



Novel Methods for Inducing Cardiac Tissue Regeneration Following Ischemic Injury

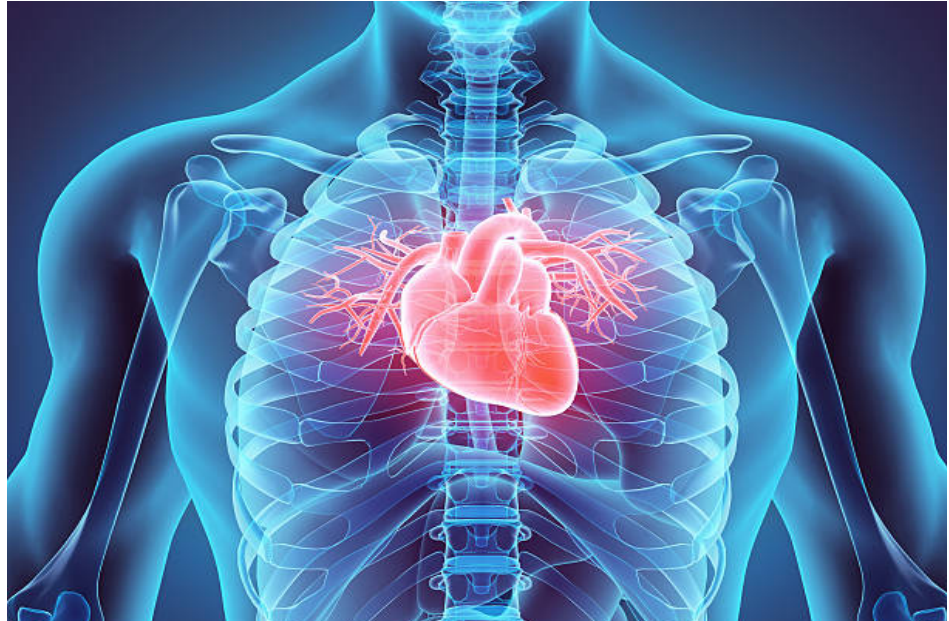
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Abstract

Cardiac disease continues to be among the most prevalent causes of death worldwide. Presently, surgeries such as angioplasties, stents, and bypasses pose many risks. To improve outcomes in treating ischemia, researchers have been pursuing minimally invasive, biocompatible treatments such as gene therapies. Gene therapies are treatments that enhance or suppress target genes to alleviate illness. There are various applications for gene therapies, including, but not limited to, the treatment of cancers, diabetes, and heart disease. Gene and stem cell therapies can regenerate cardiac tissue that is damaged due to ischemia. Furthermore, gene therapies intended to evade the immune system may decrease infection risks due to the new tissue being better accepted by the body as it is created from patients' own cells. While DNA treatments show poor results in treating cardiac illness, stem cells, such as mesenchymal stem cells and induced pluripotent cells, can differentiate into cardiomyocytes, and mRNA can be modified to express angiogenesis growth factors around the affected tissue. Although further research is needed to adapt these techniques for safe clinical use, they show potential for inducing cardiac tissue regeneration in ischemic injury. This paper conducts a review of the emerging techniques and evaluates gene therapy as a potential treatment for ischemic injury.

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Introduction

Much research has been done on various approaches to gene therapy¹ to treat an ischemic injury, yet few techniques have gone into clinical trials, and these seldom show success. This paper aims to conduct a review of a few major techniques and evaluate the potential of gene therapy as a treatment of ischemic injury.

Cardiac-related illnesses and deaths have consistently been one of the top causes of death in the U.S. and around the world², and has also been on the rise since the beginning of the COVID-19 pandemic, disproportionately affecting minority populations³. Thus the importance of researching more effective methods of treating and preventing ischemic injury is imperative. The mortality of ischemic heart injury differs by demographic, including one's age, country, and region, with lower-income countries and older people suffering higher mortality rates, with little to no improvement⁴. Since the rise of gene therapy as a potential treatment method for various illnesses in the 1990s, the field has been consistently researched⁵. The precision of the ability to modify specific genes offers great potential. However, due to obstacles such as high cost, ethical concerns, and difficulty translating into clinical applications⁵, there is still much to uncover.

Cardiac ischemic injury refers to cell damage due to diminished flow of blood, containing nutrients and oxygen, through the vessel. If ischemic injury is not caught quickly and treated in a timely manner, cardiomyocyte death can occur, with permanent effects⁶. Ironically, surgically restoring blood flow to these regions may actually cause cell death and organ failure⁷. Gene therapy thus presents as a favorable option to instead promote angiogenesis through growth factors such as VEGF-A or stem cell therapies to develop new paths of blood flow rather than risking further ischaemia-reperfusion injury (IRI).

We have decided to look not only into gene cascades that are often targeted by gene therapies, but various approaches to gene modification, including stem cell research using mesenchymal stem cells (MSC), modified mRNA (modRNA), and induced pluripotent stem cells (iPSC).

Mesenchymal Stem Cells to Treat Ischemic Injury

Myocardial infarction (MI), more commonly known as heart attack, and related injuries are a leading cause of death globally, and despite the applications of extensive novel cardiac surgery and grafting to lengthen and ameliorate life, long-term cardiac damage may persist⁸. The usage and application of mesenchymal stromal cells (MSCs) in heart tissue has become an increasingly popular candidate in the treatment of myocardial infarctions, as they exhibit factors that increase cardiomyocyte survivability⁸. There is an abundance of research aimed toward understanding the implementation of MSCs in the prognosis and improved function of heart tissue, due to their desirable trait of being able to differentiate into disparate cellular lineages⁹. It is by far one of the most promising treatments in regenerative medicine. However, it is difficult to gather results from existing clinical trials due to suboptimal reproducibility and efficacy outcomes,¹⁰ and the exact mechanism of the cell treatment is still largely unknown⁹. Previous and ongoing research has shown that the usage of mesenchymal stromal cells has proved to have significant promise in treating ischemic myopathies¹¹. Many clinical trials have shown that the application of mesenchymal stromal cells to infarcted cardiac tissue has been shown to reduce fibrosis¹², improve regional contractility¹¹, and reduce arrhythmias amongst other outcomes¹³. In clinical trials with mice, it has been demonstrated that direct application of MSCs could aid in angiogenesis and myogenesis of ischemic myocardium in murine post-acute myocardial infarction¹⁴. This is a significant effect of MSCs, since heart failure can be caused by death of large numbers of cardiomyocytes, so the induction of angiogenesis and myogenesis can prove to be a desirable outcome¹⁴. Clinical studies have also shown that application of autologous bone marrow MSCs (BMSCs) correlated to reduced scar sizes, reduced infarct sizes, improved left ventricular ejection fraction (LVEF), and a significant increase of viable tissue in patients who underwent coronary artery bypass graft surgery (CABG) compared to controls¹². One particular study aimed to investigate the direct effect of bone marrow-derived MSCs on cardiac function immediately after MI by injecting the cell sample into infarcted

intramyocardial tissues¹⁵. Although the experiment showed that the donor cells minimally remained in the myocardium after implantation, and the exact mechanism is still unknown, there was a significant reduction in infarct sizes, improved cardiac function, expression of pro-angiogenic factors, all occurring in a paracrine manner¹⁵.

Specific types of MSCs like umbilical MSCs, which promote vascular regeneration and cardiomyocyte protection, are accessible and easily expandable in experiments¹³. However, with the varying stem cell types, there are also many different constraints that have hindered more innovative research into the application of MSCs. The application of bone-marrow derived MSCs in particular include invasive or unethical harvesting procedures, and decreased proliferation among varying donors¹³. But in a more promising facet, umbilical-derived MSCs demonstrate more safe and feasible qualities, including being more easily attainable, posing less ethical concerns, and undergoing less cellular aging¹³.

Safety of MSC application is an important facet of the treatment to ensure since there are several potential health concerns surrounding the treatment, including tumor formation, organ toxicity, and ectopic tissue formation in the vasculature¹⁶. Safety profiles of intravenous MSC application in acute MI patients are successfully present, showing significant results such as reduced tachycardic episodes, improved forced expiratory volumes, and no concerns for ectopic tissue creation in the long term¹⁶. However, one study aimed to evaluate the safety of human bone marrow-derived mesenchymal cell application in patients suffering from acute MI by using a multicenter trial¹⁶. In the study, the Data Safety and Monitoring Board approved the application of MSC dosage in each experimental cohort, and primary safety assessments were carried out to monitor any adverse reactions to the treatment¹⁶. The results of this study showed that there was no apparent evidence of increased toxicity with the application of the MSCs and there the administration was well tolerated in the cohorts at all doses¹⁶. There was also evidence that the arrhythmia ratio was significantly lower in the MSC administered patients vs. the placebo group, and also showed improved function in cardiac performance and pulmonary function in comparison with the placebo group¹⁶. There was no report of long-term ectopic tissue

formation either, which showed the significant therapeutic benefits of the administration of the hMSCs overall¹⁶. Another study aimed to evaluate the safety of intravenous administration of umbilical cord-derived MSCs (UC-MSCs) as well. The experimental group involved patients with heart failure and reduced ejection fraction, and were presently treated with application of intravenous infusion of allogeneic umbilical-cord derived MSCs¹³. The results of this study showed that the UC-MSC treated patients expressed no adverse reactions to the infusion treatment and showed significant improvement in left ventricular ejection fraction over multiple time checkpoints post-administration¹³. The results of these studies show that the incorporation of MSCs as a treatment option is safe and feasible with different types of MSCs, and there were no noteworthy deleterious reactions to this treatment. The results described above also showed improvement and potential in recovery and offers a potential usage in future medicine and therapy.

Many questions are still unanswered regarding the mechanism of action of MSC treatment⁹. Studies have shown that injection of MSCs in affected heart tissues reduced infarct size and induced angiogenesis and myogenesis¹⁴. One main mechanism of action is injection of bone marrow-derived-MSCs directly into infarcted heart tissue to promote cardiac function via expansion of the cells¹¹. This is usually caused by MSCs' ability to expand and differentiate into cardiomyocyte-like cells¹³. One experiment conducted coronary artery ligations in mice and then implemented MSCs to note any changes in grafting in the ischemic myocardium and proliferation into cardiomyocytes¹⁴. This particular experiment showed results illustrating improved cardiac function after MSC implementation through the enhancement of myogenesis and angiogenesis. Other experiments involving injection of MSCs or BMCs into infarcted tissue have shown some results correlating with improved regional contractibility, and no signs of ventricular arrhythmias, worsening cardiac function, pulmonary embolisms, or cardiac tamponade¹¹. There are still suboptimal results correlating MSC transplantation and therapeutic improvement of cardiac function, since the mechanisms of action are quite complex and not fully understood⁹. But transplantation of MSCs in many

recent reviews and studies have proven to be safe¹³ and the selective study results shown could be beneficial.

MSCs have been used as a therapeutic application for many degenerative diseases, mainly due to their various differentiation potential, paracrine factors¹⁷ and secretory products like angiogenic factors, mitogenic factors, antiapoptotic factors, and growth factors¹⁸. In particular MSC's have been frequently involved in experimental models of cardiovascular diseases¹³ and express attributes that make them desirable for use in cardiac function modulation after myocardial infarction⁹ including secretions that could prevent cardiac inflammation and aid in cardiac injury repair¹⁹. Although the underlying detailed mechanism of MSC transplantation is still mostly unknown and incomplete¹⁹, and clinical trial results are still suboptimal⁹, transplantation treatments show promising effects¹⁵ regarding improvement in myocardial function in experimental models¹³. Additionally, the trajectory of evidence shows that transplanted cells have a beneficial effect on overall cardiac function after myocardial infarction²⁰ and ischemic heart disease¹⁸ due to varying different results. Transplanted MSCs from red bone marrow in adult rat specimens showed differentiation into cardiomyocytes by the presence of cardiac proteins like desmin, and also showed to form connections with native cardiomyocytes in the rat sample¹⁸. This differentiation into both vascular endothelial cells and smooth muscle cells promoted cardiac function via myogenic and angiogenic effects¹⁴. Engrafted cells harvested from a healthy human sample also showed similar results, with the stem cells strongly morphologically resembling host cardiomyocytes and also expressing desmin, myosin heavy chain, actinin, and cardiac troponin amongst others, each of which are native cardiac tissue proteins²¹.

Echocardiographic assessment of transplanted bone marrow MSCs into post-MI tissues also showed that the cells relieved the restrictive effects of left ventricular function and geometry caused by MI²⁰. Additionally, intravenous application of umbilical cord MSCs showed improved left ventricular ejection fraction and left ventricular end diastolic volume in control groups for patients with systolic heart failure or reduced ejection

fraction¹³. Novel experiments have also shown that direct injection of MSC exosomes also reduced infarct sizes, improved cardiac function, and offered protection of cardiomyocytes from hypoxia⁸.

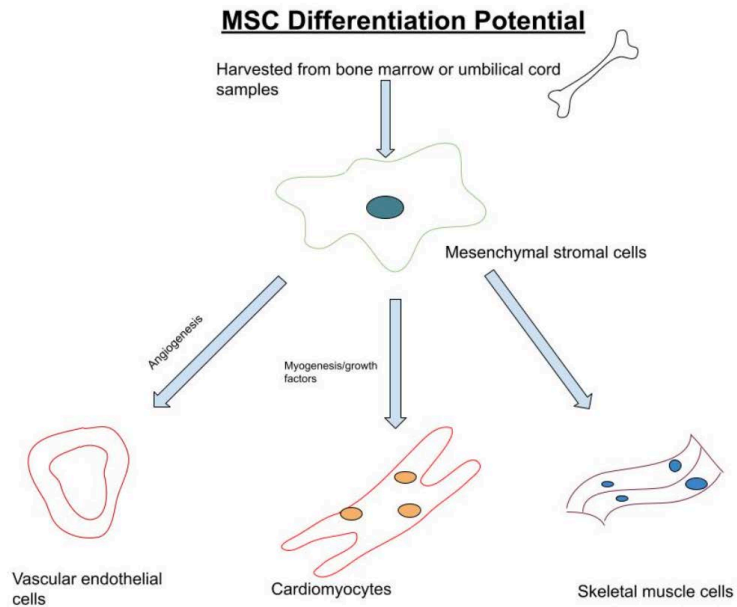


Fig 1. MSC Differentiation Potential. Based on the diagram of differentiation potential for MSC terminal phenotypes, we can see how bone-marrow MSCs or umbilical cord derived MSCs have the potential to differentiate to different cell types including cardiomyocytes, vascular endothelial cells, and skeletal muscle cells amongst other cells. This differentiation into cardiomyocytes explains why the expansion of the cells in cardiac tissue settings is very desirable. The differentiation into cardiomyocytes has been measured by proteins like desmin and troponin, and is also noted to form connections with native cardiomyocytes within the tissue.

Although much of the application of MSCs is still subject to more extensive clinical testing and development¹⁵, there is a huge promise of the already performed experiments discussed above to show safety of the clinical application and the therapeutic benefits of administration. The studies above have depicted the many potential and confirmed benefits of administration of either bone-marrow derived MSCs or umbilical cord-derived MSCs, including reduced infarct sizes, overall improved

cardiac function and LVEF, cardiomyocyte production and protection, angiogenesis, and myogenesis. Safety of administration of BMSCs and UC-MSCs were both measured with significant results in cohort groups, showing that overall, administration is safe and feasible with generally little to no adverse effects¹³. Although these studies presented significant outcomes, it has been suggested that more extensive and larger clinical trials are needed to fully understand the clinical benefits of administration of certain MSCs¹³. Due to the many measured beneficial implications of MSCs, including overall cardiac function, we can conclude that MSC transplantation can be a new therapeutic strategy for treating patients suffering from myocardial infarction¹⁴.

Modified RNA as a Method of Gene Therapy

DNA modification comes with many risks, including immunological response, and lack of specificity regarding its locus of action. Consequently, RNA therapy is becoming a further researched field, including clinical applications for modRNA and miRNA²³.

Modified mRNA (modRNA) is becoming a prevalent research topic in many different fields, such as diabetes, ischemic injury, and the mechanism of a COVID-19 vaccine²⁴. In this method of gene therapy, mRNA is injected into the heart to enhance target protein translation, which in this case is cardiomyocytes and angiogenesis to repair the heart after massive cell death²⁵. However, mRNA is short lived and cannot provide lasting effects for cardiomyocyte regeneration. They often trigger an immune response, and are also broken down by RNAases. Thus the degree of protein expression is dependent on mRNA stability²⁶ and they must be engineered to be more efficient in their expression in the cell, or be longer lasting. So far, this is achieved through four different methods: altering nucleotides, mRNA capping, untranslated region manipulation, and the length of the poly A chain²⁷.

Understanding the alterations of mRNA requires an understanding of mRNA synthesis. Often, a DNA plasmid is transcribed *in vitro* and the resulting mRNA is used for therapy. In this process, substitutions can be made in the nucleotide pairing process. Kierzek et al. showed in 2014 the

specific effects of substituting pseudouridine for uridine with the different nucleic acids, in which Ψ-A pairing demonstrated greatest enhanced stability due to hydrogen bonding, at about 0.5kcal/mol. Due to the increased stability and because pseudouridines are found naturally in the body, these modRNA are able to avoid detection by the immune system, specifically by TLR3, TLR7, and TLR8²⁸. Furthermore, it was found that VEGF mRNA whose uridines were completely replaced by pseudouridine, did not trigger the immune system and remained locally in the target tissue of cynomolgus monkeys or rats while promoting angiogenesis. In this experiment, the mRNA was injected intradermally or intravenously²⁹.

mRNA caps are methylated guanosine groups attached to the 5' end by a triphosphate group present in all eukaryotic mRNA³⁰. These give identity to the mRNA strand as well as signal for degradation when removed³¹. Thus the synthetic mRNA must also be capped adequately to increase translation of protein.

The untranslated region of RNA, called the UTR, appears on both the 5' and 3' ends of the mRNA strand, and plays a role in mRNA translation efficiency and localization³². Thus UTR of modRNA must also be manipulated adequately to optimize translation rate and half-life. For example, the addition of AU-rich elements (ARE) weakened protein expression, and the more stable UTR was able to increase transcription stability³³. Synthesizing UTR specific to the needs of the modRNA is imperative to its effective protein synthesis, and also allows for more precise control of the therapy.

mRNA is modified by extending the polyA tail to increase its translation. This modRNA is administered through intramyocardial injection to affect a large area of cardiomyocyte and non-cardiomyocyte cells²³. modRNA is able to stay longer in the cell than RNA by changing its structure with pseudouridine and evading RNAase³⁴, but is still shorter-lived than DNA, thus reducing the risk of mutation and multiple transcriptions over time²⁵. Rather, it is described as a “pulse”³⁵, producing a rapid episode of protein synthesis that can be better controlled than DNA therapy methods.

Currently, due to the temporary nature of modRNA, treatments may need to be administered multiple times to demonstrate effectiveness, which not only causes trauma on the cardiac tissue but also will become expensive²⁵. modRNA is already required in large doses³⁶, thus production or administrative costs must be reduced to make this treatment clinically feasible. Furthermore, injecting these genes into the intracardiac tissue is invasive and can be risky for patients who have already suffered injury to the heart. Further research must be done on less invasive gene and drug delivery methods that are still able to target specific cells and promote angiogenesis²⁵. One such method is cell-penetrating peptides that have been demonstrated to carry DNA, RNA, or proteins into the cell via endocytosis³⁷.

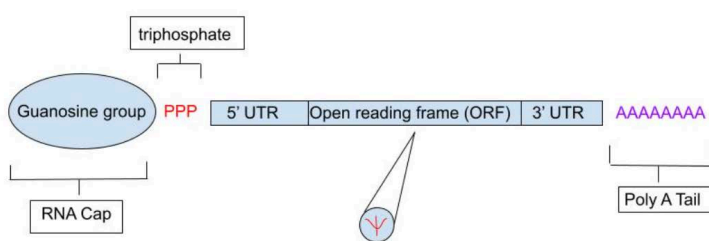


Figure 2. The Structure of a Modified mRNA. The poly A tail increases translation and allows it to remain longer within a cell.

Despite these intricacies of modifying mRNA, it has been demonstrated that modRNAs can deliver relatively quick local responses both *in vitro* and *in vivo*. Mouse hearts were able to retain the effects of a cardiac injection of modRNA for 24 hours after surgery³⁸. Furthermore, modRNA coding for VEGF-A, a growth factor that promotes revascularization was demonstrated to successfully induce angiogenesis following myocardial infarction³⁹. The modRNA injected into the site of injury promoted heart progenitor cells to differentiate into vascular cells rather than muscle cells. In this experiment, the mice injected with VEGF-A DNA and VEGF-A RNA both formed vessels, but the mice who received the RNA injection presented with vasculature that was less permeable and more similar in shape to control hearts. The vessels of VEGF-A DNA injected mice showed edema, and had higher mortality rates. These differences, likely due to the

differences in acting speed of RNA and DNA, display the potential of modRNA in the field of cardiology. Moreover, because VEGF-A, a commonly used and researched growth factor, can cause increased vessel permeability at long exposure times, the shorter “pulses” of RNA may be more effective in treating ischemic heart injury.

VEGF-A's results are much more promising with mRNA than DNA largely due to its rapid and local characteristics²⁹. This effect can be further enhanced by moving away from lipid carriers, sometimes called nanoparticles, that are often used to encapsulate proteins or genetic material. Removing this layer prevents the mRNA from entering circulation and traveling away from the target tissue, and the naked delivery of the modRNA increased protein was found to increase translation 53-226 fold³⁸. However, it must be noted that while for ischemic, local injury, removing the nanoparticles improves translation, for drugs that are aimed for general circulation, such as mRNA vaccines, the lipid nanoparticles are an essential part of drug delivery⁴⁰.

Carlsson et al. (2018)²⁹ demonstrated the effects of VEGF mRNA after further replacing the pseudouridine with 1-methylpseudouridine and injecting naked mRNA in a citrate-saline buffer in swine. The treatment resulted in efficient and long-lasting protein expression, local effects, and improvement in cardiac function at 1 week and 2 months after injection²⁹. Improvement here was defined as increase in muscle contractility and decrease in fibrosis, and were dose-dependent. These findings further support and build upon Zangi et al.'s findings in 2013³⁹ but with greater specificity in the injections' effects, as measured through bioluminescence, and with higher efficiency. With cardiac angiogenesis in both species of mice and swine, modRNA appears to be a strong candidate for carrying out cardiac regeneration after an ischemic injury. However, these studies will have to be carried out over longer periods to ensure whether the modRNA must be reinjected, and for any side effects.

While clinical trials of VEGF modRNA for cardiac regeneration are scarce, its safety and effectiveness were observed in treating patients with type II diabetes mellitus. The study was conducted in a randomized, double-blind,

placebo-controlled nature, splitting participants into three groups to receive either placebo or VEGF-A mRNA at various doses⁴¹. The results reflect that of Carlsson et al.²⁹ that demonstrated dose-dependent results. VEGF-A mRNA was able to successfully promote basal skin blood flow at injection sites of the forearm, and the results were dose-dependent. At 7-14 days after injection, vasodilation and neovascularization were induced, demonstrating the potential for VEGF-modified mRNA applications in humans⁴¹. Observed adverse events were of onsite reactions, which occurred in all participants, but with only mild severity. The effects of cardiac modRNA injection in humans as of yet unknown, though the gene therapy method is considered safe to be researched further in the context of cardiac ischemic injury. However, this clinical trial only had male participants, thus women must also be tested to ensure comparable results, and whether there are sex differences in dosing.

After the safety and biocompatibility of VEGF modified mRNA were confirmed, it was applied in the cardiac field for patients undergoing Coronary artery bypass grafting (CABG) by AstraZeneca in a study called EPICCURE. This is a very common surgical procedure that aims to revascularize the heart by attaching grafts bypassing the clogged artery. However, there are still many risks, especially for those who have a history of renal disease or stroke. Adverse events include infection, atrial fibrillation, myocardial dysfunction, which are at higher rates for those who are undergoing dialysis or have other underlying cardiac conditions such as peripheral artery disease or pericarditis⁴². AZD861, a VEGF mRNA drug in citrate-buffered saline, will be administered immediately after CABG before reperfusion at various doses to determine whether angiogenesis will be promoted, and if it will improve outcomes of the surgery³⁶. While no paper published in a scientific journal has reported the results, AstraZeneca published data on the company's website and presented it at the American Heart Association's Scientific Sessions in 2021. The treated groups demonstrated the biocompatibility and safety of AZD8601, and in agreement with the phase 1 clinical trial⁴¹, there were no infections or severe adverse events. Furthermore, because the injections in this trial were epicardial injections, they better demonstrate that VEGF mRNA is safe for

human use. But more research must be done to increase efficiency of the treatment for significant benefits in cardiac angiogenesis⁴³.

Induced Cardiomyocytes for Cardiac Regeneration

Induced cardiomyocytes (iCMs) are cardiomyocyte-like cells that are derived from the reprogramming of other cells, the most common being induced pluripotent stem cells (iPSCs)⁴⁴. Culturing iPSCs with specific media allows them to be differentiated into the target cell, in this case, iCMs. iPSCs provide a solution to the long-standing difficulty of the lack of regenerative abilities of cardiomyocytes; their ability to proliferate allows treatment to surpass the stagnant number of normal cardiomyocytes in the body, a revolutionary possibility for regenerative medicine⁴⁴. iPSCs also provide a safer alternative to potentially toxic drug treatments and can serve as excellent disease models^{44, 45}.

Though iPSCs remain the most common cell that are used in iCM derivation, cardiomyocyte-like cells have also been derived from the reprogramming of fibroblasts. Derived from direct lineage reprogramming via upregulation of genetic reprogramming factors, several studies have reported the generation of functional iCMs from cardiac fibroblasts in both *in vitro* and *in vivo* mouse models^{46, 47}. Qian et al. (2012)⁴⁶ examined the conversion of murine cardiac fibroblasts to cardiomyocyte-like cells via the expression of three cardiac-specific transcription factors (Gata4, Mef2c, and Tbx5) *in vitro* and observed a large percentage of cells that were partially reprogrammed. They applied their findings to an *in vivo* model and observed a similar rate of conversion with an even higher level of success in fully reprogramming the fibroblasts into cardiomyocyte-like cells⁴⁶. Wada et al. (2013)⁴⁷ also previously reported conversion using the aforementioned transcription factors to be successful for mouse fibroblasts *in vitro*⁴⁷. Following their *in vitro* study, they observed the generation of new cardiomyocytes from endogenous cardiac fibroblasts with the aid of the same transcription factors, and various improved aspects of overall cardiac function.

Following these discoveries, the possibility of directly reprogramming human cardiac fibroblasts (HCFs) was also studied. While success in the

conversion of HCFs to iCMs was observed, the process was observed to be more complex than the mouse model, requiring additional reprogramming factors^{47, 48}. The three transcription factors used in the *in vitro* and *in vivo* rodent models were not sufficient; the addition of Mesp1 and Myocd allowed for the generation of human iCMs *in vitro*⁴⁷. However, the iCMs derived from this process “did not beat spontaneously”⁴⁷. To differentiate into functional, beating iCMs, “human iCMs [required] coculture with murine cardiomyocytes,” unlike their mouse counterparts⁴⁷. Finally, the level of authenticity of the replicated iCMs in comparison to natural cardiomyocytes was unable to be conclusively defined⁴⁷.

Somatic fibroblasts were also found to give rise to iCMs through upregulation of cardiac gene expression, which can occur both *in vitro* and *in vivo*⁴⁹. Further studies involving human cardiomyocytes *in vitro* and *in vivo* are needed to understand the specific mechanisms required to produce an effective treatment based on this technology.

While iCMs are a very promising field of cardiac gene therapy, they are, of course, not without their limitations. Horikoshi et al. (2019)⁴⁴ observed that iCMs derived from iPSCs appear to lack the mature characteristics of normal adult cardiomyocytes. Based on the natural process of cardiomyocyte maturation, they cultured iPSC-derived iCMs with fatty acids, the basis for the fatty acid β -oxidation process that supplies mature cardiomyocytes with energy⁴⁴. Their study revealed that a fatty acid culture can promote the maturation process of iPSC-derived iCMs in order to allow them to function as fully-matured adult iCMs⁴⁴.

Another limitation observed in the field is the applicability of iCM-based treatment to chronic heart failure, a condition for which regenerative therapy is in high demand⁴⁸. While iPSCs have provided excellent platforms for disease modeling thus far, *in vivo* cardiac reprogramming studies have been focused on models in the acute stage of myocardial infarction (MI), so its potential to alleviate chronic heart failure has yet to be determined⁴⁸. However, given the marked improvement of overall cardiac function observed in numerous studies, there seems to be a definite potential for chronic heart failure reversal through treatment with iCMs.

iCMs themselves have not been observed to proliferate independently, a characteristic which would greatly improve the efficacy of regenerative therapy⁴⁸. Instead of relying on the proliferation capabilities of the source cells, such as iPSCs, development of iCMs that are able to endogenously proliferate would remove the necessity for repeated administration of iCMs. Certain critical factors have been identified in addition to the possibility of using a third iCM derivation source in the form of induced cardiac-progenitor cells (iCPCs) that would be converted into iCMs; however, further study is needed to solidify the technology and translate it to clinical application⁴⁸.

Derivation of iCMs

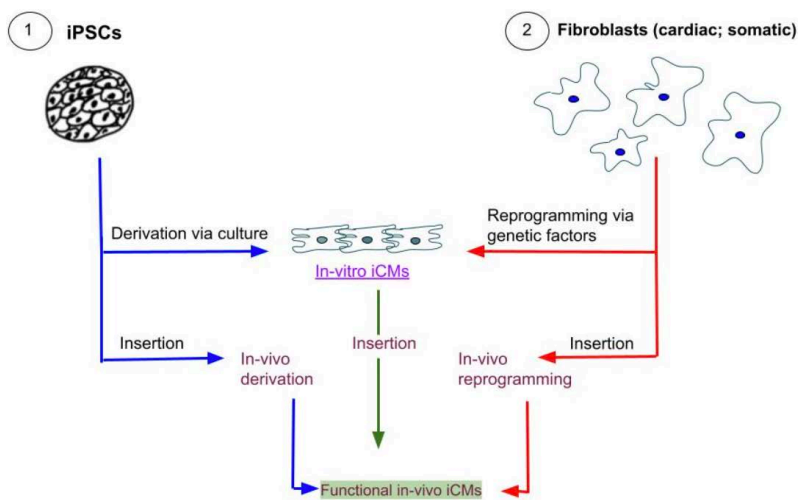


Figure 3. Common Derivation Methods of iCMs. Diagram outlining two major sources, iPSCs and fibroblasts, used for the derivation of iCMs. Details the processes by which both sources can be used to create iCMs in vitro that can be inserted for treatment. Also touches on how both of the sources can be directly inserted and used to develop functional iCMs in vivo as part of treatment regimens.

Gene Cascades to Target for Cardiac Regeneration

The manipulation of certain cardiac genes has shown significant progress in improving ischemic heart disease. Changes in the expression of certain genes influence cardiac cell proliferation and differentiation, demonstrating gene therapies' potential efficacy in treating cardiac disease. This section examines multiple therapies that concentrate on changing the expression of target genes. Two main techniques were investigated, one focusing on the transplantation of BMSCs with specific gene deletion and the other concentrating on the protein-protein interactions that influence a particular gene involved in cardiac cell signaling.

Recent studies have shown that hepcidin, an iron-regulating protein primarily in the liver, is also present in low amounts in the heart, to regulate iron homeostasis and ferritin content in cardiomyocytes⁵³. Injuries in the heart due to ischemic disease result in the destruction of cardiomyocytes, which releases a high concentration of iron into the extracellular space⁵⁴. The increased iron content outside cardiomyocytes is associated with myocardial fibrosis and with the formation of reactive oxygen species (ROS)⁵³. These conditions cause an intracellular iron deficiency that leads to the overexpression of hepcidin proteins⁵⁵. In efforts to understand how hepcidin affects ischemic heart disease and to study the possibilities of related therapies, the gene coding for hepcidin, *Hamp*, was manipulated in deletion experiments⁵⁶.

Despite the lack of knowledge on how cardiac hepcidin functions and affects heart injuries, researchers first carried out direct gene deletions of *Hamp* in mice to prevent the production of hepcidin and study its effect⁵⁶. Nonetheless, this gene therapy demonstrated unpromising results, causing scientists to quickly transition into the transplantation of either mice with bone-marrow-derived cells that are *Hamp* deficient or with myeloid cells with *Hamp* deletions. The investigation was carried out on mice that were deliberately inflicted with myocardial infarction. Those with *Hamp* expression in macrophages were shown to produce high levels of hepcidin as a result of increased interleukin (IL-6) production, which is responsible for *Hamp*'s transcription⁵⁷.

This new study on the effect of hepcidin on heart injuries revealed that this protein is related to the secretion of IL-4 and IL-13 which are each important for inducing cardiac repair⁵⁶. *Hamp* deficient mice with myocardial infarction displayed a significant reduction in infarct size and tissue fibrosis as well as increased cardiomyocyte renewal. This result was related to the lack of hepcidin that led to improved receptor functions, such as C–C motif receptor 2 (CCR2)+, in macrophages. This improvement enhanced the secretion of IL-4 and IL-13 from the macrophage increasing cardiac healing as well as cardiomyocyte regeneration after a heart injury⁵⁶.

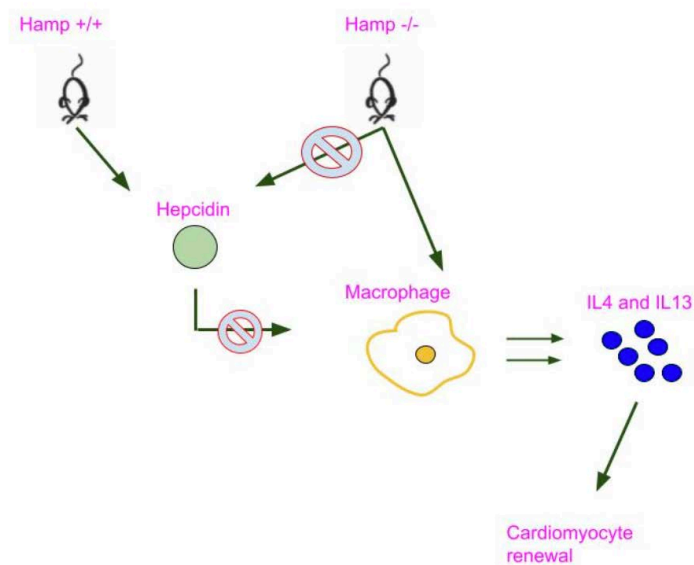


Figure 4. The Effect of the Hamp Gene on Hepcidin Production.

Hamp-deficient (-/-) mice reduced hepcidin production and induced the secretion of IL4 and IL13 responsible for cardiomyocyte renewal, while mice with Hamp gene (+/+) produced high levels of hepcidin reducing the release of IL4 and IL13.

While this research demonstrates a new finding regarding the relationship between hepcidin and IL-4/IL-13, the mechanisms of how hepcidin influences the function of certain receptors and interleukins are still not widely understood. There is limited research on the potential of using this gene therapy due to the researchers' effort to first better understand how cardiac hepcidin works in the heart and related injuries⁵⁴. Further

investigation on hepcidin is required to understand how the production of cardiac hepcidin affects certain tissues in heart cells before diving into particular therapies⁵³. The study of this protein has great potential of being utilized for improved cardiac function in ischemic heart diseases, particularly the *Hamp*-deficient BMSCs that induced cardiac repair⁵⁶.

Although not yet implemented into paradigms of gene therapy, the ongoing in-depth study of protein-protein interactions has led to the possibility of controlling the expression of specific genes, essential for signaling pathways involving cell proliferation. The detailed understanding of Wnt protein/ β -catenin pathways, responsible for cell proliferation and growth, has led to significant improvements in addressing diseases in organs such as the lungs⁵⁸. In attempts to regulate this pathway for cardiac cell regeneration, recent studies have revealed protein interactions with Wnt units that could inhibit injury-induced cardiomyocyte proliferation⁵⁹.

In a study on heart regeneration and injury repair, scientists discovered novel small molecules called cardiogens (CDMG 1 and 2) that have the ability to inhibit Wnt expression and cause β -catenin reduction downstream⁵⁹. The investigation was carried out using embryonic zebrafish with surgically-induced heart injuries. CDMGs inhibited Wnt by specifically targeting β -catenin and Tcf/Lef-mediated transcription that is needed to initiate the expression of particular genes specified by Wnt⁶⁰. The reduction in β -catenin accelerates the proliferation of damaged heart cells, leading to improved heart function. CDMG1, particularly, was effective in healing heart injuries by increased formation of cardiomyocytes and reduction in fibrotic scar tissue⁵⁹. Nonetheless, the mechanism of CDMG on particular Wnt pathways is still not well-understood. There are a wide variety of Wnt proteins and related signaling pathways that affect distinct cellular lineages and their functions. Therefore, further studies regarding what specific Wnt proteins CDMG structures inhibit and what specific genes are affected by these molecules could potentially result in new gene therapies, and ultimately improvements in medicine.

In addition to the newly discovered molecules, secreted frizzled-related proteins (Sfrp) inhibit canonical Wnt signaling pathways responsible for cell

proliferation, while activating non-canonical Wnt pathways that lead to differentiation⁶¹. This research was accomplished by culturing cardiac progenitor cells (CPC) from mice. The binding of Sfrp2 to Wnt6 reduced CPC proliferation while fostering differentiation. Inhibition of Wnt by Sfrp causes an activation of non-canonical Wnt/Planar Cell Polarity (PCP) pathway through c-Jun N-terminal kinase (JNK)⁵⁸. This activation encourages the expression of cardiac transcription factors and CPC differentiation suggesting that the regulation of Wnt proteins is a possible avenue through which clinicians can address ischemic heart injuries⁶¹. A significant limitation of this finding is the lack of comprehension of the particular pathways that the inhibition of Wnt6 affects, and what factors or genes are involved.

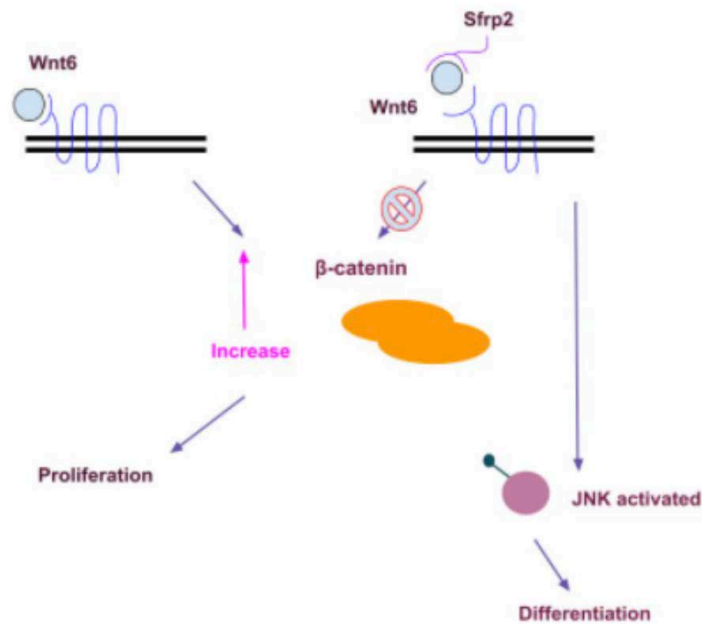


Figure 5. The Role of the Wnt Pathway in β -catenin Production. Sfrp2 binds to Wnt6 proteins to activate non-canonical Wnt pathway that results in CPC differentiation and leads to a reduction of β -catenin responsible for proliferation.

The finding of pathways related to cardiac cell induction and regeneration has demonstrated a possibility of manipulating these processes to address

heart injury. In the case of hepcidin proteins, *Hamp* deficiency has shown to be associated with iron-homeostasis and the secretion IL-4/IL-13 that help with cardiomyocyte renewal. CDMG molecules and Sfrp proteins were proven to be related to the inhibition of Wnt pathways that reduce cell proliferation but result in differentiation. However, for both cases, the pathways resulting in improved heart function are still not well-understood and just provide a glance of future research directions. There is, thus, significant room for elaboration; further studies may implicate more specific cell or gene therapies for treating ischemic heart diseases.

Conclusion

Mesenchymal stromal cells have been a popular and desirable candidate in treating function post-myocardial infarction, as they have shown promising effects in clinical trials. The administration of MSCs have shown significant therapeutic benefits contributing to patients suffering from myocardial infarction and other pulmonary and cardiac myopathies. Although more extensive and larger-scale clinical trials need to be implemented to exactly understand the mechanism and exact benefits of MSC administration, the existing studies have shown results indicating high efficacy. Studies have shown that through both UC-MSCs, patients treated with infusion of MSCs have shown outcomes which include increased myogenesis and angiogenesis, improved left ventricular ejection reaction, reduced infarct sizes in heart tissue, cardiomyocyte proliferation, and generally improved cardiac function. The difference in MSC types and their correlating benefits and consequences are also discussed, as well as the safety and feasibility of their administration onto experimental cohorts. These studies also showed little to no adverse effects to any of the treatments, and little significant worsening of conditions has been recorded. The exact mechanism of their beneficial effect is still unknown and needs to be more extensively studied, but these studies have shown promising therapeutic benefits.

Modified mRNA also demonstrates great potential to be used towards cardiac regeneration, especially expressing VEGF, as it has expressed no adverse reactions when used clinically, and successfully promoted angiogenesis. Developments have been made in the purification and evading the immune system through RNA caps, pseudo nucleotides, and the poly A

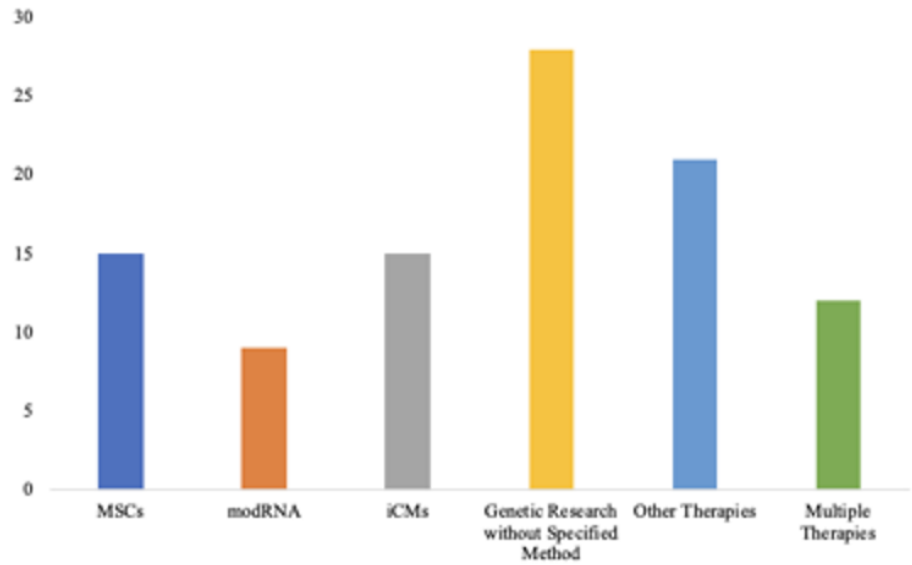
tail. However, more research must be done specifically on the ischemic injury and cardiac regeneration model for it to be used specifically in response to a myocardial infarction. Before this, an understanding of the effect of VEGF on humans, and increasing efficiency of the treatment is imperative, as there is very little data on optimal dosage concentration or repetitions that carry out the desired effects. Lastly, for modified mRNA to be a viable clinical option, the shelf life and sustainable storage of the purified mRNA in buffer must be researched to ensure minimal degradation over time. Thus, modifying mRNA shows great potential as a gene therapy mechanism, though more research must be done to adapt it to be used for human cardiac regeneration.

iCMs derived from various sources have been employed in numerous treatment approaches to cardiac illness, and continue to provide many research opportunities in the field of regenerative medicine. As observed by the evolution of iCM research over time, studies have made their way from animal models to the use of human cell sources for experimentation, suggesting that application of iCM-based treatment in clinical trials is not far off. iCMs provide a treatment option that has the potential to draw on a abundantly-sourced treatment platform, avoid the negative side-effects of toxic pharmacological treatments, and significantly expand the scope of regenerative medicine. Continued research into iCMs seems to be a very promising path to treating ischemic injury induced by cardiac dysfunction.

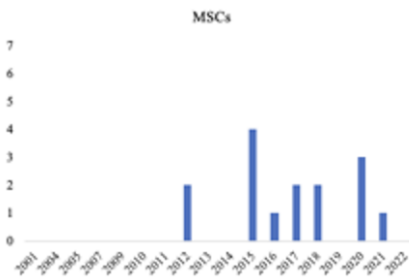
Particular genes and protein interactions related to the heart have resulted in a greater insight into cardiac regeneration to address heart injuries. Cardiac hepcidin protein reduction through *Hamp* gene deficiency was shown to be associated with iron-homeostasis and the secretion of IL4/IL13 from macrophages inducing cardiomyocyte regeneration. The inhibition of pathways related to Wnt proteins using CDMG and Sfrp molecules also revealed that cardiac progenitor cell differentiation is a possible metric with which to gauge improvement in heart injuries. However, these studies are recent research interests that have only been implemented in animal models. The particular genes, reactions, and factors involved in these pathways are still not well-understood. While there is a long way to go for a greater comprehension of how they work in the human heart, the identification of

these pathways and possible molecules provides an insight into future research directions that could potentially result in novel gene therapies.

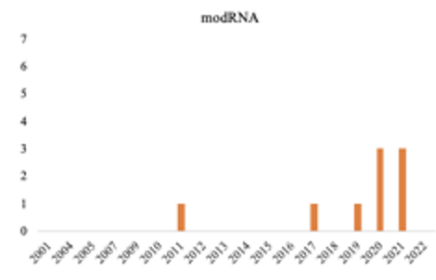
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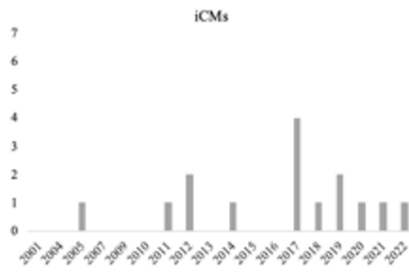
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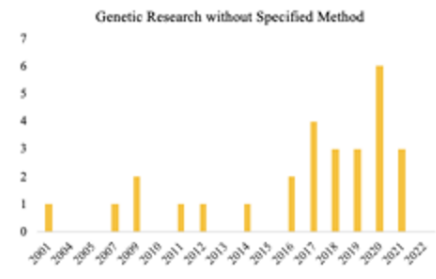
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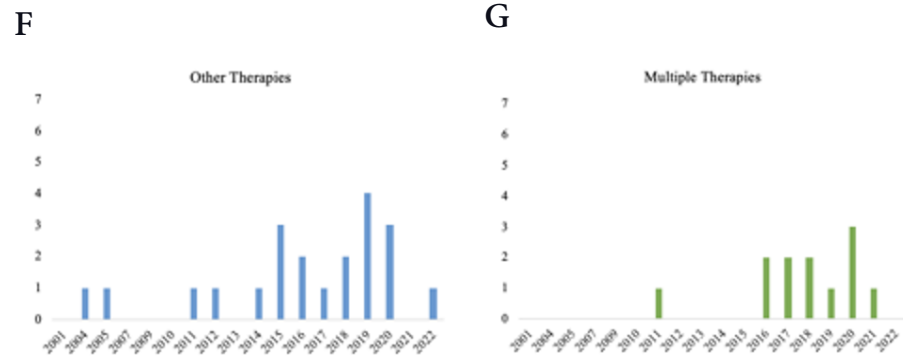


Figure 6. Overview of Amount of Research Articles Pertaining to Each Therapeutic Approach. To obtain a broad overview of how cardiac ischemic injury has been approached in research, a PubMed search of the key terms “gene therapy + cardiac regeneration + ischemic injury” was conducted on May 6, 2022. The first one hundred relevant articles were cataloged and analyzed for the specific research topic that was covered. Panel (A) shows the number of research articles that appeared by the topic overall, and panels (B)-(F) show the number of articles per topic by year of publication. “Genetic Research without Specified Method” refers to work done with specific genes that did not mention the MSCs, modRNA, or iCMs. “Other Therapies” refers to therapies used to treat cardiac ischemic injury that did not involve genetics, such as cell therapy or drug therapy. “Multiple Therapies” refers to articles that address more than one form of therapy.

Research in the field of gene therapy to alleviate cardiac ischemic injury has been studied for decades and will continue to be studied in coming years. As the current trends in the research were reviewed, all therapies covered in this article still seem to be relevant to the field. Overall, the most popularly researched therapy seems to be the direct interaction with specific genes, above any other methods mentioned. However, as mentioned, the others do not fall far behind. As the field continues to expand, it is hopeful that research will make the transition from models to clinical trials and continue to add to each of these therapies for treatment.

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