TARGETING GAP JUNCTIONS AS A MECHANISM AND POTENTIAL TREATMENT OF CARDIAC ARRHYTHMIAS

by

MARIA STROM

Submitted in partial fulfillment of the requirements
For the degree of Doctor of Philosophy

Dissertation Adviser: Dr. David S. Rosenbaum

Department of Biomedical Engineering

CASE WESTERN RESERVE UNIVERSITY
May, 2010
We hereby approve the thesis/dissertation of

_________Maria Strom_________________________

candidate for the __________Ph.D.__________________ degree *.

(signed) _____Kenneth J. Gustafson __________________________________
(chair of the committee)

______David S. Rosenbaum________________________________________

______J. Kevin Donahue___________________________________________

______Andrew M. Rollins__________________________________________

______Mark E. Dunlap_____________________________________________

________________________________________________

(date) ___September 22, 2009______________

*We also certify that written approval has been obtained for any proprietary material contained therein.
DEDICATION

This work is dedicated to my mentors, David and Kevin, who taught me to succeed with humility and fail with grace, my mother Lena, who by quiet example, taught me of diligence, poise and strength of character, and my husband Daniel, who lights up my life with love and humor.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Introduction and Background</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>Principles of Transmural Electrophysiological Heterogeneity</td>
<td>39</td>
</tr>
<tr>
<td>3</td>
<td>Gap Junction Heterogeneity as Mechanism for Electrophysiologically Distinct Properties Across the Ventricular Wall</td>
<td>63</td>
</tr>
<tr>
<td>4</td>
<td>Epicardial Gene Painting Restores Intercellular Coupling in Heart Failure</td>
<td>96</td>
</tr>
<tr>
<td>5</td>
<td>Role of Connexin Phosphorylation in Intercellular Communication</td>
<td>125</td>
</tr>
<tr>
<td>6</td>
<td>Summary, Implications and Future Directions</td>
<td>141</td>
</tr>
</tbody>
</table>

**BIBLIOGRAPHY** 153
LIST OF TABLES

Table 4.1  AdCx43 gene painting restores conduction by enhancing intercellular coupling without altering repolarization properties  108

Table 4.2  Arrhythmia susceptibility based on 3 or more nonpaced beats  109

Table 4.3  Sustained arrhythmia inducibility  110
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Schematic illustration of gap junction structure.</td>
<td>18</td>
</tr>
<tr>
<td>1.2</td>
<td>The connexin life cycle</td>
<td>19</td>
</tr>
<tr>
<td>1.3</td>
<td>Cx43 expression patterns maintain heterogeneities across the transmural wall</td>
<td>23</td>
</tr>
<tr>
<td>1.4</td>
<td>Vectors used in clinical gene therapy trials up to March 2009</td>
<td>27</td>
</tr>
<tr>
<td>1.5</td>
<td>Viral gene therapy</td>
<td>28</td>
</tr>
<tr>
<td>2.1</td>
<td>Theoretical cellular fiber</td>
<td>44</td>
</tr>
<tr>
<td>2.2</td>
<td>The composition of cells across the transmural wall determine the APD profile</td>
<td>46</td>
</tr>
<tr>
<td>2.3</td>
<td>Gap junction coupling affects the APD profile</td>
<td>48</td>
</tr>
<tr>
<td>2.4</td>
<td>Homogeneity of gap junction coupling affects the APD profile</td>
<td>49</td>
</tr>
<tr>
<td>2.5</td>
<td>APD dispersion and gradient increase when the uncoupling position crosses the EPI-MID interface.</td>
<td>51</td>
</tr>
<tr>
<td>2.6</td>
<td>Size of MID wall influences the APD profile and gradient</td>
<td>53</td>
</tr>
<tr>
<td>2.7</td>
<td>M-cell mass mediates APD dispersion, while gap junction coupling mediates the APD gradient.</td>
<td>54</td>
</tr>
<tr>
<td>3.1</td>
<td>APD dispersion and gradients are greater in the anterior left lenticular wall.</td>
<td>70</td>
</tr>
<tr>
<td>3.2</td>
<td>Conduction velocity slows in the subepicardium of the anterior left ventricular wall</td>
<td>71</td>
</tr>
<tr>
<td>3.3</td>
<td>Arrhythmia inducibility is greater in the anterior left ventricular wall.</td>
<td>72</td>
</tr>
<tr>
<td>3.4</td>
<td>Cx43 is heterogeneously expressed across the anterior left ventricular wall.</td>
<td>74</td>
</tr>
<tr>
<td>Figure 3.5</td>
<td>Localized uncoupling across the epi-mid interface increases APD dispersion.</td>
<td>75</td>
</tr>
<tr>
<td>Figure 3.6</td>
<td>Coupling profiles of the anterior and posterior left ventricular walls yield higher APD gradients across the anterior left ventricle at baseline and carbenoxolone conditions</td>
<td>76</td>
</tr>
<tr>
<td>Supplemental Figure 1</td>
<td>Epicardial cells exhibit more I_o than endocardial cells across the pLV</td>
<td>92</td>
</tr>
<tr>
<td>Supplemental Figure 2</td>
<td>Ionic current densities are similar across the aLV and pLV</td>
<td>94</td>
</tr>
<tr>
<td>Figure 4.1</td>
<td>Study design</td>
<td>99</td>
</tr>
<tr>
<td>Figure 4.2</td>
<td>Depth of gene transfer</td>
<td>103</td>
</tr>
<tr>
<td>Figure 4.3</td>
<td>Gene painting restores Cx43 protein expression</td>
<td>104</td>
</tr>
<tr>
<td>Figure 4.4</td>
<td>Gene painting restores conduction velocity</td>
<td>106</td>
</tr>
<tr>
<td>Figure 4.5</td>
<td>Gene painting restores conduction velocity when gap junction reserve is impaired.</td>
<td>107</td>
</tr>
<tr>
<td>Figure 4.6</td>
<td>Gene painting does not affect the extracellular matrix</td>
<td>111</td>
</tr>
<tr>
<td>Figure 5.1</td>
<td>Phosphorylation sites of Cx43</td>
<td>127</td>
</tr>
<tr>
<td>Figure 5.2</td>
<td>Ischemia induces Cx43 dephosphorylation.</td>
<td>131</td>
</tr>
<tr>
<td>Figure 5.3</td>
<td>Rotigaptide prevents ischemia-induced dephosphorylation of Cx43</td>
<td>132</td>
</tr>
<tr>
<td>Figure 5.4</td>
<td>Nonphosphorylated signal remains unchanged post AdCx43 gene painting</td>
<td>134</td>
</tr>
</tbody>
</table>
Targeting Gap Junctions as a Mechanism and Potential Treatment of Cardiac Arrhythmias

Abstract
by
MARIA STROM

Cardiac gap junctions play a critical role in intercellular communication, impulse conduction and propagation. Gap junction remodeling and underexpression are increasingly implicated in playing a role in cardiac disease and arrhythmogenesis. In fact, the principle ventricular gap junction protein, connexin43 (Cx43), is reduced in disease states such as heart failure (HF). Recently, we used high-resolution optical mapping to demonstrate that transmural gradients of repolarization was responsible for reentrant ventricular tachycardia in the canine model of pacing induced heart failure. Localized uncoupling at the epicardial-midmyocardial (Epi-Mid) interface provides a mechanistic link between specific changes in intercellular coupling by gap junctions to the change in spatial gradients of repolarization across the ventricular wall. While it is well established that slow conduction and increased gradients of repolarization are important to the initiation of reentrant arrhythmias, the precise role of reduced intercellular communication in arrhythmogenesis remains unresolved. Furthermore, it is unclear whether localized restoration of coupling (by gene transfer) can eradicate the substrate for arrhythmias. Herein lies the rationale for this work. Cx43 is proposed to be the mechanism and potential therapeutic target of cardiac arrhythmia mechanisms. Specifically, computer modeling, optical mapping and targeted gene transfer to enhance cellular communication are used in the investigation of the following paradigms. 1) Mechanisms of Arrhythmogenesis: Establish the proof of concept that localized uncoupling across the Epi-Mid interface is a mechanism in the maintenance of
electrophysiological heterogeneity. 2) **Enhancement of Cellular Communication:** Target gene transfer to enhance cellular communication by increasing expression of Cx43 *in-vivo*. 3) **Inhibition of Arrhythmogenic Substrate:** Determine the phenotypic consequence of increased Cx43 expression by measuring functional indices of cell to cell coupling in intact normal and diseased tissue. 4) **Arrhythmia Suppression:** Determine if targeted gene expression to restore intercellular coupling can suppress ventricular arrhythmias in failing myocardium, while not enhancing susceptibility to arrhythmias in normal myocardium (i.e. no proarrhythmic effect). The results of these studies draw a mechanistic link between altered gap junction expression and arrhythmogenic substrates, and provide an entirely novel paradigm for arrhythmia therapy based on improvement of cell to cell communication.
CHAPTER 1

Introduction and Background
MOTIVATION

Sudden cardiac death (SCD) caused by ventricular arrhythmias is the major cause of death in the developed world.\textsuperscript{1} Despite advancement in our understanding of how structural and electrophysiological changes predispose chronically diseased hearts to SCD, the precise link between mechanical dysfunction and susceptibility to arrhythmias remains unclear. Furthermore, current therapies are not curative. Suppressing life-threatening ventricular arrhythmias pharmacologically has been shown to be pro-arrhythmic. Implantable cardioverter defibrillators are effective at shocking fibrillatory rhythms back to sinus rhythm, but do not alter disease progression. Therefore, full understanding of the cellular and molecular mechanisms responsible for cardiac arrhythmias is a requirement for effective treatment and prevention strategies.

Several cardiovascular diseases are associated with remodelling of proteins encoding for ion channels, pumps, exchangers and gap junctions. Heart Failure (HF), which is characterized by impaired cardiac pumping, is one of them. This work investigates the role of intercellular coupling as a mechanism underlying the development of arrhythmias in HF. Specifically, targeting gap junctions as a mechanism and potential treatment of ventricular cardiac arrhythmias is the focus of this work.

HEART FAILURE

HF is associated with a significant reduction of quality of life, unacceptably high mortality (50\% at 5 years), and enormous cost to the health care system. Hospitalizations for heart failure account for 6.8 million hospital days per year, and the annual costs amounts to $20 – 40 billion.\textsuperscript{2} Alarmingly, HF rates are on the rise with an approximated half a million new diagnosed cases per year in the United States alone.\textsuperscript{3}
The prevalence of HF in the US is estimated at 5 million patients, while the worldwide prevalence is estimated at 22.5 million.

HF can result from any structural or functional cardiac disorder that impairs the ability of the ventricle to fill with or eject blood. The etiology of heart failure is related to the loss of a critical quantity of functioning myocardial cells after injury to the heart due to ischemic heart disease, hypertension, idiopathic cardiomyopathy, infections / inflammatory heart disease (e.g. viral myocarditis, Chagas’ disease), toxins (i.e. alcohol, cytotoxic drugs), valvular disease, or prolonged arrhythmias. The most common cause of heart failure in Western Societies is coronary artery disease with loss of functional myocardium due to prior infarction. However, the mechanisms responsible for structural and electrical remodeling during the development of heart failure are complex and poorly understood.

**Clinical heart failure**

The human heart is a highly vascularized organ and if any one of the major vessels supplying blood to the myocardium is blocked, typically due to an acute rupture of an atherosclerotic plaque leading to acute thrombosis, invariable damage occurs to the tissue bed (i.e., a prolonged ischemic event leading to subsequent cell death). The severity of tissue damage is dependent on the location and degree of vessel occlusion. If the occluding vessel is not immediately reopened, long term tissue damage can ensue and chronic left ventricular dysfunction can develop. Coronary artery disease and myocardial infarction rates continue to rise in the developed world, predominately due to increases in obesity, diabetes mellitus, and the increase in median age of the population.¹
The underlying disease causing many patients to exhibit left ventricular dysfunction is dilated cardiomyopathy (DCM) in which the heart chamber is enlarged, and the myocardial tissue is altered. DCM’s etiologies include ischemic cardiomyopathy, hereditary, infections such as viral infections leading to myocarditis, alcohol, toxins such as cobalt, drugs such as cocaine, amphetamines, or cytostatics, diabetes and thyroid disease.4

After an ischemic insult, the left ventricular myocardium remolds electrically and mechanically.5 While the initial changes in the geometry, structure and function of the left ventricle are adaptive, over time, they prove to be maladaptive. Continuous activation of the neurohormonal axis overtaxing the remaining viable myocardium, unremitting loss of functioning myocytes, and maladaptive modifications to the extracellular matrix are a few of the factors leading to left ventricular dysfunction and eventual failure.5

Infectious cardiac disease occurs when bacterial or viral agents attack the cardiac muscle, or the heart valves causing inflammation. Unfortunately, this common condition is often unrecognized making its incidence unknown. Enteroviruses, adenoviruses, and cytomegaloviruses are implicated in the pathogenesis of inflammatory heart disease causing myocarditis. Irrespective of etiology, the structural and electrical remodeling of the myocardium in heart failure makes the heart vulnerable to perilous arrhythmias.

**Animal models of heart failure**

In order to fully translate therapies into humans, proof of concept work must first be reproduced in animals that more closely resemble function, physiology and anatomy that of a human. Relevant animal models of heart failure are chosen to recapitulate
human heart failure as best as possible to mimic left ventricular dysfunction, cardiac remodeling, and increased neurohumoral dynamics. Several animal models have been developed to study the pathophysiology of the disease as well as therapeutic (pharmacologic, genetic, or surgical) strategies devised to treat it. The most commonly used instrumentation of heart failure is that of rapid ventricular pacing in the dog, and myocardial infarctions in the rat. Alternative preparations include surgically induced pressure and volume overload, and delivery of cardiotoxic agents. While no ideal animal model exists, each offers advantages from which, with careful consideration of limitations, extrapolations from experimental to clinical heart failure can be made.

Myocardial Ischemia

Myocardial infarction and or myocardial ischemia often times begets congestive heart failure (CHF). Post an ischemic insult, the LV myocardium remodels\textsuperscript{5-7}. Animal models of myocardial ischemia and infarction can be used to study the cellular mechanisms governing CHF. One of the earliest models of myocardial ischemia was that of coronary occlusion in a canine. Reimer et al described the time course of irreversible myocyte death with progressive ischemia as a wavefront that claimed subendocardial cells first and progressed transmurally to the epicardium.\textsuperscript{8} This animal model was also used to show that by reperfusing the coronaries early after ischemic injury can rescue myocardial tissue. Canine models of ischemia are not ideal, however, because the immense collateral circulation network makes it difficult to reproducibly create a necrotic lesion of the same size.\textsuperscript{9,10}

Another method employed to create chronic myocardial ischemia leading to CHF in canines is that of multiple sequential left-sided coronary artery microembolizations.\textsuperscript{11-}
Briefly, Sabbah et al. performed coronary embolization procedures over a period of ten weeks until which time the animals’ ejection fraction dropped below 35%. After 3 months, the ejection fraction further deteriorated to 21%, end-diastolic volume, and pressure increased, cardiac output was diminished, plasma norepinephrine levels increased and atrial natriuretic peptide increased. Repeated microembolizations can produce stable CHF. The level of CHF can be titrated by the number of embolization procedures. The disadvantage of the model, however, is that serial microembolization procedures are labor intensive, and may pose threat to the survival of the animal under multiple anesthesia protocols. It is also to be noted that this model does not recapitulate CHF completely without excessive embolic interventions. With that said, important information has been learned through the use of this model such as the efficacy of novel therapeutic treatments (chronic administration of endothelin receptor antagonist). as well as surgical manipulations to prevent LV dilation.

Unlike canine hearts, the coronary anatomy of the porcine heart bears a striking resemblance to human hearts. Weaver et al. systematically compared the anatomy and distribution of coronary arteries in swine to canines and human beings. Swine have scant coronary collaterals localized to the mid myocardium and sub-endocardium, unlike canines, which have extensive epicardial collaterals, making the swine an ideal animal model to mimic human CHF. In the past, three methods have been employed to induce ischemia/infarction: ameroid constriction, hydraulic occlusion, and total acute coronary occlusion. The method of ameroid constriction is used to create coronary stenosis followed by occlusion, which produces an ischemic bed of myocardium capable of meeting metabolic demands during rest but not exercise. Unlike ameroid occlusion,
hydraulic occlusion deteriorates LV wall motion at rest.\textsuperscript{15} Total acute coronary artery occlusion weakens LVS function and incites neurohormonal activation characteristic of human CHF.\textsuperscript{16}

*Dilated Cardiomyopathy*

Several large animal models of DCM have been developed. The most predominately used is that of chronic tachy-pacing induced HF first described in 1962.\textsuperscript{17} Rapid pacing (either atrial or ventricular) has been used in ovine, canine, and swine models. The model provides several advantages, including straightforward implementation, similarity to human tachyarrhythmia induced CHF, predictability in CHF development and remains a gold standard in HF research.\textsuperscript{18} To instrument this animal model, a pacing electrode must be implanted into the myocardium (atrial or more often, ventricular), and connected to a pacemaker to pace at a frequency three-to-fourfold higher than the spontaneous heart rate (between 210-240 beats/min). This pacing protocol generates severe LV dysfunction evidenced by reduced cardiac output, neurohormonal activation, a rise in systemic vascular resistance, increase in LV systolic wall stress, electrical and metabolic alterations and edematous failure,\textsuperscript{18} which are all changes that closely mimic LV dilation and dysfunction characteristic of human DCM. Importantly, the model creates reproducibly predictable CHF with severe LV dysfunction typically occurring as quickly as 3-4 weeks from the onset of rapid pacing. If a more stable, sustainable level of heart failure is desired, lower pacing rates can be used. The model’s disadvantages include rapid onset, inability to produce similar LV dysfunction as seen in patients with CHF due to ischemia, hypertension, or infection. Additionally,
unlike human CHF, the model is reversible, in that LV function partially recovers once rapid pacing is halted.\textsuperscript{19} The model of tachy-pacing induced HF is used in this work.

\textit{Other Models of HF}

Cardiotoxic agents have been used to create models of CHF. For instance, serial exposure to doxorubicin has been shown to promote HF development (reduced ejection fractions, increased LV end-diastolic diameters, elevated plasma norepinephrine and ANP levels).\textsuperscript{20} The disadvantages of using cardiotoxic agents are the variability of HF that they produce and potential systemic toxicity.

Additionally, there exist several volume overload models of HF achieved by creating high pressure from aortic constriction, aortic regurgitation, renal artery constriction, pulmonary stenosis or via mitral regurgitation. Mainly, such models are produced in smaller animals. However, a canine model of mitral regurgitation has been established\textsuperscript{21;22} in which the mitral valve chordae is damaged by a catheter to produce mitral regurgitant fractions greater than 50\%, LV dilation, neurohormonal activation, and volume overload hypertrophy.

\textbf{Electrophysiological consequences of HF}

There are numerous clinical, electrophysiological and histological changes associated with HF. Prolongation of the ECG QT interval and action potential duration (APD)\textsuperscript{23} are one of the many recognized phenotypes of failing myocardium.\textsuperscript{24-26} However, the ionic and structural changes responsible for HF-related altered repolarization and associated arrhythmia vulnerability remain poorly understood. Certain ionic channels and pumps show consistent changes in response to HF across species and models. The transient outward potassium current (\(I_{\text{o}}\)) and the delayed rectifier current
(I_K) are down-regulated in HF. Calcium handling is impaired, and proteins such as sarcoplasmic reticular Ca^{2+} ATPase (SERCA2), and ryanodine receptors are down-regulated, whereas the sodium calcium exchanger (NCX) is upregulated. Since the action potential is a reflection of the exquisite orchestration of all ionic cellular mechanisms, it has been difficult to directly show how a change in one current produces a consistent change in APD. HF also produces slow and irregular propagation from disruption of the extracellular matrix, gap junction remodeling, and other causes.

Detailed electrophysiological phenotyping of failing myocardium can be achieved via high-resolution transmural optical mapping. Left ventricular myocardial wedges from tachy-ventricular-pacing are thinner and exhibit longer APDs and QT intervals, and an increased susceptibility for arrhythmias compared to wedges harvested from normal myocardium. The APD prolongation is not uniform across the ventricular wall. There is preferential APD prolongation in mid-myocardial (Mid) compared to epicardial (Epi) cells producing marked amplification of APD gradients at the Epi-Mid interface. There exists a marked increase (by 96%) in the transmural APD gradient in HF (94 ms) relative to normal (48 ms). The formation of large gradients of repolarization in HF eventually lead to unidirectional block and reentry, which has been demonstrated to occur at the Epi-Mid interface. Therefore, enhanced APD dispersion across the transmural ventricular wall is suggested to cause reentry in HF, but the mechanism underlying the formation of these gradients remains unclear.

**Mechanisms of arrhythmias in HF**

Reentrant ventricular arrhythmias have been demonstrated in a variety of experimental and clinical HF models. Reentry is the repetitive stimulation of cardiac
Two requirements must be met in order for reentry to occur: 1) the excitation wavefront must encounter unidirectional block and 2) either the wavefront needs to be propagating sufficiently slowly, or the path of the reentrant circuit must be sufficiently long in order for all cells along the circuit to regain excitability before the circulating wave returns (i.e., the rotation time of the wavefront around the circuit must be longer than the recovery time of all segments of the circuit). Conduction speed can be influenced by the inherent excitability of the tissue (mediated by sodium channels), or the degree of cell-to-cell coupling (mediated by gap junctions). Additionally, the degree of heterogeneity of electrophysiological properties can create conditions of unidirectional block. Specifically, regions exhibiting largest differences in action potential duration can have large gradients of repolarization where one area of tissue recovers excitability before another area, leading to conditions favoring conduction block.

Other mechanisms have also been implicated as possible causes of arrhythmias in HF, including early and delayed afterdepolarizations. Spontaneous sarcoplasmic reticulum calcium releases from destabilization of the ryanodine release channel has been suggested as a sub-cellular mechanism for triggered activity in HF. Reentry remains the most commonly implicated mechanism of ventricular arrhythmias and as such, the mechanisms governing reentry are the focus of this work.

**Current therapies for arrhythmias in HF**

With deteriorated left ventricular function, the risk of ventricular arrhythmias increases and may lead to sudden death. It is entirely unclear why some patients with HF develop ventricular arrhythmias while others do not. Nevertheless, the current rationale
for treating ventricular arrhythmias in HF is two-fold: to improve quality of life and to prolong life. To do so, therapies can divided into direct therapies aimed to improve mechanical dysfunction associated with heart failure or direct therapies aimed to amerliorate/prevent arrhytmias. The former approach employs the use of vasodilators (ACE inhibitors), β-blockers (such as Metoprolol aimed to decrease cardiac demand), antihypertensives (to reduce high blood pressure), or inotropes (aimed to enhance cardiac muscle strength).

Direct therapies intended to prevent arrhythmias are effective at preventing arrhythmias, but may not prolong life, precisely because predicting which patient will suffer from sudden death is not straightforward. The clinical management of the disease is dependent on the patient’s condition, tolerance and compliance to therapeutic regiments. Yet, while careful clinical care can improve a patient’s quality of life and improve survival rate, the natural progression of the disease continues to ensue.

**GAP JUNCTIONS**

Gap junctions are principally involved in cell-to-cell communication and impulse conduction and are responsible for discontinuous propagation required for normal cardiac function. Alterations in connexin43 (Cx43, the principal ventricular gap junction protein) protein expression and distribution are thought to play a major role in many pathological states. For example, gap junction alterations cause slow conduction associated with the development reentrant ventricular tachycardia in healed myocardial infarcts. Altered gap junction function has also been associated with changes in AV nodal, His-Purkinje, and atrial conduction. Gap junctions are also decreased in number and function in the presence of dilated and hypertrophic cardiomyopathies.
Moreover, connexin proteins have a relatively short half life of 1-2 hours, allowing rapid synthesis of new gap junction proteins (i.e. remodeling) in response to various physiological stresses. This is particularly remarkable because normal cardiac function depends highly on a steady state level of gap junction proteins.

HF induces considerable gap junction remodeling manifested as 50% reduction of Cx43 expression, lateralization of gap junctions to sides rather than ends of myocytes, and redistribution of gap junctions across the ventricle. However, the mechanism of gap junction remodeling in response to disease remains poorly understood. Furthermore, the feasibility of improving intercellular communication by restoring gap junction expression in disease state has not been established.

**Gap junction composition**

Cardiac gap junctions are comprised of collections of hexameric connexin protein complexes which interconnect neighboring myocytes by forming intercellular pores. Connexins are ubiquitously expressed in a multitude of tissue types such as brain, vascular endothelium, and the cardiac myocardium.

A connexin protein is comprised of four transmembrane regions, two extracellular
loops, three intracellular domains including the N- and C-terminal tails, and a cytoplasmic loop that connects the second and third membrane spanning regions (Figure 1.1). Connexin proteins are highly conserved in structure but differences in the length and amino acid composition of the C-terminal as well as the intracellular loop provide slight variability among connexin isoforms. The C-terminal of connexin has been implicated in the ability of connexin proteins to assemble into gap junctions, disassemble and degrade. The N-terminal is responsible for voltage gating.

Molecular weight in kilodaltons (kDa) is used to identify and distinguish members of the connexin protein family. Three types of connexins are expressed in the mammalian heart, namely connexin40 (Cx40), connexin43 (Cx43), and connexin45 (Cx45). The most ubiquitous connexin protein in the mammalian heart, Cx43 is also the most abundantly expressed in the ventricle. To create a gap junction, six connexin proteins oligomerize to form a hemichannel (connexon), and two hemichannels (one from each neighboring cell) dock together to create a gap junction.

**Gap junction trafficking – the connexin life cycle**

The connexin life cycle is illustrated in Figure 1.2. Like many membrane proteins, connexins are cotranslationally threaded into the endoplasmic reticulum (ER) via the translocon and encoded start and stop transfer sequences. In contrast to most multimeric
transmembrane complexes that oligomerize in the ER, Cx43 oligomerizes in the trans-Golgi network. Once the connexin proteins are appropriately folded, they pass through intermediate compartments and then enter into the cis-Golgi network. Upon leaving the trans-Golgi network, connexins undergo post translational modification, such as phosphorylation. The extent and location of such post-translational modifications remain unclear.\textsuperscript{44}

Once the connexin proteins leave the trans-Golgi network, they are then delivered to the cell membrane via a variety of differing mechanisms. Microtubules, for instance, are believed to enhance the efficiency of transport of connexin proteins to the cell surface.\textsuperscript{44} Throughout the delivery process, connexins are gated closed, and once they are inserted into the cell membrane, they remain closed so as to prevent unnecessary transport of small molecules to the extracellular space. When in the plasma membrane, connexin are said to be “lateralized” as they are away from the intercalated disk. In this state, they are also believed to be nonphosphorylated. Once in the plasma membrane, connexin molecules diffuse freely within the lipid bilayer towards the intercalated disk. HF is associated with an increased number of lateralized connexin proteins, although the precise mechanism of lateralization has not been elucidated. Nevertheless, connexin proteins migrate towards the intercalated disk (a mechanical structure of cadherin-mediated cell-cell adhesion) and dock with other connexons from neighboring cells. Interestingly, connexin clustering at the cell surface appears to be a dynamic phenomenon: new channels congregate at the perimeter, while older channels cluster in the center of the connexin plaque.
The older channels at the center of the plaque are internalized first. Annular junctions, which are double-membrane vesicles, are believed to be specialized intracellular compartments that internalize connexin proteins. However, classical endosomal internalization may also play a role in connexin turnover. The precise pathways of connexin internalization in health and disease remain poorly understood.

**Connexin binding partners**

Scaffolding of membrane proteins is a common strategy for forming complexes of proteins, including many connexins, within membrane microdomains, such as gap junction plaques. Cx43 has been shown to bind with the PDZ containing scaffolding protein Zonula Occludens-1 (ZO-1) to provide mechanical stability of gap junctions. ZO-1 is also thought to play a role in regulating gap junction assembly. Another binding partner of connexins is β-catenin, which has been shown to co-localize and co-immunoprecipitate with Cx43. Additionally, connexins are known to directly bind to α- and β-tubulin. The specific tubulin binding domain of Cx43 is located within a 35-amino acid juxtamembrane region of the C-terminal tail. Drebrin, an actin-binding protein, has also been shown to bind of Cx43 suggesting that Cx43 is physically bridged to microfilaments. The multitude of binding partners suggest that connexin proteins are dynamically complex, and that a malfunction of just one binding partner can alter the fate of a single connexin protein.

**Role of adhesion factors on gap junction expression**

Cell-to-cell adhesion is vitally important to tissue viability and function. The intercalated disk contains groups of adhesion molecules that span the membranes of adjacent cells and provide mechanical stability. In cardiac myocytes, these adhesion
molecules are principally comprised of N-cadherins, which form fascia adheren junctions, and desmosomal cadherins, which form desmosomes. On the cytoplasmic side, N-cadherins interact with α,β, and γ catenins. Adherens junctions are thought to play a role in gap junction assembly; similarly, gap junctions are believed to be involved in adherens junctions assembly. The fact that both of these junctional entities are found at the intercalated disk suggests that their interaction might extend beyond physical proximity. Furthermore, experimental studies have correlated an up-regulation of connexin proteins with N-cadherin proteins in response to pulsatile stretch specifically at intercellular junctions. However, the precise biophysical interplay between adherens junctions and gap junctions remains poorly understood.

**Role of Cx43 expression patterns in maintaining heterogeneities across the transmural wall**

The marked amplification of APD gradients in HF (Figure 1.3, top panel) across the transmural wall has previously been attributed to differences in ionic composition of epicardial and mid-myocardial cells. Specifically, mid-myocardial cells possess relatively weak I_{KS} current, accounting for relatively longer APD of mid-myocardium compared to epicardial myocytes, and these differences are exacerbated in HF. However, under conditions of normal intercellular coupling, electrotonic interactions via gap junctions between myocytes attenuate transmural APD gradients.

Measuring Cx43 distribution across the transmural ventricular wall is accomplished by sectioning the canine left ventricle into epicardial, mid-myocardial and endocardial layers allowing for selective Cx43 analysis of each layer. Cx43 expression has been shown to be reduced by 24 ± 17% (p<0.05) in subepicardial layers compared to deeper myocardial layers. Additionally, Cx43 expression is reduced by 40 ± 3% in HF
compared with control (p<0.05). Interestingly, in HF, subepicardial Cx43-related signal is further reduced by 30 ± 18% compared with midmyocardial signal,\textsuperscript{58} corresponding to previous findings in normal LV myocardium.\textsuperscript{57} Figure 1.3 (lower panel) illustrates summary data of transmural APD gradient, and under-expression of Cx43 in subepicardial layers in HF wedges. The largest APD gradients occur precisely where Cx43 expression is lowest near the Epi-Mid interface. These changes are associated with overall reduced conduction velocity in HF compared with control, and with a marked slowing of conduction within the subepicardium of both control and HF myocardium. Since the largest APD gradients localize to the Epi-Mid interface and are highly

![Figure 1.3](image-url)

**Figure 1.3.** Cx43 expression patterns maintain heterogeneities across the transmural wall A: Action potential duration maps in normal and heart failure wedges. APD was prolonged across all layers in HF. However, the prolongation in HF was markedly heterogeneous. APD gradients were markedly increased in HF. B: APD Gradient (left) and Cx43 content (right) across transmural surface of failing myocardium. The APD gradient is greatest at the Epi-Mid interface, which is precisely where Cx43 expression is lowest.
associated with diminished Cx43 expression in the same region, the goal of this work is to enhance gap junction protein expression locally to restore function in HF.

**GENE THERAPY**

**Introduction**

Traditional antiarrhythmic drugs target one or more sarcolemmal ion channels to modify the properties of conduction and repolarization at the single cell level. Unfortunately, most antiarrhythmic drugs not only fail to prevent arrhythmias, but may actually promote arrhythmias (i.e. proarrhythmic). Presently, the only effective therapy for SCD is the implantable defibrillator, which is highly effective at terminating ongoing fibrillation, but by no means alters the natural progression of cardiac disease. There is now growing concern regarding cost and risk associated with ICD therapy such as infections, lead fracture, inappropriate shocks, and a tendency to cause progression of HF. Therefore, alternative solutions for the treatment of cardiovascular disease are needed now more than ever.

Gene therapy provides a unique approach to the treatment of human diseases. Interest in improving treatment therapies using gene transfer methodology has recently intensified, yet the fledgling field has many important obstacles to overcome before clinical strategies can be adopted. Successful programming of recombinant gene expression into cardiac myocytes has been demonstrated both in vitro and in vivo, but in spite of this, current gene transfer techniques suffer from problems related to transient expression patterns, inefficient infection by second administration of virus, inhomogeneous delivery to target tissue, potential toxicity, and loose control of non-target organ gene transfer and of the host immune response. Ongoing efforts are aimed to
resolve these problems and have resulted in improved gene transfer efficacy and homogeneity, thereby lending the hope that treatment or even a cure for cardiac disease is within reach.

**History of gene therapy**

The notion that human disease is amenable to correction at the genetic level first arose in the 1960’s, when mammalian cells were shown to be capable of incorporating and expressing foreign DNA. This was followed by the discovery that infectious agents such as papovaviruses were incredibly efficient at integrating their genetic information into the genomes of target cells. The 1970’s marked the ideation and creation of viruses specifically constructed to incorporate and express foreign/therapeutic genes with the use of recombinant DNA techniques. Such a paradigm shift not only provided the potential to offer new mechanistic insights into disease pathogenesis, but also a novel approach to therapy. In 1980, Martin Cline and colleagues transfected bone marrow cells from thalassaemia patients with plasmids containing the human globin gene and reintroduced them back into the patients. This was followed by fierce scientific, and ethical debate making it clear that gene therapy would not only have to overcome technical concerns, but ethical ones as well. The heightened excitement surrounding the field was replaced with a somber, cautious optimism as investigators trudged forward to develop viruses with therapeutic potential. Soon after, retroviruses were constructed with therapeutic genes and were demonstrated to be highly effective at infecting nearly 100% of host mammalian cells. As a natural progression, these same retroviral vectors were used to correct a genetic defect in vitro. Thereafter, new methodologies for delivering therapeutic genes to host cells (non-viral and viral) were introduced into the gene therapy
field (see full discussion of vectors below). The 1990’s marked the first approved applications of clinical gene therapy. Unfortunately, several high-profile studies with lethal adverse events stunted the burgeoning field. The safety of manipulating human DNA became the chief concern. And now, nearly two decades later, with much more stringent standards for clinical translation, new clinical trials are underway to fully test the therapeutic potential of gene therapy. To date, there are 1537 clinical gene therapy trials registered with the international registry for gene therapy clinical trials. Gene therapy is beginning to live up to its initial promise and is in a good position to make a valuable clinical impact for patients suffering with detrimental disease.

**Vectors for gene therapy**

Several potential viral and non-viral vectors have been used to deliver therapeutic genes to host tissue (summarized in Figure 1.4). Each vector has characteristic strengths and weaknesses that, upon careful consideration, can be used for specific applications. Ultimately, the nature of the human disease dictates the vector choice, as requirements for cell types, expression levels, or duration of expression to achieve a therapeutic effect may differ.

**Non-Viral Vectors**

There are many non-viral systems for delivering genes to target organs. Injection of naked DNA has been shown to effectively infect skeletal muscle. The advantages of using a non-viral approach include: ability to infect non-dividing cells, low production costs, and ability to incorporate large genes. Despite this, non-viral methods suffer from low and transient gene transfer, thereby necessitating high plasmid doses and multiple,
invasive treatments. As such, plasmid DNA is not an ideal choice vector for gene therapy, specifically to the cardiac myocardium.

**Viral Vectors**

Viruses have evolved to perform the precise task of infecting and incorporating their genetic material into target cells. Hence, they make a great vehicle for delivering potentially therapeutic genes. A virus is comprised of genetic material encapsulated by a protein coat, the capsid. A cell’s surface receptors interact with the capsid to initialize virus binding and internalization. Once in the cytoplasm, the capsid maneuvers toward the cell nucleus, releases its genetic composition into the nucleus, and the cell begins to manufacture proteins using the new gene. Figure 1.5 summarizes this process. The virus vectors most widely used in cardiac research are retroviruses/lentiviruses, adenoviruses, and adeno-associated viruses – all modified to exclude their replication capacity. The respective advantages and disadvantages of these viruses are discussed below.

**Retroviruses/Lentiviruses**

Retroviruses are RNA viruses that integrate into the host cell’s DNA (via reverse transcription in the target cell). Chromosomal integration is a requisite for gene expression, and when infecting replicating cells, retroviruses are highly efficient. Since cell division is a requirement for chromosomal integration, retroviruses are not efficient at infecting non-dividing cells such as myocytes. Moreover, there is a potential of
insertional mutagenesis, followed by secondary malignancy, as well as the potential for the gene to be passed in the germline to offspring.

Unlike retroviruses, lentiviruses are capable of stable gene transfer in non-replicating cells. Some lentivirus vectors are derived from human immunodeficiency virus type 1 (HIV-1), whose capsid is inherently directed towards CD4-expression cells (cardiac myocytes express CD4). Along with inbuilt tropism towards cardiac cells, lentiviruses can accommodate large genes (up to 8kb), and have similar infection efficiency as adenoviruses, but with longer gene expression. Despite the fact that the HIV-1 derived viruses are devoid of their replication competency, concern remains for potential wild type HIV replication.

**Adenoviruses**

Adenoviruses are double-stranded DNA viruses with stable and high infection efficiency in both non-dividing and dividing cells. Adenoviruses are characterized by their rapid gene expression kinetics with peak protein production from the therapeutic gene occurring within 7-14 days post adenoviral infection. Adenoviruses can accommodate large genes and can be produced to high titres at relatively low costs. However,
adenovirus therapy is limited by transient gene expression, and a significant immune response precludes therapeutic readministration. The immune response acts to destroy cells infected by the adenoviruses (i.e., adenoviral-mediated disease), which can lead to immune myocarditis – an undesirable side effect particularly in the cardiac arena.\textsuperscript{72} To circumvent the immune response, adenoviruses have been modified to contain little of the viral genome, which lowers immunogenicity in host tissue. Adenoviral promiscuity is another disadvantage particularly when targeting a specific organ is desired, but there are promising efforts to modify the capsid to enhance tissue-specific tropism.\textsuperscript{73} Despite these disadvantages, adenoviruses provide an attractive option for clinical cases requiring focal and speedy therapy, as well as proof-of-concept experimental bench side research. The aims of this work are investigated via the use of an adenovirus.

\textit{Adeno-associated Viruses}

Unlike adenoviruses which have characteristically transient gene expression, adeno-associated viruses (AAVs) are capable of sustained, stable, and efficient gene transfer. Additionally, they are non-pathogenic, and serotypes 1,6,8,9 have demonstrated tropism to the myocardium. Furthermore, AAVs are capable of crossing the endothelial barrier, making them suitable to deliver via coronary arteries – an approach that is less invasive than intramyocardial injection. While wild-type AAVs insert their genetic material into the host cell’s chromosome, recombinant AAVs engineered for gene therapy are integration deficient. The major disadvantage of AAVs is their limited capacity for genetic material. Being the smallest of the viruses, AAVs can only contain up to 4.7 kb of genome. The size limitation of AAVs can be circumvented by transsplicing, which allows for delivery of larger genes as smaller fragments that
recombine upon nuclear entry. Another potential disadvantage of AAVs is the slow kinetics of AAV gene expression. It takes several weeks for gene expression to occur upon treatment. However, once gene expression occurs, it is sustained for several years. As such, adeno-associated viruses make ideal candidates for clinical gene therapy in which long term expression is necessary.

**Gene therapy in heart failure**

Full understanding of the molecular mechanisms underlying HF provides the opportunity to treat HF at the genetic level. Like many diseases, HF is multi-factorial. Therefore, effective therapy will likely necessitate a combination approach as no one gene can restore a failing heart to normal function. Impaired calcium cycling, ventricular fibrosis, apoptosis, and ventricular rhythm disorders are all hallmarks of HF. Potential genetic candidates to restore these processes include, but are not limited to: SERCA2A and cardiac S100A1 for calcium cycling, IGF-1, growth hormone, and HSP70 for antiapoptosis, βTGF1, thyroid hormone receptor to inhibit fibrosis, and connexin43 to prevent perilous slowing of electrical impulse conduction. The goal of this work is to investigate mechanisms of arrhythmogensis related to altered cell-to-cell coupling, and therefore, connexin43 is the primary target for gene therapy.

**SPECIFIC AIMS**

**Gap junctions as a target for antiarrhythmic gene therapy**

Recent data from our laboratory has implicated reduced gap junction expression within the subepicardium as a factor in localized conduction velocity slowing. Therefore, gap junction remodeling in HF may be an underlying mechanism of SCD in
HF. In turn, gap junctions may represent a novel target for therapy of ventricular arrhythmias.

**Specific aims**

The general hypothesis in this body of work is that gap junction remodeling causes the decrease in conduction velocity and the enhancement of arrhythmogenic repolarization gradients associated with HF. Furthermore, improvement of conduction and elimination of electrophysiological gradients will be of therapeutic benefit. To test this hypothesis, the following specific aims are addressed:

**Aim 1:** Determine the relationship between gap junction expression patterns and electrophysiological heterogeneity across the transmural wall.

Aim 1.1. Determine if localized uncoupling can account for electrophysiological heterogeneity across the transmural wall *in-silico.*

Aim 1.2 Determine if localized uncoupling can account for electrophysiological heterogeneity across the transmural wall in intact myocardium.

**Aim 2:** Establish a causal mechanistic link between gap junction expression patterns and electrophysiological changes in heart failure.


Aim 2.2. Determine the effect of targeted gene transfer on the electrophysiological function in the heart.

Aim 2.3. Determine if targeted gene transfer can suppress arrhythmias in the failing myocardium.
REFERENCES


8. Reimer KA, Jennings RB: The "wavefront phenomenon" of myocardial ischemic cell death. II. Transmural progression of necrosis within the framework of ischemic bed size (myocardium at risk) and collateral flow. Lab Invest 1979;40:633-644


31. Schwinger RHG, Böhm M, Schmidt U, Karczewski P, Bavendiek U, Flesch M, Krause EG, Erdmann E: Unchanged protein levels of SERCA II and phospholamban but reduced Ca²⁺ uptake and Ca²⁺-ATPase activity of cardiac sarcoplasmic reticulum from dilated cardiomyopathy patients compared with patients with nonfailing hearts. *Circulation* 1995;92:3220-3228

32. Stevenson WG, Delacretaz E, Friedman PL, Ellison KE: Identification and ablation of macroreentrant ventricular tachycardia with the CARTO electroanatomical mapping system. *Pacing Clin.Electrophysiol.*


37. Marx SO, Marks AR: Regulation of the ryanodine receptor in heart failure. *Basic Res Cardiol* 2002;97 Suppl 1:I49-51


39. Peters NS, Coromilas J, Severs NJ, Wit AL: Disturbed connexin43 gap junction distribution correlates with the location of reentrant circuits in the epicardial border zone of healing canine infarcts that cause ventricular tachycardia. *Circulation* 1997;95:988-996


57. Poelzing S, Akar FG, Baron E, Rosenbaum DS: Heterogeneous connexin43 expression produces electrophysiological heterogeneities across ventricular


64. Nagata K, Marban E, Lawrence JH, Donahue JK: Phosphodiesterase inhibitor-mediated potentiation of adenovirus delivery to myocardium. *J Mol Cell Cardiol* 2001;33:575-580


70. Miller AD, Jolly DJ, Friedmann T, Verma IM: A transmissible retrovirus
expressing human hypoxanthine phosphoribosyltransferase (HPRT): gene transfer into cells obtained from humans deficient in HPRT. *Proc Natl Acad Sci U S A* 1983;80:4709-4713


CHAPTER 2

Principles of Transmural Electrophysiological Heterogeneity
INTRODUCTION

Arrhythmias in the setting of heart failure are a common cause of morbidity and mortality. Yet the precise mechanisms of arrhythmias are poorly understood. This chapter focuses on the mechanisms of cardiac electrical heterogeneity as a substrate for the genesis of ventricular arrhythmias. Computer simulations are used to describe the basic principles underlying electrical heterogeneity in the transmural wall of the heart.

A brief history

The first documented account of ventricular fibrillation was made by M Hoffa, a student of Carl Ludwig in 1849.\(^1\) The mystery of fibrillation spurred investigation of potential mechanisms which led to the implication of reentrant arrhythmias as precursors to fibrillation. Reentry is the repetitive stimulation of cardiac tissue by circulating cardiac impulse around an obstacle, the basic principle of which was demonstrated by George Mines in 1912.\(^2\)

The first transmembrane action potential was recorded from cardiac tissue in 1949 by Coraboeuf and Weidmann.\(^3\) Though originally thought to be a syncitium, Weidmann demonstrated that, in fact, electrical coupling of cardiac myocytes occurred through low resistive barriers interconnecting neighboring cells.\(^4; 5\) Such discontinuous conduction\(^6\) was determined to be an important factor for synchronized contraction, and overall cardiac function. Seminal studies by Spach et al have shown the importance of repetitive discontinuities in canine and human atrial trabecula in determining directional conduction properties.\(^7; 8\) These resistive barriers came to be known as gap junctions. The role of gap junctional conductance in normal propagation has been investigated in linear cell chains and two dimensional cellular networks experimentally\(^9; 10\) and theoretically.\(^11; 12\)
Hodgkin and Huxley described the ionic mechanisms responsible for the initiation and propagation of action potentials in the squid axon\textsuperscript{13} – a discovery that was readily applied to explain action potential generation in cardiac tissue. Most importantly, it became evident that membrane excitability was governed by passive ion fluxes, which implied the existence of ion channels. The advent of the patch-clam technique in 1976\textsuperscript{14} allowed the recording of single ion channel currents, and furthered our understanding of action potential generation and propagation in cardiac tissue. With the ever-growing body of knowledge, it is now possible to draw mechanistic links between ion channel and gap junction protein structure/function and disease pathogenesis.

However, human physiology is complex: normal function depends on the simultaneous occurrence of myriad processes. A small alteration in a fundamental process can lead to major effects on overall system behavior thereby posing a significant challenge to isolating the effect of one protein, process, or ion on physiologic or pathophysiologic function. It has become essential to integrate approaches to studying the mechanisms by which small microscopic changes lead to functional alterations at the organ level. One such approach is the use of computer models which boasts a fundamental advantage: the ability to specifically and selectively modulate a specific process in a controlled system.

**Electrical heterogeneity in the heart**

Reentry is the most common mechanism of arrhythmias and has been extensively implicated in the development of ventricular tachycardia and fibrillation. Conduction block is a requisite for reentry.\textsuperscript{15} Severe electrical heterogeneities are an important substrate for arrhythmogenesis because they can cause conduction block. Yet, the
mechanisms of heterogeneity formation which underlies the development of reentrant arrhythmias are unknown. One hypothesis is that heterogeneities in electrophysiological properties between various cell types may play a role in arrhythmic substrate formation.

Two factors influence the extent of electrical heterogeneities in the heart: 1) the ion channel heterogeneities intrinsic to different myocardial layers, and 2) the strength of intercellular coupling. Three distinct cell types characterized uniquely by their electrophysiological properties and response to pharmacologic interventions exist across the transmural wall.\textsuperscript{16; 16-21} Notably, ionic composition differences manifest as variable APD between EPI (outer most layer), MID (middle layer), and ENDO (innermost layer). Since MID cells exhibit the longest APD, and prolong preferentially than their counterpart cells, they are implicated in increasing APD dispersion.\textsuperscript{22; 23}

The strength of intercellular coupling also influences the extent of electrical heterogeneity in intact tissue.\textsuperscript{24} Intercellular coupling, which is governed in part by gap junctions, serves to attenuate electrical heterogeneities and allow for rapid and synchronous communication between cells.\textsuperscript{25; 26} Uncoupling during disease states such as ischemia\textsuperscript{27-29} or heart failure\textsuperscript{30; 31} can reveal distinct electrophysiological properties inherent to individual cells.\textsuperscript{32}

The principle gap junction protein, connexin43 (Cx43) is not homogeneously expressed across the ventricular wall. The sub-epicardium expresses significantly less Cx43 than deeper muscle layers.\textsuperscript{33} Furthermore, during disease states such as heart failure, heterogeneity in expression of Cx43 is further enhanced and is associated with conduction slowing, and enhanced APD dispersion.\textsuperscript{34} The causative relationship between Cx43 expression patterns and electrophysiological heterogeneity has not been established.
Additionally, MID cells are not uniformly distributed across the ventricular wall. Though they consistently appear within the mid-myocardium, MID cells appear to occupy variable percentages of the ventricular wall. The relationship between MID cell distribution and electrophysiological heterogeneity is unknown.

This chapter explores the role of intrinsic ionic heterogeneity, intercellular coupling, heterogeneity of coupling and the relative distribution of cell types in maintaining transmural electrophysiological heterogeneity.

**METHODS**

To investigate the role of ionic heterogeneity, intercellular coupling, localized uncoupling, and contribution of cellular layers in maintaining APD heterogeneity across the transmural wall, a multicellular fiber (Figure 2.1) was constructed of cellular elements each of Luo Rudy dynamic (LRd) model formulation. Numerically based on experimental data from guinea pig ventricular myocytes, the LRd model calculated ionic currents and concentration changes in each cell of the multicellular fiber based on Hodgkin-Huxley formalisms. Ionic pumps, exchangers, intracellular calcium stores, and appropriate buffers and buffer systems were included in the LRd model. The one-dimensional theoretical multicellular fiber was composed of a series of 180 LRd cells interconnected through resistive gap junctions.

Voltage in each cell was calculated using the finite approximation of the cable equation:

$$\frac{\partial^2 V_m}{\partial x^2} = (r_i + r_c) i_m + r_p i_p$$

(Equation 1)
Where $V_m$ membrane voltage ($V$), $x$ axial distance ($m$), $r_i$ intracellular resistance per unit length ($\Omega/m$), $r_e$ extracellular resistance per unit length ($\Omega/m$), $i_m$ membrane current per unit length ($A/m$), $i_p$ current entering the extracellular space via polarizing electrodes. Because the fiber is considered to lie in an extensive extracellular medium, and there are no electrodes, $r_e = 0$, and $i_p = 0$.

Using the Crank Nicolson method for solving partial differential equations, Equation 1 was discretized (Equation 2) to derive the equation voltage of each cell at each time point (Equation 3):

$$\frac{V_{i-1}^t - 2V_i^t + V_{i+1}^t}{R_i \Delta x} = \left[ C_m \frac{V_{i+1}^{t+1} - V_i^{t+1}}{\Delta t} + I_{ion} + I_{stim} \right] 2\pi \Delta x R_{CG}$$  \hspace{1cm} (Equation 2)

$$V_{i+1}^{t+1} = V_i^t + \left(V_i^t - 2V_i^t + V_{i+1}^t\right) f \Delta t - I_c \frac{\Delta t}{C_m}$$  \hspace{1cm} (Equation 3)

$$R_i = R_{myo} + \frac{\rho G}{\Delta x}$$  \hspace{1cm} (Equation 4)

$$f = \frac{1000a}{\Delta x^2 2R_i R_{CG} C_M}$$  \hspace{1cm} (Equation 5)

Where $C_m$ is membrane capacitance (1 $\mu$A/$\mu$F), $V_i^t$ is the membrane voltage at time $t$ and

Figure 2.1. *Theoretical Cellular Fiber*. Schematic of the one dimensional fiber comprised of serially assembled EPI, MID, and ENDO cells each of Luo-Rudy formulation interconnected by passive resistive networks such as gap junctions. The fiber is stimulated cell 1. (*cells eproduced from Shaw et. al*26)
position \(i\) (mV), \(\Delta t\) is the time increment (ms), \(I_{\text{ion}}\) is the ionic current density (\(\mu\text{A}/\mu\text{F}\)) of each cell, and \(I_{\text{stim}}\) is the stimulus current density (\(\mu\text{A}/\mu\text{F}\)). \(I_C\) is current per capacitative area (A/m\(^2\)) \(\Delta x\) is the discretization element (100 \(\mu\text{m}\)), \(a\) is the fiber radius (11 \(\mu\text{m}\)), \(R_{CG}\) is the ratio or capacitive to geometric areas (\(R_{CG} = 2\)), \(R_i\) is the lumped intracellular resistance and it is composed of myoplasmic resistivity, \(R_{\text{myo}}\) (\(\Omega\text{cm}\)) gap junctional resistance, \(rg\), (\(\Omega\text{cm}^2\)).

**Multicellular Fiber Composition**

The multicellular fiber was composed 180 ventricular cells. Cells were designated as EPI, MID or ENDO by altering the relative \(I_{Ks}\) current density as described.\(^{38}\) The relative amount of MID cells was varied from 16% to 90% of the theoretical fiber. A simplifying assumption of proportionality was made between gap junction conductance and Cx43 expression levels, without regard for the stoichiometric composition of gap junctions, phosphorylation status, or contribution of other connexin isoforms. Gap junction conductance was homogeneous throughout the fiber and varied between 0.025 \(\mu\text{S}\) (poor coupling)\(^{38}\) and 2.5 \(\mu\text{S}\) (normal coupling).\(^{39}\) Under conditions simulating regional uncoupling,\(^{33}\) \(g_j\) was held at 2.5 \(\mu\text{S}\) in the beginning of the endocardium, and was progressively varied from 2.5 \(\mu\text{S}\) to 0.025 \(\mu\text{S}\) across the remainder of the fiber at variable positions.

**Analysis**

APD was calculated using \(\text{APD}_{90}\) in each cell, and APD dispersion was defined to the difference between the longest APD and shortest APD. Furthermore, the derivative of the APD profile across the fiber, \(\frac{d\text{APD}}{d\text{Cell}}\), was calculated by averaging adjacent data points (Equation 6).
Electrophysiological heterogeneity across the ventricular wall arises when distinct cell types are coupled through gap junctions. If the myocardium was entirely comprised of identical cells, the ventricular wall would be electrophysiologically homogeneous, and the subsequent APD profile would remain constant. The effect of homogeneous ventricular composition on transmural APD is illustrated in Figure 2.2 Panel A, in which the APD profile of 3 fibers is plotted, when each fiber consists wholly of either EPI, MID or ENDO cells. The result of a fiber containing identical cells in each case is a constant transmural APD profile.

However, due to variable protein expression and electrophysiological function of different cell types, the transmural wall is electrophysiologically heterogeneous. The

\[
\begin{align*}
1 \left\{ \frac{APD_{i+1} - APD_i}{Cell_{i+1} - Cell_i} \right\} + \frac{APD_i - APD_{i-1}}{Cell_i - Cell_{i-1}} \right\}
\end{align*}
\]

(Equation 6)

Figure 2.2. The composition of cells across the transmural wall determines the APD profile. When the transmural wall is compromised entirely of a single cell type (EPI, MID or ENDO), the APD profile is constant across the transmural wall, and is equal to the APD values of EPI, MID, or ENDO steady state, single cell, APD values (bold, horizontal lines) as shown in Panel A. When the transmural wall is compartmentalized to include all three cell types, the APD profile varies dynamically across the transmural wall, with longer APD near the MID region, and shorter APD in the EPI region (panel B).
electrophysiological consequence (i.e., transmural APD profile) of introducing all three cell types into a single fiber is explored in Figure 2.2 panel B. EPI, MID, and ENDO cells are coupled at a normal level of coupling \((g_j = 2.5 \mu S)\) and experience electrotonic interactions that affect their behavior. When the transmural fiber is comprised of all three cell types, the APD profile changes dynamically exhibiting shortest APDs near the epicardial cells, and longest APDs near the MID cells.

**Gap junction coupling affects the APD profile.**

The effects of intercellular coupling on transmural APD profiles are shown in Figure 2.3. APDs along the multicellular fiber comprised of EPI, MID and ENDO cells for different degrees of gap-junction coupling are plotted in Figure 2.3, panel A. Horizontal bold lines represent APDs obtained from isolated EPI, MID and ENDO cells. Under conditions of normal coupling \((g_j=2.5 \mu S, \text{ blue line})\), the APD difference (dispersion) is substantially reduced compared to the difference in APD between isolated cells. For this level of coupling, APD dispersion is 28 msec, and the APD curve is relatively flat across the fiber. However, uncoupling by half \((g_j = 1.25 \mu S, \text{ green line})\), increases APD dispersion to 34 msec. Severe levels of uncoupling cause APDs to approach single cell isolated values, leading to a significant increase in APD dispersion of 68 msec \((g_j = 0.125 \mu S, \text{ red line})\). The dependence of APD dispersion on intercellular coupling is illustrated in Figure 2.3, panel B, in which APD dispersion is plotted vs. gap junctional conductance. It is apparent that APD dispersion is inversely related to intercellular coupling in a nonlinear fashion. Even within a physiologic range of coupling (from 2.5 µS to 0.25 µS), APD dispersion varies from 28 msec to 54.
Figure 2.3. *Gap junction coupling affects the transmural APD profile and gradient.* APDs along the multicellular fiber are shown in Panel A, with corresponding APD gradient profiles in Panel C. Bold horizontal lines denote single cell APD values of each region in Panel A. Blue line corresponds to gj = 2.5µS, the green line corresponds to 1.25µS and the red line corresponds to 0.125µS. The effect of different degrees of coupling on APD dispersion and the maximal APD gradient are plotted in panels B and D, respectively.

Progressive uncoupling also unmasks the APD gradient. The maximal gradient is determined from the plots of the derivatives of the APD profiles of Figure 2.3 panel A, found in Figure 2.3 panel C. Under conditions of normal coupling (gj=2.5µS, blue line), the maximal gradient is 8.9 msec/mm. Uncoupling by half (gj = 1.25µS, green line) increases the maximal gradient to 12 msec/mm, a level that corresponds to a value found in previous studies to be required for the development of functional block in ventricular myocardium.\(^{40, 41}\) Severe uncoupling further exacerbates the gradient to 39 msec/mm (gj = 0.125 µS, red line). The effect of uncoupling on the APD gradient is summarized in
Regional uncoupling can augment APD dispersion and unmask an APD gradient

Since intercellular coupling is reduced in the subepicardium and is associated with increased APD dispersion and gradient,33,34 the effect of regional uncoupling on the APD profile and APD gradient of the multicellular fiber was investigated by varying the degree of uncoupling and the location of the uncoupling step. The principle of APD dispersion and enhanced APD gradient under conditions of regional uncoupling is illustrated in Figure 2.4. When the fiber is homogeneously coupled (flat blue curve in Figure 2.4A), APD dispersion equals 28 ms, or homogeneously uncoupled (red curve in Figure 2.4A), APD dispersion equals 68 msec. If a portion of the fiber is uncoupled,
APDs along the fiber approach isolated single cell values (black dashed line), similar to homogeneous uncoupling. In the case of regional uncoupling APD dispersion is 58 ms, which is remarkably only 10 msec shorter than that APD dispersion under conditions of homogeneous uncoupling.

In addition to increasing APD dispersion, heterogeneous coupling also unmasks a large APD gradient. As illustrated in Figure 2.4B, when a portion of the fiber is uncoupled, the maximal APD gradient is 38 msec/mm, and occurs precisely in the same location of the maximal gradient of the homogeneous uncoupling case (compare peaks of black dashed curve and red curve). Therefore, regional uncoupling can also augment APD dispersion, and unmask an APD gradient. This is particularly relevant in understanding why arrhythmias occur in situations in which only a portion of myocardium is uncoupled (perhaps due to an ischemic event), or why conduction block occurs in regions of depressed coupling (as in the canine ventricular myocardium).

The location and degree of regional uncoupling affect APD dispersion and gradient.

Since unidirectional block occurs between sites exhibiting large APD differences, it was important to determine the role of regional uncoupling on unmasking APD differences. To that end, uncoupling position was systematically varied across the fiber for a range of gap junction conductances, which were stepped down from 2.5 µS to 0.125 µS in the epicardium using 0.05 µS steps. The location of the uncoupling step was shifted every five cells, until the entire fiber was homogeneously uncoupled. APD dispersion increased not only with increased gap junction uncoupling, but also when the uncoupling position crossed a cell-layer boundary (Figure 2.5A). Uncoupling any
Figure 2.5. APD dispersion and gradient increase when the uncoupling position crosses the EPI-MID interface. A. APD dispersion plotted as a function of uncoupling position across the transmural wall and gap junction conductance. B. Maximal APD gradient is plotted as a function of uncoupling position across the transmural wall and gap junction conductance.

A portion of the epicardium from the rest of the fiber did not produce considerable APD dispersion. For example, progressive uncoupling within the epicardium (white EPI region in Figure 2.5A) did not affect APD dispersion, which only varied from 28 msec to 30 msec. In stark contrast, once the uncoupling position crossed the cellular boundary between EPI and MID cells, APD dispersion increased dramatically for all levels of uncoupling (Figure 2.5A, gray region within MID). Uncoupling all of the MID and EPI cells from the rest of the fiber produced a maximal dispersion of 69 msec – a dispersion as large as uncoupling the entire fiber homogeneously. These data suggest that regional uncoupling in myocardium may be as perilous as uncoupling the entire myocardium. Importantly, uncoupling only a portion of the epicardium appeared to be innocuous leading to the conclusion that regional uncoupling must cross cell layers to enhance APD dispersion.

Since the development of functional block in ventricular myocardium is dependent on the APD gradient, we further investigated the role of regional uncoupling
on the maintenance of APD gradient, which was calculated for different uncoupling positions across the fiber for a range of gap junction conductances (Figure 2.5B). Severe uncoupling at any position within the fiber increases the maximal APD gradient. Uncoupling within the epicardium increases the maximal APD gradient only slightly. In marked contrast, once the uncoupling position crosses the EPI-MID border, the maximal APD gradient increases dramatically from 8.9 to 35 msec/mm. For any of the gap junction conductances, moving the location of the uncoupling step to the endocardium did not significantly alter the maximal APD gradient. As expected, the maximal APD gradient always occurred within 500 µm of the EPI-MID border (data not shown), as epicardial and midmyocardial cells have the largest APD difference in isolation. These data suggest that uncoupling across the EPI-MID cell border is a requirement to unmask an APD gradient.

**Mass of M-cell layer affects APD profile**

The results presented thus far have been obtained from a multicellular fiber consisting of fixed segment lengths of cell types. However, in intact tissue, EPI, MID and ENDO cells unlikely occupy equal percentages of the transmural wall. Evidence from optical mapping studies suggest M cells are not necessarily distributed uniformly across each transmural layer. Therefore, we investigated the effect of a variable MID layer on the overall transmural APD profile and gradient (Figure 2.6). When the MID layer occupied 20% of the multicellular fiber, APD dispersion equaled 37 msec, and the maximal APD gradient was 8.9 msec/mm (blue line in Figure 2.6A and B). Increasing the M-cell mass to 50% of the ventricular wall increased APD dispersion to 66 msec and the maximal APD gradient to 12.8 msec/mm. Notice that at this M-cell mass, APD in the middle of
Figure 2.6. Size of MID wall influences the APD profile and gradient. A. APD profile is plotted across the transmural wall with a variable MID layer. Three curves are highlighted to demonstrate how the APD profile changes with a variable MID layer occupies 20% (blue line), 50% (green line), or 90% (red line) of the ventricular wall. B. Subsequent APD gradient profiles are plotted. C. APD dispersion (difference between longest and shortest APD) is plotted against M-cell mass. D. The maximal APD gradient is plotted against ventricular M-cell mass.

The fiber prolonged significantly, but the peripheral EPI and ENDO regions were largely unaffected (green line). Not unexpectedly, when the M-cell mass occupied 90% of the multicellular fiber, APD prolonged across the entire fiber causing the APD dispersion to fall to 44 msec, and the maximal APD gradient to fall to 7.8 msec/mm (red line).

Summarized in Figure 2.6C, APD dispersion exhibits a biphasic response with M-cell mass. APD dispersion increases until M-cells occupy 63% of the ventricular wall, after which point, the rather large region of M-cells electrotonically influences the other
cell types (EPI and ENDO) to raise their APD, whereby decreasing overall dispersion. The APD gradient exhibits a less robust dependence on M-cell mass. In fact, when the M-cells occupy 30-73% of the ventricular wall, the maximal APD gradient remains constant (Figure 2.6D). These data suggest that APD dispersion is significantly affected by M-cell mass, while the maximal APD gradient may be influenced by other factors.

**APD gradient is influenced by gap junction coupling**

To assess the effect of coupling and M-cell mass on APD dispersion and gradient, the M-cell mass across the multicellular fiber was varied along with gap junction conductance. APD dispersion vs. M-cell mass is plotted for several gap junction conductances in Figure 2.7A. Uncoupling increased APD dispersion, but the overall profile of APD dispersion vs. M-cell mass remained the same. Furthermore, the minimal difference within a curve of variable M cell mass (32 msec) was larger than the maximal difference between curves of variable gap junction conductance (23 msec), suggesting that M-cell mass is the primary determinant of transmural APD dispersion. In contrast,
APD gradient is primarily governed by gap junction conductance. Figure 2.7B plots the maximal APD gradient vs M-cell mass for several gap junction conductances. In this case, the maximal difference within a curve of varied M-cell mass (5.0 msec/mm) was smaller than the minimal difference between curves of variable gap junction conductance (8.8 msec/mm), suggesting that gap junction coupling predominately influences the maximal APD gradient, and is therefore, a potent arbiter of arrhythmia substrates.

**DISCUSSION**

This work investigated the mechanisms of transmural electrophysiological heterogeneity. A computer simulation of a multicellular fiber comprised of EPI, MID and ENDO cells, interconnected by gap junctions was implemented. The model was used to demonstrate that the extent of electrophysiological heterogeneity within the transmural wall is influenced by 1) the ion channel heterogeneities intrinsic to different myocardial layers, 2) the degree of intercellular coupling, 3) the homogeneity of intercellular coupling, and 4) the relative quantity of each myocardial layer.

To prove the first mechanism of transmural electrophysiological heterogeneity, a multicellular strand was constructed of identical cells. Not surprisingly, when the fiber consisted entirely of one type of cell, the transmural APD profile was constant (Figure 2.2A). However, when all three cell types were introduced into the strand, the transmural APD profile changed dynamically (Figure 2.2B). Hence, the extent of electrophysiological heterogeneity within the transmural wall is influenced by the ion channel heterogeneities intrinsic to different myocardial layers.

The degree of intercellular coupling also had a profound influence on the manifestation of electrophysiological heterogeneity. Gap junction coupling was
systematically varied in a homogeneous fashion across the multicellular fiber containing EPI, MID, and ENDO cells. Even small changes in intercellular coupling led to marked enhancement of APD dispersion and gradient (Figure 2.3). Experiments using uncoupling agents (i.e., carbenoxolone) or coupling promotors (i.e., rotigaptide) have confirmed the effects of gap junction coupling on electrophysiological heterogeneity within intact tissue.\textsuperscript{34}

Gap junction protein expression is variable across the ventricular wall.\textsuperscript{33} Particularly, gap junction protein expression is most minimally expressed within the subepicardium, which is associated with heterogeneous transmural conduction velocity, and enhanced APD dispersion. Investigating the role of heterogeneous intercellular coupling in intact preparation is not realistic, as one cannot control the level of uncoupling, or the location of uncoupling with any specificity using traditional approaches using pharmacological agents. To that end, we employed a model in which we could specifically and selectively vary the degree and location of uncoupling to investigate how heterogeneous intercellular coupling influenced transmural APD dispersion and gradient. Uncoupling a mere portion of the fiber yielded APD dispersion and gradient values similar to those obtained under conditions of uniform uncoupling (Figure 2.4). Importantly, uncoupling across cellular layers (i.e., across the EPI-MID interface) was necessary to increase APD dispersion, and unmask an APD gradient (Figure 2.5). Taken together, these data suggest that uncoupling a portion of the ventricular wall, specifically across a cell boundary, can be as potent of an arrhythmogenic substrate as uncoupling all of the cells from one another.
While the transmural wall consists of three distinct cell types, the relative contribution of each cell type to transmural wall thickness is unknown. To this point, equal contributions of cellular layers to the manifestation of APD dispersion was an important assumption of the modeling studies. However, EPI, MID and ENDO cells are unlikely to be compartmentalized into equal thicknesses across the transmural wall in intact tissue. Since M-cells have the longest APD, we investigated the effect of M-cell mass (as a percentage of the ventricular wall) on APD heterogeneity. APD dispersion and APD gradient both exhibited biphasic relationships with M-cell mass. In general, larger M-cell masses (up to 63%) led to larger APD dispersion, at which point, the large M-cell mass electrotonically prolonged the APDs of its neighbors, whereby decreasing total dispersion (Figure 2.6C). The APD gradient also exhibited a biphasic response to variable M-cell mass, although its dependence was less steep (Figure 2.6D) indicating that the APD gradient is more heavily dependent on other factors.

Both the M-cell mass and the degree of intercellular coupling was varied to determine which had a stronger influence on APD dispersion and gradient. APD dispersion was primarily reliant on M-cell mass, but the APD gradient was more heavily dependent on the degree of intercellular coupling (Figure 2.7), suggesting that while cellular composition across the transmural wall is an important mediator of APD dispersion, the degree of intercellular coupling ultimately governs the materialization of an APD gradient capable of producing conduction block, and reentrant arrhythmias.

In this work, a theoretical single strand of tissue was used to demonstrate the principle of electrophysiological heterogeneity. The strength of this quantitative model lay in the selective and specific control of conditions. However, the model’s underlying
and simplifying assumptions limit the extrapolation and generalization of the results. Some of these limitations are discussed below.

Cellular layers are unlikely to be neatly compartmentalized and distributed across the transmural wall. In fact, there is evidence that M-cells aggregate in islands and are often interconnected by other cells of shorter duration.\textsuperscript{35} To properly simulate this condition would require further studies. The model also did not take into account the complexity of intact 3-dimensional tissue. To account for all mediators of electrophysiological heterogeneity in 3 dimensions would require 64 different simulated permutations, and robust computing power, which is beyond the scope of these studies. Nevertheless, the studies presented herein provide the theoretical framework for ionic and gap junction mediated electrophysiological heterogeneity.
REFERENCES


application to conduction and excitation in nerves. *J.Physiol.(Lond.*) 1952;117:500-544


18. Liu D-W, Antzelevitch C: Characteristics of the delayed rectifier current (I_{Kr} and I_{Ks}) in canine ventricular epicardial, midmyocardial, and endocardial myocytes: A weaker I_{Ks} contributes to the longer action potential of the M cell. *Circ.Res.* 1995;76:351-365


CHAPTER 3

Gap junction heterogeneity as mechanism for electrophysiologically distinct properties across the ventricular wall
INTRODUCTION

There is emerging awareness of the importance of transmural electrophysiological heterogeneities in disease states such as Long QT Syndrome\textsuperscript{1}, Brugada Syndrome\textsuperscript{2} and heart failure.\textsuperscript{3} Difference in ion channel composition spanning the anterior left ventricle distinguish the electrophysiological phenotype of epicardial (EPI), mid myocardial (MID), and endocardial (ENDO) myocytes.\textsuperscript{4} Previously, we have shown that the presence of steep action potential duration (APD) gradients in multicellular preparations at the EPI - MID interface can be amplified by various disease states to sufficient magnitude to form a substrate for conduction block and ventricular arrhythmias.\textsuperscript{5}

The development and maintenance of transmural electrophysiological gradients not only depends on ion channel heterogeneities intrinsic to cellular layers which span the transmural wall, but also the degree of intercellular coupling between them.\textsuperscript{6;7} Mediated by gap junctions, intercellular coupling serves to attenuate APD gradients to allow for rapid and synchronous communication between cell layers. Comprised principally of connexin 43 (Cx43) in the ventricle,\textsuperscript{8} gap junction expression patterns have been implicated in the maintenance of electrophysiological heterogeneity in the intact heart. For instance, reduced intercellular coupling during ischemia\textsuperscript{9-11} or heart failure\textsuperscript{12;13} is associated with enhanced electrophysiological heterogeneity.\textsuperscript{3} Importantly, reduced Cx43 expression at the EPI - MID interface is associated with transmural electrophysiological heterogeneity.\textsuperscript{14} Moreover, we have shown that restoring Cx43 in heart failure restores conduction velocity,\textsuperscript{15} reaffirming a role of gap junctions in the mechanism of conduction slowing in heart failure.
To date, the investigation of transmural electrophysiologic heterogeneity has focused on the transmural wall of the anterior left ventricle. However, mounting evidence suggests that the anterior and posterior LV walls are distinct. For example, differences in wall thickening,\textsuperscript{16} and stress-strain relationship,\textsuperscript{17,18} between the anterior left ventricle (aLV) and posterior left ventricle (pLV) are known to exist. Since gap junction expression is importantly influenced by stretch,\textsuperscript{19-21} gap junction distribution may vary between different regions of the heart. Interestingly, there is evidence that infarct location influences clinical phenotype and outcomes in patients. For instance, anterior wall myocardial infarction (MI) is associated with greater likelihood of ventricular fibrillation, and higher mortality than posterior wall MI.\textsuperscript{32} While the mechanisms for these differences are unknown, these data suggest that inherent electrophysiologic differences between the aLV and pLV may account for differences in electrical stability between different regions of the myocardium. In the present study, we hypothesized that conduction velocity, repolarization heterogeneities, and arrhythmia susceptibility in different LV regions can be attributable to regional differences in gap junction expression patterns.

**METHODS**

*Multiplex transmural optical mapping*

To assess the functional consequence of Cx43 expression patterns across the transmural wall in different regions of the LV, we optically mapped action potentials from cells spanning the entire transmural wall of the arterially perfused canine wedge preparation as described previously.\textsuperscript{23,24} Briefly, hearts were excised from eight male mongrel dogs (body wt, 20-25kg). The procedures used were in accordance with the
guidelines set forth by the institutional Animal Care and Use Committee. Wedges of myocardium were dissected separately from either the anterior or posterior walls of the LV in proximity to secondary branches of the left anterior descending and circumflex arteries, respectively. Wedge preparations were first allowed to equilibrate for 20 minutes while being perfused with normal Tyrode’s solution and then perfused with 100 μmol/L carbenoxolone for 10 minutes.

The optical mapping system used to measure transmural action potentials has been described previously. Briefly, electrophysiological heterogeneity across the transmural wall was assessed by recording 256 optical action potentials simultaneously from across all myocardial layers of the transmural LV wall during steady-state ENDO pacing (BCL 2000 ms at 2X diastolic threshold). Optical action potentials were recorded with spatial and temporal resolutions of 0.89mm and 0.5 ms, respectively corresponding to a total mapping area of 14 × 14 mm. APD was measured as the difference between depolarization and repolarization times as previously described. APD was measured from 16 sites per muscle layer (each site was equidistant from the epicardial surface) for each of 16 equally spaced transmural muscle layers. The average APD within each layer was calculated to generate a transmural APD profile from epicardium to endocardium. APD dispersion was calculated as the maximal difference in APD between all layers, which in every case, was given by the maximum APD difference between EPI and MID layers. Conduction velocity was measured selectively (± 15°) in the transmural direction of propagation (θTM) as a function of distance from the epicardium of each of the 16 transmural muscle layers as described. Conduction velocity dispersion was calculated as the difference between EPI and MID layers as a percentage of conduction velocities in
the MID myocardium. Functional measurements were then compared to Cx43 expression within each muscle layer of wedges from the anterior or posterior LV wall. See Expanded Materials and Methods Section for further details.

**Arrhythmia susceptibility**

Programmed electrical stimulation was performed on all wedge preparations harvested separately from the anterior and posterior walls of the LV before and after administration of carbenoxolone. After an ENDO 20-beat drive-train at a BCL of 2000 ms, an EPI premature stimulus (S2) was delivered at an S1S2 coupling interval of 500 ms. The S1S2 interval was sequentially decremented by 10 ms until refractoriness was reached or an arrhythmia was induced. An arrhythmia was defined as three or more unpaced beats.

**Transmural Cx43 quantification by immunofluorescence**

Immunofluorescence analysis of Cx43 was performed using a polyclonal Cx43 total antibody and a monoclonal Cx43-nonphosphorylated antibody (both Zymed). Images were collected from a laser scanning confocal microscope and analyzed as previously described. See Expanded Materials and Methods Section for further details.

**Theoretical multicellular fiber model**

To determine whether regional alterations in intercellular coupling could explain electrophysiological differences observed between the aLV and pLV, we constructed a theoretical multicellular fiber composed 180 ventricular cells each of Luo Rudy (LRd)
formulation\textsuperscript{7,30} interconnected through resistive gap junctions. The one-dimensional theoretical multicellular fiber contained an ENDO region (cells 1-50), a MID cell region (cells 51-140), and an EPI region (cells 141-190), as previously described.\textsuperscript{7} To determine whether localized diminishment of coupling could enhance APD dispersion, gap junction coupling was systematically varied from 2.5 $\mu$S to 0.125 $\mu$S across the theoretical fiber until 100% of the fiber was homogeneously uncoupled. To assess whether the observed gap junction protein expression could produce APD gradients, experimentally measured conduction velocities across the aLV and pLV at baseline and carbenoxolone infusion were used to determine conductance values from the theoretical relationship of discontinuous conduction.\textsuperscript{31} As such, gap junction conductance across the fiber was varied between 0.5 $\mu$S and 0.05 $\mu$S to simulate intercellular coupling at baseline and carbenoxolone conditions. APD was calculated using APD\textsubscript{90} in each cell, and APD dispersion was defined as the difference between the longest and shortest APD. Finally, the derivative of the APD profile across the fiber was calculated by averaging the slopes of two adjacent data points.

\textit{Myocyte isolation and patch clamp recording}

Since important differences in ion channel current composition between cells constitute electrophysiological heterogeneities across the aLV,\textsuperscript{4} it was important to confirm that similar ionic current differences exist across the pLV. To do so, EPI, MID and ENDO myocytes were isolated from left ventricular posterior wedge of 6 additional canines using a standard enzymatic dispersion technique.\textsuperscript{32} Single-cell action potentials as well as $I_{Ks}$, $I_{to}$ and $I_{Ca,L}$ currents were recorded. Currents are reported at peak current
potentials. We validated that 100µM of the selective inter-cellular uncoupler, carbenoxolone,\textsuperscript{33,34} had no effect on APD morphology or duration of isolated cells. For detailed solutions, see Expanded Materials and Methods Section.

\textit{Statistical analysis}

Statistical analysis of the data was performed using Student’s \( t \)-test for paired data or a single factor ANOVA. Differences in susceptibility to ventricular tachycardia (VT) between control and carbenoxolone wedges were compared using the Fisher Exact test. Summary data are presented as mean ± SEM. Differences were considered significant at \( p<0.05 \).

\textbf{RESULTS}

\textit{Electrophysiological properties of the anterior vs. posterior left ventricle}

Representative transmural APD profiles measured across the aLV (panel A) and pLV (panel C) are plotted in figure 3.1 with their corresponding APD gradient profiles (panels B, D). At baseline conditions (squares), there was a substantial difference in APDs across the transmural wall of the aLV, with the sharpest transition in transmural APD occurring between the EPI and deeper layers of tissue (panel A). Correspondingly, the maximal APD gradient was consistently localized between EPI and deeper layers of tissue (panel B). In contrast, the APD profile across the transmural wall of the pLV was relatively flat (panel C), resulting in a negligible APD gradient (panel D). When considering all experiments (panel E), transmural APD dispersion was significantly higher in the aLV compared to the pLV. Moreover, the maximal APD gradient trended
to be larger across the aLV than the pLV at baseline though this did not achieve statistical significance (panel F). Furthermore, the largest APD gradient occurred at a depth approximately 30% from the EPI surface (panel B). Importantly, this location has been shown to be precisely the region of reduced Cx43 expression.14

To determine whether inter-cellular coupling between muscle layers could explain the formation and maintenance of transmural APD gradients, wedges were perfused with 100µM of the selective inter-cellular uncoupler, carbenoxolone.33;34 A subset of experiments confirmed that 100 µM of carbenoxolone did not alter morphology or duration (p=NS) of the action potential (305±30 msec at baseline vs. 314±32 msec post carbenoxolone infusion) recorded from isolated myocytes of normal hearts. In wedges isolated from the aLV, the administration of carbenoxolone shortened EPI APD while prolonging MID and ENDO APD (panel A, circles), resulting in marked amplification of

Figure 3.1. APD dispersion and gradients are greater in the anterior left ventricular wall. Representative transmural APD profiles are plotted for the aLV(panel A) and pLV (panel C) with associated APD gradients (panels B and D) at baseline (squares) and Carbenoxolone (circles) conditions. Summary data in panel E indicated that APD dispersion is higher across the transmural wall of the aLV compared to the pLV, and is further enhanced upon the administration of the gap junction uncoupling agent, carbenoxolone. Summary data of APD gradient in panel F illustrates that Carbenoxolone amplifies the difference in APD gradient between the aLV and pLV.
the APD gradient (panel B). In contrast, in the pLV (panels C, D), carbenoxolone had no effect on the APD profile (panel C) or APD gradient (Panel D). In all experiments, carbenoxolone significantly increased transmural APD dispersion in the aLV (panel E), and APD gradient (panel F), but failed to alter APD gradients in the pLV. These data demonstrate significant differences in susceptibility to uncoupling of different regions of the heart, which implies that these regions may have distinct gap junction protein expression patterns.

In addition to repolarization properties, conduction velocity was measured in each transmural muscle layer of the aLV and compared to the pLV. The representative transmural conduction velocity profile shown in figure 3.2 reveals that under baseline conditions (panel A, squares), there is significant and selective conduction velocity slowing in the EPI of the aLV compared to deeper muscle layers. However, the transmural conduction velocity profile across the pLV was relatively flat (panel B). Summary data revealed that conduction velocity dispersion, which serves as an index of heterogeneity of conduction, was > 2.3 fold higher across the aLV compared to the pLV.

Figure 3.2. Conduction velocity slows in the subepicardium of the anterior left ventricular wall. Representative transmural conduction velocity is plotted for the aLV (panel A) and pLV (panel B) at baseline (squares) and carbenoxolone (circles) conditions. Conduction velocity is significantly slower in the epicardium compared to the mid myocardial layers in the aLV (panel A). Across the pLV, conduction velocity is uniform (panel B). Summary data in Panel C indicates that conduction velocity dispersion is higher in the aLV compared to pLV.
(Figure 3.2, panel C) at baseline. As expected, pharmacological uncoupling with carbenoxolone significantly reduced conduction velocity throughout the transmural walls of both the aLV and pLV. However, carbenoxolone significantly enhanced conduction velocity dispersion in the aLV, but not the pLV (panel C). Importantly, following carbenoxolone administration, conduction velocity dispersion remained more than 2.5 fold higher across the aLV than the pLV (Figure 3.2, panel C).

Susceptibility to inducible ventricular tachycardia was compared between the aLV and pLV. Under baseline conditions, no arrhythmias were induced in wedges harvested from either the anterior or posterior wall (Figure 3.3, panel B). In contrast, under conditions of reduced coupling with carbenoxolone, the aLV exhibited a 4.2 fold increase in susceptibility to inducible ventricular tachycardia compared to the pLV. These arrhythmias typically self terminated, lasted for ~5 seconds, and were generally polymorphic in appearance (Figure 3.3, panel A).

**Cx43 expression patterns of anterior vs. posterior left ventricle.**

Expression patterns of Cx43 across the transmural wall of the aLV and pLV are

---

**Figure 3.3.** Arrhythmia inducibility is greater in the anterior left ventricular wall. A representative example of a ventricular tachycardia is shown in Panel A. Arrhythmias were typically self terminating, lasting for 4 seconds or longer, and polymorphic in appearance. Under conditions of reduced coupling by the administration of carbenoxolone, the aLV exhibited significantly greater susceptibility to inducible ventricular tachycardia (panel B).
compared in representative immunofluorescent images shown in Figure 3.4 (panel A). In each case, Cx43 localized to the longitudinal ends of individual myocytes as reported previously.\textsuperscript{35} A representative transmural profile of Cx43 quantity in one animal shown in Figure 3.4 (panel B) demonstrates that across the aLV, Cx43 signal was selectively reduced in the EPI compared to deeper muscle layers. In contrast to the aLV, in the pLV, there were no significant differences in Cx43 expression between EPI and either MID, or ENDO layers. Averaged data from all experiments revealed that localized reduction of EPI Cx43 in the aLV produced transmural dispersion of Cx43, that was significantly greater across the aLV compared to the pLV (panel B, left inset). In other words, the average EPI expression of Cx43 across the aLV in all animals was significantly smaller than deeper muscle layers (0.87±0.34 vs. 1.1±0.3, AU respectively, p<0.05), whereas across the pLV, EPI expression of Cx43 did not significantly differ from deeper muscle layers (0.97±0.3 vs. 0.97±0.3 AU, respectively, p = NS). Interestingly, there were no significant differences in the total amount of Cx43 expression between the two regions of the ventricle (panel B, right inset). Since phosphorylation of Cx43 has been linked to proper function of gap junctions, we investigated whether the aLV or pLV exhibited differences in the relative proportions of phosphorylated to non-phosphorylated Cx43 signal. Across the aLV, non-phosphorylated percentage of total Cx43 was similar across EPI, MID, and ENDO (1.5±1.2 vs. 3.0±1.0 vs. 1.1±1.6 %, p=NS). Across the pLV, nonphosphorylated percentage of total Cx43 was also similar (1.7±1.2 in EPI vs. 1.4±0.4 in MID vs. 2.0± 0.9% in ENDO) suggesting that the electrophysiological differences observed between aLV and pLV were not influenced by non-phosphorylated Cx43.
Effect of coupling on APD heterogeneity.

The aforementioned data suggested that Cx43 expression patterns differ between the aLV and pLV. Moreover, unlike the pLV, the aLV was characterized by diminished Cx43 in regions that exhibited conduction velocity slowing and maximal APD gradients. Taken together, these data suggest that Cx43 expression patterns may be responsible for electrophysiological differences between the aLV and pLV. To further explore this hypothesis, computer simulations were performed using a one dimensional single cell chain containing ENDO, MID, and EPI cells each of LRd single-cell formulation and interconnected by gap junctions. As shown in figure 3.5, when intercellular coupling was reduced homogenously across the entire fiber from 2.5 μS (solid blue line) to 0.125 μS (solid red line), the APD profile exhibited a markedly increased transmural APD gradient, located at the EPI-MID interface.
Since our experimental data showed that Cx43 expression patterns of aLV and pLV differed as a result of reduced Cx43 expression localized to the sub-epicardial region, computer simulations were repeated to determine if uncoupling localized to subepicardium was sufficient to explain transmural dispersion of APD measured in aLV. The degree of coupling was systematically stepped from 2.5 to 0.125 μS at different depths from the epicardial surface (Figure 3.5, lower panel dashed lines). Uncoupling within the EPI (dashed blue line) failed to amplify transmural APD dispersion. However, uncoupling to a depth that spanned the EPI-MID interface (Figure 3.5, black dashed line) did produce an APD profile that was essentially identical to the one caused by homogeneous uncoupling of the entire fiber (solid red line). The simulation results are summarized in the inset in Figure 3.5 showing that transmural APD dispersion is amplified so long as the depth of uncoupling spanned
the EPI – MID interface, and uncoupling to
deep layers had little incremental effect on
APD dispersion.

To further assess whether the observed
gap junction protein expression patterns
(Figure 3.4) could produce the measured
electrophysiological changes (Figures 3.1 and
3.2), gap junction coupling was varied to
mimic the transmural Cx43 profiles of the
aLV, and pLV at baseline and carbenoxolone
conditions. Briefly, experimentally measured
conduction velocities across the aLV and pLV
at baseline and carbenoxolone infusion were used to determine conductance values from
the theoretical relationship of discontinuous conduction.31 The effect of transmural gap
junction expression patterns on the APD gradient is illustrated in Figure 3.6. Under
conditions simulating gap junction coupling across the aLV, the maximal APD gradient,
which occurred at the EPI-MID interface (data not shown) was larger on the aLV
compared to the pLV. As expected, the APD gradient was further augmented during
conditions simulating functional uncoupling by carbenoxolone. Interestingly, the
difference in APD gradient between aLV and pLV was further enhanced when coupling
was diminished to simulate carbenoxolone infusion. Taken together, these data indicate
that local uncoupling across the EPI-MID interface is an important factor in the
maintenance of APD gradients. Moreover, these data may help explain the occurrence

![Figure 3.6. Coupling profiles of the Anterior and posterior left ventricular walls yield higher APD gradients across the anterior left ventricle at baseline and carbenoxolone conditions. The effect of aLV and pLV Cx43 expression patterns on theoretical transmural APD gradient is illustrated for baseline and carbenoxolone conditions. The aLV has a larger APD gradient than the pLV, and this difference is further augmented when coupling is reduced to simulate carbenoxolone infusion.](image-url)
of unidirectional block leading to reentry at the EPI-MID interface in models of Long QT\textsuperscript{23} and heart failure.\textsuperscript{5}

**Ionic properties are not associated with electrophysiological differences in anterior vs. posterior left ventricle**

Since transmural APD differences arise from differences in transmural ionic channel compositions across the aLV, it was important to confirm that such heterogeneities were also present in the pLV. First, we verified the presence of gradient of the transient outward potassium current, $I_{to}$, between EPI and ENDO cells in the pLV that has been previously reported across the aLV.\textsuperscript{4,36} In the pLV, $I_{to}$ current density was significantly (p<0.05) larger in EPI cells (11.8±1.1 pA/pF) compared to deeper muscle layers (4.7±1.6 pA/pF); the reader is directed to the supplemental figure 1 for the current voltage graph. Importantly, current density of the slowly activating component of the delayed rectifier $I_{Ks}$ was significantly (p<0.05) smaller (0.18±0.02 pA/pF) in cells isolated from the MID myocardium than in EPI (0.4±0.05) or ENDO (0.44±0.08 pA/pF) myocytes amounting to a near 57% reduction in $I_{Ks}$ current density in the MID compared to the EPI. We measured a similar reduction of $I_{Ks}$ current density in the MID (50%) compared to EPI in the aLV. Since the duration of the action potential can also be influenced by L-type calcium current, we verified that there were no differences in $I_{Ca,L}$ current density between EPI,MID or ENDO myocytes in the pLV, and confirmed this same lack of difference in current density in the aLV. Full description of current voltage relationships may be found in the supplemental figure 2. These findings strongly suggest that the differences we report in APD gradients between aLV and pLV are attributable to differences in gap junction expression patterns, and not spatial gradients of ion channel density.
DISCUSSION

In this study, we identify significant differences in electrophysiological function between different regions of the left ventricular myocardium. In contrast to the pLV, the aLV exhibited enhanced transmural APD dispersion, heterogeneous conduction, greater susceptibility to functional uncoupling, and enhanced vulnerability to inducible ventricular arrhythmias. While these results have relevance to mechanisms of electrical instability in the heart, to our knowledge this is the first study to offer a potential explanation for regional variations in electrophysiological properties across the LV. Furthermore, these findings highlight the potential importance of differences in regional electrophysiological function across the LV when considering arrhythmia mechanisms at the level of the whole heart.

The technique of transmural optical mapping from separate myocardial segments permitted detailed and independent electrophysiological phenotyping from aLV and pLV, including the potential contribution of each segment separately to electrical stability of the LV. We found that the aLV possess significantly different electrophysiological properties than the pLV. Specifically, the aLV exhibited substantially greater transmural APD gradients than pLV (figure 3.1). Our findings from the aLV are consistent with previous reports by us\textsuperscript{14} and others\textsuperscript{24} of sharp transitions in APD within a few millimeters of the EPI, at the interface between EPI and MID layers. Additionally, the aLV and pLV also exhibited different transmural conduction velocity profiles. Unlike the pLV, in which conduction velocity was uniform, conduction velocity slowing was consistently observed in the EPI of the aLV compared to deeper muscle layers consistent with the location of increased tissue resistivity\textsuperscript{24} and reduced Cx43 expression.\textsuperscript{14}
Anterior LV segments were also more susceptible to functional uncoupling. While across the pLV, the APD gradient remained unperturbed, uncoupling by carbenoxolone infusion in the aLV significantly amplified the magnitude of transmural APD gradient to >10 msec/mm, a value previously shown to be critical for the development of conduction block and reentry.\textsuperscript{5,37} It is important to note that carbenoxolone had no effect on AP morphology or duration in isolated cells, reaffirming that our observation of transmural APD gradients amplified by carbenoxolone were most likely attributable to uncoupling. Furthermore, ventricular arrhythmias were more easily induced in the aLV after carbenoxolone perfusion, while arrhythmia susceptibility in the pLV was unchanged by carbenoxolone. These data are consistent with earlier observations that large APD gradients between EPI and MID, and conduction slowing\textsuperscript{3,23} can form substrates for conduction block and reentry. Such findings may provide potential insight to clinical outcomes that are dependant on infarct location. Specifically, the association between anterior wall MI and greater likelihood of ventricular fibrillation, and higher mortality rates\textsuperscript{22} may be due to variable electrophysiological properties across different regions of the LV.

A major focus of the present work was to ascertain potential mechanisms responsible for differing electrophysiological properties of aLV vs. pLV. To that end, we probed for major difference in the functional expression of ion channels spanning the ventricular wall and investigated the degree of intercellular coupling interconnecting cells in maintaining transmural electrophysiological heterogeneity. Previous reports in the aLV have identified important differences in the expression of the transient outward potassium current ($I_{to}$),\textsuperscript{38} the slowly activating delayed rectifier potassium ($I_{ks}$) current,\textsuperscript{39}
the late sodium current ($I_{Na,L}$)\textsuperscript{40} and sodium-calcium exchange current ($I_{Na-Ca}$)\textsuperscript{41} across cells spanning the ventricular wall. Such ionic distinctions result in a longer APD in cells of the MID wall and an increased sensitivity of these cells to APD prolonging interventions such as $I_{Kr}$ blocking drugs and bradycardia\textsuperscript{38,42}. Differences in repolarizing currents between right and left ventricles have been reported, which potentially influence clinical expression of Brugada Syndrome\textsuperscript{2} and susceptibility to drug related proarrhythmia\textsuperscript{43}. However, to this point, the existence of electrophysiological heterogeneity in different regions of the ventricle has not been addressed.

Our data indicate that ionic channel heterogeneities which underlie transmural heterogeneity of APD in the aLV were also present in pLV. Transmural gradient in $I_{Ks}$ current density and $I_{Ca,L}$ current density were compared between the aLV and pLV. These currents were examined since they are thought to play a major role in controlling action potential duration in the dog. As expected, $I_{Ca,L}$ current density did not differ between EPI, MID or ENDO cells isolated from either the aLV, or pLV. In contrast, $I_{Ks}$ current density was significantly lower in MID-myocardial compared to EPI myocytes in both aLV and pLV. Taken together, these findings strongly suggest that ion channel heterogeneities are not likely to account for the electrophysiological differences between the aLV and pLV.

Since functional uncoupling by carbenoxolone in the ALV enhanced APD gradients (by preferentially shortening APD in the EPI, while prolonging APD in the MID) but failed to affect the transmural APD profile of the pLV, we hypothesized that differences in gap junction expression patterns may explain electrophysiological differences between aLV and pLV. To test this hypothesis, we analyzed Cx43 expression
patterns across transmural layers in wedges harvested separately from the anterior and posterior free walls. Across the aLV, EPI Cx43 expression was reduced compared to deeper myocardial tissue layers. Across the pLV, however, Cx43 was evenly distributed (Figure 3.4). These expression patterns mirrored the conduction velocity profiles across the aLV, which exhibited slowed conduction in the EPI, and pLV, which exhibited uniform conduction (Figure 3.2). The relative proportion of non-phosphorylated Cx43 to total Cx43 was similar across all muscle layers of the aLV and pLV indicating that nonphosphorylated Cx43 was unlikely playing a role in regional electrophysiologic differences.

The precise mechanisms for the observed expression patterns of gap junctions remain poorly understood. It is widely accepted that gap junctions are importantly mediated by pulsatile stretch.\textsuperscript{20,21} MID and ENDO layers undergo greater segment shortening\textsuperscript{44} and strain\textsuperscript{16} than EPI fibers, which may explain the relative paucity of Cx43 expression in EPI layers compared to deeper counterparts across the aLV. Based on this, it is conceivable that different regions of the ventricular myocardium experience variable stretch forces during the cardiac cycle. Tagged MRI studies, however, reveal that during steady state conditions, the strain experienced throughout the LV myocardium is homogenous\textsuperscript{45}. Therefore, the observed heterogeneous Cx43 expression patterns across the aLV, and homogeneous expression patterns of Cx43 across the pLV cannot be explained by stretch alone. An alternative explanation for the observed expression patterns may lie in the dependence of Cx43 expression on the pattern of conduction. Patel et al reported enhanced Cx43 content distal to the site of ventricular pacing,\textsuperscript{46} suggesting that gap junction protein expression patterns are dependent on the pattern of
activation. If this were true, one might expect to see more, not less Cx43 content in the EPI of the aLV exposed to normal sinus rhythm, during which, the EPI activates last. Hence, activation patterns alone cannot explain the observed relative reduction of Cx43 and overall heterogeneous expression pattern observed across the aLV. Therefore, the mechanism responsible for heterogeneous expression patterns of Cx43 in some regions of the heart and not in others will require further study.

Computer simulations were performed to determine whether the differences in gap junction protein expression patterns between the aLV and pLV could explain the observed transmural APD gradient differences. Previously, it was shown that intercellular coupling attenuates transmural APD heterogeneities, and that uniform uncoupling can unmask electrophysiological heterogeneity.\textsuperscript{6,7} In this study, we extended this work to demonstrate that localized reduction of coupling can amplify transmural APD heterogeneity provided that the region of reduced coupling spans the EPI-MID interface (Figure 3.5). Moreover, to determine whether the observed degree of Cx43 heterogeneity could produce the magnitude of transmural APD gradients in the aLV but not the pLV, we simulated the transmural coupling profiles of the aLV and pLV to show that local uncoupling across the EPI-MID interface was indeed an important factor in the maintenance of APD gradients (Figure 3.6). These data implicate localized under-expression of Cx43 as an important mechanism of transmural heterogeneity.

There are important limitations to our modeling approach that should be considered before extrapolating the results to whole heart function. First, we made an important simplifying assumption of proportionality between total Cx43 expression and the degree of intercellular coupling without regard for stoichiometric composition, gap
junction distribution around the myocyte, or phosphorylation status. Second, in a one dimensional model, such as the one employed in this study, small changes in gap junction coupling can have a great impact on transmural heterogeneity, but in a 3-dimensional structure, higher connectivity between cells might act to minimize APD heterogeneity. Notwithstanding these limitations, the model allowed us to selectively alter inter-cellular coupling (a task unfeasible in intact preparations) to establish the principle that APD heterogeneity is profoundly dependent on the degree of cell-to-cell coupling and just as importantly, the transmural profile of coupling proteins. Finally, it is conceivable that variations of fiber orientation between transmural muscle layers (i.e., rotational anisotropy) could influence tissue resistivity in the transmural direction and, therefore, conduction along the transmural axis. However, we have previously investigated this possibility in detail using a 3D computer simulation where transmural conduction was compared with and without rotational anisotropy. We found conduction along the transmural axis was not dependent on fiber rotation orthogonal to that axis. The data suggested that transmural conduction most closely mimicked transverse conduction within a single plane of myocardium as conduction velocity and estimated space constant were similar in the transmural direction and the axis transverse to fibers within the epicardial layer. Therefore, it is unlikely that localized sub-epicardial conduction slowing was attributable to rotational anisotropy.47

Certainly there have been some discrepancies between APD gradients estimated from activation recovery intervals in situ vs. those measured in the wedge preparation.48 Therefore, it is important to consider the limitations of using the wedge preparation as an indicator of intact ventricular function. The wedge cannot fully recapitulate in-vivo
conditions because it lacks autonomic intervention, metabolic and hormonal activity. Perhaps for these reasons alone, the observed APD and Cx43 gradients have not been convincingly reproduced in situ. For example, Soltysinska et al recently investigated the transmural profile of Cx43 expression in human hearts, but failed to show a transmural gradient, presumably due to either the low number of sample points, the environmental and genetic variance of human subjects or the sensitivity of markers used. Clearly, further investigation is required to address such apparent discrepancies.

In summary, the present study demonstrates that a heterogeneous expression pattern of Cx43 has important implications to normal function of the heart. Taken together, our data suggest that regional differences in electrophysiological function may dictate susceptibility to a variety of arrhythmias. Therefore, regional Cx43 under-expression patterns may be an important mechanism underlying arrhythmia susceptibility, particularly in disease states where gap junction expression is altered.
REFERENCES


13. Peters, NS, Green, CR, Poole-Wilson, PA, Severs, NJ. Reduced content of connexin43 gap junctions in ventricular myocardium from hypertrophied and ischemic human hearts. Circulation 88:864-875.


25. Akar, FG, Roth, BJ, Rosenbaum, DS. Optical measurement of cell-to-cell coupling


39. Liu, D-W, Antzelevitch, C. Characteristics of the delayed rectifier current (I_{Kr} and I_{Ks}) in canine ventricular epicardial, midmyocardial, and endocardial myocytes: A weaker I_{Ks} contributes to the longer action potential of the M cell. Circ Res. 76:351-365.


48. Voss, F, Othof, T, Marker, J, Bauer, A, Katus, HA, Becker, R. There is no
transmural heterogeneity in an index of action potential duration in the canine left ventricle. Heart Rhythm 6:1028-34.

EXPANDED MATERIALS AND METHODS AND SUPPLEMENTAL FIGURES

Transmural optical mapping
Previous, we developed an optical action-potential mapping system\textsuperscript{1-4} capable of resolving membrane potential changes as small as 0.5 mV with 1 ms of temporal resolution from 256 sites simultaneously across the entire transmural surface of the wedge preparation. Wedges were perfused with oxygenated Tyrode’s solution containing (in mmol/l) 129 NaCl, 25.0 NaHCO\textsubscript{3}, 0.5 MgSO\textsubscript{4}, 4.0 KCL, 5.5 dextrose, and 1.8 CaCl\textsubscript{2}. Perfusion pressure was maintained at 50-60 mmHg. Wedges were discarded if collateral arteries shunted significant flow away from the preparation as evidenced by a coronary resistance $<1.2$ mmHg$\cdot$ml$^{-1}\cdot$min$^{-1}$. Preparations were immersed in temperature-controlled (36 ± 1°C) perfusate to prevent the formation of intramyocardial temperature gradients. Wedges were stabilized against a flat imaging window by application of a gentle constant pressure via a movable piston, which circumvented the need for pharmacological suppression of contraction. Preparations were determined to be stable for $>4$ h of perfusion as judged by the stability (± 5%) of coronary resistance, APD, and QT interval.

After the preparation was stained with the voltage sensitive dye 4-{$\beta$-[2(di-$n$-butylamino)-6-naphthyl]vinyl}pyridinium (di-4-ANEPPS, 15μmol/l) by direct arterial perfusion for 10 min, dye was excited by a 514 ± 5-nm light emitted by a 250-W tungsten-filament lamp. Fluoresced light was long-pass filtered at 610 nm and focused unto a 16 × 16 photodiode array (model C4675; Hamamatsu) through a high numerical aperture photographic lenses using the tandem-lens configuration (Nikon, 85 mm, F/1.4
and 105 mm, F/2.0). A 768 × 493-pixel charge-coupled device video camera (TMC-74; PULNiX, Sunnyvale, CA) was used to view and localize the mapping field relative to the anatomical features of the preparation. The anatomical reference points on the wedge were used to precisely align action potential maps with sections of tissue obtained for postoptical mapping measurements of Cx43 distribution.

**Action potential analysis and arrhythmia susceptibility**

APD was measured as the difference between depolarization and repolarization times as previously described. APD was measured from 16 sites per muscle layer (each site was equidistant from the epicardial surface) for each of 16 equally spaced transmural muscle layers. The average APD within each layer was calculated to generate a transmural APD profile from epicardium to endocardium. We used a previously described algorithm to calculate conduction velocity selectively (± 15°) in the transmural direction of propagation (θ_TM) as a function of distance from the epicardium of each of the 16 transmural muscle layers. Functional measurements were then compared to Cx43 expression within each muscle layer of wedges from the anterior or posterior LV wall.

Programmed electrical stimulation was performed on all wedge preparations harvested separately from the anterior and posterior walls of the LV prior to and post administration of Carbenoxolone. After an endocardial 20-beat drive-train at a BCL of 2000 ms, an epicardial premature stimulus (S2) was delivered at an S1S2 coupling interval of 500 ms. The S1S2 interval was sequentially decremented by 10 ms until refractoriness was reached or an arrhythmia was induced.

**Relative transmural Cx43 quantification by immunofluorescence**

Immunofluorescence analysis of Cx43 was performed as previously described.
Briefly, paraffinized tissue layers were sectioned at a thickness of 5 μm and mounted on gelatin-coated slides. Sections were deparaffinized, placed in citrate buffer, and boiled in a microwave oven for 10 min. The sections were incubated overnight with anti-Cx43 antibodies (Zymed, diluted 1:400) and then incubated with CY3-conjugated goat anti rabbit IgG (Zymed diluted 1:800) before being examined by laser scanning Confocal microscopy (×40 oil immersion lens, airy 1 pinhole). The degree of confocality was kept constant (depth of focus ≈602 μm) for each experiment to minimize overlap of Cx43 label. Each layer was analyzed from eight fields to obtain an average Cx43 quantity within a layer. To eliminate artifactual quantification of Cx43 protein, samples were discarded when the imaging plane was not parallel to the long axis of the fiber, as judged by the length-to-width ratios of myocytes < 4.7 Relative Cx43 quantity was defined as the proportion of total myocardial tissue area occupied by Cx43 immunofluorescent signal, as described previously.8 To compare Cx43 expression across experiments, each measurement of Cx43 was normalized to the mean of the total Cx43 measured for an entire experiment.
Myocyte isolation and patch clamp recording

Epicardial, midmyocardial and endocardial myocytes were isolated from left ventricular posterior wedge using a standard enzymatic dispersion technique. Single-cell action potentials were recorded using whole cell patch method. Microelectrodes were fabricated from TW150F borosilicate glass capillaries, filled with a solution of aspartic acid (120 mmol/L), KCl (20 mmol/L), MgCl$_2$ (2 mmol/L), and HEPES (5 mmol/L), CaCl$_2$ (1 mmol/L), EGTA (11 mmol/L), and brought to a pH of 7.2. Isolated myocytes were placed in a bath containing a Tyrode’s solution (in mmol/L, NaCl 137, MgSO$_4$ 1.0, KCl 5.4, glucose 10.0, CaCl$_2$ 1.8, and HEPES 10.0, pH 7.3). Action potentials were elicited in current clamp mode by injecting a square current pulse (5 ms, 1.5 - 2x threshold).

To measure the peak amplitude of $I_{Ks}$ density, cells were placed in a Tyrode’s solution containing Nisodipine (1 μmol/L) to block calcium current and calcium-activated chloride current and TTX (100 μmol/L) to block sodium current. Cells were brought from a holding potential of –70 mV to –25 mV for 25 ms to further inactivate sodium current. $I_{Ks}$ amplitude was measured as the difference between peak current and steady-state current during a 400 ms voltage step ranging from –30 to +60 mV that immediately followed.

$I_{Ks}$ currents were elicited with a 2.5 s voltage step from -30 to 60 mV from a holding potential of -40 mV and then return to -40 mV to generate outward tail currents in the presence of 5 uM E4031 to block $I_{Kr}$ and 1 uM nisodipine to block L-type Ca currents. The amplitude of tail currents was normalized to cell capacitance to obtain $I_{Ks}$ current density.
Supplemental Figure 2: Ionic current densities are similar across the aLV and pLV. Current density-voltage graphs showing that across the aLV (panel A) and pLV (panel B), the slowly activating component of the delayed rectifier K+ current (I_{Kr}), is smaller in cells from the mid-myocardial (MID) region compared to EPI, or ENDO. Peak current measured is plotted for all cell types for aLV and pLV to demonstrate that MID cells exhibit significantly less I_{Kr} than EPI or ENDO cells (panel C). Current density-voltage graphs showing no difference in L-type Ca^{2+} current density (I_{Ca_L}) between EPI, MID, or ENDO myocytes in either the aLV (panel D), or the pLV (panel E). Peak current measured is plotted for all cell types for aLV and pLV (panel F) to demonstrate no difference among the cell types. Values represent mean±SEM.

Cardiac calcium currents traces were recorded using the following extracellular solution (in mM): NaCl, 137, CsCl, 5.4, MgCl2, 1.8, CaCl2, 1.8, glucose, 10, HEPES, 10 (pH 7.4). The intracellular solution was (in mM): Cs MeSO4, 130, TEA Cl, 20, MgCl2, 1, EGTA 10, HEPES 10, Mg-ATP, 4, Tris-phosphocreatine, 14, Tris-GTP, 0.3, creatine phosphokinase 50U/ml (pH 7.2). Pclamp software (Axon Instruments) was used for generation of voltage-clamp protocols and data acquisition. All current recordings were performed at room temperature (20-22°C).
REFERENCES

1. Akar FG, Yan GX, Antzelevitch C, Rosenbaum DS: Unique topographical
distribution of M cells underlies reentrant mechanism of torsade de pointes

2. Akar FG, Roth BJ, Rosenbaum DS: Optical measurement of cell-to-cell coupling in
intact heart using subthreshold electrical stimulation. Am J Physiol Heart
Circ Physiol 2001;281:H533-42

3. Laurita KR, Libbus I: Optics and detectors used in optical mapping., in Rosenbaum
DS, Jalife J (eds): Optical mapping of cardiac excitation and arrhythmias.

4. Rosenbaum DS, Akar FG: The Electrophysiologic Substate for Reentry: Unique
Insights From From High-Resolution Optical Mapping With Voltage-
Sensitive Dyes, in Cabo C & Rosenbaum D.S. (ed): Quantitative Cardiac

5. Girouard SD, Laurita KR, Rosenbaum DS: Unique properties of cardiac action

the adult rat heart. Circ.Res. 1998;83:629-635

7. Poelzing S, Akar FG, Baron E, Rosenbaum DS: Heterogeneous connexin43
expression produces electrophysiological heterogeneities across ventricular

8. Darrow BJ, Fast VG, Kleber AG, Beyer EC, Saffitz JE: Functional and structural
assessment of intercellular communication. Increased conduction velocity
and enhanced connexin expression in dibutyryl cAMP-treated cultured

9. Libbus I, Wan X, Rosenbaum DS: Electrotonic load triggers remodeling of
repolarizing current Ito in ventricle. Am J Physiol Heart Circ Physiol
2004;286:H1901-1909
CHAPTER 4

Epicardial Gene Painting Restores Intercellular Coupling in Heart Failure
INTRODUCTION

The incidence of Heart Failure (HF) continues to rise with approximately 500,000 new cases per year in the United States alone.\textsuperscript{1} Although ventricular arrhythmias are a principal cause of cardiac mortality in HF, the mechanisms linking HF to cardiac electrical instability remain unclear.\textsuperscript{2,3} Conduction slowing is clearly a substrate for reentrant arrhythmias in HF,\textsuperscript{4} and ventricular tachycardia\textsuperscript{5} in humans, but the mechanisms responsible for producing altered conduction in HF are complex and poorly understood.

There are several potential mechanisms for conduction slowing in HF, including disruption of extracellular matrix by fibrosis\textsuperscript{6} causing nonuniform anisotropy,\textsuperscript{7} depressed cellular excitability,\textsuperscript{8} increased intercellular resistivity caused by a change in myocyte shape and size,\textsuperscript{9} and remodeling of cardiac gap junctions. Gap junctions are highly dynamic macromolecules that turn over every 1-2 hours,\textsuperscript{10} and because gap junctions are essential to intercellular propagation, they make an attractive target for gene transfer and/or cell therapies.\textsuperscript{11} HF induces considerable gap junction remodeling manifested as 50% reduction of gap junction protein expression,\textsuperscript{12-14} lateralization of gap junctions away from the intercalated disk,\textsuperscript{15,16} and redistribution of gap junctions across the ventricular myocardium.\textsuperscript{17,18} Recent data from our laboratory has implicated reduced gap junction expression within the subepicardium as a factor in localized conduction velocity slowing,\textsuperscript{14} suggesting that the epicardium may be a viable target for restoring intercellular coupling.

However, there is considerable controversy as to whether diminishment in gap junction quantity can substantially alter conduction velocity. For instance, in transgenic
mouse models, 50% reduction of Cx43 produced only modest\textsuperscript{19} to no\textsuperscript{20} changes in conduction velocity, while severe reduction of Cx43 in a conditional knockout model by 80% caused only modest impairment of conduction.\textsuperscript{21} Therefore, whether gap junction reduction at levels observed in HF is sufficient to slow conduction remains an open question.

To date, identifying the role of gap junctions in electrophysiological remodeling of HF has been challenging, in part because of the complexities of HF and the lack of specificity of drugs used to target gap junctions. However, gene transfer approaches can be used to selectively alter protein expression, investigate mechanisms of disease and identify therapeutic targets. By selectively enhancing Cx43 gene expression using the “gene painting” method previously developed in our laboratory we tested the hypothesis that gap junction remodeling causes the decrease in conduction velocity associated with HF.

**METHODS**

**Study design and statistical analysis**

The experiments were carried out in accordance with the United States Public Service Guidelines for the Care and Use of Laboratory Animals. As shown in Figure 4.1, 26 dogs were randomized to 2 groups: either tachypacing induced HF (n=13) or to non failing normal hearts (normal, n=13). Animals in both groups were further randomized to either; 1. a treatment group receiving adenovirus encoding for Cx43 (AdCx43, n=5), or 2. a sham control group receiving one of two reporter genes; E. coli β-galactosidase (Adβgal, n=3) used to assess gene penetration, or the green fluorescent protein (AdGFP, n=5) used as a functional control to AdCx43. Primary comparisons were made between
Cx43 and sham treated animals within each group. Non-failing normal animals served as a secondary control to the HF group. Animals were euthanized one week post gene transfer and their tissue was harvested for experimental measurements.

Statistical differences were assessed by ANOVA or student t-test with correction for multiple measurements, as appropriate. Chi-square test was used to determine statistical differences in arrhythmia susceptibility rates. A corrected p<0.05 was considered statistically significant. Results are expressed as mean ± standard SEM.

**Canine model of HF**

The onset of HF was initiated by rapid ventricular pacing at 240 beats/min for 4-6 weeks as described. Once the left ventricular ejection fraction, as measured weekly by echocardiography, fell below 30%, the pacing rate was titrated to 200 beats/min to maintain a stable level of HF for up to one week prior to gene transfer (Figure 4.1).
Clinical signs of HF manifested in all dogs included anorexia, lethargy, exercise intolerance, ascites, tachypnea, and muscle wasting.\textsuperscript{14}

**Gene painting procedure**

Since our previously described protocol for gene painting was completely ineffective at transferring genes to the ventricular myocardium,\textsuperscript{25} we modified that protocol by including collagenase in the protease solution, and the new solution allowed partial penetration of the gene transfer vector in the ventricles. This new gene painting solution was prepared by combing adenovirus into 20\% (w/v) polaxamer, 2.5\% trypsin and 10\% collagenase solution for a final virus concentration of $1 \times 10^9$ plaque forming units (pfu) per milliliter.

Normal and HF dogs were sedated with propofol (10mg/kg), and anesthetized with isoflurane (1\% -2\%). After sterile preparation, the chest was opened by a median sternotomy. An incision in the pericardium was used to expose the ventricle, and with manual manipulation to expose the epicardial surface of the left ventricle, the adenoviral/poloxamer/protease solution, warmed to 37\°C to achieve a gel-like consistency, was painted onto the ventricle with a round-bristle, flat paintbrush.\textsuperscript{26} The anterior left ventricle was coated twice for 60 seconds, with 60 seconds elapsed time between coats to allow adsorption. After being painted, the ventricles were exposed to air for 15 minutes to allow for virus penetration. The chest wall was closed, and animals were allowed to recover from surgery. In the HF group, pacing was suspended for the duration of the gene transfer procedure, and resumed upon full recovery from surgery.
High-resolution transmural optical mapping

To assess the functional consequences of gene transfer, we used a system for optically mapping action potentials from the epicardial surface of the arterially perfused, canine wedge preparation as described previously. Briefly, hearts were excised, and wedges of myocardium previously painted with adenoviral solution were dissected and perfused with oxygenated Tyrode’s solution (in mmol/l: 129 NaCl, 25.0 NaHCO₃, 0.5 MgSO₄, 4.0 KCl, 5.5 dextrose, 1.8 CaCl₂, and 6x10⁻³ cytochalasin-D, 36±1°C).

High resolution optical action potentials were recorded from the epicardial surface of the wedge preparation using the voltage-sensitive dye, di-4-ANEPPS (15 µmol/l) with a 1X magnification (resulting in 1.1-mm interpixel resolution) as described in detail previously.

Data analysis was performed using custom software designed for the analysis of optically recorded action potentials. APD was calculated as the time interval between local depolarization and repolarization times. Conduction velocity was calculated with respect to fiber orientation using an average of velocity vectors along the conduction path. Action potential upstroke rise time was measured and corrected for optical blurring artifact as previously described. Potential proarrhythmic effects of AdCx43 was assessed by: 1) 10 minutes of observation of spontaneous arrhythmia, 2) APD prolongation and 3) arrhythmia inducibility, which was tested using programmed stimulation at a drive cycle length of 2000 ms, with up to three premature stimuli until refractoriness was reached or a sustained ventricular arrhythmia was induced. If no arrhythmias were induced by extra-stimulus technique, burst of 10 constant cycle length stimuli were introduced starting at S1S1 cycle length of 300 ms, and decremented by 10
ms until a sustained ventricular arrhythmia was induced or 1:1 capture was not possible. Sustained arrhythmias were defined as induction of a rapid ventricular arrhythmia which persisted $\geq 10$ sec.

**Histological analysis**

Gross specimens were fixed and stained with X-gal as previously described.$^{25}$ Microscopic sections from gene painted preparations were fixed, embedded in paraffin, cut to 5µm thickness, and stained with Masson’s trichrome, as previously reported.$^{28; 34; 35}$ Extracellular matrix was assessed for inflammatory cells, fiber separation, and deposition of collagen in a blinded manner.

**Cx43 expression levels**

To assess junctional, membrane localized Cx43 signal, immunofluorescence analysis was performed as previously described.$^{36; 37}$ Briefly, formalin-fixed and paraffin-embedded tissue layers were sectioned and mounted on gelatin-coated slides. Sections were deparaffinized, placed in citrate buffer, and boiled in a microwave oven for 10 min. The sections were incubated overnight with anti-Cx43 antibodies (Zymed, diluted 1:400) and then incubated with CY3-conjugated goat anti rabbit IgG (Zymed diluted 1:800) before being examined by laser scanning Confocal microscopy. Each sample was analyzed from five fields to obtain an average Cx43 quantity. To eliminate artifactual quantification of Cx43 protein, samples were discarded when the imaging plane was not parallel to the long axis of the fiber, as judged by the length-to-width ratios of myocytes $< 4.$.$^{37}$ Relative Cx43 quantity was defined as the proportion of total myocardial tissue area occupied by Cx43 immunofluorescent signal, as described
previously.\textsuperscript{38} Furthermore, to confirm Cx43 signal was being measured from cardiac myocytes, epicardial samples were double-stained with antibodies against α-Actinin; all samples exhibited characteristic striations of cardiac myocytes.

**RESULTS**

**Transmural Viral Penetration**

The reporter gene for β-galactosidase was used to assess the effectiveness of viral penetration by the epicardial gene painting method. Previously, epicardial gene painting in the porcine achieved complete transmural gene transfer in the atria, but no effective gene transfer in the ventricle.\textsuperscript{26} In the present study, we investigated protease combinations and developed robust proteolysis using a cocktail of trypsin and collagenase to effectively penetrate to subepicardial layers of the ventricular wall. Gene painting produced successful viral gene transfer to a depth of approximately 2 mm beneath the epicardial surface in both normal and HF hearts (Figure 4.2). This corresponded to penetration of virus to 20% of the full transmural extent of the LV wall in HF hearts, achieving a depth of gene transfer to affect subepicardial zones previously shown to exhibit lowest Cx43 expression in HF.\textsuperscript{14} These data indicate that epicardial gene painting can target regions most deficient in Cx43 expression.
Effect of AdCx43 gene painting on connexin expression

To determine if gene transfer by epicardial painting using the AdCx43 virus restored Cx43 protein expression, the magnitude and spatial distribution of Cx43 protein expression was compared in each experimental group by confocal immunofluorescence. Consistent with previous reports, total Cx43 expression was significantly reduced by ~45% in HF hearts compared to normal sham controls (Figure 4.3, panel D, p<0.05). Epicardial painting with AdCx43 in the HF caused a significant increase in protein
expression (p<0.05) restoring Cx43 levels to 85% of normal control (Figure 4.3, Panel C versus Panel A).

Given the rapid turn-over and trafficking of connexins, it was important to investigate the influence of epicardial gene painting on the cellular distribution of Cx43. Figure 4.3 (panels A, B) compare representative examples of Cx43 cellular distribution patterns between normal and HF hearts. Consistent with previous reports, HF was associated with a lateralization of Cx43 around the long edge of myocytes compared to the organized expression of Cx43 at the intercalated disk characteristic of normal myocardium. Interestingly, following AdCx43 painting in HF, Cx43 expression appeared to re-localize to the intercalated disk, in an intermediate pattern between the intercalated disk distribution of normal hearts and the lateral distribution of Cx43 seen in HF (Figure 4.3, panels C vs. A). Taken together, these data suggest that not only does epicardial gene painting with AdCx43 increase Cx43 expression in cardiac myocytes in vivo, but that the protein traffics to the membrane, and specifically to the intercalated disk.

**Effect of AdCx43 gene painting on electrophysiologic function**

To assess the functional consequence of gene transfer, wedges of myocardium were harvested from left ventricular regions one week after the gene-painting procedure for optical mapping. The representative activation maps made from a normal heart (Figure 4.4, panels A) illustrate anisotropic spread of conduction on the epicardial surface. The overall pattern of activation emanating from the site of pacing was similar in every group indicating that fiber orientation was not altered by the gene painting procedure. HF hearts exhibited slowed conduction, particularly in the direction of
propagation transverse to fiber orientation (compare panel B vs. A) amounting to a ~35% reduction in conduction velocity when compared to normal hearts. However, after epicardial gene painting with AdCx43 in HF, conduction patterns and velocity appeared essentially identical to normal hearts (Figure 4.4, compare panels C vs. B) as conduction velocity was restored to a level that was ~90% of conduction velocities in normal hearts. Therefore, these data indicate that viral gene transfer of Cx43 by epicardial painting can restore conduction velocity to normal levels in HF subjects.

To investigate the quantitative relationship between conduction velocity and Cx43 expression, conduction velocity is plotted as a function of Cx43 expression normalized to Cx43 content of normal hearts(Figure 4.5). Mean data from all experimental groups are
Gene painting restores conduction velocity when gap junction reserve is impaired. Conduction velocity is plotted as a function of Cx43 expression. Cx43 expression was normalized to sham treated normal. AdCx43 enhanced Cx43 expression in failing myocardium consistent with theoretical predictions represented by the red dashed line. However, improved Cx43 expression in normal tissue failed to alter conduction velocity. Error bars are ± SDEV.

plotted along with the theoretical relationship of discontinuous conduction\textsuperscript{39} in which conduction velocity increases monotonically with progressively increased gap junction expression (red dashed line). As expected, the reduction in Cx43 expression in HF was associated with significant conduction velocity slowing (compare Normal Sham to HF Sham). AdCx43 epicardial gene painting in HF restored conduction velocity towards normal, proportionate to the increase in Cx43 protein expression. Both the diminished conduction from HF, and its restoration by AdCx43 gene transfer was predicted from the theoretical relationship\textsuperscript{39} between gap junction resistance and discontinuous conduction.

In sharp contrast, epicardial gene painting of AdCx43 in normal hearts deviated from theoretical predictions. Despite doubling Cx43 protein expression, AdCx43 applied to normal hearts failed to increase conduction velocity, suggesting that in the normal heart gap junctions are redundantly expressed, and that strategies designed to target Cx43 expression will produce conduction changes only under conditions where gap junction reserve is impaired. The similarities in Cx43 distribution in AdCx43 treated (data not
shown) to untreated normal hearts suggest that the absence of conduction change is not attributable to defective Cx43 trafficking.

To confirm that the observed changes in conduction velocity in HF were attributable to changes in Cx43 expression and not cellular excitability, action potential upstroke rise time was compared in all animals at epicardial sites located along the slow axes of propagation (Table). Rise time corrected for optical blurring was significantly shorter in HF compared to normal hearts (p<0.05), consistent with a mechanism of conduction slowing from increased axial resistivity rather than depressed excitability. Conversely, rise time was prolonged following restoration of Cx43 expression by AdCx43 in HF animals (p<0.05), consistent with a restoration of intercellular coupling and inconsistent with increased sodium channel excitability.

Since antiarrhythmic therapy has often been associated with proarrhythmic effects associated with prolongation of repolarization, we analyzed action potentials to determine if gene painting produced secondary or unexpected effects on repolarization. As previously shown in this and multiple other HF models, action potential duration was prolonged in HF compared to normal hearts (Table 4.1). However, gene painting by AdCx43 did not affect action potential morphology or duration (Table 4.1).

<table>
<thead>
<tr>
<th></th>
<th>Normal Sham</th>
<th>HF Sham</th>
<th>HF AdCx43</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Corrected Rise Time (msec)</strong></td>
<td>8.7±0.8</td>
<td>2.2± 0.8*</td>
<td>8.3 ± 0.8</td>
</tr>
<tr>
<td><strong>APD (msec)</strong></td>
<td>171± 4.0</td>
<td>195 ± 11*</td>
<td>211± 6.0*</td>
</tr>
</tbody>
</table>

Table 4.1: AdCx43 gene painting restores conduction by enhancing intercellular coupling without altering repolarization properties. Corrected rise time and APD values are given herein. The rate of rise of action potential upstrokes recorded from the epicardial surface of gene treated preparations was fastest in HF Sham tissue compared to normal. AdCx43 treatment slowed the rate of rise of the action potential in HF. While HF is associated with prolonged action potential duration, AdCx43 alone does not alter repolarization properties of the epicardium as action potential duration was not altered after treatment. * indicated p<0.05 when compared to Normal Sham.
Furthermore, we assessed inducibility of sustained arrhythmias in all preparations. Table 4.2. lists the animal number, experimental group, type of arrhythmia, its duration, and the stimulation protocol used for arrhythmias defined as 3 or more un-paced beats. Using such an inclusive definition of an arrhythmia did not elucidate any differences in arrhythmia inducibility between any of the treatment groups. However, when we considered only sustained arrhythmias induced by programmed stimulation, in the absence of Cx43 gene painting, HF was associated with significantly greater rate of inducibility of sustained arrhythmias (60% in HF VS. 10% in normal, p=0.05). Furthermore, AdCx43 preparations did not exhibit spontaneous arrhythmias and had no

<table>
<thead>
<tr>
<th>Animal</th>
<th>Group</th>
<th>Arrhythmia</th>
<th>Duration</th>
<th>Stimulation Protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>2428</td>
<td>HF Cx43</td>
<td>polymorphic VT</td>
<td>8 beats</td>
<td>PES with S3</td>
</tr>
<tr>
<td>2429</td>
<td>HF Cx43</td>
<td>polymorphic VT</td>
<td>2428</td>
<td>HF Cx43</td>
</tr>
<tr>
<td>2518</td>
<td>HF Cx43</td>
<td>polymorphic VT</td>
<td>2429</td>
<td>HF Cx43</td>
</tr>
<tr>
<td>2553</td>
<td>HF Cx43</td>
<td>polymorphic VT</td>
<td>2518</td>
<td>HF Cx43</td>
</tr>
<tr>
<td>2636</td>
<td>HF Cx43</td>
<td>polymorphic VT</td>
<td>2553</td>
<td>HF Cx43</td>
</tr>
<tr>
<td>2548</td>
<td>HF Sham</td>
<td>polymorphic VT</td>
<td>2636</td>
<td>HF Cx43</td>
</tr>
<tr>
<td>2566</td>
<td>HF Sham</td>
<td>polymorphic VT</td>
<td>2548</td>
<td>HF Sham</td>
</tr>
<tr>
<td>2613</td>
<td>HF Sham</td>
<td>alternans -&gt; VF</td>
<td>2566</td>
<td>HF Sham</td>
</tr>
<tr>
<td>2640</td>
<td>HF Sham</td>
<td>polymorphic VT</td>
<td>2613</td>
<td>HF Sham</td>
</tr>
<tr>
<td>2641</td>
<td>HF Sham</td>
<td>polymorphic VT</td>
<td>2640</td>
<td>HF Sham</td>
</tr>
<tr>
<td>2655</td>
<td>Normal Cx43</td>
<td>polymorphic VT</td>
<td>2641</td>
<td>HF Sham</td>
</tr>
<tr>
<td>2582</td>
<td>Normal Cx43</td>
<td>polymorphic VT</td>
<td>2655</td>
<td>Normal Cx43</td>
</tr>
<tr>
<td>2668</td>
<td>Normal Cx43</td>
<td>polymorphic VT</td>
<td>2582</td>
<td>Normal Cx43</td>
</tr>
<tr>
<td>2674</td>
<td>Normal Cx43</td>
<td>polymorphic VT</td>
<td>2685</td>
<td>Normal Cx43</td>
</tr>
<tr>
<td>2685</td>
<td>Normal Cx43</td>
<td>polymorphic VT</td>
<td>2674</td>
<td>Normal Cx43</td>
</tr>
<tr>
<td>2581</td>
<td>Normal Sham</td>
<td>polymorphic VT</td>
<td>2685</td>
<td>Normal Sham</td>
</tr>
<tr>
<td>2669</td>
<td>Normal Sham</td>
<td>polymorphic VT</td>
<td>2581</td>
<td>Normal Sham</td>
</tr>
<tr>
<td>2670</td>
<td>Normal Sham</td>
<td>polymorphic VT</td>
<td>2669</td>
<td>Normal Sham</td>
</tr>
<tr>
<td>2671*</td>
<td>Normal Sham</td>
<td>polymorphic VT</td>
<td>2670</td>
<td>Normal Sham</td>
</tr>
<tr>
<td>2672</td>
<td>Normal Sham</td>
<td>polymorphic VT</td>
<td>2671*</td>
<td>Normal Sham</td>
</tr>
</tbody>
</table>

Table 4.2. Arrhythmia susceptibility based on 3 or more nonpaced beats. The type, duration, and stimulation protocol used to incite the arrhythmia are listed for each animal in each experimental group * indicates a malperfused wedge with microinfarcts.
<table>
<thead>
<tr>
<th>Animal</th>
<th>Group</th>
<th>Arrhythmia</th>
<th>Duration</th>
<th>Stimulation Protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>2428</td>
<td>HF Cx43</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2429</td>
<td>HF Cx43</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2518</td>
<td>HF Cx43</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2553</td>
<td>HF Cx43</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2636</td>
<td>HF Cx43</td>
<td>monomorphic VT</td>
<td>sustained</td>
<td>PES with S3, S4, Burst Pacing</td>
</tr>
<tr>
<td>2548</td>
<td>HF Sham</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2566</td>
<td>HF Sham</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2613</td>
<td>HF Sham</td>
<td>alternans -&gt; VF</td>
<td>sustained</td>
<td>Rapid Pacing</td>
</tr>
<tr>
<td>2640</td>
<td>HF Sham</td>
<td>monomorphic VT</td>
<td>sustained</td>
<td>PES with S2, S3</td>
</tr>
<tr>
<td>2641</td>
<td>HF Sham</td>
<td>monomorphic VT</td>
<td>sustained</td>
<td>PES with S4</td>
</tr>
<tr>
<td>2565</td>
<td>Normal Cx43</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2582</td>
<td>Normal Cx43</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2668</td>
<td>Normal Cx43</td>
<td>monomorphic VT</td>
<td>sustained</td>
<td>PES with S3, Rapid Pacing</td>
</tr>
<tr>
<td>2671*</td>
<td>Normal Sham</td>
<td>polymorphic VT</td>
<td>sustained</td>
<td>PES with S2</td>
</tr>
<tr>
<td>2672</td>
<td>Normal Sham</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.3. Sustained Arrhythmia Inducibility. The type of sustained arrhythmia and stimulation protocol used to incite it is listed for each animal in each experimental group. * indicates a malperfused wedge with microinfarcts. This animal was excluded from arrhythmia inducibility.

increase in the rate of inducible arrhythmias (p=NS). In contrast, there was a trend of lower inducibility following AdCx43 gene painting in HF (20%, Table 4.3).

Effect of Cx43 gene painting on fibrous tissue content

Since collagen deposition in the interstitial space can alter tissue resistivity and conduction velocity, we compared Masson’s trichrome stained sections of HF tissue painted with AdCx43 to HF and Normal sham animals. There was no appreciable difference in collagen content between Normal and HF hearts, nor between AdCx43 painted and sham controls (Figure 4.6, panels A-B). As demonstrated previously, analysis of myocardial samples revealed no difference in the amount of collagen content
as a percent of total cross sectional area (Figure 4.6, panel D), which further implicates alteration in Cx43 (and not macroscopic disruption of the extracellular matrix) as a mechanism of conduction slowing in HF.

**DISCUSSION**

We hypothesized that gap junction remodeling plays a pivotal role in the mechanism of conduction slowing in HF. To address this hypothesis, we tested the prediction that the restoration of intercellular coupling by targeted gene transfer of AdCx43 can restore conduction velocity in the failing heart. We utilized the method of “gene painting” to enhance Cx43 in the sub-epicardium; i.e., the region having most deficient Cx43 expression in the ventricle. Targeted gene transfer to enhance Cx43 expression in failing myocardium restored conduction velocity to near normal levels,
strongly implicating gap junction remodeling as a major cause of conduction slowing in HF. In contrast, targeted gene transfer to enhance Cx43 expression in normal myocardium failed to improve conduction, suggesting that Cx43 overexpression affects electrophysiological properties only under conditions where gap junction reserve is impaired. Taken together, these data suggest that epicardial gene painting may provide a unique approach for targeting arrhythmia substrates in HF.

In the present report, we demonstrate for the first time, the in vivo manipulation of Cx43 expression in myocardium for the purpose of restoring electrophysiological function in heart disease. Manipulating conduction in cell culture models by gene transfer has been explored in the recent past using varying techniques. Kizana et al recently applied lentiviral vectors encoding for mutant Cx43 to cell monolayers to slow conduction for the purpose of applying this technique to uncouple regions of slow conduction in disease states. There has also been significant work in the field of microRNAs and their role in disease pathogenesis, particularly in their ability to silence ion channel genes by suppressing translation. Particularly, miR-1 expression has been linked to Cx43 depression and conduction slowing. Using an antisense inhibitor to eliminate miR-1, Yang et al were able to indirectly upregulate Cx43 protein expression.

**Epicardial gene transfer by gene painting**

HF is associated with an overall reduction in Cx43 protein expression with further underexpression of the Cx43 in the subepicardium. Such expression patterns are associated with reduced conduction velocity, amplification of repolarization gradients across the ventricular wall, and an increased propensity to arrhythmias. Whether
remodeling of cardiac gap junctions by HF underlies the mechanism of arrhythmogenesis in HF has been controversial. To establish causality between altered gap junction expression and reduced conduction velocity, we sought to restore Cx43 expression in order to recover function in the failing myocardium. The epicardial painting approach was employed to specifically target the ventricular epicardium, because this region exhibits lowest Cx43 expression. Viral gene transfer successfully penetrated into ~20% of the ventricular wall, reaching the region previously noted to be maximal Cx43 deficiency in HF canine myocardium (Figure 4.2). Previous work in the porcine model reported complete transmural atrial gene transfer post epicardial gene painting but no effective ventricular gene transfer when painting with dilute concentrations of trypsin. In the present study, we modified this procedure to include more robust proteolysis with a combination of trypsin and collagenase to effectively penetrate to subepicardial layers of the ventricular wall both in normal and HF canine hearts. This approach, has potential relevance to gene transfer for a variety of diseases, although confirmation of effective delivery would be needed in other animal and disease models, as species and disease etiology differences may exist.

In addition to effectively delivering the Cx43 gene to the target, the epicardial painting method also circumvented pitfalls associated with systemic administration or intramyocardial injection of viral genes. Previously, strategies to deliver genes to the heart employed either direct delivery to the myocardium, or intravascular approaches through the coronary arteries. Direct intramyocardial injection has limited utility in promoting localized gene transfer in larger animal models, and the needle lacerates the myocardium, damages tissue along the needle track and creates small local areas of scar,
potentially creating anatomical regions for conduction block. Intracoronary delivery of viral vectors, on the other hand, has more clinical applicability especially when the myocardium is not readily accessible. Yet this method has been shown to be ineffective unless coupled with adjuvant therapies that increase vascular bed permeability or perfusion pressure.\textsuperscript{45, 46} The epicardial painting approach, in contrast, directed the Cx43 gene directly to the target zone.

An important limitation to our approach for other applications is that regions of functional alteration may extend well beneath the epicardium making the epicardial painting approach unsuitable for applications that require reaching deeper muscle layers. Areas of slowed conduction and block responsible for intramural reentrant circuits,\textsuperscript{47} for example, would be unaffected by the epicardial painting method. The results presented in this paper, however, provide the conceptual framework for delivering a gene of interest directly to the epicardium via gene painting. Targeting the mid-myocardium, or endocardium will necessitate development of new gene delivery methods.

**Role of gap junctions in mechanism of conduction slowing in heart failure**

In the present study, confocal immunofluorescence indicated that gene painting with AdCx43 restored protein content in the failing heart to \( \sim 85\% \) of levels observed in normal, non-failing myocardium (Figure 4.3). Furthermore, the newly formed Cx43 protein was properly targeted as most of the signal was seen at the intercalated disk in response to AdCx43 gene transfer. Previously, we\textsuperscript{14} and others\textsuperscript{16} have reported that pacing induced HF causes substantial conduction velocity slowing. Therefore, it is not surprising that in the present study we found that HF was associated with significant reduction of conduction velocity compared to normal sham-treated animals.
Additionally, rise times of the action potential were faster in HF than controls, consistent with conduction slowing attributable to intercellular uncoupling (i.e. gap junction remodeling) in this model. Epicardial gene painting with AdCx43 in HF enhanced Cx43 protein content and significantly improved conduction velocity, restoring it to ~90% of normal levels (Figure 4.4). Taken together, these findings indicate that HF-induced remodeling of gap junctions, and specifically abnormal quantity and cellular distribution of gap junctions is an important mechanism of conduction slowing in HF.

In addition to gap junction remodeling, it is important to consider other factors which may contribute to conduction velocity slowing in HF. In the present study, high resolution optical mapping of hearts painted with adenovirus revealed a pattern of uniformly anisotropic propagation across all experimental groups (Figure 4.4, panels A-B), indicating that gene painting did not affect conduction by altering fiber structure. Additionally, failing hearts painted with AdCx43 exhibited action potential upstroke times similar in duration to that of normal, non-failing hearts, consistent with an effect attributable to improved intercellular coupling. Had AdCx43 increased conduction by enhancing sodium channel excitability, one would predict more rapid rather than slower action potential upstrokes following Cx43 gene transfer. Previous reports indicating that this model of HF does not alter sodium current density is consistent with our findings. Also, there was no appreciable macroscopic disruption of the extracellular matrix, as measured by collagen content in AdCx43 treated preparations (Figure 4.6). Finally, it is possible that the effect of Cx43 gene transfer on conduction may be greater in the rapid pacing model of HF compared to other HF models because alterations of extracellular matrix from fibrosis is not a prominent feature of the rapid pacing model. As such,
the results presented herein must be extrapolated with caution to human HF which can include fibrotic changes.48

**Relationship between Cx43 expression and function in disease**

The relationship between cardiac gap junction quantity, intercellular coupling, and conduction velocity has been controversial. According to theory of discontinuous conduction,39 conduction velocity decreases monotonically with progressively increased axial resistivity (i.e. reduced gap junction expression) as an exponential function. An important assumption of modeling studies is that intercellular coupling is proportional to the magnitude of Cx43 expression, with no regard to the molecular composition of the gap junction, the distribution pattern of the Cx43 protein around the myocyte, compensatory changes in other connexin isoforms, or the phosphorylation status of the Cx43 protein. Despite these limitations, the theoretical prediction provides a framework for understanding the relationship between gap junction protein expression (i.e., resistive coupling) and conduction velocity.39; 49

To date, the principal verification of the aforementioned theoretical relationship arose from experiments in transgenic mice. A murine model of the heterozygous null mutation for Cx43+/− producing 50% reduction of Cx43 quantity exhibited conduction velocity slowing of 25% relative to wild type littermates,19; 50 which is consistent with theoretical predictions.50 In contrast, more severe depletion of Cx43 (by 95% of wild type) in a conditional knockout Cx43−/− slowed conduction (by 55%)51 to a lesser extent than predicted. A number of mechanisms were proposed to explain why the latter experimental measurements departed from theoretical predictions including the possible
contribution of other connexin isoforms, or a greater role of capacitive coupling in murine myocytes because of their small volume.

Our experiments using gene transfer in the adult canine heart can provide novel insights to the relationship between Cx43 expression and conduction because: 1. The range of Cx43 under-expression (50% of normal) parallels human disease, 2. Canine myocyte volume is comparable to humans, and 3. Gene transfer is achieved in adult hearts without exposure to abnormal Cx43 levels during heart development which can influence cardiac morphogenesis. In the present study, a 50% depletion of Cx43 protein by HF slowed conduction to an extent consistent with theoretical prediction. Moreover, gene painting with AdCx43 enhanced epicardial Cx43 in HF, and restored conduction velocity, in a manner that also closely followed the theoretical relationship between conduction velocity and intercellular coupling (Figure 4.5). These data suggest that the newly formed Cx43 protein post-gene transfer decreased intercellular resistance in HF by being properly trafficked to create functional gap junctions at the intercalated disk, thereby reducing axial resistivity. The exact mechanism of how newly formed Cx43 proteins function to enhance conduction in the setting of HF remains to be elucidated.

Interestingly, in normal myocardium, although AdCx43 painting doubled Cx43 protein content, conduction velocity was not altered (Figure 4.5). Therefore, it appears that under conditions of physiologic Cx43 expression, gap junctions are redundantly expressed, and generating supra-physiologic quantities of Cx43 via gene transfer produces no physiologic effect. There are several potential explanations for this observation. It is possible that a normal cell requires a specific threshold of connexin proteins to sustain normal propagation and enhancing protein content past the threshold is
redundant. This might be attributable to established physical limitations at the intercalated disk dictating the necessary threshold of functioning Cx43 proteins. It is well recognized that cell adhesion is a requisite for gap junction formation; therefore, electrically functional gap junctions are restricted to structurally sound cellular interfaces comprised of desmosomes, adherens junctions and intermediate filaments. Given the high turnover rate of connexin proteins, it is possible that the physical space at the intercalated disk is saturated in normal conditions, and despite the fact that a cell’s machinery produces more proteins, there may be no vacancy at the intercalated disk to accommodate redundant proteins. Further studies are required to elucidate the mechanisms of this apparent “gap junction reserve” in normal myocardium.

Clinical implications

In the present study, targeted and selective enhancement of Cx43 gene expression in the canine ventricle was used to demonstrate that gap junction expression is redundant in the normal heart, and that gap junction remodeling plays a significant role in conduction slowing in HF. The etiology and pathogenesis of human HF is complex, hence our results must be extrapolated to human HF with caution. However, our results establish gap junctions as a viable target to potentially mitigate or retard a potent arrhythmogenic substrate. This markedly contrasts conventional approaches with antiarrhythmic drugs which where generally not developed to target substrates with specificity, and suffer from undesirable proarrhythmic side effects associated with prolongation of repolarization. Moreover, the gene therapy approach can be targeted to specifically affected regions of the heart, thereby avoiding potentially undesirable systemic effects or effects on untransduced regions of the heart. For example, we
recently demonstrated that regional administration of KCNH2 gene to critical and localized zones in ventricular tachycardia circuits can effectively suppress arrhythmias without prolonging the QT interval. To successfully improve conduction velocity in human HF, it will be necessary to titrate the magnitude, and duration of enhanced protein content to produce a homogeneous and sustainable effect. Moreover, a precise HF gene therapy approach will likely depend on the mechanism of HF and may require a combination of molecular targets. But this goal is realistic. In cases requiring focal therapy, epicardial gene delivery methods may provide a unique approach for targeting arrhythmia substrates in heart disease.
REFERENCES


24. Kääb S, Nuss HB, Chiamvimonvat N, O'Rourke B, Pak PH, Kass DA, Marban E, Tomaselli GF: Ionic mechanism of action potential prolongation in


CHAPTER 5

Role of Connexin Phosphorylation in Intercellular Communication
INTRODUCTION

Gap junctions are expressed in nearly every mammalian cell type. Their ubiquitous expression illustrates the importance of cell-to-cell communication in cell and tissue biology. Defective, or altered, gap junction expression or function is associated with many disease states.\textsuperscript{1-3} Moreover, gap junction formation and degradation is a highly dynamic process with turnover rates on the order of 1-2 hours.\textsuperscript{4, 5} Therefore, the regulation of gap junction formation and degradation is a critical component of cell-to-cell communication. In addition to their dynamic generation/degradation properties, gap junction function is highly amenable to changes in voltage, pH, and phosphorylation.

Connexin 43 is a phosphoprotein capable of phosphorylation by several kinases, and dephosphorylation by phosphatases. The C-terminus of Cx43 is the principle region of phosphorylation. The majority of phosphorylation sites are serine residues, though threonine and tyrosine residues are also phosphorylated (Figure 5.1). The literature contains conflicting reports regarding the role of Cx43 phosphorylation on gap junction gating. For example, phosphorylation of Ser368 has been reportedly associated with a decrease in dye coupling in cultured fibroblasts.\textsuperscript{6} In intact rat hearts, however, dephosphorylation of Ser368 is associated with decreased electric coupling in the setting of ischemia.\textsuperscript{7} Most recently, Axelsen et al. demonstrated that Ser368 undergoes dephosphorylation during ischemia – a process that was prevented with the use of rotigaptide.\textsuperscript{8}

Rotigaptide is a novel antiarrhythmic peptide analogue that enhances gap junction intercellular conductance between ventricular myocytes without changing membrane conductance.\textsuperscript{9} Previously, we showed that rotigaptide is able to prevent conduction
Figure 5.1. Phosphorylation sites of Cx43. To date, 19 different phosphorylation sites have been identified on the C-terminus. Figure modified from Lempe et al, The International Journal of Biochemistry & Cell Biology 36 (2004) 1171–1186.

slowing during acidosis in the isolated guinea pig heart. In accordance with an effect on gap junction intercellular conductance, rotigaptide has been shown to reduce dispersion of action potential duration in isolated rabbit and guinea pig hearts. Interestingly, rotigaptide has also been shown to reduce the inducibility of ventricular tachycardia (VT) during acute ischemia in dogs, supporting an antiarrhythmic effect associated with the effect on cell-to-cell coupling. However, precisely how rotigaptide alters gap junction intercellular conductance is unknown.

In addition to changes during acute insults like ischemia, connexins also remodel in chronic disease states. Heart failure (HF) is associated with significant changes to the Cx43 protein, including overall downregulation of the protein, lateralization of the protein away from the intercalated disk, and an increase in the amount of
nonphosphorylated Cx43 signal. While rotigaptide is effective in preserving intercellular conductance of existing connexin proteins (that may malfunction in response to acute ischemia), chronic conditions such as HF necessitate a strategy to create new proteins to replace downregulated gap junctions. One such strategy is generation of Cx43 via an adenovirus encoding for Cx43 (AdCx43). We have previously shown that AdCx43 applied in the setting of HF nearly doubles Cx43 quantity (Chapter 4). Interestingly, AdCx43 had a similar effect in normal tissue. However, the molecular composition of newly formed protein remains completely unknown.

Therefore, this study aims to determine 1) the level of nonphosphorylated Cx43 under conditions of ischemia or HF, and 2) how pharmacological modification via rotigaptide or genetic manipulation via AdCx43 affects the nonphosphorylated isoform. The results of this study suggest that pharmacological and genetic modification of gap junction proteins is a promising novel approach in antiarrhythmic therapeutics development.

**METHODS**

**Ischemia and Cx43**

Male guinea pigs (600-880 g) were anaesthetized with 30 mg/kg ip, pentobarbital sodium (Abott Laboratories), tracheotomized and ventilated on room air using a Harvard rodent ventilator (tidal volume: 5 ml, frequency: 60 per min). The aorta was exposed and cannulated as described previously. Hearts were perfused in the Langendorff mode at 31.9±0.1°C with an oxygenated (100% O2), filtered (45 μm) modified Tyrode’s solution containing (in mmol/l): CaCl₂, 1.25; NaCl, 140; KCl, 4.5; dextrose, 5.5; MgCl₂, 0.7; HEPES, 5, and 5.0 diacetyl monoxime; titrated to a pH of 7.4 with NaOH. Additionally,
one canine heart was excised, and a wedge of myocardium from the secondary branch of
the left anterior descending artery was dissected and perfused with oxygenated Tyrode’s
solution (in mmol/l: NaCl 129, NaHCO₃ 25.0, MgSO₄ 0.5, KCl 4.0, dextrose 5.5, CaCl₂
1.8, and 6×10⁻³ cytochalasin-D, 36±1°C). Perfusion pressure was maintained at
approximately 50 mmHg during baseline conditions by adjusting flow accordingly.
Cardiac rhythm was monitored by volume conducted ECG using 3 silver disc electrodes
fixed to the chamber in positions corresponding to limb leads I, II, and III.

Guinea pigs were randomized to two groups: a rotigaptide-treated (n=5) or a
vehicle-treated (n=5) group. In both groups, hearts were initially allowed to equilibrate
for 15 minutes at baseline pacing (150 beats per minute (bpm), Baseline) At the end of
the Baseline period, either 50 nM rotigaptide or vehicle was added to the perfusate for 15
minutes. Then, coronary flow was reduced by 75% to induce global low-flow ischemia
for 20 min (Treatment + Ischemia).

Heart failure and Cx43

To assess Cx43 protein in the setting of HF, tissue homogenates were harvested
from tachypacing induced HF (n=10) or non failing normal hearts (normal, n=10).
Animals from both groups contained either: 1. a treatment group receiving adenovirus
encoding for Cx43 (AdCx43, n=5), or 2. a sham control group receiving adenovirus
encoding for the green fluorescent protein (AdGFP, n=5). Primary comparisons were
made between Cx43 and sham treated animals within each group. Non-failing normal
animals served as a secondary control to the HF group.

Cx43 expression levels

Western immunobloting was performed on ventricular tissue samples as previously
described. Briefly, left ventricular homogenates were sampled from Langendorff
perfused guinea pig hearts during treatment (n=5), and treatment+ischemia (n=5). To allow for paired analysis, each animal served as its own control (i.e., tissue samples were taken from different vascular territories prior to and post ischemia perfusion). Phosphatase inhibitors (Sigma) were added to the lysis buffer for the purpose of maintaining phosphorylation state. A commercially available polyclonal rabbit antibody to Cx43 (Zymed), which reacts with Cx43 independent of phosphorylation state was used. In addition, an immuno blot for calsequestrin was used concurrently as a loading control. Lanes were loading with 17 μg of total protein, and values were averaged between columns to account for pipetting errors. Cx43 protein content was analyzed using ImageQuant TL (Molecular Dynamics). The signal intensity from bands at ~43kDa corresponding to Cx43 protein were summed to calculate total Cx43 protein content, whereas the higher molecular weight bands were attributed to phosphorylated Cx43 as described7.

To assess junctional Cx43 signal, immunofluorescence analysis was performed as previously described.4,17 Briefly, anti-total-Cx43 antibodies and anti-nonphosphorylated Cx43 at serine 368 (both Zymed, diluted 1:400) were used followed by CY3-conjugated goat anti rabbit IgG or FITC-conjugated goat anti-mouse secondary antibodies (both Jackson IR diluted 1:800) before being examined by laser scanning Confocal microscopy. Each sample was analyzed from five fields to obtain an average Cx43 quantity within a sample. To eliminate artifactual quantification of Cx43 protein, samples were discarded when the imaging plane was not parallel to the long axis of the fiber.17 Relative Cx43 quantity was defined as the proportion of total myocardial tissue area occupied by Cx43 immunofluorescent signal, as described previously.18
RESULTS

Ischemia induces Cx43 dephosphorylation

To confirm that ischemia leads to Cx43 dephosphorylation, guinea pig hearts and one wedge preparation from a canine heart were subjected to 20 minutes of low flow ischemia. As expected, at baseline conditions, Cx43 is primarily phosphorylated, therefore, there is relatively little nonphosphorylated Cx43 signal (Cx43 NP). Notice that there is significantly less green signal (Cx43 NP) than red signal (Cx43 P) in merged (Cx43 Total) images of baseline in both panels A and B of Figure 5.2. However, after 20 minutes of ischemia, there is substantially more nonphosphorylated Cx43 signal (green) in both guinea pig, and canine hearts (panel A and B, respectively Figure 5.2, green signal, Cx43 NP). Interestingly, much of the nonphosphorylated Cx43 is localized to the same cellular locations as phosphorylated Cx43, suggesting that during 20 minutes of ischemia, Cx43 undergoes dephosphorylation, but remains stationary, suggesting that dislocation of Cx43 from the intercalated disk may occur at a later time. These results suggest that low flow ischemia caused changes in Cx43 phosphorylation consistent with the time course of ischemia-induced uncoupling.\(^7;19\)

Figure 5.2. Ischemia induces Cx43 dephosphorylation. Representative confocal immunofluorescent images show phosphorylated (Cx43 P red) and nonphosphorylated Cx43 (Cx43 NP, green) at baseline and ischemic conditions. Cx43 NP increases during ischemia. Merged images show that in the short time course of 20 minutes, Cx43 NP localizes to the same locations as phosphorylated cx43. Panel A guinea pigs, Panel B is the same experiment in canine.
Rotigaptide inhibits ischemia-induced Cx43 dephosphorylation

To explore a potential mechanism for rotigaptide’s effect during ischemia, guinea pig hearts were treated with either vehicle, or rotigaptide prior to ischemia induction. Representative confocal immunofluorescence images revealed that total Cx43 protein expression and cellular distribution remained the same prior to, and after global low flow ischemia during vehicle and rotigaptide perfusion (Figure 5.3, panel A) suggesting that rotigaptide’s ability to preserve conduction during ischemia\textsuperscript{20} was likely attributable to an effect on gap junction conductance. To further support the conclusion that rotigaptide’s
effect was potentially attributable to inhibition of Cx43 dephosphorylation, western blots revealed a marked change in the amount of dephosphorylated Cx43 during global low-flow ischemia induction (Figure 5.3, panel B). As expected, ischemia resulted in a substantial increase in dephosphorylated Cx43, amounting to a near 16% enhancement of dephosphorylated Cx43. Interestingly, rotigaptide prevented ischemia-induced dephosphorylation of Cx43, suggesting a possible molecular mechanisms for the maintenance of intercellular coupling by rotigaptide.

**Heart failure and Cx43 dephosphorylation**

To determine the quantity of functional Cx43 under conditions of HF, the relative contribution of nonphosphorylated Cx43 to total Cx43 signal with and without AdCx43 gene painting was assessed. Representative confocal images (inset) and the relative contribution of nonphosphorylated signal to the total Cx43 signal is plotted in the stacked column of Figure 5.4. As expected, HF was associated with substantially higher nonphosphorylated Cx43 than normal controls (0.15 ± 0.05 AU vs 0.04± 0.01 AU, p<0.05). Interestingly, HF animals gene painted with AdCx43 exhibited a similar level of nonphosphorylated Cx43 signal as their HF sham counterparts (0.16 ± 0.07 vs 0.15 ± 0.05 AU, p = NS) which suggests Cx43 protein enhancement following AdCx43 gene painting results from an upregulation of primarily phosphorylated and therefore, functional Cx43. Normal animals treated with AdCx43 exhibited an enhancement of total Cx43 protein that was also due to functional (i.e, phosphorylated) Cx43 protein as the total amount of nonphosphorylated protein did not differ from the normal sham animals. Taken together, these data suggest that even though HF is associated with increased nonphosphorylated Cx43 signal, AdCx43 gene painting increased functional
Figure 5.4. *Nonphosphorylated signal remains unchanged post AdCx43 gene painting.* Confocal immunofluorescent images show phosphorylated (P, red), nonphosphorylated (NP, green) and total (merged) images for normal and HF hearts treated with AdCx43 or sham controls. Summary data below illustrates a stacked column where the contribution of nonphosphorylated Cx43 to total Cx43 is represented by the dark gray bars. As expected, HF hearts exhibited higher quantities of nonphosphorylated Cx43. However, Nonphosphorylated Cx43 quantity remained unchanged post AdCx43 treatment in either HF or normal hearts.

Cx43 expression in cardiac myocytes *in vivo*, allowing the protein to traffic to the membrane, and specifically to the intercalated disk in both HF and normal animals. These data further suggest that AdCx43 produces Cx43 that is uniquely processed from endogenously generated protein.
DISCUSSION

In the present study, we investigated the state of Cx43 phosphorylation during ischemic perfusion both with and without rotigaptide. Cx43 dephosphorylation has been implicated as an important mechanism of impaired junctional coupling during ischemia.7 To date, Cx43 dephosphorylation and its implication for gap junction gating has been unclear. Of the 19 different phosphorylation sites identified in the C-terminal domain of Cx43, Ser368 is at the center of the phosphorylation controversy. Reports linking a reduction in dye coupling of cultured fibroblasts with phosphorylation of Ser3686; 21 have been challenged by others reporting that dephosphorylation of Ser368 promotes electrical uncoupling during ischemia in isolated rat hearts.7 Recently, Axelsen et al. demonstrated that pre-treatment with rotigaptide prevented dephosphorylation of Ser297 and Ser368 during ischemia.8 The findings of the present study are consistent with the time course of Cx43 dephosphorylation and uncoupling7; 22 described previously for ischemic conditions. Prior to ischemia induction, phosphorylation status remained stable over time as expected (data not shown). Moreover, the ability of rotigaptide to suppress ischemia-induced Cx43 dephosphorylation suggests a potential molecular basis for rotigaptide’s effect on conduction velocity, spatial synchronization of repolarization, and alternans.20 Importantly, confocal immunofluorescent evaluation of tissue revealed no changes in total Cx43 quantity or distribution patterns, which further underscores the role of Cx43 dephosphorylation as a mechanism of gap junctional uncoupling. We can not exclude from our data that rotigaptide could have also influenced intracellular trafficking of Cx43, or have had additionally effected signaling pathways which modulate gap junctions.
We also examined the state of Cx43 phosphorylation after AdCx43 gene transfer in both HF and normal hearts. While HF was associated with an increased level of nonphosphorylated signal, which is consistent with recent reports,\textsuperscript{14, 15} AdCx43 did not alter the total amount of nonphosphorylated Cx43. Interestingly, AdCx43 had a similar lack of effect on nonphosphorylated Cx43 quantity in normal hearts. Importantly, AdCx43 increased the total amount of Cx43 protein, suggesting an increase in phosphorylated/functional Cx43 protein. This enhancement in protein content was associated with an improvement in conduction velocity in HF (Chapter 4). Even though the role of phosphorylation status on conduction velocity is controversial, it has been demonstrated that gap junctional communication decreases upon dephosphorylation of Cx43.\textsuperscript{7, 20} The lack of appreciable difference in the amount of nonphosphorylated Cx43 signal post AdCx43 painting in HF suggests that AdCx43 created \textit{functional} protein that improved gap junctional communication. Furthermore, these data suggest that AdCx43 generated protein must be distinctly processed from endogenously created protein.

Endogenously generated Cx43 protein is believed to be delivered to the plasma membrane via microtubules upon which, the protein undergoes phosphorylation (through processes not fully elucidated) as it migrates towards the intercalated disk.\textsuperscript{2} However, recent evidence suggests that connexin vesicles can be delivered directly to the intercalated disk.\textsuperscript{23} Therefore, it is plausible that AdCx43-generated Cx43 protein is directly trafficked to the intercalated disk by microtubules whereby circumventing the traditional pathway of endogenously generated Cx43 protein. This would explain why AdCx43 gene transfer resulted in more phosphorylated Cx43. We cannot rule out, however, that the antibody we used against Serine368 was too specific and
underestimated the amount of nonphosphorylated Cx43. Nevertheless, the newly formed protein was associated with improved conduction velocity, leading us to conclude that AdCx43 generated functional protein. The precise mechanism of AdCx43-mediated Cx43 formation requires further investigation.

Regulation of gap junctions is an incredibly complex process occurring through multiple signaling pathways and resulting in (de)phosphorylation of multiple sites. Nevertheless, there is enough corroborating data to suggest that HF and ischemia are associated with dephosphorylation of Cx43, though the precise mechanisms remain unknown. For the time being though, it seems that rotigaptide and adenoviral therapy can provide a unique approach for improving intercellular communication by retaining gap junctional conductance or by improving the total quantity of functional protein.
REFERENCES


CHAPTER 6

Summary, Implications and Future Directions
SUMMARY

The role of gap junctions in creating substrates for arrhythmias has been a subject of intense research for many years. Gap junctions, principally comprised of Connexin43 (Cx43), serve to couple neighboring myocytes in ventricular myocardium, allowing for the rapid transport of chemical messengers, and ionic currents essential for impulse generation and propagation. Gap junctions are dynamically generated and degraded on the order of 1-2 hours\(^1\), and their protein expression and function is highly amenable to changes in the extracellular environment, particularly in response to pathologic insults. Still, the precise role of gap junctions in the maintenance of arrhythmogenic electrical heterogeneity and conduction slowing in health and disease remains an open question. Furthermore, restoration of electrophysiological function by improving intercellular coupling in heart disease has been largely unaddressed.

Important differences in ionic composition of cells isolated from different chambers of the heart are vital for normal, synchronous cardiac function. Furthermore, three distinct cell types within the ventricle have been well characterized by their electrophysiologic response to pharmacologic interventions.\(^2\) Despite ionic heterogeneities present across the transmural wall, strong electrical coupling masks electrical heterogeneity.\(^3\) To investigate the principles of transmural electrical heterogeneity, a theoretical single cell strand was constructed (Chapter 2). The model was used to demonstrate that the extent of electrophysiological heterogeneity within the transmural wall is influenced by 1) the ion channel heterogeneities intrinsic to different myocardial layers, 2) degree of intercellular coupling, 3) homogeneity of intercellular coupling, and 4) the relative quantity of each myocardial layer.
The functional consequence of transmural electrophysiological heterogeneity in different regions of the ventricle remained unknown. Moreover, the role of gap junction coupling on conduction velocity, repolarization heterogeneities, and arrhythmia susceptibility in different regions of the heart was an open question. Previously, it was demonstrated that a localized reduction of Cx43 was associated with decreased conduction velocities and enhanced repolarization gradients.\(^4\) We demonstrated significant differences in Cx43 expression and electrophysiological function between different regions of the left ventricular myocardium (Chapter 3). In contrast to the posterior wall, in which Cx43 is homogeneously expressed, a heterogeneous expression pattern of Cx43 across the anterior left ventricle was associated with heterogeneous transmural conduction, enhanced transmural APD dispersion, greater susceptibility to functional uncoupling, as well as an enhanced propensity to inducible ventricular tachycardia. These data suggested that the anterior and posterior walls of the canine left ventricle are electrophysiologically distinct, and that relative Cx43 expression patterns are responsible for maintaining these differences.

The functional consequence of heterogeneous Cx43 expression in heart failure (HF) has been previously described.\(^5\^-7\) However, elucidating the precise role of gap junctions in electrophysiological remodeling in HF has been challenging due to the complex nature of the disease, and the lack of specificity in (un)coupling agents used to target gap junctions. In this report, we used gene transfer to selectively alter protein expression as a tool to investigate mechanisms of arrhythmias and identify a therapeutic target to help mitigate HF (Chapter 4). Targeted gene transfer of AdCx43 enhanced Cx43 expression in failing myocardium, and restored conduction velocity to near normal
levels, whereby strongly implicating gap junction remodeling as a major cause of conduction slowing in HF. However, targeted gene transfer enhanced Cx43 expression in normal myocardium, but failed to improve conduction, suggesting that Cx43 overexpression affects electrophysiological properties only under conditions where gap junction reserve is impaired. Taken together, these data suggest that epicardial gene painting to restore Cx43 may represent a unique approach for targeting substrates of ventricular arrhythmias in HF.

In addition to total protein expression level, Cx43 dephosphorylation has been implicated as an important mechanism of impaired junctional coupling.\textsuperscript{8, 9} The state of Cx43 phosphorylation in pathologic conditions (ischemia or HF) with and without treatment (rotigaptide or AdCx43, respectively) was investigated (Chapter 5). Rotigaptide, an antiarrhythmic peptide analogue that enhances gap junction intercellular conductance between ventricular myocytes without changing membrane conductance,\textsuperscript{10} prevented ischemia induced dephosphorylation of Cx43.\textsuperscript{11} Interestingly, though HF was associated with an increased level of nonphosphorylated Cx43, AdCx43 did not affect total nonphosphorylated Cx43 quantity. Nor did AdCx43 alter the total amount of nonphosphorylated Cx43 signal in normal, non diseased tissue. These data suggest that either AdCx43 enhanced protein production without affecting degradation processes, or that AdCx43 generated protein was distinctly trafficked from endogenous Cx43.\textsuperscript{12} The precise mechanism of AdCx43-mediated Cx43 formation requires further investigation.

**IMPLICATIONS**

By establishing Cx43 as an important arbiter of electrophysiologic function in the normal and diseased heart, this work has important clinical implications. The role of gap
junctions in maintaining electrophysiological heterogeneity has been previously unknown. An important contribution of the work was showing that in addition to total depression of Cx43 signal, localized diminishment of coupling across cellular layers can enhance electrophysiological heterogeneity and therefore create the substrate for arrhythmias. This new knowledge can potentially have important implications on arrhythmia management in diseased hearts. For example, in a reentrant arrhythmia, the EPI-MID interface could be acting as a source of unidirectional block in a patient with recurrent tachycardia. A priori knowledge of arrhythmogenic substrates predisposing the heart to ventricular tachycardia could potentially help guide ablation therapy, and prove to be time and cost effective.

Additionally, this work demonstrated that a heterogeneous expression pattern of Cx43 has important implications to normal function of the heart. Taken together, our data suggest that regional differences in electrophysiological function may dictate susceptibility to a variety of arrhythmias. There is evidence that infarct location influences clinical phenotype and outcomes in patients. For instance, anterior wall myocardial infarction (MI) is associated with greater likelihood of ventricular fibrillation, and higher mortality than posterior wall MI.13 While the mechanisms for these differences are unknown, these data suggest that inherent electrophysiological differences between the anterior and posterior left ventricular myocardium may account for differences in electrical stability between different regions of the heart. Therefore, regional Cx43 under-expression patterns may be an important mechanism underlying arrhythmia susceptibility, particularly in disease states where gap junction expression is altered.
Additionally, in the present study, targeted and selective enhancement of Cx43 gene expression in the canine ventricle was used to demonstrate that gap junctions are a viable target to potentially mitigate a potent arrhythmogenic substrate. Targeted gene therapy markedly contrasts conventional approaches with antiarrhythmic drugs which suffer from lack of specificity, and undesirable proarrhythmic side effects associated with prolongation of repolarization. Importantly, the gene therapy approach can be targeted to specifically affected regions of the heart, thereby avoiding potentially undesirable systemic effects or effects on untransduced regions of the heart.\textsuperscript{14} To successfully improve conduction velocity in human HF, it will be necessary to titrate the magnitude, and duration of enhanced protein content to produce a homogeneous and sustainable effect. Moreover, a precise HF gene therapy approach will likely depend on the mechanism of HF and may require a combination of molecular targets.

In other words, targeted gene therapy has the potential to revolutionize the current standard of care and management of cardiac diseases. For instance, gene therapy could be instrumental in annihilating arrhythmic substrates in cases requiring focal therapy. An example of a potential application of this is coupling gene therapy with revascularization therapy. One might envision a situation with a patient being rushed to the catheter lab with an MI, and upon opening the previously occluded vessel, the interventionalist could also inject an adenovirus for Cx43, or a combination of molecular targets to help the previously damaged tissue recover faster. Hence, gene delivery methods may provide a unique approach for targeting arrhythmia substrates in heart disease.
FUTURE DIRECTIONS

In this work, we investigated the principle of transmural electrophysiological heterogeneity and its dependence on the composition of myocardial layers, as well as the degree and homogeneity of intercellular coupling. Simplifying assumptions were made regarding the distribution of myocardial layers, which were modeled as distinct layers. More robust models mimicking physiologic myocardial layer distribution to include gradients of cells, or even islands of cells are necessary to understand the mechanism of electrophysiological heterogeneity in the setting of 3 dimensional complexity.

The role of intercellular coupling in maintaining arrhythmogenic substrates in the setting of HF were elucidated in this work. Enhanced Cx43 protein content improved conduction velocity in HF, implicating gap junction coupling as an important mediator of conduction. It is well recognized that an ischemic insult (which alters intercellular coupling) in the setting of existing HF is highly proarrhythmic. However, it is unknown how an enhanced amount of Cx43 affects the changes induced by ischemia, or other conditions affecting intercellular coupling, such as during acidosis, or administration of uncoupling agents such as Carbenoxolone. Upregulating Cx43 with an adenovirus may be able to protect against uncoupling events during ischemia. It would be important to test the degree to which arrhythmias can be prevented in these settings.

As shown in Chapter 4, enhanced Cx43 expression failed to alter conduction in the normal heart. These data suggest the existence of sufficient reserve of Cx43 protein, and that improving protein expression levels beyond reserve levels may not produce functional change. If this is true, a normal heart with supraphysiological Cx43 expression would be protected against ischemic injury more so than a normal heart with
normal Cx43 expression. It would be instructive to see if the changes in electrophysiological function in response to ischemia/acidosis/Carbenoxolone are similar in preparations treated with AdCx43 than those with normal Cx43 expression levels. One might predict a normal heart with supraphysiologic level of Cx43 would be less susceptible to uncoupling interventions because it does not follow the theoretical relationship between intercellular resistance and conduction velocity (Figure 4.5). Specifically, a more preserved level of function would be predicted than in conditions that strictly follow the theoretical relationship.

The effect of ischemia or intercellular uncoupling on the spatial organization of cellular alternans in general, and discordant alternans in particular has been investigated recently. Specifically, it has been demonstrated that discordant alternans can be suppressed by increasing gap junction intercellular communication.11 However, it is entirely unknown how ischemia/acidosis/Carbenoxolone affects the threshold of discordant alternans in the setting of upregulated Cx43 proteins. One might predict that improved Cx43 protein expression in the normal heart might increase the heart rate threshold for discordant alternans. Moreover, HF is associated with a higher threshold in discordant alternans. However, how the spatial organization of cellular alternans in the setting of HF with improved Cx43 protein expression is entirely unknown. One might predict that enhanced Cx43 expression via gene transfer would increase the heart rate threshold for discordant alternans in HF.17

The work presented in Chapters 3 and 4 primarily investigated the consequence of improved Cx43 protein expression in the failing and nonfailing heart. The mechanism of improved protein expression in the setting of limited infection efficiency remains largely
unaddressed. Since gap junctions form when two abutting cells contribute a connexin, it is plausible that one infected cell can create connexin proteins, assemble them into a connexon hemipore, deliver it to the intercalated disk, and signal to its neighboring cell to produce a connexon hemipore to create a gap junction. However, it remains unclear whether moderate infection efficiencies and the enhancement of Cx43 expression described herein is the result of juxtacrine (cell contact-mediated) and or short-range paracrine signaling from the neighboring cells.

Furthermore, the generation of adenovirus-mediated protein is not well understood. Are adenovirus-mediated Cx43 proteins trafficked to the membrane in which they are free to migrate to the intercalated disk following the classical pathway of gap junction formation,\textsuperscript{18} or are they delivered directly to the intercalated disk via microtubules?\textsuperscript{12} In other words, are adenovirus-mediated Cx43 proteins distinct from endogenously generated proteins? Such questions require further detailed investigation in simplified, well controlled, cell-culture systems.

In this work, adenovirus was strategically directed at the epicardium. However, demonstration of efficient transmural and/or whole heart gene transfer is necessary prior to clinical translation. Moreover, gene painting methods are suitable to reaching physically accessible targets, but deeper myocardial layers remain largely unaffected. Therefore, it becomes important to develop new gene delivery methods to target deeper muscle layers.

In summary, exploration of the following aims will help elucidate the mechanisms of disease-mediated cardiac dysfunction, and in turn, may translate to effective clinical therapies:
1. Use targeted gene transfer to enhance cellular communication to mitigate arrhythmogenic risks in normal and HF hearts.

   *Hypothesis 1:* Enhanced Cx43 expression in ventricular myocardium will be protective against ischemia-induced conduction slowing

   *Hypothesis 2:* Enhanced Cx43 expression in ventricular myocardium will increase the heart rate threshold for discordant alternans

2. Determine the mechanism of adenovirus-mediated protein enhancement

   *Hypothesis 1:* Adenovirus mediated protein enhancement occurs through a physiologic pathway that is distinct from endogenous Cx43 protein.

   *Hypothesis 2:* Adenoviral-mediated production of Cx43 protein in one cell causes enhanced Cx43 production in its neighbor.

3. Develop gene transfer methodology to target the entire ventricular wall

   *Hypothesis:* Enhanced Cx43 protein across the ventricular wall will serve to eradicate arrhythmogenic repolarization heterogeneities.
REFERENCES


BIBLIOGRAPHY

Ai, X, Pogwizd, SM. Connexin 43 downregulation and dephosphorylation in nonischemic heart failure is associated with enhanced colocalized protein phosphatase type 2A. Circ Res 96:54-63.


Antzelevitch, C. and Fish, J. Electrical heterogeneity within the ventricular wall. Basic Res.Cardiol. 96, 517-527. 2001.


Girouard, SD, Laurita, KR, Rosenbaum, DS. Unique characteristics of optically recorded action potentials. SPIE 2132:347-357.


Guerrero, PA, Schuessler, RB, Davis, LM, Beyer, EC, Johnson, CM, Yamada, KA, Saffitz, JE. Slow ventricular conduction in mice heterozygous for a connexin43 null


Johnson, CM, Green, KG, Kanter, EM, Bou-Abboud, E, Saffitz, JE, Yamada, KA.


Kizana, E, Chang, CY, Cingolani, E, Ramirez-Correa, GA, Sekar, RB, Abraham, MR,


Liu, D-W, Antzelevitch, C. Characteristics of the delayed rectifier current (I_{Ks} and I_{Ks}) in canine ventricular epicardial, midmyocardial, and endocardial myocytes: A weaker I_{Ks} contributes to the longer action potential of the M cell. Circ.Res. 76:351-365.


Lowe, JE, Reimer, KA, Jennings, RB. Experimental infarct size as a function of the amount of myocardium at risk. Am J Pathol 90:363-379.


Marx, SO, Marks, AR. Regulation of the ryanodine receptor in heart failure. Basic Res Cardiol 97 Suppl 1:149-51.


Miller, AD, Jolly, DJ, Friedmann, T, Verma, IM. A transmissible retrovirus expressing human hypoxanthine phosphoribosyltransferase (HPRT): gene transfer into cells obtained from humans deficient in HPRT. Proc Natl Acad Sci U S A 80:4709-4713.


Oxford, EM, Everitt, M, Coombs, W, Fox, PR, Kraus, M, Gelzer, AR, Saffitz, J, Taffet,


Peters, NS, Coromilas, J, Severs, NJ, Wit, AL. Disturbed connexin43 gap junction distribution correlates with the location of reentrant circuits in the epicardial border zone of healing canine infarcts that cause ventricular tachycardia. Circulation 95:988-996.

Peters, NS, Green, CR, Poole-Wilson, PA, Severs, NJ. Cardiac arrhythmogenesis and the gap junction. J.Mol.Cell.Cardiol. 27:37-44.


Pu, JL, Boyden, PA. Alterations of Na\(^+\) currents in myocytes from epicardial border zone of the infarcted heart - A possible ionic mechanism for reduced excitability and postrepolarization refractoriness. Circ.Res. 81:110-119.


Reimer, KA, Jennings, RB. The "wavefront phenomenon" of myocardial ischemic cell death. II. Transmural progression of necrosis within the framework of ischemic bed size (myocardium at risk) and collateral flow. Lab Invest 40:633-644.


Rudy, Y, Quan, W. A model study of the effects of the discrete cellular structure on electrical propagation in cardiac tissue. Circ.Res. 61:815-823.


Sasano, T, McDonald, AD, Kikuchi, K, Donahue, JK. Molecular ablation of ventricular


Schwinger, RHG, Böhm, M, Schmidt, U, Karczewski, P, Bavendiek, U, Flesch, M, Krause, EG, Erdmann, E. Unchanged protein levels of SERCA II and phospholamban but reduced Ca\(^{2+}\) uptake and Ca\(^{2+}\)-ATPase activity of cardiac sarcoplasmic reticulum from dilated cardiomyopathy patients compared with patients with nonfailing hearts. Circulation 92:3220-3228.


Shaw, RM, Rudy, Y. Ionic mechanisms of propagation in cardiac tissue - Roles of the sodium and L-type calcium currents during reduced excitability and decreased gap junction coupling. Circ.Res. 81:727-741.


Spach, MS, Miller, WT, Dolber, PC, Kootsey, JM, Sommer, JR, Mosher, CE. The functional role of structural complexities in the propagation of depolarization in atrium of the dog: Cardiac conduction disturbances due to discontinuities of effective axial resistivity. Circ.Res. 50:175-191.


Stevenson, WG, Delacretaz, E, Friedman, PL, Ellison, KE. Identification and ablation of macroreentrant ventricular tachycardia with the CARTO electroanatomical mapping system. Pacing Clin.Electrophysiol. 21:1448-1456.


Viswanathan, PC, Shaw, RM, Rudy, Y. Effects of I_{Kr} and I_{Ks} heterogeneity on action potential duration and its rate dependence - A simulation study. Circulation 99:2466-2474.
Voss, F, Opthof, T, Marker, J, Bauer, A, Katus, HA, Becker, R. There is no transmural heterogeneity in an index of action potential duration in the canine left ventricle. Heart Rhythm 6:1028-34.


Wickham, TJ, Carrion, ME, Kovesdi, I. Targeting of adenovirus penton base to new receptors through replacement of its RGD motif with other receptor-specific peptide motifs. Gene Ther 2:750-756.

Wiegerineck, RF, Verkerk, AO, Belterman, CN, van Veen, TA, Baartscheer, A, Opthof, T, Wilders, R, de Bakker, JM, Coronel, R. Larger cell size in rabbits with heart failure increases myocardial conduction velocity and QRS duration. Circulation 113:806-813.


