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Review

Cardiac Stem Cell Therapy: Stemness or Commitment?

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Cardiac stem cell therapy to promote engraftment of de novo beating cardiac muscle cells in cardiomyopathies could potentially improve clinical outcomes for many patients with congestive heart failure. Clinical trials carried out over the last decade for cardiac regeneration have revealed inadequacy of current approaches in cell therapy. Chief among them is the choice of stem cells to achieve the desired outcomes. Initial enthusiasm of adult bone marrow stems cells for myocyte regeneration has largely been relegated to paracrine-driven, donor cell-independent, endogenous cardiac repair. However, true functional restoration in heart failure is likely to require considerable myocyte replacement. In order to match stem cell application to various clinical scenarios, we review the necessity to preprime stem cells towards cardiac fate before myocardial transplantation and if these differentiated stem cells could confer added advantage over current choice of undifferentiated stem cells. We explore differentiation ability of various stem cells to cardiac progenitors/cardiomyocytes and compare their applicability in providing targeted recovery in light of current clinical challenges of cell therapy.

Key words: Stem cells; Cardiomyocytes; Differentiation; Myocardial infarction; Regenerative medicine

INTRODUCTION

During mammalian development, embryonic heart expands through proliferation of its constituent cell types of which 60% are cardiac fibroblasts, 30% force-generating heart muscle cells (cardiomyocytes), and the remainders, vascular endothelial and smooth muscle cells (31). Proliferative capacity of those cardiomyocytes rapidly declines and becomes terminally differentiated shortly after birth (2). Although reports suggest the presence of progenitor populations in the heart (67), their ability to support basal turnover of cardiomyocytes is debatable (56). Critically, when large numbers of cardiomyocytes are lost as in myocardial infarction, intrinsic cardiac regeneration is invariably poor. Current effective therapy to prevent total congestive heart failure demands orthotropic transplantation, but shortage of donor organs, high costs, and complications of immunosuppressants continue to plague the rehabilitation process.

In recent years, application of cell-based therapy has gained considerable interest as an alternate means to "remuscularize" the injured heart and to overcome limitation of organ shortage. Early preclinical studies provide evidence of fetal, and neonatal cardiomyocytes proliferate and survive when injected in the heart (59,76,96). More importantly, these cells repopulated scars, buttressing wall thickness and eventually improved left ventricular function (76,126) with signs of electromechanical integration (92,134). These initial results suggested that cardiomyocyte transplantation may be a promising avenue to replace fibrotic scar. However, use of fetal and neonatal cardiomyocytes poses significant clinical hurdle due to their limited supply and ethical concerns. These have fueled the search for alternative cardiogenic source for myocardial regeneration (Table 1).

The first experimental attempts to restore cardiac function in an injured myocardium utilized multipotent cells from skeletal muscle and bone marrow. Although not bona fide heart stem cells, accessibility, scalability, and autologous source, make them viable candidates (39). Unfortunately, experiments in rodents demonstrated that myoblasts transplanted to infarcted hearts survived but failed to convert to cardiomyocytes (89) and demonstrated no electromechanical coupling (93). However, some groups (36,112) demonstrated positive effects on heart function through poorly understood mechanisms. Furthermore, randomized, double-blind,

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Table 1. Various Types of Stem Cells With Applications in Cardiac Therapy

Type	Source	Markers	Differentiation to CM	References
HSC	BM	$CD90^+$, $CD45^+$, $CD117^+$, $CD29^+$	Treatment with 5Aza-dC	(90)
MSC	BM	$CD44^+$, $CD90^+$, $CD106^+$, $CD71^+$, $CD45^{low}$, $CD133^+$, $CXCR4^+$	Treatment with 5AzadC and coculture with CM	(115)
EPC	BM/PB	$CD34^+$, $CD31^+$, $CD146^+$, vWF^+, $CD45^{low}$, $VE\text{-}CAD^+$, $CXCR4^+$	Treatment with 5Aza-dC	(57)
VSEL	BM	$Sca1^+$, $CD34^+$, $Nkx2.5^+$, $GATA4^+$, $CD45^-$, $Oct4^+$, $CXCR4^+$	–	(121)
ESC	ICM	$Isl1^+$, $Nkx2.5^+$, $Oct4^-$, $GATA4^+$	Spontaneous and GF-induced differentiation	(116)
$Isl\text{-}1^+$	Heart	$Isl1^+$, $Nkx2.5^+$, $GATA4^+$, $\alpha\text{-}MHC^+$	Spontaneous differentiation	(55)
$Sca\text{-}1^+$	Heart	$Sca1^+$, $Nkx2.5^+$, $cTnI^+$	Treatment with 5Aza-dC	(81)
CSC	Heart	MDR^+, KDR^+, $CD31^+$, $cTnI^+$, $Nkx2.5^-$	Spontaneous differentiation and low frequency electromagnetic waves	(72)
$c\text{-}Kit^+$	Heart	$cKit^+$, $Isl1^+$	Treatment with 5Aza-dC	(109)

HSC, hematopoietic stem cell; MSC, mesenchymal stem cell; EPC, endothelial progenitor cell; VSEL, very small embryonic-like stem cell; ESC, embryonic stem cell; CSC, cardiac stem cell; BM, bone marrow; 5Aza-dC, 5-aza-2′deoxycytidine; GF, growth factor; CM, cardiomyocyte.

placebo-controlled clinical trial failed to show improvement in several key parameters of cardiac function after a follow-up period of 6 months (71).

Bone marrow-derived stem cells, on the other hand, have been demonstrated to differentiate into various cell types including cardiomyocytes. Some groups (10,30,46) suggested bone marrow cells transdifferentiated to cardiomyocytes, whereas others (3,7,79,80,113) attributed such observations to cell fusion between host and transplanted cells. Furthermore, clinical trials often with conflicting results accentuated the controversy (98). Positive effects from bone marrow stem cell transplant have been relegated to poorly understood paracrine effect, possibly by its direct effect on the myocardium or by contributing to increased vascularization (39).

Although the field has witnessed considerable controversies over the past decades, cell-based cardiac therapy nevertheless remains a mainstream experimental concept that can revolutionize management of heart failure. In this review, we expound probable stem cell populations for cardiac repair, and we discuss the concept of milieu-directed differentiation versus predifferentiation of bone marrow-derived stem cells and human pluripotent stem cells towards muscular lineages in exacting post-transplant benefits in the infarcted myocardium. Extensive literature on stem cells for cardiac repair has been reviewed elsewhere; we selectively discussed literature on the subject of stem cell cardiomyogenesis in relation to functional benefits.

CARDIOMYOGENIC DIFFERENTIATION OF STEM CELLS

Traditionally, stem cells consist of two broad categories of adult stem cells and embryonic stem cells (ESCs). While adult stem cells are derived from postnatal somatic tissues and are generally considered to be multipotent, their embryonic counterparts are derived from the inner cell mass of blastocyst-stage embryos and are pluripotent (114). Recent advancements in stem cell biology has enabled reprogramming of differentiated somatic cell types into a pluripotent state similar to ESCs via induced expression of selected pluripotent genes to yield induced pluripotent stem cells (iPSCs) (108). In the following sections, we discuss cardiogenic ability of those stem cells, and if predifferentiating them to cardiac lineages for exogenous cell-based therapy is warranted.

Bone Marrow-Derived Stem Cells and Cardiogenesis

Human bone marrow is the major source of adult stem cells. Bone marrow contains a complex assortment of progenitor cells, including hematopoietic stem cells (HSCs), side population (SP) cells, mesenchymal stem cells (MSCs) or stromal cells, and multipotent adult progenitor cells (MAPCs), a subset of MSCs (47). There is considerable evidence to suggest that those stem cell populations harbor putative cardiac transdifferentiation potential (22,65,82,128). Although some of the apparent transdifferentiation events has later been attributed to technical artifacts or previously overlooked phenomena, such as cell fusion and “ectopic” stem cells (3,120).

Mesenchymal Stem Cells

In the past decade, emphasis of in vitro targeted differentiation towards myocytes has been focused on MSCs, mainly for their ease of isolation and expansion in culture. Makino et al. (65) was among the first to report that immortalized murine MSCs exposed to 5-aza-deoxycytidine (5-aza-dC), an inhibitor of DNA methylation, resulted in appearance of spontaneously beating foci. Furthermore, those cells also expressed hallmark markers of cardiac

phenotype. Similarly, Shiota et al. (102), Antonitsis et al. (5), and many other groups (42,86,91) also demonstrated cardiac differentiation of MSCs utilizing 5-aza-dC based on phenotypic observations but without contracting foci.

To obviate safety concerns of 5-aza, we induced cardiogenic differentiation from human MSCs using a combination of insulin, dexamethasone, and ascorbic acid to generate cardiomyocyte-like cells (CLCs). They were found to express multiple sarcomeric proteins [cardiac troponin I (cTnI), sarcomeric tropomyosin, and cardiac titin] that are associated with cardiomyocytes. Furthermore, CLCs showed a nascent cardiomyocyte phenotype with cross-striated myofibrils characterized by α-actinin-positive Z bands (99). However, they did not beat spontaneously. Similarly, Schittini et al. (95) utilized conditioned medium from human cardiac explants (HCEs) as a potential source of paracrine factors for differentiating MSCs. Their proteome analysis indicated that HCEs release macromolecules, including cytokines, growth factors, and myocardial and metabolism-related proteins, which trigger differentiation of MSCs to CLCs (95), without contracting foci. Recently, Behfar and colleagues (8) demonstrated that human MSCs could be guided to cardiopoiesis by recombinant cocktail of transforming growth factor-β1 (TGF-β1), bone morphogenetic protein-4 (BMP4), activin A, retinoic acid, insulin-like growth factor-1 (IGF1), fibroblast growth factor-2 (FGF2) and α-thrombin, and interleukin-6 (IL-6). These transformed human cells expressed NK2 transcription factor-realted locus 5 (Nkx-2.5), T-box transcription factor 5 (TBX5), mesoderm posterior 1 (MESP1), and myocyte enhancer factor 2C (MEF2C) and demonstrated synchronized contractions with rhythmic calcium transients in response to external electrical stimuli. More recently, Shinmura et al. (101) reported that treatment of human MSCs with piolitazone, a peroxisome proliferator-activated receptor γ (PPARγ) activator, significantly enhanced the cardiomyogenic transdifferentiation (BM vs. pBM: $1.9 \pm 0.2\%$ $n=47$ vs. $39.5 \pm 4.7\%$ $n=13$, $p<0.05$) (101).

Peran et al. (83) also demonstrated transdifferentiation of human adipose-derived stem cells to cardiomyocytes using an intracellular extract obtained from adult human heart tissue. More interestingly, Rebelatto et al. (87) utilized two nitric oxide (NO) agents [*S*-nitroso-*N*-acetylpenicillamine (SNAP) and diethylamine NONOate (DEA/NO)] to induce bone marrow-derived mesenchymal stem cells (BM-MSCs) and adipose tissue-derived stem cells (ADSCs) towards cardiogenic lineage. Gene and protein studies demonstrated presence of cardiac markers (87) without spontaneous contractions.

While extensive studies have focused on the use of soluble factors to direct differentiation, there is growing evidence illustrating potential to modulate stem cell differentiation via precise engineering of shape or underlying mechanical property. Tay et al. (111) demonstrated that spatially defined geometries or physically tuned substrate stiffness (28) could modulate cardiac differentiation of MSCs in the absence of any induction factors. Moreover, long-term electrostimulated human MSCs was found to induce cardiac gene expression and form a striated muscle photype (35). Collectively, these results suggest that cardiac differentiation within a conducive environment is feasible, providing support for in situ myocardial differentiation. However, identification of cardiomyocytes is based on gene and/or protein expression as spontaneous contractions in most of the cases is not evident.

Hematopoietic Stem Cells

HSCs are known to be mobilized and homed to injured myocardium following infarction (118). A number of clinical trials have been performed using undifferentiated hematopoietic stem cells, which have demonstrated a modest effect in patients. Although several studies have shown that HSCs differentiated in vivo into smooth muscle, endothelial cells, and cardiac myocytes (62,105), their transdifferentiation into cardiac myocytes in vitro has not been definitively proven.

Jackson and colleagues isolated an enriched population of HSCs from the bone marrow, which when injected in myocardial infarction mice model showed only 0.02% cardiomyocytes of donor origin (46). In another study, Orlic et al. (82), reported a remarkable regeneration following direct injection of HSCs in mouse infarct model. The authors demonstrated that transplantation of c-Kit$^+$ cells occupied more than two thirds of the infarcted zone by day 9 posttransplantation accompanied with improved myocardial functioning. However, subsequent studies by other groups (7,79,80) failed to reproduce similar findings. Moreover, the reported number of newly formed cardiac myocytes has, for the most part, extremely low, and transdifferentiation of HSCs into cardiac myocytes has been suggested to occur not to any measurable extent in non-primates (6). Furthermore, whether these stem cells that expressed myofiber structural proteins engrafted to become mature cardiac myocytes, functionally integrated into the myocardium, is still not clear (53). Over the years, controversies have been raised on whether the observed cardiac differentiation of HSCs was actually due to transdifferentiate or cell fusion (6,20). Nevertheless, most groups agree that myocardial transplantation of HSCs improves cardiac function in the infarcted mouse heart.

Pluripotent Stem Cell-Derived Cardiomyocytes (PSC-CMs)

The ability of pluripotent stem cells (embryonic/induced) to differentiate into a variety of cell types makes

them an excellent choice for exogenous cell therapy. Although currently most of the performed studies are preclinical in nature, current approval from the US-FDA to use derivatives of embryonic stem cells in clinical trials suggest a very promising future for pluripotent stem cell therapy. Furthermore, the recent success in reprogramming of human somatic cells into ESC-like pluripotent cells has a potential to make personalized cell-based therapies plausible.

Since the mid-1980s, it is well established that mouse ESCs differentiate into cardiomyocytes during in vitro differentiation via embryoid bodies (EBs) (25). Similar differentiation ability of human embryonic stem cells towards cardiomyogenic lineage is also well documented (23,54,77,123,125). Ironically, their pluripotency has been a major hindrance of those stem cells due to their capacity to form teratoma in vivo. Thus, developing tightly regulated and controlled protocols to differentiate these pluripotent stem cells towards cardiac phenotype becomes mandatory and their purification highly desirable.

Numerous laboratories, including our own, have been able to successfully derive cardiomyocytes from human as well as induced pluripotent stem cells, using either EB-based (48,69) or guided differentiation protocols (54,125). Gene expression studies and immunohistochemical studies have demonstrated expression of early cardiac-specific transcriptional factors (Nkx2.5, GATA4, MEF2c, Tbx-5), sarcomeric proteins [α-actinin, cardiac troponins, myosin heavy chain (MHC), atrial and ventricular myosin light chains (MLC2v and MLC2a), desmin, and tropomyosin], gap junction proteins as well as other cardiac proteins (49,69,77,97,123). At the ultrastructural level, hESC-CMs show clearly identifiable sarcomeres and intercalated discs (97,123). However, clinical applications demand a preparation of highly purified cardiomyocytes, which preferably derived using a well-defined and xenogenic-free culture system.

In the past few decades, a number of protocols have been employed to differentiate hESCs to high purity cardiomyocytes with varying success. Early developmental studies indicated critical role of anterior endoderm for cardiac induction (32,106). Coculture of hESCs with END-2 cells, a visceral endoderm-like derivative of P19 cells, induced cardiogenesis (78) confirming the role of endoderm for cardiac induction. The group also identified small molecule inhibitors of p38 mitogen-activated protein (p38MAP) kinase that at optimal concentration resulted in 80% of EBs contracting rhythmically (38). Modifications to their protocol resulted in preparations of 20–25% cardiomyocytes (97). Interestingly, coculture with END-2 cells predisposes EBs to generate relatively more human fetal ventricular cell-like phenotype (~85%) (21,77).

On the other hand, Laflamme's group utilized TGF-β superfamily members, activin A and BMP4, to guide hESCs towards cardiac phenotype. In their approach, hESCs are sequentially treated with activin A and BMP4 in RPMI-B27 medium. Cells are subsequently maintained in RPMI-B27 medium for an additional 2–3 weeks in the absence of any growth factors. Spontaneously contracting areas are observed approximately 10 days postinduction and could be enzymatically dispersed at the third week with typically >30% cardiomyocytes (54). The efficiency of the protocol is not limited to hESCs but works comparatively well with hiPSCs (97,108). Although this protocol generates cardiomyocytes efficiently, its reproducibility across different hESC lines is still unclear (21).

The third protocol developed by Keller's group not only exploits TGF-β family members but also demonstrates the importance of canonical Wnt signaling in cardiogenesis. In Keller's staged protocol, the combination of activin A and BMP4 induces primitive streak-like population and mesoderm development. Wnt inhibitor, DKK1, is later added to specify cardiac mesoderm and finally vascular endothelial growth factor (VEGF) added to promote expansion and maturation of cardiovascular lineage. This protocol reportedly generates populations consisting of 40–50% cardiomyocytes. Sorting the differentiating cultures for early cardiovascular progenitors further enhanced the efficiency of this protocol. Kinase insert domain receptor/fetal liver kinase (flk1/KDR)^{low}/ckit^{neg} cells selected on days 5–6 of differentiation when plated as monolayer gave rise to 57% cTnI-positive cells (125). The other population (KDR^{high}/ckit^{+}) gave rise to progenitors of hematopoietic and vascular lineages (125).

In spite of those defined protocols, majority of laboratories rely on spontaneous differentiation to generate cardiomyocytes. Spontaneous cardiac differentiation can occur in regular fetal bovine serum containing medium as early as day 10, although efficiency may vary drastically depending on the cell lines used. However, different research groups have developed variations to this basic protocol by adding growth factors or small molecules that further enhance EBs towards cardiac lineage. While some groups provide a serum shock by reducing serum concentrations from 20% to 2% in media (129), other have utilized "spin EBs" (EBs generated from precise cell numbers) to enhance cardiogenic differentiation (14). A summary of some of these methods is presented in Table 2.

DIFFERENTIATED OR UNDIFFERENTIATED CELLS FOR CARIDAC REPAIR?

In the current section, we focus on the abilities of these cells (undifferentiated vs. differentiated) in attenuating adverse ventricular remodeling. While early studies by Sakai et al. (94) and Chiu (19) showed that bone marrow stem cells performed better than fibroblasts in cardiac cell therapy, one major question remains unanswered is whether cardiac differentiation of bone marrow stem

Table 2. Variations in Embryoid Body-Based Differentiation Protocols for Generation of Pluripotent Stem Cell-Derived Cardiomyocytes

Group	Cell Lines	Protocols	Outcomes	References
Burridge et al.	HUES7, BG01, NOTT1, NOTT2	Forced-aggregation of defined numbers of hESCs in V-96 plates, with activin A and bFGF	Differential beating efficiencies in all cell lines (range 1.6–9.5%). Defined media increases efficiency to 23%. Interline variability may be seen.	(14)
Xu et al.	H1, H7, H9, H9.1, H9.2	20% FBS containing media and various differentiating factors	25% beating by d8 increased to 70% by d16. Contractions observed till d70.	(123)
Zhang et al.	iPSC lines	20% FBS containing media for first 10 days followed by 2% FBS media	Beating efficiency varied from 1% to 10% for various iPSC lines.	(129)
Mohr et al.	H9	Microwell for EB formation, initial 10 days in 20% FBS followed by 2% FBS containing medium	300–400 μm microwells EBs generated maximum beating by d30.	(73)
Gai et al.	H9 and iPSC	20% FBS medium for 6 days; followed by 10% FBS medium with various cardiogenic inducers	5-Azacytidine enhanced efficiency, DMSO had no effect and low serum and BMP2 marginally improved efficiency.	(33)
Gaur et al. and Mehta et al.	H9 and iPSC lines	Cardiac differentiation with p38MAPK inhibitor	p38MAPK inhibitor enhanced beating efficiencies by twofolds.	(34,69)
Lee et al.	iPSC lines and H7, HES-3	StemPro-34 with a combinations of various growth factors	Beating 14 days postinduction.	(56)
Kim et al.	SNUhES3 and SNUhES4	BMP2 addition post attachment of EBs	Long-term culture enhanced CM differentiation.	(51)

cells is important for cardiac repair. Similarly, clinical trials performed using undifferentiated adult stem cells demonstrated their ability to help in functional recovery in patients, it is unclear if predifferentiating them to cardiogenic lineage could yield better outcomes.

Adult Stem Cell-Derived Cardiomyocytes in Cardiac Repair

Most of the clinical studies performed so far have used bone marrow-derived mononuclear cells (BMCs) for treating patients with acute or chronic infarcts [reviewed by Dimmeler et al. (24)]. In clinical setting >1,000 patients have been treated with bone marrow-derived cells—either unfractionated (most studies) or enriched in progenitor subpopulations—in numerous clinical trials worldwide (66). However efficacy [gauged by left ventricular ejection fraction (LVEF) or magnetic resonance imaging (MRI)] has been inconsistent and primary end points in the majority of large randomized controlled studies have not been met (66). Instead a modest but significant benefit was seen in meta-analysis of all published studies (1,64). A number of plausible explanations have been given for these modest effects, which include (a) impaired functional activity of BM-derived cells in heat failure patients, (b) engraftment and short-term homing in patients (18), (c) paracrine effects from transplanted cells, and (d) increased stiffness of the ischemic areas of the ventricular wall from increase cell mass (39). Furthermore, in vivo cardiomyogenic differentiation abilities of these stem cells are extremely low, long-term beneficial effects are elusive (18,39). Indeed, Pozzobon et al. (85) failed to detect differentiation of $CD133^+$ bone marrow stem cells (BMSCs) to cardiomyocytes either in vitro and in vivo.

It could be suggested that milieu-directed differentiation may provide better answers to long-term beneficial effects. Following demonstration of such ability in cardiac resident stem cells in response to cardiac injury to form three major cardiovascular cell types, SCIPIO and CADUCEUS trials (70) were initiated to evaluate the concept of milieu-directed differentiation. In SCIPIO trial (clinical trials.gov: NCT00474461), patients with ischemic cardiomyopathy received an infusion of autologous cardiac stem cells (CSCs) isolated from atrial appendage, while LV dysfunction patients in CADUCEUS trial (clinical trials.gov: NCT0089336) received cardiosphere-derived cells (CDCs) (117). While the recently completed CADUCEUS trial did not improve LVEP (65a), initial results from 16 patients in SCIPIO trial (12) have shown improvements. In 14 CSC-treated patients, LVEF increased from 30.3% to 38.5% at 4 months, which did not change in control patients. Furthermore, in seven patients there was a significant reduction in infarct size by 24% at 4 months and 30% by 1 year. These initial results in patients are very encouraging, and suggesting that intracoronary infusion of autologous CSCs is effective in

improving LV systolic function and reducing infarct size in patients although role of cardiomyogenesis by those CSCs remains to be determined.

Despite a large pool of safety data and preclinical application of BMSCs in cardiac repair, their use in experimental infarction models has not been without problems (13,16). Injection of BMSCs into infarcted areas may differentiate into fibroblasts or form intramyocardial calcifications (61,127). Furthermore, efficiency of in vivo cardiac differentiation of BMSCs is debatable. To avoid such uncertainty, a number of groups have utilized differentiated adult stem cells in expecting better recovery in preclinical models. Our group transplanted differentiated CLCs into peri-infarct borders of infarcted rodent myocardium. In comparison to undifferentiated MSCs, CLCs prevented negative remodeling and improved hemodynamic recovery (110). Furthermore, superiority of CLCs over MSCs was demonstrated in load-independent measurements of the end-systolic pressure–volume relationship and preload recruitable stroke work (100). Similarly, Behfar et al. (8) demonstrated that guided cells when delivered into infarcted myocardium of nude mice showed superior functional and structural benefits as compared to their unguided counterparts. They further demonstrated that transplantation of these cells significantly improved left ventricular fractional shortening (unguided BM vs. guided BM: $-4.8 \pm 2.1\%$ vs. $5.2 \pm 1.5\%$). Potapova et al. (84) demonstrated that cardiac differentiated hMSCs repaired a full thickness canine right ventricular defect as compared to unmanipulated hMSCs. In a recent study, Li et al. (61) compared the efficacy of induced BMSCs (iBMSCs) and uninduced BMSCs (uBMSCs). iBMSCs were cocultured with rat cardiomyocytes before being transplanted into border regions of rodent cardiac scar. Echocardiography results indicated near normal LVEF in iBMSC injected rats, but a lower LVEF in uBMSC groups at 4 weeks (iBMSC vs. uBMSC: 80% vs. 53%, $p < 0.01$). Fractional shortening improved significantly from $22.9 \pm 3.4\%$ to $45.6 \pm 2.9\%$ in iBMSCs, whereas the improvements were not significant in PBS treated or uBMSC treated groups. While Langendorff perfusion studies indicated significant improvements in coronary flow, left ventricular end diastolic pressure (LVEDP) and dP/dt, histopathological examinations indicated iBMSC engrafted and gave rise to new myocardium (61). These results suggest that predifferentiation of MSCs towards cardiogenic lineage may have a promising effect on cardiac function in animal models in comparison to naive MSCs. However, a more robust paracrine signaling in those modified cells than their naive counterparts cannot be totally discounted since the extent of benefits achieved thus far are rather similar to beta-blockers or angiotensin-converting enzyme (ACE) inhibitors interventions reported previously (88).

While the above studies have demonstrated short-term efficacy of differentiated MSCs, long-term studies are warranted. Recently, Smits et al. (103) addressed the issue of long-term restoration in infarcted animal model. Isolated cardiomyocyte progenitor cells (CMPCs) from fetal and adult human hearts have demonstrated efficient differentiation to cardiomyocytes, endothelial cells, and smooth muscle cells in vitro (103,104). Intramyocardial injection of (undifferentiated) human CMPCs and differentiated CMPC-CMs to regenerate the infarcted mouse heart and their effects on cardiac function in long-term follow-up were evaluated. While end systolic volume (ESV) and end diastolic volume (EDV) lowered significantly, LVEF increased significantly in both the cell-treated groups in comparison to PBS-treated animals (103). Furthermore, PBS-injected group showed larger infarct in comparison to CMPC-CM injected animals ($43.38 \pm 0.05\%$ and $32.29 \pm 0.01\%$ of the LV circumference), suggesting cell injection prevented adverse remodeling. While both cell-treated groups demonstrated reduction in infarct size, only CMPC-treated animals led to more robust neovascularization in the border zone as compared to PBS controls (478 ± 56 vs. 287 ± 57 vessels/mm^2) (103). These findings suggest that predifferentiation of CMPCs (CMPC-CMs) may not be more advantageous as no additional benefits were observed. Unlike other bone marrow-derived stem cells, CMPCs are committed to cardiac cell lineages (9,103); more studies with precommitted cardiac cells are required to ascertain their additional benefits to cardiac outcome. In summary, it could be suggested that precommitment of naive cells towards cardiac phenotype/progenitors demonstrate better functional recovery in short and medium term studies. However, question on how those cells provide better benefits than naive cells remains unanswered currently.

Pluripotent-Derived Cardiomyocytes in Cardiac Repair

In contrast to adult stem cells with limited cardiomyogenic differentiation, ESCs are double-edged swords due to their pluripotency and unlimited differentiation potential. When undifferentiated ESCs are transplanted in vivo, they can form teratomas, benign tumors derived from all three germ layers (the endoderm, ectoderm, and mesoderm). Their allogenic origin carries a risk of immune rejection and finally ethical issues associated with their derivations need to be addressed. Despite these shortcomings, the first clinical trial with cells derived from allogeneic ESCs has commenced in the United States, in patients with spinal cord injury.

The tumorigenic potential of ESCs could be greatly reduced when cells are predifferentiated in vitro before implantation, thus cardiac differentiation of embryonic stem cell invariably becomes mandatory. Generation of pluripotent stem cells following reprogramming of

somatic cells via viruses, plasmid, or proteins was considered to obviate ethical as well as immunological issues of ESCs. However, human iPSCs face significant biological and technical hurdles such as teratoma formation, insertional mutagenesis, low efficiency of nuclear reprogramming (31,66), genetic abnormalities (37), and potential immunogenicity (132) that hamper their current clinical applicability. However, methods to expedite the generation of myocytes from somatic cells by direct reprogramming may be interesting (45). However, the argument of transplanting fully differentiated against partially differentiated (progenitors) cells is far from settled. Although there are encouraging preclinical studies indicating functional efficacy with ESC-based cardiac repair (17,54,58,116,119,125), their development is lagging behind those of adult stem cells.

Cardiomyocytes in Cardiac Repair

Laflamme's group demonstrated that human ESC-derived purified cardiomyocytes mixed with a prosurvival factors could partially remuscularize the infarct zone and preserve regional and global cardiac function in a nude rat infarct model (54). Furthermore, teratomas were absent and practically all the surviving graft cells were cardiomyocytes. Gepstein's group compared efficacy of undifferentiated hESCs, hESC-CMs (microdissected from beating EBs), and noncardiac hESC derivatives in infarcted rat hearts (17). While injected undifferentiated hESCs showed teratomas as expected, hESC-CMs did not show teratomas but formed small, but viable, human myocardial tissues. Importantly, the hESC-CMs transplanted hearts demonstrated better preservation of LV dimensions and global cardiac function as compared to controls up to 8 weeks posttransplantations. Mummery's group reported modest improvements in cardiac function of murine acute infarct models at 4 weeks, but the beneficial effects were no longer significant at 12 weeks (119). Cumulative results suggest that short-term benefits are apparent, with studies demonstrated engraftment and homing of hESC-CMs, but not other noncardiac hESC derivatives (17,54,119). The studies also provide indication that engrafted cells do not survive for long period in the infarcted myocardium. Recently, Zhao et al. (131) demonstrated genetically modified mESCs with C2A domain of synaptotagmin I promotes binding to dead or dying cardiomyocytes, but not live cardiomyocytes, through phosphatidylserine, a well-known surface marker for apoptosis. They suggested that this may effectively increasing numbers of grafted cells within the injured myocardium. Nevertheless, its applicability in hESCs still awaits further validation.

Interestingly, most studies have been performed on acute/subacute myocardial infarction (MI) model. However, such pluripotent-derived cardiomyocytes are most likely technically more amendable for patients with chronic MI. Hence it would be worthwhile to evaluate the efficacy of hESC-CMs in a chronic MI model. Fernandes et al. (29) performed intramyocardial injection of hESC-CMs at 1 month following ischemia–reperfusion in nude rats. Histopathological evaluation at 3 months demonstrated human cardiomyocytes survived and increased sarcomere organization but showed no improvement in ventricular structure or function. This indicates that, like their adult counterparts, hESC-derived CMs may be more effective when provided in acute or subacute phase of MI rather than chronic MI (29,43,60).

While most of the studies with hESC-CMs demonstrate engraftment and modest functional recovery, testing of hESC-CMs in a murine/rat xenotransplant model have several limitations. Apart from the immunogenicity issues, rapid beating rates of rat hearts may mask arrhythmias generated by pacemaker activity (54). Despite their ability to graft and form electrochemical junctions and beat synchronously with rat cardiomyocytes in vitro (50,124), ESC-CMs exhibit immature electrophysiological properties in relation to mature adult CMs. Specifically, ESC-CMs have significantly longer action potential duration, more depolarized maximum diastolic potential, less equilibrated resting membrane potential, and less organized gap junctions (41,77). Furthermore, significant proportions of ESC-CMs exhibit automaticity resembling pacemaker cells, implying potential arrhythmogenesis in transplanted syncytium (50,77,124). Recently, Liao and colleagues (63) demonstrated that intramyocardial injection of mESC-CMs in murine MI model showed a significantly higher incidence of inducible ventricular tachyarrhythmias (54%) in comparison to MI group (21%). Cardiac MRI showed similar improvement in LV ejection fraction in MI + ESC-CM group compared with MI group at 1 week ($30.3 \pm 5.2\%$ vs. $12.4 \pm 1.4\%$, $p < 0.05$) and 3 weeks ($27.0 \pm 4.8\%$ vs. $10.6 \pm 2.8\%$, $p < 0.05$) post-MI. Furthermore, invasive hemodynamic assessment at 4-week showed significant improvement in LV + dP/dt in MI + ESC-CM group ($2{,}539 \pm 389$ mmHg/s, $p < 0.05$) compared with MI group ($2{,}042 \pm 406$ mmHg/s). However, one of the limitations of the study was isolation of mESC-CM clusters that were heterogenous (atrial 35% and ventricular 65%), which may lead to local electrical heterogeneity and arrhythmia (63). The most probable way to address this issue may be generating cellular homogeneity through purified cardiomyocyte subtypes. Recently, Zhang and colleagues (130) demonstrated that retinoic acid (RA) signaling regulates the fate specification of atrial and ventricular myocytes during cardiac differentiation. Noggin + RAi (RA antagonist) were reported to promote ventricular specification whereas Noggin + RA promoted atrial specifications. The study thus suggested that regulation of retinoid signals

could generation relatively homogenous populations of atrial and ventricular-like myocytes from hESCs. While the END2 cell coculture protocol could yield 85% ventricular cardiomyocytes (21), inhibition of the NRG (neuregulin)-1β/ErbB signaling pathway increased the proportion of nodal-like cells (133). Although subtype specification protocols for cardiomyocytes are available, it is unclear how these individual myocyte subtypes would perform in an experimental model in vivo.

Fully differentiated cells lack the ability to divide further, thus numbers of cells required are likely to be much higher (11), especially in large animal models. Furthermore, to generate large numbers of pure cardiomyocytes, purification protocols utilized for enrichment need to be more robust. However, current available purification protocols like genetic/fluorescent selection (4,44,52), Percoll gradient (122), or mitochondrial dye-based sorting (40) have their limitations. While genetic manipulations have limited success in hESCs/iPSCs (4,21), mitochondrial dye-based selection may be clinically more feasible (21). Recently, two surface molecules, signal regulatory protein α (SIRPA) and vascular cell adhesion molecule 1 (VCAM1), have been suggested to be expressed on cardiomyocytes surface and could be effectively utilized for sorting out cardiomyocytes from a pool of different cell types (26,27).

Cardiac Progenitors in Cardiac Repair

An alternative option may be to explore cardiac progenitors, which are capable of dividing while differentiating towards desire cell types. A clinical trial initiated by Advanced Cell Technology employed ESC differentiated retinal pigment epithelial cells for ocular diseases (www.advancedcell.com), and the PISCES trial by the ReNeuron Group (www.reneuron.com) utilized a clonal neural stem cell line derived from fetal cortical brain tissue for stroke patients. These indicate that defined progenitors may have a place in targeted cell therapy. Nevertheless, clinical outcomes of those trials are still outstanding. GRNOPC1 trial by Geron, Inc. (www.geron.com), utilizing ESC-derived precursors to oligodendrocytes (OPCs) for spine injury too was initiated but has been prematurely terminated for financial reasons, recently.

Tomescot et al. (116) demonstrated that combined administration of BMP2 and SU5402, a FGF receptor inhibitor, to hESCs could trigger them to cardiomyogenic lineage. These cardiac committed cells [expressing Tbx6, insulin gene enhancer protein 1 (LIM homeodomain transcription factor; Isl1), forkhead box H1 (FoxH1), Nkx2.5, Mef2c, and α-actin] were transplanted in immunosuppressed rats following coronary ligation. Assessment 2 months posttransplantation demonstrated presence of human cardiomyocyte (stained with human β-MHC) in rat hearts that populated the scar region with no teratomas (116). Later the same group (11) reported isolation of an early population of cardiovascular progenitors, characterized by expression of octamer binding transcription factor 4 (Oct4), stage-specific embryonic antigen-1 (SSEA-1) and MESP1, following treatment with BMP2. These multipotent progenitors were able to generate cardiomyocytes as well as smooth muscle and endothelial cells. Transplantation of these cells ($SSEA1^+$ cells derived from Rhesus ESCs) into the infarcted myocardium of immunosuppressed nonhuman primates differentiated into ventricular myocytes and reconstituted 20% of the scar tissue, with no teratomas (11).

While the above reports have demonstrated cell populations capable of cardiac differentiation, till date there is no specific cell surface determinants that are specific for heart stem cells or progenitors and most studies rely on utilizing mixed surface determinants (67). Laugwitz et al. (55) demonstrated the existence of a post natal cardiac progenitor population marked by the expression of the LIM homeodomain transcription factor Isl1. These $Isl1^+$ progenitors could differentiate into functionally mature cardiomyocytes [based on endothelial progenitor (EP) studies]. More interestingly, these cells label the second heart field and its derivatives as demonstrated by lineage-tracing studies (15,75,107). These studies provide evidence that isl1 marks progenitors with cardiac origins, with clonogenic and self-renewing abilities (75). Recently, Laugwitz et al. demonstrated that $Isl1^+$ cells could be isolated from both human and mouse iPSCs. Injecting mouse Isl1 cells obtained from differentiation of miPSCs directly into the left ventricular wall of adult nude mice demonstrated engraftment and distribution throughout the myocardium. Most of the cells expressed myofibrillar proteins in an unorganized manner, some were found to form organized sarcomeric structures. Furthermore, these cells costained for smooth muscle actin, CD31 and vascular endothelial (VE)-cadherin (endothelial markers). These results suggest that cardiovascular progenitors were able to generate three cardiovascular cell types when exposed to the cardiac environments in vivo (74). More recently, Mauritz and colleagues (68) demonstrated that mouse-induced pluripotent-derived $Flk1^+$ cells, when injected in ischemic myocardium of mice, resulted in a favorable myocardial remodeling and improved left ventricular function (68). These studies provide a proof-of-concept that cardiac committed progenitors may be a valuable option for cardiac repair. However, isolating these progenitor populations can be technically challenging, and detailed examination is warranted to further characterize their partially differentiated status. Further studies that compare cardiac progenitors versus fully differentiated cardiomyocytes would enable formulation of rationale for deployment of each cell type in specific clinical requirements.

CONCLUSIONS

The choice of ideal cell types for cardiac cell therapy has long been debated. Over the past decade, initial enthusiasm of adult bone marrow stem cells for myocyte regeneration has largely been relegated to paracrine-driven, donor cell-independent cardiac reparation process. Preventing negative LV remodeling and supporting angiogenic revascularization by any suitably functioning adult stem cell may be suffice in acute MI or refractory angina pectoris. However, delivering cost effective cellular interventions that surpass current risk-adjusted benefits in optimally managed heart failure patients may require replacement of lost myocytes. Primitive stemness of embryonic stem cells and induced pluripotent stem cells that enables bona fide cardiac differentiation makes for logical choice in myocyte replacement therapy. However, transplanted cardiomyocytes that form isolated niches in infarcted myocardium may present fertile substrate for arrhythmias, even though vast clinical experience gleaned from adult bone marrow stem cells failed to show increased arrhythmogenicity following cell therapy. Current hurdles in upscaling and purifying cardiomyocytes are formidable. Evolving work on identifying cardiac progenitors and precommitting adult stem cells to cardiac lineages are warranted to broaden our appreciation of functional restoration in cell therapy from being mere cellular transplantation.

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