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At a Crossroad: Cell Therapy for Cardiac Repair

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Heart failure is a highly prevalent, debilitating, and costly condition with generally poor clinical outcomes [1]. Aside from heart transplantation, which is an available treatment option for only a small fraction of patients due to donor organ shortage [2], there is no effective therapy that can reverse the course of this disease. A single episode of myocardial infarction (MI) may result in the loss of 1 billion cardiomyocytes or more (~25% of total cardiomyocytes) [3]. Given the limited intrinsic capacity of the adult heart to repair itself, the goal of cardiac regenerative medicine has centered on strategies to remuscularize the diseased heart.

Conceptually, the functional regeneration of an infarcted heart would entail the replacement of lost myocardium by aligned, electrically coupled, and mature new cardiomyocytes that beat in synchrony with the host myocardium. Beyond achieving this remarkable result, the avoidance of procedure-related complications and other potential adverse events such as tumor formation or cardiac arrhythmia is paramount for the therapy to be considered a success. While the process of finding the most appropriate cell type and delivery approach to achieve this objective has been the holy grail of cardiac regenerative medicine, a growing body of literature has now documented our initial efforts in this area. From these studies, the encouraging finding is that cell transplantation into the diseased heart (via intracoronary, transendocardial, or direct epicardial injection) appears to be reasonably safe. Furthermore, the practicalities of harvesting, expanding, and re-introducing cells back into the patient do not seem too cumbersome. However, the sobering reality we have learned is that tremendous roadblocks exist in achieving significant improvement in long-term cardiac function and *bona fide* remuscularization after cell transplantation.

We believe the field of cardiac regeneration is at a crossroad. While ongoing debate regarding the most appropriate cell type, timing, route of delivery, and clinical setting will be addressed by further experimentation in animal models and patients, we need to consider

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whether the premise of cell transplantation as a treatment strategy for diseased hearts is still fundamentally sound and viable for further exploration. As we now enter the second decade of research on cell-based therapy for cardiovascular disease, it is instructive to revisit some of the key findings from published cell transplantation studies in order to better understand what is needed to move the field forward. We will briefly summarize the efforts related to the transplantation of autologous non-cardiac cell populations such as skeletal myoblasts and bone marrow-derived cells (BMCs), as well as recent trials on resident cardiac stem/progenitor cells (Figure 1). We will also discuss whether pluripotent stem cells such as human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) will be able to move into the clinical arena in the next decade, and the advantages and disadvantages of these cells in comparison with autologous adult-derived cells. Ultimately, our pursuit of cardiac regeneration will be looked upon by future generations as akin to Ponce de Leon's search for the Fountain of Youth, or as one of the greatest success stories in modern medicine. It is our hope that the combined efforts of many dedicated cardiovascular investigators in this area will eventually lead to a durable therapy that can reverse the rising incidence of ischemic heart failure.

Skeletal myoblasts

The initial observation that skeletal myoblasts can be harvested and cultured *ex vivo* from muscle biopsies and then transplanted into an infarcted animal heart sparked the interest of basic and translational investigators that cell-based therapy may be a potentially viable strategy for cardiac remuscularization [4]. The appeal for using skeletal myoblasts as a donor cell source is their autologous origin, ability to rapidly expand in culture, and propensity to generate muscle cells by spontaneous differentiation. Furthermore, these cells appear to engraft into the injured heart with remarkable efficiency and undergo *in situ* differentiation into striated muscle bundles. While the earliest clinical studies conducted in small numbers of patients using autologous skeletal myoblasts reported significant improvements in cardiac function [5], a subsequent trial with a larger number of participants found no demonstrable benefit (as measured by ejection fraction) and a high prevalence of ventricular tachyarrhythmia requiring the implantation of defibrillators [6]. This early effort on skeletal myoblast transplantation illustrates our capability to move quickly from basic discovery to human studies in the cell therapy arena. However, the finding of potentially lethal arrhythmia in the remuscularized hearts suggests the requirement of cell-to-cell coupling between the transplanted graft and endogenous cardiomyocytes to minimize electrical heterogeneity within the diseased heart. Indeed, the introduction of connexin 43, a cell junction protein involved in cardiomyocyte coupling, into mouse skeletal myoblasts reduced arrhythmia following cell transplantation [7].

Bone marrow-derived stem cells

The finding that hematopoietic cells may harbor greater developmental plasticity than previously suspected had spurred the subsequent investigation of these cells for regenerative studies in other tissues including the heart [8]. Studies on hematopoietic cells have most often utilized unselected mononuclear cells isolated directly from the bone marrow or from peripheral blood, as well as a more refined subset of marrow-derived or circulating cells termed endothelial progenitor cells. This latter subset has been reported to induce neovascularization in animal models [9], and are enriched in cell populations possessing the cell surface markers CD34, CD133, and/or receptors for vascular endothelial growth factor. Conceptually, the introduction of autologous cells that exhibit stem cell features into a damaged heart is highly appealing. Furthermore, results from clinical trials have supported the safety and feasibility of intracoronary delivery of bone marrow and circulating stem cells. However, despite encouraging results in animal models, the efficacy of bone marrow

cell transplantation in patients has been modest overall and inconsistent between studies [10–17]. Nevertheless, these initial studies have illustrated three key issues that warrant further consideration: 1. The low retention rate of bone marrow cells in the heart, 2. The questionable efficiency of cardiomyocyte transdifferentiation, and 3. The uncertain mechanisms of functional improvement by the delivery of bone marrow cells.

It appears that the chosen cell type, as well as the route of administration may play a role in the issue of low cell retention in the heart after transplantation. One human PET imaging study demonstrated that CD34+ cells homed to the infarcted myocardium with a roughly 10-fold greater efficiency compared to unselected BMCs after intracoronary cell transfer [18]. Additionally, emerging data suggests that direct intramuscular injection may be slightly more effective than intracoronary delivery [19, 20]. For intracoronary injections, the requirement for diapedesis through the coronary arterial wall may account for the limited amount of cell retention. Hence, innovative strategies aimed at increasing the targeting efficiency of transplanted cells and methods promoting cell survival following transplantation into the heart would prove highly beneficial [21].

The efficiency of cardiomyocyte transdifferentiation from bone marrow-derived cells still poses significant challenges. While the initial findings appeared highly encouraging, subsequent studies identified several confounding issues, such as cell fusion and imaging artifacts that may account for some, if not all, of the apparent hematopoietic cell transdifferentiation into cardiomyocytes [22–25]. While it is not improbable that bone marrow cells can transdifferentiate into cardiomyocytes with the introduction of appropriate epigenetic modifiers, the exact conditions and molecular factors required to achieve this *in vivo* are far from clear.

Despite the low efficiency of cell retention and cardiomyocyte transdifferentiation, there appears to be a modest, but statistically significant (~3–5%), rise in ejection fraction after bone marrow cell transplantation compared with control [14, 26]. Head to head comparisons and meta-analyses suggest this effect has not been reserved to a particular cell type, although most studies used unselected bone marrow mononuclear cells rather than mobilized circulating cells or other selected cell populations [27–29]. There is a growing consensus that the beneficial effects are mediated by paracrine action from either the process of cell injection alone, regardless of cell type, or specific factors secreted by the transplanted cells, or both [30, 31]. The presence of these factors may exert a favorable remodeling effect, augment neovascularization, and/or stimulate the expansion of endogenous cardiac progenitor cells. In support of this, Loffredo et al. showed recentlythat bone marrow-derived c-kit+ cells stimulate cardiomyogenesis by increasing the number of stem/progenitor cell-derived cardiomyocytes [31]. Furthermore, the number of proliferative BrdU+ cardiomyocytes increased in bone marrow c-kit positive but not c-kit negative cell-treated hearts. These results suggest that the identification of specific paracrine factor(s) and the targeted cell population mediating endogenous cardiomyogenesis may allow us to achieve at least as comparable a regenerative response as bone marrow cell transplantation, but in a cell-free manner.

Mesenchymal stem cells

Mesenchymal stem cells, or MSCs, are multipotent stromal cells that can differentiate into a variety of mesodermally derived tissues and constitute another potential candidate for cell-based therapy. For cardiac applications, they have been most commonly isolated from the bone marrow or adipose tissue and defined by their ability to adhere to plastic culture dishes during in vitro propagation. In vitro, MSCs may be induced to exhibit cardiomyocyte-like features in the presence of the demethylating agent 5-azacytidine or when co-cultured with

cardiomyocytes [32–34]. They also enjoy the potential advantages of possessing low immunogenicity, as well as the ability to home to the site of injury within the myocardium [35]. The combination of these attributes, in theory, could allow for intravenous allogeneic delivery of cell therapy via an 'off the shelf' approach without the requirement for administering concomitant immunosuppression.

In this regard, the results from the recently reported POSEIDON study showed that transendocardial allogeneic MSC transplantation was associated with a favorable safety profile when compared to autologous MSCs transplantation [36]. Also, in an earlier randomized trial, the intravenous application of allogeneic MSCs after acute MI resulted in an improvement in global symptom score at 6 months and a small but significant improvement in ejection fraction in patients with large MI's [37]. As with bone marrow-derived stem cell transplantation, the low frequency of MSC engraftment and cardiomyogenic differentiation in the heart suggests that the functional improvement observed in preclinical models and human trials is likely related to paracrine effects of the injected cells as opposed to generation of de novo cardiomyocytes [38]. Several other ongoing clinical trials including PROMETHEUS, TAC-HFT, ADVANCE, and PRECISE will add important information as to safety and efficacy of bone marrow or adipose derived MSC therapy in cardiac disease.

Endogenous cardiac stem cells

While the lesson from bone marrow cell transplantation studies may be found in the presence of a paracrine-mediated effect in cardiac repair, the goal of achieving cardiomyogenesis by direct cell introduction into the injured heart remains elusive. As the search for a true cardiomyogenic cell population continues, several laboratories have reported an endogenous population of cardiac stem/progenitor cells (CSCs) residing in the postnatal heart. Adult CSCs have been isolated based on the expression of surface markers or functional features such as c-Kit, Sca-1, MDR-1/ABCG2 (a.k.a. side population), and aggregational properties (i.e. cardiosphere) [39–42]. The capacity of these CSCs to self-renew in a clonal fashion *in vitro* and differentiate into multiple cardiovascular lineages both *in vitro* and *in vivo* suggests the potential therapeutic benefit of these cells following transplantation into the injured heart [43]. The first study (SCIPIO) evaluating the safety and feasibility of c-Kit+ CSCs in a clinical context was recentlyreported [44]. The encouraging finding is that harvesting and *ex vivo* expansion of c-Kit+ CSCs appears to be feasible, and no overt toxicity has been found thus far. While not pre-specified as the primary endpoints, LV systolic function increased and infarct size reduced following intracoronary infusion of autologous *ex vivo* expanded c-Kit+ cardiac cells in ischemic heart failure patients. Further studies will be needed to determine whether the functional benefit achieved is due to new cardiomyogenesis or paracrine effects as observed in bone marrow cell transplantation. In addition, as the identity and the cardiomyogenic potential of cardiac c-Kit+ cells becomes clarified between different groups [45, 46], it will be important to have additional confirmation of the results in SCIPIO by other investigators in order to help sustain the interest of cardiovascular clinicians and scientists in this approach.

In this regard, a prospective, randomized Phase 1 study of cardiosphere-derived cells (CDCs) (CADUCEUS) was recently reported [47]. Unlike the SCIPIO study where cardiac cells were further purified based on their expression of c-Kit, cardiosphere-derived cells are collected from aggregating cells following right heart biopsy and *ex vivo* expansion. They express c-Kit in ~20% of the cells [48]. When introduced into patients with systolic heart failure, CDC-treated patients showed an apparent reduction in scar size and a corresponding increase in heart muscle mass. Regional contractility was increased as well. Interestingly, the end-diastolic and end-systolic volume, and the overall left ventricular ejection fraction

(LVEF) were not changed compared to control patients. It remains to be seen whether the transplantation of CDCs results in new cardiomyocyte generation, given the reported cardiomyogenic potential of these cells, or whether an endogenous cardioprotective mechanism becomes activated to prevent myocyte loss. While LVEF is an imperfect measurement of systolic function, the lack of a significant increase in this parameter, despite the reported reduction in scar size and increase in heart muscle mass, suggests that even if new cardiomyocytes were generated from the transplanted CDCs, their ability to contribute to positive force generation during systole is somewhat limited. Further studies that address the extent of CDC engraftment in the transplanted heart, the degree of their cardiomyocyte differentiation, and the extent of electrical coupling with native cardiomyocytes will help clarify the apparent disconnect between the increase in muscle mass and the lack of improvement in LVEF.

Pluripotent stem cells

Thus far, our efforts to regenerate the diseased heart have focused on identifying a population of cells that is both autologous and most likely to harbor cardiomyogenic potential. The immunocompatibility and the relative accessibility of autologous cells have been chosen over cardiomyogenic efficiency. This bias has been deliberate since it allows us to move from preclinical studies to human trials in a relatively short period of time. As we speculate on where cell therapy for cardiac disease might be in another decade, it is worth revisiting our original goal of *in vivo* cardiomyocyte replacement. There is a general agreement that pluripotent stem cells such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have unambiguous ability to differentiate into most, if not all, major cell types within the body. Since their initial discovery in 1998 [49], human ESCs have been touted as one of the most promising cell sources for regenerative therapies. The ability of human ESCs to self-renew indefinitely *in vitro* and differentiate into cell types of interest with the supplementation of selected growth factors has stimulated the development of various differentiation strategies to increase the efficiency of cardiomyocyte generation and purification [50].

One of the main reasons for the need to generate a high degree of cardiomyocyte purity is the alarming frequency of teratoma formation when undifferentiated ESCs are introduced into the heart [51, 52]. Indeed, the issue of tumorigenicity has raised the threshold for FDA approval of human ESC products so high that one well-recognized company in this area had to eliminate its entire program. There are currently active trials on Stargardt's disease and adult macular degeneration using human ESC-derived retinal epithelial cells. It remains to be seen whether teratoma formation will be an issue in these early applications of human ESC-derived products.

For cardiac diseases, a number of human ESC-derived cardiomyocyte (hESC-CM) preclinical studies have been published [21, 53]. In these studies, the transplantation of hESC-CMs into the injured rodent heart has resulted in small-sized grafts that are largely electrically isolated. As a consequence, the functional benefit observed early (4–6 weeks) after transplantation was not sustained at 12 weeks or beyond. An encouraging finding from these studies is that no teratoma formation was observed despite the presence of ~20% non-cardiomyocytes within the cardiomyocyte-enriched cell population that underwent transplantation. However, since these studies were performed as xenografts in immunocompromised rodent hosts and the number of the engrafted cells has been very small, it remains to be seen if teratomas would be found when a larger number of these cells are transplanted into humans.

Assuming the teratoma concern can be eliminated in the near future, a patient who undergoes hESC-CM treatment would still require immune suppression given the allogeneic source of the engrafted hESC-CMs. Despite intensive efforts to generate patient-specific hESCs by somatic cell nuclear transfer (SCNT), there has so far been no *bona fide* hESC line created by this approach. The interest in pursuing the generation of patient-specific human ESCs from SCNT waned when it was discovered by Takahashi and Yamanaka that the forced expression of Oct4, Sox2, Klf4, and c-Myc can induce a pluripotent cell phenotype from somatic cells [54, 55]. These so-called induced pluripotent stem cells (iPSCs) exhibit properties highly similar, but not identical, to hESCs and can readily generate cardiomyocytes by *in vitro* differentiation using protocols similar to those used for hESCs. While the generation of iPSCs from patients' own fibroblasts should, in principal, obviate the need for immunosuppression when cardiomyocytes derived from these cells are transplanted, a recent study reported the triggering of an immune response against autologous iPSC-derived teratoma in mice, raising the possibility of rare antigen expression in iPSCs that is not normally expressed in ESCs [56, 57]. It would be important to clarify whether these antigens are unique to teratoma *per se* or are present in all iPSC-derived cells. If these antigens are expressed ubiquitously in iPSCs and their progenies, the advantage of autologous human iPSCs over allogenic hESCs would be limited.

An additional consideration that will undoubtedly play a role in pluripotent stem cell-based therapy for cardiac disease is the phenotype of the iPSC/ESC-derived cardiomyocytes. Myocytes in the heart are naturally diverse, with highly specialized physiological attributes and regional diversity that are essential for normal cardiac function. Currently, all protocols for human iPSC/ESC differentiation generate a mixture of atrial and ventricular cardiomyocytes and nodal-like cells. These cells are largely immature and do not resemble the mature rod-shaped, and often bi-nucleated, cardiomyocytes found in an adult mammalian heart. Their fetal ion channel expression and electrophysiological properties may potentially be arrhythmogenic if electrically coupled with endogenous mature cardiomyocytes. If so, toxicity issues will significantly diminish their translational potential in clinical applications [58, 59]. In this respect, efforts addressing factors that regulate ventricular vs atrial vs nodal-specific differentiation will be highly valuable [60]. Furthermore, understanding the key roadblocks that prevent electromechanical maturation of *in vitro* differentiated iPSC/ESC-derived cardiomyocytes will help to minimize cardiotoxicity from cardiomyocyte transplantation. Ultimately, it will be important to determine the exact degree of cardiomyocyte maturation needed to enable the most optimal engraftment, expansion, and maturation after transplantation. It might be the case that transplanting cardiomyocyte progenitor cells can lead to greater cell engraftment and survival in the diseased heart but these cells mature poorly and form a heterogeneous population of cardiovascular cells that becomes arrhythmogenic. On the other hand, transplanting fully mature cardiomyocytes may result in poor overall engraftment and survival due to their greater demand for oxygen and cell-cell contact despite being more phenotypically compatible with endogenous adult cardiomyocytes. Additional studies in large animal disease models using human ESC/iPSC-derived cardiac progenitor cells, immature cardiomyocytes, and mature ventricular cardiomyocytes should help to resolve some of these dilemmas.

Direct cardiomyocyte reprogramming

The remarkable success of somatic cell reprogramming into iPSCs has generated a renewed interest in direct cell lineage reprogramming since the discovery of MyoD [61]. Indeed, reports of fibroblast conversion into neurons, blood, and liver cells by transcription factor overexpression have been published recently [62–67]. The advantage of somatic cell transdifferentiation into another adult cell without an intermediate state of pluripotency is

that it may circumvent the risk of teratoma formation associated with pluripotent stem cell-derived cell transplantation. In this regard, Ieda et al. reported the reprogramming of murine postnatal cardiac and tail tip fibroblasts into cardiomyocyte-like cells by overexpressing a combination of three cardiac transcription factors - Gata4, Mef2c, Tbx5 [68]. Using fibroblasts from αMHC-GFP transgenic mice, approximately 6% of virally infected cells were double positive for GFP and cardiac Troponin-T. In rare instances, spontaneous calcium transients were noted in infected but not control cardiac fibroblasts. While *in vitro* reprogrammed fibroblasts may constitute a novel source of transplantable cardiomyocytes without the risk of teratoma formation, we believe the reprogramming efficiency must improve significantly (e.g. up to greater than 50% conversion into cardiomyocytes from a starting pool of fibroblasts) for this strategy to be therapeutically relevant, given the issues of cell retention and survival after transplantation discussed above and the lack of ability of these cells to proliferate after transplantation. Nevertheless, the prospect for cellular reprogramming to play a role in cardiac regenerative therapy is quite intriguing and the development of a robust and reproducible protocol for direct conversion of fibroblasts into cardiomyocytes will be important to move the field forward. If this can be achieved, we envision the possibility that one day we might directly reprogram scar fibroblasts in the injured heart without the need for cell transplantation. The recent reports that *in vivo* reprogramming of scar fibroblasts into cardiomyocytes appears to be more efficient than *in vitro* reprogramming is a promising first step towards making this a clinical reality [69, 70].

Future perspective

In the past decade, we have witnessed tremendous excitement among basic and translational scientists towards therapeutic strategies that involve direct cell transplantation into the injured heart. From the wealth of preclinical and clinical data gathered, we gained a greater appreciation for the inherent challenges in survival, retention, cardiomyogenic differentiation, and functional integration of cell transplantation. While no durable therapy has arisen from these efforts thus far, the knowledge we gained with regard to cell procurement, processing, and delivery will be very useful for ongoing and future cell transplantation studies and should improve our chances of success with this approach.

Important issues that will continue to require major investigative efforts include the identification of the most effective strategy for cell engraftment and survival, the most efficient delivery technology to accomplish this goal, the most relevant cell type for transplantation that can achieve cardiomyogenesis to the level that directly contributes to positive force generation, and the improvement in hard clinical endpoints such as reduction in mortality and recurrent hospitalization. With regard to translational clinical studies, the challenges of objectively quantifying improvement in cardiac contractility and function in humans will require appropriate trial design and incorporation of technologies that are least susceptible to observer bias. In this regard, it is noteworthy that many of the bone marrow trials reporting improvement in LVEF employed echocardiography as the method of functional assessment whereas studies utilizing cardiac MRI showed less or no improvement after bone marrow cell transplantation. This suggests that meticulous execution of a double-blinded trial design and the incorporation of cardiac MRI in the assessment of LVEF will be optimal in future trials. Given the relatively small change in LVEF after cell transplantation, the absence of data on mortality or major adverse cardiovascular events, and the small number of patients studied in each trial thus far, future clinical studies will likely shift away from patients with acute myocardial infarction towards patients with ischemic heart failure or vascular insufficiency where the benefit from cell therapy may be greater. As we continue the noble pursuit of cardiac regeneration, it will be important to maintain objectivity in the reporting of preclinical and clinical outcomes in order prevent the creation of unrealistic expectations from the public, particularly given the low societal tolerance for medical errors.

We believe the future prospect for cardiovascular cell therapy remains bright. The finding of an effective therapy that can remuscularize a damaged heart will not only be a remarkable achievement in modern medicine–it will be the most effective, if not the only way that we can reverse the growing incidence of heart failure worldwide.

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Non-standard Abbreviations and Acronyms

αMHC	alpha myosin heavy chain
ABCG2	ATP-binding cassette sub-family G member 2
BMC	bone marrow-derived cell
CDC	cardiosphere-derived cell
CSC	cardiac stem cell
ESC	embryonic stem cell
GFP	green fluorescent protein
hESC-CM	human embryonic stem cell-derived cardiomyocyte
iPSC	induced pluripotent stem cell
LV	left ventricular
LVEF	left ventricular ejection fraction
MDR-1	multidrug resistance gene-1
MI	myocardial infarction
MSC	mesenchymal stem cell
PET	positron emission tomography
Sca-1	stem cell antigen-1
SCNT	somatic cell nuclear transfer

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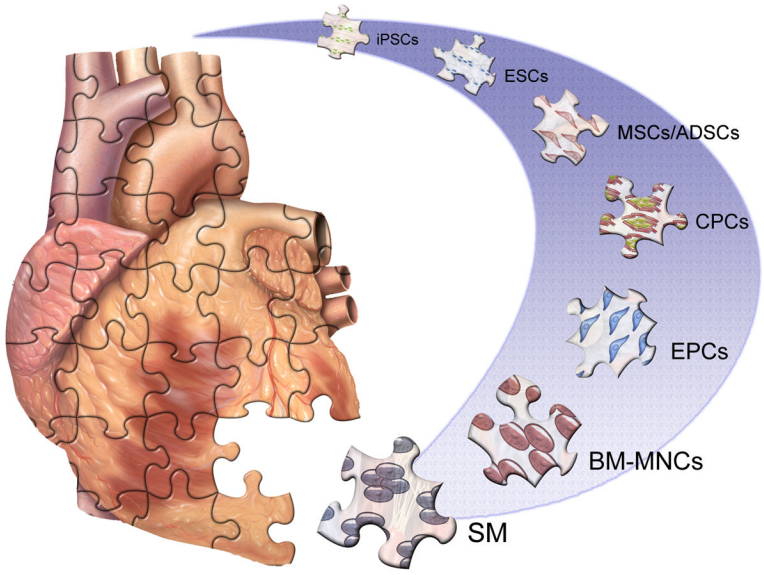

Figure 1. Candidate cell types for cardiovascular regenerative therapy

A variety of cell sources with differing cardiomyogenic potential and developmental origins are under active investigation for cardiac cell therapy after myocardial infarction. BM-MNCs - Bone marrow mononuclear cells. CSCs -Cardiac stem cells. EPCs - Endothelial progenitor cells. ESC - Embryonic stem cells. iPSC - Induced pluripotent stem cells. MSCs - Mesenchymal stem cells. SM - Skeletal myoblasts.