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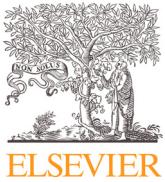Gude, Natalie A
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Cardiac regenerative therapy: Many paths to repair ☆

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ABSTRACT

Cardiovascular disease remains the primary cause of death in the United States and in most nations worldwide, despite ongoing intensive efforts to promote cardiac health and treat heart failure. Replacing damaged myocardium represents perhaps the most promising treatment strategy, but also the most challenging given that the adult mammalian heart is notoriously resistant to endogenous repair. Cardiac regeneration following pathologic challenge would require proliferation of surviving tissue, expansion and differentiation of resident progenitors, or transdifferentiation of exogenously applied progenitor cells into functioning myocardium. Adult cardiomyocyte proliferation has been the focus of investigation for decades, recently enjoying a renaissance of interest as a therapeutic strategy for reversing cardiomyocyte loss due in large part to ongoing controversies and frustrations with myocardial cell therapy outcomes. The promise of cardiac cell therapy originated with reports of resident adult cardiac stem cells that could be isolated, expanded and reintroduced into damaged myocardium, producing beneficial effects in preclinical animal models. Despite modest functional improvements, Phase I clinical trials using autologous cardiac derived cells have proven safe and effective, setting the stage for an ongoing multi-center Phase II trial combining autologous cardiac stem cell types to enhance beneficial effects. This overview will examine the history of these two approaches for promoting cardiac repair and attempt to provide context for current and future directions in cardiac regenerative research.

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The Cardiac Stem Cell (CSC) field

The field of adult mammalian CSC biology and cell therapy emerged nearly twenty years ago [1,2], generating great excitement about the possible existence of resident CSCs and their therapeutic potential for treating heart disease. Original adult CSCs were identified and isolated using criteria established to define hematopoietic stem cells (HSCs) such as expression of c-Kit and Sca1 surface proteins in the absence of hematopoietic lineage markers [2]. The discovery of adult neuronal stem cells and *de novo* neurogenesis in the adult mammalian brain, another presumably non-regenerative organ [3], validated the premise for finding adult progenitors in the heart. A highly active phase of cardiac research ensued amid disagreements over the ability of these cells to transdifferentiate into functional myocardium [4–6]. Despite this controversy, the idea that the adult mammalian heart might possess heretofore unrecognized regenerative potential sparked important novel cardiac research directions and collaborations [7,8], including numerous clinical trials for cardiac cellular therapy. Multiple independent laboratories have identified and character-

ized bone marrow [9,10] and CSC populations based on an array of properties including stem cell protein expression, dye exclusion [11], growth characteristics in culture [12], or stem cell and cardiac gene expression profiles [13]. Circulating or resident endothelial progenitors [14–17], pericytes [18,19] and epicardial cells [20,21] are a few examples of nonmyocyte cell types investigated for cardiac regenerative potential. Adoptive transfer experiments in preclinical small and large animal models using various candidate stem/progenitor cells have revealed that the cells are exerting beneficial effects, whether by creating new myocardial tissue, stimulating endogenous repair mechanisms, or preserving injured myocardium through secretion of cardioprotective factors [22–25].

CSC controversy

The field of myocardial proliferation has always been controversial, but came under increased scrutiny following disparate claims of cardiomyocyte turnover in adult mammalian heart [26,27] and disagreement regarding the contribution of adult cardiac interstitial cell populations to cardiomyogenesis [28–35]. Studies from multiple laboratories replicating many aspects of the original CSC findings remain unchallenged [36–42], lending credence to discoveries and characterizations enumerated above. Even CSC skeptics acknowledge existence of cardiac c-Kit+ cells and demonstrated

that they have endothelial lineage potential [28]. Although the very laboratory that launched the adult CSC field came under scrutiny resulting in retractions and expressions of concern for several publications [43], many important discoveries and research directions have their origins in the CSC field, including cardiac exosomes [44–46], the cardiac secretome [47], and the concept that modest endogenous repair mechanisms within the heart can be augmented through potentiation of endogenous repair [48–50] or enhancement of exogenously applied cell therapies [37,51,52]. Important tools and methodologies such as induced pluripotent stem cells (iPSCs) [53], embryonic stem cells (ESCs) [54,55], tissue reprogramming [56–58], stem cell lineage tracing models [28,38,42,59], dual recombinase lineage tracing models [60,61] and gene editing [62,63] have been developed for probing cardiac biology and revealing the molecular mechanisms underlying cardiomyocyte differentiation and heart disease. Tissue engineering approaches combine material sciences with stem cell biology to devise tissue compatible patches as delivery systems for beneficial factors to damaged hearts [64–68]. Finally, genomic and RNA sequencing technologies are uncovering novel molecular relationships among cardiac tissues at the bulk, single cell, and single nucleus levels, highlighting the heterogeneity of cardiac interstitial cells and iPSCs [53,69–73], and molecular dynamics of cardiomyocytes in response to injury [74]. The genesis of myocardial regenerative medicine has roots in the cardiac stem cell field and many important scientific problems remain to be resolved including functional roles of endogenous cardiac interstitial cells. Regardless of whether there is a 'true' cardiac stem cell, the diversity of cardiac interstitial cell subpopulations exhibits a spectrum of cell types and plasticity that continues to defy arbitrarily simple categorizations such as "stem" or "progenitor" cell.

Cardiac aging

Aging is a major risk factor for cardiovascular disease, leading to compromised cardiac function and reparative capacity at the tissue, cellular and molecular levels [75]. Yet most experimental models addressing cardiac failure and repair rely on non-aged animals and tissues. While findings based on these models provide fundamental insights into cardiac therapy, they do not fully capture the health challenges faced by most adult or aging patients requiring treatment for heart disease. Endogenous myocardial survival and repair decline with aging, exhibiting features of cellular senescence and aging, such as a Senescence Associated Secretory Phenotype (SASP), as well as inability to proliferate, self-renew and differentiate into functional tissue. Compromised renewal capacity of aged myocardium presents a significant challenge for cellular therapeutic approaches that rely upon endogenous responses of the host tissue to mediate repair [76]. 'Next generation' cellular therapeutic approaches are confronting the problem of aging with *ex vivo* enhancements such as genetic or bioengineering approaches [77,78]. Modeling cardiac aging at the cellular and organismal levels, along with continued research into mechanisms underlying age-related cellular and molecular dysregulation are all necessary to design effective preventative measures and treatments for cardiac disease in aging patients.

Clinical consequences

The original c-Kit cell therapy model utilizing bone marrow cells [1] prompted an early rush to clinical cardiac cell therapy in part due to easy accessibility of the cells. High patient variability and lack of standardization among these early efforts produced modest overall outcomes, although some patients responded better than others, and unanticipated benefits were observed. Recent meta-analysis of clinical trials for cell-based therapies unrelated

to the disputed CSC studies reveals that these treatments are safe and can improve clinical outcomes in patients with refractory angina [79]. Several cardiac cell therapy trials using non-cardiac autologous cells demonstrate varying degrees of improvement [77] but overall, meta-analyses indicate that adult bone marrow therapy improves cardiac patient survival, and infarct repair in humans receiving CSC therapy is better than in large animal pre-clinical models [80,81]. Regarding autologous cardiac-derived cell therapies, the SCIPIO Phase I clinical trial utilizing cardiac c-Kit+ CSCs derived from patients meeting specific functional criteria established safety, feasibility, and yielded lasting functional improvement in several patients [82]. Adoptive transfer of autologous cardiosphere derived cells likewise proved safe in the CADUCEUS trial [83]. Although Phase II trials for SCIPIO remain unrealized, the CONCERT-HF trial [84] combining autologous cardiac c- Kit+ CSC and mesenchymal stem cells has resumed. In summary, cardiac cell therapy holds promise as a treatment for cardiac disease, but remains limited in terms of observed functional improvement, evidence of actual myocardial regeneration in patients, and increased skepticism from the scientific and clinical communities [85]. Innovative approaches such as the combinatorial cell therapy used in CONCERT-HF, and enhancement strategies to potentiate survival, engraftment and beneficial properties of adult human cardiac progenitors may overcome these limitations [52]. Meanwhile, clinical outcomes for ongoing studies will play an important role in deciding the fate, so to speak, of autologous cardiac cell therapy in the treatment of heart disease.

Myocyte cell division

Myocyte cell cycle and proliferation are longstanding topics of cardiac research gaining renewed attention as part of the effort to understand and promote myocardial repair [86]. Although robust proliferative capacity exists in non-mammalian vertebrates and neonatal rodent hearts, cell division is all but absent in adult mammalian cardiomyocytes. Animals models in which adult cardiomyocyte counts are increased, whether through transgenic overexpression of cell cycle proteins, or manipulation of hormonal or metabolic function, likely mediate their effects by prolonging and enhancing perinatal myocyte proliferation rather than driving cell cycle completion in fully mature myocytes. [50,87–91]. Furthermore, agents that increase proliferation in immature myocytes may manifest alternate phenotypes in adult cells. For example, transgenes promoting cell division in embryonic and neonatal cardiomyocytes exert protective effects in adult cardiomyocytes, contributing more to myocardial preservation than actual regeneration following pathologic insult [92,93]. To complicate matters further, many methods that rely on detection of canonical cell cycle markers as evidence of proliferation do not represent cell division in postmitotic adult cardiomyocytes [94,95]. Cells often increase their DNA content in response to stress or for other non-replicative purposes, such that incorporation of BrdU into chromosomal DNA will not distinguish between S-phase of the cell cycle, DNA repair, or an increase in ploidy unrelated to cellular division [96]. Likewise, phospho-histone H3 labeling, frequently used to denote mitosis, is expressed in non-proliferating cardiomyocytes exposed to hypertrophic stress [97], while Aurora Kinase B can be detected in binucleating cardiomyocytes [98]. Newer studies adding more precise cellular markers such as midbody or IQGAP3 positioning to define cell cycle progression are challenging the claims of actual cardiomyocyte division given that aborted cytokinesis, endoreplication, endomitosis and endo-duplication can generate positive signals for classic cell cycle markers [98,99]. Genetic approaches such as cardiomyocyte- specific Mosaic Analysis with Double Markers (MADM) or hypoxia lineage tracing mouse models detect a very low rate of cardiomyocyte proliferation in

the postnatal mouse heart and following pathological challenge [100,101]. Finally, reports of cardiomyocyte turnover rates in humans vary widely depending on methodology used and interpretation of results [26,102]. Regardless of the detection method, cell division in mature mammalian cardiomyocytes clearly does not occur at sufficient levels to repair cardiac damage. A better understanding of cardiomyocyte cell cycle status, progression and withdrawal is required to identify molecular targets for prompting proliferation in these cells [103].

Perspective

Identification and characterization of a specific cardiac interstitial cell expressing c-Kit receptor tyrosine kinase in the absence of hematopoietic lineage markers launched the field of adult CSC research. Somewhere along the way, c-Kit itself became the lightning rod for controversy surrounding cardiac cell therapy. Stem cell properties including self-renewal, pluripotency and clonogenicity of c-Kit cardiac stem/progenitor cells have been successfully demonstrated by some groups [104] but not others [105] leading to an over-emphasis on the c-Kit marker itself while blurring the distinction between c-Kit as one of many stem cell markers, and cardiac c-Kit biology in general. Mouse models for investigating c-Kit, including genomic c-Kit mutants, transgenic and genetic c-Kit lineage reporters, or dual-recombinase-mediated cell tracking lines are all valuable tools for elucidating cardiac c-Kit biology. However, due to limitations particular to each system, none exclusively capture the full significance and role of c-Kit expressing cells within the developing and adult mammalian heart [38,106,107]. Further refinement and alternative models which incorporate specific markers of stem/progenitor cells are necessary [28,38,42,60,61,108,109]. Various studies demonstrating cardiac c-Kit expression and activity in non-stem cell populations [28,38,42,59] including myocytes, substantiate heterogeneity within the cardiac c-Kit+ cell population and re-focus the discussion on what defines a stem cell beyond surface selection markers [110,111]. Indeed, recent studies demonstrating upregulation of c-Kit expression in "dedifferentiating" adult mammalian cardiomyocytes suggest a somewhat fluid relationship between stemness, post-mitotic commitment and stem cell marker expression [112–115] echoing but not equivalent to lower vertebrate or mammalian neonatal myocardial plasticity.

Similarly, consensus regarding true measures of cardiomyocyte proliferation remains unclear.

Reliable authentication of cell cycle status is essential for understanding the basic biology of and potential for proliferation in adult cardiomyocytes. False positive signals based on traditional measures of cell cycle interpreted as evidence for cell division substantiate the need for new methods and tools that distinguish between true cell division and incomplete progression through the cell cycle. For example, the cardiac specific FUCCI reporter mouse provides a testable real time readout of cardiomyocyte cell cycle status in vivo and in vitro. Together with more conventional measures of cell cycle, the cardiac FUCCI model illustrates that adult cardiomyocytes appear to be poised at the G1/S cell cycle restriction point [116]. Taken further, combining FUCCI with newly described, more precise markers of cell cycle status will help distinguish between actual cell division and other dynamic ways in which cardiomyocytes respond to changes in their environment. These distinctions will be critical to determining where cardiomyocytes get stuck in the cell cycle under various conditions [117], and identifying the cellular and molecular mechanisms that impede cell cycle progression with the goal of revealing potential targets for promoting cell cycle completion in these cells.

Increased recognition of the heterogeneity and plasticity of cardiac myocyte and non-myocyte populations [115] has prompted multiple studies using single cell analysis and individual cell profiling [118]. In the last two years, transcriptomic analysis has progressed from RNA-seq of bulk cardiac insterstitial populations [70], to single cell transcriptional profiling of interstitial and primary cultures of cardiac origin [119,120], and even single nucleus profiling of cardiomyocytes [74]. Single cell transcriptional profiling allows molecular assessment of both heterogeneity and inter-relatedness of cardiac interstitial cell populations as well as the cardiomyocyte pool during development, homeostasis and in response to injury, while transcriptomic analysis of individual isolated cardiomyocyte nuclei overcomes many limitations imposed by cardiomyocyte size and viability following isolation, and facilitates assessment of nuclear content as it pertains to cardiomyocyte biology. More recently, single cell western technology paired with transcriptional profiling aims to capture heterogeneity of protein expression within cardiac populations. Finally, recent findings reveal nonmyocyte ploidy as a previously unrecognized aspect of interstitial cell heterogeneity with possible implications for functional divergence [121].

Taken together, these advances in technology permit deeper investigation into the cellular and molecular biology of cardiac cell populations, and move scientific understanding past fixed perspectives that prevent progress toward cardiac regenerative therapies.

Conclusion

Cardiac disease remains the primary cause of death among humans [122], representing a costly, multifaceted public health dilemma that cannot be resolved with a single minded approach. Reversing these dire statistics requires an integrative program of increased support for basic and clinical research, health education, preventative medicine and regenerative therapies together with

Table 1

Trajectory of cardiac regeneration research as represented by select studies.

Adult mammalian cardiac regeneration			
	Past	Present	Future
Cardiomyocytes (CMs)	Measuring CM cell cycle, overexpressing cell cycle proteins to promote CM proliferation [88,89,123–125]	Retrenching adult CM perspective [86] Renewed interest in CM proliferation for cardiac repair. Identifying new stimuli for CM cell cycle [50,58,90,95,101,126] Developing new tools to assess CM cell cycle progression [98,99,116]	Developing new tools to accurately pinpoint CM cell cycle progression and molecular and cellular targets for promoting CM proliferation [103] Investigating CM heterogeneity using single cell molecular and biochemical analyses [74,127]
Cardiac Stem Cells (CSCs)	Discovery of adult CSCs [2] Development of cardiac cell therapy [10,80,82,83,128]	Retrenching adult CSC perspective [43,86,106,111,129–131] Novel research directions arising from CSC field [44,45,55–57,67,132,133] Advancing combinatorial CSC clinical trials [22,24,84,134]	Potentiating CSCs to improve therapeutic outcomes [135] Investigating CSC heterogeneity using single cell molecular and biochemical analyses [72]

currently available treatments. Reviewing the trajectory of cardiac regeneration research provides perspective and suggests potential areas for innovation to better understand basic cardiac cell biology in an effort to promote myocardial repair (see Table 1). Development of multiple reparative therapies whether based on cardiomyocyte proliferation, stem cell applications, tissue engineering, or some combination therein, is key to moving cardiac regenerative medicine forward. Therefore, research into every aspect of cardiac survival and repair is valid and necessary, and cooperation among researchers crucial if we are ever to succeed in pushing the human heart out of its resolutely postmitotic state. Revisiting old questions with new perspectives and tools such as bioinformatics, innovative animal or cellular models, or cell free systems will provide the fresh insights and interpretations necessary to create effective regenerative therapies for the treatment cardiac disease.

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