Stem Cells Research for the Enhancement Cardiac Regeneration: The Current Role of Multi- and Pluri-Potent Cells in Injury Repair

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By
Veronica M. Meriweather
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Thesis Approval(s):

_____, Steven Houser PhD Temple University Dept. of Physiology

_____, Michael Autieri PhD Temple University Dept. of Physiology

_____, Rosario Scalia MD, PhD Physiology

ABSTRACT

The study of cardiac regeneration can have many forms in which it is defined. It can not only be the ability to add new myocardium to dead or dying tissues, but also include the prevention of cardiac tissue degeneration, reversal of tissue remodeling, and the maintenance of systolic and diastolic function in the incidence of tissue damage, which can lead to subsequent heart failure progression. The use of stem cells for cardiac regeneration represents a growing field of new therapies for patients with end stage cardiac disease. Various studies have noted promising results in the recovery and reparation of these tissues. Cumulatively, their goals have become the identification of the most suitable cell type, as well as how to maximize functional efficiency and cost effectiveness for practical application. Many protocols simply do not ensure adequate cell engraftment, viability, and ultimately the return of normal tissue function. Investigators seek to determine how these processes can be enhanced or manipulated to promote cardiac regeneration in hopes of eventually making their clinical use a standard practice.

DEDICATION

Dedicated to my highly supportive group of family and friends that continually push me to achieve my goals and encourage me to set new ones.

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CHAPTER 1

INTRODUCTION

1.1 The Need for New Cardiovascular Disease Therapies

The development of the field of cardiac regeneration came out of necessity because of the prevalence of heart disease. Heart disease encompasses a wide spectrum of heart defects including coronary heart disease, heart attack, congestive heart failure, and congenital heart disease. Even with the ever expanding list of drug treatments available, if a patient has progressed to the end stages of the cardiac disease, the next step is usually to transplant a new heart. The key most important limitation in heart failure transplant is the inadequate number of qualifying hearts available. Donors must meet a number of legal minimum requirements to be applicable and the recipient too must meet certain qualifications in order to be considered for the transplant due to the limited number of hearts available. But even when proper donor hearts are located and recipients are found to be acceptable, heart transplant does not insure a lifetime of complete cardiovascular health. Donor hearts can fail due to cardiac allograft vasculopathy or be rejected by the recipient's immune system. And suppression of the immune system to prevent rejection can easily lead to infection or the development of cancerous formations. After transplant, the risk for cardiovascular problems such as high blood pressure and atherosclerosis can increase. And finally, aftercare regimens become more stringent. The ultimate achievement in cardiovascular research would be for the heart to be able to repair itself so that transplant intervention is made unnecessary and in doing so, post-operative complications would minimize or no longer be a factor.

1.2 Why Look Towards Regeneration?

Worms, reptiles, fish, and amphibians have all been shown to possess the power of regeneration in many tissues which humans are not capable of. The ability to regenerate vital tissues can mean the difference in quality of life or between life and death. In humans, continual large scale cell turnover or regeneration only occurs in a few tissues such as skin, gastro-intestinal lining, and blood cells, etc (1). Its processes are regulated by communication between the needs of those cells and the needs of the local microenvironment, giving cells cues for the enduring transition from proliferation through apoptosis (2).

CHAPTER 2

STIMULI FOR CARDIOMYOCYTE TURNOVER

2.1 Assessing Regeneration

The process of in-house cardiomyocyte regeneration is governed by cellular capacity to reenter the cell cycle, and/or proliferate and differentiate into electromechanically integrated and therefore, functional adult cardiomyocytes. An accurate way to assess cell cycle progression, and thus potential regeneration, is by the presence of certain molecular or directly observable markers of cell differential or mitotic processes. As cardiac stem cells proliferate in normal cardiac turnover, three structural protein markers become apparent during its mitotic progression: Ki67, an indicator of proliferation; as well as, aurora B kinase which phosphorylates histone H3 (3). Adult cardiomyocytes that are not dividing are characterized by their lack of Ki67 and tumor suppressor protein, p16 INK4a. And aged adult cardiomyocytes that are nearing apoptosis have upregulated p16 INK4a, indicating the cell will no longer be able to replicate or grow (3). Cardiac regeneration is not always prompted by cardiomyocytes or their stem cells. When non-cardiac stem cells are used to initiate cardiac regeneration they do so by releasing paracrine mediators that influence cardiomyocytes or by themselves being stimulated to directly differentiate into adult cardiomyocytes.

Myocardial infarction (MI) is challenged with cell loss, systolic and diastolic dysfunction, and tissue remodeling. These symptoms can be visualized as apparent tissue and vasculature degeneration, poor pumping ability, failure of the chambers to fill properly, chamber dilation, and areas of weakness with compensatory wall hypertrophy to stabilize the remaining tissue. The degree and efficiency of cardiac regeneration is

governed by resolution of any or all of these symptoms. For these reasons, regeneration will be referred to as the development of new myocardial tissue or those mechanisms which preserve or repair existing tissues and functions.

2.2 Inflammation in Cardiac Injury

Signaling molecules influence the extent to which the body can repair itself. Any degree of tissue damage elicits a response that promotes chemotaxis of inflammatory mediators to the site of injury. The acute response to injured myocardium releases a plethora of signaling molecules including proinflammatory cytokines and ROS, which are directly related to the death of cardiomyocytes and ultimately, the degeneration of myocardium after MI (4, 5). But their chief purpose is to aid the injured myocardium by performing protective, compensatory, and reparative processes. Significant reductions in blood supply to the heart, as is the norm is cardiac disease, can generate an infarct. The presence of this infarct causes the release of several regulatory molecules. Interleuking-1 (IL-1) stimulates matrix metalloproteinase (MMP) transcription, inhibits collagen synthesis, and upregulates cellular adhesion molecules (CAMs); IL-6 upregulates CAMs, and it also has a negative inotropic effect; IL-10 suppresses inflammation and stimulates collagen deposition; Monocyte Chemotactic Protein-1 (MCP-1) chemoattracts macrophages and activates them, leading to myofibroblasts infiltration which deposits collagenous scar tissue ; Transforming Growth Factor-β (TGF-β) suppresses inflammation, promotes the transformation of fibroblasts into myofibroblasts; and finally, Tumor Necrosis Factor-α (TNF-α) which upregulates inflammatory mediators, has a negative inotropic effect, upregulates CAMs, causes neutrophil chemoattraction, stimulates MMP transcription, and inhibits collagen synthesis (6). And even though

fibrotic collagen deposition and scar formation are protective compensatory mechanisms to prevent cardiac rupture, they can reduce the functionality of the myocardium by inhibiting excitation-contraction coupling at that site (6). A balance in inflammation must be maintained because chronic inflammation or a significant spike in inflammation can further myocardial damage.

2.3 Modulating Signaling to Enhance the Host Regenerative Environment

The efficiency of stem cell therapy is dictated by molecular communication between the host environment and the either native or foreign stem cells. Presumptively, being able to regulate cellular proliferation, differentiation, angiogenesis, apoptosis, migration and morphogenesis will enhance the efficacy of stem cells in regenerating myocardium. By augmenting or omitting proteins that regulate these processes, it could be possible to optimize the microenvironment in which regeneration takes place. Adult cardiomyocytes can then largely be reactivated to enter the cell cycle and restore performance by hyperplastic remodeling, rather than hypertrophic, as a form of compensatory repair upon injury.

This type of alteration is illustrated by one such study that utilized plasmids encoding VEGF, which when injected into ischemic pig hearts, they produced approximately a 5x increase in the number of mitotically active cardiomyocytes and subsequently increased hyperplastic repair (7). In a second study, intravascular injection of G-CSF induced bone marrow derived hematopoietic stem cells to mobilize by downregulating SDF-1 and upregulating its receptor, CXCR4 (8). Another study by Kawaguchi et al. showed that when cardiac stem cells expressing high levels of GATA-4 were co-cultured with adult cardiomyocytes, the adult cells displayed enhanced IGF-1

expression. GATA-4 is a transcription factor that regulates genes of myocardial differentiation and function and IGF-1 promotes cell growth and development. These findings contributed to an increase in contractility and a reduction in apoptosis, showing a possible correlation to ventricular tissue survival and attenuated systolic dysfunction (9). Many regenerative therapies can be aided by altering these and other signals in the host microenvironment alone, or they can be strengthened by manipulating stem cells themselves, or with the addition of structural distribution systems.

CHAPTER 3

STEM CELL UTILITY IN THE HOST ENVIRONMENT

3.1 Stem Cell Basics and Breakthroughs in Research

Pluri- and multi-potent stem cells are the most undifferentiated of immature cell types. They are found in embryonic, fetal, and adult tissues of the body. In order to be classified as having stemness, a cell must have three key properties: It must be unspecialized, capable of long term proliferation, and it must differentiate into specialized cell types (10). Some cells, such as those found in bone marrow, proliferate continuously. While others, for the most part, experience cessation of this process beyond a certain point in their lifetime (1). When and how a stem cell differentiates, are determined by intercommunication of intrinsic signals of the cell with extrinsic signals of the environment. Stem cells from a given tissue usually differentiate into specialized cells of that same tissue, though this is not a concrete rule. When subjected to the proper stimulation, these cells can be induced to differentiate into cells of an alternative tissue. Not only do they develop into specialized tissues of the body in utero, but throughout the body in adulthood, minute populations exist specifically for reparation and renewal of worn or damaged tissues and are stalled in their development so that they may diverge into many cell types when needed. Theoretically, when called upon, they can be used to jump start regeneration and repair of tissues, including those that are considered by most to no longer replicate.

Significant historic breakthroughs in stem cell research began about 30 years ago. In 1981, embryonic stem cells were first derived from mouse embryos. Since then the field has continually expanded, leading from the derivation of human embryonic stem

cells from donated IVF embryos in 1998 to the development of an embryonic-like induced pluripotent stem cell from somatic cells in 2006 (10). The progressive breakthroughs in stem cell research over the years has demonstrated the plethora of resources in which these cells reside and has established some of their primary activities in organ systems. These resources and their conceivable clinical applications are of particular importance to the field of cardiac regeneration because general heart disease is the leading cause of death amongst men and women in the US (11).

3.2 Using Stem Cells to Modulate the Host Environment

The goal in the use of stem cells is not only to repair anatomical and functional losses from the primary injury, but to prevent the secondary injury from inflammatory cells and their cytokines. Introducing stem cells alone can alter host signals enough to generate some level of recognizable shift toward injury repair. Direct intramyocardial injection of bone marrow mesenchymal stem cells increases the levels of signaling molecules like IL-10, B-cell leukemia-lymphoma-2 (BCL-2); vascular endothelial and basic fibroblast growth factor (VEGF and bFGF) and decreases proinflammatory associated proteins TNF-α, IL-1β and BCL-2-associated X protein (BAX) (12). By simply introducing stem cells into an environment, the prevalence of certain signals is too altered, leading to a certain degree of improvement in cardiac function, attenuated fibrosis, and increased capillary density. Using stem cells in this manner is cardioprotective, thwarting further myocardial injury and functional degeneration. Inflammation typically prompts scar formation, but mesenchymal stem cells (MSC), while beneficial for regeneration, have also been found to contribute to the development of myocardial scarring, which as previously stated, further reduces function (13).

Without intervention, this effect is regularly seen as a consequence of inflammation.

3.3 Normal Cell Turnover in the Human Heart

For years, it has been considered common knowledge that myocardium does not spontaneously regenerate itself. Adult cardiomyocytes were simply thought not to reenter the cell cycle and proliferate for cellular turnover and tissue repair. Animal studies have demonstrated that mammalian cardiomyocyte mitosis is highest during embryonic stages and gradually decreases as an organism approaches birth. This is thought to be due to a change in the method of cardiomyocyte growth from hyperplasic to hypertrophic. And within a relatively short period of time, mitosis of cardiac cells, if present, was thought to be virtually undetectable (14). But this has been shown to not necessarily be the case. Recently, low levels of cardiomyocyte division have been found in adult mice and then in humans.

Adult cardiac tissue still maintains small pockets of cardiac stem cells that are normally responsible for only low levels of regeneration. The activities of these cells do not usually produce a large enough effect to have any benefits in tissue remodeling, reversal of dysfunction, or preservation of current function to prevent overall deterioration (3). But there are some benefits behind the negligible amount of innate regeneration of the heart. The incidence of cancer in the heart is extremely rare as indicated by the presence of tumor suppressor protein, p16 INK4a during cardiomyocyte mitosis. This protein prevents progression beyond G1 in the cell cycle in the event of DNA replication mistakes. Ironically, the heart is protected from a disease that would be highly detrimental within such an important instrument, as is the heart (3).

3.4 The Ideal Stem Cell

Casting choice stem cells with minimal post-introductory complications will provide generous engraftment and long term viability to ensure maximal recovery. One of the major problems is that stem cell retrieval and administration to the host can significantly increase immune responses that could render treatment ineffective or additionally damaging. All methods of delivery will result in at least a minor inflammatory response. Injecting large quantities of stem cells, even autologous cells with no immunogenic properties, will trigger this response (15). But an autologous donor would be the most desired in order to significantly cut down the risks associated with an inflammatory response. These risks range from fibrotic capsule development to cellular rejection to Host vs. Graft Disease (HVGD). If obtaining the proper type of stem cell from the host is not possible for any number of variables, a syngenic sibling would be equally beneficial, with lastly an allogenic donor to be considered because of their noted immunogenic nature upon transplantation.

3.5 How Do Stem Cells Exercise Their Influence?

Stem cell injection provides a promising means for producing regenerative effects in the heart. Many studies report that even when engraftment is low, stem cells are still capable of inducing enough regeneration to relieve some of the symptoms of myocardial infarction associated with cardiac disease. The common observation with stem cell administration is that the much of cardiac improvement is mediated by the secretion of trophic factors (15). These factors can still activate the regenerative capabilities of adult cardiomyocytes and the other small pockets of stem cells in the heart. Several studies in animal models of cardiac injury indicate that stem cells secrete soluble factors influencing myocardial protection, neovascularization, cardiac metabolism, contractility,

cardiac regeneration, and cardiac remodeling (16). The dispensed cells respond to the environmental cues of a distressed heart. Stem cell growth medium also contains these same secreted mediators that attenuate ischemic injury to promote tissue repair (17). Hematopoietic cytokines such as G-CSF, GM-CSF, SCF, Flt-3, and erythropoietin have been recorded as having effects on proliferation, differentiation, maturation, and lineage commitment in bone marrow stem cells which in turn, affect non-hematopoietic tissues of cardiac regeneration, improving tissue function and structure, and encouraging survival (18). The tissue, stem cells, and factors work hand in hand.

Myocardial infarction is one of the many types of injury that incites crosstalk between cardiomyocytes and their environment. Characteristics of MI that stimulate communication are ROS, hypoxia, mechanical stress, inflammation, cell-to-cell interactions, ECM, and other soluble signals. In turn, these stimulants cause the release of some well characterized signaling molecules that modulate cell survival, neovascularization, homing and migration, differentiation, contractility, remodeling, immunomodulation and other cell activities. Survival is dictated by VEGF, IGF, HGF, Tbeta4, PGF, EPO, SDF1, Survivin, GCSF; neovascularization by VEGF, bFGF, PDGF, Ang1, Tbeta4; migration and homing by SDF1, VEGF, HGF, Tbeta4, FGF, GCSF; differentiation by IGF, HGF, VEGF, TGFbeta; contractility by VEGF, Tbeta4, FGF, HGF; remodeling by TGFbeta, IL10, MCP1, Tbeta4, MMP, TIMP, IL1, TNFalpha; and immunomodulation by TSG6, IL10, IL4, PGE2 (6).

3.6 Noteworthy Stem Cell Types

An abundance of stem cell types have been explored for use in cardiac regeneration. But only a few have been highlighted for their potential implications or

actually displayed cardiac improvements that deem them worthy of further exploration. They are classified into three groups: embryonic stem cells, somatic adult stem cells, and an intermediate set of fetal stem cells. They are additionally broken down, again, into embryonic stem cells; as well as, endothelial progenitor, hematopoietic, and mesenchymal stem cells from a number of sources including bone marrow, umbilical & peripheral blood, amniotic membrane or fluid, placental tissue; induced pluripotent stem cells; skeletal muscle progenitor cells; unrestricted somatic cells, adipose stem cells, coronary artery stem cells; and cardiac stem cells. Embryonic stem cells come directly from the developing blastocyst within the embryonic mass itself. Intermediate stem cells are neither embryonic nor adult. They are fetal in origin and come from locations such as placental tissue, the umbilical cord blood, or are sloughed off into the amniotic fluid or within the amniotic membrane. Bone marrow, peripheral blood, skeletal muscle, induced pluripotent, unrestricted somatic, adipose, coronary artery derived and resident cardiac stem cells are all considered somatic stem cells. Induced pluripotent have been reprogrammed to have embryonic stem cell-like properties even though they are actually adult by nature. The primary cells that are sought after from bone marrow, peripheral blood, or fetal tissues are mesenchymal stem cells.

3.6.A Embryonic Stem Cells

Embryonic stem cells are the ideal source for all tissue regeneration. They are easily cultivated in a long term laboratory setting, which is beneficial because large numbers of stem cells must be injected in order to get a regenerative response (10). They also have a pluripotent capability to differentiate into any cell types including cardiomyocytes which can electromechanically integrate with existing tissue (10). There

are two major flaws in using embryonic stem cells; the first being that the use of these cells for tissue regeneration is of ethical concern. The second issue is that it is highly probable that these cells will lead to the formation of teratomas (19). No reliable standard protocol has been established for the use of embryonic stem cells. Current protocols rely on the formation of embryoid bodies that then spontaneously generate cardiomyocytes, which occurs less than 1% of the time. With only a limited number of cardiomyocytes generated, these cells must then be selected out and then enriched so that large numbers can be injected. The process is both monotonous and time consuming (20).

Even with these concerns, research has continued with the purpose of expanding the functionality of embryonic stem cells for use in cardiac regeneration. The coadministration of leukemia inhibitory factor (LIF) and bone morphogenic protein-2 (BMP-2) can induce embryonic stem cells to upregulate cardiomyocyte markers. In one study, these stimulated and partially committed cells were injected into a mouse MI model intramyocardially, and lead to an improvement in stem cell engraftment and they differentiated into cardiomyocytes and endothelial cells, which improved capillary density and LV function (21). In another study, when embryonic stem cells overexpressing microRNA1 were injected into an infarct, they promoted survival by attenuating apoptosis through suppression of the PTEN/Akt pathway. The preservation of myocardium showed that treated mice had an improvement in fractional shortening and ejection fraction (22).

3.6.B Induced Pluripotent Stem Cells

The engineering of induced pluripotent stem cells is a major breakthrough in stem cell research. They can be host or donor adult cells that have been reprogrammed to resemble embryonic stem cells for similar application, but without the same ethical concerns. To confirm that adult stem cells have been induced into an embryonic stem cell like state, cells are characterized by their new embryonic-like morphology, stem cell factors, and staining for embryonic antigens (23). Since they can be autologous, these cells are able to be sourced easily from the host which hinders the opportunity for allogenic immune response (19). Multiple adult cell types can be induced to become embryonic-like. This is achieved by introducing four genes for pluripotency, Oct3/4, Sox2, Klf4, and c-Myc, into somatic cells and confirming the cell's new embryonic-like status by its ability to spontaneously form into a morula (19, 24, 25). Once the process is completed, the induced cells have the same pluripotent capabilities as embryonic stem cells. These cells then go on to differentiate into cardiomyocytes, as well as electromechanically integrate with host tissue just as embryonic stem cells do (19, 23). Unfortunately, genetic modification of these cells also lead to the same 3 germ layer teratoma formations as seen with embryonic stem cell injection and a severe immune response to counter (19, 23). Y Zhang et al. demonstrate how this tumorogenicity is independent of stem cell dosage, transplant duration or cardiomyopathy. He found that tumorogenicity of engrafted cells is induced by the administration of the undifferentiated population within the bulk of induced cells, rather than the differentiated population (26).

Once it was discovered that undifferentiated cells were the primary causes of tumorogenicity, concerns were reduced by separating out undifferentiated cells that are not committed to the cardiac lineage before cell transplant. Mauritz et al. did this by

identifying and highlighting Oct4 expressing cells, which are an indication that a cell still has pluri- potential, and then removing these cells before injection to prevent the known disadvantage (27). In this study, Flk-1 was removed from the equation to inhibit angiogenesis in hopes of preventing tumor development due to lack of a nutritive source. Differentiated Flk-1 $^{-}$ cells still enhanced induced cell engraftment, prevented reverse remodeling and they improved cardiac function. The lack of Flk-1 was beneficial but the degree of improvements in Flk1 $^{-}$ weren't as prolific as Flk1 $^{+}$ cells (27).

Li et al. suggests that the poor engraftment and long term survival of induced pluripotent stem cells could be boosted by transfecting these stem cells with CREG. Since CREG is an inhibitor of apoptosis and inflammation and enhances differentiation, CREG altered cells might be able to maintain a more stable position in the myocardium long enough to engraft and differentiate to aid in tissue regeneration (28).

Overall, studies demonstrated that a number of somatic cell types including fibroblasts and skeletal muscle cells, can be used to create induced cells. These cells then go on to become cardiomyocyte-like and contribute to cardiomyogenesis, while restoring pre-ischemic contractile performance, ventricular wall thickness, and electric stability and achieving in situ regeneration of cardiac, smooth muscle, and endothelial tissue (25).

3.6.C Skeletal Muscle Myoblasts

Skeletal muscle myoblasts were proposed because of their general myogenic potential and physiologic ties to cardiac tissue (23). They produce a number of cardioprotective factors such as HGF, IGF2, NGF, and VEGF. And unlike myocardium, skeletal muscle can undergo considerable regeneration after insult or injury (15). Skeletal muscle myoblasts have been found to contribute to reparative regeneration (29, 30), but

other studies have shown that the use of these cells for cardiac repair after MI can lead to arrhythmogenesis which just adds insult to the already weekend state of a diseased heart (31). Skeletal muscle myoblasts have also been reprogrammed for use as induced pluripotent stem cells partly because they intrinsically possess ¾ of the embryonic transcription factors required for pluripotent stemness. The fourth transcription factor, c-Myc, was found to be mostly negligible with respect to cardiac tissue regeneration, but without it, myoblast transformation to an induced state was less efficient (23).

Even though skeletal muscle myoblast have been found to be arrhythmogenic, M Gmeiner et al. has found some benefits to their use. This group intramyocardially injected autologous skeletal muscle myoblasts that overexpress placental growth factor (PGF) into a MI model, and enhanced transplant survival and myocardial perfusion, reversed remodeling and reduced fibrosis (32).

Wnt has been used to enhance skeletal muscle myoblasts, encouraging them to partially differentiate into cardiomyocytes which can go on to spontaneously contract. And when intramyocardially injected, Wnt11 myoblasts have been found to differentiate 4x more and survived longer in ischemic myocardium than without Wnt. But there was no observable change in infarct size or wall thickness (33).

In another study, delaying skeletal muscle myoblast transplant after scar formation following MI reduced engraftment and further efficacy for the use of regeneration. When transplanted immediately, cell engraftment was significantly improved. These trends were attributed to the fact that hepatocyte growth factor (HGF), is highest right after MI. HGF regulates cell growth, motility, and morphogenesis and as levels decreased over time, so does the efficiency of myoblast engraftment (34).

3.6.D Mononuclear Stem Cells

Mononuclear stem cells used for applications in cardiac regeneration come from placental and amnion tissues, umbilical cord and circulating blood, and bone marrow. These mononuclear stem cells are a CD34+ heterogenous mixture composed primarily of endothelial progenitor cells, hematopoietic stem cells, and mesenchymal stem cells. Endothelial progenitor and hematopoietic stem cells have more limited applications in cardiac regeneration than do mesenchymal stem cells.

3.6.D1 Hematopoietic Stem Cells

Research has placed more emphasis on mesenchymal stem cells over hematopoietic stem cells because they are found in much lower quantities in bone marrow. More importantly, hematopoietic stem cells do not differentiate into cardiomyocytes or vascular endothelium, thus, significantly limiting their applicability but their secreted cytokines have been found to affect many cell process so their use in regeneration should be further explored (18, 35).

3.6.D2 Endothelial Progenitor Cells

Endothelial progenitor stem cells are thought to be difficult to characterize because in some studies, a specific set of markers can not be identified, making them incapable of being acknowledged as a cell responsible for cardiac repair (19, 35). Another group found that they could effectively characterize endothelial progenitor cells and were able to decipher that they are mobilized by erythropoietin and as expected promote the generation of new vasculature, aiding in myocardial perfusion and possibly the reduction of infarct size (36). This creates some controversy as to the accuracy of this study because blood vessels can also be generated from mesenchymal stem cells (37) and

endothelial progenitor cells share a lot of the phenotypic and functional characteristics of hematopoietic stem cells due to their common ancestor, the hemangioblast (19, 38). Naftilan and Schuening believe that endothelial progenitor cells could possibly even be differentiated from hematopoietic stem cells, suggesting yet again that their use in cardiac regeneration has not completely been established (19).

3.6.D3 Mesenchymal Stem Cells

Mesenchymal stem cells are extensively researched because can easily undergo genetic manipulation via vector transduction, and be easily fractionated from the other mononuclear cells in culture (19, 39, 40). Unlike embryonic or induce pluripotent stem cells, mesenchymal stem cells are not tumorogenic, classifying them as a safer option for therapy. But mesenchymal stem cells too are not without their flaws. They are known to have a low efficiency for transformation into cardiomyocytes, indicating that more cells must be retrieved from large sources like bone marrow and then cultured (41). Mesenchymal stem cells not only transdifferentiate into cardiomyocytes and vascular endothelium, but they can also differentiate into the fibroblasts that cause scar formation upon cardiac injury. This contributes to the idea that just as mesenchymal stem cells are beneficial to regeneration; they are also capable of contributing to dysfunction of the myocardium, which furthers injury by encouraging remodeling (13).

3.6.D3-1 Bone Marrow Mesenchymal Stem Cells

Bone marrow stem cells are the most researched and clinically applied stem cell type. The benefit of using bone marrow is that it is the largest source of stem cells in the adult body. The volume needed for regeneration varies depending upon the mode of cell delivery to the host, but generally, large numbers of stem cells must be obtained in order

to produce the desired effects, and those numbers can be more easily obtained from bone marrow extraction. These cells are also easily accessed and processed, although the method of acquisition is fairly invasive (20). Being that bone marrow contains 3 different stem cell types, bone marrow stem cells are capable of differentiating into many cell types including cardiomyocytes and vascular endothelial cells, which aid in maintaining cardiac function and reducing infarct size via neovascularization (20). Thus, their use promotes long term viability of the myocardium in the event of ischemic injury. But bone marrow mesenchymal stem cells typically do not engraft as desired (42, 43) and therefore, their survival upon transplant is still less than ideal. So methods continue to be explored to augment the value of these cells in cardiac repair.

There are many methods by which bone marrow mesenchymal stem cells are introduced into the host system; one of the simplest and least invasive, being intravenously. But by this method, bone marrow mesenchymal stem cells largely become trapped in the pulmonary microcirculation. This brings forth no detrimental effect on the lung, yet it also minimizes benefits attributed to homing to the site of injury, and it has been reported that by this method, there are no paracrine signaling benefits to the repair of cardiac tissue or function (43). Mesenchymal stem cells generally are known to have beneficial qualities that aid in repair by other administration methods, but a less invasive method such as intravenous injection would be ideal. Future prospects would look to bypass entrapment in the lungs and enhance homing to the infarct region, or promoting trophic factor release, upon intravenous injection, for a paracrine response.

The age of the donor of stem cells has been proven to have some bearing on the outcome of transplant. N. Madhur et al. found that using donors of a considerably

younger age for transfer of bone marrow derived mesenchymal stem cells, are more beneficial in older patients than using cells from older donors within their same age range (44). With these findings in mind, the success of autologous transfer repair from older patients would be significant reduced. Thus, using donated cells such as in the above study would be the reasonable option for the typically older patients in need of such repair. But while using younger donor cells increases the likelihood of restoration and recovery, it also heightens the potential for immune response.

Whether or not to sort out cells for various applications was debated because doing so may or may not have any bearing on the effectiveness of transplant since mesenchymal stem cells are so abundant in bone marrow. Krausgrill et al. compared efficacy of using the bulk assembly of bone marrow mononuclear cells to strictly sorting out bone marrow mesenchymal cells and examined how the desired effects of each cell type could be enhanced. They found that intramyocardial injection with variable doses of platelet derived growth factor-BB (PDGF-BB) treated mononuclear cells or mesenchymal stem cells, attenuated the loss of cardiomyocytes as time progressed and in a dose dependent manner (45). PDGF-BB is a growth factor that enhances cell migration and angiogenesis and is an inhibitor of apoptosis in cardiomyocytes (46, 45). Treated mesenchymal stem cells persisted in the heart longer than treated mononuclear cells in reperfused hearts, but survived even longer with permanent ligation (45). The conclusion here is that focusing research on mesenchymal stem cells rather than the collection of mononuclear cells sourced from bone marrow provides more benefit to repair when directly injected intramyocardially. And by treating these cells with high doses of PDGF-

BB, cardiomyocyte survival was promoted, by curbing apoptotic death even in the presence of H_2O_2 reactive oxygen species (46).

Wnt is a signaling molecule that regulates cell proliferation and differentiation into a cardiomyocyte cell line. There are two pathways by which the Wnt family proteins affect cardiomyogenesis: the non-canonical and canonical pathways. The non-canonical pathway of Wnt signaling by Wnt 4, 5, and 11 is cardiogenic, demonstrated by Wnt11 overexpression in bone marrow mesenchymal stem cells (BM-MSC) causing their differentiation into cardiomyocytes by direct injection. Z. He et al. showed that these cells can also be cardioprotective using paracrine signaling. When injected into an infarct border zone or exposed to hypoxic conditions in vitro, these cells have been found to secrete more than 3x the normal levels of Wnt11 and 1.5x the normal levels of TGFβ-2. Apoptosis was attenuated when cardiomyocytes were either co-cultured with BM-MSC (Wnt11) or introduced to the Wnt11 cell medium. In vivo studies showed an improvement in contractility and infarct size, as well as, a reduction in cell death (47). These findings also showed that Wnt induced mesenchymal stem cell differentiation was mediated by upregulating GATA-4 (47).

Although overexpression of Wnt in MSC leads to differentiation into cardiomyocytes and improved cardiac function, inhibition of Wnt has also shown to be cardioprotective as well. By the canonical pathway, Wnt 1, 2a, 3a, and 8a all restrain cardiogenesis by preventing differentiation. Saraswati et al. demonstrated this by implanting polyvinyl alcohol sponges subcutaneously, and administered the Wnt inhibitor pyrvinium daily. The results showed that the production of granulation tissue, a collagen-rich tissue which forms at the site of an injury, was more organized and

vascularized than expected with injury. In the same study, a single intramyocardial injection of pyrvinium into an infarct proved to reverse cardiac remodeling and an increased number of Ki67+ cells around the infarct, indicating an increase in cell proliferation (48).

Another study found that preconditioning of a rat heart with low-level laser irradiation 3 wks after MI and then injecting the host with bone marrow mesenchymal stem cells produced upregulated vascular endothelial growth factor (VEGF), increased the activity of superoxide dismutase (SOD) and inhibited the production of malondialdehyde (MDA) (49). VEGF is a promoter of angiogenesis and increasing SOD will lead to a reduction of reactive oxygen species ROS (50). Reducing ROS is one of the many goals in cardiac regeneration because they reduce adhesion, leading to poor engraftment and fibrotic scarring following tissue damage (50). MDA is an indicator of lipid peroxidation which is a major cause of damage upon reperfusion after ischemia. Therefore, reductions in MDA lead to a decrease in reperfusion injury. Finally, transplanted bone marrow mesenchymal stem cells had an increased survival and capillary density improved after laser preconditioning (49).

Survival of bone marrow mesenchymal stem cells has also been shown to increase by activating the JAK2-STS3 pathway by administering rosuvastin (ROSU) along with stem cell injection after acute MI. This method also results in reduced cardiac dysfunction and differentiation of transplanted cells was observed (21).

To find the best bone marrow derived mesenchymal cell type for treatment after MI, one study used various concentrations of bone marrow mesenchymal stem cells alone or those that were partially differentiated into cardiomyocyte-like cells and

intramyocardially injected them after MI. Shim et al. ultimately found that partially committed cardiomyocyte cells were more beneficial than undifferentiated mesenchymal stem cells in enhancing engraftment, viability, and functional improvement after MI. They also showed that this effect was dose dependent (50, 51).

And finally, chronic, low dose administration G-CSF could mobilize bone marrow mesenchymal stem cells to prevent the deterioration of the heart due to coronary artery disease induced ischemia (52).

Mesenchymal stem cells can also be found in peripheral and umbilical cord blood sources, placental tissue, and amnion. With them all being mesenchymal stem cell sources; they have a lot of the same advantages as using bone marrow mesenchymal stem cells. But they apparently can have varying degrees of efficiency in cardiac regeneration depending upon which of the several possible sources mesenchymal stem cells are obtained from (39).

3.6.D3-2 Placental and Umbilical Cord Stem Cells

Placental tissue and umbilical cord blood mesenchymal stem cells could be considered more attractive than bone marrow stem cells when received from an allogenic source, because newborns have limited immunity, so recipients of donated cells don't always need an exact human leukocyte antigen (HLA) match (40, 57). Using cord blood affords a supply of these stem cells that is more accessible and less immunogenic that of bone marrow, resulting in fewer cases of Graft vs. Host Disease and it is less invasive for the donor, resulting in a more manageable recovery (40). Placental derived stem cells come from 4 tissue regions: amniotic epithelial, amniotic mesenchymal, chorionic mesenchymal, and chorionic trophoblastic tissues, which diverge into amniotic epithelial

cells, amniotic mesenchymal stromal cells, and chorionic mesenchymal stromal cells. These placental progenitor cells differentiate into many cell types but their development toward vascular endothelial cells are what aid in cardiac regeneration by increasing myocardial perfusion after MI (53, 54).

Conditioning of placental stem cells with hyaluronan mixed ester of butyric and retinoic acid (HBR) and injecting them intramyocardially after MI, has been found to result in a 40% smaller infarct scar and reverse remodeling. This method produces a thickening of ventricular walls and improvement in contractile function. Perfusion also increases by the improved capillary density, giving way to an up to 45% similarity between infarct and undamaged myocardium (53).

3.6.D3-3 Peripheral Blood Stem Cells

Peripheral blood stem cells circulate in low numbers, so their use in stem cell therapy initially seems like a fruitless prospect when compared to using bone marrow. But, with the aid of G-CSF, bone marrow stem cells could be dose dependently mobilized into the peripheral blood and then procured for cultivation and transplant (55, 56). In clinical trial, injecting peripheral blood stem cells, erythropoietin, and G-CSF simultaneously, was cardioprotective by a combinatory effect. G-CSF mobilizes bone marrow stem cells into the peripheral blood (55), while erythropoietin plays a role in improving cardiac function by limiting the progression of infarct development and apoptosis on cardiomyocytes, as well as promoting neovascularization by endothelial cells and/or their progenitors (55). Santoso et al. found that this method gives mixed results for improvement, as seen by the persistence of ventricular dilation, yet cells engrafted and were viable months after injection. Ejection fraction and perfusion were

increased significantly and restenosis did not occur in those patients with preexisting vascular disease. This study proved to be a safe and reasonably effective method for the treatment of MI with the use of peripheral blood cells (55).

3.6.D3-4 Amnion Derived Stem Cells

Amniotic fluid or membrane contains numerous progenitor cell types expressing known pluripotent cell markers CD29 and CD90, and transcription factors genes such as Oct4, Sox2, and Nanog (57). They too represent an alternative source for the well noted mesenchymal stem cells with lessened ethical questionability. For donation, these cells are collected by amniocentesis or the membrane is peeled from the placenta after neonate birth and then those mesenchymal cells are isolated from the bulk cells for use. Amnion derived stem cells have been shown to give promising results in studies of cardiac regeneration. Co-culture of amniotic membrane cells with adult cardiomyocytes has shown that these cells will differentiate into cardiomyocytes and functionally integrate with them (58). These results were duplicated in in-vivo studies. On occasion, mesenchymal stem cells from unlikely sources such as menstrual blood, umbilical cord blood, and the placental chorionic plate have been shown to have greater efficiency of differentiation than even the highly regarded bone marrow derived cells (58). But collectively, bone marrow has been found to be the most beneficial source for application for all of the characteristics that comprise cardiac regeneration. The source of these mesenchymal cells seems to play a role in the regenerative effects and the degree of those effects (39).

Amniotic fluid stem cells have also been shown not only to differentiate into cardiomyocytes, but also endothelial cells and when injected intramyocardially, they

produced a favorable effect of reverse remodeling by reduction in LV chamber and improved cardiac function, reduced fibrosis at the infarct, increased capillary density, and improved wall thickness (57, 59). The reduction in infarct size is thought to be due to the secretion of Thymosin β-4 (Tβ-4) which promotes wound healing (17).

3.6.E Unrestricted Somatic Stem Cells

Unrestricted somatic stem cells were recently isolated from umbilical cord blood. They are newly identified $CD45^-$ blood stem cells that have pluripotent capabilities and can differentiate into multipotent mesenchymal stem cells. They have a dose dependent reparative effect on MI after intramyocardial injection. Markers for cardiomyocytes, smooth muscle cells, and endothelial cells can also be found in the infarct region after injection. These cells eventually differentiate into cells that play a part in cardiac tissue repair and improved perfusion; as displayed by improvement in cardiac function and capillary density, and inhibition of fibrosis. (60).

3.6.F Adipose Stem Cells

Adipose-derived stem cells have been found to engraft and differentiate into a smooth muscle cell phenotype through various methods of delivery. The larger size of adipose cells compared to other proposed cell types is of importance in intravascular injection due to the potential for occlusion with large cell numbers. Administering these stem cells after MI does lead to an increase in vascular density, but with little functional improvement (61). Long term benefits of these cells for their use in cardiac disease has not been studied but the promise of neovascularization to increase myocardial perfusion has the potential to improve cardiac function with time by promoting tissue survival.

Adipose derived stem cells have also been found to enhance engraftment when used cell sheets in rats with chronic MI. They reduce mortality and prevent further tissue remodeling, which in turn maintains the integrity and function of tissue (62). They also release VEGF, bFGF, and SDF-1α which are essential for angiogenesis (62, 63). Even with limitations on their differential capacity, adipose stem cells are still therapeutic in cardiac regeneration due to paracrine signaling, which promotes vascular repair and bone marrow stem cell recruitment to the infarct (63). Bai et al. used intramyocardial injection of adipose stem cells in acute MI model to generate local engraftment and increase expression of bFGF and IGF1 (64).

S-nitroso-N-acetyl-D, L-penicillamine (SNAP) treatment of adipose derived stem cells enhances them with nitric oxide to increase the expression of cardiac markers, VEGF, CD34 in bone marrow mesenchymal stem cells. These cells improve cardiac function and vascular density, and reduced oxidative stress (65). In a similar fashion, increasing nitric oxide by creating endothelial nitric oxide synthase (eNOS) expressing adipose tissue derived stem cells allowed them to differentiate into myocytes and vascular endothelial cells. Shi et al. intramyocardially injected eNOS expressing cells to induce eNOS expression in existing vascular endothelium and smooth muscle. These cells mobilized vascular endothelium and smooth muscle cells in the heart, contributing to angiogenesis and infarct size was significantly smaller than without eNOS (66).

Dedifferentiated adipose cells are another adipose cell type that is capable of enhancing cardiac regeneration. When introduced into an infarct, they have been found to engraft and redifferentiate into cells of cardiac and endothelial lineage, and increase vascular density (67).

3.6.G Coronary Artery Derived Stem Cells

The identification of a coronary artery derived stem cell could aid in cardiac regeneration by being yet another in-house source for enhancing perfusion of myocardial tissue and minimizing infarct development. They are multipotent cells that are identified by their cell markers c-kit and the VGF receptor, Flk-1 (68). There is limited information on the use of coronary artery derived stem cells but these multipotent cells should be looked into further for their intrinsic role in potential reparation of atherosclerotic damage leading to ischemia and vascular dysfunction.

3.6.H Cardiac Stem Cells

Cardiac stem cells are inclined to differentiate into cardiomyocytes, vascular smooth muscle and endothelium. They only exist in small pockets within cardiac tissue and it has been reported that these pockets contribute to only about 1% or less cardiomyocyte turnover annually. Coupled to the myocardium's regular rate of apoptosis, this regeneration is not nearly enough to repair tissue damage on its own (3). It has been shown that intramyocardial and intracoronary injection of cultivated cells into an infarct does improve cardiac function (9, 69). Along with their direct differentiation into adult myocytes, these in-house stem cells have also been reported to release paracrine signals to further regeneration of adjacent somatic cells (9). Depending upon how they are obtained, using these cells prompts some of the same donor-host difficulties associated with heart transplant and their use is prolonged because they must be cultured to obtain the required numbers for use. These stem cells also display significant variability in their multipotent and differential capacities, calling for the need to sort cells according to their clonogenic capabilities (9, 69).

Just as any other stem cell types, cardiac stem cell potential can be enhance through the use of signaling molecules. Ellison et al. showed how endogenous porcine cardiac stem cells can be induced to higher differential activity. Coadministration of IGF-1 and HGF through the infarct-related artery in a single dose ranging from 0.5 - 2 μg HGF and 2 - 8 μg IGF-1 significantly increased their cardiomyogenic potential. It also improves cardiomyocyte survival, and reduces fibrosis and hypertrophy. The dual factor and stem cell method reduces infarct size and improved left ventricular function after acute MI as well (70).

Another method that increases stem cell differentiation into cardiomyocytes is by using the physical and molecular communication of gap junctions between adjacent adult cardiomyocytes and cardiac stem cells. These junctions allow miRNA-499 from adult cardiomyocytes to cross gap junctions to stimulate cardiac stem cell differentiation into an adult phenotype. By overexpressing miRNA-499 in the stem cells before intramyocardial injection, they are predisposed to conditions which promote the maturation of all injected cells rather than strictly the stem cells that are in contact with adult cells. While the injection of untreated cardiac stem cells result in improved LV function, miRNA-499 treated stem cells present 50% larger and differentiated at a much higher level than without treatment, contributing to more cardiomyogenesis (71).

Another way cardiomyogenesis can be enhanced is by treating cardiac stem cells with ephrin A1. Adult cardiac cells express ephrin A1 ligand, while cardiac stem cells express ephrin A2. This enhancement results in increased motility in vitro and migration to acutely damaged myocardium in vivo after acute MI. Cardiomyogenesis and vasculogenesis lead to a 2x increase in myocardial regeneration, improved function, and

reduced arrhythmogenesis. Thus ephA1 activated cardiac stem cells have enhanced homing ability (72).

CHAPTER 4

OPTIMIZING DELIVERY

4.1 Cell Transplant

The delivery of stem cells to MI animal models is largely performed in 4 ways: intravenous infusion, myocardial injection, intracoronary infusion, and engineered supporting materials (73). The best mode of delivery seems to vary by study parameters. Intracoronary and intramyocardial injection methods are the most direct ways of reintroducing cardiac stem cells back into damaged myocardium. When comparing the two, intracoronary injection produces equivalent or better results in reversing the remodeling process and curbing cardiac dysfunction (74). This is due to intracoronary injection's ability to distribute cardiac stem cells more evenly within the infarct area (74). Boomsa R.A. et al. used femoral vein injection, where mesenchymal stem cells in mice were able to home in on intact myocardium after induction of myocardial infarction. This method of distal vascular injection proved itself to be a practical application for stem cell delivery and in turn improved systolic function to near normal levels after MI, although ventricular remodeling was unaffected by this procedure (75). But while vascular injection worked for this study, others have observed difficulty injecting mesenchymal stem cells in this manner. Intravenous injection is considered the safest and least complicated of the methods because it is minimally invasive in comparison (73). But complications of this method are that cells can largely become entrapped in the respiratory microcirculation (76). Intramyocardial injection seems the most direct way to apply stems cells to an infarct. It has the benefit of not needing to rely on mobilization or homing to produce the desired effect (73). Induced pluripotent stem cells have been

noted to engraft at the infarct and peri-infarct regions and go onto to survive in the host tissue environment by this method (23). But other studies show that although there may be some improvement in cardiac function, cell viability is low and thus engraftment is hindered (77). Performing either intracoronary or intramyocardial injection is considered a highly invasive method and has the potential for scar tissue formation, arrhythmia due to that scar tissue, regional calcification, and micro-infarction from tissue damage potentially brought on by inflammation (15, 31). In contrast, other studies found no arrhythmia, thrombus formation, distal embolus formation, nor injury to the artery associated with intracoronary injection (55).

Intramuscular injection into skeletal muscle tissue could be a better alternative to the other two methods since studies show that the majority of cells injected by this method do not migrate to the heart, yet they produce regenerative effects, presumably by action of trophic factors (15).

4.2 Safety of Delivery

Many studies have shown improvement in cardiac tissue structure and function within the few weeks that these studies are performed. But very few studies have assessed the long term effects of stem cell use. In one such long term study, a clinical trial assessed the long term safety of autologous peripheral blood hematopoietic stem cell transplant for the treatment of ischemic cardiomyopathy over a 4 to 8 year period. This study found improvement in cardiac function, shown by a lowered physical limitation NYHA classification, within 6 months of transplant, which persisted over the length of the study. There were no long term consequences due to transmyocardial injection of either the stem cells or G-CSF, and by using peripheral blood, stem cell collection was

safe and easy (78). Similar functional NYHA classification results were seen in a 1 year study involving percutaneous injection of autologous skeletal muscle myoblast (79).

4.3 Cell Numbers

Stem cell numbers required to stimulate the regenerative capacity of cardiac cells were found to be significantly different depending on the stem cell type used and the mode of delivery. Cell dosage can range from as few as 10,000 cells all the way up to tens of millions. Yanling et al. explains that umbilical cord blood mesenchymal stem cells need a minimum 4 million cells for intramyocardial injection and a minimum 16 million cells for intracoronary injection (80). Shabbir et al described a method in which bone marrow mesenchymal stem cells were injected directly into the skeletal muscle in a hamster heart failure model presenting with degeneration of myocytes, inflammation, fibrosis, calcified lesions, ventricular dilation, and up to 35% systolic dysfunction. With injection doses of stem cells ranging from 0.25, 1, and 4 million cells, improvement in cardiac function was found in all three groups. But the most advantageous dosage was the highest at 4 million cells, displaying the greatest improvement in cardiac function and reverse remodeling (81). Significant migration of stem cells to the myocardium was not observed and again it was concluded that the improvement seen was due to release of trophic factors from mesenchymal stem cells that in turn promoted myocardial repair.

There seems to be a correlation between increased cell number and functional improvement. But caution should be used against administering excessively large numbers of stem cells to follow this trend because it can lead to arrhythmias and sudden death (80).

Henning et al. did a study, again to directly compare the 3 most common methods of stem cell delivery of cord blood cells in different doses to MI models. When comparing intracoronary, intramyocardial, and intravenous injection of human mononuclear umbilical cord blood stem cells 2 hours after LAD, all 3 methods proved to be advantageous to improvement in the size of the infarct. The most beneficial method and dosage for reducing infarct size was intramyocardial injection of $4*10^{6}$ cells. The dosage by intracoronary injection reduced infarct size by 80%. And finally, intravenous injection of $16*10^{6}$ cell reduced infarct size by around 76% (82). This study shows that varying methods may need to vary cell numbers to be the most effective. With intravenous injection, significantly more stem cells are necessary as they cells must circulate through the systemic vasculature. But even so, intravenous injection, while the simplest and safest of all methods, still proved to be the least effective even with the high number if cells injected.

4.4 Single Dose vs. Multiple Doses

Stem cells and their chemical modulators are regularly introduced into the system by injections. Some therapies have found that a single large dose is capable of producing beneficial results, while others employ repeated injections at regular intervals. Repeated intracoronary injection of peripheral blood stem cells after G-CSF stimulation has been shown to be safe and effective method to improve contractility without consequence (83).

Gavira et al. showed that a single intramyocardial injection of an averaged $329.6*10^{6}$ skeletal muscle myoblasts was not as effective as using 3 doses for the treatment of MI. Improvement was seem in engraftment, cardiac function, vasculogenesis, and in reduced infarct size (29). This method proved to be dose

dependent and even with multiple injections, via a highly invasive method, hearts displayed improved cardiac function beyond the known risks when followed over a 7 month period.

As well as aiding in the inhibition apoptosis and infarct development, erythropoietin, this time in synthetic form, when injected in a single large dose, has been shown to be a safe method of therapy that mobilized endothelial progenitor cells that could possibly lead to angiogenesis (84). Prunier et al. concluded that chronic administration of darbepoietin was maximally beneficial at mobilizing endothelial progenitor cells when administration by multiple lower doses. After issuing various doses of darbepoietin intraperitoneally, once a week for 8 weeks, they found a reduction in the MI area/area at risk (AAR) for myocardial necrosis ratio, as well as the LV chamber size; with an increased hematocrit. The results were all maximized at the highest dose of 1.5 micrograms/kg of darbepoietin. Other results that only manifested at this high concentration showed an increase in endothelial progenitor cells, capillary density and improvement of cardiac function (85).

Each of the above mentioned cell types proved to be advantageous to cardiac regeneration, while showing varying degrees of potential depending upon the cell number and type, method of administration, drawbacks and ethical consequences, and most importantly, the state of the subject at the time of therapeutic intervention. As of yet, there is no consensus amongst the scientific community as to what the best dosage is for what type of stem cell, and by what method of delivery. The conclusion so far seems to be on an individual study basis, depending upon the host environment. Most studies

suggest that higher cell numbers, with higher cardioprotective factor concentrations, and direct intramyocardial injected produce the most favorable of results.

CHAPTER 5

GELS, SCAFFOLDS, AND SHEETS TO AID CELL DELIVERY

One of the key problems with reparative stem cell therapy is that even when some of the damaged tissue is regenerated, cardiac function is not significantly improved to due poor engraftment and thus long term viability. Another category of stem cell delivery utilizes injectable gels or mechanical support systems. Gels, scaffolds, and sheets resolve the issues of engraftment and survival by affording mechanical support for the maintenance of tissue structure and function; thus preventing further tissue remodeling and improving function. The names of these systems are sometimes used interchangeably depending upon their key structural components. But their use sets the stage for potential long term regeneration and repair (20, 42, 86).

5.1 Gels

Hydrogels are composed from native and foreign materials such as extracellular matrix elements like collagen, fibrin, and hyaluronic acid, and foreign sourced alginate or modified peptides (20, 86). They are natural or biosynthetic injectable polymers that functionally resemble the extracellular matrix. Since direct injection of stem cells alone or with suspension doesn't result in maximal cell engraftment and viability, hydrogels are injected to introduce some level of physical stabilization of tissues; and deliver drugs, stem cells, or paracrine mediators directly to the damaged tissue (86, 20). Hydrogels support the myocardium by preventing physical deformations of the heart like remodeling, and reduce wall stress (20). Wen-Yu Lee et al has used a methyl cellulose hydrogel system to cultivate amniotic fluid mesenchymal stem cell and create cell bodies of a similar size and shape before intramyocardial injection (87). Paired with stem cells

and/or influent signals, hydrogels aim to create optimal conditions for cardiogenesis.

Another approach for the delivery of stem cells and cardioprotective proteins is by intrapericardial injection of gelfoams. Gelfoams are considered to be "non-toxic, inexpensive, non-immunogenic, and biodegradable" (88). They follow some of the same principles associated with the use of hydrogels. Studies are currently being done to test their safety and effectiveness in regeneration.

5.2 Scaffolds

Scaffolds patches are made from substances such as pullulan polysaccharide and dextran and can also be seeded with stem cells to enhance engraftment and provide support. They form a structural support that can also be used to deliver cells and their mediators.

A study by C. L. Visage et al. showed that a scaffold seeded with bone marrow mesenchymal stem cells can produce a 4.6% increase in engraftment over myocardial injection alone. They also showed improved migration and modest VEGF upregulation (42). A study by Xiong et al., attached fibrin patches seeded with vascular smooth muscle and endothelial cells to the region of infarct and improved vascular infiltration, leading to an improvement in contractile function (89). In the same manner, Yeh at al. used a combination of hydrogel and scaffold material to improve cellular adhesion. They cultured human amniotic fluid stem cells onto a methylcellulose hydrogel coated with collagen, and applied it to the myocardium. This resulted in an improvement in the survival of transplanted cells, leading again to enhanced vascular infiltration, reverse remodeling, and an overall improvement in cardiac function (90).

Extracellular matrix component, fibrin glue, and adipose derived stem cell can be used to enhance engraftment, and improve cardiac function beyond that of injecting adipose stem cells alone. Further, this method can improve vascular density, showing that the addition of a scaffold material such as fibrin prevents stem cell washout and promotes attachment to encourage survival and regenerative effectiveness (90=90).

5.3 Cell Sheets

Cell sheets are comprised of manufactured layers of stem cells and are also used in conjunction with scaffold systems. They have already been proven to repair some of the damage associated with ischemic injury, alleviating the adjacent cell death observed with intramyocardial injection (91). But by increasing the number of cell sheets applied to an infarct, the effects are multiplied with each additional layer (92).

CHAPTER 6

THE FUTURE OF MYOCARDIAL REGENERATION

Every tissue of the body is sculpted into its properly formed organ by the extracellular matrix scaffold. Cardiac matrix is composed from fibrillar collagen, basement membrane, proteoglycans, and associated proteins which provide a framework for structural integrity of cardiomyocytes and supports the vasculature, while giving cues for cellular activities (93, 94). The next step in stem cell research regarding regeneration could be cultivating stem cells in the natural state of a supportive matrix to construct partial or whole organs. Renal, gastrointestinal, brain matter, vascular, and other tissue matrix scaffolds for clinical purposes are currently in limited use, although cardiac applications are not yet available. These natural architectural scaffolds have the beginning tools for organ assemblage because of their viscoelasticity, supportive nature, plus cell engraftment to and cell survival upon its structural proteins (95). But what allows the seeded stem cells to proliferate, differentiate, and promote vascularization are growth factors like VEGF, bFGF, and TGF beta.

H C Ott et al. attempted to mimic the process of cardiac development while negating the use of stem cells. They proceeded to decellularize a whole heart to the point of nearly undetectable levels of DNA evidence, leaving just the EMC scaffold perfectly shaped as the intact heart. What was left consisted of epicardial fibers, epicardial basement membrane, and vascular basement membrane. The construct was complete and unchanged in its morphology, but left variations in its resistive and elastic properties. The matrix was then seeded and cultivated with neonatal cardiomyocytes, fibrocytes, endothelial cells and smooth muscle cells. The result resembled that of a true developing

heart with vessel formation, electromechanically active cardiomyocytes, and the beginnings of contractile function with phenylephrine or electrically paced stimulation (96). Left to mature under the appropriate conditions, models such as this could potentially create a viable heart to be tested for use in vivo. Innovations such as this bring cardiac regenerative research one step closer toward being able to secure more options for patients by limiting the need for extensive wait, donor matching, and immunologic risk.

A similar study attempted this method in hopes of repairing just the infarct region rather than regrowing the heart as whole. This method differs from others patch methods in that it uses decellularization of the tissue to leave the entire construct, rather than applying individual matrix components. By using a fraction of decellularized heart matrix and applying to it a fibrin hydrogel seeded with mesenchymal stem cells, a patch is made that can then be attached to an infarct region. The end result of this method is similar, with an increase in cellular migration, neovascularization, upregulation of paracrine signals, and an improvement in cardiac function with reverse remodeling (97).

Genetic engineering of future generations is a far fetch idea that is considered by some to be highly unethical. Engineering the perfect child, also known as creating a "designer baby", is currently being implemented in the form of gender selection to allow couples the ability to plan a family in hopes of preventing genetic disorders specific to a certain gender or simply to balance their family according to social or cultural preferences. But to take this a step further, what if it were possible to create a permanent societal resolution to cardiac disease? This is being explored in the form of genetically "engineered" murine progeny that resist cardiac disease.

Yamada et al. did a study in which they injected ROSA26 mouse embryonic stem cells into a C57BL/6J mouse blastocyst to produce a chimera that resisted infarct development even after permanent coronary occlusion, and any deterioration in function observed was resolved within 1 month of obstruction. Normal physiologic and anatomic compensatory changes associated with ischemic injury did not present due to an increase in stem cell repair as demonstrated by a significant increase in adipose tissue and it's derived stem cells, and upregulation of Ki67, c-Kit, and stem cell antigen-1 (Sta-1) (98).

CHAPTER 7

DISCUSSION

The best way to maximize cardiac regeneration using stem cells is to promote engraftment, long term survival of transplants, and functional integration with native myocardium. The heart itself seems to only produce low level intrinsic regeneration making an external method of repair a necessity. There are a wide array of potential sources for stem cells in the use of cardiac repair and regeneration. Donor cells can come from the host that is intended to use them, which seems to be the ideal scenario. Or donor cells can come from an alternative source that is a genetic match or of non-familial origin but of reasonable compatibility. Either way some level of immunologic response may be elicited depending on the method in which the cells are used. The goal is to limit chronic inflammation which can lead to extensive oxidative stress and continued tissue damage. Limiting tissue damage by employing immune suppressants leaves the body susceptible to infection or the development of cancer.

With an embryonic stem cell's ability to proliferate in an almost unlimited capacity and with their ability to differentiate into every cell type, they seem to be the most reasonable source for not only cardiac repair but repair of any damaged tissue or organ of the body. But the lack of a consistent, standard protocol for their cultivation, ethical issues surrounding the use of an embryo, and tumorogenicity leave many limitations on what health benefits can be achieved for its use by the general public. Induced pluripotent stem cells, although a legitimate alternative to embryonic stem cells, should also be excluded from viable options because of the issue of tumorogenicity. A potential way to limit the issue of tumorogenicity would be to partially differentiate these

cells to cardiac cell precursors before administration. But more studies should be done to determine if the reduced tumorogenicity is enough to outweigh the risks in clinical trials. The next most reasonable cell type for use would have to be mesenchymal stem cells because of their abundant sources. These stem cells don't have the proliferative potential of embryonic or induced pluripotent stem cells have, but it may not be needed because of paracrine signaling.

The use of stem cells for myocardial repair is most effective soon after injury. Bone marrow is a significant and genetically matched source of stem cells that can be tapped for large quantities of cells for sooner use than others. But it still requires an invasive method of cell retrieval. Fetal tissue derived stem cells are less immunogenic and could theoretically be cultivated before hand and maintained at storage facility for use by the general population. Most studies show that benefits of mononuclear cells are largely accredited to mesenchymal stem cells, so as to maximize regeneration these cells should be sorted out, and cultivated to obtain the necessary numbers for use. Inducing them to partially differentiate into cardiomyocyte-like cells before administration would also be advantageous.

Currently, intravascular injection is an unreliable method because of potential pulmonary entrapment or vascular occlusion with adipose stem cells. Direct intramyocardial injection or intracoronary injection produces the most consistent outcome. And while intramyocardial produces the best results overall, intracoronary injection disperses cells better allowing more even distribution and is somewhat less invasive than its counterpart. Dose dependent improvement is usually seen by any

method of administration. The exact dose varies on an individual basis and multiple low doses are ideal.

As of today there is no definitive method that investigators can agree upon to maximize cardiac regeneration and repair. The conclusion seems so far to be on an individual study basis depending upon the host environment. Cumulatively, studies have shown mixed results. Protocols for the use of any of these stem cells have not been refined enough to give consistent preferential results without complication or consequence.

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