cardiac muscle. The image shows 7-micron thin paraffin section of a rat heart stained for connexin-43. (**g**) Cartoon representing the proteins present in the adherens junction of the intercalated disc. Cadherin proteins transverse the plasma membrane and interact with a number of proteins within the cytoplasm. These interactions facilitate coupling to the actin cytoskeleton. Three-dimensional representation is also shown, where the protein plaques are depicted as one unit interacting with the actin filaments

Similar to the case of mixed cell populations (Sect. "Mixed cell populations"), a complete differentiation of ESC into cardiomyocytes may not be the ideal path for cell transplantation. As mentioned earlier, myocytes have a limited mitotic capacity, which insinuates obvious problems when one transplants the heart with a pure myocyte population. Furthermore, it is questionable as to whether pure populations can sufficiently engraft to the myocardium, as it appears that cardiac fibroblasts are essential for this process [55]. In one such study, GFP-labeled ESC-derived cardiomyocytes were detected in the hearts of only 22% of the treated mice, versus 63% of mice treated with an equal number of ESC-derived cardiomyocytes plus fibroblasts [55]. Furthermore, the presence of fibroblasts has been shown to decrease endothelial cell death and increase cell proliferation in vitro, which suggests that these cells can also promote vascularization of the injured myocardium [20]. Additional evidence of the benefits of a heterogeneous cell population has been documented in studies of engineered heart tissue, where the constructs made from mixed cell populations improved cardiac function to a greater extent than the ones made from a purified myocyte populations [66].

Although ESC can be used as a reliable source of new cardiac myocytes, there are a number of studies that focus not on ESC-based regeneration of cardiac muscle, but on paracrinal signaling between the host tissue and injected ESC [62, 67]. For example, TGF-beta/BMP2 proteins are secreted by both cardiac myocytes and fibroblasts following myocardial infarction [68]. In vitro, application of TGF-beta and BMP upregulated expression of cardiac-specific transcription factors in ESC, and embryoid bodies showed an enhanced cardiac differentiation [62]. In vivo, transplanted ESC integrated with host myocardium and contractile performance was enhanced when the TGF-beta/BMP pathway was intact. Notably, the phenomenon was absent when inhibitors of the pathway were administered. The ESC-conditioned media has been shown to contain antiapoptotic and antifibrotic factors and to protect cardiomyocyte-like cells from oxidative stress in vitro [69]. Therefore, functional improvement of ischemic myocardium seen in ESC engraftment studies, can also be a result of reduced atrial natriuretic peptide signaling and/or collagen production [67].

Injury to the myocardium may initiate the release of factors that act as a homing signal for injected cells. This possibility received experimental support when ESC-derived cardiomyocytes injected into the tail vein had been shown to translocate to ischemic areas of the heart [70]. Furthermore, an in vitro study observed enhanced ESC-derived cardiomyocytes migration toward cells that overexpress TNF (tumor necrosis factor), a cytokine likely to be present at the site of injury [70].

Recently, reprogramming of somatic cells, via retroviral transduction, has yielded a new cell population that characteristically resembles ESCs [71, 72]. Most importantly, these induced pluripotent stem cells (iPS) are able to differentiate into cells from all three germ layers. This discovery may bring about the development of patient-specific stem cells, thereby reducing the risk of immune rejection and avoiding ethical concerns. However, additional studies are necessary to assess the risk and possibly eliminate the use of retroviral vectors for gene delivery. Since iPS resemble ESC in protein expression, it is expected that myocardial engraftment will also be similar to what has been observed in ESC transplantation studies.

Engineered Tissue Constructs

Many of the above mentioned cell sources can serve as precursors to cardiac myocytes. The studies discussed so far either injected these cells into the infarct region or delivered them via the circulatory route. Alternatively, one can try to recreate cardiac muscle in vitro (Fig. 1c, d), and then use it to surgically repair the diseased myocardium. Several laboratories are successfully moving toward this goal [48, 66, 73–76]. For example, engineered heart tissue grafts (EHTs) have been developed by seeding neonatal cardiomyocytes in circular molds with collagen and Matrigel, and then culturing the formed rings in a mechanical stretch device [73]. Furthermore, these EHTs can be stacked together in various geometries to increase graft size and then stitch the graft to an infarcted heart. Epicardial mapping has shown that paced EHTs propagate electrical signals to the surrounding myocardium, and that these grafts do not lead to arrhythmia [74]. Moreover, echocardiograph and MRI indicated reduced left ventricular dilation and end diastolic volume. Recent modifications to EHT generation, using a mixed cell population with fibroblasts, smooth muscle cells, and endothelial cells, have resulted in superior contractile performance over cardiomyocyte samples alone [66]. Moreover, the creation of EHT from ESC and successful engraftment of these ESC-EHT into the working myocardium has also been reported [74].

Alternatively, three-dimensional cell layers can be created by seeding cardiomyocytes on a polymer-coated dish that serves as a biodegradable scaffold [75]. This polymer is temperature sensitive and the entire cell sheet can be detached by simply lowering the cell culture surface temperature. The multiple cell sheets are then fused together to create a small thin piece of synchronously beating, cardiac-like tissue. In a recent study, implantation of such a mini cardiac graft over an area of burned epicardial muscle appeared to restore wave propagation through the injured

area [76]. However, if one takes into account the provided values on the conduction velocity and the size of the burn, the spatiotemporal resolution of the recordings was not sufficient to make such a conclusion, therefore additional studies will be needed to ascertain it.

Given that the main reason for extensive necrosis following myocardial infarction is the lack of blood flow to the myocardium, it makes sense to pre-vascularize the tissue intended for transplantation. For this reason, studies are underway to engineer *vascularized* three-dimensional myocardial grafts [20, 77] using special biodegradable scaffolds and perfusion systems. One such system is designed to house cardiomyocytes, fibroblasts, and endothelial cells in a construct that provides stability and permits cellular ingrowths [20]. The end result resembles a native myocardial tissue that contracts in synchrony and exhibits a high degree of muscle maturation.

Mechanical and Electrical Engraftment of Transplanted Cells with the Host Myocardium

Despite significant efforts, it remains difficult to confirm and functionally characterize the engraftment of newly derived cardiomyocytes into the host tissue. Part of the problem is that it is hard to visualize the engrafted cells, or even more importantly, to clearly distinguish them from the host cells. Different methods have been used in an attempt to do so. A number of studies have used GFP- or CFP- expression to label the injected cells [78–82], but in only a few studies do ESC-derived cardiomyocytes (ESC-CM) appear to be structurally integrated [79, 82]. Immunohistochemistry is another common tool used to distinguish between the host cells and engrafted ESC-CM. Anti-human mitochondrial antibody, for example, can be used to identify human ESC injected into a rat heart [83]. The human stem cells differentiated into a cardiac lineage, as shown by α-actinin colocalization with staining against human mitochondrial antigen [83]. The images shown in this study not only clearly demonstrate ESC-CM presence, but also the fact that engrafted cells are aggregated into clusters. Such clusterization highlights an important question as to whether transplanted cells form functional syncytium with the host cardiac muscle, or simply reside at the injection site. To the best of our knowledge, there are no systematic studies that address how transplant type (i.e., stem cell source being xenogeneic, allogeneic, isogeneic to the host) affects the degree of implanted cell clustering. The latter will be an interesting question to address in future studies.

To address the issue about the *functional* engraftment, efforts are usually made to verify the presence of intercalated disc protein expression in the area containing ESC-CM (Fig. 1e). For the new cells to be well coupled, staining for intercalated disc proteins is expected to be observed along the membranes of transplanted cells, ideally, in a pattern that outlines intercalated disc appearance in the adult heart muscle (Fig. 1f). In most cases, however, the connexin-43 staining is very blurred or absent between the islands of transplanted stem cells and the rest of the myocardium [55, 80, 81, 83]. One can also try to discern electrical connectivity between the host and engrafted cells by using a dye transfer technique [84, 85]. While demonstration of electrical coupling in vivo remains a challenge, several in vitro studies clearly show that it does occur [81, 83]. Documented evidence of electrical coupling in vivo, albeit at a macroscopic level, is presented in two studies in which ESC injected into the left ventricle successfully paced the hearts of animals in which the atrioventricular node was disabled [81, 83]. One study used an optical mapping approach and observed episodes of a new ventricular ectopic rhythm, which had a substantially different morphology compared to junctional or paced rhythms. Mapping and pathological examination confirmed that the source of this new ventricular ectopic rhythm was the site of cell transplantation [83]. In addition, documented electrical activation propagation throughout a cardiomyocyte ESC-CM co-culture, indiscriminate of whether cardiomyocytes or ESC-CM were stimulated, was observed using a microelectrode array [83]. As exciting as these findings are for the future of cell-based pacemakers, it is important to take into account possible paracrinal or fusion events that may also play a role in the observed positive effects.

An interesting insight came from a recent study in which not stem cells, but fetal myocytes were employed as a cell source for intramyocardial graft. Specifically, GFP-labeled murine fetal myocytes were injected into cryoinjured areas and adjacent myocardium of cryoinjured mouse ventricle [86]. The hearts were sliced a week later and microelectrodes were impaled to stimulate action potentials in viable slices. Transplanted fetal myocytes could be distinguished clearly from host tissue by their green fluorescence and their electrophysiological properties. Out of the transplanted cells that were surrounded by viable tissue, 82% were electrically coupled to the myocardium and developed electrophysiological properties that resembled those of mature cells. Comparatively, the cells surrounded by cryoinjured tissue did not electrically integrate and retained their fetal phenotype. This study provides useful insight for stem cell transplantation, as many ESC studies inject cells directly into the infarcted area. Importantly, in addition to the coupling itself, the above-discussed study also suggested that immature cardiac cells might be more prone to develop into the adult cardiac phenotype when surrounded by viable host cardiomyocytes.

Incomplete Coupling and Ensuing Heterogeneity Pose a Risk of Arrhythmogenic Substrate

As alluded to earlier, exogenous cell transplantation can cause heterogeneity of the myocardial substrate that may predispose it to an arrhythmia. This negative outcome was the main reason for discontinuation of early clinical trials which employed skeletal myoblasts [24]. This heterogeneity effect is clearly documented in vitro using co-cultures of cells of interest and neonatal rat cardiomyocytes [87, 88]. As an example one can cite a dose-response study that examined MSC and rat neonatal myocyte networks, and observed delayed conduction velocities and an increased inducibility of reentrant arrhythmias by high-frequency stimulation [87]. These effects occurred despite the presence of functional gap junctions, functionality of which was confirmed by a dye transfer technique, and was attributed to the presence of unexcitable MSC.

When conduction velocity slows down, the activation of the regions containing a mixture of engrafted cells and myocytes is delayed. This also delays the recovery of these regions. Therefore at high heart rates or premature impulses, wavefronts encounter refractory tissue in the graft area, leading to localized block of impulse propagation and susceptibility to reentry increases. This is the main mechanistic explanation for the increased arrhythmogenicity of engrafted tissue based on the reentry concept. In addition, there is concern regarding triggered activity or increased automaticity from the areas of stem cell engraftment. The spontaneous excitability of ESC is well documented and is easily observed when one allows ESC to aggregate into embryoid bodies using the hanging-drop protocol (Sect. "Embryonic Stem Cells (ESC)"). When beating areas of EBs are dissected, they exhibit heterogeneous electrophysiological properties and are prone to triggered arrhythmias [88]. The contractions of ESC-derived myocytes depend on transsarcolemmal calcium influx rather than calcium-induced calcium release from the sarcoplasmic reticulum, which is an expectable outcome given their early developmental stage [89]. Lack of expression of such calcium-handling proteins, as phospholamban and calsequestrin [90], further attests to the immature capacity of the sarcoplasmic reticulum in the ESC. This observation is unsurprisingly similar to that of fetal excitation–contraction coupling; wherein intracellular calcium oscillations, rather than transmembrane ion currents, evoke small membrane depolarizations that have the ability to trigger L-type calcium channel-driven action potentials [91]. Lastly, immature human ESC have been shown to express prominent Na^+ and HCN currents, but lack the inward rectifier potassium current which clamps the value of the resting membrane potential [92], thus setting the stage for spontaneous cell activation. These and other studies that point to automaticity of ESC-derived cardiomyocytes in the early stages of their differentiation [93–95], also suggest that spontaneous automaticity of these cells will diminish with progression to a more mature cardiomyocyte phenotype.

The Need for Better Integration

Visualization of stem cell grafts, even in the most successful engraftment studies, usually shows islands of structurally separate cells. Such incomplete integration not only limits functional impact of the grafts, but also, as detailed in the previous section, is highly arrhythmogenic. In order to better understand how we can help newly arrived cells to become welcomed and fully functional members of the cardiac syncytium, one needs to know how the network of cardiac muscle cells is formed and maintained. The latter is the subject of our next three subsections.

Intercalated Disc Structure

In order to establish a functional syncytium, cardiomyocytes are coupled via the intercalated discs (Fig. 1g). The intercalated discs are comprised of fascia adherens junctions and desmosomes, which serve as mechanical junctions, as well as gap junctions that permit electrical coupling. Gap junctions are made up of two connexin hemichannels, each composed of six connexin proteins, that link cardiomyocytes together by establishing a functional channel that allows the passage of ions between the two cells [96]. Therefore, gap junctions serve to electrically connect neighboring cardiomyocytes and enable rapid, synchronized electrical activation of cardiac muscle [97, 98]. In close proximity to gap junctions are the adherens junctions and desmosomes; these connections mediate adhesion between cardiomyocytes by way of the cadherin proteins, which interact with the cytoskeleton via the catenin proteins [99, 100]. Adherens junctions link the intercalated disc to the actin cytoskeleton via classical cadherin proteins, while desmosomes connect to the intermediate filament system via desmosomal cadherins [101, 102]. Within the adherens junction, the N-cadherin protein binds to either β or γ-catenin proteins, which in turn, complex to α-catenin in order to link to actin filaments [103, 104].

Mechanical Junctions Guide the Formation of Electrical Junctions

A bulk of studies have suggested that electrical coupling is established between cells only after mechanical junctions

are in place [104–107]. The so-called 'redifferentiation model' was used in many of these studies. This procedure involves dissociating adult rat cardiomyocytes, and then examining the newly formed intercalated discs as they are reestablished in vitro. It was determined that N-cadherin colocalized with β-catenin at newly formed cell–cell contact sites, and colocalized with α- and γ-catenins at both the cell–cell contact sites and within the spreading areas of the cardiomyocytes [104]. Importantly, connexin-43 was found at cell contact sites later, only after the adherens junctions were in place. A later study corroborated these observations by following a similar protocol using a GFP-tagged N-cadherin [107]. However, the adherens junctions are not the only prerequisite to initiate functional expression of connexin-43, as another type of mechanical junctions, the desmosome, also appears to form prior to gap junctions. Within five days of cardiomyocyte isolation, N-cadherin, the catenins, and desmoplakin were all present at sites of cell-cell contact. Once the adhesion proteins organized into zipper-like structures within the intercalated disc, connexin-43 was then progressively incorporated and gap junction size and number increased in the following days [106].

Changes in Expression of Intercalated Disc Proteins and their Effect on Cardiac Coupling

Homozygous mutations in the N-cadherin protein result in embryo lethality by gestation day10; severe cardiovascular defects observed in the embryos further validate the vital role of N-cadherin in the heart [108]. To overcome embryonic lethality, a Cre/lox conditional knockout was generated to examine the role of N-cadherin after development of the myocardium was complete [109]. It was noted that a knockout of the N-cadherin gene also resulted in loss of α-, β- and γ-catenin, as well as desmoplakin and p120. Overall, the intercalated disc appeared disassembled with modifications in adherens junction and desmosome structure, and a decrease in connexin-43 expression. The mutant mice exhibited impaired cardiac function, with most animals dying due to spontaneous ventricular arrhythmia and sudden cardiac death. Functional effects were further investigated; mutant mice exhibited abnormal ventricular conduction, decreased conduction velocity, and tachycardia with stimulation [110]. Taken as a whole, diminished N-cadherin presence leads not only to disruption of mechanical coupling, but also to disintegration of electrical junctions.

The proper maintenance of electrical junctions is a key part of cardiac function, an effect which can be clearly seen when gap junctional proteins are modified. Although animals are able to survive till birth with a null connexin-43 mutation, these animals die shortly after due to cardiac malformation [111]. For this reason, cardiac-specific knockouts of connexin-43 have been developed to examine effects postnatally [112]. Similar to the observations noted in N-cadherin knockouts, the hearts of cardiac connexin-43 knockout mice exhibit slow ventricular conduction velocity, spontaneous ventricular arrhythmia, and sudden cardiac death [112, 113]. As an alternative approach, heterozygous connexin-43 null mice have also been developed [114, 115]. They are phenotypically identical to control except that connexin-43 expression is 50% less. When acute ischemia is induced, connexin-43± animals experience spontaneous ventricular arrhythmias and ventricular tachycardia more frequently than controls [114].

Modulation of Intercalated Disc Proteins as a Means to Facilitate Engraftment Efficiency

Surprisingly a small number of studies have attempted to modulate, either pharmacologically or genetically, the intercalated disc proteins of either host or grafted cells as a means to facilitate engraftment efficiency. There are two main reasons for that. First, regenerative medicine is a relatively new field and the use of stem cells for solid tissue grafts has become the subject of mainstream research only recently. The second reason is the complexity of the task ahead. Developmental biology gives us an appreciation of how complex and finely tuned adhesion strength, specificity, and timing have to be for proper assembly of multicellular tissues. So, while it is easy to hypothesize that "stickier" stem cells may engraft more efficiently, it is much harder to understand how alterations in the expression of proteins involved in adhesion and coupling can be used for this purpose.

For example, let us look at the potential upregulation of N-cadherin, the major adhesion protein in cardiac muscle. As alluded to above, an array of proteins are present in the intercalated disc, each of which plays a key role in establishing connections between cardiomyocytes. One of these proteins, p120, has proven to be a central player in the cadherin trafficking process. In fact, p120 binds to an N-cadherin prototype, while β- or γ-catenin attach only after the protein is processed into its mature form, which involves phosphorylation and cleavage of its proregion [116]. p120 becomes phosphorylated through its interaction with cadherin at the plasma membrane, which enhances the affinity between the two proteins and acts to stabilize cadherin in the plasma membrane [117]. Moreover, knockdown of the p120 protein results in a decrease in cadherin protein expression and loss of cell–cell adhesion, in a variety of different cell types [118]. For this reason, it is thought that p120 functions to control the

turnover of the cadherin proteins. If p120 were to become saturated and unable to further assist in the trafficking of N-cadherin to the plasma membrane, it seems plausible that an abundance of cadherin protein may be found in the cytoplasm. Excess N-cadherin distributed throughout the cytoplasm has been documented in both in vivo experiments [119], as well as within our own in vitro studies, in which N-cadherin was constitutively overexpressed in an embryonic stem cell line (NCadESC). Indeed, we found that the excess N-cadherin protein found in NCadESC is not localized to the plasma membrane, but is found intracellularly. Moreover, the plasma membranes of these cells show a much weaker N-cadherin staining. Paradoxically, however, NCadESC adhere to each other much better than the cells from parental ESC line and exhibit elevated levels of connexin-43. The exact mechanisms behind increased cell–cell adhesiveness of NCadESC are puzzling and remain to be explored further. One possibility is that other cadherins are expressed in larger amounts or form more stable complexes at cell-to-cell junctions. Alternatively, increased cytosolic levels of N-cadherin can trigger pathways that lead to elevated expression of proteins that stabilize the existing N-cadherin complexes at adherens junctions. One such protein mentioned above, p120-catenin [116, 118], was found to be present in higher amounts in the membranes of NCadESC. All in all, these findings reconfirm our earlier statement that efforts to upregulate cell adhesion as a means to improve engraftment might involve multiple players. Nevertheless, an embryonic stem cell line which exhibits an increased cell–cell adhesion and elevated amounts of gap junctional proteins holds promise for improved engraftment with host tissue and calls to be explored further.

We conclude that modulation of adhesion and coupling proteins in cells that are employed for cardiac tissue transplantation presents a poorly explored but exciting research opportunity. This field may help to facilitate cell engraftment and alleviate arrhythmogenicity of cardiac grafts, thus advancing stem cell-based therapy for cardiac disease.

Acknowledgments We thank Drs. Ara Arutunyan and Zaruhi Karabekian for helpful discussions. Financial support from the American Heart Association Mid-Atlantic Affiliate (0715335U to NG) and the National Institutes of Health (HL076722 to NS) is gratefully acknowledged.

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