Published in final edited form as:

Regen Med. 2008 March ; 3(2): 189–198. doi:10.2217/17460751.3.2.189.

Multipotent Stem Cells in Cardiac Regeneration

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Summary

The potential for stem cells to ameliorate or cure heart diseases has galvanized a cadre of cardiovascular translational and clinical scientists to take a “first-in-man” approach using autologous stem cells from a variety of tissues. However, recent clinical trial data show that when these cells are given by intracoronary infusion or direct myocardial injection, limited improvement in heart function occurs with no evidence of cardiomyogenesis. These studies illustrate the great need to understand the logic of cell-lineage commitment and the principles of cardiac differentiation. Recent identification of stem/progenitor cells of embryological origin with intrinsic competence to differentiate into multiple lineages within the heart offers new possibilities for cardiac regeneration. When combined with developments in nuclear reprogramming and provided that tumor risks and other challenges of embryonic cell transplantation can be overcome, the prospect of achieving autologous, cardiomyogenic, stem cell-based therapy may be within reach.

Keywords

cardiac regeneration; adult stem cells; myocardial infarction; cardiac progenitor cells; cardiac development

Background

Cardiovascular disease remains the most common cause of mortality in the Western world [1]. This is due, in part, to the fact that the endogenous capacity for myocardial regeneration is inadequate to recover from substantial injury. In recent years, the concept of using stem or progenitor cells as the basis for cardiovascular therapy has gained considerable interest. This was initially fueled by the finding of developmental plasticity in hematopoietic and bone marrow-derived cells [2]. Given the autologous source of these donor cells and the intense interest from clinical cardiologists to identify novel cardiac therapies, a number of clinical studies have been undertaken with mixed results. This early experience illustrates the high degree of efficiency in our ability to translate scientific discoveries from bench-to-bedside, which is in itself, encouraging. Nevertheless, it also highlights our profound lack of understanding of the biological basis of cardiomyocyte development from cardiovascular stem/progenitor cells and the means by which cell-based cardiac therapy is most likely to succeed. This review will briefly summarize the preclinical studies using autologous stem cells (mainly

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Figure 1 adapted from Wu et al, *Cell* (2006) 126:1137-1151.

bone marrow derived) in experimentally-induced myocardial infarction that have led to the recent clinical trials. The remainder of this article will focus on emerging data from developmental and stem cell biology that describe the origin and properties of embryonic cardiovascular progenitor cells, which may one day offer new opportunities for translational studies. Only with a comprehensive understanding of the biological properties of cardiac progenitor cells and the mechanism of their lineage-specific differentiation will we be able to realize the true promise of cardiovascular cell-based therapy.

Adult Stem Cell Preclinical Studies

Bone Marrow-derived Stem Cells

Much of the recent interest in adult stem cells has been driven by successes gained from hematopoietic cell-transplantation that began in 1970's. The ability of circulating or bone marrow-derived hematopoietic stem cells (HSCs) to reconstitute hematopoietic lineages in autologous or sublethally-irradiated hosts has been well described. In the late 1990's, a set of intriguing papers suggested the possibility of bone marrow-derived cell plasticity contributing to the repair of skeletal muscle, hepatocytes, glial cells, and neurons [3-7]. Further refinement showed that HSCs (marked by Lin⁻/c-kit⁺ or side population) were responsible for the transdifferentiation of bone marrow cells into mature cardiomyocytes, smooth muscle cells, and endothelial cells in murine models of infarction [8,9]. Moreover, local injection of lin⁻/c-kit⁺ HSC's into peri-infarct regions resulted in improved post-infarct left-ventricular function (PI-LVF) [8]. These apparent successes were followed by additional work demonstrating the effect of enhanced HSC mobilization with infarction [10]. Treatment of mice with subcutaneous stem cell factor (SCF) and granulocyte stimulating factor (G-CSF) prior to and after left anterior descending coronary artery (LAD) ligation improved survival and PI-LVF in mice. Most notably, a 114% increase in ejection fraction was demonstrated 26 days following infarction in G-CSF treated mice. These dramatic results in animal models have also generated interest in using other multipotent bone marrow-derived cells in myocardial repair including mesenchymal stem cells (MSCs). Direct myocardial injection of autologous MSCs was shown to engraft infarcted myocardium and improved PI-LVF in animal models of ischemia [11, 12].

The enthusiasm for these initial studies has been tempered by data demonstrating that one mechanism by which bone marrow derived progenitor cells repopulate damaged tissue is via fusion with endogenous cells [13-16]. Therefore, what was previously thought to represent *de novo* cardiomyogenic differentiation from HSC's may have been due largely, if not entirely, to fusion events between HSC's and endogenous cardiomyocytes. Furthermore, attempts to replicate the repopulation of ischemic myocardium with transplanted labeled-HSC's have been unsuccessful [17,18].

Although the ultimate fate of transplanted HSC's is subject to debate, post-infarction hemodynamics seem to improve in nearly all cell-based myocardial reconstitution studies including those using HSCs [17]. The basis for this remains poorly understood. *De novo* cardiomyocyte differentiation from HSC's is unlikely to explain the treatment effect [16,19]. Although generally a low frequency event, cell fusion may contribute to myocardial repair by either reprogramming differentiated cells into cells with greater developmental potency or by preventing cellular apoptosis [20]. In addition, transplanted HSC's may exert their effect by stimulating angiogenesis and infarct healing via paracrine effects on surrounding myocytes and possibly resident cardiac progenitor cells [21-23]. This alternative hypothesis remains to be rigorously examined in myocardial repair but has been suggested in mouse models of post-infarct neurogenesis [24].

Adult Cardiac Stem Cells

The adult cardiac stem cell hypothesis was introduced in recent years following the report of evidence for *de novo* cardiomyocyte formation in the injured adult heart that may be mediated by stem/progenitor cells [25,26]. Unfortunately, there has been no validated cardiac-specific surface marker available for the unambiguous isolation of cardiac stem/progenitor cells to-date. Though established in the hematopoietic lineage as a marker for stem cells, c-Kit is expressed widely in germ cells, neurocrest derivatives, and melanocytes [27]. The reported adult cardiac stem cells in mice are marked by c-Kit, Sca-1, or their ability to efflux Hoechst dye due to the expression of ATP-binding cassette transporter (a.k.a side population) [28-30]. These cells exhibit distinct characteristics with regards to their surface marker expression and biological properties (see Murray et al for a detailed review) [31]. The c-Kit positive stem cell was reported to be clonogenic and self-renewing and capable of *in vitro* differentiation into cardiomyocytes, smooth muscle cells, and endothelial cells. Both c-Kit and Sca-1 positive cells have been shown to engraft infarcted myocardium and differentiate *in situ* when these cells were transplanted into the peri-infarct region in rodents with experimentally-induced infarcts. Although the exact stimuli that are present in the myocardium to support differentiation of these stem cells are largely unknown, it appears that at least some of the cells have adopted a differentiated cardiomyocyte fate by fusion with endogenous cardiomyocytes [29]. Since adult cardiac stem cells are expected to be rare, they need to undergo extensive *in vitro* expansion before transplantation in order to achieve detectable level of engraftment. Long-term cell culture has been reported to epigenetically modify gene expression and biological properties of a variety of cell types which may result in phenotypic drift [32]. Hence, investigators from different groups are actively working to identify signaling molecules that regulate self-renewal of these adult cardiac stem cells with the hope that growth factor infusion may expand these cells *in situ* and provide alternative modes of stem cell therapy. One might predict that conserved pathways such as Notch, Wnt, and BMP that regulate stem cell self-renewal in other lineages may play important roles.

Recently the isolation of adult human cardiac stem cells based on the expression of c-Kit has been reported [33,34]. These cells appear to be phenotypically similar to their murine counterparts. Following transplantation into the infarcted myocardium of nude rats, these cells were shown to differentiate into cardiomyocytes as well as smooth muscle and endothelial cells [33]. Although our understanding of the molecular phenotype and biological properties of these cells remain limited, it appears that they can be expanded *in vitro* and may soon be tested in clinical trials.

Adult Stem Cell Clinical Trials

Despite the limited data on the mechanism of benefit for bone marrow-derived cell therapy following myocardial infarction in animal models, the promise for myocardial regeneration was felt to be sufficiently great to justify the costs and, more importantly, potential harm to patients in clinical trials. A variety of approaches have been undertaken in these trials which can be grouped by the mode of cell delivery to the infarcted region: mobilization of hematopoietic cells from the bone marrow by systemic G-CSF administration, intra-coronary administration of *ex vivo* expanded mononuclear cells from bone marrow biopsies, and direct implantation of bone marrow-derived mononuclear cells (BMMC's) into ischemic myocardium by either direct epicardial or endocardial injection.

G-CSF Mobilization

Although the administration of G-CSF has had conflicting results in animal models of myocardial infarction, several clinical trials looking at the effect of G-CSF on PI-LVF have been undertaken [10,35]. MAGIC was the first such trial, but was prematurely terminated due

to angiographic restenosis in 7 of 10 patients that received G-CSF at 6 months [36]. Two subsequent studies looking at the safety of G-CSF administration post-infarction did not have such high rates of restenosis [37,38]. Timing of G-CSF administration may be the reason for these discordant safety profiles. In MAGIC, G-CSF was given prior to percutaneous coronary intervention (PCI), but in the other two trials, G-CSF was given after PCI. Of the safety trials, only one showed enhanced PI-LVF with G-CSF at 4 months [38]. REVIVAL-2 and GCSF-STEMI were designed as randomized, blinded trials powered to assess the effect of post-PCI G-CSF on PI-LVF [39,40]. Neither of these trials reported significant rates of restenosis with G-CSF, but neither trial demonstrated any changes in infarct size or PI-LVF 3-6 months following G-CSF administration. Take together, G-CSF administration following infarction is unlikely to result in myocardial regeneration.

Intra-Coronary Administration

The first reports of intra-coronary (IC) administration of bone marrow-derived mononuclear cells (BMMC's) to determine safety were reported by Strauer et al and the TOPCARE-AMI Investigators [41-44]. In both studies, patients were administered BMMC's via the culprit vessel 5-7 days following ST-elevation myocardial infarction (STEMI). Neither trial demonstrated any adverse changes in PI-LVF or ventricular geometry as assessed by repeat angiography 3-4 months following BMMC administration. Furthermore, TOPCARE-AMI showed that PI-LVF was not adversely affected for up to 12 months following IC BMMC administration and did not report any dysrhythmia.

The apparent safety of IC BMMC administration subsequently led to five major trials designed to assess the effect of BMMC treatment on PI-LVF. BOOST, REPAIR-AMI, and Assmus et al reported 2.5-6% improvement in PI-LVF at 3-6 months in patients receiving IC BMMC compared to controls [44-46]. By contrast, Janssen et al and the ASTAMI investigators were unable to demonstrate improvement in PI-LVF following IC BMMC administration [47,48]. Furthermore, subsequent results of the BOOST trial were unable to show a difference in PI-LVF 18 months from the time of therapy, suggesting that if BMMC's have an effect on PI-LVF, this effect may be transient [49]. The reason for these disparate results remains unclear but may include difference in BMMC handling, injection technique, number of cells administered, and time of administration. Indeed, the number of cells transplanted is highly variable from trial to trial ranging from 0.7×10^6 CD34⁺ cells in ASTAMI to 9.5×10^6 CD34⁺ cells in BOOST [44,48]. Advances in cell isolation techniques such as use of mobilization factors and subsequent apheresis may improve progenitor cell yield in future trials [50]. Additionally, the timing of BMMC administration following infarction seems to have a large effect on outcome [45]. Of note, REPAIR-AMI was the largest of the intracoronary cell therapy trials and the only one to specifically assess clinical outcomes. These investigators demonstrated a modest reduction in the composite endpoint of death, recurrent infarction, and need for rehospitalization for heart failure at 4 months and 1 year after administration of IC BMMC's [43,45].

Intra-Myocardial Administration

The feasibility and safety of intra-myocardial transplantation of bone marrow cells by direct epicardial injection was first established in a study of eight patients with recent myocardial infarction who received CD133 positive cells (also known as AC133 positive) in the peri-infarct area at the time of coronary artery bypass grafting (CABG) [51]. This mode of cell delivery is limited to patients undergoing CABG, a specialized population with greater frequency of diabetes, multi-vessel disease and reduced systolic function. Recent advances in catheter technologies have made possible percutaneous delivery of cells to the myocardium by trans-endocardial approaches. Only a few clinical studies thus far have assessed the safety and feasibility of percutaneous intra-myocardial cell delivery. In patients with chronic refractory

angina, local cell therapy seems to reduce the frequency of angina, improve functional capacity and ejection fraction at 3 months post injection in patients with end-stage ischemic cardiomyopathy, [52-54]. While promising, these studies are small and uncontrolled and the mechanism for the apparent clinical improvement remains to be elucidated.

Opportunities and Challenges for Adult Stem Cell Therapy—Although cell-based therapy, in principle, holds great promise for the treatment of cardiovascular disease, recent clinical trials have produced conflicting results with unclear mechanisms of benefit. One may argue that many of the current standard of care treatments began as therapy with an unclear target of action and that cell-based therapy may follow as well. Yet, it makes intuitive sense that a clearer understanding of the basic mechanism of cardiovascular stem cell biology could improve the likelihood of success in clinical studies. Future opportunities for making cell-based therapy a clinical reality will include identifying the ideal stem/progenitor cell for myocardial regeneration, the optimal timing for cell therapy, the safest approach to cell delivery that results in high engraftment efficiency and robust expansion *in situ*, and the best adjunctive medical therapy that would allow for engrafted cell survival and long-term safety. In this regard, one recent trial looking at the efficacy of post-MI IC BMMC infusion was prematurely terminated due to cardiac complications at the time of bone marrow harvest and ineffectiveness of the treatment [55]. Another study reported ventricular tachycardia (VT) storm within days of BMMC infusion and ongoing VT up to one month after IC BMMC [56]. While these reports are contrary to the generally low rate of adverse events following IC BMMC's in other larger trials, they do serve as a reminder that no treatment is without risk and the slightest lapse in our consideration for patient safety may lead to catastrophic outcomes. Nevertheless, autologous cell-based therapy remains a promising field particularly if the underlying mechanism for benefit can be identified and independently validated.

Embryonic Cardiovascular Stem Cells—Looking ahead to future prospects for cardiac regeneration, the recent discovery of multipotent cardiac progenitor cells from the developing heart offers interesting new possibilities for cell-based therapy [57-59]. Embryologically, the cardiac lineage is derived from the mesoderm with signaling contributions from both endoderm and ectoderm (for a detailed review see Solloway and Harvey 2003 [60]). The mesoderm is marked broadly by the expression of transcription factor Brachyury T. Subsequent differentiation into precardiac mesoderm results in the expression of mesoderm posterior 1 which has been shown to contribute to the three main cardiac lineages (i.e. cardiomyocytes, smooth muscle and endothelial cells) *in vivo* [61]. Commitment to a cardiac-specific fate in the precardiac mesoderm coincides with the expression of homeodomain transcription factors Nkx2.5 and Isl-1. Expression of these factors in the earliest heart forming structure, the cardiac crescent, marks the boundary of the first and second heart fields which give rise to the mature left ventricle and right ventricle, respectively. While Isl-1+ cardiac progenitor cells retain some capacity for vascular differentiation giving rise to endothelial and smooth muscle cells of the aorta and pulmonary artery and the proximal coronary vessels, Nkx2.5+ cells become lineage-restricted to cardiomyocytes and smooth muscle cells during the looping stage of cardiac development (embryonic day 9.0 in mice) [57,58]. These embryonic cardiac progenitor cells have bona fide capacity to spontaneously differentiate from a single cell into two (or three in the case of Isl-1) main cardiac lineages given their assigned roles in early heart formation. Their differentiation can also be controlled by growth factors shown to regulate cardiomyocyte formation during embryonic development.

Since it will be difficult, if not impossible, for human fetal tissue to become the cell origin for clinical cardiac regeneration, the availability of an alternative tissue source that can recapitulate the developmental characteristics of a human embryo will be essential if embryonic cardiac progenitor cells are to be clinically relevant. In this regard, two sources of cells that have the potential to give rise to embryonic cardiac progenitor cells have recently emerged. The first is

human embryonic stem (ES) cells whose ability to develop into cells from all three germ layers when transplanted ectopically into immune-deficient mice has been consistently demonstrated [62]. The similarity between ES cells and embryos in their stage-specific gene expression and developmental potency enables one to apply principles of embryonic development to enhance ES cell differentiation into specific cell types (for a detailed review see Wobus and Boheler 2005 [63]). When differentiated *in vitro*, human ES cells can easily form cardiomyocytes that exhibit electrophysiological characteristics similar to fetal cardiomyocytes [64]. In translational studies, cardiomyocytes derived from human ES cells engraft the infarcted myocardium in nude rats and provide functional benefit at four weeks post-transplantation [65,66]. Despite the improvement in ejection fraction, an index of heart function, the mechanism of benefit is probably unrelated to the presence of human myocardium since the volume of engrafted cells per heart is generally small and no functional electrical coupling is apparent [66]. Additional experimental refinements will be needed to improve the size of the transplanted graft and the formation of electrical coupling between endogenous and transplanted cardiomyocytes. Human ES cell-derived cardiac lineage populations, including cardiac progenitor cells, may be a viable option for clinical studies if important issues such as teratoma formation, immune-rejection, engraftment efficiency, and cardiomyocyte-specific differentiation can be resolved.

Since autologous cells are most likely immune tolerant, the availability of patient-specific ES cells would greatly reduce the need for post-transplant immunosuppression. Derivation of such cells is hampered by the need for a large number of donor human oocytes and the unfavorable political climate for somatic cell nuclear transfer (also known as therapeutic cloning). In this regard, the recent discovery of fibroblast-derived induced-pluripotent stem (iPS) cells has drawn rapidly growing interests from scientists and politicians alike as these cells offer a potential alternative to embryo-derived human ES cells. Through introduction of four defined factors (Oct3/4, Klf4, Sox2, and c-Myc) Takahashi and Yamanaka first demonstrated the ability of these genes to revert a fully differentiated adult mouse tail fibroblast to an ES cell-like cell that is capable of forming teratomas in ectopic transplantation [67]. This finding was subsequently confirmed independently by two other groups and further extended by Yamanaka's own group to show the ability of these cells to generate a new living mouse derived entirely from iPS cells [68-70]. Since these cells were generated from reprogramming of fibroblast nuclei by retroviral-mediated introduction of the four factors, viral oncogenesis (and contribution by c-Myc, a known oncogene) will greatly limit the clinical utility of these cells. Indeed, Okita et al observed that up to 20% of chimeric iPS mice went on to develop tumors due to reactivation of c-Myc [68]. Nevertheless, human iPS cells have recently been generated by the introduction of these factors into differentiated human skin fibroblasts [71,72]. When transplanted into immunodeficient mice, these cells can differentiate ectopically into cells of all three germ layers. Future studies will focus on direct side-by-side comparison of differentiated progenies from embryo-derived ES cells with iPS cells, and determine whether tumor formation will be a significant limitation to translation of iPS cell-derived cells in clinical studies. If these issues can be overcome, one may envision a scenario where a patient with ischemic cardiomyopathy may provide his or her skin fibroblasts for derivation of patient-specific iPS cells. These cells can then be expanded *in vitro* and differentiated to generate cardiac progenitor cells or cardiomyocytes for autologous cell transplantation to replace lost cardiomyocytes (Figure 1).

Conclusion

The combined efforts of basic, translational, and clinical scientists for the past nine years have led to the dawning of the era of cell-based cardiovascular regenerative therapy. Our tremendous ability to translate novel advances in basic research into clinical trials is high encouraging, yet we need to recognize that there exists a large knowledge deficit with regards to our

understanding of the mechanism of cardiac lineage differentiation and the origin and biology of cardiac stem/progenitor cells. If cell replacement therapy is to play a significant role in the armamentarium of a cardiovascular clinician, we must objectively evaluate experimental and clinical data and appropriately advance basic concepts into clinical studies when they are sufficiently mature. Only with a clear understanding of the mechanistic basis for the expected therapeutic benefit will the true promise of cardiac stem cell therapy be realized.

Executive Summary

The changing paradigm of cardiovascular stem cell therapy

- Initial animal studies showing developmental plasticity of hematopoietic stem cells (HSCs) have led to multiple clinical trials testing the efficacy of bone marrow-derived cell transplantation in acute and post myocardial infarction.
- Subsequent studies demonstrated no appreciable transdifferentiation of HSC's to cardiomyocytes and found that cell fusion may explain the apparent differentiation event.
- Results of human clinical trials examining the effects of bone marrow-derived mononuclear cells (BMMC's) on post-infarction left ventricular function have shown a small but statistically significant improvement despite no documented evidence for new cardiomyogenesis.
- Recent identification of cardiovascular stem cells from adult and embryonic sources offers new types of cells for translational studies.
- Clinically relevant sources of embryonic cardiovascular progenitor cells can be derived from human embryonic stem cells and patient-specific induced pluripotent stem cells.
- Future cardiac stem cell therapy will depend on a clearer understanding of the basic mechanisms governing cardiac lineage differentiation from adult and embryonic stem cells.

Unresolved Issues

- For bone marrow-derived cell therapy, the optimal cell type, the best cell number, the best timing to transplant cells, the safest route of cell delivery as well as the long-term safety following infarction are all unknown.
- The mechanism by which transplanted bone marrow-derived cells improve post-infarct left ventricular function remains unknown.
- Multiple markers have been reported to label resident adult cardiac stem cells. The exact nature of the relationship between these cell types needs further investigation.
- It is unclear whether transplantation of embryonic cardiovascular progenitor cells can lead to myocardial differentiation *in situ* and functional improvement.

Future Perspective

- Bone marrow-derived cells will likely be studied in a large-scale clinical trial despite the limited benefit shown in smaller studies. It is anticipated that clinical endpoints such as the rate of patient survival, rehospitalization, and revascularization, as well as the frequency of angina and patients' functional status will all be closely monitored

- There needs to be a clear demonstration of *in vivo* cardiomyogenesis from transplantation of stem/progenitor cells of any source. This should not be merely from immunofluorescent co-localization of a few rare transplanted cells with markers for cardiomyocytes but the appearance of a full thickness myocardium
- The ability of embryonic cardiovascular progenitor cells to differentiate into all three major lineages in the heart suggests their potential for cardiac regeneration in translational studies. These cells may be isolated from *in vitro* differentiation of human ES cells or induced pluripotent stem (iPS) cells.
- The derivation of human iPS cells may allow one to generate autologous embryonic cardiovascular progenitor cells for regeneration while minimizing the need for chronic immunosuppression. However, these cells cannot contain retro- or lentivirus and the risk of tumor formation must be eliminated before clinical studies can be undertaken.

Acknowledgements

The authors would like to thank Dr. Joy Wu for critical reading of the manuscript.

This work was supported by NIH grant HL081086 and the GlaxoSmithKline Education and Research Foundation.

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Figure 1.

Derivation of Autologous Cardiac Lineage Cells from Induced Pluripotent Stem (iPS) Cells. The therapeutic potential of embryonic cardiovascular stem cells may be harnessed by deriving iPS cells from skin fibroblasts of a patient through the introduction of the four defined factors (or their chemical mimetics). The desired cardiac lineage cells can then be generated from *in vitro* differentiation of iPS cells.