Myocardial Regeneration for Humans
— Modifying Biology and Manipulating Evolution —

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Cardiovascular disease remains the leading cause of death worldwide and developing novel therapies to treat and cure the disease remains a high priority in the healthcare research community. Adult stem cells were successful in entering numerous clinical trials over the past 15 years in attempts to regenerate the heart. First-generation adult stem cell therapies for myocardial regeneration were highly promising in small animal models but realized benefits in humans were far more modest. Consequently, second-generation therapeutic approaches in early implementation phases have focused on enhancing cellular properties with higher survival and regenerative potential. Genetic programming dictates cellular fate, so understanding genetic composition and responses at the gene level to influence the outcome of the cell is essential for successful outcomes in regenerative medicine. Genetic editing is at the forefront of scientific innovation and as basic scientific research continues to expand upon understanding eukaryotic regenerative themes, a clearer vision of the possible future therapeutic approaches can be realized. Ultimately, enhancing biology and manipulating evolutional selection of cellular properties will be critical to achieving clinically relevant and biologically meaningful cardiac regeneration.

Key Words: Cardiomyopathy; Gene therapy; Regeneration; Stem cells

The “fountain of youth” is a mythical spring that restores youthfulness and heals disease of anyone who drinks or bathes within its water, and legends of these waters date back as far as ancient Greece in stories by Herodotus. In reality, the closest approximation to such a mythical fountain where residents exhibit long-term properties of youthfulness is Yuzurihara, Japan, known as “the village of long life”. Villagers routinely live into their 80s and 90s while retaining youthful skin, hair and energy levels while living free of disease and sickness. Preservation of youth in the village is attributed to a combination of a low-calorie diet, high in hyaluronic acid and plant estrogens, and regular physical activity. Residents of Yuzurihara are blessed with healthy lives that many perceive to be of mythical proportions because so much of the world is afflicted with sickness and disease, in mid-life, and well before age 80–90.

Heart disease is the number 1 cause of morbidity and mortality and is a growing worldwide epidemic. Development of heart disease can progress gradually over time with age and is exacerbated by concurrent health complications such as diabetes, obesity, hypertension, or habitual stressors such as smoking, poor diet, or drug/alcohol abuse. The most common cause of heart disease leading to heart failure (HF) is rooted in loss of functional cardiac tissue. Although traditional pharmaceutical approaches prolong survival and help to slow decline in the face of advancing HF, the need to reverse heart disease and restore normal heart function remains a major public healthcare challenge.

Replacement of pathophysiologic cardiac tissue involves multiple underlying mechanisms, including promotion of endogenous repair, reduction of fibrotic tissue, improvement in vascularization, formation of new myocytes, and enhancement of cardiac contractility. Experimental approaches have focused on stem cell therapies to regenerate the heart. Several clinical trials showed modest improvement, prompting novel experimental approaches focusing on modifying biology through combinatory stem cell therapy for dual enhancement, genetic modification to enhance cellular properties prior to injection, and enhancement of the cardiac microenvironment to stimulate endogenous cell and tissue myocardial regeneration.

Beyond biological modification to treat heart disease, future approaches could involve experimental manipulation of evolution, entwining breakthrough technology and theoretical assessment of regenerative models to enhance biological properties by leaps and bounds towards cardiac regeneration. This review will first examine current experimental models of biological modification to enhance stem cells, myocytes, and the microenvironment towards myocardial regeneration (Figure 1). Discussion will then shift to visionary approaches to manipulating evolution and improving cardiac regeneration based on naturally found regenerative eukaryotic models and ongoing research related to DNA modification. These discussions serve as a platform for the short- and long-term shaping of regenerative medicine, with a particular focus on cardiac health and longevity.
**Modifying Biology**

**Enhancing Stem Cells**
Adoptive transfer of stem cells is currently a widely accepted approach to regenerating the myocardium. First-generation stem cell therapy used transfer of either allogeneic or autologous single stem cell populations. Clinical trials revealed that adoptive transfer of in vitro expanded, unmodified adult stem cells does not restore damaged myocardium to normal functionality but may confer modest beneficial effects.7–13 One of the most promising adult stem cell clinical trials involved cardiac stem cells (CSCs) that are endogenous to the heart.14 The 2009 SCIPIO trial (NCT00474461) used autologous CSCs to treat patients who had undergone elective coronary artery bypass graft surgery post myocardial infarction (MI) and had left ventricular ejection fraction (EF) <40% at 4 months after intervention.14 SCIPIO’s 2-year follow-up showed intriguing improvement in EF of 12% in treated patients (from baseline 30% EF to 42% EF) compared with 3.7% improvement in the placebo treatment group.15 These sustained functional effects were more promising than prior clinical trials but also demonstrated that advanced therapeutic solutions are necessary to return the failing myocardium to normal function.

Stem cell enhancement techniques currently being explored include combinatorial stem cells, preconditioning, pharmacological treatment, and genetic modification. Combinatorial stem cell techniques using a mixture of CSCs and mesenchymal stem cells (MSCs) are underway in 2 active US clinical trials, CONCERT-HF (NCT02501811) and TAC-HFT II (NCT02503280). Another combinatorial approach involves the coculture of MSCs, cardiac progenitor cells (CPCs), and endothelial progenitor cells (EPCs) in a unique approach that allows the 3 cell types to adhere in a cluster formation prior to injection.16 A third twist on the classic combinatory approach is fusion of 2 stem cells into a novel chimeric cell, as demonstrated with improved EF post-MI in a mouse model after injecting mouse CPC/MSC chimeras into the MI border zone.17 Combinatory stem cell approaches reveal improved functional output compared with single stem cell types, but have not yet demonstrated restored EF or cardiac output to normal physiologic status.

Preconditioning enhances stem cells in vitro prior to reintroduction into the pathologically damaged heart. Preconditioning treatment is typically performed with growth factors, hypoxic shock or antiaging reagents intended to improve cellular engraftment, survival and differentiation potential for augmentation of myocardial repair and regeneration. Cytokines associated with MSCs used in preconditioning include hypoxia-inducible factor 1α (HIF1α),18 stem cell derived factor 1 (SDF-1),19 vascular endothelial growth factor (VEGF),20 fibroblast growth factor 2 (FGF-2),21 hepatocyte growth factor (HGF),22 insulin growth factor (IGF),23 and transforming growth factor α (TGFα).24 Alternatively, preconditioning has been performed with hypoxia,25,26 hydrogen peroxide,27 β-adrenergic signaling,28 and pharmacological treatment, with reagents such as trimetazidine,29 diazoxide,30 and 5-azacytidine.31 CHART-1 (NCT01768702) is a US clinical trial using preconditioned bone marrow stem cells to treat ischemic HF; results have indicated a positive improvement trend with treatment but a statistically significant difference was not reached.32 Preconditioning stem cells prior to injection is a useful but temporary means to improve cellular therapy by augmenting short-term survival and cell behavior, but results have not demonstrated complete regeneration of the heart or normal physiology after treatment. A more advanced therapeutic approach is warranted.

Genetic modification of stem cells is a vanguard therapeutic approach that seeks to amplify particular trait(s)
within stem cells intended to increase repair and the regenerative response after adoptive transfer. Improvement of cellular survival and/or proliferation after injection into the damaged myocardium has been effected with MSCs engineered to overexpress Bcl-2,33 AKT34 or survivin,35 whereas CPCs have been engineered with AKT,36 as well as with Pim-1, a pro-survival serine/threonine kinase downstream of AKT.37–39 Vascularization of the infarcted and border zone regions of damaged myocardium was increased using MSCs enhanced with VEGF and Ang1,40 and CPCs have been enhanced with Notch,41 and EPCs have been enhanced with endothelial nitric oxide synthase and heme oxygenase-1.42 Such examples of engineered stem cells display additional improvement over non-pretreated cells but, as with so many approaches, they do not restore damaged myocardium to normal function after treatment. As an alternative or concurrent intervention to stem cell therapy, enhancement of the myocardial microenvironment can be also directly targeted through small molecule intervention.

**Enhancing the Cardiomyocyte**

The most conceptually direct way to improve cardiac function after injury is through enhancing cardiomyocyte survival, function, division and proliferation. Through the latter part of the 20th century, researchers studied the unique cellular characteristics of cardiomyocyte function under normal physiological and stress pathological conditions, revealing the basic biology of the sarcomere and the limited proliferative capacity of an adult mammalian myocyte.43,44 In the 21st century, researchers continue to explore the basic biology of the cardiomyocyte in ongoing efforts to determine how to enhance myocytes when responding to physiological disease so the heart will regenerate the necessary cellular population to repair and replace damaged tissue with healthy, viable tissue.

The enhancement of cardiomyocytes has primarily focused on improvement of cellular survival after damage or injury, in addition to increased contractile velocity and force. Early studies with activated or overexpressed AKT in cardiomyocytes demonstrated superior cell survival in response to ischemia-reperfusion injury in mice.46–48 Working downstream of AKT, Pim-1 overexpression exhibits dramatic improvements in cardiomyocyte survival and prevents extensive damage after MI.49,50 Another concern with the failing heart is impairment of both diastolic relaxation and reuptake of calcium into the sarcoplasmic reticulum (SR) during diastole. The SR calcium ATPase 2a (SERCA2) protein transfers calcium from the cytosol of a cardiomyocyte into the lumen of the SR during relaxation. Overexpression of SERCA2a in adult human cardiomyocytes from failing myocardium tissue results in both increased SERCA2a protein expression and pump activity, as well as faster contraction and relaxation velocity, demonstrating faster calcium reuptake into the SR.51 SERCA2a gene therapy for cardiomyocytes reached clinical trials but overall results were disappointingly neutral using viral vector delivery to myocytes with the goal of improved functional output in patients with advanced HF.52 Genes S100A1 and S100A4 have also been shown to enhance contractile function in the ischemic heart, because S100 is part of the family of calcium-modulated proteins known to regulate myocardial contractility.53,54 These examples of gene therapy targeting cardiomyocyte preservation are promising but remain ongoing because large-scale clinical trials have only just begun to branch into gene therapy to treat the failing myocardium55 and are hampered by high cost and bureaucratic, and safety concerns.

**Enhancing the Myocardial Microenvironment**

The microenvironment supporting cardiomyocytes includes vasculature, interstitial cells and extracellular matrix, which each can serve as additional targets to be enhanced in an effort to improve the repair and regeneration of the myocardium after injury. Approaches include engineering scaffolds, matrigel infused with cells, and secretome byproduct injection. Engineering scaffolds have focused mainly on either scaffold-free cellular sheets or synthetic porous scaffolds that cells can populate to patch the damaged myocardium with the intent of increasing contractility and cardiac output.56,57 An alternative to scaffolds, hydrogels can create a synthetic microenvironment for cells in vitro and thereafter introduced into the myocardium as a patch or injected into the damaged region of the heart.58 Secretome byproduct injection arose as a regenerative medicine model from ongoing stem cell research. The word “secretome” is a generalized term referring to the entirety of the byproduct produced and secreted by cells and includes proteins, growth factors, cytokines, chemokines, microRNAs and similar soluble factors. Beneficial growth factors in the protection of cardiomyocytes and reduction of fibrosis in the infarct region include VEGF, TGF-β, SFRP-1, -2 and -4, Smad-5, endothelin, and epiregulin.60,61 Additional mobilization factors such as HGF, LIF, SDF-1, SCF and VE-cadherin,62 as well as miRNAs such as miR-15, miR-17, miR-20a, miR-103, miR-133a, miR-199a, miR-210, miR-210 and miR-451, improve myocardial structure and function after ischemia or infarction.63,64 All the aforementioned approaches provide limited protective effects for the damaged myocardium, but efficient regeneration/replacement of cardiomyocytes still remains an unattainable goal. Collectively, methodologies focused on enhancing stem cells, cardiomyocytes or the microenvironments within the heart have not demonstrated successful regeneration for restoration of normal cardiac structure and function. Research results have demonstrated that biological enhancement of stem cells augments their reparative potential, which is certainly a promising direction to pursue for further advances to stem cell-mediated regeneration. While ongoing studies pursue further refinement of stem cell modification(s) to bolster their regenerative action, even more advanced methodologies are emerging on the horizon of regenerative cardiac research. Taking lessons from other organisms, humans may benefit by incorporating regenerative biology through natural selection and evolutionary advancement.

**Manipulating Evolution**

**Eukaryote Regeneration Phylogenics**

The 3 primary kingdoms model of the organization of life is based on gene similarities and categorized into 3 main branches: bacteria, archae and eukaryote.67 This classification is based on the genetic composition of plants, protist, fungi, and animals, within the eukaryote dominion, that share more genome similarity than life classified within the other 2 dominions. Evolution of eukaryote life has continued for more than 500 million years, with some of the oldest persistent life forms including fish (500 M+) plants (400 M+), and amphibians (300 M+). Primates date back 75 M years, while the genus Homo is 2.5 M years old and our
Eukaryote Evolution, Ploidy and Regeneration

Figure 2. Evolution of eukaryote cell-based organisms over the past 500 million years. As life has evolved, the frequency of polyploidy has increased. An additional characteristic of polyploid organisms is an enhanced capacity to regenerate. Underlying mechanism(s) of regeneration, as related to polyploid DNA content, remain the focus of investigation.

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anatomically modern *Homo sapiens* subspecies dates back a mere 200,000 years. Through Darwinian evolution, natural selection dictates which characteristics are meaningful and necessary for optimal survival of an organism. One evolutionary trait associated with regeneration is duplicative DNA material contained within a nucleus through either genome duplication or polyploid chromosome content. Higher order ploidy content observed in fungi, plants and selected organs and tissues in animals is a widespread evolutionary phenomenon associated with adaptation to physiological and pathological conditions including age, disease and coping strategies in response to environmental factors.\(^{68,69}\) The meaning behind polyploidy remains an ongoing area of debate beyond its correlation with evolution,\(^{70}\) and the extent of benefits behind polyploid genomic content remains to be fully elucidated.

Polyploidy was first observed in plants over 100 years ago and is an advanced evolutionary trait of adaptation and survival,\(^{71}\) evidenced by polyploid genomic content in over 70% of flowering plants.\(^{72}\) Plants are continuously responding to DNA damage imposed by UV rays from the sun, extreme weather conditions as global warming continues to alter the earth’s climate, serving as a food source for multiple species with little means to defend against depredation and, for a number of plants, a continuous life cycle. With multiple environmental stresses facing plant life on a daily basis, regeneration is a necessity for survival. Therefore, plants have evolved to gain an adaptive advantage through polyploidy, frequently driven by endoreplication.\(^{72}\) Not only do regenerative plants display polyploid cells, but fish and amphibians are also known to possess polyploid eukaryote cells.

In fish, polyploidy or genome duplication occurred in a number of families and has evolutionarily evolved over the past few 100 million years.\(^{73}\) Although it is unclear how polyploidy or genome duplication occurs in fish, the majority of polyplloid fish reproduce sexually\(^{74}\) and many polyplloid fish are from hybrid species.\(^{75}\) One benefit found in cultured salmonid species with polyploid content is increased survival.\(^{74}\) Likewise, the zebrafish has undergone complete genomic duplication and this species is capable of regenerating organs or entire limbs after amputation.\(^{75}\) The zebrafish has become a well-known experimental model for cardiac repair, because it has been found to regenerate 20% of the heart after ventricular resection.\(^{76}\) Amphibians, salamanders in particular, regenerate limbs throughout their life, which is attributed to polyploid content,\(^{77}\) as well as an adaptive immune system.\(^{78}\) Although frogs possess a regenerative capability that diminishes into adulthood as compared with salamanders,\(^{79}\) a number of frog species possess polyplloid DNA content and are more resistant to parasites.\(^{79}\) Among reptiles, few polyploid animals have been identified and self-sustaining 4n reptiles are still a theoretical concept generated through hybrid breeding.\(^{80}\) Regeneration in reptiles is also limited,\(^{81,82}\) suggesting a correlation between regenerative capability and ploidy/genomic duplication.

The only tetraploid mammal discovered thus far is the viscacha rat,\(^{83}\) which is unique as the only polyploid mammal identified to date. During development, mammals normally possess polyplloid cells that commit to various cell types.\(^{84}\) Into adulthood, multiple mammalian organs retain populations of polyploid cells, most notably the heart,\(^{85,86}\) liver,\(^{69,86,87}\) and skin.\(^{88}\) Although these organs possess polyplloid cells and have limited regenerative capacity, the relationship between ploidy and regenerative capacity of the organ remains unclear and seems highly variable.

The liver, one of the few tissues capable of regenerating in a postnatal mammal, contains mono- and binucleated polyploid somatic cells with ploidy content up to 16n.\(^{69}\) The relationship between hepatocyte ploidy and regenerative capacity of the liver remains an intriguing area of ongoing research, as hepatocytes undergo chromatin reduction during extensive division and liver regeneration.\(^{88}\) In comparison, the liver comprises cardiomyocytes that transition from single nuclei diploid cells in neonates to binucleated tetraploid adult cells.\(^{69}\) Binucleated cardiomyocytes are typically unable to complete the cell cycle. However, humans show replacement of approximately 50% of car-
diomyocytes over a lifetime, prompting debate over the source of these new cells from preexisting cardiomyocytes or other cell type such as a stem cell population. The true significance of polyploidy in regeneration remains obscure, but there is no dispute that polyploidy and regenerative capacity are correlated in eukaryote evolution (Figure 2). Recognizing these relationships should foster investigations into mechanism(s) behind the regenerative capacity inherent in polyploid cells and how these characteristics can be introduced to diploid cells.

Using Genetics in Regeneration
As the role that ploidy plays in regeneration is unraveled, further understanding of genes activated or silenced during age, stress, or injury must also be explored. Environmental stress affects gene expression in cardiomyocytes and hepatocytes, particularly those involved in stress pathways. Mammalian polyploid cells also exhibit transcriptional activity consistent with increased resistance to apoptosis, DNA damage, and abiotic stress, as well as potential for proliferation, differentiation and senescence that ultimately influence regenerative potential, suggesting a link between ploidy and tissue reparative responses. One of the major challenges in using gene therapy and modifying gene expression or DNA content is lack of basic scientific understanding and the significance of genomic content. Only 15 years has passed since the human genome was mapped, representing groundbreaking progress towards gaining basic understanding of the underlying complexity of DNA content and how eukaryote cells function. Advanced genomic sequencing has allowed researchers to identify and map the evolution and diversity of mammals and also led to technological advancements in potential future therapeutic approaches using genetic editing techniques. These genetics-specific therapies are at the forefront of medicine and have early developmental status. As basic scientific discoveries in genetics continue to expand, novel and revolutionary approaches to regenerate the heart become foreseeable.

Future Directions
First-generation therapeutic approaches to regenerate the heart focused on adaptive transfer of various stem cells or cardiomyocytes in amplitude numbers. In the advancement of therapeutic approaches to regenerate the heart, biological modification to enhance stem cells or cardiomyocytes or enhancement of the microenvironment became the second-generation approach to regenerating the heart. These enhancement approaches are not mutually exclusive but could be combined for further synergistic effects desired for restoration of normal myocardial function in the pathologically damaged heart. In advancing the basic science and biological understanding between human and animal models, the discovery of genetic information and assessing which traits are necessary and sufficient to regenerate the heart will be achieved. As small modifications and enhancements to cellular therapeutics are the current clinical model of treating HF, future models may expand into gene therapies and multi-level enhancements to increase regeneration and improve heart function. To determine which enhancements will be the most beneficial, analyzing mononuclear polyploid cells naturally occurring in regenerative tissues is one methodical approach. It is in these discoveries and successful applied therapies that we may come closer to life with the fountain of youth.

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