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Heart regeneration in mouse and human: a bioengineering perspective

Barry Fine[1] and Gordana Vunjak-Novakovic[1,2]

In this short review, we draw parallels and stress differences between heart regeneration in mice and human, from a bioengineering perspective. As the prevailing dogma that the adult heart is completely post-mitotic is starting to change, there are multiple opportunities for augmenting the limited but definitive turnover of cardiomyocytes, to the extent necessary developing clinically relevant modalities for enhancing heart repair. We discuss some of the most promising among these new directions: mobilization of paracrine signaling by therapeutic cells, cell-free therapy of the heart using extracellular vesicles, and direct reprograming of endogenous cells. These new directions share the cell-free, mechanistic approach to heart repair that could be translated into the clinic faster and safer than the traditional cell therapies.

Addresses
[1] Department of Medicine, Columbia University, New York NY 10032, United States

[2] Department of Biomedical Engineering, Columbia University, New York NY 10032, United States

Corresponding author:
Vunjak-Novakovic, Gordana (gv2131@columbia.edu)

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Introduction

Historical autopsy studies along with early experiments using thymidine incorporation to measure DNA synthesis heralded the sobering reality that the heart is primarily post-mitotic [1,2]. New cardiomyocytes are rare in the adult heart. Over 50 years later, the inability of the heart to regenerate results in 31% of all deaths worldwide [3]. Clinical cardiovascular research has primarily focused on generating tools to mitigate cardiomyocyte loss and promote residual muscle performance. However, a new wave of research is focusing on both intrinsic and extrinsic pathways to promote cellular renewal. Mechanisms have remained elusive, cells are varied and methods are not uniform. Despite these shortcomings, the field is quickly moving forward and clinical trials in humans have begun.

The scale of the clinical problem is enormous, as cardiovascular disease remains the leading cause of death in the world. Following myocardial infarction, contracting cells are lost and are replaced by a fibrotic response orchestrated by fibroblasts and fortified by collagen. The resulting decrease in pumping capacity leads to deleterious alterations in neurohormonal cascades that provide acute hemodynamic stabilization, but chronically result in cardiac remodeling and heart failure. At the most advanced stages of heart failure, the only available option is heart transplantation, representing a most radical form of heart renewal. While transplantation can be curative at end-stages of heart failure, only 10% of patients with end stage heart failure will receive a heart transplant.

Heart regeneration in lower vertebrates

Some of the most optimistic work in cardiac regeneration comes from lower vertebrates that appear to tolerate and repair significant injury. Teleost fish (zebrafish) is one such organism that has shown enviable capacity to regenerate all the organs including brain, spinal cord, appendages and the heart. In a seminal study by Poss *et al.* [4], removal of 20% of the ventricle by apical resection resulted in cardiomyocyte proliferation leading to the replacement of myocardium 60 days following surgery. However, the replacement was not achieved through stem cell processes as two different fate mapping studies indicated that existing cardiomyocytes dedifferentiated and proliferated [5,6].

Heart regeneration in mammals

Genetic ablation studies using an X linked conditionally lethal allele in fetal mouse hearts demonstrated regenerative capacity for replacing up to 50% of the heart [7]. The post-natal mouse retained substantial regenerative capacity on its first day of life. In experiments similar to those conducted in zebrafish, the ventricular apex was resected in P1 mice [8]. Cardiomyocyte proliferation was robust and ventricular tissue was restored within three weeks. Similarly, in P1 mice subjected to LAD ligation MI, up to 95% of lost tissue has been replaced within three weeks [9]. Lineage tracing experiments parallel to those in zebrafish demonstrated that the cardiomyogenesis occurred from proliferating cardiomyocytes. This renewal capacity however was quickly extinguished, such that by P7, the murine heart had lost its ability for therapeutic cardiomyocyte proliferation. Also, a burst of DNA

synthesis observed at P4 was found to be not associated with cell division [10], likely representing the change from cell division to hypertrophic growth. At this time, human data of the same kind are lacking. However, there are reports of significant ventricular recovery after MI during the immediate post-natal period, indicating that there may be intrinsic reparative potential similar to that seen in mice [11].

Adult cardiomyocytes have only a minimal capacity for self-renewal. Studies in rodents initially using thymidine incorporation and then refined with fate mapping showed that less than 0.0005% of cardiomyocytes synthesize DNA per 24 hours and that in a young adult, only approximately 1% of cardiomyocytes per year turn over [10,12].

To determine the capacity of self-renewal in adult human, the seminal paper by Bermann *et al.* [13••] took advantage of the C14 atmospheric spike in the 1950's and 60's due to nuclear testing, to perform what is essentially a human pulse chase experiment. Cells were dated by comparing C14 levels in DNA to that of the atmosphere. Cardiomyocytes were sorted based on troponin staining and diploid DNA content to control for an increase in new DNA per cell due to polyploidization. These cells were found to be younger than the patient, though just barely, and the estimated turnover per year of cardiomyocytes was approximately 1% in 20-year olds, falling to 0.4% in 75-year olds. Experiments in mice using a combination of multi-isotope imaging mass spectrometry and lineage tracing demonstrated that new cardiomyocytes were generated at a similarly low rate and were derived from pre-existing cardiomyocytes [14]. Myocardial infarctions in these animals further revealed that the increase in DNA synthesis observed after injury did not result in completed cell division and that only 3.2% of cardiomyocytes went on to generate new cells.

The regenerative cell

The stem cell revolution that blossomed over the past 20 years has generated intense interest in elucidating whether stem cells can be used for regenerating an injured heart. A number of different cell types have been the focus of intense research and are summarized in Figure 1.

Endogenous stem cells

The c-KIT positive cardiac progenitor cell (CPC) has received significant attention as a potential reparative cell. These cells were isolated and described by initial groups as a resident cardiac progenitor cell [15]. Several investigators reported an incredible cardiomyogenic potential of these cells isolated from adult mammalian hearts. c-KIT + CPCs from humans were reported to give rise to cardiac, endothelial and smooth muscle cells and to repair infarcted myocardium in rodents [16,17]. A Phase II clinical trial in humans was found to be safe and initially positive [18].

The validity of c-KIT positive cells as an endogenous stem cell however was surrounded by controversy. Careful lineage tracing experiments in mouse revealed that c-KIT positive cells generate cardiac endothelial cells and do not contribute to the generation of new cardiomyocytes [19•]. This finding was further confirmed using multiple reporters in the c-Kit mouse locus, demonstrating that these cells generate endothelial cells both at baseline and in response to injury [20]. There have also been several retractions and expressions of concern over the scientific conduct surrounding several significant publications in the field. In response, the NIH halted further enrollment in CONCERT-HF, a phase II clinical trial of c-KIT positive cells with and without mesenchymal stem cells for chronic heart failure [21] (NCT02501811).

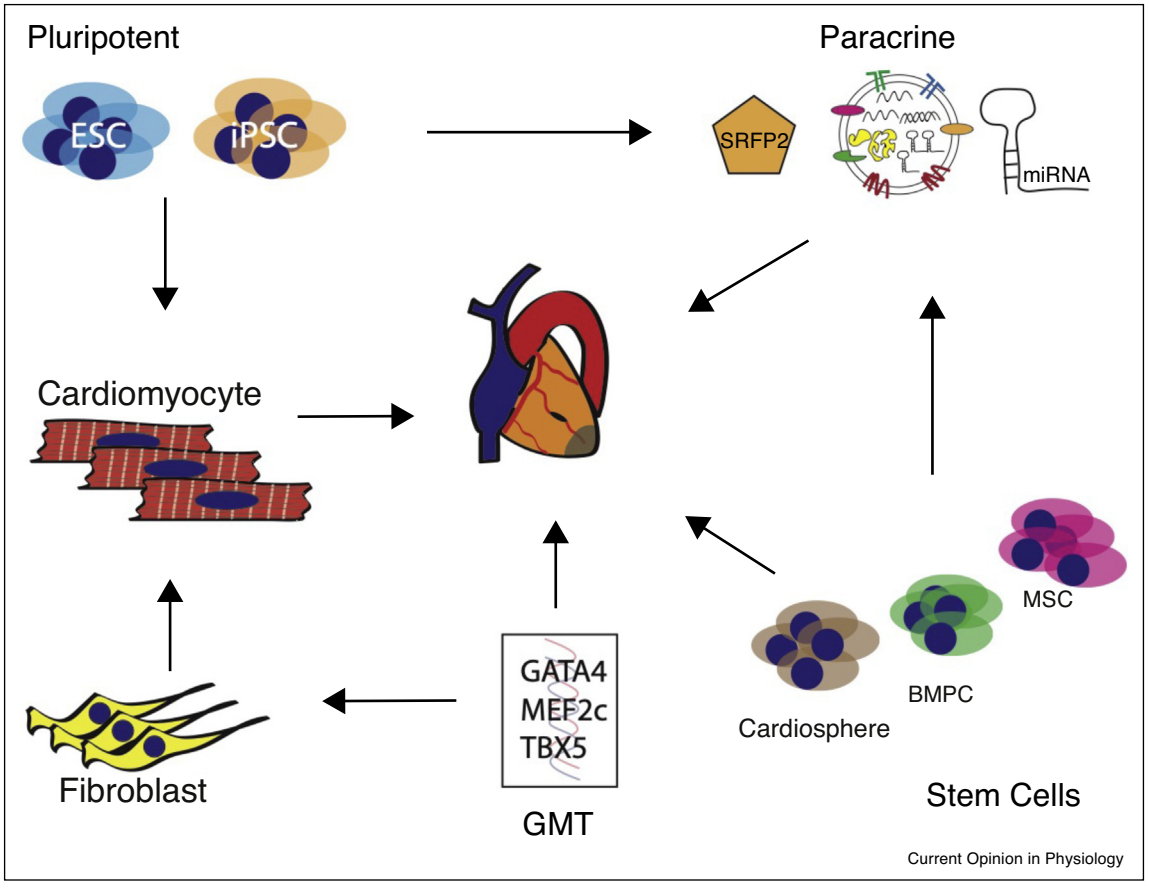
Cardiospheres

Cardiospheres have been reported as another cardiac progenitor population with both clonogenic and multilineage potential. These cells form a heterogenous suspension spheres that can be isolated from explant culture biopsies [22]. Intracoronary administration of cardiospheres in animal models resulted in improved ventricular performance and reduced scar formation [23,24]. A small phase I clinical trial using intracoronary delivery of cardiospheres showed evidence of scar mitigation (by MRI) in post-MI patients [25,26]. The exact identification of the therapeutic stem cells in cardiospheres remains ambiguous and the production of autologous cardiosphere requires an invasive procedure and has a 10–15% failure rate. It also is a timely process that would not be feasible during an acute myocardial infarction. Allogeneic cardiospheres have been developed with promising results in animal models; however, a commercial trial utilizing allogeneic cardiospheres was terminated early, after interim analysis showed a low likelihood of a positive outcome (NCT01458405).

Paracrine effects

The field of cardiac regeneration was fostered early on by the report that bone marrow derived cells [27] and mesenchymal stem cells [28] were able to generate cardiomyocytes in response to injury. This theory of transdifferentiation by which one cell type transforms into another lineage however, was not supported by well-designed fate mapping studies [29,30]. Other evidence pointed to cell fusion as a possible confounder in the original studies [31]. Multiple *in vivo* injury models have demonstrated mild improvement in several measures of left ventricular function after various modes of injection of bone marrow cells, despite the evidence that these cells do not remain at the injection site [32,33]. Initially the prevailing theory was that these cells improved the signaling milieu around an injury bed and released pro-survival cytokines [17]. Inhibitors of Wnt signaling in

Figure 1



Modes of Cardiac Regeneration. Pluripotent stem cells (iPSC's and ESC's) can differentiate into cardiomyocytes *in vitro* for tissue engineering (upper left), and secrete paracrine factors that can be isolated and delivered into the heart (upper right). Fibroblasts can be converted to cardiomyocytes either *ex vivo* or *in vivo* by GMT (lower right). Various types of stem cells have been implanted into damaged myocardium (lower right), and their mechanisms of action seem to involve paracrine factors (upper right). Cell-free factors such as exosomes, Wnt antagonists (SRFP2) and miRNA's have been applied to the heart (upper right).

particular have been identified as a possible mediator of signaling in murine mesenchymal stem cells [34•]. Similarly, studies comparing the functional benefit of various stem cell populations demonstrated that cardiospheres exhibited high levels of paracrine pro survival factors such as stromal derived factor (SDF-1) and vascular endothelial growth factor (VEGF) [35].

Despite the lack of clarity about the effects and mechanisms of action of injected stem cells, human trials were started in earnest and thousands of patients have been enrolled in numerous studies [36]. The indication spanned from acute myocardial infarction to non-ischemic heart failure, and study designs focused on local cell delivery through intracoronary injection. Two of the larger placebo controlled clinical studies using bone marrow cells in the acute myocardial infarction setting did not show benefit [37,38]. A recent Cochrane meta-analyses of 41 trials involving 2700 patients demonstrated that for treatment of acute myocardial infarction, bone marrow derived cells do not show a benefit [39]. More optimistically, a follow-up Cochrane analysis of another 38 trials involving 1900 patients with chronic ischemic cardiomyopathy did show positive signal in terms of safety, reductions in death and the number of heart attacks, although the level of confidence was still considered low [40].

Induced pluripotent stem cells (iPSC)

The laboratory manipulation of stem cells took a leap forward with the isolation of embryonic stem cells (ESC) from the inner cell mass of a blastocyst in 1998 [41] and then with the reprogramming of a fibroblast into an induced pluripotent stem cell (iPSC) by application of four transcription factors in 2006 [42]. The ability to culture these cells *in vitro* in an undifferentiated state, and to expand and differentiate these cells into nearly any cell type has generated a novel field of human models of disease that can be used to study the safety and efficacy of

tissue replacement. These advances have been accelerated by the confluence of rapid genome editing, the ability to derive autologous iPSC's from nearly any individual with any disease state, and advances in tissue engineering.

Cardiac differentiation protocols have been refined and cardiomyocyte populations derived from iPSC's have exhibited critical components of cardiac physiology including calcium flux, ion channels, sarcomeres, transverse tubules and excitation-contraction coupling [43]. *In vivo* experiments in porcine models of acute myocardial infarction, implantation of either ESC [44] or iPSC [45•] derived cardiomyocytes resulted in engraftment and generation of new cardiomyocytes accompanied by improved indices of cardiac function. ESC derived cardiomyocytes were shown to engraft in non-human primates though with a signal for increased ventricular arrhythmias [46•]. The first iPSC cardiomyocyte trials in humans have been announced in Japan, in which sheets of iPSC derived cardiomyocytes will be implanted on infarcted myocardium in patients with advanced heart failure [47].

The race to iPSC therapeutics has been tempered by several concerns regarding their therapeutic potential. iPSC's by their very nature have the ability to generate all three germ layers and if left uncontrolled, can form a teratoma. More concerning however is the potential for alterations in oncogenes and tumor suppressors during culturing that may promote a more malignant process. Recently, p53-dominant negative mutations were reported in iPSC lines and were selected for with passaging [48]. Tumorgenicity assays will have to be carefully performed on each cell line before any therapeutic intervention and long-term safety studies in large animals will need to be performed.

A second concern is the immaturity of cells differentiated from iPSC's. iPSC cardiomyocytes are largely at the neonatal stage of development in terms of gene expression and function. Improvements in maturation will be important to generate truly functional cardiac tissue units that can contribute to cardiac output and withstand high-pressure states. Tissue engineering combining both three dimensional structure and electromechanical stimulation has demonstrated significant improvements in the maturity of engineered cardiac muscle from iPSC's [43,49]. While high level of maturity is critical for disease modeling and studies of drugs, large animal experiments will need to determine the most clinically suitable maturation state for therapeutic grafting.

Cell-free cell therapy of the heart

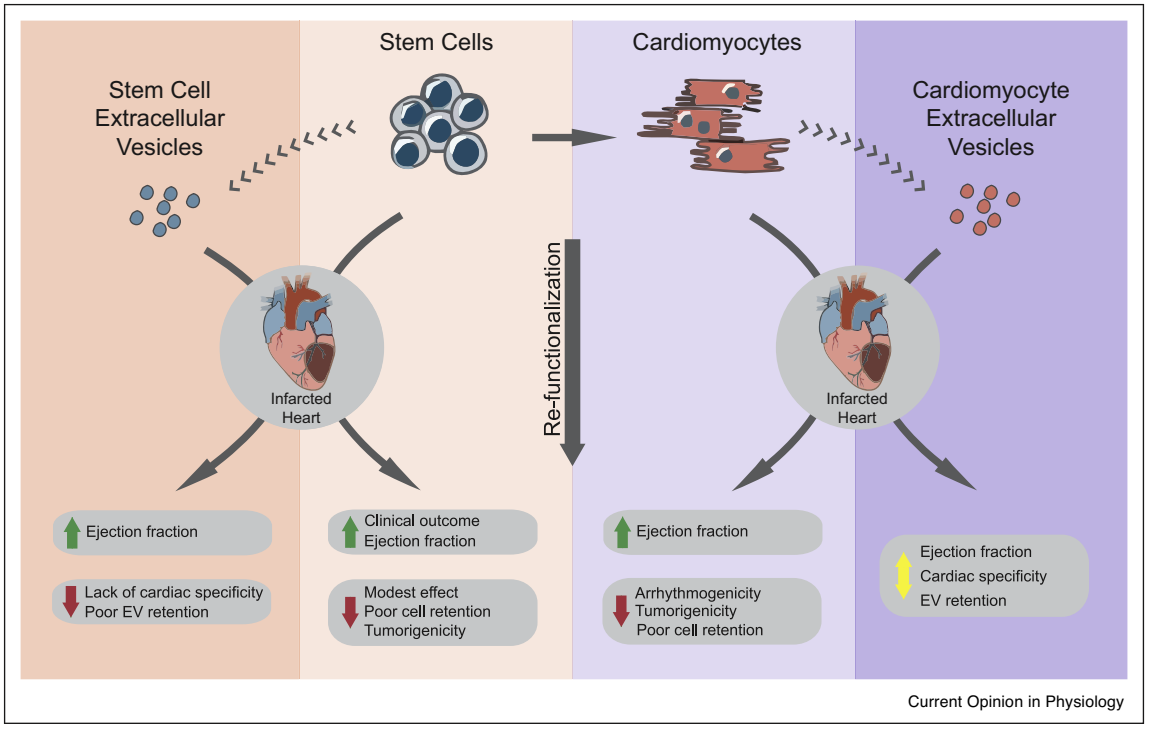
With the shift in stem cell therapeutic mechanisms towards paracrine effects [50], there has been renewed focus to identify the exact mediator of that pro-survival signal. An ideal therapy would be free of any cellular component, focused on the direct mechanism of action, and thus improve both the safety and efficacy profile of treatment while obviating the need for immunosuppression. Studies in rodents showed that cardiomyocytes communicate with neighboring cells via secreted proteins, nucleic acids and lipids and that extracellular vesicles (also known as exosomes) play key roles in this intercellular communication [51]. Injection of extracellular vesicles secreted by various types of cardiogenic cells into infarcted hearts and the cargo they carry have been investigated in animal models of myocardial infarction. We have demonstrated that extracellular vesicles secreted by human iPS cell-derived cardiomyocytes have therapeutic potential when implanted in a sustained delivery patch in a myocardial infarction rat model [52•] (Figure 2). Extracellular vesicles from other cell types including mesenchymal stem cells [53], ESCs [54] and cardiospheres [55,56] have shown benefit in animal models of myocardial infarction.

Extracellular vesicles are a complex mixture of proteins, RNA and miRNA. Because of their role in heart development and their ability to affect large transcriptional changes, there has been a focus on miRNA's in cardiac regeneration. Just in the past year, several studies have demonstrated the link between miRNA and endogenous cardiac proliferation. Intracardiac injection of miR-19a/b was shown to enhance cardiomyocyte proliferation after myocardial infarction in mice leading to improved cardiac function [57]. Using an AAV vector to express miRNA-199a, Gabisonia *et al.*, demonstrated improvement in global and regional contractility due to cardiomyocyte proliferation [58••]. Unfortunately, this particular miRNA resulted in a myoblastic phenotype that caused arrhythmic death. Antagonism of miRNA is also being explored as miRNA may control the switch from proliferative to hypertrophic responses postnatally. In particular, loss of miR-128 in mice promoted cell cycle entry and cardiomyocyte proliferation [59], resulting in decreased fibrosis and improved cardiac function after myocardial infarction.

Direct reprogramming

In vitro work with fibroblasts using combinatorial expression of 20 transcription factors revealed that the combination of Gata4,Mef2c and Tbx5 (GMT) was able to reprogram dermal and cardiac fibroblasts into cardiomyocytes [60••]. The potential of this approach is to directly reprogram fibroblasts into cardiomyocytes and functionally repopulate scar. Importantly, unlike iPSC's, these cells did not traverse developmental stages such as mesoderm or cardiac progenitor stages and thus are less likely to represent malignant risk. Direct delivery of GMT into infarcted murine myocardial infarctions resulted in high levels of fibroblast reprogramming generating electrically coupled cardiomyocytes [61,62], with improved ventricular function and reduced scar size. Other transcription factors including Hand2 [63] and combinations of microRNA's miR-1, miR-133, miR-208 and miR-499 [64] have

Figure 2



Therapeutic potential and challenges of cell and extracellular vesicle (EV) based therapies. Cell-based therapies using either stem cells or differentiated CMs have shown clinical utility in their ability to refunctionalize the injured heart. When directly injected into the heart, both stem cells and CMs have resulted in the refunctionalization of the heart and improved clinical outcomes. However, many challenges arise from the use of cell therapies. Specifically, stem cell therapies only demonstrate modest effects, whereas CM therapies have a high propensity to induce arrhythmias. Stem cell EVs have also been able to refunctionalize the injured heart, mediating the regeneration of the heart after myocardial infarction. However, the cargo of stem cell EVs is not specific to cardiac processes. We hypothesize that iCMs, unlike naive iPS cells, secrete EVs carrying CM-specific cargo that can target the myocardium, providing protection from injury and promoting recovery after myocardial infarction (reproduced with permission from Nature Biomedical Engineering, Ref. [44]).

also shown promise for generating new cardiomyocytes from fibroblasts *in situ*. Direct reprogramming of fibroblasts using small molecules has also been reported and may open a door to facile generation of large scale cardiomyocytes in the future [65].

Conclusion

The prevailing dogma that the heart is completely postmitotic is starting to change. We are recognizing that the adult mammalian heart does indeed have some myocyte turnover, rather small but definitive. Exciting new pathways are now emerging for broadening that limited potential into clinically relevant modalities for directing and enhancing heart repair. Some of the exciting new opportunities for heart repair include mobilization of paracrine signaling, utilization of cardiogenic, vasculogenic and cardioprotective molecules contained in the cargo of cell secreted extracellular vesicles for cell-free heart therapy, and endogenous repair by direct reprograming. These recent efforts focus on bioactive factors rather than exogenous cells, and on mechanistic understanding of the signaling pathways, and could thus allow faster and safer translation into the clinic than the traditional cell therapies.

Conflict of interest statement

Nothing declared.

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- • of special interest
- •• of outstanding interest

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