A Novel Needleless Liquid Jet injection Methodology for Improving Direct Cardiac Gene Delivery: An Optimization of Parameters, Aav Mediated Therapy and investigation of Host Responses in Ischemic Heart Failure

Anthony Samuel Fargnoli
University of Pennsylvania, fargnoli2@gmail.com

Follow this and additional works at: http://repository.upenn.edu/edissertations

Part of the Biomedical Commons, Nanoscience and Nanotechnology Commons, and the Surgery Commons

Recommended Citation
http://repository.upenn.edu/edissertations/1273

This paper is posted at ScholarlyCommons. http://repository.upenn.edu/edissertations/1273
For more information, please contact libraryrepository@pobox.upenn.edu.
A Novel Needleless Liquid Jet injection Methodology for Improving Direct Cardiac Gene Delivery: An Optimization of Parameters, Aav Mediated Therapy and investigation of Host Responses in Ischemic Heart Failure

Abstract
Heart disease remains the leading cause of mortality and morbidity worldwide, with 22 million new patients diagnosed annually. Essentially, all present therapies have significant cost burden to the healthcare system, yet fail to increase survival rates. One key employed strategy is the genetic reprogramming of cells to increase contractility via gene therapy, which has advanced to Phase IIb Clinical Trials for advanced heart failure patients. It has been argued that the most significant barrier preventing FDA approval are resolving problems with safe, efficient myocardial delivery, whereby direct injection in the infarct and remote tissue areas is not clinically feasible. Here, we aim to: (1) Improve direct cardiac gene delivery through the development of a novel liquid jet device approach (2) Compare the new method against traditional IM injection with two different vector constructions and evaluate outcome (3) Evaluate the host response resulting from both modes of direct cardiac injection, then advance a drug/gene combination with controlled release nanoparticle formulations.

Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Bioengineering

First Advisor
Charles R. Bridges

Second Advisor
Jason Burdick

Keywords
device, gene therapy, heart failure, liquid jet injection

Subject Categories
Biomedical | Nanoscience and Nanotechnology | Surgery

This dissertation is available at ScholarlyCommons: http://repository.upenn.edu/edissertations/1273
A NOVEL NEEDLELESS LIQUID JET INJECTION METHODOLOGY FOR IMPROVING DIRECT CARDIAC GENE DELIVERY: AN OPTIMIZATION OF PARAMETERS, AAV MEDIATED THERAPY AND INVESTIGATION OF HOST RESPONSES IN ISCHEMIC HEART FAILURE

Anthony Samuel Fargnoli

A DISSERTATION

in

Bioengineering

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2014

Supervisor of Dissertation

__________________________
Dr. Charles R. Bridges MD ScD
Chairman and Professor of Thoracic and Cardiac Surgery

Graduate Group Chairperson

__________________________
Dr. Jason Burdick PhD, Professor of Engineering

Dissertation Committee

Dr. Kenneth B. Margulies MD (Chair), Professor of Medicine
Dr. David B. Weiner PhD, Professor of Pathology and Laboratory Medicine
Dr. Shu Yang PhD, Professor of Engineering
Dr. Scott L. Diamond PhD, Professor of Engineering
Dr. Robert J. Levy MD, Professor of Medicine
A NOVEL NEEDLELESS LIQUID JET INJECTION DEVICE METHODOLOGY FOR IMPROVING CARDIAC GENE THERAPY: AN INVESTIGATION OF DELIVERY PARAMETERS AND THEIR ASSOCIATED HOST RESPONSES IN ISCHEMIC HEART FAILURE

2014

Anthony Samuel Fargnoli

This work is licensed under the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 License. To view a copy of this license, visit http://creativecommons.org/licenses/by-
Acknowledgment:

There are many individuals to recognize and thank for supporting me through this extraordinary endeavor as it was a lifelong dream to obtain a doctorate from Penn. One of the greatest lessons I’ve learned from a wise professor at Penn during my master’s studies was to “savor the journey” and reflect on to all of the experiences and individuals that made it worthwhile along the way. First and foremost my family was my bedrock from an early age in providing love, guidance, and a stable foundation all of which I owe most of my success. Thank you Grandmother and Mother for instilling the educational values at a very early age through teaching me patience, dedication and going beyond what was expected. To the countless teachers and mentors I’ve been blessed with in my formative years in private and public schools, I thank you for your time and inspiring me to grow in new ways I never thought possible. When I came of age, thank you Father for supporting me through my college career at the University of Miami.

I owe most of my early career development and will be forever grateful to the University of Pennsylvania staff and faculty in providing world class training and research opportunities. I still can recollect the long commuter train rides and classes while still running to cover operating rooms in the Department of Surgery. It was there I learned the values of persistence, dedication and excellence in the pursuit of higher knowledge. On from there, I sincerely thank Dr. Charles Bridges for providing me with my best one to date, the opportunity to both take an active leadership role in a cutting edge laboratory while pursing these academic studies in conjunction. Our research lab has bonded on level beyond most, having grown achieving many milestones and the greatest was moving our laboratory to Charlotte NC. It is here I must recognize my best professional confidant, Dr. Michael Katz who has provided unwavering support and
assistance with my studies. The frequent travel for both business and PhD endeavors was truly an enriching experience and I'll always cherish the first class upgrades. Coming back to campus and having stimulating discussions with my committee members Dr. Margulies, Dr. Weiner, Dr. Yang and Dr. Levy was truly inspirational and helped me grow as an investigator to focus and develop research objectives. I also thank Dr. Diamond for providing precise, yet thorough questions on my research approaches which forced me to challenge my own research. These frequent interactions and travel would not have been possible without the support of my family, I have to definitely thank my Stepfather for all of the 4AM rides to catch the 1st outbound Charlotte flight and the 7:30PM “on the dot” US Airways arrivals in Terminal A. Two key figures in my life are my brothers and I could not be where I am and who I am today without them. Thank you Michael for showing how to live life in its fullest, seize opportunities, and keeping me thoroughly entertained with your worldly travels and racing hobbies. Thank you Anthony Joseph for teaching me patience, how to have fun, and for letting me be there for you through your life journey from helping build award winning science projects to soccer teams. Saving the best for last, meeting the love of your life in a Princeton cafe and getting married through the journey was certainly the best of all – thank you Alyson Mary for making it all worthwhile. You are my true inspiration and the best wife any man could want.
ABSTRACT

A NOVEL NEEDLELESS LIQUID JET INJECTION DEVICE METHODOLOGY FOR IMPROVING CARDIAC GENE THERAPY: AN INVESTIGATION OF DELIVERY PARAMETERS AND THEIR ASSOCIATED HOST RESPONSES IN ISCHEMIC HEART FAILURE

Anthony S. Fargnoli
Charles R. Bridges

Heart disease remains the leading cause of mortality and morbidity worldwide, with 22 million new patients diagnosed annually. Essentially, all present therapies have significant cost burden to the healthcare system, yet fail to increase survival rates. The heart transplant, which is the gold standard for patients suffering from disease, will never meet clinical demand due to the shortage of viable donors. Therefore, the investigation of novel treatments is a significant unmet need in the health system with vast opportunity. Since the decoding of the human genome, the rapid expansion of the biotechnology field has ushered in new tools for therapeutic development. These new therapies can be tailored to act at the cellular level to address the root cause of disease progression or in other cases replace/repair those affected by disease.

One key employed strategy is the genetic reprogramming of cells to increase contractility via gene therapy, which has advanced to Phase IIb Clinical Trials for advanced heart failure patients. It has been argued that the most significant barrier preventing FDA approval are resolving problems with safe, efficient myocardial delivery, whereby direct injection in the infarct and remote tissue areas is not clinically feasible. Briefly, widely accepted intracoronary (IC) infusion via catheterization is limited by poor transfer across intact endothelial barriers, while direct needle injection is essentially a dead end approach due to injury, inefficient transfer, and high incidence of the immune response. Here, we aim to: (1) Improve direct cardiac gene delivery through the
development of a novel liquid jet device approach (2) Compare the new method against traditional IM injection with two different vector constructions and evaluate outcome (3) Evaluate the host response resulting from both modes of direct cardiac injection, then advance a drug/gene combination with controlled release nanoparticle formulations.

We plan to execute as follows. Briefly, animals will be assigned to one of the 6 resultant groups: 2 delivery methods x 2 AAV vector constructs = 4 treatment groups +2 Saline delivery control groups. Then each animal will be assessed for quantitative gene transfer, cardiac function and various immunohistochemistry assays to assess inflammation and adaptive immune responses. Separately, a forward thinking drug/gene therapy concept will be advanced with controlled release polymer formulations.

The work presented in this dissertation represents the advancement in the field of cardiac gene delivery techniques toward improving outcomes in future pre-clinical and clinical studies. The evaluation of synergistic relationships between route selection, vector configuration and their respective impact on the host response will improve delivery science.
## TABLE OF CONTENTS

**ACKNOWLEDGMENT:** ....................................................................................... III

**ABSTRACT** .......................................................................................................... V

**LIST OF TABLES:** .............................................................................................. XI

**LIST OF FIGURES:** ............................................................................................ XII

**CHAPTER 1** ......................................................................................................... 1

*Introduction: Heart Disease and Emerging Gene Therapy Applications* ...................... 1

1.1 Heart Disease Burden and Treatment Options .......................................................... 1

1.2 Cardiac Gene Therapy Applications Strategies ......................................................... 3
   1.2.1 Overexpression of target gene .................................................................................. 4
   1.2.2 Specific gene blockade ............................................................................................ 4

1.3 Heart Failure Gene Targets ....................................................................................... 7
   1.3.1 The calcium cycling proteins .................................................................................. 7
   1.3.2 The β-adrenergic signaling cascade ......................................................................... 10
   1.3.3 Other Ischemic Heart disease targets ...................................................................... 11

1.4 Therapeutic Vectors ............................................................................................... 13
   1.4.1 Non-viral vectors .................................................................................................. 13
   1.4.2 Viral vectors ........................................................................................................ 14

1.5 Conclusions ........................................................................................................ 19

**References** ........................................................................................................ 20

**CHAPTER 2** ....................................................................................................... 27

2.1 Introduction ....................................................................................................... 27

2.2 Direct Cardiac Gene Delivery ................................................................................ 29
   2.2.1 Intrapericardial Injection ....................................................................................... 30
   2.2.2 Endocardial Injection .......................................................................................... 31
   2.2.3 Intramyocardial Injection .................................................................................... 32

2.3. Transvascular Gene Delivery .............................................................................. 35
   2.3.1 Selective Coronary Catheterization with Antegrade Intracoronary Delivery ............. 35
   2.3.2 Nonselective (indirect) Intracoronary Delivery ..................................................... 37
   2.3.3 Selective Coronary Sinus or Coronary Venous Catheterization with Retrograde Delivery .................................................. 39
   2.3.4 Ex vivo Gene Delivery ......................................................................................... 40
CHAPTER 6 ............................................................................................................................ 96

6.1 Introduction ........................................................................................................................... 96

6.2 The S100A1 Target: Molecular Actions & Previous Studies ............................................. 96
    6.2.1 The multi-potent effects of S100A1.................................................................................. 96
    6.2.2 Previous Results from Animal Studies ............................................................................. 99

6.3 Methods & Materials ............................................................................................................. 99
    6.3.1 Echocardiography Procedures ....................................................................................... 100
    6.3.2 Surgical Procedures & AAV Delivery .............................................................................. 101
    6.3.3 Quantitative Real Time Poly Chain Reaction (QPCR) of ssAAV9.S100A1 Genome ...... 101
    6.3.4 Histology Protocols: Quantitative S100A1 Protein Analysis in Myocardial Cross Sections via Immunohistochemistry with Confocal Microscopy & Masson’s Trichrome Staining for Infarction Area .......................................................................................................................... 102
    6.3.5 Host Response to S100A1 Gene Therapy .................................................................... 105

6.4 Results ................................................................................................................................. 106
    6.4.1 Operative & Delivery Results ......................................................................................... 106
    6.4.2 Echocardiography Measures of Cardiac Performance .................................................. 106
    6.4.2 Quantitative PCR Detection of AAV9.S100A1 Therapeutic DNA .......................... 109
    6.4.3 Quantitative Myocardial S100A1 Proteomic Levels & Confocal Microscopy ............. 111
    6.4.4 Host Responses to Direct ssAAV9.S100A1 Expression ................................................ 114

6.5 Discussion ............................................................................................................................ 116

References .................................................................................................................................. 119

CHAPTER 7 .............................................................................................................................. 122

7.1 Introduction ......................................................................................................................... 122

7.2 Methods & Materials ........................................................................................................... 125

7.3 Results ................................................................................................................................. 126
    7.3.1 Operative & Delivery Results ......................................................................................... 126
    7.3.2 Echocardiography Results ............................................................................................. 126
    7.3.3 Quantitative PCR Detection of scAAV9.S100A1 DNA ................................................... 128
    7.3.4 Quantitative Cross Sectional S100A1 Proteomic Expression ........................................ 130
    7.3.5 Host Response to scAAV9.S100A1 Therapy ................................................................. 131

7.4 Discussion ............................................................................................................................ 133

References .................................................................................................................................. 138

8.1 Introduction .......................................................................................................................... 140

8.2 Methods and Materials: ...................................................................................................... 142

8.3 Results ................................................................................................................................. 148
    8.3.1 Process Capability ......................................................................................................... 148
    8.3.2 Particle Characterization .............................................................................................. 149
8.3.4 Process Limitations ........................................................................................................ 152
8.3.5 In-Vitro Myocyte Transfection ...................................................................................... 153

8.4 Discussion ........................................................................................................................... 155

8.5 Conclusions and Future Direction ..................................................................................... 157

References: ................................................................................................................................. 157

CHAPTER 9 ................................................................................................................................. 161

9.1 Summary .............................................................................................................................. 161

9.2 Limitations and Future Direction ........................................................................................ 170

9.2.1 Specific Aim#1 - Define and optimize engineering parameters for the liquid jet injection
device for the cardiac application in an acute rodent model and ex vivo large animal setting to
define a safe, yet effective operating transfer range. ............................................................... 170

9.2.2 Perform a two phase therapeutic delivery study to the beating heart in an acute rodent
model to evaluate the liquid jet approach against traditional methods in practice. The methods
to evaluate against are: A. Standard IM Injection B. Non-selective Intracavitary Infusion C.
Intracoronary Infusion .............................................................................................................. 172

9.2.3 Perform a chronic ischemic infarction 10 week model study to evaluate delivery of a
single dose $1.2 \times 10^{11}$ GC of ssAAV9.S100A1 vector featuring a head to head format
comparing liquid jet vs. IM injection. ...................................................................................... 174

9.2.4 Specific Aim #4 - Two key testing parameters that may significantly alter host responses
are offered: A. Using double stranded vector equivalent B. Co-delivery of anti-inflammatory
drugs to minimize host response during initial delivery phase................................................. 176

9.3 Conclusions .......................................................................................................................... 178

References .................................................................................................................................. 179
LIST OF TABLES:

Table 6.1  Echocardiography and Outcome Data for ssAAV9.S100A1....................106
Table 6.2  Pro-Inflammatory Blood Cytokine Data at 10 Weeks Post MI..................114
Table 7.1  Echocardiography and Outcome Data: scAAV9 Pilot Study.......................126
Table 7.2  Pro-Inflammatory Blood Cytokine Data at 10 Weeks Post MI: scAAV9.S100A1 ..........................................................131
Table 7.3 Compiled Outcome Results: ssAAV9 vs. scAAV9 Per Group....................132
LIST OF FIGURES:

Figure 1.1 Cardiac Gene Manipulation Strategies .......................................................... 3
Figure 1.2 Cardiac Gene Therapy Targets for Heart Failure ......................................... 7
Figure 1.3 Calcium Handling Targets in Myocardium .................................................. 8
Figure 1.4 Other Targets for Ischemic Heart Disease .................................................. 12
Figure 1.5 Viral Vector Mediated Gene Transfer ......................................................... 18
Figure 2.1 Diagram of Cardiac Gene Delivery Techniques by Route ......................... 28
Figure 2.2 Direct Myocardial Gene Delivery: Left, (A) Pericardial; Middle, Based (B) Catheter, Endocardial; Right, (C) Intramyocardial Sites ................................................. 29
Figure 2.3 Catheter Based Antegrade Catheter Infusion Delivery Approaches: Selective (A) Left, (B) Right, Non-Selective ................................................................. 35
Figure 2.4 Advanced Cardiopulmonary Bypass Based Gene Delivery Systems: (A) Top, Single pass delivery (B) Bottom, Closed Loop Recirculation Delivery with MCARD ........ 44
Figure 3.1 MCARD, Advanced Dual Perfusion Transvascular Delivery Featuring Complete Cardiac Isolation ................................................................. 55
Figure 4.1 Gene gun delivery is demonstrated featuring gold particles coated with vector DNA being accelerated and dispersed at the target tissues ......................... 62
Figure 4.2 Liquid delivery is demonstrated featuring DNA vector suspended in a liquid formulation being accelerated and dispersed at the target tissues .......... 64
Figure 4.3 The DermoJet Needleless Liquid Jet Device ................................................ 66
Figure 4.4 Mounted Device to Control and Optimize Delivery Distance ..................... 68
Figure 5.1 Cardiac Gene Delivery Methods (A) IM Injection (B) Liquid Jet Injection Concept (C) Intra left ventricular cavitary Infusion [LVIC] (D) LVIC-OCCL Infusion featuring dual occlusion of aortic and pulmonary vessels .............................................. 76
Figure 5.2 Acute Study Results Per Group (Left to Right) IM Injection, Liquid Jet Delivery, LVIC-OCCL and LVIC Infusion. Rhodamin B dye retention demonstrated in myocardial cross sections (top) and systemic exposure in the liver sections (bottom) .... 82
Figure 5.3 Acute Model Results: (A) Liquid Jet and LVIC-OCCL demonstrate significantly greater retention versus both IM and LVIC groups p<0.05 (B) All groups result in systemic exposure (C) Direct methods, IM and Liquid Jet combined demonstrate lower overall exposure versus Infusion methods or LVIC-OCCL and LVIC combined ........................................ 83
Figure 5.4. Left Ventricular Cross Section GFP Expression Distribution Per Delivery Method. Top to Bottom, IM Injection, Liquid Jet, LVIC-OCCL and LVIC AAV9 groups. Expression profiles presented in the Middle and Basal LV sections (left to right) per group with composite % area transfected with GFP………………………………………..86

Figure 5.5. AAV9.GFP Survival Model Study Results. (A) Liquid Jet and LVIC-OCCL groups demonstrating greater overall expression versus IM and LVIC groups, p<0.05. (B) Liquid jet group presented with a much lower GFP liver exposure score compared with the other groups, p<0.05…………………………………………………………………87

Figure 5.6. Ex Vivo Ovine Methylene Blue Results for translational potential of needleless liquid jet injection (A) Injection points located on the epicardial surface demonstrate the jet’s local, yet dispersive profile along the adjacent surface contours and (B) robust transmural delivery retained per injection site within the endocardial surface…………………………………………………………………………………………...88

Figure 6.1 Multi-target effects of S100A1 Protein………………………………………….97

Figure 6.2 Echocardiography of Left Ventricular Function………………………………..99

Figure 6.3 Quantitative Proteomic S100A1 Methodology Featuring IHC & Confocal Microscopy; 32 individual zones (YELLOW) throughout the myocardium…………………103

Figure 6.4 Masson’s Trichrome Staining for Infarct scar at 10 Weeks: (A) Typical IM gene therapy animal with large (blue) infarct zone while the Liquid Jet therapy group (B) demonstrated much lower scar and thicker myocardium…………………………………108

Figure 6.5 Quantitative PCR Results: IM-S100A1 Group (Red) Demonstrated significantly higher detection in the Liver, while the Liquid Jet-S100A1 group (Green) was more cardiac specific with higher detection in the heart…………………………………………………………………………………109

Figure 6.6 Quantitative Proteomic Analysis: Both IM-S100A1 and Liquid Jet S100A1 groups yielded significantly higher fold overall S100A1 in the myocardial cross sections at 10 Weeks. IM scored higher as compared with Liquid Jet, all p<0.05………………111

Figure 6.7 S100A1 Expression Variability. IM Injection results in a higher degree of variation in the myocardium following after analyzing the standard deviation in the 32 zones per animal……………………………………………………………………………...112

Figure 6.8 Confocal Microscopy Images: Cross sectional myocardial S100A1 expression (maroon) with labeled nuclei (blue) by route: (A) IM Injection (B) Liquid Jet Delivery. Liquid Jet has a less intense, more disperse pattern of expression as compared with IM……………………………………………………………………………..112

Figure 6.9 Pro-Inflammatory CD38 Marker Expression reveals significant fold higher presence in both the Liquid jet and IM groups *p<0.05. The magnitude was nearly 2 fold higher in the IM group †p<0.05 suggesting a higher degree of immune activation………114
Figure 7.1  Host Response model depicting the difference between tolerance and immunity. Multiple signaling events contribute to adaptive immune response through antigen recognition (Signal 1), Co-stimulation (Signal 2) and the production of systemic inflammatory markers (Signal 3)……………………………………………………………………122

Figure 7.2  QPCR analysis revealed significantly higher transfer levels in both the cardiac regions and collateral liver. The IM Group demonstrated significantly higher expression * p<0.05 for both regions, with the liver superseding † p<0.01 other tissues……………………………………………………………………………………128

Figure 7.3  The second iteration of S100A1 proteomic analysis revealed significantly higher overexpression in all treatment groups * p<0.05. Comparatively, both Self Complementary vector treatment groups scored higher † p<0.05 than corresponding Single stranded groups. The IM-SC group scored much higher overall than all others §p<0.05………………………………………………………………………………………129

Figure 7.4  Typical representative image capture comparing single stranded versus self-complementary S100A1 expression (A) IM single stranded Group (B) IM self-complementary Group demonstrates higher S100A1 expression……………………………130

Figure 7.5  CD38 Expression trends higher inline with overall viral mediated S100A1 expression. As found previously, overexpression of S100A1 increases immune signaling * p<0.05. The IM group as compared with Liquid Jet was significantly higher †p<0.05………………………………………………………………………………………131

Figure 8.1.  The water oil water double emulsion nanoparticle production process work flow sequence to generate high quality anti-inflammatory formulations………………………………………………………………………………142

Figure 8.2.  SEM Characterization Sizing Results. A. Narrow size distribution and high quality spherical shaped yield example in A at 2µm scaling. B. Close up 500 nm scaling image indicates narrow size distribution in the 200-350 nm range consistently for all manufactured yields. Nanoparticle average size by drug and polymer combination. C. Aspirin nanoparticles of either 50:50 or 65:35 type had greater size (p<0.05) versus prednisolone………………………………………………………………………………………149

Figure 8.3.  Nanoparticle Zeta Potential Colloidal Stability Testing Results indicate that the PLGA50:50 nanoparticles are more stable in solution versus the PLGA65:35 types……………………………………………………………………………………………150

Figure 8.4.  Controlled release study results demonstrate that aspirin particles overall release faster than prednisolone type……………………………………………………………………………………………………………………………151

Figure 8.5.  In Vitro Fluorescent Imaging at 48 hours post transfection. All 4 particle systems exhibited safe and robust uptake in myocytes while not interfering with plasmid uptake and subsequent GFP expression. Yellow signal indicates co-existence of GFP and nanoparticle in: A. PLGA65:35 Aspirin B. PLGA50:50 Aspirin C. PLGA50:50 Prednisolone D. PLGA65:35 Prednisolone……………………………………………………………………………………………………………………………………………………………153
CHAPTER 1

Introduction: Heart Disease and Emerging Gene Therapy Applications


1.1 Heart Disease Burden and Treatment Options

An estimated 83.6 million American adults suffer from numerous cardiovascular diseases (CVD). Mortality data show that CVD accounted for 31.9% of all deaths in 2010, or 1 in every 3 deaths in the United States. That is 4.4 times more than the deaths attributable to cancer. The total direct and indirect cost of CVD in the USA for 2010 is estimated to be $315.4 billion. By comparison, the estimated cost of all cancers and benign neoplasms was $201.5 billion [1]. These data clearly show that cardiovascular diseases are still the leading cause of mortality and morbidity in the US and developed countries. In the United States, coronary artery disease is the underlying cause of CVD in 65-75% of cases [2]. Following the initial myocardial insult after a period of time, the unfortunate fate for the majority of chronic heart disease patients is full blown heart failure despite significant accumulated, yet ineffective clinical interventions. Heart failure (HF) imposes a significant burden on the global healthcare system with annual costs alone exceeding $32 billion. The incidence and death rate is appalling, with 22 million additional patients diagnosed per year with 50% mortality within 5 years. In many of these patients, HF develops as a consequence of post infarction remodeling and associated progressive dilatation extending to remote areas distant from the original infarct [2].
Although cardiac transplantation is the gold standard for the treatment of end-stage heart disease, up to 30% of patients unfortunately die waiting for a matched donor heart. Even more disturbing, as many as 60,000 patients per year could benefit from such therapy [3,4]. The most frequently used technology at present for these patients is mechanical pump-based assist devices, which have been quite useful both as a bridge to transplantation [5,6], and as destination therapy using both left ventricular assist devices (LVAD) [7,8] and the total artificial heart [9]. Despite significant improvements mitigating some of the limitations of LVAD, most notably thromboembolic events, it appears that mechanical cardiac assist technology will face increasing scrutiny given its cost to benefit ratio. Thus, given the shortcomings of the best available therapies, there is a significant unmet need for newer, more efficacious and more cost-effective therapies for severely afflicted patients.

There are two major emerging alternative treatment paradigms to improve long term outcomes in both early and late stage heart disease, namely replacement (i.e. cell therapy) or genetic reprogramming of native cells with gene treatments. The focus in late stage management is improving contractility, whereby the opportunity to arrest infarct expansion and global LV remodeling has past. Therefore, the transfer of therapeutic genes for the treatment has emerged as the more attractive strategy given its impact on directly enhancing contractility within the myocytes. Extensive pre-clinical studies have provided solid proof of concept data indicating gene therapy’s clinical potential, whereby the expression of selected transgenes in the myocardium enhances contractility, restores global function, and in some cases completely reverses chronic HF. Although promising, as this body of research will explore, a number of problems
must be addressed in order to achieve more successful outcomes and avoid the deleterious events the field has experienced in the early 2000’s.

1.2 Cardiac Gene Therapy Applications Strategies

Currently, there are primarily two strategies described for cardiovascular gene therapy manipulation (Figure 1.1).

![Strategies of Genetic Manipulation](image)

**Figure 1.1 Cardiac Gene Manipulation Strategies**

The most commonly applied strategy features overexpression of a target gene, which may involve either replacement of a missing or dysfunctional gene as in X-linked recessive disorders such as the heart failure associated with or Becker’s cardiomyopathy, an autosomal recessive gene defect such as those associated with alpha sarcoglycan deficiency in the limb girdle muscular dystrophies. More commonly, heart failure may not have a defined genetic basis (ischemic cardiomyopathy) yet certain genes are consistently downregulated (e.g., SERCA2a). The second group of strategies
relates to inactivation of dominant negative gene function involved in disease etiology or progression (other names: gene silencing or gene blockade) [10,11,12].

1.2.1 Overexpression of target gene

A gene’s physiological function may be impaired or downregulated as a result of a mutation or a pathological process. Therefore, the restoration of function through exogenous delivery to replace the deficient gene seems quite logical. In this case, full-length or partial cDNA encoding the deficient gene is delivered to the target tissues using a vector system capable of expressing the therapeutic protein [10]. Several steps in the gene overexpression process may be modulated, including the transcription, RNA splicing, translation, and posttranslational modification of a protein.

1.2.2 Specific gene blockade

1.2.2A Antisense oligodeoxynucleotides (ODN)

ODN are used as inhibitors of specific gene expression without any change in function of other genes. Single stranded ODN may be delivered either by direct administration (as a pharmacological agent) or by transfection with a vector encoding the ODN. The ODN binds to the target mRNA transcript and prevents translation. This mechanism of action is based on the presence of two forms of ODN: the RNase H-dependent ODN, which induce the degradation of mRNA, and the steric-blocker ODH, which physically blocks the progression of mRNA translation. Concerning cardiac applications, the antisense ODH approach has been tested to prevent restenosis after balloon angioplasty [13]. Treatment with antisense ODN directed against VEGF receptors could prevent VEGF-mediated arteriogenesis [14]. Systemic delivery of an
antisense ODN induces silencing of miR-208a in the myocytes, thus improving cardiac function and survival in hypertensive-induced heart failure (HF) in rats [15].

1.2.2B Decoy-based gene therapy

Synthetic double-stranded (ds) DNA with high affinity may be introduced into target cells as a “decoy” or alternatively described as a cis-regulatory element, which binds to a sequence-specific DNA factor and changes gene transcription. Transfection of dsODN will result in the attenuation of the cis-trans interaction of cell surface receptors, and remove the trans-factor from the endogenous cis-element resulting in inhibition of gene expression [12]. dsODN containing binding sequences (decoy) for transcriptional factors involved in the activation of pathogenic genes. Transfection of the decoy ODN prevents the binding and trans-activation of the genes regulated by the target transcriptional factor [10].

The decoy strategy is very attractive for several reasons: the synthesis of the sequence specific decoy is relatively simple and can be targeted to specific tissues, the knowledge of the exact molecular structure of the target transcription factor is unnecessary; it has been shown to be more effective than antisense technology [12]. Some restrictions of this method however are: short half-life, lower uptake efficiency and degradation by endocytosis and nucleases. Using mice and monkey models it was found that E2F decoy transfection prevented intimal hyperplasia in cardiac allografts [16]. In a report of Yamasaki et al, the successful in vivo transfer of NFkappaB decoy ODN to inhibit vascular stenosis in balloon-injured porcine coronary arteries was demonstrated [17].
1.2.2C Short interfering RNA (siRNA)

Gene silencing via siRNA technology is a novel strategy with great therapeutic potential. siRNA is a short dsRNA molecule that induces sequence specific posttranscriptional gene modification. This mechanism is called RNA interference (RNAi). Recently, this strategy was used for the treatment of HF and the results showed that the restoration of cardiac function was most likely through the reduction of hypertrophy [18]. Once transfected into a cell, the siRNA incorporates into the nuclease complex, where they then interrupt translation of targeted genes. Successful left ventricular intracavitary delivery of DNA/siRNA complexes by means of sonoporation was demonstrated in murine hearts [19]. The incorporation of siRNA into terminally differentiated adult rat cardiac myocytes using adenovirus has also been reported [20].

1.2.2D Ribozymes

Another strategy used to inhibit the disease process at the transcriptional level is the use of ribozymes. Ribozyme gene therapy aims to turn off a mutated gene in a cell by targeting the mRNA transcripts copied from the gene. Therefore, protein synthesis by the target RNA may be specifically inhibited by ribozymes. This process involves three steps: (1) Delivery of RNA strands engineered to function as ribozymes (2) Specific binding of the ribozyme RNA to mRNA encoded by the mutated gene and (3) Cleavage of the target mRNA, preventing it from being translated into a protein. Several studies have used ribozymes to limit neointimal hyperplasia with smooth muscle cell proliferation in response to balloon angioplasty. Ribozymes against c-myb mRNA [21] and transforming growth factor [22] prevented development of restenosis.
1.3 Heart Failure Gene Targets

Gene therapy targets generally aim to: increase contractility, attenuate adverse remodeling and inhibit apoptosis. A schematic demonstrating the therapeutic aim and their associated specific targets is presented in (Figure 1.2).

![Therapeutic Targets: Heart Failure]

**Figure 1.2 Cardiac Gene Therapy Targets for Heart Failure**

**1.3.1 The calcium cycling proteins**

Ca\(^{2+}\)-cycling has been found to be critically dysregulated in chronic HF and, provides an important role in excitation-contraction coupling. Depicted in (Figure 1.3) is the excitation contraction complex in the myocyte including all elements.
To understand the Ca\(^{2+}\) handling defects in heart failure, we need to briefly describe the processes occurring in cardiac excitation–contraction coupling. During the cardiac action potential, Ca\(^{2+}\) enters the cell through depolarization-activated Ca\(^{2+}\) channels as an inward Ca\(^{2+}\) current, which contributes to the action potential plateau. Ca\(^{2+}\) entry triggers Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR). This allows Ca\(^{2+}\) to bind to the myofilament protein troponin C, which then switches on the contractile process. For relaxation to occur there is a decline in intracellular Ca\(^{2+}\) concentration, allowing Ca\(^{2+}\) to dissociate from troponin. This requires Ca\(^{2+}\) transport out of the cytosol by pathways involving SR Ca\(^{2+}\) -ATPase, sarcolemmal Na\(^{+}\) / Ca\(^{2+}\) exchange, sarcolemmal Ca\(^{2+}\) -ATPase or mitochondrial Ca\(^{2+}\).

**1.3.1A SERCA2a**

Deficient SR Ca\(^{2+}\) uptake during myocyte relaxation has been identified in failing hearts from both humans and animals, and is associated with a decrease in the expression and activity of sarcoplasmic reticulum calcium ATPase (SERCA2a). This
protein is a Ca\(^2+\)-ATP-dependent pump of the sarcoplasmic reticulum that has a critical role in Ca\(^2+\) regulation, known as SERCA2a. The overexpression of SERCA2a has been demonstrated to increase contractility and normalize calcium cycling in failing human cardiomyocytes [23]. A number of animal studies with a variety of models of HF has demonstrated that overexpression of SERCA2a has a positive inotropic effect, improves oxygen utilization, attenuates the progression of HF and prolongs survival. SERCA2a gene transfer was found to substantially decrease incidence of ventricular arrhythmias and reduce infarct size in a model of ischemia/reperfusion [24]. A lentiviral vector-mediated SERCA2a intracoronary delivery after myocardial infarction in rats resulted in favorable molecular remodeling with improving systolic and diastolic function six months later [34]. An improvement in left ventricular diameter, fractional shortening, and Ejection Fraction was also demonstrated in a tachycardia-induced HF model [25].

A first-in-human clinical trial Calcium upregulation by percutaneous administration of gene therapy in cardiac disease’ (CUPID) involving gene transfer of SERCA2a cDNA via a rAAV1 vector in patients with advanced HF has been undertaken in a randomized, double-blind, placebo-controlled study; although the results were somewhat difficult to interpret due to the absence of a clear dose-response. At 12 months, SERCA2a treated patients in the highest dose cohort demonstrated a consistent trend in clinical symptomatic improvement and in functional capacity [26].

1.3.1B S100A1

In cardiomyocytes, S100A1 plays an important role in increasing SERCA2a activity. This effect is achieved through diminishing diastolic SR Ca\(^2+\) leak, augmenting the systolic open probability of the ryanodine receptors, leading to an overall gain in SR Ca\(^2+\) cycling. Also, S100A1 regulates SERCA2A-Phospholamban function, resulting in a
balanced enhancement of SR Ca2+ release and uptake. S100A1 is downregulated during the development of HF [27]. Thus, in theory, S100A1 may be a promising factor in the treatment of HF. In a rat model of HF, significant cardiac recovery was demonstrated after 8 weeks in AAV6/S100A1 treated animals [28]. A study in a postinfarction pig model after 14 weeks revealed improvement in dP/dt and ejection fraction, and also restoration of high-energy phosphate homeostasis in failing myocardium [29].

1.3.1C Phospholamban (PLN)

PLN regulates the homeostasis of SR Ca2+ mediating slower cytosolic Ca2+ decay in cardiomyocytes, which translates into diastolic relaxation. Phosphorylation of PLN suppresses its inhibitory effect. AAV-mediated overexpression of a mutant (“pseudophosphorylated”) form of PLN improved LV function and mitigated adverse remodeling in post-MI rats [30]. Silencing of PLN expression after tachycardia-induced HF in sheep increased ejection fraction and decreased LV end-diastolic area [107]. A study in a volume-overload HF proved that adenovirus encoding antisense PLN preserved LV contractility and normalized LV mechanoenergetics [31].

1.3.2 The β-adrenergic signaling cascade

The β-adrenergic receptor (βAR) signaling system plays an important role in the control of cardiac function, mediating the inotropic, chronotropic and lusitropic responses to the sympathetic neurotransmitters [32,33]. Therefore it represents an attractive molecular target to improve heart function. Two important components of the βAR system include the β-receptors and the regulatory G protein-coupled receptor kinases (GRKs). Dysregulation of the βAR pathway, including downregulation, uncoupling of second-messenger systems, and upregulation of βAR kinase (βARK1, GRK2), has been
shown to be a hallmark of HF. βARs are regulated by GRK2, a member of a G protein-coupled receptor kinase family that phosphorylates and inactivate these receptors [34]. βARKct, a competitive inhibitor of GRK2, has the potential to resolve βAR downregulation and desensitization associated with HF [34,35]. Thus, inhibiting the activity of GRK2 or lowering its expression appears to offer a novel means to enhance cardiac function.

1.3.2A The βARKct Peptide

In a rabbit model of HF induced by myocardial infarction it was first demonstrated that the βARKct transgene improved heart function and delayed development of HF [36]. Inhibition of myocardial βARK1 via Ad/βARKct delivery before creation of acute coronary ischemia, may represent a new strategy for cardiac protection [37]. Long-term βARKct expression in the rat is by reversed LV remodeling and a normalization of the neurohumoral status of chronic HF animals [38]. The high level of βARKct expression in pressure-overload heart hypertrophy can preserve adenyl cyclase activity and βAR density and also improve cardiac function and cell morphology [39]. Based on the above results and earlier results derived from transgenic animal models, it appears that delivery of βARKct could be beneficial in the setting of IHD and HF.

1.3.3 Other Ischemic Heart disease targets

Current gene therapy research efforts in IHD include: stimulation of angiogenesis; limitations of reperfusion injury through the use of antioxidant therapy and endothelial nitric oxide synthase; and cardioprotection by using anti-apoptotic proteins [40]. These are summarized in (Figure 1.4). Nevertheless much of the previous research was devoted to the study of angiogenesis.
One major focus of gene therapy for ischemic heart disease is neovascularization of fibrous post-infarct or poorly-perfused (hibernating) myocardium. Therapeutic angiogenesis can be achieved by gene transfer of vascular endothelial growth factor (VEGF), hepatocyte growth factor, fibroblast growth factor, and hypoxia-induced factor 1α. VEGF has five isoforms which act on tyrosine kinase receptors, FLK-1 and FT1. This protein factor has been shown to stimulate endothelial cell proliferation, migration and vascular permeability and to affects fibroblast and smooth muscle growth [40,41,42]. Preclinical gene therapy studies with VEGF in various large animal models of myocardial ischemia have demonstrated stimulation of angiogenesis and improvement in fractional shortening [43], reduction of infarct size and peri-infarct fibrosis [44].
addition, it has been noted that there is an appearance of apoptosis-resistant cardiomyocytes in the border zone [45] and improvement of myocardial viability [46].

1.4 Therapeutic Vectors

Choosing the right vector for cardiovascular applications is one of the most challenging aspects. The availability of vectors for gene transfer has improved significantly over time. The ideal vector would have the following characteristics: it must be cardiotrophic, result in long-term expression, minimize the risk of cellular immune response, have a large coding capacity and have high transduction efficiency [41]. The main challenges to the vector are as follows: (i) Escaping the neutralizing effects of specific antibodies and non-specific adsorption to other blood components (ii) overcoming the endothelial barrier and penetrating the vascular wall for diffusion through the extracellular matrix; and (iii) Uptake into the cell at the level of the plasma membrane and efficient trafficking to the nucleus and (iv) synthesis by the host of the complimentary DNA strand for single stranded delivery vectors followed by transcription and translation of the transgene [47]. A number of different vectors have been used to achieve myocardial gene transfer, modified or selected to enhance the probability of overcoming each of these challenges. All vectors can be classified into two main categories, the non-viral and recombinant viral.

1.4.1 Non-viral vectors

Non-viral vectors are grouped as plasmid DNA, liposome-DNA complexes (lipoplexes), and polymer-DNA complexes (polyplexes). Oligonucleotides are also considered non-viral vectors [48]. In 1990, Lin and associates injected plasmid DNA into the left ventricle and demonstrated that the lacZ gene could be introduced and
expressed in cardiac myocytes [49]. Although non-viral vectors have the major advantage of production in relatively large quantities at low cost while at the same time possess fewer toxic or immunological problems, their transfer efficiency is generally poor independent of delivery route [50,51]. Nevertheless, a large number of human cardiac clinical trials are based on plasmid-mediated gene transfer investigating angiogenesis in myocardial ischemia [52-54]. A major advantage of this approach is that it avoids many of the biosafety concerns associated with viral vectors. However, the level of transgene expression and the efficiency of gene transfer (percent of target cells expressing the transgene) are low and expression is restricted to the zone of the injection site. DNA complexes are relatively more efficient [55].

There is however a major discrepancy between the data obtained in vivo and in vitro. In addition, these complexes are unstable and thus quickly removed by phagocytes when delivered, especially through intravascular delivery systems. An additional shortcoming of these vectors (e.g. oligonucleotides) is their short biological half-life due to intracellular degradation and non-specific binding [56]. The demonstration of plasmid gene transfer opened a new era of cardiovascular pharmacotherapy. Despite numerous efforts to enhance efficiency through modification, direct myocardial plasmid injection basically remains a proof of concept tool only [57].

1.4.2 Viral vectors

Successful cardiac gene therapy applications demand both efficient myocardial transduction initially and long-term transgene expression. Many authors strongly believe that only viral vectors appear to meet these demands in terms of performance [58,59]. Compared to non-viral vectors, viruses have an evolutionary advantage in their
interactions with the cellular surface receptors, directly leading to more efficient intracellular trafficking of packaged DNA to the nucleus. Furthermore, their protein capsid protects the message from degradation in lysosomes [47,57,60,61]. Some viral vectors are able to integrate into the host genome, whereas others remain episomal. Integrating viruses result in persistent transgene expression while viruses in episomal form lead to long term expression in predominantly non-dividing tissues (e.g., adult myocardium) but only transient expression in rapidly dividing tissues (e.g., the hematopoietic system). It should be noted that for some disorders, short -term expression in a relatively small proportion of cells would be sufficient or even desirable (e.g., angiogenesis post myocardial infarction) whereas other pathologies might require long-term expression (e.g., autosomal recessive cardiomyopathy).

1.4.2A Lentiviruses

These vectors were initially developed for HIV therapy. Lentiviral vectors can infect non-dividing cells, cause long-term expression and do not typically induce an inflammatory or immune response. The major limitation is the risk for mutagenesis and oncogenesis [58]. The new generation of lentiviruses, containing a mRNA and a nuclear import sequence have been used for successful myocardial transduction, although expression is usually short-term [62,63]. Fleury et al. in a study with rat cardiomyocytes in vivo succeeded in obtaining persistent GFP transfer for up to 10 weeks [64]. In another study the transduction efficiency of lentiviral vector-mediated SERCA2 gene transfer was about 40% and the positive physiological effect persisted six months later [65].

1.4.2B Adenoviruses
Adenoviral vectors have historically been the most frequently used transfer system in experimental and clinical studies. This is attributed to the vector’s known advantages such as: the ability to transduce non-dividing cells, ease of manufacture in very high titers, the possibility to achieve high levels of transgene expression and a large transgene cloning capacity. However, their use is limited clinically due to transient gene expression, and their inability to integrate the genome into the cellular chromosomal DNA. In addition, adenoviral vector particles are highly immunogenic and cause inflammatory and toxic reactions in the host. This is due to the fact that the adenovirus stimulates both the innate and adaptive immune systems. Using a rat model, it was confirmed that adenovirus was several orders of magnitude more efficient in transducing myocytes than plasmid DNA expressing the same construct [66,67]. Later, it was shown that the direct intramyocardial injection of replication-deficient adenovirus can program gene expression in large animal in vivo. However, the authors noted a robust T cell-mediated immune response against the vector and limited distribution of the reporter gene [68].

Simultaneously, several groups confirmed the possibility to achieve significant cardiac gene expression after catheter-mediated delivery of adenovirus encoding phospholamban and the β2-adrenergic receptor [68,69]. Using adenovirus to deliver selected transgenes, enhanced cardiac performance several weeks after gene transfer has been demonstrated [70]. Despite sophisticated modifications in an attempt to attenuate the host immune response to the adenovirus, the risk is too high to advocate the use of this delivery vector for clinical cardiovascular applications.

1.4.2C Adeno-associated viruses
Adeno-associated virus (AAV) is a small (20 nm), non-enveloped virus that belongs to the dependovirus genus of the parvovirus family. AAVs have a single-stranded DNA genome. The viral genome is approximately 4.7 kb in length, and is composed of two major open-reading frames which encode Rep (replication) and Cap (capsid) proteins [71]. For an infection to occur, AAV requires co-infection with a helper virus such as adenovirus. This allows the viral genome to replicate episomally, and leads to synthesis of the AAV proteins. AAV is one of the smallest viruses, with a capsid mean diameter of 22 nm. The first AAV2 infectious clone was created in 1982 by Samulski and colleagues [72]. Several years later, it was established that AAVs can express foreign genes in mammalian cells [73]. One of the major advantages of AAV vectors is that in multiple animal models and humans, it has been demonstrated that after reaching a steady state level, AAV expression may last for years with an absence of a significant immune response to the transgene [74]. Moreover, AAV vectors can be engineered to provide a wide range of cell type tropism with the ability to transduce both dividing and non-dividing cells. Due to their biological properties and advantages over other viral vector systems, AAV has gained great popularity in the last decade in many clinical trials. Seventy five clinical trials using AAV have been initiated over the past 15 years [60] with ~10% indicated for cardiovascular diseases [75].

The process of AAV endocytosis and intracellular trafficking is complex and cannot be underscored in understanding problems with clinical outcomes. Despite the availability and diversity of AAV vectors, several biological barriers appear to limit the effectiveness of AAV mediated gene therapy [76,77]. Understanding the fundamental basis of these barriers has led to the establishment of methods to improve the efficiency of rAAV-mediated gene delivery [61,77]. Clarification of the processes by which a virus
first enters and traffics through a cell helps to understand the life cycle of the virus and its ability to act inside the cardiac muscle. The transport activity of AAV is mainly determined by selective receptor-mediated vesicle transcytosis [78]. This intracellular route does not appear to alter the properties of the AAV. The entry of AAV vectors into the cell involves several steps illustrated in (Figure 1.5).

(1) Binding to the membrane receptor/co-receptor, or attachment factors; (2) Endocytosis of the virus by the host cell occurs in distinct membrane compartments, called clathrin-coated pits, which can be internalized to form clathrin-coated vesicles. Clathrin-independent endocytosis involves the uptake in caveolae, membrane lipid rafts and microdomains; (3) Following endocytosis the AAV vectors are compartmentalized into early endosomes. This is the distribution station in the endocytic pathway; (4) Early endosomes then mature into late endosomes that are degraded by fusion with the lysosome later, secretory vesicles, and the material that will be recycled back to the
plasma membrane; (5) Some separate viruses can escape lysosomal degradation via acidification of the endosome and it is a necessary prerequisite for the release of the AAV; (6) These AAVs particles are then trafficked into the nucleus, where viral uncoating leads to single-stranded DNA release. The single stranded DNA is then converted to double stranded DNA, and finally to concatamers and or integrated into the host genome.

AAV transport can be blocked by neutralizing antibodies, temperature, and physical and chemical inhibitors through a time and dose-dependent process. In vivo studies have noted that several serotypes of AAV are able to cross vascular endothelium with different efficiencies [79]. It is known that AAV2 has a relatively poor tropism for vascular cells, although reasonable levels of transduction have been achieved in cardiac myocytes [80]. Local delivery AAV2 led to transduction of underlying vascular smooth muscle cells and sequestration of AAV in the extracellular matrix around endothelial cells thus preventing cell binding and entry. The potential of AAV6 vector for cardiac gene therapy was achieved through the use of VEGF to increase vascular permeability [81].

1.5 Conclusions

The growth in CVD gene therapy applications has been dramatic in recent times given successes in other disorders. However, physicians and translational scientists still must address key safety and efficacy problems associated with viral vector mediated gene transfer. The adeno-associated virus platform to date offers the best means to achieve safe and long term gene expression as indicated now with over 3 years of safety data from the Phase I/IIb CUPID trials. Despite this promise, the level of interest in
regenerative cell therapy applications will continue to grow unless the problems of efficient delivery and avoidance of undesirable immune responses can be achieved. Next generation viral and non-viral vector applications are being co-developed alongside smart device based delivery systems to increase cardiac specificity and limit off target effects. The remaining chapters of this body of work are dedicated to advancing cardiac gene therapy through the development of a novel, cardiac specific delivery system. This body of research essentially asserts that delivery strategy selection and execution drives outcome with any given high risk/reward gene therapeutic.

References


[23] Davia K, Bernovich E, Ranu HK, del Monte F, Terracciano CM, MacLeod KT. SERCA2a overexpression decreases the incidence of aftercontractions in adult rabbit ventricular myocytes. 2001; J Mol Cell Cardiol 33:1005-1015.


CHAPTER 2
Cardiac Gene Therapy Delivery Systems: Present and Future Applications

2.1 Introduction

An important prerequisite for introducing cardiac gene therapy into clinical practice is the development of simple and efficient gene delivery techniques. During the last two decades, we have witnessed the development of several experimental gene delivery strategies with potential therapeutic value for the transition from the preclinical phase to clinical trials. Yet, efforts at gene transfer will require solutions to several problems. These problems include delivery of the vector to the target tissue, improved safety and efficacy, prevention of complications, creation of new delivery devices and techniques, and improved geographical specificity of gene delivery to areas of therapeutic interest while simultaneously minimizing systemic spillover.

Although cardiac tissue-specific promoters may mitigate collateral organ gene expression, only a true cardiac specific gene delivery method can diminish the biodistribution of vector capsids to extra cardiac organs. Extra cardiac exposure results in unsafe levels of exposure to antigen presenting cells. Antigen presenting cells provide another mechanism to increase the potential for a T-cell mediated immune response to the vector capsid. Ideally, the most optimal gene-delivery system should be combined with an appropriate vector. Recombinant AAV vectors have rapidly evolved as tools for cardiac gene therapy. For numerous target diseases, they offer advantages over other viral vector systems. Multiple AAV serotypes have been isolated in recent
years. Among all AAV serotypes, AAV2 vectors have been successfully used in several experimental approaches such as protection from ischemia/reperfusion injury, inotropic therapy and beneficial effects on neoangiogenesis. Recent reports demonstrate that serotype 6 and 9 facilitate relatively stable cardiac gene expression and are superior to others in the heart, likely due to enhanced cellular internalization and nuclear uncoating in cardiomyocytes. Successful solutions to these and other challenges will undoubtedly help to achieve transmural, homogeneous, high-density cardiac gene transfer [1, 2].

There are many published methods to transduce myocardium; yet, most of these approaches have shown inefficient transduction and thus failed to demonstrate effective therapy when applied to disease involving the whole heart [3]. Existing methods of gene delivery can be classified by the site of injection, interventional approach and the physiology of the cardiac circulation (Figure 2.1)

Figure 2.1 Diagram of Cardiac Gene Delivery Techniques by Route
In this chapter, we describe both the most common and innovative gene delivery methods and attempt to outline future developments in this dynamic field.

2.2 Direct Cardiac Gene Delivery

Direct gene delivery methods have been utilized for at least two decades and some authors continue to reference and utilize them in their preclinical cardiac gene therapy studies. Numerous methods have also successfully been translated for use in clinical trials. The most relevant of them are usually classified as either an open technique, that is to include the surgical opening of the chest or a closed technique, i.e., transcutaneous or minimally invasive. A minimally invasive application is desirable and can also be achieved via a subdiaphragmatic or thoracoscopic approach. (Figure 2.2) depicts the three major approaches and their results which are described in detail in the following subsections.

![Diagram of Direct Myocardial Gene Delivery](image)

**Figure 2.2 Direct Myocardial Gene Delivery:** Left, (A) Pericardial; Center, (B) Catheter Based Endocardial; Right, (C) Intramyocardial Sites
2.2.1 Intrapericardial Injection

The rationale underlying intrapericardial gene delivery is related to the advantage of the anatomical connection between the pericardium and the myocardium, and the accessibility of the pericardial sac for percutaneous vector delivery [4] (Figure 2.2A). Zhang et al. made a percutaneous puncture of the pericardium at the left costoxiphoid angle of the anterior chest with injection of Ad.CMV.lacZ. After three days, significant lacZ expression was observed in the epicardium, myocardium, and endocardium of neonatal mice. The authors concluded that intrapericardial injection is an efficient technique to achieve transmural gene expression. Unfortunately, these results appear to be age-specific and are far less efficient in adult (rather than neonatal) animals. Furthermore, the group later found that at two months the expression only persisted in atrial tissue and not in ventricles, the presumed target area to address the majority of cardiomyopathic diseases. Additionally, this method was associated with high levels of hepatic transduction [5].

Fromes et al. performed intrapericardial injections with a transdiaphragmatic approach, using adenovirus encoding β-galactosidase in rats. The staining observed was exclusively restricted to the pericardial cell layers; however, injecting a mixture of proteolytic enzymes with the virus led to an increase of transgene expression to 40% of the myocardium at day seven. As expected when using an adenoviral vector, the expression decreased to 0.5 % at day 28. The authors also found positive β-galactosidase activity in distant organs [6]. March et al. used a hollow, helical-tipped penetrating catheter for vector delivery in the canine myocardium. The catheter was introduced percutaneously and advanced into the pericardial space through the apex of the right ventricle. All of the animals tolerated the procedure without incident,
demonstrating the feasibility of localized cardiac gene delivery via a catheter-based pericardial approach [7]. The administration of Ad2.CMV.LacZ into the pericardial sac produced extensive transfection of the visceral and parietal pericardium and doxycycline pretreatment increased this effect [8].

2.2.2 Endocardial Injection

The feasibility of fluoroscopy-guided, percutaneous endocardial vector injection was demonstrated by Gwon et al. in the porcine heart [9] (Figure 2.2B). Sanborn et al. used the same approach with a coaxial catheter for endocardial delivery of adenovirus encoding VEGF in the porcine myocardium. Regional VEGF expression was found to be significantly greater in targeted zones as compared with non-targeted zones [10]. Several authors believe that the electromechanical mapping-guided approach allows for better deployment of the tip of the catheter around areas of ischemia [11-13]. However, this system and the equipment needed to execute the procedure are quite complex and expensive.

In a porcine model, Grossman et al. compared endomyocardial and epicardial microsphere injection, finding that endomyocardial injection performed with the Stiletto system was associated with 43% microsphere retention, compared with 15% after epicardial injection. Reduction of injectate volume (10 µL) resulted in significantly improved retention compared to 100 µL injection, a typical volume used in clinical trials. These authors also found evidence of significant viral transfection in the liver and spleen after injection of adenovirus encoding β-galactosidase [14]. The study of Naimark et al. highlights the importance of enhancing the biocompatibility of the catheter for endocardial and epicardial gene delivery [15]. Despite the ease of implementation
advantage of catheter-based endocardial and epicardial injection, there is an increased potential for complications associated with ventricular perforation, cardiac tamponade, endocardial thrombosis and intramyocardial hematoma, especially in patients with chronic ischemia who have thinned and scarred myocardium [9].

While there has been demonstration of the feasibility of percutaneous endocardial gene transfer and gene expression, many questions still exist regarding this approach [10]. For instance, in patients with ischemic cardiomyopathy, should vector/transgene administration be targeted to ischemic areas alone or to “border zone” to stimulate collateral flow? What quantity represents the ideal dose and how many injections are necessary, especially given that the patients have multiple areas of ischemia?

2.2.3 Intramyocardial Injection

The majority of successful preclinical studies have involved direct administration of vector. This technique allows for the application of a high concentration of vector directly at the target site. Several groups have demonstrated the feasibility of delivering transgenes to the heart via direct intramyocardial injection of plasmid DNA [16-19] (Figure 2.2C). Although these studies have been encouraging because plasmid DNA may be expressed for up to six months by cardiomyocytes adjacent to the area of injection, estimates of the number of myocytes that can be transfected in vivo have been as low as 60 to 100 cells per injection [16]. This low efficiency has made it difficult to measure the physiological effects of gene expression in myocytes, making it unlikely that clinically significant effects will result [20]. The low transduction efficiency of plasmid DNA vectors leads to the search for improved gene transfer efficiency with direct
injection of an adenovirus vector. Hearts transfected with an adenovirus vector containing the β-galactosidase gene showed significantly increased β-galactosidase enzymatic activity compared with hearts injected with β-galactosidase plasmid. Unfortunately, the gene expression persisted for only one week after injection and it included acute inflammatory response, which the authors considered to be related to the injury produced by direct injection as well as a cellular immune response against the adenovirus itself [20].

The studies of French et al. [21] demonstrated for the first time, in a porcine model, a number of important points relevant to this technique: 1) direct intramyocardial injection of replication-deficient adenovirus is 140,000 times more efficient than injection of an equal number of genome copies of recombinant plasmid DNA and can program recombinant gene expression in the cardiomyocytes of a large animal species; 2) the impact of this procedure on cardiac function appears to be negligible; 3) the amount of recombinant protein produced increases with the amount of virus; 4) the expression of recombinant genes following intramyocardial injection is similar in the left and right ventricles; 5) the percentage of cardiomyocytes expressing β-galactosidase in the needle track adjacent to the injection, but rarely are lacZ positive cells detected farther than 5 mm from the injection site [21].

In a canine study using adenovirus encoding chloramphenicol acetyl transferase, peak gene expression was noted at two days and decreased by an order of magnitude 14 days after direct single myocardial administration. In this study, there was not significant transduction of distant organs and no documented changes in global or regional LV function [22]. However, the feasibility of adenovirus-mediated gene transfer has been limited by the cellular immune response which causes myocardial
inflammation and results in transient recombinant gene expression [23].

Svensson et al. showed that stable β-galactosidase expression can be achieved without evidence of myocardial inflammation or myocyte necrosis after substituting rAAV for adenovirus vector-mediated gene transfer [24]. Furthermore, in an landmark study, Tomiyasu et al., succeeded in augmenting cardiac function in cardiomyopathic hamsters with heart failure by transfecting cardiac muscle with the β2-AR gene after direct intramuscular injection. Echocardiographic examination revealed that stroke volume and cardiac output were significantly elevated at two to four days after β2-AR gene transfer [25].

Rengo et al. studied myocardial gene transfer to post-myocardial infarct rats with intramyocardial direct injection. In order to stop beating of the heart (for 2.5 min), adenosine was injected and both ascending aorta and pulmonary artery were clamped. A volume of 4x10\(^{11}\) total particles of rAAV6-βARKct was injected in the LV free wall. The investigators found robust transgene expression in the LV at 12 weeks after delivery. A significant finding was that βARKct (gene of interest) significantly improved cardiac contractility and reversed LV remodeling in this heart failure model [26].

In summary, the direct gene delivery approach was the first among others that helped establish the therapeutic efficacy of cardiac gene therapy. Furthermore, the use of this method in some experimental models resulted in successful therapeutic myocardial angiogenesis, and focal treatment of cardiac arrhythmias through effects on cellular electrophysiology; thus, making this platform widely used. Finally, this approach has been successfully utilized in Phase I/II clinical trials demonstrating its potential therapeutic relevance.
2.3. Transvascular Gene Delivery

There are many potential therapeutic targets that are inaccessible directly in vivo and, hence, require the transvascular administration of gene delivery vectors. A few candidate therapeutic applications include essential hypertension and pulmonary hypertension. Effective therapy in these diseases will likely require a gene delivery method capable of globally transducing the myocardium and selected other tissues and organs [36-38]. This paradigm is particularly valid in heart failure gene therapy where most authors agree that gene transfer should be as diffuse and homogeneous as possible [39,40].

2.3.1. Selective Coronary Catheterization with Antegrade Intracoronary Delivery

To date, one of the most preferred gene delivery methods involves catheter-based, percutaneous infusion of vector into the coronary arteries (Figure 2.3A). The benefits of this technique include its minimal invasiveness, the possibility of transgene delivery to all four myocardial chambers, and the delivery of vector genomes using
endovascular coronary catheterization—a procedure for which there is established clinical experience. Early reports using simple antegrade intracoronary delivery achieved very limited myocardial transduction efficiency [22,39,41,42]. The variability in transduction was due to a number of factors that included differences between animal species, biocompatibility of catheter and vector, different pharmacological agents used to permeabilize the vasculature, and vector-related variables such as vector serotype and titer [4].

Using intracoronary perfusion in explanted hearts, Donahue et al. reported highly effective gene transfer to the heart and identified critical parameters influencing the efficiency of intracoronary gene transfer. These included exposure time, high coronary flow rate and perfusion pressure, the use of crystalloid solution as opposed to whole blood, virus concentration, and temperature [37]. Several authors believe that aside from these factors, a major deficiency in intracoronary gene transfer is the short residence time of vector within the coronary circulation [39,43,44]. Attempts to resolve this shortcoming have resulted in a number of strategies. Logeart et al showed that brief interruption of coronary flow is required to obtain significant myocyte transduction during single-pass delivery in the isolated rat heart model [42].

In other experiments, this laboratory obtained similar results in vivo, when adenoviruses were delivered downstream of an occluded artery and when occlusion was maintained for 30 seconds following adenovirus injection [42]. This procedure raises questions as to the potential role of ischemia in enhancing gene transfer by increasing microvascular permeability. The authors also demonstrated that selective catheterization of the coronary venous sinus, which was transiently occluded and retroperfused with saline during their procedures, increased the pressure inside post-capillary venules,
which in turn improved gene transfer by increasing vector residence time in coronary vessels [39].

Hayase et al. used the technique of antegrade intracoronary gene delivery with concomitant coronary venous blockade. The myocardium was preconditioned with one minute of occlusion of the left anterior descending and circumflex arteries. Quantitative β-galactosidase analysis showed that gene expression was improved after selective coronary venous blockade [40]. Donahue et al. found that decreasing perfusate Ca²⁺ concentration or pre-treating with serotonin or bradykinin significantly decreased the exposure time necessary to achieve widespread transfection [45]. To prolong viral exposure time, Ding et al. pharmacologically induced transient cardiac arrest, while occluding the aorta and obstructed venous return to the heart. Cardiac arrest of two minutes allowed for transfection of 18% of cells, whereas an extended time to five minutes resulted in a cardiac transfection of about 43% of cells [43].

Increasing perfusion pressure and flow augments myocardial expression perhaps by increasing the fenestration width between capillary endothelial cells, permitting better viral transendothelial transfer and enhancing virus-myocyte interaction [46, 47]. Emani et al used an apparatus consisting of a constant flow infusion pump with pressure transducer and examined the effects of altering intracoronary flow rate, while obtaining a seal between the catheter and the coronary lumen. The results indicate that efficient cardiac transgene expression is dependent upon the infusion flow rate and requires an intraluminal seal. Excessive flow rate is associated with myocardial injury [48].

2.3.2 Nonselective (indirect) Intracoronary Delivery
Hajjar et al observed that adenovirus infusion into the left ventricle during brief aortic clamping results in efficient adenoviral gene transfer, perhaps due to the resulting high perfusion pressure inside the coronary vessels (Figure 2.3B). Later, to achieve diffuse cardiac gene transfer in vivo, the authors developed a catheter-based technique in rodents. In this approach, a catheter was inserted in the LV apex and advanced beyond the aortic valve. A high concentration of an adenoviral preparation was then injected through the catheter while the aorta and pulmonary artery were cross-clamped, distal to the catheter tip for a period of 10 to 40 seconds.

This method achieves grossly homogeneous transduction of cardiac myocytes throughout the left and right ventricles. By cross-clamping both the pulmonary artery and the aorta, the left ventricular end-diastolic pressure does not increase because blood return to the left ventricle is minimal. This method relies on the creation of a transcoronary myocardial perfusion gradient for vector delivery. This allows perfusion of the virus at relatively low downstream pressure, and the endocardium can be efficiently transfected. It is noteworthy, according to the authors, that aortic occlusion during aortic valvuloplasty is well tolerated in ill patients for periods of time comparable to those required for gene transfer in animal models [36,49].

Maurice et al used a similar technique in the leporine model, whereby a catheter was placed into the LV chamber through the apex of the heart. The adenovirus solution was injected while the aorta was cross-clamped for 40 seconds. After six days, the authors found global myocardial β-galactosidase expression in both ventricles. However, three weeks later, β2-AR over-expression was minimal [50]. This method shunts the virus down the coronary arteries, and global transgene expression is possible; yet, there is a risk of systemic ischemia and acute LV overload during the aortic cross-clamping,
and time must be limited [51]. This data was supported by Parsa et al., who showed in a rabbit model with cross-clamping of the aorta, a significant decrease in $dP/dt^{\text{max}}$, which they explained, indicates a negative effect on cardiac contractility after aortic occlusion with elevation of afterload [46]. Kaspar et al. also used aortic and pulmonary artery cross-clamping for indirect coronary delivery of AAV encoding GFP. Gene expression was evaluated at four time points up to one year after vector delivery, revealing 20-32% transmural gene expression in the left ventricle [52]. Eckhart et al. showed that in vivo myocardial gene delivery in rabbits using this LV/cross-clamp delivery method of either the β2AR transgene or a gene encoding βARKct can enhance cardiac function in normal hearts as well as failing hearts [53]. Variations of this method may include clamping of the aorta without pulmonary artery occlusion, occluding the distal rather than ascending aorta, and the use of hypothermia to prolong cross-clamp times [4].

2.3.3. Selective Coronary Sinus or Coronary Venous Catheterization with Retrograde Delivery

The feasibility and efficacy of percutaneous retrograde gene delivery by selective pressure-regulated retroinfusion of the coronary veins has been demonstrated by Boekstegers et al using a constructed apparatus, consisting of a pump unit, extracorporeal circuit, and retroinfusion catheter and suction device. The authors demonstrated advantages of retrograde delivery compared to antegrade and confirmed the results from several groups that blocking the venous outflow and coronary ischemia can significantly increase viral transfection of the myocardium [44, 54]. These authors believe that selective coronary retroinfusion prolongs adhesion time of the vector and increases endothelial permeability.
This finding has led to an important advance in this field of delivery, although this method does not reduce transduction of extra cardiac organs like the liver and lung [55]. Also, Hou et al. showed that a single retrograde coronary venous administration resulted in efficient regional myocyte transfection of human Del-1 and GFP. The authors believe that the coronary venous approach offers minimal washout and allows for controlled dwell times for longer exposure [56]. Kaye et al. developed the V-Focus delivery system for a minimally invasive percutaneous procedure, which was designed to isolate the coronary circulation from the systemic. This system includes percutaneous intervention catheters, extracorporeal pump-oxygenator circuit, infusion pump and monitors.

According to the authors, this system achieves superior myocardial gene expression in contrast to intracoronary delivery and is associated with lower systemic expression [57, 58]. Although the authors represent this method as a “closed loop” recirculation, careful analysis of the authors’ reported quantitative PCR results indicates that the vector genome concentration was 26 times higher in the liver than in the heart and thus isolation of the heart was not achieved as claimed [59].

In summary transvascular gene delivery provides the ability to obtain homogenous and efficient cardiac gene expression accomplished with rapid dilution of vector in circulating blood with significant extra cardiac expression. Surgical manipulations included temporary aortic/pulmonary clamping or coronary arterial/venous occlusion but both have significant clinical limitations and would require more translational research, preferably in large animals, prior to considering clinical application.

2.3.4 Ex vivo Gene Delivery
Even though there is a shortage of donor organs, over 2,000 heart transplants are performed annually in the United States. Ex vivo, myocardial gene delivery to the donor heart before reimplantation is an active area of investigation. Such an approach is clinically relevant in the setting of clinical heart transplantation and in surgical treatment of heart failure. Strategies to improve cardiac allograft function could potentially increase the longevity of current allografts, modulate the host immune response for prevention of allograft rejection, treat ischemia-reperfusion injury and make available additional marginal organs for transplantation that are currently not utilized [64]. Multiple studies have demonstrated the ability to transfact cardiac allografts with intracoronary, intramuscular, and transported delivery of vectors prior to transplantation [60,65-67]. Because gene delivery is carried out in the donor heart, total body virus exposure is limited in the recipient, thus making it clinically favorable [68]. Using intracoronary perfusion in explanted hearts, Donahue et al. reported highly effective gene transfer to the heart [37].

In the study of Griscelli et al., recombinant adenoviruses encoding β-galactosidase were injected into the coronary vessels of the harvested non-beating hearts of piglets at a dose 1010-1011 pfu. The hearts were maintained in contact with the vector-containing solution for one hour at 40C and the coronary vascular bed was then washed out by injecting 100 ml of cardioplegia solution and the last virus titer in the coronary sinas effluent was three logs below that of the infused viral solution. Gene transfer to allografts was evaluated four days after heterotopic reimplantation. In four out of 11 animals, transgene expression was detected in all cardiac areas, PCR analysis revealed minimal collateral organ transfection [60], and gene expression was not enhanced by the exclusion of blood [47]. Shah et al. performed ex vivo perfusion of
cardiac allografts with adenovirus encoding transgenes that enhance β-AR signaling [69]. The group demonstrated that five days after heterotopic transplantation, left ventricular systolic and diastolic performance was significantly increased in transfected grafts compared with controls. Further, Svensson et al. showed that 15 minutes of perfusion with AAV.CMV.lacZ was sufficient to result in transduction of 40% of cardiomyocytes after four weeks [24].

2.3.5 CPB and Cardioplegic Arrest

Another possible application of gene therapy is its use during operations with extracorporeal circulation or cardiopulmonary bypass (CPB), which has become a routine cardiac procedure. In fact, in 2009 more than one million cardiac procedures that depend upon CPB were performed worldwide. Potentially widespread clinical application would involve intracoronary (antegrade or retrograde) gene transfer in the setting of CPB and cardioplegic arrest. The ability to augment myocardial performance with gene transfer could potentially reduce the need for long-term inotropic or mechanical support in the post-bypass setting, avoiding the complications associated with these interventions [64].

High-risk patients undergoing revascularization or valve replacement with coexisting severely reduced ventricular function and inherited forms of cardiomyopathy might particularly benefit from concomitant gene therapy. Bridges et al. and Davidson et al. first hypothesized that cardiopulmonary bypass may facilitate cardiac-selective gene transfer using recombinant replication-deficient adenovirus [70,71]. The absence of a significant influence of cold temperatures on transgene expression in an in vivo model with CPB was described by Jones et al. [72]. They also demonstrated the presence of
crystalloid cardioplegia compared with blood cardioplegia within the coronary circulation had no effect on transgene expression and hypothesized that endothelial contact with cardioplegia and the associated relative ischemia likely increased endothelial permeability [73]. Ikeda et al. evaluated the feasibility of restoring δ-sarcoglycan deficiency in cardiomyopathic hamsters after injection of a cardioplegia solution containing an adenoviral vector encoding δ-sarcoglycan into the aortic root [74]. At one and three weeks after transfection, immunostaining showed extensive restoration of deficient membrane proteins with significantly less progression of LV dysfunction compared with controls. Davidson et al. demonstrated the feasibility of myocardial gene delivery during CPB with cold, hyperkalemic cardioplegic arrest in the porcine model [71]. (Figure 2.4A).
Unlike previous studies that utilized a single-pass perfusion technique, Bridges et al. were the first to create an isolated “closed loop” recirculating model of vector-mediated cardiac gene delivery in the large animal heart using cardiopulmonary bypass with an antegrade delivery approach, allowing for vector recirculation for 20 minutes [70]. Later, they used CPB with high-pressure retrograde coronary sinus infusion with multiple-pass
recirculation of vector through the heart and washed out of the cardiac circuit prior to weaning from CPB, which limited extracardiac gene expression (Figure 2.4B). They were able to show an increase of several orders of magnitude in cardiac marker gene activities compared with controls. Furthermore, there was minimal gene expression in the liver and other collateral organs [75]. These results validate this surgical technique as a potentially clinically translatable approach for cardiac gene therapy in carefully selected patients.

In summary, despite the allure of ex-vivo and CPB-mediated gene delivery approaches, it should be noted that one cannot exclude possible attendant morbidity. Morbidity may be related to technique-associated complications as well as the additional CPB time required. Finally, these methods have not been translated to clinical trials, and the only evidence we have that they are effective is founded on the basis of data derived from experimental studies. Therefore, it is difficult to judge objectively about the likely clinical efficacy of these gene delivery platforms.

2.4 Cardiac Gene Delivery in Clinical Trials

A significant proportion of patients with myocardial ischemia and congestive heart failure remain refractory to pharmacological therapies and unsuitable for percutaneous or surgical interventions. Trials involving gene therapy in patients with ischemic heart disease have been undertaken to stimulate angiogenesis. Rosengart et al reported a Phase I clinical trial involving 21 patients with clinically significant coronary artery disease that utilized adenovirus encoding human VEGF 121 cDNA with a goal to induce therapeutic angiogenesis. The vector construct was administered by direct myocardial injection into an area of reversible ischemia either as an adjunct to coronary
artery bypass grafting or as sole therapy via a mini-thoracotomy. In both groups, coronary angiography and stress sestamibi scans suggested improvement in angina class patients after therapy [80,32,33].

Percutaneous catheter-based myocardial gene transfer of naked plasmid DNA encoding phVEGF-2 was tested in human subjects by Vale et al. [81]. After the completion of electromechanical mapping, the injection catheter was introduced percutaneously via a femoral arteriotomy across the aortic valve into the LV and the needle was advanced 4 to 6 mm intramuscular to administer six injections of plasmid DNA into the ischemic myocardium. All patients experienced reduced angina, reduced nitroglycerin usage and improved myocardial perfusion by SPECT-sestamibi scanning. Also, results of single intracoronary administration of Ad5-FGF4 show evidence of favorable anti-ischemic effects in patients with stable angina pectoris [82]. In this study the transgene was infused over a period of 90 seconds through subselective catheters into all major patent coronary arteries that could be engaged. Nevertheless, it should be noted that these Phase I studies have not measured the amount of recombinant protein produced; hence, it has not been possible to make accurate, quantitative determinations of the relative efficiency of gene transfer, and a placebo-induced therapeutic effect cannot be excluded.

Hajjar et al and Jaski et al performed the first-in-human Phase I/II clinical trial with single intracoronary infusion of AAV1.SERCA2a in advanced heart failure. Standard percutaneous catheter engagement technique with the coronary arteries was performed. This usually involves delivering two-thirds of the dose to the anterolateral and one-third to the posterolateral myocardium, based on the coronary anatomy. Of the nine patients,
several demonstrated symptomatic, functional and biomarker improvements from baseline to month six. The Phase II results largely showed similar results [83,84].

2.5 Conclusions

Gene delivery technology and science has improved expeditiously as demanded by the growing pipeline of therapies for various cardiovascular diseases. Translational problems have surfaced through clinical implementation and are associated with dosing at sufficient levels to achieve therapeutic benefit while remaining within safety limits. The ideal method for a particular cardiac gene therapy application maximizes therapeutic expression with the least required dose and delivery efficiency at the cardiovascular tissue is paramount. Despite significant advancements, there is still great room for improvement to increase both safety and efficacy through developing more advanced systems alongside the clinical science. Minimally invasive catheter based systems remain at the forefront due to their ease of use in the clinic, but must address problems associated with compromised vessels. As reviewed in this chapter, advanced catheterizations systems including pharmacologic agents to increase vector distribution from the primary infusion site have been implemented. It is expected this trend will continue given that 80% or more of advanced patients present with moderate to severe coronary artery disease. Higher risk surgical mediated delivery methods featuring cardiopulmonary bypass at present are the only known means to achieve the highest possible transduction through the vascular anatomy, but at the cost of complexity. Furthermore these systems would be only reserved for a limited number of patients undergoing cardiac surgery for a separate indication.
Chapter 3 will describe the rationale for a unique contribution to the direct myocardial delivery repository, which is currently limited to standard IM injection. Direct injection methodology are primarily reserved for local, peri-infarct area but could also be implemented for other therapies provided the risk/reward and clinical strategy are feasible.

References


3.1 Specific Aims and Hypotheses

Despite a well characterized gene transfer vector in AAV, high levels of transfer in larger species have been problematic both due to scale (i.e. 100 times greater myocardial mass) and more restrictive anatomical barrier factors. Transfer problems in general are not realized in murine models, since successful AAV mediated gene transfer can be achieved with a simple tail vein injection. As summarized in detail in Chapter 2, cardiac gene delivery systems basically fall into two major categories:

1. Transvascular Based Infusion Methods (i.e. access and transfer of AAV through capillary network vessels into myocardium)
2. Direct Myocardial Injection Methods (i.e. AAV directed right into myocytes through mechanical means)

By physician standards, either of these categories can be considered minimally invasive with recent advances in image guided catheterization. Also as outlined in previous studies, each specific technique within its respective category has its own advantages and disadvantages. Transvascular methods, particularly antegrade intracoronary infusion, are still the mode of choice given for AAV products since they are routinely utilized in the clinic by interventional cardiologists. These following requirements however have not been met completely with current transvascular techniques:

(1) Limit or stop leakage of AAV in the system and demonstrate greater gene transfer in the heart versus collateral organs
(2) Provide a solution for patients pre-screened with antibodies to AAV

(3) Allow for global myocardial gene transfer with the highest possible efficiency within safety limits (e.g. not damage delicate vessels).

In response to the problems with other transvascular methods described in (1) (2) and (3), our lab has developed the most sophisticated transvascular gene delivery system, molecular cardiac surgery with recirculating delivery (MCARD). (**Figure 3.1**)

![Figure 3.1 MCARD, Advanced Dual Perfusion Transvascular Delivery Featuring Complete Cardiac Isolation](image)

MCARD was inspired from the need to address these demands from larger organisms: higher cardiac specific transduction efficiency and to reduce systemic exposure. MCARD is a dual perfusion system featuring complete cardiac isolation in situ, which recirculates a high concentration of AAV (i.e. cardiac circuit is bloodless) with complete protection from the system circulation. It offers the unique ability to deliver
AAV through the much more favorable dimensions of coronary venous system, while also not working against the beating heart (e.g. CPB arrests the heart). When comparing the MCARD vs. standard intracoronary infusion delivery methods with the same vector dose, greater than 400 fold higher expression was found in the MCARD animals with no immune response [16,17]. Despite unprecedented transduction performance and the ability to minimize the risk for collateral expression, it is clear that the cost of invasiveness of this system relative to catheter based approaches will limit MCARD as an option for many patients unless substantial risk/benefit is demonstrated. This type of delivery method would most likely be indicated for Class III-IV heart failure patients receiving adjunctive therapy.

On the other hand, the direct methods by and large are seemingly confined to needle based systems. Due to the simple ease of use, needle injection into heart muscle was the first method performed at the large animal and human scale for gene therapy. Once the heart is exposed via thoracotomy or accessed via catheterization, control and selection over the desired injection sites is attainable.

Despite this advantage, it is well known now that several critical disadvantages with AAV needle injections have resulted in a clear bias in favor of vessel infusion techniques. The problems of scale and immunogenicity have seemingly placed a dead end with using this approach for a clinical application. With regard to scale, this is evidenced by the fact that 4-5 injections were found sufficient to achieve beneficial therapeutic expression levels in small animal models (heart weight = 3-6 g) versus greater than 200 to achieve a substantially reduced expression profile in larger species (heart weight = 350 – 600 g). Limitations in volume injection per site, the number of available sites to inject, and adverse immune reactions have been documented by various investigators [18]. Furthermore there is an often overlooked problem of
inadvertent systemic leakage most likely attributable to myocardial wall perforation during the injection process.

**Given the limitations of the most advanced transvascular and needle injection methods, there is a need for a direct viral vector cardiac specific delivery system that would offer greater myocardial gene transfer per injection. Understanding the immune consequences as a function of delivery and vector selection has not been explored in detail. The scope of this work seeks to expand both the methodology and delivery science of direct myocardial injection.**

**Global Hypothesis:** We hypothesize that liquid jet injection technology can offer an alternative delivery platform with superior cardiac specificity competitive with intracoronary infusion and superior to needle injection. In addition, evaluating the role of delivery profiles in the context of the host response with regard to delivery and vector selection can improve outcome. Specifically, this work will test four hypotheses that are described in detail below. The overall goal of the work is to obtain a better understanding of how to address both the problem of scale and immunogenicity associated with direct intracardiac injections, especially within the ischemic disease setting since this is the intended clinical application.

**Specific Hypothesis #1:** The mechanical liquid jet device approach can be optimized for a cardiac gene therapy application to maximize cardiac gene transfer as measured per myocardial cross sectional area, while reducing unwanted systemic exposure as measured by collateral liver.

**Specific Aim 1:** Define and optimize engineering parameters for the liquid jet injection device for the cardiac application in an acute rodent model and ex vivo large animal setting to define a safe, yet effective operating transfer ranges.

Optimization is defined as in vivo tolerance whereby significant cardiac damage is
avoided, yet sufficient myocardial transfer is achieved suitably. This aim will be achieved with ex vivo testing, quantitative fluid mechanics evaluation of the jet, and limited non-survival animals.

**Specific Hypothesis #2:** The mechanical liquid jet device approach will result in increased myocardial retention, cardiac specificity (i.e. heart transfer to collateral organ exposure), and wider range delivery area versus IM injection, while in the range of the best intracoronary delivery approach in a rodent model.

**Specific Aim 2:** Perform a two phase therapeutic delivery study to the beating heart in an acute rodent model to evaluate the liquid jet approach against traditional methods in practice. The methods to evaluate against are: A. Standard IM Injection B. Non-selective Intracavitary Infusion C. Intracoronary Infusion featuring Occlusion. The first phase of the study will evaluate immediate retention following 10 minutes of delivery in a non-survival model followed by a repetitive second phase study using AAV9.GFP marker therapy in the normal heart.

**Specific Hypothesis #3:** Liquid jet injection will achieve efficacious therapeutic AAV9 mediated S100A1 gene therapy over standard IM injection, while minimizing collateral organ exposure and minimizing the immune host responses. It is argued that a disperse, more homogenous expression pattern with the same magnitude range as IM delivery will yield greater efficacy.

**Specific Aim 3:** Perform a chronic ischemic infarction 10 week model study to evaluate delivery of a single dose $1.2 \times 10^{11}$ Vgp of ssAAV9.S100A1 vector featuring a head to head format comparing liquid jet vs. IM injection. Outcome assessment will be a comprehensive, compiled data set of: (1) AAV9.S100A1 genome copy detection (2) Quantitative proteomic S100A1 (3) CD38 Histological marker of Immune activation in cardiac tissue (4) Serological markers of global inflammation via
cytokine panel. In this aim there will be 4 assigned groups: A. IM Injection with Saline B. Liquid Jet injection with Saline C. IM injection ssAAV9.S100A1 and D. Liquid Jet injection ssAAV9.S100A1

**Specific Hypothesis #4:** Altering therapeutic vector and delivery strategy can significantly alter outcome and in vivo performance.

**Specific Aim 4:** Two key testing parameters that may significantly alter host responses are offered: A. Using self-complementary double stranded vector equivalent B. Co-delivery of anti-inflammatory drugs to minimize host response during initial delivery phase. The first part of this aim will be achieved by a simply repeat of the animal protocol in Aim#3, however this time with a more potent double stranded or self complementary scAAV9.S100A1 vector. The second part explores a theoretical drug/gene combination approach with controlled release formulations in vitro.

### 3.2 Overview of Research and Impact

The motivation for advancing the topic of improving direct delivery of AAV mediated gene products is an important research discipline, especially since enrollment in cardiac gene therapy trials have been and are expected to increase in the near future. Despite significant advancements in the field overall, most of the attention in gene therapy is geared toward vector engineering and target identification but not delivery. Chapter 1 outlined all of the key therapeutic mechanisms, targets, and rationale for AAV mediated gene therapy, whereby S100A1 presents with significant potential to translate in future clinical trials. Chapter 2 provides a thorough review and key insightful conclusions on state of the art cardiac gene transfer systems and concepts within the context of clinical applications.
This research seeks to provide a clear, yet comprehensive approach to designing, building and testing a new direct cardiac injection technique as well as identify key delivery variables with existing systems in its evaluation. Chapter 4 summarizes the rationale and technical development phase of the needleless liquid jet injection concept. Chapter 5 is the heart of this work, which characterizes the refined concept in both an acute retention and AAV mediated marker gene expression model. It is here where the technology is evaluated against existing methods with key insights offered for both future development of cardiac gene delivery applications. Chapter 6 presents a comparative head to head assessment of liquid jet injection against standard IM delivery in a chronic ischemic myocardial infarction model featuring the ssAAV9.S100A1 therapy. The translational aspects of direct cardiac delivery are thoroughly evaluated in terms of outcome, whereby cardiac performance, transduction efficiencies and host response endpoints are paramount.

From there, two novel therapeutic concepts are explored to improve direct cardiac delivery: (1) The use of self-complementary (scAAV9.S100A1) and (2) Co-delivery of anti-inflammatory drugs with AAV products to increase efficacy. Chapter 7 provides a complete pilot study assessment with the same methodology in Chapter 6 to explore whether or not a different vector with S100A1 may result in an improved outcome. Chapter 8 conceptualizes a forward thinking, theoretical concept of a controlled release drug/gene combination approach with a feasibility test in vitro. Finally, Chapter 9 capstones key points for future cardiac gene therapy applications with respect to delivery science. The work ultimately advances the body of knowledge with the aim to improve outcomes in cardiac gene therapy, specifically when using direct administration routes.
4.1 Introduction

The previous chapters provided a solid foundation describing the rationale for a new direct injection cardiac gene delivery method. In this chapter, we briefly review the core science of ballistic delivery, its track record in similar applications, and the executed development path in this dissertation. Needleless liquid jet injection is characterized as a ballistic delivery based approach. The technology was originally developed in the early 1960’s and applied for these simple medical applications: mass routine vaccinations, administration of local anesthetics, tuberculin testing and dermatology treatments of minor skin lesions. Closely related, the “gene gun” concept has a solid foundation in molecular biology. In this chapter, the literature highlighting key results is briefly discussed in Section 4.1.1 below for the gene gun, while section 4.1.2 delves into the related, but fundamentally different liquid jet application. Finally beta testing and modification of the concept for translation into the cardiac anatomy for a gene therapy application is detailed in sections 4.3 and 4.4, respectively.

4.1.1 Gene Gun Ballistic Delivery Principles

Particle bombardment (also called Gene gun or ballistic DNA transfer) utilizes heavy metal particles introduced with a high velocity pressurized inert gas into the target cell (Figure 4.1).
Naked DNA can deposit onto these particles, and after entering the cell they are gradually released. Acceleration can be achieved by a high-voltage electric spark or a helium pressure gun. For optimal gene expression the following parameters have been taken into account: (1) the properties, density and sizes of gold particles for bombardment; (2) the DNA doses, and (3) the discharge voltage for optimal gold particles penetration. Using a sub-microgram amount of DNA per bombardment, 1000 to 10,000 copies of DNA can be delivered to each target cell [1,2].

The fundamental principal of the gene gun is the transfer of DNA-coated particles into the cell through the holes in the plasma membrane resulting from the initial injection. Some authors believe that it may be possible to employ a gene gun to transfect cells that are relatively resistant to other delivery systems [3]. In this method DNA or RNA adhere
to metal particles (gold or tungsten) then the DNA-particle complex is accelerated and shot into the target tissue. Uncoated metal particles could also be delivered through a solution containing DNA surrounding the cell thus picking up the genetic material and proceeding into the living cell.

Despite some interest in translation, the primary problem with the gene gun is the technical maintenance cost in addition to complexing vectors to relatively large metallic particles. Given the stability and formulation advantages of modern viral vectors, ballistic delivery of active therapeutic is more cost effective through liquid jet delivery.

4.1.2 Liquid Jet Delivery Principles

A ballistic method jet injection is performed using a high-speed pressurized gas, usually CO2 but the driving gas can be changed per application. The injection is comprised of piercing center and surrounding microjets, whose effects create pores in cellular membranes to facilitate intracellular gene transfer (Figure 4.2).
The penetration power depends on three factors: the applied pressure of the gas, the dispersion factor of the jet as a function of the nozzle bore and the tissue’s mechanical properties [4]. Levels of gene expression by jet injection were found to be 50-fold higher than by conventional needle injection in one preliminary study [5]. The jet injection gene transfer is usually well tolerated without side effects provided that the settings are optimized. This method is described only for non-viral based techniques of gene transfer and uses a high pressure.

Macromolecules of varying chemistries and properties can be delivered ranging from stable active compounds, DNA and to a lesser degree proteins. Pressure used is typically less than 3-4 bars with resultant velocity profile of the droplets ranging from 100 m\(\text{s}^{-1}\) to 300 m\(\text{s}^{-1}\). The velocity of the fluid in the jet injection contributes to the
distribution in the tissues, whereas the diameter of the jet and the injected volume limit the penetration depth [6]. The efficiency of this method depends on nozzle diameter (150-300 µm), velocity of the liquid jet and distance between the nozzle and surface of the tissue [7].

The penetration of the injected molecules inside the cells subsequently results in direct uptake, maximizes bioavailability in the tissue environment for additional molecules, and worst case inadvertently reaches off target organs via systemic circulatory penetration. The high pressure of the jet creates a hole in the tissue surface; the depth of this hole is increased due to the accumulation of fluid. The use of intramuscular jet injection of DNA combined with electroporation was demonstrated to be feasible in a mouse model [8]. We and others could not find publications on the applications of this method for cardiac gene therapy [9].

4.2 Device Description & Specifications

The handheld needleless liquid jet (DERMOJet™) was purchased from Robbins Instruments (Chatham, NJ). The device itself was invented by Dr. A. Krantz in France with the medical device manufacturer Akra DermoJet (Pau, France). The device (Figure 4.3) is comprised of a stainless steel design with polycarbonate reservoir with dimensions: 8 ounces (weight), 6.5 inches (length), and 11/16th inches (Diameter) injector head. The exit orifice is 0.0079" (32 gauge; 235µm) diameter in a jewel shape design. The size transmission limit is crystalline particles up to 200 microns.
The device is actuated by an internal spring mechanism which drives a piston that subsequently accelerates a fixed volume fluid load. Injection is executed by a loading lever mechanism which sets the spring lock element, while the push button trigger releases for the acceleration of drug through the orifice. The ejection orifice features an internal jewel shaped configuration for a dispersive effect. The ejection volume is fixed at 100μL at the ideal retention limit of most tissues. Various mechanical components in the device are tunable, most notably the spring and orifice characteristics.

4.3 Beta Testing

4.3.1 Manual Injection

My first experience testing the device after priming was to assess both the level of penetration and inflammatory response first hand. At the manufacturer’s settings, I injected sterile saline into my palm approximately 0.5cm above the skin surface. An immediate, sharp burst provided transfer beneath the dermal layer with a small pinhole entrance site marked by spot bleeding. The inflammation and pain from the injection peaked at 24 hours, and diminished after 48 hours. It was determined that if left at this

Figure 4.3 The DermoJet Needleless Liquid Jet Device

The device is actuated by an internal spring mechanism which drives a piston that subsequently accelerates a fixed volume fluid load. Injection is executed by a loading lever mechanism which sets the spring lock element, while the push button trigger releases for the acceleration of drug through the orifice. The ejection orifice features an internal jewel shaped configuration for a dispersive effect. The ejection volume is fixed at 100μL at the ideal retention limit of most tissues. Various mechanical components in the device are tunable, most notably the spring and orifice characteristics.

4.3 Beta Testing

4.3.1 Manual Injection

My first experience testing the device after priming was to assess both the level of penetration and inflammatory response first hand. At the manufacturer’s settings, I injected sterile saline into my palm approximately 0.5cm above the skin surface. An immediate, sharp burst provided transfer beneath the dermal layer with a small pinhole entrance site marked by spot bleeding. The inflammation and pain from the injection peaked at 24 hours, and diminished after 48 hours. It was determined that if left at this

Figure 4.3 The DermoJet Needleless Liquid Jet Device

The device is actuated by an internal spring mechanism which drives a piston that subsequently accelerates a fixed volume fluid load. Injection is executed by a loading lever mechanism which sets the spring lock element, while the push button trigger releases for the acceleration of drug through the orifice. The ejection orifice features an internal jewel shaped configuration for a dispersive effect. The ejection volume is fixed at 100μL at the ideal retention limit of most tissues. Various mechanical components in the device are tunable, most notably the spring and orifice characteristics.

4.3 Beta Testing

4.3.1 Manual Injection

My first experience testing the device after priming was to assess both the level of penetration and inflammatory response first hand. At the manufacturer’s settings, I injected sterile saline into my palm approximately 0.5cm above the skin surface. An immediate, sharp burst provided transfer beneath the dermal layer with a small pinhole entrance site marked by spot bleeding. The inflammation and pain from the injection peaked at 24 hours, and diminished after 48 hours. It was determined that if left at this
current setting transfer would not be ideal since the piercing jet did not provide adequate
distribution but only a deep penetrating bolus in a locale. Subsequent manual tests at
greater distances revealed much less piercing, but it was interesting to note significant
fluid retention under the surface of the skin with seemingly no pain from the injection was
observed. This transfer distance whereby retention was achieved at the lowest level
was about 24 inches from the nozzle.

4.3.2 Explanted Rodent Hearts

Methylene blue dye was loaded into the device and fired directly on the surface
of explanted rodent myocardium samples, which are generally 1-2 cm thick transmurally
across the left ventricle. A clear “jet track” line was noted, with less than 1mm
distribution from it. It was noted that retracting the device at 2 inch increments resulted
in more surface coverage and in some cases complete penetration. At greater distances
this effect was erratic and a “spray” behavior was noted indicating only superficial layer
penetration. Given that the cardiac gene therapy application requires both maximal
transfer without damage and consistency, several modifications to the device were
needed for application.

4.4 Device Modifications for the Cardiac Gene Therapy Application

Following the very early experiments described previously, it was readily
determined that optimizing both the pressure and distance settings would permit ideal
transfer within safety limits. Very early acute model rats suffered severe cardiac
bleeding events and or arrest with distances less than 8 inches at the manufacturer
settings. Safety limits were defined as tolerance of at least 6 consecutive 100µL
injections without significant EKG changes or morbidity.
4.4.1 Pressure and Velocity Optimization

The original experiments indicated that the device as is would not be suitable for any cardiac application since the potent jet was piercing with high force and simultaneously providing limited distribution. Advancing to distances greater than 24 inches from the target organ were required to remotely obtain anything of value in terms of dye transfer, thus it was imperative to modify the settings. Since the device was actuated by a spring, we worked with the manufacturer to cut the constant down by 80% of the original length. The original jet velocity was measured and confirmed by the factory at just over 330 m/s out of the nozzle. The modified device yielded roughly 110 m/s exit velocity with a 100-200kPa driving pressure range. As a means to control the distance between the exit nozzle and the target, the device was mounted to an arm apparatus as shown in (Figure 4.4).

![Mounted Device to Control and Optimize Delivery Distance](image)
References


CHAPTER 5

Needleless Liquid Jet Injection: Validation Studies in Normal Hearts


5.1 Introduction:

Heart failure (HF) remains a significant burden to the global healthcare system with annual costs exceeding $32 billion [1]. Besides the limited number of donor organs available for transplantation, current medical and device therapies have not significantly reduced disease burden. Through careful scientific investigation and clinical development, gene therapy has emerged as a potential strategy to directly treat myocyte dysfunction at the molecular level. A number of genes have been identified to impact these critical disease pathways - which if expressed robustly in the myocardium - enhance contractility, promote survival, and in some cases completely reverse chronic HF [2-4]. Adding to this momentum, novel microRNA and angiogenesis targets promoting genetically induced regeneration are accelerating through various developmental stages [5,6]. Therefore, the recent basic scientific progress has bolstered an already thriving pipeline of viral mediated gene therapeutics and it is expected that new clinical trials will be forthcoming.

A pivotal milestone for the cardiovascular gene therapy field was the successful launch of the Calcium Up-regulation by Percutaneous Administration of Gene Therapy in Cardiac Disease (CUPID) trial featuring adeno-associated viral vector (AAV) encoding the contractility enhancing sarcoplasmic endoreticulum ATPase (SERCA2a) gene [7]. This key trial has provided reason for cautious encouragement as favorable results were achieved in the high dose group versus placebo [7,8]. Despite these encouraging results
in the high dose group, achieving efficacy with lower doses remains a concern given that no improvement was found in patients receiving less than the maximum 1 x 10^{13} DNAase resistant viral particles (vp) [8]. Delivery efficiency is the main concern, whereby higher doses are needed to achieve sufficient expression levels, which inherently increases the risk for adverse events. Therefore, more efficient delivery systems would most likely improve clinical outcomes.

The administration route currently in clinical trials is antegrade intracoronary infusion, given its safe and effective implementation in clinical practice [9]. From a therapeutic AAV transfer perspective however, standard infusion approaches are critically limited by these factors: (1) Prohibitive physical barriers (e.g. pre-capillary sphincters and endothelial barriers) preventing viral vector diffusion into the interstitial compartment [10] (2) Plaques and inflammatory elements in primary vessels, which are common in HF patients with advanced coronary disease (3) neutralizing antibodies and blood components which limit viral particle bioavailability [11] (4) Inadvertent systemic exposure via general circulation and finally (5) Exclusion of many patients seropositive for AAV. Sophisticated modifications to percutaneous catheter infusion (e.g. single or concomitant retrograde/antegrade balloon occlusion systems [12] and even surgical closed loop recirculation systems [13]) have been advanced recently to increase efficiency at the cost of complexity. Some of these systems may be implemented in future trials, however most would either exclude high risk patients or impose additional safety concerns.

An alternative to infusion methods in an effort improve efficiency in terms of cardiac specificity is direct myocardial delivery. Direct injection methods circumvent the blood and anatomical barriers by directly administering product into the myocardium, which additionally offers the advantage of precise site selection. For this reason direct
intramuscular (IM) injections were attempted with adenovirus in the very first series of angiogenesis trials [14], but yielded poor results. Cited problems with this route are limited distribution per site and inflammatory responses [15,16]. There has been little work to date to improve direct myocardial delivery applications despite these concerns and limitations.

In this study we present a novel approach to improve direct cardiac gene delivery using a needleless liquid jet methodology. Similar ballistic delivery methods were utilized in very early DNA transfection studies via gene gun [17,18], but to date none have attempted delivery of AAV therapeutics as a potential clinical strategy. The liquid jet application is essentially a device concept that accelerates and disperses the therapeutic at a targeted myocardial site. The core hypothesis offered is that this approach with optimized settings could result in: increased therapeutic retention in the initial delivery phase, achieve significantly more total myocardial expression per dose, and in addition yield a more homogenous profile around the injection site. Overall, this would increase efficiency in terms of transduced muscle per delivery site unit and decrease the likelihood of an immune response.

This proof of principle study optimizes the needleless liquid jet strategy for cardiac applications and evaluates the method in a series of rodent experiments. In each set of experiments, the liquid jet method is compared with three existing methods: Intramuscular injection (IM), Single pass LV intracavitary infusion (LVIC) infusion, and LV intracavitary infusion with aortic and pulmonary artery occlusion (LVIC-OCCL). In the first series of experiments we evaluate each methodology’s immediate myocardial retention profile in an acute model. In the second series we utilize a highly cardiotropic AAV9.EGFP marker gene construct to simulate a more realistic long term gene expression scenario. Finally, a preview of the needleless liquid jet method’s translational
potential in the large animal setting is performed with post-harvest specimens. Key translational aspects for direct injection and infusion approaches are then discussed for future directions to improve cardiac gene delivery technologies.

5.2 Materials and Methods:

Animal Care and Handling

40 rats were procured from Charles River Laboratories and handled in accordance with IACUC guidelines at the Cannon Research Center at Carolinas Healthcare System. Normal Sprague Dawley rats (males, weight 300 g) were obtained for all experiments.

Needleless Liquid Jet Injection Device Optimization & Specifications

A DermoJet™ liquid jet device (Robbins Instruments, Chatham NJ) was acquired and modified for cardiac delivery applications. The spring actuator length was reduced by 80% to 1.9 cm, which resulted in a significant driving power reduction. A super speed camera (Zeiss International, Thornwood NY) at the University of Pennsylvania Complex Fluids Laboratory was used in a custom analysis suite to quantify jet fluid exit velocity and to compute estimated driving pressure. The nozzle jet velocity was measured at 110 m/s based on a 30,000 images per second capture rate. The driving pressure ranged from 150 – 250 kilopascals (kPa). Prior to the modification, the factory setting results were 330 m/s velocity at greater than 550 kPa.

The next key parameter to optimize for a surgical application was the injector head distance normal to the target cardiac plane. To execute, the pen configuration injector was mounted to a stainless steel arm apparatus secured to a solid base. For alignment
with the cardiac targeted surface plane, an optical system consisting of a field laser and
digital level were utilized in conjunction to ensure accuracy between injections.

The safe liquid jet delivery distance range was determined by performing a limited
number of acute studies with methylene blue dye injection. Safety criteria were defined
as tolerance of 6 consecutive projected needleless injections without visible bleeding
and EGC changes. Confirmation of hits was validated via marks by surface retention of
dye. The optimal distance range was determined between 8-10 inches (i.e. nozzle tip to
heart surface). This range was found effective where sufficient dye was retained
transmurally, but no incidence of major cardiac damage was observed.

**Rhodamin B Dye Preparation for Acute Studies**

Rhodamin B Dye (Sigma Aldrich, St. Louis MO) was prepared in (50 µg/mL) working
aliquot concentration. Rhodamin B dye was selected based on easy formulation and
high performance in muscle tissue types for the acute retention purpose.

**AAV9.EGFP Viral Vector Production for Survival Studies**

All Recombinant AAV9.EGFP was sourced from the University of Pennsylvania Vector
Core (Philadelphia, PA). Briefly, vector production, harvest, purification and testing were
done from cell lysates by the Penn Vector Core. The recombinant vector used in this
study contains an AAV serotype-9 viral capsid and a single strand: ~4.5-kb DNA
containing the EGFP marker gene driven by a cytomegalovirus immediate-early
promoter/enhancer, a hybrid intron, and a bovine growth hormone polyadenylation
signal. Manufacturing process details can be found
([http://www.med.upenn.edu/gtp/vectorcore/](http://www.med.upenn.edu/gtp/vectorcore/)). Following production, viral preps were
pooled from individual lots and aliquoted in sterile vials each containing a dose of $2 \times 10^{11}$ vp in 500µL volume.

**Acute Model Protocol: Retention of Rhodamin Dye**

The endpoint selected for retention in the cardiac cross sections was % myocardial area positive for Rhodamin B dye level shortly after cardiac delivery. Since global, homogenous distribution is the ideal outcome for contractility genes, this measure essentially determines what areas of myocardium are accessed per route.

All rats were anesthetized with ketamine IM, intubated and placed on a Harvard Apparatus ventilator and maintained with isoflurane 1-3%. A left thoracotomy was performed and the pericardium excised to expose the beating heart. Each animal was then assigned to 1 of 4 acute delivery groups (n=6 each) [IM Injection, Needleless Liquid Jet, LVIC-OCCL, LVIC)] to receive a single 500µL fixed dose Rhodamin B Dye. A 10 minute waiting period commenced after delivery. The heart was then harvested and sectioned immediately following.

**Delivery Route Descriptions**

(Figure 5.1) provides a conceptualized illustration of all 4 cardiac delivery approaches of IM Injection, Needleless Liquid Jet injection, Left ventricular single pass infusion (LVIC) and Left ventricular infusion with concomitant occlusion of the aorta and pulmonary vessels (LVIC-OCCL) applied in the model studies.
The technical delivery details for execution were as follows:

**IM Injection**

A 1 mL 30 gauge syringe was loaded with the 500µL of volume. Injection sites on the left ventricular apex, anterior wall, and lateral wall were marked with a surgical pen. Two injections were performed in the mid-ventricular plane for each of the anterior and lateral walls, with a single injection in the apex for 5 total injections. Injections were spaced about 5 mm.

**Needleless Liquid Jet Injection**
500µL delivery volume was loaded into the chamber with a residual capacity of at least 200µL additional to ensure the 5th shot maintained the same kinetics as per DermoJet™ manufacturer instructions. A digital level and laser was used to ensure adequate degree and plane alignment with the middle left ventricular plane. Five separate shots, each containing 100µL, were fired perpendicular to the LV surface. Hits were confirmed by detection of pinhole piercing on the epicardial surface with surrounding coloration changes.

LVIC-OCCL Infusion

Silk suture was placed in two locations, the proximal aortic arch and the left pulmonary artery. Following, a 16 gauge needle introducer was used to catheterize the left ventricle from the apex. A catheter was secured in place with a syringe containing the 500µL of therapeutic. Just prior to infusion, concomitant occlusion of both snares with slow infusion was conducted over a 10 second period. The occluding sutures were then released after the 10 second period.

LVIC Infusion

A 16 gauge needle introducer was used to catheterize the left ventricle from the apex. A catheter was secured in place with a syringe containing the 500µL of therapeutic. Slow infusion in the left ventricle over 10 seconds was performed without interruption.

Survival Model Protocol: Expression Profile Following Delivery of AAV9.EGFP

All rats were anesthetized with ketamine IM, intubated and placed on a Harvard Apparatus ventilator and maintained with isoflurane 1-3%. A left thoracotomy was performed and the pericardium excised to expose the beating heart under aseptic conditions.
conditions. Each animal was then assigned to 1 of 4 delivery groups (n=4 each) following the same delivery protocol for [IM Injection, Needleless Liquid Jet, LVIC-OCCL, LVIC]) groups. The therapeutic was a single 500 µL fixed dose 2 x 10^{11} (vp) of single stranded adeno-associated virus encoding green fluorescent protein (ssAAV9.EGFP).

Following delivery, the incision was closed and each animal was recovered and monitored to 6 weeks. At the 6 week peak expression endpoint, the harvest commenced.

**Tissue Harvest and Cross Section Preparation**

For each Rhodamin Dye animal, middle and basal left ventricular cardiac sections and a single liver cross section was acquired. Similarly, for each AAV9.EGFP therapy animal, the left ventricle was sectioned in 2 plane regions: Middle and Basal regions to assess the degree of AAV mediated EGFP dispersion per route. A single liver section was also acquired. All cross sections were snap frozen in OCT. Ten micron sections were cut on a cryostat and mounted on glass slides for further analysis.

**Immunohistochemistry Staining for EGFP Detection**

Tissue from rats receiving the ssAAV9.EGFP was stained with a rabbit polyclonal Anti-GFP antibody (Abcam ab-290). Bound antibody was detected with an ImmPRESS anti-Rabbit kit (Vector Labs MP-7401) and visualized with ImmPACT VIP chromagen (SK-4605). Cells were counter stained with methyl green.

**Cross Section Image Capture**

Images of the entire cross section were obtained using an Olympus BX40 microscope with a DP72 camera and CellSens software with fixed acquisition settings at 4 x
magnification. Multiple images at 4x were stitched to reconstruct a complete 2D cross section for heart and liver per animal per region. Slides of unstained cross section tissue from rats receiving Rhodamin B were imaged under 540nm fluorescence. Basic light microscopy was used with the same approach as described above for the EGFP sections, however without fluorescence as positive GFP detection was indicated by brown-purple stain hues.

**Imaging Analysis Protocol**

Following acquisition, all myocardial and liver cross section images were standardized with a custom Java programmed macro in ImageJ software to apply uniform brightness, contrast and sharpness settings. Quality control checks of pixel density, resolution, and image size were done prior to analysis. All images were saved into Grayscale 16 bit format and blinded for analysis.

**Cardiac Cross Section Analysis**

Each cross section was loaded into ImageJ. For the Rhodamin B fluorescent images only, each cross section was first subtracted from saline control templates to minimize the background effect. The Iterative self-organizing data analysis technique (ISODATA) algorithm was utilized to determine the threshold for positive therapeutic (Rhodamin B or EGFP positive pixels). Briefly, the algorithm mathematically derives the statistically validated threshold based on the normal distribution sampling of all pixel intensity values and arrives at an optimal detection value [19].

Following ISODATA identification, the 16 bit Grayscale images were converted into binary, whereby all pixel values above the computed threshold were scored positive. Two myocardial contours were drawn, one for the outer epicardial border and another for
the inner endocardial surface. The percentage positive therapeutic area was defined as follows for the cross sectional myocardium:

\[
\text{% Score} = \frac{\# \text{Positive Therapeutic Pixels}}{\# \text{Pixels in Outer Area} - \# \text{Pixels in Inner Area}} \times 100
\]

Liver Cross Section Analysis

The cross sectional images were handled the same as described above, however scoring was focused on therapeutic intensities in the liver to gauge systemic uptake. First, as in above the percentage positive liver area was determined. Second, the grayscale Integrated density was determined by multiplying the gray value (1-255) for each pixel:

\[
\sum_{1}^{\text{Total (+) Pixels}} \text{Gray Value} \times \text{Pixel Unit}
\]

The final exposure score was computed as the mean gray value of all positive pixels determined from the algorithm (Integrated Density/Total Positive Pixels) X % affected area.

Both the Rhodamin and EGFP populations were evaluated in two separate left ventricular planes, middle and basal respectively. For simplicity, an overall score combining both planes was used to characterize the Rhodamin model. Similarly, an overall score was compiled for the EGFP groups by combining both LV sections for a total cardiac score, but presently separately to assess total myocardial coverage.

**Needleless Liquid Jet Evaluation in Explanted Ovine Myocardium**
To obtain a basic translational perspective on the optimized device, a basic methylene blue injection experiment was executed with post-harvest ovine left ventricle. 15 liquid jet injections each containing 100 µL were fired from the device setup at a 4 inch distance to account for the greater thickness. Qualitative epicardial and endocardial wall penetration as well as coverage were observed.

Data Analysis and Statistics

All data are reported as mean ± SEM, per delivery group in each respective study. Single way ANOVA was used to determine difference across all 4 delivery groups. T-testing with Bonferroni correction was used for comparison between multiple groups.

5.3 Results:

Rhodamin B Dye Acute Model Analysis

(Figure 5.2) illustrates the typical distribution profile per method from the Rhodamin B retention analysis. The images demonstrate both the degree of myocardial coverage and regional cross section peak intensities (e.g. mild red to hot yellow) as a function of delivery route. These results were consistent between subjects per group; supporting the claim that delivery route had a major influence on retention profile.
IM injection resulted in highly focal, heterogeneous cardiac delivery with a moderate degree of systemic exposure. By comparison, the liquid jet group demonstrated a more disperse, yet homogeneous pattern with higher intensities along the epicardial surface, but none has high as in the IM sections.

Conversely, the infusion groups demonstrated that increased retention is driven by induced pressure and residence time as there was a dramatic difference between the LVIC-OCCL and LVIC groups.

Quantitatively, the Liquid Jet [52±6] % (+) myocardial area group performed in a similar range with the LVIC-OCCL group [58±5] % (+). Both the Liquid Jet and LVIC-OCCL methods resulted higher distribution levels (p<0.01) compared with the IM [31±8] % (+) and LVIC [35±5] % (+) (Figure 5.3A). All groups presented with varying degrees of
systemic liver exposure, (Figure 5.3B) IM [37±7] arbitrary Rhodamin Units (AU), Liquid Jet [26±5] (AU), LVIC-OCCL [45±7] AU and LVIC [46±7] AU. The Liquid Jet group was the most cardiac specific.

When comparing direct (i.e. combined IM/Liquid Jet) versus infusion (i.e. combined LVIC/LVIC-OCCL), higher systemic liver exposure (Figure 5.3C) [46±5] AU as compared with direct injection methods [31±4] AU was found p<0.05.

Figure 5.3. Acute Model Results: (A) Liquid Jet and LVIC-OCCL demonstrate significantly greater retention versus both IM and LVIC groups p<0.05 (B) All groups result in systemic exposure (C) Direct methods, IM and Liquid Jet combined demonstrate lower overall exposure versus Infusion methods or LVIC-OCCL and LVIC combined
AAV9 Mediated EGFP Expression Analysis at 6 Weeks Post Delivery

Despite the profound size (Rhodamin dye 20-50Da; AAV 4 x 10^6Da), diffusive properties and functional differences between dye and AAV, it was found that the overall distribution patterns for each delivery group nearly matched those results found in the acute study.

A key number of findings were determined, specifically with respect to the EGFP distribution throughout the LV anatomy, indicative of methodology characteristics. (Figure 5.4) depicts representative whole left ventricular cross sections per group (i.e. higher basal and lower middle LV plane regions), whereby GFP detection is indicated with (brown/purple) stain while white/blue regions are devoid of any detectable expression. The results are summarized by group as follows:

IM Injection Group

The GFP distribution was localized around the primary injection sites, but in comparison to dye results the size and bioactivity of AAV permitted greater myocardial coverage in the middle region. The middle left ventricular region score was significantly higher [46±2] % (+) than the basal region [13±12] % (+) p<0.05. This difference was most likely attributable to the consistent application of AAV into the middle left ventricular zone. Based on this difference, it is likely that not much product diffuses into regional distances beyond the site of injection. The GFP systemic liver exposure score was relatively high at [71±10] AU.

Liquid Jet Injection Group
The needleless jet offered a robust, yet homogenous pattern of GFP expression with nearly equal levels in the basal and middle left ventricular sections indicating the jet profile’s dispersive effects per shot. The scores were [54±8] % (+) in the middle and basal [56±9] % (+) regions. The intensity was generally greatest in the epicardial regions of site impact, although significant expression was found in the remote posterior wall regions indicating complete myocardial penetration. Moreover, the systemic liver score was low at [27±3] AU.

LVIC-OCCL Infusion

This route offered the highest degree of expression in terms of both intensity and distribution of cross sectional GFP coverage. The score in the middle left ventricular was the highest at [71±6] % (+). The basal region also scored high [50±14] % (+) and presented with a homogenous pattern. These model results recapitulate the importance of driving local pressure and residence time to maximize delivery through the coronary anatomy.

LVIC

The difference between expression levels between the anatomical regions was the most dramatic here, with the middle region scoring [41±6] % (+) while the basal region [5±3] % (+), p<0.05. These results suggest that very little retention in remote areas is achieved without increased pressure and residence time.
Figure 5.4. Left Ventricular Cross Section GFP Expression Distribution Per Delivery Method. Top to Bottom, IM Injection, Liquid Jet, LVIC-OCCL and LVIC AAV9 groups. Expression profiles presented in the Middle and Basal LV sections (left to right) per group with composite % area transfected with GFP.
Overall when combining both the middle and basal regions, AAV mediated GFP delivery in the heart was more effective with the Liquid Jet [54±5] % (+) and LVIC-OCCL [60±8] % (+) methods, both p<0.05 as compared with IM [30±9] % (+) and LVIC [23±9] % (+) (Figure 5.5A). The Liquid Jet group was the most cardiac specific resulting in a lower liver score versus the other 3 groups, p<0.05 (Figure 5.5B).

**Figure 5.5.** AAV9.GFP Survival Model Study Results. (A) Liquid Jet and LVIC-OCCL groups demonstrating greater overall expression versus IM and LVIC groups, p<0.05. (B) Liquid jet group presented with a much lower GFP liver exposure score compared with the other groups, p<0.05

**Ex Vivo Ovine Myocardial Liquid Jet Injections**

The selected epicardial injection distance for the proposed clinical equivalent was determined at 4 inches. This strike distance was found optimal since maximum myocardial coverage and retention transmurally, but not through the endocardium.
surface (i.e. complete LV wall retention). The figure below depicts primary impact sites shown on the epicardial surface (Figure 5.6A) and transmural retention shown from the reverse endocardial surface (Figure 5.6B). As expected the greater scale size factor imposes concerns for direct delivery.

**Figure 6**

![Figure 6](image)

**Figure 5.6.** Ex Vivo Ovine Methylene Blue Results for translational potential of needleless liquid jet injection (A) Injection points located on the epicardial surface demonstrate the jet’s local, yet dispersive profile along the adjacent surface contours and (B) robust transmural delivery retained per injection site within the endocardial surface.

**5.4 Discussion:**

Achieving sufficient myocardial expression through any selected vector, dose and delivery route configuration is balanced by the clinical implementation risks [20,21]. As demonstrated in this study, the choice of delivery method alone can significantly impact the type of cardiac distribution and collateral organ expression profiles realized over time. The ideal cardiac gene delivery method would facilitate sufficient transduction levels in as much myocardium possible effectively achieving global rescue with the
minimum required dose. The resultant expression profile would also have to be sustainable over time and not induce host responses. Fundamentally this study demonstrates that route efficiency is paramount in achieving maximum myocardial expression, but also offers insight into distribution profiles. The initial delivery is largely influenced by physical transport principles (i.e. pressure, residence time, dispersion trafficking, mechanical resistance) that are independent of AAV characteristics and therefore must be considered with any given delivery strategy. AAV serotype and capsid engineering must also be considered within these limits in obtaining desired expression patterns.

Two unresolved problems with direct delivery wherein the needleless liquid jet approach may be beneficial are to improve initial therapeutic retention and diminish subsequent host responses associated with highly focal expression. With regard to the first problem, other studies have confirmed that a significant portion of the IM injection volume is either lost into the systemic circulation or back through the injection site itself [22-24]. This finding likely explains the variable results achieved between patients, since the beating heart represents a challenging dynamic target featuring accentuated changes in the structural wall thickness during injection [25]. Our results in this study demonstrated that the IM method consistently had a higher degree of systemic delivery as exemplified in the liver versus the liquid jet method. Our application of liquid jet technology demonstrates that a tunable pressure/dispersion profile can promote maximally retained therapeutic per unit volume across a wider surface area within a range of given thickness. With a more sophisticated instrumentation setup, it would be possible to actively adjust driving parameters with real time system feedback and or image guidance.
The second and more challenging problem to resolve for direct delivery methods is the risk for adaptive immune responses. Large animal studies utilizing IM injection have reported high incidence of myocarditis and T cell mediated immune responses [26]. In addition to the inflammation generated from multiple needle sticks, comprehensive studies have raised key concerns regarding the highly localized gene expression profile in muscle [26,27]. It has been postulated that both inflammation resulting from injury at the time of delivery as well as the highly focal expression profile are interrelated in triggering subsequent immune responses long after the initial delivery period [28]. Both innate and adaptive immune responses are major concerns especially since advanced heart failure patients present with moderate to severe degrees of chronic inflammation and fibrosis [29]. This study did not evaluate levels of induced inflammation, but it is hypothesized that the jet’s impact should be less inflammatory versus repeated needle sticks in the same area. Expounding upon this point, other reports confirm that pharmacodynamic effects with IM injections saturate to a biological limit [30,31], whereby expression levels beyond the therapeutic threshold provide no clinical benefit and actually increase risks.

Although intriguing, the liquid jet injection approach would need extensive modification to be positioned in the clinic as a standalone intervention. Certainly our results are very limited to effectively targeting 1 gram of transmural myocardial mass in the rodent. In on our development course, we have identified optimized settings for a thickness level (e.g. transmural rodent cross section at 0.5cm). The settings in this particular application are most likely not optimal for larger animals. Our basic explanted heart dye application demonstrated this point and underscored the problem of scale. Given that 1 g of mass was covered with 5 injections, it is very likely that >250-500 sites
would be needed to achieve the same level of coverage in a human left ventricle. This is certainly not feasible with the existing setup. Future model studies will be needed to determine if further optimization is possible to reduce the number of sites as well as establish safety limits with a more sophisticated prototype.

Infusion approaches will remain on the forefront in trials but must address the problem of increasing driving pressure and navigating anatomical/disease barriers while remaining within safe limits. In the future there could be a gradual shift to direct methods, since >50% of the general population has pre-existing immunity to one or more AAV serotypes, preventing enrollment [32]. Therefore, improved direct delivery methods would most likely be a better choice for these patients. It is conceivable that future applications could include modifying the various driving parameters with a single injector for angiogenesis products that require local delivery and a multiple injector array adapted to supporting instrumentation for global delivery for heart failure applications. Either approach could be further developed as an adjunctive or primary minimally invasive surgical procedure, or along with microinjection devices with minimally invasive endocardial delivery applications.

ACKNOWLEDGEMENTS

This work was supported by the National Heart Lung & Blood Institute R01 grant #2R01HL083078-05. Gracious support was received from the Gene Therapy Resource Program in providing all rAAV vectors for the animal studies. In addition we received support from the Heineman Research Foundation in Charlotte for seed funding. We also acknowledge Tracy Walling MS in the Cannon Research Center for assisting with all the
histology work and Dr. Paulo Arratia at the Complex Fluids Laboratory for the device measurements.

References:


CHAPTER 6

A Comparative Evaluation of Direct Injection Methodology: Liquid Jet Injection Increases the Efficacy of Therapeutic S100A1 Expression in Limiting Ischemic Myocardial Infarction

6.1 Introduction

Chapters 4 & 5 established the core bioengineering concepts and physical limits for needleless liquid jet injection delivery of AAV therapeutics to the myocardium. Given that the injections were well tolerated with excellent results in normal subjects, the research focus then shifted to determine if whether or not the delivery profile could rescue from ischemic myocardial infarction. The therapeutic selected for the study was serotype 9 single stranded AAV S100A1 (ssAAV9.S100A1), which has been established as a potent therapeutic in driving contractility and restoring cardiac function. As discussed in previous chapters, AAV is the primary vector of choice in clinical trials where long term therapeutic expression is desired. Therefore it was logical to evaluate the direct injection methodology in an ischemic infarction setting with a gene that could rescue from acute MI and restore contractility. This chapter describes a very thorough evaluation of direct delivery methods and their outcomes with a potent transgene encoded with a high quality vector. A key focus is placed on the quantitative aspects of gene therapy, which are seldom explored in detail including genome copy number, therapeutic induced protein levels, and finally a careful look into the immunological aspects of direct delivery of AAV to the myocardium.

6.2 The S100A1 Target: Molecular Actions & Previous Studies

6.2.1 The multi-potent effects of S100A1
The lead candidate in clinical trials at present is the AAV1.SERCA2a therapeutic, which seeks to restore calcium handling through long term SERCA2a expression to drive calcium handling in the sarcoplasmic reticulum in failing myocytes. As described in Chapter 1, SERCA2a is further along the regulatory pathway; however the S100A1 target has certainly provided very intriguing results to date and is well positioned for clinical trials. The S100A1 target is multi-faceted in aim, improving not only calcium handling, but also shown to accentuate function in these areas: anti-apoptotic mechanisms, mitochondrial energetics, structural protein binding enhancements and is also hypothesized to have a role in directing regeneration [1].

S100A1 is a member of the S100 protein family, which represents the largest group among EF-had Ca\(^{2+}\) binding proteins prevalent in vertebrates [2,3]. In addition to vigorous effects on calcium handling, these S100 proteins have a variety of key biological functions including but not limited to: cell proliferation, differentiation, survival, motility, cytoskeletal dynamics, nitrous oxide homeostasis [4-6]. S100A1 expression is the highest in the myocardium, with moderate levels found in the skeletal muscle, kidneys and nervous tissues. Within myocardium, S100A1’s presence is only in myocytes and not fibroblasts or endothelial cells, suggesting a prime role in contractility. It has a role in both diastolic and systolic function through influencing both SERCA2a and secondary calcium gating ryanodine receptor 2 (RyR2) as shown in (Figure 6.1) [7-10].
From detailed pre-clinical studies, it is evident that S100A1 has multiple binding sites within the cytoplasmic domain featuring separate effector mechanisms in both energetics and structural integrity of myocytes. With regard to energetics, S100A1 in cardiac mitochondria enhances the effects of the enzyme mitochondrial ATP synthase, which is the most important enzyme for ATP production. In addition to synergistic activity with the synthase, S100A1 also works on the adenosine nucleotide translocator, mediating exchange of ADP and ATP between the mitochondrial matrix and the cytoplasm, thus increasing the availability of ATP for use [11-13]. Thirdly, S100A1’s effects on myocyte viability via structural integrity have been documented through activity on microfilaments. S100A1 has been detected and found to play a significant role in the sarcomere unit. Periodically, it appears at the Z and the M line as well as within the I and A bands [14-16]. The key function is within the I band, whereby S100A1 interacts with a very large integral structure protein titin. Titin has a key role in cardiac mechanics, specifically with the preservation of passive tension in myocytes [16].
6.2.2 Previous Results from Animal Studies

The first attempts with S100A1 overexpression were with an adenovirus vector in a rodent heart failure model via coronary infusion at 12 weeks. The study reported reversal of post ischemic deterioration and preservation cardiac function [17]. Serial studies in swine led by Dr. Patrick Most revealed S100A1’s clinical potential both with AAV6 and AAV9 serotypes, demonstrating high expression levels and rescue from post MI [18,19]. Summarizing, it is anticipated that S100A1 gene therapy for ischemic cardiomyopathy will gain significant traction moving toward a Phase I trial in the next 1-3 years.

6.3 Methods & Materials

Protocol Summary

32 rats received a baseline echo and infarct creation via left anterior descending artery (LAD) ligation and were divided into 4 separate groups (n=8 ea.): 2 Control consisting of Needle injection (IM) and Liquid Jet Injection (LJ), both receiving equal amount of saline and 2 Experimental consisting of IM and LJ each receiving $1.2 \times 10^{11}$ vg of ssAAV9.S100A1. IM injections were performed with three separate 100uL in the left ventricle with a 30G needle, while the LJ device fired three separate 100 µL injections projected at the exposed left ventricle from 25 cm above the thoracotomy. Following 10 weeks, the rats were evaluated with echocardiography for LV function, Masson’s Trichrome staining for infarct area, QPCR for AAV9.S100A1 genome detection, and IHC staining for S100A1 protein. In addition to function and therapeutic endpoints, host response assessment was conducted on 10 week blood and cardiac tissue specimens via cytokine panel and the CD38 immune marker.
6.3.1 Echocardiography Procedures

All rats were subjected to baseline and follow up 10 week post myocardial infarction echocardiography as follows. Rats were induced and maintained with 1-5% isoflourance and placed supine on a surgical table. The VisualSonics Vevo5000 Echocardiography small animal unit was utilized to capture all images. Briefly, 1.7 – 2.2 cm depth, 15-20MHz frequency, 60-70% Gain, and other visual parameters were adjusted for optimal view. Long axis and M-mode views to assess ventricular wall motion score were obtained for each animal. 3 individual data sets were acquired and averaged for 1 overall score set reported for each animal. This ensured minimal variability per run, an example is shown in (Figure 6.2).

Figure 6.2 Echocardiography of Left Ventricular Function
6.3.2 Surgical Procedures & AAV Delivery

An SpO₂, Harvard Apparatus Ventilator, and ECG devices were calibrated prior to surgery. Following induction and intubation, all rodents were placed in the left decubitus position and prepared for a mini left thoracotomy while under complete monitoring of heart rate, ECG, SpO₂ and respiratory parameters. The heart was exposed and the left anterior descending artery was identified. The mid line of the artery was marked with a surgical pen, then 7.0 prolene sutures were used to ligate the artery. Infarct creation was confirmed by both discoloration and ECG changes. Following infarction, each animal received 3 separate 100µL injections of either saline or ssAAV9.S100A1 therapeutic by either IM injection or the Liquid Jet. All animals were recovered and monitored until the 10 week endpoint. The heart was harvested, with myocardial cross sections and individual samples prepared for assay testing.

6.3.3 Quantitative Real Time Poly Chain Reaction (QPCR) of ssAAV9.S1001 Genome

All servicing was performed blinded by the Molecular Profiling Core at the Cannon Research Center. Three separate cardiac zones were harvested from each animal: Anterior Wall, Borderzone, and the Lateral Wall. The liver organ was selected as the primary endpoint target to quantify systemic exposure and was also collected for each animal. Once samples were transferred to the facility a routine protocol with robotic instrumentation was used for processing. Briefly, whole DNA was isolated from 3 left ventricular and 1 collateral organ liver specimens using the Chemagen MSM1 kits to set up the QPCR assays for viral AAV9.CMV.S100A1 DNA. To examine the expression of S100A1, customized kits designed by Abiomed Systems generated the S100A1 primers and then laboratory equipment was prepared to execute the experiment. The
level of ovine GAPDH DNA was used to calculate normalized expression with the ΔΔCt method to obtain QPCR values. Absolute genomic copies of S100A1 DNA were quantified with the standard normal curve built from serial dilutions. Data was reported as number of viral genome copies (GC) per 100 ng of whole DNA. Data analysis was performed for each delivery group combining data from all 3 cardiac zones, with the liver scored separately. All samples reported out of range, either undetectable at the limit of transfer or those >100,000 GC per 100 ng DNA were removed from the analysis.

6.3.4 Histology Protocols: Quantitative S100A1 Protein Analysis in Myocardial Cross Sections via Immunohistochemistry with Confocal Microscopy & Masson’s Trichrome Staining for Infarction Area

6.3.4.1 Quantitative S100A1 therapeutic protein levels in myocardium

Samples of Rat Heart Tissue

Sections were prepared from formalin fixed and paraffin embedded heart tissue blocks. All Immunofluorescence staining, image processing, and quantification were performed by the CaresBio Resources Inc. (Shelton, CT) service provider in a blinded manner.

Immunofluorescence (IF)

Immunofluorescence staining was performed on tissue samples. Briefly, slides were subjected to heat induced antigen retrieval in citrate buffer (pH 6.0) and or in proteinase. Slides were incubated for 1 hour/ overnight with primary antibodies CD38, S100A1), after blocking with nonspecific antigenicity blocker. Corresponding fluorescent conjugated secondary antibodies were applied for 1 hour at room temperature. 4, 6 -Diamidino-2-phenylindole (DAPI) were included with the secondary antibodies to
visualize nuclei. The staining, imaging and image analysis were performed by CaresBio Laboratory, LLC (Shelton, CT, USA).

Image Acquisition and Analysis

Briefly, slides were scanned using a customized, computer-controlled microscope (with xy-stage and z controller) a Zeiss LSM780 confocal microscope (Carl Zeiss GMBh, Jena, Germany) with ×40 and X20 objective. Every case had 32 images including 16 images per section. Images were analyzed using an image analysis software based on MATLAB (R2011b, MathWorks), developed by CaresBio Laboratory. The final result is the average of 32 images per case what was compared with average baseline to calculate times of increase. Image analysis algorithms were applied to the images generated from microscopic slides of tissues stained with secondary antibody and isotype controls to generate the background score. Control/Baseline was used to generate the algorithm to differentiate between the signals and signal to noise ratio. The same algorithm has been applied to all the images. This provided the option for separation of single stained only areas-and double/triple-stained areas from secondary only and or- isotype only stained areas by means of subject specific thresholding. A good separation of single- and double-stained pixels from secondary antibody only and or isotype control (background) stained pixels was achieved. Automatic background subtraction was performed. Significant differences in relative areas stained and mean specific intensity for the stains between control and treatment groups in rat tissue were calculated.

Quantitative analysis of the IHC cross sections stained for S100A1 was performed with confocal microscopy detection and normalization to untreated controls to
remove the effect of endogenous levels. A 32 array grid, featuring 8 random but separate zones defined in the anterior, lateral, posterior and septal wall regions were obtained for analysis at 20x magnification. (Figure 6.3) best characterizes the method, whereby the yellow squares represent individual regions of interests zoomed out at 4x magnification. A composite overall expression score normalized to control (i.e. 1.0 background endogenous S100A1) was computed for each animal.

Figure 6.3 Quantitative Proteomic S100A1 Methodology Featuring IHC & Confocal Microscopy; 32 individual zones (YELLOW) throughout the myocardium were analyzed

6.3.4.2 Masson’s Trichrome Staining for Infarct Size Characterization

Formalin fixed paraffin embedded tissues were sectioned at 4 microns on a Leica RM2125 microtome, picked up on Plus slides and air dried overnight. Slides were then baked in a 60 degree oven for 20 minutes to remove excess paraffin before being deparaffinized and with xylene and rehydrated with several changes of graded alcohol to
distilled water. H&E sections were stained with Mayer’s hematoxylin for 20 minutes, rinsed with running tap water and stained with Eosin Y for 5 seconds followed by several rinses in 95% alcohol. Masson’s Trichrome slides were stained with Weigert’s Iron Hematoxylin for 10 minutes followed by a rinse in tap water. The rest of the staining was done with a Masson’s Trichrome kit purchased from Polyscientific (Bay Shore, NY cat# k037 ). The slides were then dehydrated with several changes of graded alcohol and cleared with xylene before being coverslipped with Permount.

6.3.5 Host Response to S100A1 Gene Therapy

The IM route has documented evidence of inducing both inflammatory and adaptive immune responses that can dramatically reduce efficacy and lead to poor outcomes. As described in the previous chapters a significant aim of the dissertation presented was to investigate these both in the myocardial tissue and global response via blood testing. The analysis was divided into two subparts, cardiac tissue and whole blood.

Cardiac Tissue Immunohistochemistry for CD38 Expression

The CD38 protein is a marker of cell immune activation, specifically described at the crossroad of innate and immune activation [18]. The same preparation of tissue and quantitative methodologies described in section 6.3.4 were utilized, except with an anti-CD38 antibody obtained from AbCam Scientific.

Whole Blood Cytokine Panel

This analysis was utilized to obtain a clear picture of the host’s global inflammatory profile at 10 weeks post myocardial infarction and gene delivery. Analyte concentrations were measured and quantitated using a Bio-Plex Suspension Array System (Bio-Rad, Hercules, CA, USA). A Bio-plex multiplex premixed rat cytokine, chemokine and growth factor panel was used. Analytes including EPO, G-CSF, GLP-1,
GM-CSF, GRO KC, IFN-γ, IL-1α, IL-1β, MCP-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p70, IL-13, IL-17A, IL-18, M-CSF, MIP-3α, RANTES, TNF-α, and VEGF were examined. These assays use color-coded bead sets, each of which is conjugated with analyte-specific antibodies and designed in a capture sandwich immunoassay format. The samples were diluted using with the supplied diluent as directed. The antibody-coupled beads were mixed and incubated with diluted plasma samples, or with the standards. Thereafter, unbound proteins were removed by washing in the Bio-Plex Pro wash station (Bio-Rad) and a biotinylated detection antibody was added to the beads. Each captured analyte was detected by the addition of a reporter molecule, streptavidin-phycoerythrin. The contents of each well were drawn into the Bio-plex array reader (Bio-Rad) where precision fluidics align the beads and lasers excite them in order to quantitate the captured analytes. Analyte concentrations were automatically calculated with Bio-Plex Manager software by using a standard curve derived from the recombinant standard provided with the assays. A key focus was placed on these markers which were more relevant for the particular analysis of innate and adaptive immune response following gene therapy: IFN-γ, IL-1α, IL-4, IL-6, IL-10, IL-17, RANTES, TNF-α.

6.4 Results

6.4.1 Operative & Delivery Results

The overall survival rate from induction to 10 weeks endpoint was 93%. A limited number of animals (n=3) were lost during infarct creation and replaced such that each group tallied at least (n=8). The IM injections were tolerated without major incidence, but minor bleeding events were noted and managed appropriately. The liquid jet injections were also tolerated without any major incidence, although some minor bleeding and discoloration in the impacted area was noted as in previous studies with this method.

6.4.2 Echocardiography Measures of Cardiac Performance
(Table 6.1) below summarizes all major data obtained to determine left ventricular function in addition to global left ventricular remodeling parameters associated with the progression following ischemic myocardial infarction.

Table 6.1 Echocardiography and Outcome Data for ssAAV9.S100A1

<table>
<thead>
<tr>
<th>Data Point:</th>
<th>Baseline</th>
<th>10 Weeks Post MI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight [g]</td>
<td>409±21</td>
<td>346±9</td>
</tr>
<tr>
<td>Heart Rate [bpm]</td>
<td>355±12</td>
<td>344±13</td>
</tr>
<tr>
<td>End Diastolic Volume Index [mL/cm²]</td>
<td>0.6±0.1</td>
<td>0.6±0.1</td>
</tr>
<tr>
<td>End Systolic Volume Index [mL/cm²]</td>
<td>0.2±0.03</td>
<td>0.2±0.04</td>
</tr>
<tr>
<td>Ejection Fraction [%]</td>
<td>71±2</td>
<td>68±2</td>
</tr>
<tr>
<td>Epicardial 2D Area [cm²]</td>
<td>0.7±0.1</td>
<td>0.7±0.1</td>
</tr>
<tr>
<td>LV Wall Thickness [mm]</td>
<td>1.5±0.06</td>
<td>1.4±0.05</td>
</tr>
</tbody>
</table>

* p<0.05; Baseline to 10 Weeks
† p <0.01; vs. Other 3 Groups at 10 Weeks

Single way ANOVA revealed no significant difference in any data point at baseline. However, as expected the effects of both the infarction and potential therapeutic action of S100A1 led to a number of differences observed at the terminal 10 week post MI point. First confirming model behavior, all 4 groups exhibited an increase in LV
remodeling points, all * p<0.05 for: End Diastolic and End Systolic Indices, Ejection Fraction, 2D Epicardial Area and a decline in LV wall thickness suggestive that the ischemic MI induced these changes. These are consistent results and provide solid evidence that the infarction promoted the typical dysfunction and compensatory changes following the initial myocardial insult at time zero. Second, as compared in order with the [IM Saline]; [Liquid Jet Saline] and [Liquid Jet-S100A1] groups, the IM-S100A1 had a more progressive remodeling course suggestive of more severe LV dysfunction with End Systolic Volume Index at [1.0±0.2 μL/cm²] vs. [0.7±0.2 μL/cm²]; [0.7±0.1 μL/cm²] and [0.5±0.1 μL/cm²] respectively and greater End Diastolic Index at [1.7±0.2 μL/cm²] vs. [1.2±0.2 μL/cm²]; [1.3±0.1 μL/cm²] and [1.3±0.2 μL/cm²] respectively. These data points did not pass the single way ANOVA, however two key points did, Ejection Fraction and Global Myocardial LV thickness.

The Liquid Jet-S100A1 preserved the most baseline function at [60±3]% and was greater p<0.01 than the other 3 groups in terms of overall performance; IM Saline [47±3]%, Liquid Jet [46±3]% and IM-S100A1 [43±4]%.

There was a much greater degree of structural integrity in the treated Liquid Jet S100A1 group with a greater overall wall thickness p<0.05 [1.5±0.1 mm] versus all 3 other groups.

The infarct sizing characterization yielded very striking results. In support of the functional data, The IM-S100A1 exhibited extremely profound deterioration in the left ventricular cross sections. The infarct size in this group revealed a much greater affected zone [40±4] %, p<0.05 area versus the IM Saline [33±6]%, Liquid Jet Saline [20±6]% and Liquid Jet S100A1 [17±5]%. (Figure 6.4) depicts the extreme, featuring a typical IM S100A1 (A) compared with a typical Liquid jet S100A1 (B).
The Liquid jet S100A1 group overall had the lowest scar size, which was not expected but intriguing. Overall, there was a clear difference between the delivery groups with the same therapeutic dosing/gene strategy thus confirming the method was the difference in outcome both in terms of function and LV integrity.

6.4.2 Quantitative PCR Detection of AAV9.S100A1 Therapeutic DNA

The analysis was divided into two major anatomical zones. The first was the Cardiac zone comprised of 3 harvested sites per animal (Anterior Wall, Borderzone, Lateral Wall) to determine the degree of therapeutic transfer (i.e. 8 animals by group x 3 specimens = 24 Cardiac). The second collateral liver (i.e. 8 animals by group x 1 liver specimen = 8 Liver).

The IM S100A1 group demonstrated robust detection in all 32 cardiac and liver specimens, however 10 were greater than 100,000. This suggests that there was very high focal expression in the myocardium and a high degree of systemic exposure in the liver as found in the Chapter 5 marker study. After the removal of these outliers, the

Figure 6.4 Masson’s Trichrome Staining for Infarct scar at 10 Weeks: (A) Typical IM gene therapy animal with large (blue) infarct zone while the Liquid Jet therapy group (B) demonstrated much lower scar and thicker myocardium
overall cardiac zone score for the IM group was \([13128\pm2774]\) GC per 100 ng DNA, while the systemic liver score was significantly higher \(p<0.05\) at \([65051\pm15836]\) GC per 100 ng DNA.

The Liquid Jet S100A1 group on the other hand demonstrated an overall lower score in terms of focal expression as 14/32 specimens did not have detection, however those that did scored in the range \((n=10)\), but lower than the IM group at \([10147\pm3572]\) GC per 100 ng DNA. The collateral liver analysis revealed minimal detection \([40\pm40]\), with two samples with readings near zero. (Figure 6.5) summarizes the data by delivery method. For assay validity, non-therapy controls were run and yielded undetectable readings (data not shown).

![Figure 6.5](image)

**Figure 6.5** Quantitative PCR Results: IM-S100A1 Group (Red) Demonstrated significantly higher detection in the Liver, while the Liquid Jet-S100A1 group (Green) was more cardiac specific with higher detection in the heart.

These results confirm what was found in the Chapter 5 marker study: (1) IM delivery results in highly focal levels of viral mediated expression (2) Inadvertent infusion
and or in combination with poor myocardial retention occurred resulting in lower cardiac specificity. The greater cardiac specificity of the liquid jet is most likely attributable to the tuning of the jet properties, whereby microjet penetration is sufficient enough to perforate, but remain in the myocardium with reduced volume entry into the left ventricular cavity. Although not confirmed with additional biodistribution testing, there is most likely collateral residual expression in the thoracic and adjacent lung tissues with the liquid jet’s dispersion and impact effects.

6.4.3 Quantitative Myocardial S100A1 Proteomic Levels & Confocal Microscopy

The overall proteomic S100A1 detection assay utilizing advanced confocal microscopy applications featuring immunofluorescent detection yielded more detailed findings beyond viral mediated detection. Two key findings were overall fold higher expression and a difference in distribution per method.

In terms of overall S100A1 expression as measured by normalization to saline controls [1.0±.05], both the IM [5.0±0.1] and Liquid Jet [4.3±0.1] gene therapy groups yielded dramatically higher fold S100A1 in S100A1 Fluorescence Intensity Fold Ratios. As expected, AAV9 mediated viral vector transfer of S100A1 raised baseline levels and or restored depleted S100A1 reserves lost in ischemic MI. Another key finding inline with QPCR data was that the IM had roughly 1 fold greater overall S100A1 gene expression (Figure 6.6).
Due to the variability inherent with both delivery and the ischemic model, a logical fixed 32 zone sequences was utilized to determine overall expression, but the overall score does not address the problem of heterogeneous expression as found previously with the IM group.

In an effort to characterize the degree of S100A1 expression per zone, the standard deviations derived per animal resulting from the 32 individual zones were analyzed. It was found, as expected, that the Liquid Jet had a more homogenous S100A1 as measured by a lower variability score in the S100A1 expression profile [13.5±1.5] versus the IM group [20.1±0.5], p<0.05. (Figure 6.7)
The confocal microscopy images verified these findings with vibrant images indicating the degree and distribution pattern of S100A1 expression typically found by route (Figure 6.8). These are depicted in the figure below.

Figure 6.7 S100A1 Expression Variability. IM Injection results in a higher degree of variation in the myocardium following after analyzing the standard deviation in the 32 zones per animal.

Figure 6.8 Confocal Microscopy Images: Cross sectional myocardial S100A1 expression (maroon) with labeled nuclei (blue) by route: (A) IM Injection (B) Liquid Jet Delivery. Liquid Jet has a less intense, more disperse pattern of expression as compared with IM.
6.4.4 Host Responses to Direct ssAAV9.S100A1 Expression

Given the dramatic difference in outcome between the delivery groups, as asserted in this dissertation as well as compelling evidence suggests that highly focal cardiac expression may contribute to triggering maladaptive immune responses. The host response was approached from both a local tissue inflammatory CD38 marker in part A, and a complete physiologic cytokine panel B. Part A seeked to determine local risk profile for an adaptive T cell mediated immune response via CD38 activation, while B sought to determine if the immune system was activated via evidence of elevated cytokines.

6.3.4A The CD38 Inflammatory Marker

Given the difference in both LV performance and cardiac structure, it was anticipated that the IM-S100A1 group suffered from the compounded effects of myocardial infarction in addition to a host response. Both groups experienced a significant level of CD38 expression throughout the myocardial cross section against Control specimens [1.0±0.03]. The IM-S100A1 group [7.2±0.8] was nearly twice as high as the Liquid Jet-S100A1 group [3.8±0.2] in CD38 Fluorescence Intensity Fold Ratio (Figure 6.9).
The Whole Blood Global Pro-Inflammatory Cytokine Panel

The cytokine results are clearly summarized in Table 2 with a number of key findings. The IM-S100A1 group exhibited a significantly higher amount of a variety of key circulating pro-inflammatory cytokines involved in innate and adaptive immune responses. These were IL-1α, CCL5 (RANTES), TNFα, and IFNγ. Higher trends were also noted with IL-4, IL-6 and IL-12.

<table>
<thead>
<tr>
<th>CYTOKINE:</th>
<th>BLOOD LEVELS [picograms/mL]</th>
<th>Controls</th>
<th>IM - S100A1</th>
<th>Liquid Jet - S100A1</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td></td>
<td>113±29</td>
<td>319±127*</td>
<td>197±21</td>
</tr>
<tr>
<td>CCL5 (RANTES)</td>
<td></td>
<td>156±22</td>
<td>262±55*</td>
<td>211±29</td>
</tr>
<tr>
<td>TNFα</td>
<td></td>
<td>124±26</td>
<td>256±50*</td>
<td>189±94</td>
</tr>
<tr>
<td>IFNγ</td>
<td></td>
<td>79±15</td>
<td>242±77*</td>
<td>54±11</td>
</tr>
<tr>
<td>IL-4</td>
<td></td>
<td>37±8</td>
<td>67±22</td>
<td>28±4</td>
</tr>
<tr>
<td>IL-6</td>
<td></td>
<td>46±11</td>
<td>136±65</td>
<td>109±95</td>
</tr>
<tr>
<td>IL-12</td>
<td></td>
<td>54±14</td>
<td>105±49</td>
<td>39±13</td>
</tr>
</tbody>
</table>

* p<0.05; AAV.S100A1 Delivery Group vs. Saline Controls

The IM-S100A1 group exhibited a significantly higher amount of a variety of key circulating pro-inflammatory cytokines involved in innate and adaptive immune responses. These were IL-1α, CCL5 (RANTES), TNFα, and IFNγ. Higher trends were also noted with IL-4, IL-6 and IL-12.
Thus, both inflammatory signals were found in the cardiac zones and the peripheral blood system indicating the AAV mediated gene therapy via direct injection induces a number of responses. It is clear that these responses, inline with the poor outcome data, were more profound in the IM group.

6.5 Discussion

The results from this study solidify key points identified in the Chapter 5 marker study. Major novel contributions include revealing some key risk factors for direct AAV mediated gene therapy post myocardial infarction. First, all of the major principles of liquid jet injection were re-affirmed in this study with a bona fide AAV9.S100A1 transgene vector platform, namely:

- Liquid jet can achieve a disperse, long term gene expression profile in the disease setting of acute myocardial infarction
- S100A1 is a robust transgene that promotes long term rescue of myocytes in the ischemic MI setting.
- A homogenous expression profile similar to infusion approaches is possible
- Liquid Jet Injection more cardiac specific than IM since optimized settings control the degree of penetration into higher risk areas (i.e. left ventricular cavity)
- Synergistically, a cardiac specific, homogenous gene expression profile and minimal collateral organ expression is at much less risk for host responses

Reflecting these key points above in the context of this rodent study, Liquid Jet injection results achieved a disperse, cardiac specific S100A1 profile compared with the heterogeneous profile of the IM route. The conclusion that delivery with ssAAV9.S100A1 therapy is more efficacious with the liquid jet route is more than justified.
given the compiled data sets. The difference in ventricular function, geometry, and disease burden were dramatically different between the treatment groups. The IM S100A1 group suffered deleterious effects in the left ventricle leading to poor outcome, most likely attributable to an adaptive immune response that exacerbated left ventricular remodeling, despite seemingly adequate levels of S100A1 overexpression. In particular the infarct size measurements were striking, whereby nearly double affected zone was found between groups. The non-homogenous expression profile as found in the proteomic analysis in combination with much higher levels of systemic exposure via QPCR data are more than likely the causes.

In essence, there were 3 main stimuli events in this model that factored into the clinical outcome and host responses found at 10 weeks: A. Induced Myocardial Infarction B. Injury from Delivery C. AAV mediated S100A1 expression and D. Inflammatory risk factors. The interaction of all 4 of these determined the outcome per group. A goal of this dissertation was to determine the relationships. With respect to infarction it is understood that there is inherent variability in any given ischemic model, however it is very unlikely that variation alone in this category factored in the outcome based on robust numbers in each group.

With respect to injury, it was beyond the means of this particular study to determine the level of injury at the cellular level per route, especially in the context of acute MI. However despite this key limitation, a few points can be introduced for future discussion. It has been challenged by this dissertation that one of the advantages of Liquid Jet injection is that it is safer than the IM route. This was visually evident in the surgeries, whereby less bleeding was noted with the jet injections which only left visible welts on the epicardial surface. As asserted previously, any given delivery method results in a
level of injury, but the degree of injury is paramount to consider in the actual delivery application.

Numerous studies have made the case that host inflammation resulting from delivery itself can result in aggressive adaptive immune responses against AAV mediated transgenes [19]. While the immunological possibilities are complex, it is logical to conclude there is a synergistic role of delivery in an already inflammatory post MI environment. Although not supported with additional data, it was very intriguing to find that the Liquid Jet Groups (treated and untreated) had smaller infarct zones overall. This observation would counter an argument that the system is more deleterious alone versus IM injection.

Additional insight for future studies with S100A1 or AAV mediated products is the degree of overexpression. The CD38 inflammatory expression data indicates that greater expression levels increase the risk for maladaptive immune responses. In this case study, it appears that delivery route, more than the vector/transgene with direct injection contributed to the poor outcome in the IM group, but these factors may have different relative significance with other vector/transgene systems. In general, numerous gene therapy studies selecting the IM route have confirmed that very little successful transfer and or reactions occur in damaged muscle [20]. Similar problems, but to a lesser extent, have been found in Hemophilia trials, whereby infusion of the same AAV dose in the liver was much more efficacious than IM delivery [21,22]. Thus, the case for liquid jet injection for cardiac gene therapy applications with AAV product is solidified. Expanding on the discussion of adequate expression levels, optimal gene therapy treatment would utilize the minimum amount of vector such to achieve therapeutic expression in the myocardium. From the standpoint of adequate expression levels, it was interesting to note that despite very high S100A1 expression as measured by both
QPCR and proteomic analysis, the IM S100A1 was spiraling down the path of chronic heart failure. The Liquid jet group on the other hand despite a fold lower, was efficacious. It is asserted by this dissertation that the difference can be found with the host response, in that once the responses drive beyond a threshold to complete systemic activation the therapy itself becomes toxic.

Summarizing, these results communicate an important concept that there is most likely a delicate balance between successful therapy (i.e. tolerance) with AAV mediated products versus complete immune activation. The conclusions in this study assert that outcome can most definitely be mitigated with route, vector and dose selection. The next two Chapters 7 and 8 are dedicated to exploring additional options in navigating this balance. Chapter 7 repeats the study design as presented in this chapter, except with a double stranded AAV9 vector.

References


CHAPTER 7

Alternative Strategies for Enhancing Direct Myocardial Gene Delivery in Ischemic Myocardial Infarction: A Case Study Featuring Self Complementary Vector

7.1 Introduction

The previous chapter revealed some key findings with the use of ssAAV9.S100A1 therapy through two different direct routes of administration. Despite the strong compelling case for Liquid Jet Injection as the superior route and the confirmation of multiple host responses in the IM group, there was no conclusion on whether or not it was the intensity of the expression profile, vector capsid interactions, or high systemic exposure that caused the aggressive host responses in the IM group. Given that both groups had a high expression profile in terms of cross sectional area and intensity, it would appear less likely that S100A1 was the problem given the discrepancy in outcome at 10 weeks. As described in previous chapters, AAV infection imposes risks to the system that have been acknowledged in the field for decades, but more recent studies have elucidated the means [1].

In particular with AAV in muscle, a diagram model of the host response is presented below in (Figure 7.1). Numerous studies have unraveled more detailed information on the risks of AAV mediated gene therapy in terms of host response. In order to improve clinical outcomes, one major goal the field is focusing upon is correlating the upfront risks of delivery with systemic priming, whereby ultimately the immune system tips into therapeutic tolerance or mounts a response. It is believed that understanding these interactions throughout the process of therapy can mitigate the risks.
AAV is less immunogenic than adenovirus because it avoids directly interacting with and transducing antigen presenting cells. However, in the presence of inflammation or when systemic exposure penetrates a threshold, these key cells can be activated against both AAV and transgene antigens and or signals derived from the cells that contain them [2].

In our case study example presented in Chapter 6, it is very likely the IM S100A1 group with single stranded vector suffered a deleterious TH1 adaptive immune response since the risk factors were present, recapitulating: (1) High levels of IFN$\gamma$, TNF$\alpha$ in the blood (2) CD38 markers all present in higher proportion versus the other 3 groups (3) Inflammation from both infarct and delivery events were present at time zero and beyond. Not shown in the diagram is the contribution of early inflammation, dubbed Signal 0, which has a role in determining whether or not Signal 2 occurs.

Given that ischemic myocardial infarction and subsequent therapeutic target areas are very high risk, this Chapter describes an alternative vector strategy, the self-
complementary AAV9 vector or double stranded DNA vector which has several advantages over the single stranded version.

One of the key advantages of S100A1 besides its potent effects is its transgene cassette size at only 330 base pairs (bp). The cloning capacity of AAV cassette is only 4,440 bp total, however much of the space is designated for the inverted terminal repeats, promoter and other elements, thus limiting transgene packaging to around 3100 base pairs. Therefore other transgenes over a certain size such as SERCA2a could never be packaged into a double stranded vector. The double stranded vector has two key advantages that might increase performance in higher risk myocardium. The first advantage is leveraging the key molecular biological advantage double has over single stranded versions that is skipping the DNA template conversion step.

Described briefly here since vector engineering is beyond the scope of this research, all AAV viruses package single stranded DNA sequence cassettes that must first convert to double stranded DNA. This step precedes which precedes therapeutic expression and if conversion fails so does the therapy. Self-complementary vector was developed to address the key rate limiting step of single stranded DNA to double stranded after the virus successfully transfects a cell. This process of template synthesis was which was once thought highly efficient, however key studies and applications in clinical scenarios prove otherwise [3,4]. Furthermore, even after successful template synthesis conversion, studies have reported instability events whereby the newly converted strand is degraded rapidly in vivo, negating any therapeutic potential of the original transfection [5].

The second hypothesized advantage, although controversial and one point this research seeks to determine is whether or not double stranded vector with the same dose/transgene should be less inflammatory. Single stranded DNA infections have a
long established research track record with vaccinations, or induced immune responses [6]. The following logic is that within inflammatory environments and or high concentrations of product (i.e. vaccination delivery), this would increase the risk of an adaptive response.

Supported by a solid body of evidence, the double stranded vectors because of these cited reasons leads to both faster and higher levels of gene expression in muscle [7]. One study in rodent hearts reported 10 to 15 fold higher copy number vector transfer along with a more robust marker gene expression profile [8]. This study cited the concept of a saturation point, whereby maximum possible copy number increased with local injection but reached an upper limit in terms of actual cellular transduction.

Therefore, this Chapter 7 was dedicated to exploring the concept of using the same gene therapeutic intervention, however constructed with a self-complementary vector. The central hypothesis challenged is twofold:

(1) That for one or both routes of direct injection, self-complementary vector would offer increased expression levels and similar or better outcome at 10 weeks post MI

(2) Due to eliminating the conversion step risk factor, the therapy overall would be less inflammatory

7.2 Methods & Materials

7.2.1 Protocol Summary

20 rats received a baseline echo and infarct creation via left anterior descending artery (LAD) ligation and were divided into 2 separate groups (n=10 ea.): 2 Experimental consisting of, IM and LJ each receiving $1.2 \times 10^{11}$ vg of scAAV9.S100A1. IM injections were performed with three separate 100uL in the left ventricle with a 30G needle, while the LJ device fired three separate 100 uL injections projected at the
exposed left ventricle from 25 cm above the thoracotomy. Following 10 weeks, the rats were evaluated with echocardiography for LV function, Masson’s Trichrome staining for infarct area, QPCR for AAV9.S100A1 genome detection, and IHC staining for S100A1 protein. In addition to function and therapeutic endpoints, host response assessment was conducted on 10 week blood and cardiac tissue specimens via cytokine panel and the CD38 immune marker.

**Essentially this study is a repeat of the previous Chapter 6, however featuring delivery of only scAAV9.S100A1 vector. All echocardiography, surgical, animal handling, and tissue harvest/analysis were as is the previous.**

7.2.2 Grouping & Data Analysis

Previous data from the 2 control groups [IM Saline, Liquid Jet Saline] and 2 ssAAV9.S100A1 treatment groups [IM S100A1, Liquid Jet S100A1] were used to assess various endpoints in comparison to the results presented in Chapter 5. Specifically, the control groups were not repeated and used for reference to evaluate: LV mechanics, S100A1 and CD38 proteomic expression normalization, and cytokine panels. The genome copy detection via QPCR is however completely original.

7.3 Results

7.3.1 Operative & Delivery Results

One animal in each the IM group and Liquid Jet group was excluded due to complications with either the MI or delivery, thus grouping IM scS100A1 (n=9) and Liquid Jet scS100A1 (n=9) for the analysis. Delivery parameters were executed as in the previous study without major incidence.

7.3.2 Echocardiography Results
The key hemodynamic data from this follow up study are presented in (Table 7.1).

**Table 7.1 Echocardiography and Outcome Data: scAAV9 Pilot Study**

<table>
<thead>
<tr>
<th>Data Point</th>
<th>BASELINE</th>
<th>10 WEEKS POST MI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IM - Sa.</td>
<td>Liquid Jet-Sa.</td>
</tr>
<tr>
<td>Weight [g]</td>
<td>409±21</td>
<td>406±32</td>
</tr>
<tr>
<td>Heart Rate [bpm]</td>
<td>355±12</td>
<td>346±9</td>
</tr>
<tr>
<td>End Diastolic Volume Index</td>
<td>0.6±0.1</td>
<td>0.7±0.1</td>
</tr>
<tr>
<td>End Systolic Volume Index</td>
<td>0.2±0.03</td>
<td>0.2±0.05</td>
</tr>
<tr>
<td>Ejection Fraction [%]</td>
<td>71±2</td>
<td>68±2</td>
</tr>
<tr>
<td>Epicardial 2D Area [cm²]</td>
<td>0.7±0.1</td>
<td>0.8±0.1</td>
</tr>
<tr>
<td>LV Wall Thickness [mm]</td>
<td>1.5±0.06</td>
<td>1.4±0.05</td>
</tr>
<tr>
<td></td>
<td>587±16*</td>
<td>610±22*</td>
</tr>
<tr>
<td>Heart Rate [bpm]</td>
<td>306±31</td>
<td>317±9*</td>
</tr>
<tr>
<td>[mL/cm²]</td>
<td>1.2±0.2*</td>
<td>1.3±0.1*</td>
</tr>
<tr>
<td>[mL/cm²]</td>
<td>0.7±0.2*</td>
<td>0.7±0.1*</td>
</tr>
<tr>
<td>Ejection Fraction [%]</td>
<td>47±3*</td>
<td>46±3*</td>
</tr>
<tr>
<td>Epicardial 2D Area [cm²]</td>
<td>1.4±0.1*</td>
<td>1.5±0.1*</td>
</tr>
<tr>
<td>LV Wall Thickness [mm]</td>
<td>1.2±0.1*</td>
<td>1.2±0.1*</td>
</tr>
<tr>
<td>Infarct Size [% LV Area]</td>
<td>33±6</td>
<td>20±6</td>
</tr>
</tbody>
</table>

* p<0.05; Baseline to 10 Weeks  
† p <0.01; vs. Control Saline Groups  
§ p<0.01; vs All Other Groups

There was no difference between the groups at baseline. At 10 weeks however, the most striking results were found in the IM SCS100A1 group, whereby dramatic LV functional improvement was found as compared to the single stranded data as found in Chapter 5 Table 1. This group unlike the analogous with the single stranded, preserved significant baseline function at [60±2]% and was found superior to both IM Saline [47±3] and Liquid Jet Saline [46±3]%. This group also demonstrated the lowest degree of global geometric LV remodeling changes with: (1) Lower 2D Epicardial Area [1.1±0.1]
cm² $p<0.05$, versus all other groups IM Saline [1.4±0.1] cm², Liquid Jet Saline [1.4±0.1] cm², and the Liquid Jet SCS100A1 group [1.5±0.1] cm². (2) Trending lower End diastolic and End systolic dimensions. It is important to note however that this particular group also started with lower indices, but the point is the difference in direction from the single stranded group which dramatically worsened in the direction of chronic heart failure. On the other hand, the Liquid Jet SCS100A1 group basically confirmed similar results found in the previous study, with a significantly retention of LV functionl [EF+57±4%], $p<0.05$ versus IM Saline and Liquid Jet Saline.

Structure wise, both treatment groups featured severe MI zones with IM SCS100A1 [30±13]% and the Liquid Jet SCS100A1 [32±9]% LV affected area. Despite these affected zones however, both treatment groups had greater average LV wall thickness (IM SCS100A1 [1.4±0.04] mm; Liquid Jet SCS100A1 [1.5±0.1] than both IM Saline [1.2±0.1] and Liquid Jet Saline [1.2±0.1], suggestive of rescue.

Summarizing, both the IM and Liquid Jet herapy groups in this pilot study evaluating scAAV9.S100A1 were efficacious within study limits.

### 7.3.3 Quantitative PCR Detection of scAAV9.S100A1 DNA

The same methodology for analysis were that were used in Chapter 5 were also applied here. The fold expression with the self-complementary vector was much higher compared with the single stranded results in Chapter 5 (Figure 7.2). In comparison to the single stranded vector results, there are a number of key points that can be drawn. First, the large change in the magnitude of copy number in both groups was profound, more so in the IM group as expected with the focal expression profile. The IM SCS100A1 group presented with [304276±36753] GC per 100 ng DNA in the cardiac
regions, but also had a distinctly higher presence in the liver that superseded all group specimens tested at $[665279\pm39818]$ GC per 100 ng DNA, $p<0.01$.

By comparison, the Liquid Jet (i.e. compared with IM SCS100A1) cardiac regions had nearly 10 times less copy number, with $[36753\pm5844]$ GC per 100 ng DNA. However unlike the previous single stranded set, a sizeable amount was found in the Liver at $[39818\pm3246]$ GC per 100ng DNA.

Fold detection wise, the IM group experienced a roughly 23 fold increase in the cardiac zones comparing double to single stranded vector transduction. The Liquid jet likewise was 3.6 fold higher in the cardiac but ever more so in the liver at nearly 1000 fold. Recall there was minimal detection in the liver with the single stranded vector with this route previously.

**Figure 7.2** QPCR analysis revealed significantly higher transfer levels in both the cardiac regions and collateral liver. The IM Group demonstrated significantly higher expression * $p<0.05$ for both regions, with the liver superseding † $p<0.01$ other tissues.
Summarizing, double stranded vector in terms of transduction performed higher than the reported target of 10-15 times fold in comparison to single stranded vector, but particularly with the IM group the cardiac specificity is an issue.

7.3.4 Quantitative Cross Sectional S100A1 Proteomic Expression

Given the accentuated increase in copy number it was expected protein amount would also increase with the self-complementary vector. This was true for both the IM and Liquid Jet groups. The IM SCS100A1 group scored very high levels of overexpressed S100A1 protein [8.8±0.3] S100A1 Fluorescent Units, in fact significantly p<0.05 higher than the other 3 delivery/vector combinations (Figure 7.3).

Proteomic Data: Myocardial S100A1 Expression Per Vector/Route Combination

Figure 7.3 The second iteration of S100A1 proteomic analysis revealed significantly higher overexpression in all treatment groups * p<0.05. Comparatively, both Self Complementary vector treatment groups scored higher † p<0.05 than corresponding Single stranded groups. The IM-SC group scored much higher overall than all others § p<0.05.
The Liquid Jet SCS100A1 group reached [6.1±0.3] levels, higher than both single stranded IM [5.0±0.1] and Liquid Jet [4.3±0.1].

Confocal microscopy images revealed what was found in the analysis at 20 times magnification. (Figure 7.4) demonstrates the typical intensity in S100A1 expression found when comparing single versus self-complementary vector results.

7.3.5 Host Response to scAAV9.S100A1 Therapy

In line with both an increase in copy number and S100A1 protein detection, CD38 marker also increased. As found in Chapter 5, the Liquid Jet SCS100A1 [8.0±0.3] CD38 Fluorescent Intensity Units was significantly p<0.05 lower than the IM SCS100A1 which scored the highest at [10.7±0.5]. (Figure 7.5) plots the results.
Blood Cytokine Panel Results

(Table 7.2) summarizes the cytokine analysis determining the global immune effects of self-complementary vector driven overexpression.

Table 7.2 Pro-Inflammatory Blood Cytokine Data at 10 Weeks Post MI: scAAV9.S100A1

<table>
<thead>
<tr>
<th>CYTOKINE:</th>
<th>BLOOD LEVELS [picograms/mL]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
</tr>
<tr>
<td>IL-1α</td>
<td>113±29</td>
</tr>
<tr>
<td>CCL5 (RANTES)</td>
<td>156±22</td>
</tr>
<tr>
<td>TNFα</td>
<td>124±26</td>
</tr>
<tr>
<td>IFNγ</td>
<td>79±15</td>
</tr>
<tr>
<td>IL-4</td>
<td>37±8</td>
</tr>
<tr>
<td>IL-6</td>
<td>46±11</td>
</tr>
<tr>
<td>IL-12</td>
<td>54±14</td>
</tr>
</tbody>
</table>

* p<0.05; AAV.S100A1 Delivery Group vs. Saline Controls
Elevated profiles of IL-1\(\alpha\) and CCL5 (RANTES) were found in both the IM and Liquid Jet groups. There was however no major increase in TNF\(\alpha\) or IFN\(\gamma\) as found in the IM single stranded group that experienced an increase in all 4. Another unique finding was that the double stranded results yielded an increase in IL-12, which was not found previously. Based on these results, overall, it appears the double stranded vector, despite much higher fold S100A1 expression, is less inflammatory for IM Injection. For Liquid Jet Injection on the other hand, it appears more inflammatory but does not result in poor outcome at 10 weeks.

In terms of outcome based on cardiac mechanics and host response, a penultimate outcome overview is described in (Table 7.3).

**Table 7.3 Compiled Outcome Results: ssAAV9 vs. scAAV9 Per Group**

<table>
<thead>
<tr>
<th>GROUP</th>
<th>CARIAC FUNCTION PROFILE</th>
<th>THERAPEUTIC PROFILE</th>
<th>HOST RESPONSE PROFILE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Function EF%</td>
<td>Integrity LV WT</td>
<td>LV Scar %</td>
</tr>
<tr>
<td>Liquid Jet S100A1</td>
<td>60±3%</td>
<td>1.5±0.1</td>
<td>17±5%</td>
</tr>
<tr>
<td>IM S100A1</td>
<td>43±4%</td>
<td>1.1±0.1</td>
<td>40±4%</td>
</tr>
<tr>
<td>Liquid Jet SC-S100A1</td>
<td>57±4%</td>
<td>1.5±0.1</td>
<td>32±9%</td>
</tr>
<tr>
<td>IM SC-S100A1</td>
<td>60±2%</td>
<td>1.4±0.04</td>
<td>30±13%</td>
</tr>
</tbody>
</table>

**7.4 Discussion**

The two major limitations of this study are the single dose of 1.2 x 10\(^{11}\) viral particles and the lone 10 week follow up timepoint. We will review the compiled data within the context of these two limitations. Despite these constraints, the compiled
studies do offer some very interesting concepts that can be explored for future direct myocardial delivery studies featuring AAV mediated overexpression for ischemic MI.

Several key topics for discussion will be: the pharmacodynamic effects of viral mediated S100A1 therapy by vector/route selection, saturation limits with the IM route regardless of dose, and factors that elevate host response risk profiles.

As argued by this dissertation, the ideal direct delivery mediated gene treatment paradigm for ischemic MI would do the following: (1) Transfect a sufficient % of myocardium to rescue failing myocardium and simultaneously reduce disease burdened cells in affected fibrosis area (2) Tip the host response in favor of tolerance rather than a deleterious adaptive immune response (3) Minimize collateral overexpression in non-target organs with an optimized direct route of administration. (4) As a function of (1), (2) and (3) selection of the correct vector/dose that would achieve the objective within safety limits. Essentially we argue that a solution is not likely an optimized vector construct or the perfect device arrangement, but a systematic approach exploring the interactions between them.

One key element of a systematic approach includes an understanding of the pharmacodynamics of AAV mediated cardiac gene therapy. This has become a significant area of interest, with a number of studies designed to elucidate the ideal copy number required for an efficacious delivery profile or exploring relationships between exposure and downstream expression levels [9]. In this study as in previous studies [10], self-complementary vector at the same dose level and route results in dramatic differences in: therapeutic viral DNA transfer, mRNA expression levels, and subsequently therapeutic protein production. For IM delivery, our results demonstrated a 23 fold difference with the same dose. For Liquid Jet, less dramatic, but still significant elevated at roughly 4 fold. Thus, it is a reasonable conclusion that the same therapeutic
benefit in our study could have been achieved with a lower dose of self-complementary AAV given that only a 4 fold overall increase in S100A1 expression was found to be efficacious in the ssAAV9.S100A1 Liquid Jet S100A1. Therefore, future dose response studies including both vectors would reveal more important findings on kinetics.

These observations on the performance differences between the vector constructs leads our discussion into two key directions with regard to dose/vector selection when targeting failing myocardium. First, after breaching an inherent biological capacity threshold, additional copy numbers may not provide benefit in terms of additional therapeutic protein production and should be considered carefully. Secondly, direct routes (i.e. whether IM, needleless microinjections) a cardiac muscle region via saturation limit prior to inducing host response risks. This upper limit is analogous to those that are sought with routine vaccinations, whereby a successful cardiac gene therapy administration is the antithesis.

Expanding on the first point above, it was found that the overall fold protein S100A1 ratio in the IM Groups (i.e. increase from 5.0 to 8.3 fold) did not correlate with the 23 fold increase in DNA copy number, the basis for expression. This suggests internally either the cells can only generate so much S100A1 protein from mRNA or additional regulators exist. From a clinical perspective, this indicates an important concept on a per gram myocyte basis scaled up to larger organisms. Therefore, it is plausible that there is a suitable effective range of vector which in effect reaches the biological upper limit, but does not provide benefit beyond that with increased copy number. This range may depend on the health of the area, since diseased muscle areas are more difficult to transfec [11], but ultimately an upper limit is reached readily with direct injection methods since there are minimal barriers to circumvent as opposed to transvascular delivery [12]. This study demonstrates that at least 2,000 – 10,000 copies
per 100 ng DNA of encoded S100A1 DNA in rodent myocardium seemed to be sufficient in rescuing from acute myocardial infarction with raising basal expression levels 4 fold. Expanding upon the second major point, we and others assert that the same therapy providing benefit beyond the window can dramatically increase the risk of host responses. In our case studies this concept may have been realized even though two groups were efficacious, but immunologically activated.

This concept was exemplified in this study with two very different vector transduction efficiencies at the same dose level. The group with the highest cardiac specificity was the single stranded Liquid Jet, the only group (Table 7.3) with both a positive cardiac and host tolerance outcome measures. This group featured the lowest overall fold S100A1 protein level by comparison with the other 3, but also had the least host response as measured by CD38 and the systemic cytokines. It is very likely that very minimal off target exposure in the liver reduced these risks given that delivery, vector and dose variables were controlled. However it is important to note when switching to self-complementary vector this level in the Liquid Jet group increased nearly 1000 fold. Although striking and not in proportion with the IM group changes, this is likely explained by the difference in single stranded genome conversion between in the liver. In other AAV mediated gene studies it was found that liver cells have much more difficulty with single strand synthesis likely explaining the results in our studies [5-7]. Therefore, collateral exposure is a function of both delivery and vector selection and must be considered in risk assessment.

The most striking outcome discrepancy in the compiled results is clearly with the IM route when comparing single versus self-complementary vector. It appears one plausible conclusion is that the difference in outcome when comparing transduction and protein levels with IM are: (1) the single strand to double strand conversion step is very
inflammatory with a robust, heterogeneous IM expression profile and (2) Self complementary vector has a significant time advantage in establishing vital expression in the early post MI period.

As described previously, strong evidence supports the claim that the single stranded genome conversion process in vivo after the viral nuclear docking step is not without risk and can trigger immune activation. The difference between the IM and liquid jet expression profiles established in Chapter 5 is spatial myocardial distribution. It is reasonable to assert that the highly heterogeneous, overexpressed regions in muscle containing unconverted viral origin single stranded DNA triggered the difference. The liquid jet methodology essentially disperses this risk under a theorized immunological threshold. The second intriguing explanation may be timing, whereby self-complementary has a key advantage in the early stage of acute MI. This is due to the fact that self-complementary vector as designed delivers fully capable therapeutic DNA that immediately generates S100A1 protein. Therefore, is very likely that in addition to being less inflammatory, the scAAV9 vector generated efficacious levels much faster. Due to the 10 week timepoint limitation, no conclusion can be made as to whether or not any of the 2 efficacious groups with elevated risk profiles (Liquid Jet SCS100A1 or the IM SCS100A1) would be remain so over time.

There are many studies that demonstrate efficacy for periods up to 3 months, then show rapid losses of both therapeutic expression and outcome measures due to aggressive immune responses. For this reason, a number of gene therapy trials have actively acknowledged the risks of systemic exposure and inflammation by incorporating immunosuppression in an effort to keep the host under the critical threshold of immune activation [13].
Summarizing, outcome is a function of the efficacious target expression balanced by the risk of delivering the sufficient vector payload to reach a stable range, yet not breach tolerance. Host response problems are clearly identifiable when doses are dramatically increased beyond a range such that therapeutic outcome has saturated or systemic exposure becomes too high.

This study illustrated these concepts and support further temporal experiments with additional vector configurations, whether self-complementary or those with different promoters/transgenes etc. Chapter 8 explores a means to reduce the host response risks upfront with a combination drug/gene therapy concept. It is anticipated that the first critical post AAV delivery window has a major role in determining therapeutic benefit.

References


vectors to overcome the rate-limiting step to transduction in vivo. Gene Ther 10: 2112–2118.


CHAPTER 8

A drug/gene combination strategy to enhance direct myocardial AAV delivery


8.1 Introduction

Acquired heart disease from myocardial infarction (i.e. heart attack) remains the leading cause of mortality and morbidity worldwide, with 22 million new patients diagnosed annually. Essentially, all approved pharmacologic and device systems impose significant cost burden to the health system, yet fail to increase survival rates. [1-3] Cardiac transplantation, which is the gold standard for patients, will never meet the clinical demand due to the chronic shortage of viable donors [4,5]. Therefore, new therapeutic approaches to manage the disease burden represent a significant unmet need. Recently, sophisticated molecular profiling tools combined with a deeper knowledge base derived from disease models have ushered in a new era of biopharmaceutical development for heart disease. This has resulted in the development of a more potent class of therapies designed to act at the myocyte level, whereby therapeutic action is achieved primarily through DNA, RNA and or microRNA genetic reprogramming. [6]

Various gene therapy concepts have been applied successfully in animal models demonstrating increased contractility, repaired myocardium, and or regenerated new vessels to reduce myocardial infarction reoccurrence. [7-10] Independent of the targeted gene mechanism, the most common means to achieve these aims are with either bioengineered viral or non-viral vector biologics, since the uptake and success rates of naked molecular therapies is very poor in vivo. [11,12] The most effective gene
products today have shown remarkable promise, but at the same time have also presented more risks and complicated translational issues, especially when compared with traditional pharmaceutical compounds.

Despite the availability of effective transgene-vector systems, one major rate limiting problem is with achieving safe and efficient myocardial gene transfer in the clinic. [13,14] Due to size scale and more complex membrane barriers, these issues do not emerge in smaller animal studies yet are a major challenge in larger organisms. [15,16] Although the preferred route of administration in clinical trials, it remains controversial whether or not minimally invasive catheter infusion approaches can yield sufficient therapeutic expression levels that significantly improve outcomes in the clinic. [17] Another major problem with these systems is restricting therapeutic expression to the heart and minimizing off target effects. In fact, published large animal data has demonstrated a greater than 2000 fold higher presence in collateral organs versus the heart [18-20]. Alternatively, direct myocardial delivery methods can largely restrict therapeutics to the heart if safely administered.

Direct myocardial delivery methods (e.g. needle injection, sonoporation) can offer greater cardiac specificity of gene therapeutics compared to percutaneous infusion approaches. The key unresolved problem is with the limited distribution of gene therapeutic per delivery site requiring many injections. [21] Increasing the number of injections has the adverse effect of triggering inflammation in the myocardium, thus limiting the availability of additional injection sites and jeopardizing the retained therapy. The immune response to gene therapy products, especially notorious with the viral mediated products, is complex but several key studies have demonstrated a clearer relationship between inflammation and the increased risk of an adaptive immune response. [22,23] Therefore it is postulated the use of an anti-inflammatory drug co-
delivered with the gene therapy product could: (1) Address the inflammation to minimize the adaptive immune response and promote therapeutic tolerance (2) increase trafficking and uptake in a more favorable microenvironment and (3) potentially permit more injection sites.

This concept of a direct injection drug/gene approach has yet to be translated into the heart, whereby problems exist with increasing uptake and extending the half-life of anti-inflammatory drugs at the site of injection beyond the peak acute inflammatory window of 48 hours. In addition to the timing issue, the anti-inflammatory load must not interfere with vector trafficking or the subsequent gene expression efficiency. Numerous studies have explored of advanced non-viral vectors to increase in vivo performance by means of transfection alone. [24,25] However, none have attempted to use anti-inflammatories at the injection site co-delivered with a higher risk, but optimal gene transfer vector to provide a more promising clinical strategy.

This study summarizes the development and parameter testing of a reliable nanoscale anti-inflammatory formulation production process for co-delivery with gene products. The development phase features aspirin and prednisolone, two widely utilized anti-inflammatories and incorporates them into two common FDA approved poly lactic glycolic acid (PLGA) polymers. [26] Complete nanoparticle characterization, process tolerance limits and an in vitro feasibility assessment in harvested myocytes are offered to evaluate the concept of a drug/gene combination therapy.

8.2 Methods and Materials:

Poly-Lactic Glycolic Acid Nanoparticle Production Process

Pre-Processing Steps:
A water oil water (w/o/w) double emulsion process outlined in (Figure 8.1) was executed to generate aspirin (99% pure, Sigma Aldrich USA) and prednisolone (99% pure, Sigma Aldrich USA) loaded poly-lactic glycolic acid (PLGA) nanoparticles (NPs).

First, initial drug load water phase stocks of 1-3 mg/mL aspirin and 0.1-0.4 mg/mL prednisolone were created by dissolving in 1% poly vinyl alcohol (PVA) solution. In the case of prednisolone due to its poor water solubility, a 10% Ethanol (w/w%) was added. These doses were selected based on body weight and pharmacokinetic data for the rodent species. The second step or oil phase was generated in a separate vial, with PLGA, input mass range (20-120 mg) of one of either types (50:50, 65:35 i.e. % of lactic: glycolic acid chains) dissolved in 2.5 mL of Dichloromethane. For the in vitro study only, production runs were carried out as described in the methods above except 100 µg of Rhodamin B dye powder was added to the first drug water phase.

**Process Steps:**

The first emulsion was created by adding 1 mL of aspirin or prednisolone drug PVA 1% solution dropwise to the oil phase polymer in a 5 mL glass vial under probe...
sonication. After 3 minutes, this resultant emulsion was then added dropwise to a larger outer water phase containing 15 mL of PVA 1% to create the double emulsion. The double emulsion was then placed in a fume hood and stirred gently for at least 24 hours to facilitate solvent evaporation and particle formation. Separation was achieved with via ultra-centrifugation at 30,000g for 35 min at 10C. The resultant particle pellets were washed to remove residual drug/polymer, then freeze dried overnight. Four nanoparticle compositions were generated with the reaction: PLGA (50:50 Aspirin), PLGA (65:35 Aspirin), PLGA (50:50 Prednisolone) and PLGA (65:35 Prednisolone).

**Post-Processing:**

All yields were weighed then stored in sterile cryovial containers at -20C.

**Scanning Electron Microscopy (SEM) Analysis & Characterization**

Approximately 5 mg of each freeze dried NP sample was prepared for SEM with gold sputter coating, and then imaged on a JEOL SEM unit at 1.00 kV between 10-20,000 x. Multiple images were taken from separate locations on the field with focus in the range of 1 um to 500 nm.

**Stability Testing**

**24 Hour Formulation Stability Test**

Saline stability re-constituted particles tests were also conducted. Ten mg of freeze dried nanoparticles were dissolved and probe sonicated in sterile 0.9% saline water and allowed to settle over a 24 hour period. Repeat droplets were first dried and sputter coated for loading into the SEM.

**Zeta Potential Colloidal Stability Measurements**

A sample of each particle composition was prepared in water and added to testing cuvettes per manufacturer instructions of the Zetasizer Nano ZS instrument.
(Malvern Instruments, UK). Triplicate runs were averaged to represent a single data point for multiple samples from the same production lot.

*Controlled Release & Loading Efficiency Analysis*

A UV-vis spectrophotometer (NanoDrop, National Instruments) was used to generate two separate standard curves for serial dilutions of known drug concentrations. The wavelength consistent with aspirin detection was 275 nm and 235 nm for prednisolone.

**Drug loading efficiency calculations**

To compute loading efficiency, the amount of either aspirin or prednisolone encapsulated in the nanoparticle formulations was determined by measuring the residual amount in the supernatant following centrifugation relative to the initial load in the water phase. Percentage was derived as the mass amount of drug remaining in the supernatant following separation.

*Controlled Release Analysis*

High quality yields were selected based on the most potent drug formulation of 5 wt.% PLGA aspirin and 1.5% PLGA prednisolone were manufactured per process specifications. Each particle formulation was prepared for controlled release studies as follows: (1) 20 mg of particle was dissolved into 10 mL of 42C (2) Samples for spectrophotometry analysis were removed with a syringe 450nm filter at 12 hours, 1, 2, 3, 4, 5 days (3) The sample volume was replaced and the process repeated for each interval up until the final point (4) Triplicate UV-vis spectrophotometry measurements against the standard curve for each drug were performed on each sample to determine the percentage released for each run.

*In Vitro Testing Protocol*

*Neo-natal Rat Cardiac-myocyte harvesting*
Day 0 to 3 neonatal pups are used and the pups were euthanatized by decapitation and the heart was immediately removed with forceps. The atria and great vessels were removed and the left ventricular tissue was minced and subjected to a trypsin-based disaggregation procedure in a 6 well plate with ethanol cleaned scissor, rinsed with HBSS with 1% P/S/G, and place in a 50ml conical tube containing 10ml of Trypsin solution for shaking (200rpm) at 37C for 15 min. Cells were then centrifuged at 660rpm at 4c for 5 minutes. The supernatant was discarded and the cells were resuspended in 20 ml of media and pre-plate for 1-3 hours in the incubator. Harvested cells were collected with centrifuge spin at 660rpm for 5 min at room temperature. Cell pellets for experiments were then placed in the culture media and counted using 0.4% Trypsin blue.

**Plasmid GFP DNA & Tagged Nanoparticle Preparation**

A 10 mg master aliquot of eGFP plasmid DNA was obtained from Invitrogen and handled according to manufacturer’s instructions. Under sterile conditions working yields for each well was created with, 6µg of DNA was diluted into 100 µL of RNAase free water. Separately 10 mg of each of the process output 4 resultant particle systems [PLGA50:50-Aspirin, PLGA50:50-Prednisolone, PLGA65:35-Aspirin, PLGA65:35 Prednisolone] tagged with Rhodamin B was finely crushed and mixed into 20 mL of phosphate buffer saline. To remove residual dye not bound within the particle structure, the particle solution was placed into a dialysis membrane submerged in an outer bath of PBS at 37. Prior to well transfection, 10 µg of each nanoparticle solution in 500µL was placed in individual aliquots.
DNA and Nanoparticle Well Transfection

On the day before transfection, cells were placed in 12 well plates with each well seeded at a density of 500,000 myocytes in 1mL of DMEM (GIBCO) media without antibiotics. The transfection complexes were then prepared:

Complex 1 – One 6µg of DNA aliquot was diluted into 100µL of media in an individual eppendorf vial. Complex 2- 8µL of Lipofectamine (Invitrogen) was diluted into 100µL of media. Then, complexes 1 and 2 were mixed together and permitted to incubate at room temperature for at least 20 minutes. After 20 minutes the contents of the individual eppendorf yields were then transferred into each well. The plate was gently rocked then placed back in the incubator until the first 24 hour imaging time point.

For the nanoparticle treatment wells as designated, the 500µL filtered sterile solution was added via syringe to each well with a 450nm to prevent aggregates from transferring.

Follow up Fluorescent Microscopy

The first set of images was taken at 24 hours post transfection. Media was removed from each well prior to imaging and replaced prior to returning to the incubator. The remaining set of images was taken at the 48 hour time point.

Fluorescence images were acquired using a Nikon Eclipse TE2000-U fluorescence microscope equipped with a Plan Fluor ×20/0.50 objective (Nikon, Tokyo, Japan). Microscope controlling and image processing were carried out using Image-Pro Plus 4.5.1.27 (Media Cybernetics, Bethesda, MD, USA).
Statistical Analysis

All SEM and nanoparticle characterization data was loaded into GraphPrism software suite for statistical testing. Single way ANOVA was utilized to determine differences in nanoparticle subtypes. Individual paired t-tests were used to compare across individual groups. Bonferroni corrections were applied for significance testing.

8.3 Results

8.3.1 Process Capability

Over 45 nanoparticle production yields were obtained over the development course with the optimal ranges. The process volumes were held in a fixed ratio, featuring water phase #1 at 1mL, the oil phase at 3mL, and the outer water phase #2 at 15 mLs. Pilot runs in greater amount adhering to this proportion scale yielded the same quality particles. Briefly, basic guidelines for each process phase:

Water Phase: Aspirin 1-3 mg dissolved in PVA 1% or Prednisolone 0.02-1 mg in 10% ethanol PVA1%. It was noted that adding additional solvents to increase drug load in this phase resulted in failure to maintain particle integrity and stability.

Oil Phase: The process was very flexible in terms of changing the amount of polymer added to the system and was stable in the range of 20 – 120 mg of either PLGA type.

Outer Water Phase: The PVA in the system acts as a vital stabilizer that can be readily increased. Increases beyond 2% tended to inhibit the amount but not the quality of generated nanoparticles. Thus, a working range of 0.5-2% of PVA stabilizer in the outer water phase is suitable for accommodating various drug/polymer complexes with good stability.
8.3.2 Particle Characterization

The results presented here summarize the characterization for each of the 4 resultant nanoparticle types acquired from 5 consecutive runs. Polymer load was fixed at 60 mg, aspirin 3 mg and prednisolone at 1mg respectively.

SEM images from the various runs for each nanoparticle type were loaded into ImageJ software for analysis. The process consistently yields uniform, spherically shaped formulations (Figure 8.2AB). The size distribution was very narrow, of high quality and was as follows: PLGA50:50 Prednisolone [234±9 nm], PLGA65:35 Prednisolone [228±7 nm], PLGA50:50 Aspirin [323±13 nm] and PLGA65:35 Aspirin [302±7 nm]. ANOVA indicated significance between the groups, specifically it was determined that aspirin contributed to larger particles as both PLGA50:50 and PLGA 65:35 types were significantly larger than their matched prednisolone counterparts. (Figure 8.2C) This difference in size was most likely attributable to both higher aspirin mass content and charge of the first water phase in the reaction since size was unaffected by the addition of more polymer (data not shown).
Yields were very consistent and proportional to polymer mass input in the range of 75-80% recovery upon final harvest. The average yields per polymer/drug type based on 60 mg input were: PLGA50:50 Prednisolone [46±1 mg], PLGA65:35 Prednisolone [45±2 mg], PLGA50:50 Aspirin [48±1 mg] and PLGA65:35 Aspirin [47±2 mg].

Production yields with increased or decreased polymer loading revealed the same results (data not shown).

Loading Efficiency results were uniform for all 4 nanoparticle types, independent of drug or polymer and were: PLGA50:50 Prednisolone [88.9±0.01 %], PLGA65:35...
Prednisolone [88.2±0.01 %], PLGA50:50 Aspirin [89.0±0.01 %] and PLGA65:35 Aspirin [88.8±0.01 %].

Stability Analysis

Positive nanoparticle visualization was realized on the SEM 24 hours after re-constituting freeze dried product in saline for all 4 polymer configurations. The particle shape and size was retained. The stability of the nanoparticles in suspension was moderate to good in the range of -30 to -53 mV. A score much less than -30 indicates a stability issue with a pharmaceutical dispersion, while any score higher than -60 indicates maximum. The potential scores by nanoparticle type shown in (Figure 8.3) were as follows: PLGA50:50 Prednisolone [-47±5 mV], PLGA65:35 Prednisolone [-31±1 mV], PLGA50:50 Aspirin [-45±0.5 mV] and PLGA65:35 Aspirin [-32±0.9 mV]. Statistical tests revealed that the PLGA50:50, independent of drug load was superior compared with the 65:35 type.

![Nanoparticle Colloidal Stability Measure by Type](image)

**Figure 8.3.** Nanoparticle Zeta Potential Colloidal Stability Testing Results indicate that the PLGA50:50 nanoparticles are more stable in solution versus the PLGA65:35 types.
Controlled Release of the Nanoparticle Formulations

(Figure 8.4) shows a graphical depiction of the release over the span of 5 days. It was evident that the aspirin released faster overall as compared with prednisolone. This is most likely due to a combination of factors including size, stability and charge. The PLGA50:50 Aspirin type had the fastest release profile.

![Nanoparticle PLGA Formulation Release Analysis](image)

Figure 8.4. Controlled release study results demonstrate that aspirin particles overall release faster than prednisolone types.

8.3.4 Process Limitations

The high quality in terms of particle shape uniformity, yield, surface charge and release properties were critically limited by a number of key variables. Therefore, production with major deviations with the water phase I input (data not shown) resulted in lower quality profiles featuring aggregation and wider size ranges. The first major critical variable was the concentration of the loading drug in the first water phase, which
was largely limited by the inherent solubility at room temperature. In the case of aspirin, without solvents added, the maximum concentration was 3 mg/mL directly at the solubility limit. Runs at the 5-10 mg/mL range resulted in aggregation and lower quality. In the case of prednisolone, it was anticipated that on a per gram basis at least 1 mg/mL would be required to achieve high quality in addition to a realistic dosing paradigm for a rodent heart with target of 1 gram mass. This was achieved suitably with 10% Ethanol, however concentrations greater than 25% in an attempt to load more drug distorted the process (data not shown). The PLGA and PVA stabilizer system as presented here is therefore open to excipient manipulation provided that solubility and other attributes of the selected drug are addressed such to prevent deviations in overall quality which may or may not be desired depending on the application. We anticipate this platform would be open for further experimentation by professional formulation scientists tailored to each specific PLGA/drug selection for the intended direct injection application.

8.3.5 In-Vitro Myocyte Transfection

All wells were checked for viability and it was determined that none indicated any major media discoloration or visual evidence of contamination. The following 5 groups all had positive detection of GFP (green) in at least 2/3 replicate wells at both 24 and 48 hours, with a greater degree of cells positive as expected at 48 hours. Figure 5 depicts independent uptake of both GFP plasmid and nanoparticle co-signal. The absorption clusters were confirmed in the center of myocytes. The multiple DNA and nanoparticle infection groups yielded nanoparticle presence (red) or both (yellow) at the 24 and 48 post transfection: I. Control GFP DNA only (data not shown) II. GFP and PLGA65:35 Aspirin (Figure 8.5A) III. GFP and PLGA50:50 Aspirin (Figure 8.5B) IV. GFP and
PLGA50:50 Prednisolone (Figure 8.5C) V. GFP and PLGA65:35 Prednisolone (Figure 8.5D).

Figure 8.5. In Vitro Fluorescent Imaging at 48 hours post transfection. All 4 particle systems exhibited safe and robust uptake in myocytes while not interfering with plasmid uptake and subsequent GFP expression. Yellow signal indicates co-existence of GFP and nanoparticle in: A. PLGA65:35 Aspirin B. PLGA50:50 Aspirin C. PLGA50:50 Prednisolone D. PLGA65:35 Prednisolone.
8.4 Discussion

This study presents two key findings that have broad implications for the advancement of cardiac gene therapies. First a reproducible, simple to use lab scale process was developed to generate anti-inflammatory nanoparticles of very high quality for co-administration with gene products in a regulatory friendly PLGA platform. Although only two anti-inflammatory drugs were utilized in this feasibility assessment, it is anticipated that any other drug indicated for injection into muscle could be introduced by modification of the first drug water phase. Also the process offers an easy means to adjust the polymer content in the oil phase for the desired degradation/release profile, along with increasing the amount of stabilizer. Therefore this system can provide a platform to guide future pre-clinical studies to investigate reliable clinical interventions to address the role of inflammation on the relative performance of gene products in muscle tissue.

The potential role of inflammation should not be overlooked, particularly in myocardial tissue where the most common delivery scenario is in ischemic regions, which are characterized by a high degree of inflammation and fibrosis. The second key finding in the final test was that PLGA uptake and release of anti-inflammatory agents in myocytes does not interfere with the absorption and trafficking of the GFP plasmid. Muscle tissue has a high risk of developing an adaptive immune response to gene products. Wilson et al described in detail the host response after AAV delivery by route of administration and more specifically the role of inflammation. [27] A key finding with AAV mediated gene transfer was that the host either induces tolerance or an adaptive immune reaction through a series of complex interactions. [28-30] A prime risk factor in these interactions that was found to trigger adaptive immune responses were
inflammatory cytokines and signals either already present in tissue or induced at the
time of delivery. [31] It has been postulated that with attenuation of innate inflammatory
response signals, the immune system has a much lower risk for mounting maladaptive T
cell responses.

Using the example of AAV, once vector capsid antigens are cleared from the
system, typically 12-16 weeks after delivery, there is a good chance for therapeutic
tolerance. The risk is that an adaptive immune response will destroy those cells
expressing the transgene of interest well before these antigens are cleared. Use of anti-
inflammatory agents to mitigate the innate response to injury is likely to result in
enhanced long term gene expression. Intravenous delivery approaches are associated
with a lower level of induced inflammation but are also very inefficient. In contrast, the
IM route in the heart remains attractive because greater cardiac specificity can be
achieved, especially for angiogenesis or regenerative therapies that require a more local
delivery profile. Yet IM delivery is associated with a more robust innate immune
response due to associated tissue injury.

Direct injection into healthy or ischemic myocardial regions introduces the gene
product into a highly inflammatory region, which likely explains the poor cardiac gene
therapy results with IM interventions. Early studies by Snyder [32] et al reported that
very little successful transfer occurs in damaged muscle in the inflammatory
environment. Numerous examples have validated these observations in gene therapy
trials. In hemophilia trials for example it was found that IM injection into skeletal muscle
resulted in transient therapeutic gene expression and an adaptive CD4+ immune
response. [33,34] However, delivery of the same product infused into the liver has
resulted in better outcomes and limited reactions. Muscular dystrophy trials have
encountered similar difficulties and have attempted to utilize immunosuppressant drugs
and other agents to limit responses after multiple IM injections compromising patient safety. [35]

8.5 Conclusions and Future Direction

In this proof of concept study, GFP plasmid was utilized to simulate a therapeutic construct understanding that naked DNA is likely to be at the lower end in terms of transduction efficiency. For more practical gene therapy applications, it is anticipated that viral vectors encoding the gene of interest could readily be combined with particles containing potent anti-inflammatory drugs. The hypothesis offered is that with the right formulation, the anti-inflammatory agent would be released at the sufficient level over the critical post-delivery inflammatory period to provide an optimal viral vector trafficking microenvironment. There would be a predicted increase in transduction efficiency, minimize the innate and adaptive immune response to the vector and/or transgene and promote long term gene expression in the target tissues.

This strategy of course would not be without its own limitations and would require much more experimentation to determine the best matched drug and release profile for co-administration into the heart. More complex approaches in managing the host response following therapy have been applied, however it may turn out that simply addressing the innate immune response at the time of delivery may be a meritorious approach to advance successful clinical translation.

References:


CHAPTER 9

Summary, Limitations and Future Direction for Needleless Liquid Jet Delivery for Cardiac Gene Therapy Applications

9.1 Summary

Recurrent or a single major acute myocardial infarction (MI) event results in a significant decline in cardiac function in the early stages, progressive decline through left ventricular remodeling and maladaptive physiology changes in the mid stage, then ultimately in end stage heart failure whereby high cost/low reward (i.e. excluding transplants) options still fail to extend survival. The burden this problem presents to the healthcare industry as whole does not need more awareness, but more effective solutions.

Cardiac gene therapy applications are expected to meet or exceed growth projections and will remain a strong research area in an effort to develop new targets, engineered vectors to encode them and finally as argued by this dissertation their optimal delivery route for the given dose required. Demand is the main reason cardiac gene therapy will be a stronghold in modern medicine for years to come. This demand is driven by 3 primary factors: (1) The chronic shortage of donor hearts will never meet demand for 22 million end stage patients (2) State of the art surgeries and the latest line of drugs are palliative and are no longer cost efficient for government and managed care payers (3) The incidence of heart disease, specifically from coronary artery disease, is expected to increase significantly with aging populations in the United States, Europe, Asia and other developing nations whom adopt western habits.

Chapter 1 describes the overall advantage of cardiac gene therapies as compared with the standard of care. Regardless of the specific mechanism, the goal is to treat the
myocardial disease permanently at its root cause by administering therapeutic molecular biologics as the medicine. These strategies either restore function and or attenuate maladaptive cellular regulation with a sustainable encoded fix. The excitation contraction coupling, angiogenesis, and other related survival mechanisms are well established. With the continual growth of computation power, microarrays and bioinformatics the number of targets for myocardial dysfunction will also likely increase over the next 10-15 years as massive genetic databases are being built [1]. On the basic science front, more detail knowledge is being developed with microRNA regulation, therefore new strategies to induce cell directed repair/regeneration have already found some early success [2].

In addition to the explosion of new strategies, Chapter 1 also illustrates that viral mediated genome transfer can result in safe, long term outcomes as evidenced by a longer track record of other AAV gene therapy trials. The field has simply learned much more with a more prudent scientific approach, whereby human trial data and a growing network of investigators have blossomed. Bioproduction engineers and scientists therefore will continue to be motivated to develop, safer, more effective transfer vehicles engineered for the intended cardiovascular disease now that safety and efficacy can be realized with careful administration [3].

Given the availability of targets and improved transfer vehicles, Chapter 2 introduces the key problem of efficient myocardial gene delivery. This dissertation and a growing body of evidence argues that this is the rate limiting problem that must be solved in the clinic [4]. Overall, cardiac gene delivery systems have lagged in terms of development as compared with targets and vector engineering. As presented in our detailed development of a liquid jet concept, it is clear there are major discrepancies between animal model and actual clinical applications. The first major difference is anatomy, not
only in terms of size scale but additional transport barriers that exist in humans such as
pre-capillary sphincters [6]. The second are co-morbidities common to heart disease
patients, such as chronic inflammation in the targeted area compounded by diseased
transport vessels necessary for AAV trafficking into the myocardial compartment. These
are never captured in large animal models that claim to resolve the scale issue and must
be considered.

Delivery strategies as reviewed in Chapter 2 fall into two major categories: (1)
Transvascular or those that seek to leverage the capacity of the patient’s cardiac vessels
for transfer (2) Direct methods which apply physical means of fluid, pressure, electrical
current etc. to drive AAV directly into beating myocardium. The primary goal is to
transfect the highest % of myocytes possible with the least degree of systemic exposure
that would raise risk profiles. The majority of gene therapy trials because of safety and
an established preference with interventional cardiologists, select the percutaneous
antegrade catheter infusion system.

As leaders in the cardiovascular gene delivery space for the last 10 years, our
group has demonstrated that the route of administration is the prime factor that will
determine outcome, since transfecting a high % of myocytes is an absolute requirement
for demonstrable efficacy in terms of cardiac rescue with AAV mediated excitation
contraction coupling genes. The CUPID Phase II trial results featuring AAV1.SERCA2a
do not show a statistically significant improvement in echocardiography and other
improvement metrics are trivial at best [6,7]. Based on analogous large animal and a
limited number of post mortem clinical specimens, evidence suggests that antegrade
single pass “slow” intracoronary infusion transduces only 1-3% of myocytes [7,8].
Moreover, the distribution is limited to the axis of the primary infusion vessel. Other
published large animal data with the same construct resulted in a much higher % of liver
transfection versus the heart, even with attempts to recapture vector leaving the cardiac system with a perfusion circuit (i.e. V-Focus device) [9, 10]. It is important to note that these ovine subjects did not have either the transport or disease barrier conditions, suggesting that intracoronary infusion without modification may never be a viable route despite its safety.

Given the shortcomings of single pass antegrade, the field has employed extensive bioengineering means to develop a variety of catheter modifications such as different lumen and or balloon occlusion configurations that have resulted in a slight increase in efficacy.

For upcoming AAV1.SERCA2a trials, a shift has occurred toward favoring retrograde systems that leverage more favorable dimensions and less restrictive transport barriers on the venous side cardiac anatomy. Our laboratory designed an ideal system leveraging the venous side at the most extreme level, surgically isolating the heart in situ with cardiopulmonary bypass, featuring a high pressure retrograde recirculation of AAV vector throughout the Molecular Cardiac Surgery with Recirculating Delivery (MCARD) system [11]. The MCARD system with AAV1.SERCA2a demonstrated the highest reported cardiac specificity (i.e. up to 2000 fold more cardiac expression versus liver) and rescued in later stage ovine ischemic cardiomyopathy [12]. The obvious limitation of MCARD is its relative complexity and available use in a limited number of patients already undergoing adjunctive surgical therapy.

Given the state of cardiac gene delivery systems, it is clear that there is a major bias toward transvascular systems and this research sought to contribute to the less visited, but increasing important direct injection routes [13]. As described in Chapters 2 and 3, this bias is primarily due to the poor performance of other direct methods in terms
of distribution and resultant induced inflammation, which is known to trigger immune responses [14].

Chapter 3 describes the complete aims in this dissertation to develop a translatable direct myocardial injection methodology leveraging liquid jet device technology. The challenge was adapting the DERMOJet™ device for both a rodent and ovine application. The hypothesized advantages of liquid jet delivery were: (1) increase myocardial retention (2) limited collateral spillover provided tuned device settings (3) wider coverage of myocardial tissue mass per injected unit volume (4) Less inflammatory; both in terms of the initial punctures and subsequently generated homogenous expression profile and (5) Would potentially provide a solution for 50-60% patients excluded from AAV therapy trials due pre-existing antibody titer. Ideal delivery with this proposed route as stated was to project and disperse AAV through myocardium in a powerful, yet safe jet stream that would be retained.

Liquid jet injection technology as discussed in Chapter 4 is not at all novel and had previously been utilized in both scientific and basic medical applications including vaccination [15]. Within the realm of gene therapy, there was no prior literature describing the concept for AAV mediated therapy with contractility transgenes, thus the inspiration for this work.

Preliminary experiments with the DERMOJet™ revealed the need for optimization. As described in Chapter 4, ex vivo muscle and mock demo testing of various materials characterizing the jet profile revealed a potent laser like injection track with minimal dispersion from close range (i.e. 1-3 inches from the surface). The nozzle velocity was roughly 330 m/s with a 500KPa driving pressure inside the chamber. These factory ratings were tuned for deep skin/muscle penetration, which of course serve
vaccination applications well. It was determined that if this approach would work, optimization was required.

The device settings were altered through working the engineering team of the manufacturer to reduce the power rating via actuator spring size reduction. Then, through a series of optimization experiments in beating heart non survival models, it was determined that 110 m/s velocity and a driving pressure of 100-250KPa would provide the proper dispersion. Another key variable was identifying optimum target distance range to achieve safety and maximum myocardial dispersion simultaneously. This range was determined with simple methylene blue studies. The ideal range was between 7.5 to 9 inches from the thoracotomy open heart for the rodent application, and 2-4 inches for the ex vivo ovine heart application. Once optimization was achieved, several key rodent model experiments were executed in Chapters 5 to both validate the system and compare with other delivery approaches.

Chapter 5 was the heart of this work, essentially validating the liquid jet methodology and simultaneously exploring key variables in the new approach against others in practice. As described, important clinical translation aspects were described in the experimental series. The first was retention, whereby needle injection and especially transvascular methods were not well characterized from a quantitative basis. We selected a simple fluorescent dye, much smaller than AAV, to assess retention after 10 minutes post-delivery in an acute model. The Liquid jet performed well in terms of retention with limited uptake in the live and a disperse, homogenous myocardial coverage profile. As expected, the IM group had a very robust retention around the injection track, but resulted in inadvertent systemic infusion. Comparing the left ventricular infusion with versus without occlusion, two key points were validated as argued previously in this dissertation. The transvascular systems must increase
residence time and driving pressure variables in order to both increase cardiac specificity and limiting systemic exposure. The cost is the level of invasiveness, but the concepts were demonstrable.

The follow up live AAV9.GFP survival model largely revealed the same expression profiles as found with the acute retention series. This permits conclusion that the initial delivery event largely determines the expression profile that will be achieved in the future. It was here that a number of key points were determined for the overall aims with liquid jet injection for cardiac gene therapy, namely: (1) Liquid jet injection with optimized settings can achieve roughly the same, but not as intense, level of the best available transvascular approach (2) Limit the degree of systemic exposure (3) Achieve more myocardial coverage per injection. Point #3 is arguably the most important, since limiting the number of injections is key for direct myocardial gene delivery. This topic will be expanded upon in the next section discussing the limitations.

Although the validation experiments revealed positive concept testing results, the next series of experiments described in Chapters 6 and 7 tested the methodology's validity in an ischemic myocardial infarction model. It cannot be underscored that the differences between normal and ischemic myocardium are immense. The induced model insult analogous to the clinic has a significant impact on subsequent cardiac performance and host responses. Separately from intervention, the ischemic myocardial insult promotes a complex myocardial degeneration process marked by inflammatory events, limited repair mechanisms [16]. Due to the limited capacity of cardiac repair, a number of compensatory mechanisms locally in the myocardium and neurohormonal physiology commence. These temporal changes from initial insult to chronic failure must be considered when optimizing any delivery strategy. This was a prime challenge for the liquid jet injection method, but certainly attainable with careful design.
Essentially for Chapter 6, the focus of the liquid jet injection’s applicability was to test whether or not it would be efficacious with an AAV therapeutic encoding a bona fide transgene within the clinically relevant maladaptive post MI environment. We selected a high cardiotrophic AAV9 vector and arguably the most promising S100A1 transgene, which is rapidly approaching clinical trial. The four groups were 2 Control saline and 2 single dose $1.2 \times 10^{11}$ vp ssAAV9.S100A1 treatment. The head to head delivery assessment featured liquid jet against traditional IM injection. This chapter ultimately did not incorporate a comparative infusion group since these results translate poorly to the clinical problem as argued in the discussion of Chapter 5.

The results in Chapter 6 were striking featuring a major discrepancy in outcome across delivery groups. The IM S100A1 group performed considerably worse in terms of overall LV function and presented with a degraded LV structure. The affected infarcted area was double, with full blown heart failure in nearly all 8 group subjects. The Liquid Jet group achieved a 4.3 fold higher S100A1 expression profile, greater than control (1.0) but slightly less than the IM group at 5.0 overall. Despite an overall score of 5, the proteomic analysis variability in the cross section indicated that some regions likely increased this average due to intensity, but the overall degree of expression as in % of transfected myocardium may have been lower. In addition, the results indicated that the pattern of expression and the degree of systemic exposure contributed to the major declines found in the IM animals. The host response testing revealed that the IM group most likely suffered a deleterious immune response as hypothesized based on much higher CD38 tissue marker and systemic inflammatory cytokine profile.

Following the results of the first major efficacy study, Chapter 7 explored a new approach with S100A1 gene therapy with the use of the double stranded DNA or self-complementary vector scAAV9.S100A1. Since a vector control group was not utilized,
the same dose was selected for this study based on the hypothesis that a selective in vivo dose response would be elucidated with higher copy number. The use of double stranded vector is intriguing with several advantages. The first main advantage is conversion efficiency, whereby DNA is ready to drive gene expression just after nuclear transfection, whereas with single stranded vector a key conversion step is needed. The second key advantage, as argued by this dissertation, was that in highly focal muscular expression patterns the single stranded step might be inflammatory.

The efficacy results to support these claims and is valid given that the IM SCS100A1 performed dramatically better than the single stranded group. It therefore can be concluded within the limits of this study that on a per dose basis self-complementary vector should be used for direct injection applications since they achieve the desired level of therapeutic copy number more efficiently. A major limitation of the efficacy studies presented is the imprecise cause of the adaptive inflammatory immune responses in rank order IM S100A1, IM SCS100A1, and Liquid Jet SCS100A1. The host immune response is a complex interaction of induced innate inflammation caused by delivery route, biological state of target tissues (i.e. healthy vs. remodeling from acute MI), AAV viral vector antigen payload risk, S100A1 expression profile/distribution, and collateral organ (i.e. liver) expression. It is acknowledged that the specific root cause of the immunological host response was beyond the limits of this study; however Chapter 8 explored an often overlooked aspect of these risks, the initial delivery event.

Cardiac gene therapy methods, especially direct injection techniques, cause mild to moderate levels of inflammation that can increase the risk for the host response. Chapter 8 explores the concept of a drug/gene therapy approach, whereby gene therapy product would be co-delivered with an anti-inflammatory drug at the time of delivery to:

1) Promote a more favorable AAV trafficking environment
2) Reduce stress on cells
that need to generate therapeutic expression in the microenvironment (3) Induce
tolerance by ensuring the host immune response up to 1 week post delivery (i.e. vector
clearance rate is high by this point) is beyond the window of co-stimulation, which is
critical for adaptive host responses. For this theoretical application, a poly lactic glycolic
acid polymer nanoparticle production process was developed. The rationale was that a
nanoformulation would: (1) increase local drug uptake in the myocardium due to size
advantage (2) Not interfere with vector trafficking and expression mechanisms (3)
Extended release of anti-inflammatory over the innate immune response period through
6 days. Much of the work entailed the anti-inflammatory process development aspects,
whereby a robust set of operating parameters were identified to engineering 200-300 nm
particles encapsulating a variety of anti-inflammatory drugs. Aspirin, prednisolone and
solumedrol have been tested in the system and the in vitro work illustrated the potential
for this combination approach.

The remaining sections discuss the key limitations and future studies for each
specific aim, followed by an overall conclusion on the body of work.

9.2 Limitations and Future Direction

9.2.1 Specific Aim#1 - Define and optimize engineering parameters for the liquid
jet injection device for the cardiac application in an acute rodent model and ex
vivo large animal setting to define a safe, yet effective operating transfer range.

9.2.1.1 Limitations

Adapting the liquid jet injection approach for cardiac gene delivery required
detailed experiments and select modifications of key driving device parameters. A
problem is that these same pressure settings that performance well in one given
myocardial region can also permanently injure another with a more advanced disease
stage. In a controlled, consistent rodent model application however it was possible to obtain excellent results due to this level of precision. This of course would not be in the clinic, whereby populations of patients with a diverse set of co-morbidities will have different levels of affected areas and geometry. Therefore settings would need to be flexible enough over an acceptable range to remain safe. Given these findings, the key limitation of the current results is that this development process would have to be done at a much higher level of sophistication for a clinical application.

First as presented, the size scale factor is a major problem as it is very unlikely that a single pen injector design would be suitable for an AAV mediated therapy. This is especially true for cardiac contractility genes, whereby global myocardial expression over roughly 150 – 300 mg of myocardial mass is needed in the left ventricular zone. Our rodent model demonstrated efficacy, but the target was only 1 mg reached with 3 injections. Therefore, scaling estimates with this primitive system would require at least 300 injections to reach the same efficacy level, which would not be feasible with a single device.

It is also likely that primate and human host responses are more aggressive than the rodent model, therefore inflammatory events would have to managed even more carefully with a selective pressure system approach.

9.2.1.2 Future Direction

A second generation device configuration would most likely go in two directions: (1) A multi array port of injections with selective control over pressure settings featuring real time feedback or (2) A hybrid microneedle array concept for direct injection that would penetrate then slightly pulse pressurize for maximal distribution. Concept #1 would ideally leverage an imaged guidance setup, whether it be echocardiography or high fidelity MRI to use structural data to map desired target areas. The
conceptualization is that ischemic areas are typically much thinner than remote healthy zones, therefore different injection ports would require the correct programmed pressurized settings. These settings could be optimized with a series of engineering modeling and cadaver harvests over a range of cardiac mechanical properties (i.e. optimize pressure settings for levels of thickness, disease vs. non-diseased structures).

Another key concept not explored in this dissertation due to cost was the use of a more optimal driving gas. Many of these liquid jet systems are pressurized by carbon dioxide gases instead of room air/oxygen [17]. Although minimal, there is a risk of air penetration into the system warranting the use of C02. The selected gas, may or may not alter transfection rates but would be interesting to explore. For safety reasons, C02 gas would be the obvious option that is readily available in the clinic. In the future, if warranted, it is also conceivable that the liquid approach could be adapted to a minimally invasive catheter system that would delivery from the endocardial surface.

Exploring Concept #2 of a microneedle system, these have been tried with different transdermal systems with various adjunctive configurations but have not been translated into the heart [18]. It is conceivable that the liquid jet concept could be engineered to perform more consistently with an image guidance capability but this would be difficult to execute. If engineered correctly however this system would have the advantage of more precision in select zones at the cost of complexity.

9.2.2 Perform a two phase therapeutic delivery study to the beating heart in an acute rodent model to evaluate the liquid jet approach against traditional methods in practice. The methods to evaluate against are: A. Standard IM Injection B. Non-selective Intracavitary Infusion C. Intracoronary Infusion

9.2.2.1 Limitations
As concluded in Chapter 5, the sequential validation rodent studies evaluating the liquid jet strategy revealed a number of key considerations for myocardial delivery overall; with a keen focus on animal model to translational perspectives. A number of key points were highlighted for each delivery class when analyzing model results. The first is scale, and was demonstrated effectively with the Liquid Jet concept. The targeted myocardial LV mass is roughly between 1-2 mg in the healthy condition. Our results demonstrate that with optimal settings, 300 microliters of volume was sufficient to cover the entire cross sectional space with Liquid Jet or Infusion with occlusions.

The ovine (i.e. more resembling the human application) mass target is at least 150-200 mg, but much less coverage is achieved with a single injection. This result in the demand for 100-350 injections and this reaches the higher risk range for both injury and priming for immune responses. IM delivery on the other hand is worse in that the highly focal expression profiles achieved would not promote an overall increase in contractility at the larger scale that is needed and surprisingly results in higher systemic exposure than expected due to poor retention [19].

The conclusion with the infusion approaches (e.g. primary infusion with or without anatomical occlusions) was that a means to increase the driving pressure and residence time of vector in the cardiac anatomy is required to increase expression levels. This concept clearly demonstrates the problem of the endothelial barriers, which prevent viral vector trafficking into the interstitial. Although recognized, implementing this at much larger scale is very difficult and requires complex methodology. Banding the pulmonary artery and aorta would never be clinically feasible. Our laboratory to date has presented the only competent means to achieve higher residence time and pressure, which can only be done safely under cardiopulmonary bypass conditions [20].
The concept of increasing pressure locally around a peripheral artery or vein however, is attainable with balloon occlusion catheters. Despite their success in large animal models, the disease vessel integrity breach and other complications that would result (i.e. especially on the arterial side) would not be justified with the benefits. Balloon occlusion on the venous side with primary infusion through the greater cardiac vein the most applicable strategy. Groups have shown increased AAV uptake with retrograde balloon infusion with various pressure and occlusion settings versus antegrade. These approaches are likely to have a higher translational impact. This is due to the fact that venous side anatomy has both increased dimension and less disease factors [21].

9.2.2.2 Future Direction

Marker gene delivery studies featuring multiple approaches most definitely would be strengthened by incorporating a wider dose range and using different settings for said approaches. For example altering injection volumes, pressure, occlusion times, device design may increase therapeutic AAV transfer. Elucidating these mechanisms in more detail requires multiple level designations for each method, this study only featured one dose with one set of delivery parameters for each.

9.2.3 Perform a chronic ischemic infarction 10 week model study to evaluate delivery of a single dose $1.2 \times 10^{11}$ GC of ssAAV9.S100A1 vector featuring a head to head format comparing liquid jet vs. IM injection.

9.2.3.1 Limitations

Although very striking differences were found between the IM injection and Liquid Jet groups versus controls, there were major limitations in this well executed efficacy study. The first was using a single fixed dose and at one time (i.e just after infarct). Given the many inherent variability factors that can be different between animal subjects, having one or ideally two more dose arms would have complemented the data sets.
tremendously. Moreover, analyzing how the transgene rescues at different stage of disease would be more clinically relevant. The analogous clinical situation in this model was just after MI, but more than likely AAV.S100A1 therapy would be for advanced disease stages. This is true especially in the context of evaluating why the IM injection group performed so poorly which can only be postulated was due to an inflammatory response caused by too much collateral expression and or single stranded vector conversion signals. It is anticipated this response might be different after the remodeling and after inflammatory courses secondary to MI reach more of a steady state condition in the later disease stages.

Another major limitation in this study beyond its scope was the lack of precision with regard to the immunology assays. Typically, gene therapy studies incorporate the use of two key assays that were not used due to cost. The first is the neutralizing antibody titer, which determines the circulating levels of antibody specific to the AAV9 capsid antigens. The second is the T cell mediated response precisely determined by ELISPOT, whereby a determination can be made on whether responses were generated to the capsid antigens or viral mediated S100A1 protein. The protein encoded in this case was human and overexpression of foreign transgenes in different hosts is more likely to cause responses than the antigens, although this was not confirmed. It was assumed that the immune reactions would be complex and the focus was more on outcome in terms of cardiac function with an associated overall response regardless of source.

Last but not least, the single 10 week timepoint was the most impactful limitation in that all conclusions can only be made at 10 weeks. This concern is twofold given that: (1) LV remodeling and cardiac dysfunction changes over greater time periods from initial MI (2) Expression profiles, although less likely to change with AAV mediated therapies
are still variable (3) Immune response are unpredictable (i.e. the 3 efficacious groups might have suffered a response with a reversal in cardiac preservation at 2, 4 or 6 months later).

Item #3 is of particular concern since it is backed by findings in many studies that show efficacy in the 1-2 month timeframe, then a steep drop off and reversal. These studies would typically show a decrease in gene expression levels accompanied by a deleterious CD8 T cell mediated response. Therefore, the delivery conclusions were more valid in this time frame, however long term impact can only be evaluated with successive timepoints.

9.2.3.2 Future Direction

Multiple dose levels at different points of intervention (i.e. baseline, 3 weeks post MI 12 weeks post MI), more precise immune response assays, and longer term timepoints (i.e. 3 months, 6 months post MI) would provide a more clear means to evaluate aims in greater detail.

9.2.4 Specific Aim #4 - Two key testing parameters that may significantly alter host responses are offered: A. Using double stranded vector equivalent B. Co-delivery of anti-inflammatory drugs to minimize host response during initial delivery phase.

9.2.4.1 Limitations

Given that the only difference in the follow up pilot study with the self-complementary double stranded scAAV9.S100A1 vector was molecular, the already compiled limitations as stated in 9.2.3.1 hold for part A of this aim as well. The performance of self-complementary vector may or may not be different if applied beyond the baseline/Post MI period. This pilot study only tested whether or not it would perform better in terms of transduction and cardiac efficacy. Despite significant changes in
efficacy found in the IM injection group between the two vectors constructs, there was no means to determine if these events would be maintained with elevated cytokine profiles. With respect to the Part B aim of co-delivery of anti-inflammatories with AAV product, the concept was largely limited to a primitive proof of concept in vivo application. Much more detailed in vivo work would have to be performed to determine the correct drug, drug and tuned PLGA release profile that would maximize AAV therapy. The case study only featured DNA plasmid in neonatal myocytes which have different properties.

9.2.4.2 Future Direction

There are numerous parameters independent of delivery that can alter the host response profile in vivo. Follow up studies changing dose levels, promoters in the AAV constructs, co-expression of regulatory elements, and changing serotype might yield more useful in myocardial muscle in the early to late post MI periods. AAV1, AAV6 and AAV9 all have high functional reported efficacy in cardiac muscle but there have not been comparative studies assessing outcome in terms of the host response. Given that the serotype affects efficiency independent of method on a per dose basis, it is conceivable that the more efficient vector would be less inflammatory given that dose can be lowered to reach the same target expression level. Counter to that argument, it might also be that one set of antigens of the capsid is more responsive versus others, this would be measured with ELISPOT assays at a fixed dose with the same construct. Despite the best efforts to engineer the AAV delivery vehicle to minimize expression, there is no way to stop innate immune activation following direct myocardial delivery whether it be with a needle, liquid jet or other physical method. An anti-inflammatory co-formulation strategy is novel from the sense of it being locally directed to the myocardium rather than systemically. Numerous gene therapy trials incorporate systemic use of steroids and or immunosuppression to knockdown inflammatory host
responses. This dissertation argues that a local blockage may actually be more effective and better for the patient's overall health given the side effects of the current means. However, identifying the correct drug with the precise release profile must be determined to thoroughly test this strategy in future in vivo rodent study.

9.3 Conclusions

Improving clinical outcomes in present and future AAV mediated gene therapy trials for post myocardial infarction induced heart failure patients will require a systematic approach. Delivery as shown in the study series in this dissertation was proven to be a prime factor that can determine outcome along with proper dose, vector and gene selection. Delivery considerations in terms of safety and efficacy may be defined patient to patient, whereby the goal is to rescue the sufficient percentage of cells that would reach desired expression levels indicative of improved measurable outcomes. These measures must be defined within safety limits that promote host tolerance, or the cardiac gene therapy will fail. Gene therapy as a whole field is a risky enterprise due to this risk/reward complex. In the case studies where therapy fails it is most certain that there was not enough therapeutic expression or maladaptive responses mounted negating any therapeutic effect.

The inspiration of this dissertation was to advance the seemingly dead end direct delivery arm of cardiac gene delivery technology with a novel concept. The liquid jet concept was reduced to practice in a rodent model, but as stated many more technical challenges would need to be surmounted in order for it to become a viable therapeutic strategy.

In terms of market need the liquid jet technology would at minimum be suitable as an adjunctive surgical therapy with a controlled device, or perhaps a minimally
invasive application where the transgene expression need to be localized (i.e. angiogenesis genes). Therapeutic formulation manipulation with either altering vector molecular biology (i.e. as in our case example with double stranded vector) and co-administration with working pharmacologic compounds to address secondary problems offer solutions to manage these risks clinically. Since the goal is to treat diseased and healthy at risk myocardial areas with the least possible dose resulting in an efficacious expression profile, it is argued that delivery studies will remain a key research area to improve outcomes.

References


