MODELING ATRIAL FIBRILLATION USING
HUMAN EMBRYONIC STEM CELLS

Zachary Laksman

A thesis submitted in conformity with the requirements for the
Degree of Masters of Science
Institute of Medical Sciences
University of Toronto

© Copyright by « Zachary Laksman » « 2015»
Abstract

Thesis Title: Modeling Atrial Fibrillation Using Human Embryonic Stem Cells

Degree: Masters of Science, Institute of Medical Sciences, University of Toronto

Convocation: November 2015

Background: Atrial fibrillation (AF) is the most common clinical arrhythmia and is associated with significant morbidity and mortality.

Hypothesis: “Atrial” cardiomyocytes generated from hESCs will recapitulate the key hallmarks of human AF in a 2 dimensional tissue model, as well as demonstrate the expected electrophysiologic changes in response to drugs.

Methods and Results: Our “atrial” cardiomyocytes were 80-90% positive for cardiac troponin, were enriched for the atrial markers ANF and KCNJ3, and lacked ventricular markers (MYL2 and IRX4). The “atrial” cells mimicked the human electrophysiologic phenotype with the majority (90%) demonstrating atrial like action potentials. Optical mapping of multicellular cell sheets was performed on rotors at baseline, after induction of rotor formation and with the addition of commonly employed antiarrhythmic drugs.

Conclusions: We have successfully generated a human model of AF in vitro and demonstrated its utility in screening for known, and unpredicted effects of commonly employed anti-arrhythmics.
Acknowledgements

I would like to thank Dr. Peter Backx and Dr. Gordon Keller for their support and mentorship. It has been a great privilege to work with scientists of the highest caliber, and people who have earned my greatest esteem and respect.

I would like to thank my beautiful daughters Kaiya, Nava, Arielle, and Samara.

Thank you to all my family and friends for their help and understanding.

Finally, thanks to my wife Jodi who remains the strongest person I have ever met. Thank you for continuing to believe in me and support me unconditionally.

I would like to dedicate this thesis to my late father Sander Laksman.
Technical Contributions and Acknowledgement

I acknowledge the following individuals for their contributions to my MSc project:

Stephanie Protze, PhD. and Jeehoon Lee, PhD candidate
Dr. Protze and Jeehoon’s work in the development and validation of the differentiation protocol used to derive the “atrial” cells under study laid the foundation upon which this project was based. They performed the qRT-PCR and aldefluor stains reported in this thesis as a part of these efforts.

Wallace Yang
Worked tirelessly to optimize and improve the optical mapping rig and its requisite signal processing.

Dr. Mark Gagliardi, PhD
Provided assistance and expert consultation in all aspects of tissue culture and directed differentiation of hPSCs

Dr. Roozbeh Aschar-Sobbi, PhD
Provided expert consultation on nearly all aspects of experimental cardiac electrophysiology

Farzad Izaddoustdar
Assisted in signal processing and technical aspects related to optical mapping
Table of Contents

Abstract ii
Acknowledgements iii
Technical contributions and acknowledgements iv
List of abbreviations vii
List of Tables viii
List of Figures ix

Chapter 1 Background 1
1.1 Atrial fibrillation (AF) – clinical burden 1
1.2 Normal cardiac physiology 1
1.3 AF management 3
1.3.1 Rate control vs. rhythm control of AF 3
1.3.2 AF ablation 5
1.4 Basic electrical properties and mechanisms underlying AF 7
1.5 Anti-arrhythmic drugs: dofetilide and flecainide 11
1.6 Wave theory 12
1.7 Rotors 13
1.7.1 Physiology of rotors 14
1.7.2 Clinical relevance of rotors in AF 16
1.8 Embryonic stem cells and induced pluripotent stem cells 18
1.8.1 Directed Differentiation 20
1.8.2 Retinoic acid signaling 23
1.8.3 Molecular markers of atrial and ventricular cardiomyocytes 24
1.9 Disease modeling using hPSCs 25
1.9.1 cLQTS 26
1.9.2 acquired LQTS 29

Chapter 2 Research Aims and Hypotheses 33

Chapter 3 Materials and Methods 35
3.1 Differentiation protocols 35
3.2 Flow cytometry and cell sorting 36
3.3 Molecular markers 37
3.3.1 qRT-PCR 37
3.3.2 Immunostaining 37
3.4 Cardiac Electrophysiology 37
3.4.1 Patch clamping 37
3.4.2 Micro Electrode Array 39
3.4.3 Optical mapping 40
3.4.3.1 Signal processing 42
3.4.4 Intracellular recordings of cell sheets 45
Chapter 4 Results
4.1 Differentiation protocols of “atrial” and “ventricular” cardiomyocytes 46
4.2 Molecular markers of “atrial” and “ventricular” cells 51
4.3 Single cell electrophysiology studies of “atrial” and “ventricular” cells 53
4.4 MEA studies of “atrial” and “ventricular” cell sheets 57
4.5 Optical mapping studies of “atrial” and “ventricular” cell sheets 59
4.5.1 Optical mapping the effects of antiarrhythmic drugs: flecainide and dofetilide 65

Chapter 5 Discussion
5.1 Molecular markers of “atrial” and “ventricular” cardiomyocytes 73
5.2 Single cell electrophysiology 73
5.3 Modeling atrial disease using hPSCs 75
5.3.1 APD restitution and heterogeneity 78
5.3.2 Conduction velocity 79
5.3.3 Effects of dofetilide on “atrial” cell sheets 80
5.3.4 Effects of flecainide on “atrial” cell sheets 81

Chapter 6 Future Directions
6.1 Validation of current findings 87
6.2 Study AF remodeling 90
6.3 Model other types and features of acquired AF 92
6.4 Introduce genetic variation as a determinant and model of AF 94

Chapter 7 References 99

Chapter 8 Appendix 122
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAD</td>
<td>Anti-arrhythmic drug</td>
</tr>
<tr>
<td>AF</td>
<td>Atrial fibrillation</td>
</tr>
<tr>
<td>ANF</td>
<td>Atrial natriuretic factor</td>
</tr>
<tr>
<td>AP</td>
<td>Action potential</td>
</tr>
<tr>
<td>APA</td>
<td>Action potential amplitude</td>
</tr>
<tr>
<td>APD</td>
<td>Action potential duration</td>
</tr>
<tr>
<td>APD50</td>
<td>Action potential duration at 50% of repolarization</td>
</tr>
<tr>
<td>APD90</td>
<td>Action potential duration at 90% of repolarization</td>
</tr>
<tr>
<td>Cav1.3</td>
<td>Alpha 1D subunit of the L-type voltage dependent calcium channel</td>
</tr>
<tr>
<td>CM</td>
<td>Cardiomyocyte</td>
</tr>
<tr>
<td>CV</td>
<td>Conduction velocity</td>
</tr>
<tr>
<td>Cx</td>
<td>Connexin</td>
</tr>
<tr>
<td>DMP</td>
<td>Diastolic membrane potential</td>
</tr>
<tr>
<td>dv/dt\text{max}</td>
<td>Maximum action potential upstroke velocity</td>
</tr>
<tr>
<td>EAD</td>
<td>Early after depolarizations</td>
</tr>
<tr>
<td>EB</td>
<td>Embryoid body</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
</tr>
<tr>
<td>ERP</td>
<td>Effective refractory period</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic stem cell</td>
</tr>
<tr>
<td>FDA</td>
<td>U.S. Food and Drug Administration</td>
</tr>
<tr>
<td>FFT</td>
<td>Fast fourier transform</td>
</tr>
<tr>
<td>GJ</td>
<td>Gap junction</td>
</tr>
<tr>
<td>hPSC</td>
<td>Human pluripotent stem cell</td>
</tr>
<tr>
<td>ICD</td>
<td>Implantable cardioverter defibrillator</td>
</tr>
<tr>
<td>iPSC</td>
<td>Induced pluripotent stem cell</td>
</tr>
<tr>
<td>KCNJ3</td>
<td>Potassium inward rectifying channel, subfamily J, member 3</td>
</tr>
<tr>
<td>LQTS</td>
<td>Long QT syndrome</td>
</tr>
<tr>
<td>MEA</td>
<td>Micro electrode array</td>
</tr>
<tr>
<td>MYL2</td>
<td>Myosin regulatory light chain-2</td>
</tr>
<tr>
<td>NCX</td>
<td>Sodium Calcium exchanger</td>
</tr>
<tr>
<td>OAPD</td>
<td>Optical action potential duration</td>
</tr>
<tr>
<td>PS</td>
<td>Phase singularity</td>
</tr>
<tr>
<td>PV</td>
<td>Pulmonary vein</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Real-time quantitative reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoic acid</td>
</tr>
<tr>
<td>RMP</td>
<td>Resting membrane potential</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>RP</td>
<td>Refractory period</td>
</tr>
<tr>
<td>SAN</td>
<td>Sinoatrial node</td>
</tr>
<tr>
<td>SR</td>
<td>Sinus rhythm</td>
</tr>
<tr>
<td>TDR</td>
<td>Transmural dispersion of repolarization</td>
</tr>
<tr>
<td>TdP</td>
<td>Torsades-de-pointes</td>
</tr>
</tbody>
</table>
List of Tables

Table 1: Characteristics of APs recorded from the protocol generating “atrial” cardiomyocytes compared to the protocol used to generate “ventricular” cardiomyocytes.
List of Figures

Figure 1: Flow cytometric analyses of markers of undifferentiated ES cell
46
Figure 2: Scheme of the protocol used to differentiate hESCs into “atrial” and
“ventricular” cardiomyocytes
47
Figure 3: qRT-PCR results comparing relative expression of ALDH1A2 and
CYP26A1 in low induction (BMP4 3 ng/ml and Activin A 2 ng/ml) compared
to high induction (BMP4 10 ng/ml and Activin A 6 ng/ml)
48
Figure 4: Flow cytometric analyses of low induction vs. high induction with
respect to PDGFRα and Aldefluor staining
49
Figure 5: Flow cytometric analyses of important markers used in the
optimization and validation of the directed differentiation protocol (CD56,
Pdgfr-α, CD90, SIRPα, cTNT).
51
Figure 6: qRT-PCR-based expression analyses of markers of atrial and
ventricular cardiomyocytes in “atrial” and “ventricular” differentiation
protocols
52
Figure 7: Fluorescent immunostaining of cTNT and the ventricular specific
marker MLC2v in “atrial” and “ventricular” differentiation protocols
53
Figure 8: Typical patch recordings of APs generated from “atrial” and
“ventricular” differentiation protocols
54
Figure 9: Showing presence of cardiomyocyte types with representative APs
and their prevalence in the “atrial” differentiation protocol
54
Figure 10: Showing presence of cardiomyocyte types with representative
APs and their prevalence in the “ventricular” differentiation protocol
55
Figure 11: Comparing the AP characteristics of cells generated from the “atrial”
differentiation protocol compared to the “ventricular” differentiation protocol.
56
Figure 12: Representative patch clamp recordings of an anode break in
“atrial” cardiomyocytes
57
Figure 13: Representative MEA recordings of FPDs from “atrial” and
“ventricular” cell sheets
58
Figure 14: MEA recording demonstrating pacing and capture of a cell sheet
58
Figure 15: Time sequence imaging of an “atrial” cell sheet in “SR”
60
Figure 16: Representative example of typical OAPs generated from an
“atrial” cell sheet
60
Figure 17: On the left, an APD restitution curve of an “atrial” cell sheet
generated by plotting OAPD vs. rate. On the right, a recording of electrical
alternans generated in an “atrial” cell sheet during burst pacing.
61
Figure 18: Time sequence imaging of an “atrial” cell sheet in “AF”
61
Figure 19: APD maps of “SR” and “AF” of “atrial” cell sheets
62
Figure 20: Representative activation maps of “SR” and “AF” of “atrial” cell
sheets
64
Figure 21: Representative conduction velocity maps of “SR” and “AF” of
“atrial” cell sheets
64
Figure 22: Representative examples of OAPDs and the effects of dofetilide
and flecainide on OAP morphology in “atrial” cell sheet
65
Figure 23: Effect of rotor induction and dofetilide on cycle length and APD in “atrial” cell sheets

Figure 24: Effect of rotor induction and dofetilide on conduction velocity in “atrial” cell sheets

Figure 25: Effect of flecainide on cycle length and APD in “atrial” cell sheets

Figure 26: Effect of flecainide on conduction velocity in “atrial” cell sheets

Figure 27: Effect of rotor induction and dofetilide on conduction velocity in “atrial” cell sheets as a function of the distance from the origin of the electrical wavefront

Figure 28: Effect of flecainide on conduction velocity in “atrial” cell sheets as a function of the distance from the origin of the electrical wavefront

Appendix Figure 1: Example of an intracellular recording of a cell sheet using high resistance micropipettes

Appendix Figure 2: Serial activation maps generated after the induction of a rotor in an “atrial” cell sheet demonstrating the initiation of two rotors after the application of flecainide

Appendix Figure 3: Optical APs recorded from hiPSC derived cardiomyocytes expressing Arclight.
Chapter 1
Background

1.1 Atrial Fibrillation – clinical burden

Atrial fibrillation (AF) is the most common clinical arrhythmia, currently affecting 1-2% of the total population\(^1\). AF is the most common arrhythmia necessitating hospital admission, and is a major and growing burden on the health of Canadians and our health care system. Age is an important determinant of AF occurrence with a 25% lifetime risk of developing AF after the age of 40. As the proportion of our populations get older, along with a rise in a set of parallel AF risk factors, the prevalence of AF is expected to double over the next 4 decades\(^2\). A diagnosis of AF is associated with a doubling in all-cause mortality, a five-fold increase in stroke, an accelerated development of heart failure, and a substantially poorer quality of life\(^3,4\). In addition to the costs of identifying and treating cardiac risk factors leading to AF\(^5-8\), costs directly attributed to AF exceeded $20,000/year/patient in 2010\(^9\). Total costs are expected to accelerate with more widespread use of expensive, and invasive, AF ablation ($20k/procedure)\(^10\), which can be seen as palliation rather than a cure, touting success rates of less than 60% after 1 year even in highly selected patient populations\(^11\).

1.2 Normal Cardiac Physiology

To begin to discuss the pathophysiology that predisposes patients to AF, we must first review normal cardiac physiology, and the link between its conduction system and force generating contraction. While all myocytes within the heart have the capacity to conduct electrical impulses through gap junctions, the heart’s pump function under normal conditions is tightly regulated by a specialized conduction system. When an electrical
impulse (action potential) stimulates myocardium it contracts. This process, which is fundamental to all muscle physiology, is known as excitation-contraction coupling. The conduction system of the atria and ventricles are designed to undergo regular and orderly depolarization/repolarization sequences in order to maximize cardiac efficiency while minimizing arrhythmias. The sinoatrial node (SAN) acts as the primary pacemaker of the heart, depolarizing spontaneously to determine and drive the rate of cardiac contraction. This normal physiologic rhythm is called sinus rhythm (SR). The electrical wavefront generated by the SAN first initiates the coordinated contraction of the right and left atria. Atrial contraction ejects blood in the ventricles, the bottom chambers of the heart, through the atrioventricular valves, contributing as much as 30% towards the final cardiac output ultimately ejected by the ventricles through the semilunar valves. After atrial depolarization, the electrical wavefront then invades the atrioventricular node, which serves as a gateway to the specialized conduction system of the ventricles called the His-Purkinje system. When AF is present, atria no longer beat regularly (i.e. atria "fibrillate"). AF is easily detected on electrocardiographic recordings (ECGs) as rapid, "irregularly-irregular" QRS complexes (from ventricles) along with irregular ("random") baseline signals (from atria)\textsuperscript{12,13}. Within the atria, AF is characterized by the appearance of abnormal electrical re-entry circuits which are self-sustained sequences of depolarization-repolarization cycles called rotors or wavelets\textsuperscript{14-16}. Despite cyclic patterns seen with re-entry, the electrical patterns in AF usually have “random” signatures due to wave-breakup/conduction blocks, thus the term "wavelets" has been proposed\textsuperscript{14}.Crudely speaking, though important for AF discussion, the propensity towards re-entry (rotors/wavelets) depends on an electrical
property called the “atrial wavelength” (i.e. λ) which is the product of the effective refractory period (ERP), which is ~action potential duration (APD), and the conduction velocity (CV), which is the speed of spreading action potentials (APs) in atria. Re-entry however does not occur without initiation by processes called “kindling” or "triggering". Both the substrate of AF, the atrial wavelength, and the triggers of AF are potential targets for therapy.

1.3 AF management

Although atrial beating normally makes relatively minor contributions to the heart's overall pumping efficiency (approximately 15%), AF nevertheless has important clinical consequences. For one, because ventricles are electrically-entrained by the atria via the AV-node, AF leads to elevated pumping rates of the heart (typically >120 beats/min when AF is untreated)\(^\text{14}\) which severely impairs cardiac output regulation, leading to patient fatigue, and promoting “tachycardia induced” cardiomyopathy. The loss of sequential atrial contractions also causes blood pooling within atrial appendages resulting in thrombus formation, embolization and stroke, which is the major cause of morbidity and mortality in AF patients\(^\text{17,18}\). Antithrombotic therapy is thus recommended for a majority of patients. The decision regarding initiation of anticoagulation balances the patient specific risks of bleeding against the treatment and patient specific benefits of antiplatelet and/or anticoagulants \(^\text{19}\). This decision is made independent of the choice regarding the management of the underlying arrhythmia.

1.3.1 Rate control vs. rhythm control

There are two main strategies for the electrical management of AF, rhythm control and rate control. Despite the intuitive attraction of restoring sinus rhythm, several trials have
failed to show clinical benefit of this strategy over rate control. The rate control strategy involves the use of AV nodal blocking agents, such as beta blockers, calcium channel blockers, and digoxin, to slow impulses through the AV node while allowing uninterrupted fibrillation of the atrial chambers. Several studies have shown non-inferiority of rhythm control compared to rate control in terms of survival benefit, while studies targeting a higher risk population of heart failure patients similarly did not show clinical benefit to the rhythm control strategy. Because of this clinical trial data, rhythm control is only indicated for patients who suffer from symptoms of AF, or those who cannot receive adequate rate control and suffer from tachycardia induced cardiomyopathy.

It is worth dissecting some of the trial data that led to our current guideline recommendations further when considering avenues for future research and novel therapeutics. Prior to the AFFIRM trial in 2002, a study which arguably has had the most significant impact on the clinical management of AF, first line therapy was rhythm control with an implicit belief that patients would benefit from fewer symptoms, better exercise tolerance, a lower risk of stroke, eventual discontinuation of long-term anticoagulant therapy and better survival. In this trial of over 4000 patients over the age of 65, and including 25% of patients with decreased left ventricular systolic function, the rhythm control strategy was left to the discretion of the primary physician and included anti-arrhythmic drugs (AADs) associated with increased mortality, particularly in this type of high risk population group. The study determined that there was a trend towards worse survival and increased hospitalizations in patients on a rhythm control compared to a rate control strategy. Further complicating the picture, patients on rhythm control had a
higher propensity towards discontinuing their anti-coagulation. Interestingly, 37.4% of patients in the rhythm control group remained in AF, and 34.6% of patients in the rate control group were in sinus rhythm, clearly demonstrating the ineffectual nature of the era’s anti-arrhythmic strategy, and supporting the concept that this was not truly a comparison of successful rhythm control compared to rate control. Furthermore, crossover between groups was moderately large with 15% of rate control patients and 38% of rhythm control patients crossing over to the other treatment group 21. Later, an “on-treatment” analysis of the AFFIRM trial showed that the presence of sinus rhythm was in fact associated with a lower risk of death 26. Further post-hoc analyses of rhythm vs. rate control strategies have shown a consistent pattern of improvement in quality of life measures in patients who achieve and maintain sinus rhythm 27,28. In the PIAF and HOT CAFE trials for example, 6-minute walk times and maximal treadmill workloads were increased 20,29. In the Canadian Trial of Atrial Fibrillation, quality of life measures were significantly improved at 3 months from baseline in patients who achieved sinus rhythm 30.

1.3.2 AF ablation

From the aforementioned results, two main streams of thought have become pervasive in the field. The first continues to target symptoms with little emphasis on the electrocardiographic documentation of sinus rhythm or AF. The second centers on a belief that to date, there has never been a true comparison of rate vs. rhythm control on “hard” outcomes such as hospitalization, progression of left ventricular dysfunction, or mortality, since we have not had a therapy that can successfully maintain a high proportion of patients in sinus rhythm to act as a reasonable on-treatment group. One of
the hopes for a more effective and durable therapy has been the invasive management of catheter ablation. Remarkably, the first catheter ablation of AF was performed in 1981 by Dr. Scheinman using high energy DC shocks to destroy the AV node as a form of rate control. Since that time, the technology has rapidly evolved to include radiofrequency energy that heats the tip of the catheter to a much higher temperature and in a considerably more controlled fashion. The most significant advancement was subsequently made in 1998 when Dr. Haissaguerre found that pulmonary vein (PV) ectopy, originating from the muscular sleeves that extend from the atrial myocardium, are frequent triggers for AF that can be successfully targeted for ablation. They found that 94% of the ectopic foci responsible for initiating AF originated from the PV in a group of 45 patients. Long term success rates from this procedure were 33% off AADs with an additional 13% of patients being free of AF on AADs. In 2001, it was Dr. Haissaguerre again who described a novel approach to PV isolation that targeted the proximal insertion points of the PVs to the left atrium, thus decreasing the most notable complication of PV ectopy (PV stenosis), and providing benefit to patients who did not have inducible PV ectopy during their electrophysiology study.

Since 2001, incremental improvements in catheter and mapping technology have certainly improved safety and efficacy of the procedure, however the success rates remain suboptimal. The procedure predominantly targets the triggers of AF, and to date, there has been little convincing evidence that ablation can successfully target the underlying substrate. Therefore, the best results from ablation are in those with paroxysmal AF of short duration, absence of cardiac structural disease and comorbidities, and without signs of atrial remodeling, such as dilatation or fibrosis, as seen on MRI using late gadolinium
A meta-analysis of 19 studies that targeted this relatively small population demonstrated a 68% success rate at 1 year and 60% success at maintaining sinus rhythm at 3 years. Despite the steady improvements in catheter ablation techniques, they remain expensive, high risk procedures, which target only patients with lone paroxysmal AF, a relatively small subpopulation of the growing population of patients with AF. Furthermore, while AF invariably begins as isolated short-lived episodes called "paroxysmal AF", each paroxysm accelerates atrial remodelling thereby promoting "persistent AF" (i.e. "AF-begets-AF")

As discussed previously, persistent AF is much less amenable to the current ablation strategies, with abysmal outcomes and a lack of consensus regarding the ability of any ablation strategy to modify the underlying substrate. For these reasons, and the fact that there is growing evidence that maintenance of sinus rhythm is an important consideration of outcome studies, there is renewed interest in the development of AADs for the treatment of AF. An effective drug would not only be applicable to a wider population, but may benefit the population of patients waiting up to 1 year for their AF ablation in decreasing AF related remodelling which has been shown to predict worse outcomes with ablation.

1.4 Basic electrical properties and mechanisms underlying AF

To discuss the future of AAD therapy for AF, we must first revisit the concepts alluded to earlier, namely the basic mechanisms underlying atrial re-entry. As already mentioned, AF generally is believed to require a vulnerable substrate that generally requires a kindling process to initiate semi-random electrical activity featuring underlying "re-entry" events. The fundamental appreciation of the functional determinants of AF are critical
for understanding the mechanism of action of current AADs. Current dogma proposes that the generation (triggers) and maintenance of continuous or fibrillatory activity in the atria depends on the underlying properties of the atrial (and related regions like the pulmonary veins) substrate and the balance between the determinants of refractoriness and excitability, or the imbalance thereof. As discussed earlier, the propensity towards re-entry in the atria depends on the atrial wavelength (i.e. \( \lambda \)) which is the product of the effective refractory period (ERP), which is \( \sim \) action potential duration (APD), and the conduction velocity (CV), which is the speed of spreading APs in atria (\( \lambda = \text{ERP} \times \text{CV} \)). If \( \lambda \) is small relative to the physical dimensions of atria\(^{44} \) then re-entry circuits are more likely, and hypothetically the atria could accommodate a larger number of simultaneous reentry circuits.

The action potential, a key component of the atrial wavelength, constitutes changes in the membrane potential of cardiomyocytes. The membrane potential is established by an unequal distribution of electrically charged ions across the sarcolemma and predominantly by the presence of conducting ion channels in the sarcolemma. Opening and closing of the ion channels allows ionic currents to flow across the membrane culminating in the generation of the action potential. The direction of current depends on the electrochemical gradient of the corresponding ions, and the conductivity of channels that carry the current alters at different membrane potentials.

The atrial action potential is comprised of 5 phases. Depolarization (phase 0), a short-lived hyperpolarization (phase 1), the plateau (phase 2), and repolarization (phase 3) to resting potentials (phase 4). Activation (phase 0) is primarily driven by the depolarizing inward Na+ current (\( I_{Na} \)). Upon reaching threshold, the rapid influx of Na+ through
voltage-gated sodium channels causes further rapid depolarization of the membrane (typically to \(+30\) to \(+40\) mV), which in turn activates the transient outward potassium current (\(I_{\text{to}}\)), causing very rapid, but short-lived repolarization of the membrane potential (phase 1). Activation of L-type Ca\(^{2+}\) current (\(I_{\text{CaL}}\)) follows with simultaneous activation of several repolarizing voltage-gated K\(^{+}\) channels (\(I_{\text{K}}\)). The plateau phase (phase 2) of the action potential and therefore the action potential duration (APD) is governed by a delicate balance of \(I_{\text{CaL}}\) and \(I_{\text{K}}\), with membrane repolarization being driven ultimately by inactivation of L-type Ca\(^{2+}\) channels and progressive activation of \(I_{\text{K}}\) (phase 3). The resting potential (typically at \(-70\) to \(-80\) mV) is maintained by the inward rectifier current \(I_{\text{K1}}\) and Ca\(^{2+}\) extrusion is carried out primarily by the electrogenic Na\(^{2+}\)/Ca\(^{2+}\) exchanger (NCX) current (\(I_{\text{NCX}}\)). NCX exchanges 3Na\(^{2+}\) ions for each Ca\(^{2+}\) ion with one net positive charge moving in the direction of sodium transport. Ion channels that are enriched in atrial cardiomyocytes compared to other types of cardiomyocytes contribute to the atrial specific action potential morphology. In contrast to atrial and ventricular myocytes, SAN and AVN myocytes demonstrate slow depolarization of the resting potential during phase 4, a property related to the relative absence of \(I_{\text{K1}}\) allowing inward currents (predominantly \(I_{\text{f}}\)) to depolarize the membrane potential. Slow depolarization during phase 4 inactivates most sodium channels and thus depolarization is mainly achieved by \(I_{\text{CaL}}\) and T-type Ca\(^{2+}\) currents. \(I_{\text{Na}}\) reductions and increases in several K\(^{+}\) currents are linked to AF. APD heterogeneity can be altered by either pacing or vagal stimulation leading to regional differences in muscarinic-activated K\(^{+}\) currents, \(I_{\text{K,Ach}}\). It is important to note that changes in resting membrane potentials, which
are determined by "background" $K^+$ currents (i.e. $I_{K1}$ and $I_{K,Ach}$) can also contribute to AF by modulating $I_{Na}$ inactivation and availability.

Conduction Velocity is a measure of the spread of depolarization between electrically coupled regions within the atria and is determined primarily by sodium current ($I_{Na}$) densities/kinetics and gap junction (GJ) channel properties. In atria, $I_{Na}$ is largely generated by Nav1.5 pore-forming $\alpha$-subunits, coassembled with various $\beta$-subunits ($Nav\beta$) and other proteins. GJs are clusters of closely packed channels that directly connect the cytoplasmic compartments of adjacent cells allowing the passage of ions and small molecules. Each of the neighbouring cardiomyocytes contributes 1 connexon, of which 21 members have been identified. In the atria, GJ channels are formed by both Connexin40 (Cx-40) and Connexin-43 (Cx-43) proteins which co-localize to a considerable extent. Cx-40 is specific for the atria, and important for atrial conduction. Targeted deletion of Cx-40 in mice generates a phenotype of diminished atrial conduction velocity (up to 30%) Somatic mutations in the Cx-40 gene (GJA5) in humans is associated with a heritable form of AF, thought to be related to reduced intercellular electrical coupling. Taken together, changes in the expression (reductions), distribution and phosphorylation (reductions) of GJ proteins and $I_{Na}$ are associated with AF. Note the contributors to RP and CV are chamber specific and reflect the concert of ion channels and gap junctions specific to the chamber of interest.

Since shorter wavelengths promote AF induction and maintenance, many anti-arrhythmics target RP and thus wavelength prolongation in order to suppress AF. Alternatively, and less important for this discussion, AADs can act by suppressing AF.
triggers. For the purpose of this study, we will discuss two anti-arrhythmics, dofetilide and flecainide, in further depth.

1.5 Anti-arrhythmic drugs: dofetilide and flecainide

Dofetilide is a class III anti-arrhythmic which selectively inhibits the rapid component of the time-dependent outward potassium current ($I_{kr}$) $^{75}$. Studies in dogs demonstrated prolongation of the ERP and APD in a dose dependent manner, as well as dofetilide’s ability to facilitate conversion of electrically induced fibrillation $^{76,77}$. Dofetilide has no effect on the maximum rate of depolarization and does not influence conduction within the His-Purkinje system or within the myocardium$^{75}$. Data from placebo controlled trials demonstrated the efficacy of dofetilide $^{78}$ with a 30% conversion rate to sinus rhythm, and a 60% success rate at maintaining sinus rhythm out to 1 year, compared to 20% in the placebo group. In patients with structural heart disease, dofetilide had no effect on mortality, and decreased the risk of hospitalization and progression of congestive heart failure $^{79,80}$. As predicted however, since $I_{kr}$ is an important contributor to the repolarization of ventricular cells, many patients were exposed to the pro-arrhythmic nature of ventricular APD prolongation, which can lead to QT prolongation and put patients at risk for torsades-de–pointes. In clinical trials, the incidence of torsades was as high as 5% leading to a significant decrease in the clinical uptake of the drug, as well as explicit warnings and regulations regarding its use $^{81,82}$.

Flecainide is a class Ic antiarrhythmic agent, classically considered to slow CV with little effect on ERP $^{83,84}$ This in itself would not be predicted to have favourable effects on AF as it would decrease wavelength and increase AF susceptibility. Flecainide however has been shown in dog models to increase the atrial refractory period in a rate dependent
fashion thus increasing wavelength at high rates \(^{58}\). This has been attributed to the drug’s effect on decreasing atrial APD accommodation during periods of increased heart rate, possibly related to an effect on repolarizing potassium current, as well as decreasing the heterogeneity of atrial activation \(^{85}\). Flecainide has been shown in placebo controlled trials to decrease AF recurrence once converted to sinus rhythm \(^{86,87}\). Flecainide has known pro-arrhythmic properties and has been associated with increased mortality in patients with a history of ischemic heart disease, and left ventricular dysfunction \(^{88-90}\). Increased inducibility of ventricular arrhythmias in animal models of infarction and ventricular dysfunction have been reproducibly observed \(^{91}\). This has been attributed in part to paradoxical amplification of flecainide induced conduction slowing in depolarized tissue producing dispersion of conduction \(^{91}\).

1.6 Wave theory

To this point, the discussion regarding AF substrate has ignored a critical concept in arrhythmia induction and perpetuation by presuming that there is an anatomic obstacle at the center of our reentrant circuit. To fill in this gap in our developing theorem of AF, once again historical context will shed light on the evolving concepts. In fact, studies of fibrillation and its mechanisms date back to the turn of the 20\(^{\text{th}}\) century when Mayer demonstrated sustainable circulatory activity in isolated rings of the contractile bell of the rhyzostomous Scyphomedusa (jellyfish) and turtle ventricular muscle \(^{92}\). By applying electrical pulses at one end of the ring, Mayer induced activation wavefronts that circulated uni-directionally around the ring. This was reproduced by Mines and Garrey and then described as the circus movement hypothesis of reentry by Lewis in which he described a reentrant arrhythmia as a wavefront that circulated back to its partially
Wave theory continued to develop in the 1960s and 1970s, driven predominantly by technological innovations such as high-speed cameras and the application of computer or numerical modeling. In 1973 Alessie experimentally modeled reentry in the absence of an anatomical obstruction thus introducing the concept of reentry around a functional obstacle or a rotor. Using microelectrode recordings in an isolated dog atrial preparation, they demonstrated sustained reentry in healthy muscle. This led to the leading circle model of functional reentry which relied on a center of refractoriness around which the rotating wave circulates, maintained in its state of refractoriness through ongoing bombardment of centripetal wave fronts. In contrast to anatomical reentry, the leading circle hypothesis proposes that there is no fully excitable gap as the circulating wavefront must encroach on its own tail.

1.7 Rotors

Interestingly, circulating wavefronts are not unique to cardiac tissue. The concept of rotors underpinning cardiac fibrillation stems from the field of nonequilibrium thermodynamics. The discovery of the initial phenomenon birthing this field is attributed to Boris Belousov who observed in the 1950s oscillations of colours in a colourless solution of potassium bromate, cerium sulfate, malonic acid and citric acid in sulfuric acid. Later, Anatoly Zhabotinsky rediscovered this reaction sequence and ultimately disseminated the concept widely at a conference in 1968. The oscillatory patterns observed in the “BZ” reaction are seen across many natural phenomena including the growth pattern of amoeba.
1.7.1 Physiology of rotors

A rotor is similar to the leading edge model of functional reentry with a critical difference, the curved wavefront must meet with its wavetail at a singularity where the tissue is not refractory. Rotors are hypothesized to be the drivers of cardiac fibrillation and can be represented in 2-dimensional (2D) space as spiral waves or in 3-D space as scroll waves. The wavefront represents cells that have undergone full excitation (depolarization) and are returning to their rest state (repolarization). Gray et al demonstrated a phase singularity (PS) using optical mapping experiments of fibrillation in 1998, and generated phase maps which could track the spiral, its curvature, and its tip over time and space. This was a dramatic step forward in the study of rotor dynamics allowing its visualization and quantification. They demonstrated that the PS anchored the rotor, while the spiral wave rotated around it. A rotor however, unlike the leading circle model, allows for changes in the location of the PS dependent on subtle changes in the rotor’s environment, or chaotic unpredictable behavior generating complex shapes.

Principal to the rotor theory is the curvature of the rotating wavefront which controls the velocity of the impulse and the dynamics of the reentrant wavefront. The leading front is curved, in contrast to the leading circle model of reentry, which gives the activation a spiral shape. An important concept to consider is the source/sink dynamics of a propagating wavefront. For excitation to occur, a depolarizing wave (the source) must carry sufficient current to bring membrane potentials to threshold for firing (activating voltage-gated sodium channels) in electrically vulnerable “downstream cells”, which act as a sink. The geometry of the wavefront influences the source/sink ratio. Compared to a planar wavefront, the leading edge of a convex wavefront will have
relatively few cells driving depolarization of many cells, shifting the balance to a greater sink than source current. This results in reduced rate of voltage rise ahead of the wavefront, increasing the time to sodium channel activation, effectively slowing CV. The reverse is true in concave wavefronts, where CV is higher than that of a planar wavefront. The curvature of the spiral wave is proposed to increase progressively towards the center, where at the tip, the curvature achieves a critical value whereby the core becomes inaccessible to excitatory activity. This steep wavefront slows the conduction velocity to a critical level thus forming the PS. This allows for the meandering nature of rotors, as the core is unexcited, but is not required to be refractory and therefore can subsequently be excited\(^\text{103}\). Finally, the rotor is not dependent on a fixed wavelength, as is required by anatomic reentry and the leading circle model. In fact, electrotonus is predicted to shorten the action potentials of cells near the core\(^\text{104}\). This final point is of particular importance given the previous discussion of the effect of antiarrhythmics on wavelength.

In the rotor model of fibrillation, wavelength is variable and dynamic, and thus medications are predicted to have variable effects along the curvature of the wavefront as the complement of currents at play will vary depending on the location.

The initiation of a rotor relies on wavebreak after the interaction of an electrical impulse or wavefront with an obstacle\(^\text{105}\). This process can occur in a homogeneous medium when transient heterogeneity is introduced into the system. Classically this is performed with an S1-S2 protocol where a first wave (S1) is followed by a second wave (S2) oriented perpendicularly to S1\(^\text{106}\). If S2 hits S1 before it has completely repolarized, S1 acts as a barrier to S2 propagation resulting in a rotating spiral wave. Alternatively, at high stimulation rates, electrical and or calcium alternans can be induced where action
potential duration and or the amplitude of calcium transients demonstrate beat-to-beat variation despite a constant stimulation frequency\textsuperscript{107}.

Alternans is a risk factor for cardiac arrhythmias including atrial fibrillation, and in some cases can act as a prognostic tool for arrhythmia risk stratification and therapy\textsuperscript{108} \textsuperscript{109} \textsuperscript{110}.

In cardiomyocytes, beat-to-beat regulation of the membrane potential (Vm) and cytosolic calcium are bi-directionally coupled and are dependent on the kinetics of APD restitution. APD restitution refers to the dependence of an action potential on the preceding diastolic interval to influence its duration. At rapid rates, APDs continue to shorten along their restitution curves until they reach a critical point where the curve becomes so steep that self-sustaining oscillations of APDs can occur\textsuperscript{111} \textsuperscript{112} \textsuperscript{113} \textsuperscript{114}. This underlying mechanism has been hypothesized to be related to the time-dependent recovery of ion channels from inactivation\textsuperscript{115}. Similarly, the calcium transient and conduction velocity have restitution kinetics which have been identified as potential contributors to alternans and fibrillation\textsuperscript{116}.

1.7.2 Clinical relevance of rotors in AF

Although not universally accepted, rotor theory has become an important mechanistic explanation for AF as well as a potential therapeutic target. Multiple clinical observations have supported the existence of focal AF drivers in a subset of patients. These include the ability of a single ablation lesion to terminate persistent AF that is resistant to cardioversion\textsuperscript{117}, spatiotemporal stability and localized regions of stable frequencies using dominant frequency mapping\textsuperscript{118} \textsuperscript{119} \textsuperscript{120} and reproducible vectors of AF propagation over time\textsuperscript{121}. The main limitation to developing a mechanistic understanding of rotors in human AF however has been the resolution of current mapping systems, namely their
ability to differentiate the principal components of the signal from the disorganized activation patterns. This has required significant mathematical post-processing to identify areas of spatiotemporal reproducibility.

Recently data from a novel mapping system employing monophasic action potential recordings and a physiologic noise filter has led to a rotor centred ablation strategy. The Focal Impulse and Rotor Modulation (FIRM) mapping system purports to reveal stable electrical rotors in a majority of individuals with AF. Using direct contact gold electrodes attached to 64 pole basket catheters, both the right and the left atria are mapped simultaneously. Electrodes are 4-6 mm apart along each spline and separated by 4-10 mm between splines. The software developed to interpret the tracings advertises its ability to examine patient specific AF electrograms based upon rate-dependent refractoriness, or as previously described, restitution, and conduction slowing. Rotors are identified as PS and are targeted for ablation if they are stable for minutes. The rationale for localized ablation lesions has been based on the theory of ablation of microreentrant focal atrial tachycardias, for which ablation has proven successful.

The initial clinical trial, the CONFIRM trial, demonstrated a dramatic benefit in terms of freedom from AF compared to conventional ablation (82.4% vs. 44.9%, p = 0.001) after a median 273 days. This benefit has been shown to persist over 3-4 years follow up and has been replicated in a multicenter experience recently reported. While there is certainly growing data to support rotors as drivers of AF, the community of clinicians and scientist are still mixed with respect to their belief in the ability of focal ablation lesions to terminate AF and prevent recurrence. The hesitation is centered around the concept that rotors are not stable, and ablation would promote anatomical re-entry and not
necessarily prevent recurrence of functional re-entry. There is however no published literature to date reporting the lack of efficacy using the FIRM technology and software, and thus critical appraisal to date has been only anecdotal. What we have certainly learned from our growing clinical experience mapping rotors is that they can be demonstrated using a variety of technologies and are likely important targets for therapy. A powerful tool for the mechanistic understanding of AF and development of novel targets would incorporate what we have learned about atrial wavelength and its response to medications over the past century, the mathematical and clinical observations pointing to rotors as the major drivers of AF in a model that carries the cell and tissue specific factors already highlighted to be important in AF initiation, propagation and recurrence. Such a tool has only recently become available with the advent and application of human pluripotent stem cell technology. While disease modeling using human pluripotent stem cells would not be a novel proposition, as its application and validation have disseminated rapidly to multiple inherited and acquired diseases in multiple organ systems including the heart, there are several unique challenges to building a model of AF using human pluripotent stem cells. First however, one must review the source of pluripotent stem cells, the nature of directed differentiation, and the applications of this technology to date.

1.8 Embryonic stem cells and induced pluripotent stem cells

Embryonic stem cells (ESCs) cells are derived from totipotent cells of the developing embryo. They are capable of unlimited proliferation while maintaining their undifferentiated state. Human blastocyst-derived pluripotent stem cell lines were first derived in 1998. Cleavage stage embryos produced by in-vitro fertilization were
donated by individuals after informed consent. Fourteen inner cell masses were isolated and 5 ES cell lines derived from 5 separate embryos. These cell lines had normal karyotypes, expressed surface markers that characterize primate embryonic stem cells, and lacked markers defining early lineages. Specifically, the cell surface markers that characterize undifferentiated ES cells including stage-specific embryonic antigen (SSEA)-3, SSEA-4, TRA-1-60, TRA-1-81 were expressed, while the cell lines did not stain for SSEA-1. After 5 months in culture, these cells retained their ability to derive all three embryonic germ layers; endoderm, mesoderm and ectoderm. When injected into severe combined immune-deficient (SCID)-beige mice, each injected mouse formed a teratoma that included gut epithelium (endoderm), cartilage, bone, smooth muscle and striated muscle (mesoderm) and neural epithelium, embryonic ganglia and stratified squamous epithelium (ectoderm). When grown in vitro to confluence, ES cells differentiated spontaneously.

Since the introduction of human stem cell lines, a second source for pluripotent stem cells has arisen. In 2006, Takahashi and Yamanka demonstrated that adult skin fibroblasts in mice can be reprogrammed into ES like cells by using a retroviral vector to force the expression of 4 transcription factors: Oct3/4, Sox2, Klf-4 and c-Myc. Subsequent studies were able to recapitulate these findings using the same 4 transcription factors in human fibroblasts and showed that these cells, human induced pluripotent stem cells (hiPSCs), were capable of self-renewal, could remain undifferentiated in culture, and could give rise to all somatic cell types. The potential to generate an unlimited number of any of the hundreds of cell types in the human body has innumerable applications in the fields of biomedical research and regenerative medicine. The potential however
cannot be actualized without the ability to control and drive the directed differentiation into the cell type required.

1.8.1 Directed differentiation

Three basic differentiation methods have been developed and applied for the differentiation of ESCs; the formation of 3-D aggregates known as embryoid bodies (EBs), the culture of ESCs as cell sheets, and the culture of ESCs directly on stromal layers. While early protocols using fetal calf serum were difficult to reproduce and were generally poorly optimized for the generation of a single cell lineage, several advances including the use of serum-free media with specific inducers, and the development of reporter ESCs to monitor progression through the developmental and differentiation steps have advanced the field. With these new tools, it became possible to approach the problem of directed differentiation through the lens of developmental biology, applying insights from other model systems towards the goal of recapitulating human developmental milestones in vitro. For the purpose of this discussion, the focus will be primarily on the EB culture method of generating cardiomyocytes for disease modeling and regeneration.

Understanding the steps taken by early cardiac progenitors in the developing mouse embryo has assisted in the identification of critical signalling pathways, gene expression profiles and surface markers that direct in vitro differentiation of hESCs. Perhaps the most important event in embryogenesis lies in gastrulation, when uncommitted epiblast cells migrate through a transient structure known as the primitive streak and exit as either mesoderm or definitive endoderm. Brachyury, a T-box transcription factor, is up-regulated as the epiblast cells enter the primitive streak, and then rapidly down regulated
as the newly formed mesodermal cells exit the primitive streak. Specification of distinct subpopulations is controlled both temporally and spatially suggesting different signalling environments responsible for lineage specification. Members of the TGFβ family including BMP4 and Nodal as well as the Wnt family are essential pathways during these developmental steps. These agonists work in concert with regional expression of inhibitors to create domains that are conducive to specifying germ layer induction. Subsequent studies demonstrated that the same signalling pathways can be manipulated in vitro to regulate primitive streak development, setting the stage for germ layer induction.

The early stages of mesoderm induction can be monitored by the up regulation of fetal liver kinase-1 (Flk-1) and PDGF receptors. The induction of early cardiac progenitors requires initially Wnt signalling and then its subsequent inhibition in order to specify the mesoderm, while using various concentrations of BMP4 and activin may generate different subpopulations. Cardiomyocytes are derived from the lateral plate mesoderm and in two waves of development of cells marked by the VEGF receptor Flk-1 and the transcription factor Nkx2.5. Mesodermal cells migrate to the anterior region of the embryo and organize into the cardiac crescent. Ultimately the cardiac crescent fuses to become the primitive heart tube which subsequently undergoes a complicated series of changes, or looping, resulting in heart chamber formation. The differentiation protocols have improved tremendously over the last decade resulting in cardiomyocyte yields of 80-90%.

The study of the electrophysiologic properties of ES derived cardiomyocytes has given us significant insights into their developmental stage, and has become an important
functional assay in the search of differentiating cells towards further cell type specificity. While many aspects of the human pluripotent stem cell (hPSC) derived cardiomyocytes are comparable to adult cardiomyocytes, there are several notable differences. Principally it is believed that these differences are attributable to the immature nature of these cells, as they beat spontaneously, have unorganized sarcomeres, and have a different complement of ion channels compared to adults. In particular, the excitation-contraction machinery appears to be immature with hPSCs lacking clear T-tubules, and displaying disorganized sarcomeric striations and immature Ca$^{2+}$ handling. Time has been shown to be an important aspect of maturation, with cells cultured for extended periods of time acquiring modifications in current densities and properties, increased expression of a “more mature” host of ion channels, and significant improvements in cell morphology. No studies to date however have been able to demonstrate a dramatic impact on the relatively immature AP shape, or the $I_{k1}$ expression felt to be at least in part responsible for the relatively depolarized resting membrane potential compared to adult cardiomyocytes.

We have also learned that standard protocols to generate CMs from hESCs produced mixed populations of atrial-, pacemaker- and ventricular-like cardiomyocytes. The majority of cells derived carry a ventricular like action potential (AP) morphology (60-90%), with a minority of cells exhibiting atrial or nodal like AP morphologies. There has been a substantial amount of evidence, both in vivo and in vitro, implicating retinoic acid (RA) signalling as an important player in atrial specification.
1.8.2 Retinoic acid signaling

RA signalling has been shown to regulate anterior-posterior polarisation of the heart. RA treatment of mouse and chicken embryos leads to oversized atria and smaller or missing ventricles, while the opposite is true for embryos in which RA signalling is inhibited at critical time points. Furthermore, studies of mouse embryonic stem cells indicated that RA signalling promotes the expression of atrial-specific genes. Finally, exogenous treatment of RA has been shown to direct the differentiation of hESCs into atrial like myocytes.

RA is synthesized from vitamin A (retinol) through a chain of 2 oxidative sequences. In the second step, which is thought to be rate limiting, all-trans retinaldehyde is converted to all-trans RA by any of three related aldehyde dehydrogenases, Raldh1, Raldh2, and Raldh3. Previous studies have implicated Raldh2 as the main Raldh involved in early cardiac development. Our group hypothesizes that early cardiac progenitors that express Raldh2 are programmed to synthesize and thus respond to RA, and that atrial precursors and not ventricular precursors would thus express Raldh2. Since RA readily diffuses across cell membranes, ventricular progenitors would have to “protect” themselves from RA being synthesized by neighbouring cells, and could do so by expressing an enzyme that degrades RA. Cyp26a1, a member of the Cyp26 family of cytochrome p450 enzymes, converts RA to metabolites that are not bioactive. Upregulation of Cyp26a1 has been shown to attenuate RA signaling in the prospective rostral spinal cord of zebra fish, thus limiting the downstream signaling involved in the expression of hox genes, and demonstrating its functional role in determining the hindbrain-spinal cord boundary.
The potential therefore exists to drive cardiac differentiation to specific cell fates as would be required for disease modeling of atrial tissue. The validation of the successful generation of a specific cell type will require multiple modalities in order to verify the cell’s molecular and physiologic fingerprint. The tools that are currently relied on most heavily are the quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) targeted analysis of RNA expression, flow cytometry, immunostaining and patch clamping. Important for the purposes of this study will be the ability to differentiate the two major cell lineages of cardiomyocytes in our cultures, atrial like cells and ventricular like cells.

1.8.3 Molecular markers of atrial and ventricular cardiomyocytes

Recently, comparative proteomic and transcriptomic analysis of human fetal atria and ventricular samples has been performed identifying and validating chamber enriched and chamber specific proteins \(^{163}\). Our group has focused on the following atrial enriched markers: atrial natriuretic factor (ANF), a potassium inward rectifying channel (KCNJ3), Connexin-40 (Cx-40), and the alpha 1D subunit of the L-type voltage-dependent calcium channel (Ca\(_V\)1.3). ANF is encoded for by the gene natriuretic peptide A (NPPA) and belongs to a family of natriuretic peptides involved in fluid and electrolyte homeostasis. Mutations in NPPA have been associated with familial AF. KCNJ3 or Kir3.1, associates with other G-protein-activated potassium channels to form a heterotetrameric pore forming complex. These multimeric G-protein gated inwardly rectifying potassium (GIRK) channels are important in atrial electrophysiology and can be activated by muscarinic M2 receptors. Cx-40 is encoded for by the gene GJA5. Connexins form gap junctions in the heart through which charged ions flow between neighbouring cells thus
facilitating action potential propagation. The two most highly expressed connexins in the heart are Cx-40 and Cx43, however Cx-40 is expressed in atrial cardiomyocytes and not ventricular cardiomyocytes, and has been associated with a heritable form of AF. Cav1.3 is highly expressed in atrial compared to ventricular tissue, contributes significantly to the repolarization process in human atria, and has been linked through genome wide association studies to AF in humans\textsuperscript{164}.

In a similar fashion, our group has utilized the absence of ventricular markers myosin regulatory light chain-2 (MYL2) and Iroquois-class homeodomain protein 4 (IRX4) to mark atrial cardiomyocytes and their precursors. Ventricular myosin light chain-2 (MLC-2v) refers to the ventricular form which plays a critical role in embryonic cardiac development and function as well as representing one of the earliest markers of ventricular specification\textsuperscript{165}. IRX4 expression is restricted to the ventricular precursors during the embryonic formation of the linear heart tube, and is absent from both atrial and outflow tract precursors\textsuperscript{166}.

1.9 Disease modeling using hPSCs

The careful dissection and directed differentiation of cardiomyocyte subtypes is a relatively new and important step forward in the field of disease modeling using hPSCs. Prior to this advance, investigators have generated predominantly ventricular like cells and thus focused on diseases of the ventricles. The first patient specific disease model to be studied in depth was the long QT syndrome in 2010 when hiPSC derived cardiomyocytes were clearly shown to have the capacity to recapitulate the clinical disease in a dish. Since then there has been an explosion of interest and innovation around this novel technology\textsuperscript{167} and its application in the study of innumerable cardiac
diseases from inherited arrhythmia syndromes to cardiomyopathies\textsuperscript{168-170}. Disease
modeling with hPSCs has become an important part of the armamentarium for gene
discovery, its subsequent functional analysis, and drug testing. For the purpose of this
discussion, the lessons learned in modeling congenital and acquired long QT syndrome
will be reviewed in further detail, as they will act as the building blocks upon which this
thesis was designed and carried out.

\subsection{1.9.1 cLQTS}

The congenital long QT syndrome (cLQTS) is a life threatening disease that represents a
leading cause of sudden cardiac death in the young\textsuperscript{171}. The electrocardiographic features
of the disease are QTc prolongation and T wave abnormalities at rest, and failure of the
QTc to shorten with exercise and epinephrine\textsuperscript{172}. The QT interval represents the
depolarization and repolarization phases of the cardiac action potential, as previously
reviewed. Decreases in repolarizing outward potassium currents or increases in
depolarizing inward sodium or calcium currents can lead to prolongation of the QT
interval, and not surprisingly then, mutations in genes encoding ion channels have been
identified as the most common pathogenic variants. Approximately 1 in 2500 healthy
live births will have an abnormally long QT interval and have congenital LQTS,
transmitted via an autosomal dominant inheritance pattern\textsuperscript{171}.
Beta-blocker therapy is the primary treatment for most patients with LQTS and offers
substantial protection from fatal cardiac events\textsuperscript{173-176}. Patients who are intolerant or
refractory to beta-blockers can be offered left cardiac sympathetic denervation\textsuperscript{177}.
Patients who have cardiac events while on beta-blockers, who have suffered a cardiac
arrest, or who are deemed sufficiently high risk, can be offered an implantable
cardioverter defibrillator (ICD)\textsuperscript{178-181}. ICD therapy however has lifelong implications, and complications are common especially in young patients who may have the device for >20 years. In a recent review of an academic tertiary center’s outcomes for primary prevention ICDs, 35% of patients had ICD related morbidity, and none of their LQTS patients received appropriate shocks for LQTS related arrhythmias \textsuperscript{182}.

The majority of efforts at improving risk stratification in patients with LQTS have focused on our rapidly improving understanding of genetics. Consequently LQTS has become one of the best-understood and characterized monogenic genetic diseases, serving as a model for the investigation of genotype-phenotype interactions. This mechanistic basis and understanding of disease has afforded clinicians an improved patient specific management strategy. This has also given researchers a unique depth to the understanding of a monogenic disease that often carries a clear, distinct, and easily measurable phenotype. Mutations in ion channel genes causing QT interval prolongation do so by prolonging the action potential duration. Delayed repolarization can facilitate early after depolarizations (EADs), a process that has been proposed to relate to L-type calcium channel reactivation, and is dependent not only on time but also on membrane potential \textsuperscript{183 184}. It is however widely accepted that torsades-de-pointes (TdP) is triggered by EADs under conditions of a prolonged QT interval \textsuperscript{185}. Drugs that further prolong the APD can aggravate the underlying QT prolongation and put patients at risk for sudden cardiac death.

The first inherited cardiac disorder to be modeled was a patient with type-2 LQTS due to a missense mutation in the KCNH2 gene affecting the pore-forming region of the HERG channel \textsuperscript{186}. This mutation leads to a significant reduction of the rapid component of the
delayed rectifier potassium current ($I_{kr}$). After an iPS line was generated and differentiated into beating cardiomyocytes, intracellular recordings revealed APD prolongation compared to control cells as well as a significant reduction in $I_{kr}$ current. A significant proportion (66%) of LQTS iPSC derived cardiomyocytes displayed EADs, whereas control cells did not. The additional stress of specific $I_{kr}$ blockers prolonged the APDs further and incited an increased number and complexity of EADs. Application of potential therapeutic agents including calcium channel blockers and pinacidil, a KATP-channel opener, resulted in APD abbreviation and elimination of EADs.

The observations in this iPS model of LQT2 are striking, however the implications are limited by the investigation of a tissue based arrhythmia at a single cell level. Under normal physiologic conditions, there is dispersion across the ventricles during repolarization\textsuperscript{187}. While the cellular basis for this continues to be debated, one theory that has gained popularity relies on the gradient of cell types across the myocardial wall. From epicardium to myocardium, and possibly M cells in between, ventricular cardiomyocytes differ in terms of their repolarization properties. The ionic determinants of the differing properties of these cell types has also been proposed to underlie the enhanced transmural dispersion of repolarization (TDR) under conditions of prolonged QT interval that can predispose patients to TdP and sudden cardiac death. An increase in TDR may be essential for the development of TdP, serving as a functional reentrant substrate for its maintenance, as well as critically facilitating the propagation of an EAD to generate the first initiating beat\textsuperscript{188,189}. In the absence of TDR in canine and rabbit ventricular wedge preparation models, TdP does not develop in the setting of QT prolongation despite frequent phase 2 EADs. Further evidence dissecting the surrogate
outcome of QT prolongation and EADs from the clinically relevant outcome of TdP is the observation that the incidence of TdP is not proportional to the extent of QT prolongation in drug-induced or acquired LQTS. Cardiologists have long since learned the perils of treatment paradigms based on surrogate outcomes. Perhaps the most notable lesson has come from the Cardiac Arrhythmia Suppression Trial (CAST), designed on the premise that suppression of ventricular ectopy after a myocardial infarction reduces the incidence of sudden cardiac death. Based on a well documented observation that ventricular ectopy was associated with sudden death after myocardial infarction, antiarrhythmics were commonly prescribed to suppress them until the CAST trial definitively showed that this management strategy was associated with increased mortality. Despite the clear limitations of single cell physiology studies and surrogate outcomes, we have gained significant insights into the utility of hPSC technology for disease modeling and drug screening from recent studies of congenital and acquired LQTS.

1.9.2 acquired LQTS

New drug development suffers from low phase II clinical success rates, and a daunting price tag of 1.8 billion dollars and 12 years of development, testing, and regulatory processes spanning the time from discovery to commercial launch. The need for new technology is highlighted further by the fact that the attrition rate of new therapeutics for any disease condition is 89% after preclinical animal testing, with 1/3 being ineffective and an additional 1/3 having safety issues, with cardiac toxicity being the major factor in this attrition. Much of this attrition rate has been attributed to the poor predictive power of animal models. Several high profile drug withdrawals have led to mandatory
pre-clinical screening policies to detect compounds which may prolong the QT interval and predispose patients to TdP \(^{194}\). So called acquired QTc prolongation is much more common than congenital and typically ascribed to drugs that incidentally block the I\(_{Kr}\) channel, but can exert this effect through any number of mechanisms, and thus prolong the ventricular action potential duration \(^{195,196}\). Current FDA mandated methods rely on cell lines artificially expressing single cardiac ion channels, or whole animal heart and purkinje assays \(^{197}\). These screens have obvious and important limitations, with poor sensitivity and specificity profiles for human translation, and a host of untestable electrophysiologic toxicities that are not considered \(^{194}\). Pluripotent stem cell assays have been shown to act as efficient, reproducible screens for the efficacy of new compounds as well as the safety profile of compounds in development \(^{198}\). With the advent and explosion of genetic engineering techniques, hPSCs carry the unique privilege of being the only human model system that can incorporate the breadth of genetic diversity of patient populations, while also holding the promise to personalize medical therapy and risk stratification at the level of a single patient.

The first major effort towards the development of hPSC based drug screening was performed at the single cell level in hiPSCs derived from healthy subjects and patients considered to be at higher risk of developing drug induced QT prolongation. The high risk population drew from cohorts of patients with congenital long QT syndrome, familial hypertrophic cardiomyopathy and familial dilated cardiomyopathy \(^{197}\). Cardiomyocytes generated from high risk hiPSCs demonstrated increased susceptibility to drug induced APD prolongation, early and delayed after depolarizations compared to control. Subsequently, investigators from the same group generated a library of genome edited...
iPSC derived cardiomyocytes, with mutations again representing the highest risk patient populations, and compared the effects of drugs to isogenic controls. The application of genetically modified human cell lines demonstrated clear utility in efficient high throughput screens for small molecules and chemical compound testing. Of course, the same limitation ascribed to the LQT2 model previously described applied to single cell drug screening assays, screening for a surrogate markers (EADs) of the actual feared clinical outcome (TdP).

The atria and the ventricles share the same fundamental electrical properties at a single cell level that impact the action potential profile, and at a tissue level when considering electrical wave propagation and repolarization. Accordingly similar principles apply to understanding arrhythmias in the two chamber types, specifically, the determinants of wavelength, and the clinical patterns of arrhythmogenesis including fibrillation involving reentry, conduction block, and spiral wave formation (i.e. rotors). However, there are key differences in the physiology and pathophysiology of atria and ventricles, principally their electrophysiologic signatures and their cell to cell communication, that can be exploited for therapy, and tested for safety. Unfortunately, our understanding of the mechanisms of atrial arrhythmias remains dismal and treatment approaches are grossly ineffective and unsafe. One of the major barriers in the pipeline of drug development has been the lack of appropriate models. The challenges of interspecies differences in receptor subtypes, distribution and signaling are compounded by significant differences in the ion channels and gap junctions that work in concert to dictate the physiology and pathophysiology involved in human AF. The major objective of our studies is to develop a novel platform, using human pluripotent stem cells (hPSCs), to
study the pathophysiology of AF in order to identify novel targets for therapy and as a platform to study the mechanisms required for proper treatment and prevention of AF.
Chapter 2

Hypotheses and Research Aims

This study proposed to generate an in vitro model of AF using human pluripotent stem cells. Building on the work to date from the Keller lab and published literature, we generated cardiomyocytes from hESCs that carry the molecular and electrophysiologic hallmarks of atrial cells. The differentiation scheme developed relies principally on retinoic acid signaling in driving the atrial cell fate, as well as fine tuning of the BMP and Activin signaling pathways in order to generate cells that mimic early atrial precursors. Once validated at the single cell level, using hESC derived ventricular-like cells as controls, we have moved forward in generating a simple tissue model in the form of a multicellular sheet. The atrial chambers are thin compared to the ventricles, with a mean thickness of 1-2 mm compared to ~15 mm, and thus multicellular sheets may be a reasonable approximation of atrial tissue. This platform has the potential to facilitate optical mapping of the electrophysiologic properties of the atria under physiologic and pathophysiologic conditions. Thus our hypothesis is that multicellular sheets of hESC derived atrial cardiomyocytes will serve as a reliable model of human AF and has the potential to act as a tool to understand the mechanism of action of anti-arrhythmic drugs, and to study and screen for novel therapeutics. Based on this hypothesis, the objectives of this study are the following:

1. Generate atrial like cardiomyocytes from hESCs through the manipulation of Activin and BMP signaling pathways and the addition of retinoic acid

2. Generate atrial like tissue in the form of cell sheets

3. Induce atrial fibrillation in the cell sheets
4. Develop an optical mapping setup, and the requisite signal processing software, capable of monitoring the electrophysiologic properties of cell sheets during sinus rhythm and AF.

6. Test the effects of AADs that are commonly used for the treatment of human AF on the cell sheets using the acquisition setup and signal processing.
Chapter 3

Materials and Methods

3.1 Generation of cardiomyocytes from hESCs

HES3 NKX2-5<sup>egfp/w</sup> cells were maintained on irradiated mouse embryonic feeder cells in hESC media consisting of DMEM/F12 (50:50; MEdition, Hedndon, VA) supplemented with 20% knock-out serum replacement (SR), 100 μM nonessential amino acids, 2mM glutamine, 50U/ml penicillin, 50 μg/ml streptomycin (Invitrogen Grand Island, NY), 10<sup>-4</sup> M B-mercaptoethanol (Sigma, St Louis, MO), and 20 ng/mL hbFGF (R&D Systems, Minneapolis, MN) in 6-well tissue culture plates. Cells were passaged to new feeders as single-cell suspensions following dissociation with TrypLE (Life technologies). Embryoid bodies for differentiation were generated after feeder depletion and growth of ES cell colonies to 80% confluence. Single cell suspensions were formed after dissociation with TrypLE. Embryoid bodies were formed by plating small aggregates at a concentration of 500,000 cells/ml in 2 ml basic media containing StemPro34 (Invitrogen), 2mM glutamine, 4 X 10<sup>-4</sup> M monothioglycerol (MTG), 50 μg/ml ascorbic acid (Sigma), and 0.1 ng/ml BMP-4 (R&D Systems). The aggregates were incubated at 37°C in a hypoxic environment of 5% CO2, 5% O2, and 90% N2 on a rotator set at 70 rpm for 24 hours. Following this, the aggregates were spun in a centrifuge at 300 rpm, washed using IMDM, and then re-suspended in the induction media.

The following concentrations of factors were used for the ventricular (control) differentiation protocol: BMP-4, 10 ng/ml; human bFGF, 5 ng/ml; activin A, 6ng/ml; IWP2, 0.5 ng/ml; and human VEGF, 10 ng/ml. This is referred to subsequently as 10B/6A, control, or “ventricular” differentiation protocol. The factors were added with
the following sequence: days 1–3, BMP4, bFGF and Activin A; days 3–5, VEGF and IWP2; day 5-12 VEGF, and days 12-20 only backbone media as previously described. Media changes were performed by centrifuging the EBs at 800 rpm and washing the EBs with IMDM. Cultures were maintained in a hypoxic 5% CO2/5% O2/90% N2 environment for the first 12 days and then transferred to a normoxic 5% CO2/air environment.

The atrial differentiation protocol was carried out as above, with the following changes; BMP-4 and Activin were titrated down to 3 ng/ml and 2 ng/ml respectively. On day 3, retinoic acid (RA) was added to the culture at a concentration of 0.5 ng/ml. Other than the addition of RA, this protocol only differed from the control protocol in terms of the concentrations of BMP4 and Activin used at T3 employing lower concentrations. This is referred to in text and figures as 3B/2A +RA, or “atrial” differentiation protocol.

3.2 Flow Cytometry and Cell Sorting

For cell-surface antigens, staining was carried out in PBS with 3% FCS. For intracellular antigens, staining was carried out on cells fixed with 4% paraformaldehyde in PBS. Staining was done in PBS with 3% FCS and 0.5% saponin (Sigma). Cells were stained at a concentration of \(2.5 \times 10^6\) cells/ml with anti-KDR- APC (R&D Systems; 1:10) and anti-PDGFRA– PE (R&D Systems; 1:20), anti-SIRPA–PE-Cy7 (clone SE5A5; BioLegend; 1:500), anti-CD90-APC (BD Pharmingen, 1:2000anti-CTNT (clone 13-11; Thermo NeoMarkers; 1:400), goat anti-mouse IgG–APC(BD; 1:200). Cells assayed for Aldefluor (STEMCELL Technologies) were prepared based on manufacturers instructions. Incubation with the Aldefluor reagent was 45 minutes. Stained cells were analyzed on an LSRII flow cytometer (BD Biosciences). For FACS, the cells were sorted.
at a concentration of $10^6$ cells/ml in IMDM/5% FCS using a FACSARiaTMII (BD Biosciences) cell sorter (SickKids-UHN Flow Cytometry Facility). Data were analyzed using FlowJo software (Treestar).

### 3.3 Molecular Markers

#### 3.3.1 Quantitative Real-Time PCR (qRT-PCR)

Total RNA was prepared with the RNAqueous-Micro Kit (Ambion) and treated with RNase-free DNase (Ambion). RNA (500 ng to 1 μg) was reverse transcribed into cDNA using random hexamers and Oligo(dT) with Superscript III Reverse Transcriptase (Invitrogen). qRT-PCR was performed on a MasterCycler EP RealPlex (Eppendorf) using QuantiFast SYBR Green PCR Kit (Qiagen). Expression levels were normalized to the housekeeping gene TATA binding protein (TBP).

#### 3.3.2 Immunostaining

Immunostaining was performed using the following antibodies: Polyclonal rabbit anti-MLC2V (Synaptic systems, 1:1000) with the secondary antibody anti-rabbit rabbit IgG Cy3 (Jackson ImmunoResearch), and mouse anti-CTNT (Thermo NeoMarkers; 1:100) with the secondary antibody anti-mouse IgG Alexa647 (Invitrogen). DAPI (Life Technologies, SlowFade Gold) was used to counterstain nuclei. The stained cells were visualized using a fluorescence microscope (Leica CTR6000) and images captured using the Leica Application Suite software.

### 3.4 Cardiac Electrophysiology

#### 3.4.1 Single Cell Electrophysiology

Spin EBs resulting from control and RA-treated differentiations were dissociated at day 20 to single cells using type B collagenase and TrypLE. They were sorted based on
DAPI-/SIRPA+/GFP+/CD90- surface markers as previously described, generating a pure population of cardiomyocytes. Single cells were plated on matrigel-coated coverslips. Electrophysiological measurements were performed 7-10 days after dissociation and plating.

Spontaneous action potentials (APs) were recorded using the patch clamp technique in the whole cell configuration. APs were recorded at room temperature (22-23 °C), and were not corrected for the calculated liquid junction potential. Pipettes were pulled from borosilicate glass (with filament 1.5 mm OD, 0.75 mm ID, Sutter Instrument Company) using a Flaming/Brown pipette puller (model p-87, Sutter Instrument Company) and heat polished. The resistance of these pipettes was 8-10 MΩ when filled with recording solution. Micropipettes were positioned with a micromanipulator (Burleigh PCS-5000 system) mounted on the stage of an inverted microscope (Olympus IX70). Seal resistance was 2-15 GΩ. Myocytes were placed into and perfused with a bath solution of Tyrode’s containing (in mmol/L) 140 NaCl, 2.5 KCl, 1.5 CaCl$_2$, 1 MgCl$_2$, 10 HEPES and 5.5 D-glucose with pH adjusted with NaOH to 7.4. Standard AP internal solution was used to fill the pipettes which consisted of (in mM) 100 K aspartate, 10 KCl, 3 NaCl, 5 NaHCO$_3$, 3 KHPO$_4$, 1 MgCl$_2$, 5 MgATP, 10 HEPES, 75 μM EGTA, with pH adjusted to 7.2 with KOH.

Membrane potential was controlled with an Axon headstage (CV 203BU) connected to an Axopatch 200B voltage-clamp amplifier (Axon Instruments, Foster City, CA). Data was digitized (Axon Digidata 1320A) and acquired using Axon Clampex software (pClamp version 8.2.0.232). Prior to cell attachment and formation of a GΩ seal, electrode potential was adjusted to a baseline of zero current. An agar-salt bridge was
used as the reference electrode. Rupture of the cell membrane patch was achieved using pressure disruption by brief suction. Following rupture of the cell membrane, the membrane capacitive transient was elicited by small depolarizing voltage pulses. Data was analyzed using Clampfit (Molecular Devices, Sunnyvale, CA, U.S.A).

### 3.4.2 Micro Electrode Array (MEA) electrophysiology

MEA chips were cleaned, autoclaved, and coated with dilute matrigel overnight at 37 °C. hESC-CMs were digested using collagenase B at a concentration of 250 U/ml in Hank’s balanced solution (Life technologies) overnight at a concentration of 5 million cells/ml. TrypLE was used to create single cell suspensions which were sorted based on DAPI-/SIRPA+/NKX2-5 GFP+/CD90- surface markers as previously described, generating a pure population of cardiomyocytes. Single cells were plated on standard 60 electrode MEAs at a concentration of 1-2 million cells per 150 μL drop in backbone media plus 1 ng/ml ROCK inhibitor (Y-27632 Dihydrochloride Hydrate, Toronto Research Chemicals, cat.no Y100500) generating cell sheets that were 1-1.5 cm in diameter. Extracellular recording was performed using a MEA1060INV MEA amplifier (Multi Channel Systems, Reutlingen, Germany). Output signals were digitized at 10 kHz by use of a PC equipped with a MC-card data acquisition board (Multi Channel Systems). Standard measurements were performed in IMDM supplemented with NaCl (final concentrations in mmol/L: 140 NaCl, 3.6 KCL, 1.2 CaCl2, 1 MgCl2, 10 HEPES and 5.5 D-glucose). During recordings, temperature was kept at 37 °C. Data were recorded using Cardio2D+ (Multi Channel Systems,) and analyzed off-line with Cardio2D (Multi Channel Systems).
3.4.3 Optical Mapping Electrophysiology

150 μL of 100% concentrated matrigel drops were placed in the centre of 35 mm tissue culture treated petri dishes (Falcon). The dishes were kept on ice for 30 minutes and then the matrigel was removed leaving a thin coat of matrigel with a diameter of 1 cm. The petri dishes were incubated at 37 °C overnight. hESC-CMs were digested using collagenase B at a concentration of 250 U/ml in Hank’s balanced solution (Life technologies) overnight at a concentration of 5 million cells/ml. TrypLE was used to make single cell suspensions which were sorted based on DAPI-/SIRPA+/NKX2-5 GFP+/CD90- surface markers as previously described, generating a pure population of cardiomyocytes. Single cells were plated on the matrigel coated petri dishes at a concentration of 1.25 million cells per 150 μL drop in backbone media plus 1 ng/ml ROCK inhibitor (Y-27632 Dihydrochloride Hydrate, Toronto Research Chemicals, cat.no Y100500) generating cell sheets of 1 cm diameter. To improve the stability of the cell sheets and maintain confluence, various percentages of CD90+ cells generated from the sort were spiked into the cell sheet, from 5-10%. Cell sheets were incubated under normoxic conditions at 37 °C for 2-3 weeks prior to imaging. Once the differentiation was optimized to generate 80-90% cardiomyocytes consistently, cells were digested into single cells, using the aforementioned techniques, and plated directly on the matrigel as cell sheets without sorting. This ultimately had the effect of generating confluent cell sheets with greater consistency.

Optical mapping was performed using the voltage sensitive dye AminoNaphthylEthenylPyridinium (Di-4-ANEPPS)(Life Technologies). Di-4-ANEPPS is an amphiphilic compound with two hydrocarbon chains that allow it to anchor into the
plasma membrane. When bound to the membrane, the chromophore aligns perpendicularly to the membrane/aqueous interface and undergoes reduction in emission intensity (~10% per 100 mV depolarization) when excited at 540 nm and monitored at 680 nm emission

The incubation was optimal when cold Di-4-ANEPPS (on ice) was applied to the cell sheets and then incubated for 30 minutes at 10 μM. Blebbistatin, an inhibitor of the adenosine triphosphatases (ATPases) associated with class II myosin isoforms in an actin-detached state, was employed to stop cardiomyocyte contraction in an effort to avoid motion artifact. Blebbistatin has high specificity for Myosin II and has been demonstrated previously to be an effective excitation-contraction uncoupler, immobilizing cardiac preparations without affecting AP morphology and intracellular calcium handling. Blebbistatin (Sigma) was used at a concentration of 10 μM.

Imaging was performed in IMDM supplemented with NaCl (final concentrations in mmol/L: 140 NaCl, 3.6 KCl, 1.2 CaCl2, 1 MgCl2, 10 HEPES and 5.5 D-glucose). The Di-4-ANEPPS and blebbistatin were washed and replaced with the same IMDM containing 2 μM Di-4-ANEPPS. The petri dish was placed on a plate warmer constructed using copper piping sandwiched by 2 aluminum plates. The copper piping was connected to a water bath heater and pump with rubber tubing. Sylgard was used to coat the aluminum plate where a well had been created and sized for the 35mm petri dish. This generated even heating across the medium in the petri dish to a temperature of 36-37°C.

The tissue was illuminated using a mercury light source (X-Cite Exacte, Lumen Dynamics, Mississauga, ON, Canada) with a 525 ± 50 nm band-pass filter. Fluorescent light was collected using a 645 ± 75 nm band-pass filter. Images were captured using an
electron multiplying charge coupled device (EMCCD) camera (Cascade 128+, Cascade Evolve, Photometric, Tucson, AZ, U.S.A) connected to an Olympus MVX-10 upright microscope (Center Valley, PA, U.S.A) equipped with a 0.63x c-mount adapter and a 0.38x lens relay. Frames were captured at 1408 fps, at 2x2 binning and 64x32 pixels resolution, or 522 fps at 1X1 binning, using Image Pro Plus (Media Cybernetics, Rockville, MD, U.S.A) software. Stimulation and sensing electrodes were constructed from platinum wire coated with Sylgard for electrical insulation. Stimulation electrodes were designed in a bipolar configuration in order to achieve point stimulation. These electrodes were connected to Pulsar 6i & 6b stimulators (FHC Inc, Bowdoinham, ME, U.S.A) for programmed stimulation. Capture threshold was determined either by direct visualization of cell sheet contraction, or simultaneous optical mapping of signals to confirm rate. We were able to obtain 100% capture on the majority of cell sheets using 40mV with a pulse duration of 2 ms. Burst (“on and off”) pacing at a cycle length of 50 msec for 1-2 minutes was used for AF induction. AF induction was defined by rotor initiation in a cell sheet that had demonstrated nodal activation at baseline. Sequences of 2048 frames were captured serially to maximize data acquisition while minimizing photobleaching.

3.4.3.1 Signal Processing and Data

Optical action potential duration (OAPD) measurements were performed using ImageJ, an open source image processing program designed for scientific multidimensional images. Images were analyzed using minimal processing as defined as an averaging of the image sequences which were then appended, and the unprocessed images were subtracted from the processed images. This processing, in addition to removing the
background, enhanced the pixel intensity changes associated with depolarization to aid in analysis. In other words, for every pixel, let \( I(x,y) \) be the input. Let \( O_1 \) be the first stage of output. Then \( O_1(x,y) = I_{ave}(x,y) - I(x,y) + 10000 \), where \( I_{ave}(x,y) \) is the time average of pixel(x,y) and \( I(x,y) \) is the intensity of the individual pixel.

Optical APs were generated by measuring stacks over regions of interest (ROIs) using both raw data and minimal processing as previously defined. When recordings had suitable signal to noise ratios, 1 pixel was used to generate optical APs. When the signal to noise ratio was less favourable, ROIs were selected, up to an area of 10 pixels, over a region of the cell sheet that was in a single phase, meaning the activation wavefront would cross the ROI at the same time in each pixel included.

In order to generate activation maps and conduction velocity maps, recordings were analyzed using Scroll software (courtesy of Sergey Mironov). Processed images were subjected to temporal and spatial filtering to reduce high frequency noise. The activation time (the time of the first derivative of the fluorescence signal corresponding to the steepest segment of the optical AP) was defined for each pixel first, followed by generation of the activation map and the isochronal activation map. The activation time delay between two adjacent neighbours in the vertical, or horizontal components divided by the distance between the two neighbours was used to calculate the vector gradient for each pixel. The resultant local gradient vector was then reciprocated to obtain the conduction velocity vector magnitude, and inversed to gain conduction velocity vector direction. The resultant vector velocity map was then used to guide ROI analysis of conduction velocity. The cell sheet was divided into tertiles based on their distance from the focus of electrical generation and propagation in the cell sheet (either nodal or the
centre of a rotor, identified as the phase singularity). ROIs were taken across each tertile, and then combined to generate a mean and standard deviation of conduction velocity across the cell sheet.

Generating APD maps: Advanced Signal Processing

Due to the low S/N ratios, further processing was required. The first filtering was spatial. Either of the following were used: 7x7 kernel averaging or bandwidth filtering using a 2D FFT. The 2D FFT implementation was that given by ImageJ. The bandwidth used was 8 - 64 pixels. Let O2 be the 2nd output stage.

O2(t) = spatial filtering(O1(t))

A temporal filtering was also required. Pixel-by-pixel, this was done by taking the FFT of O2. Zeroing the coefficients associated with higher frequencies k as well as the negative frequencies, and then reconstructing the signal with an IFFT. The k = Cutoff frequency was chosen to be as high as possible that will enable a good estimation of the action potential duration. The cutoff frequency ranged from 5Hz to 58Hz. In all cases, this meant including the fundamental frequency and several of its harmonics to many of its harmonics. Let O3 be the third output stage.

O3(x,y) = IFFT(0 to cutoff frequency of O2(x,y))

Depending on the amount and type of filtering the baseline of O3 may have a low frequency component. A filtering is performed by using the rolling ball algorithm to establish the baseline. In this variation of the rolling ball algorithm, the height of the ball is scaled to the signal swing O3 as the ball is moved temporally beneath the signal. The radius of the ball is tweaked between 128 to 256 time points to establish a baseline that was manually checked for accuracy.
O4(x,y) = rolling ball baseline filtering(O3(x,y))

The APD map was generated from O4. The measurement was taken from the beginning of the upstroke (moving away from the baseline) to the first occurrence of the data point less than half of the peak value.

3.4.4 Intracellular Recordings of Cell Sheets

Micropipettes were pulled from borosilicate glass (thin walled pipettes measuring 1.2 mm outer diameter, 0.75 mm internal diameter, and thick walled pipettes measuring 1.2 mm outer diameter and 0.4 mm internal diameter, WPI, Sarasota, FL, U.S.A) using a Flaming/Brown pipette puller (model p-87, Sutter Instrument Company, Novato, CA, U.S.A). Pipettes were filled with 3M KCl solution and the resistance was ~30 MΩ for thin walled pipettes and 80-100 MΩ for thick walled pipettes. High resistance microelectrodes were required so as to minimize cellular damage, and minimize K+ leakage. Pipettes were pulled with wispy, long shanks that allowed for flexibility of the pipette when impaled in thin tissue. A vacuum chamber was used after filling to remove bubbles. Pipettes were positioned using course manipulators (Newport, Irvine, CA, U.S.A) and recordings were acquired at 1000 Hz using an Axopatch 200B amplifier with a MiniDigi 1A digitizer and Axoscope acquisition software (Molecular Devices, Sunnyvale, CA, U.S.A). Impalement of tissue was performed by first positioning the microelectrode on the surface of the tissue which was evident by a drop in the potential recording.
Chapter 4

Results

4.1 Generation of cardiomyocytes from hESCs

We employed the HES3 line of hESCs which had sequences encoding enhanced GFP (eGFP) introduced into the NKX2-5 locus by homologous recombination (NKX2-5\textsuperscript{egfp/w}) which has previously demonstrated its utility and reliability in faithfully reporting endogenous Nkx2.5 expression\textsuperscript{208}. The homeobox gene Nkx2-5 is the earliest known marker of vertebrate heart development, expressed in early cardiac progenitor cells and through adulthood\textsuperscript{209}. This line was chosen to facilitate quantification of cardiac differentiation and purification of cardiomyocytes when performing single cell analyses. This human ES cell line expressed cell surface markers that characterize undifferentiated human cells including stage-specific antigen (SSEA)-3, SSEA-4, TRA-1-60, TRA1-81. These cells did not stain strongly for SSEA-1 (s 1)\textsuperscript{126}.

![Figure 1: Flow cytometric analyses of the frequencies of cell surface markers used routinely to identify undifferentiated hESCs; stage specific embryonic antigen 3 and 4 (SSEA3, SSEA4) and two human EC cell antigens Tra-1-60 and Tra-181 also used to mark undifferentiated hESCs. SSEA1 is expressed on differentiated hESCs. Flow cytometry performed after 5 days of hESC culture on MEFs at the time point when cells are dissociated for the T0 of differentiation. Here we show, based on gates set on the unstained sample on the far left, high frequencies of makers of undifferentiated hESCs and a low frequency of a marker of differentiated hESCs.](image-url)
To generate cardiomyocytes from hESCs, we employed the embryoid body staged differentiation protocol that involved the formation of a primitive streak like population defined by T (BRACHYURY) expression (days 2-4), the induction and specification of cardiac mesoderm (MESP1; days 3 and 4), and the expansion of cardiovascular lineages. This protocol uses the combination of Activin and BMP at the primitive streak and mesoderm stage, and then the addition of the WNT inhibitor IWP2 as well as retinoic acid (RA) to the developing cardiomyocytes (Figure 2). As previously mentioned, RA signaling is crucial for atrial chamber development in vivo, and its activation has been shown to effectively drive atrial cardiomyocyte differentiation in mouse and human ESCs.

![Figure 2](image-url)

Figure 2: Scheme of the protocol used to differentiate hESCs towards the cardiomyocyte lineage highlighting the three main stages of development: 1) mesoderm induction, 2) cardiovascular specification and 3) maturation. Protocols for the generation of “ventricular” and “atrial” cardiomyocytes differed in terms of the concentration of Activin and BMP4 used (high vs. low respectively) and whether or not retinoic acid (RA) was added to the cultures at T3. RA is added to the low Activin and BMP4 induction protocol in order to generate “atrial” cardiomyocytes as discussed in the text.

Our group has gone on to show that the manipulation of the BMP and Activin signaling pathways can generate two distinct cardiac precursors as defined by their expression of
either Raldh2 (“atrial” precursors), or upregulation of Cyp26a1 (“ventricular” progenitors) in a temporal pattern consistent with developmental cues (Figure 3). The first protocol uses lower levels of BMP4 and Activin during mesoderm induction (3B/2A), while the second uses higher concentrations (10B/6A) as discussed in the Methods section. The 3B/2A protocol displayed a higher relative expression of RALDH2, the main Raldh involved in early cardiac development. The 10B/6A protocol produced a population of cells that upregulated, Cyp26a1, the cytochrome p450 enzyme that degrades RA.

![Graphs showing qRT-PCR results](image)

Figure 3: qRT-PCR results comparing the relative expression of ALDH1A2 and CYP26A1 over time in a protocol incorporating low levels of BMP4 and Activin (3B/2A) and a protocol with higher BMP4 and Activin (10B/6A). This highlights the different cell types generated through the manipulation of the strength of signaling in these pathways. While both protocols were previously demonstrated to make cardiomyocytes at high frequencies, the 3B/2A protocol generated a population of cells with significantly higher expression of ALDH1A2 (the gene encoding RALDH2) than the 10B/6A protocol. In parallel, the 10B/6A protocol generated a population of cells with significantly higher CYP26A1 (the cytochrome p450 enzyme that degrades RA). These graphs highlight the importance of the careful manipulation of the BMP4 and Activin pathways in directing differentiation of a cell population poised to generate and employ RA (3B/2A), in contrast to a cell population that upregulates an enzyme to protect itself from RA exposure (10B/6A). Unpublished data reproduced with permission from Dr. Stephanie Protze and Jeehoon Lee.

To validate the function of the two systems, our group employed a commercially available assay known as Aldefluor \(^{211}\). Briefly, cells are incubated with a fluorescent aldefluor substrate that when oxidized by an aldehyde dehydrogenase can no longer
efflux from a cell and becomes effectively trapped. The amount of fluorescence exhibited reflects the activity of aldehyde dehydrogenase in the cell. The addition of 4-diethylaminobenzaldehyde (DEAB), which is a potent inhibitor of Aldh enzymes, prevents the enzymatic oxidation of this product thereby providing a negative control for fluorescence. Our group demonstrated that the activity of aldehyde dehydrogenases was restricted to a population of cardiac precursors exposed to lower concentrations of BMP4 and Activin, suggesting that this population of cells not only expresses Raldh2, but that the enzyme is functional in these cells and not in the “ventricular” precursors (Figure 4).

Figure 4: Flow cytometric analyses plotting the frequencies of aldefluor positive cells against PDGFRα positivity (a marker of cardiac progenitor cells). Using the aldefluor assay, cells that express high levels of aldehyde dehydrogenase will fluoresce, in this study used as a potential functional marker of RALDH2 activity. DEAB, a potent inhibitor of Aldh enzymes, serves as a negative control. Once again, two protocols are compared incorporating either low levels of BMP4 and Activin (3B/2A) or higher BMP4 and Activin (10B/6A). While both protocols generate a high frequency of PDGFRα+ cells (an early marker of cardiomyocyte differentiation efficiency), only the 3B/2A protocol generates a high frequency of aldefluor+ cells. This functional assay reiterates the importance of precise manipulation of the BMP4 and Activin signaling pathways in patterning cells that have the machinery necessary to generate and thus utilize RA. Unpublished data reproduced with the permission of Jeehoon Lee and Dr. Stephanie Protze.
In order to monitor and optimize the efficiency of the differentiation protocol, several surface makers were monitored at key commitment stages. The induction of cardiac mesoderm was monitored by the temporal expression of CD56, KDR (Flk-1) as well as PdgfR-α. CD56/NCAM marks the differentiation of early mesodermal progenitors from hESCs. These mesodermal progenitors however are multipotent, giving rise to many mesodermal lineages including cardiomyocytes. KDR/Flk-1 has demonstrated its utility in marking the induction of cardiac mesoderm in mouse and human ESCs, however it is also expressed in different mesoderm populations. PdgfR-α is co-expressed with Flk-1 in the cardiac mesoderm at embryonic stages and is found on cardiac progenitor cells in the cardiac crescent. Optimizing the induction steps to generate greater than 60% Flk-1+ PdgfR-α+ cells generates cultures of highly enriched cardiomyocytes. The earliest time point at which we monitor mesodermal induction is at day 3 or day 4, looking for the emergence of a double-positive population (>80%) of CD56+ and PdgfR-α+ cells. The generation of cardiomyocytes was quantified using flow cytometry by staining of cardiac troponin T (cTNT) and the surface marker SIRPA, which has previously been shown to uniquely mark the cardiomyocyte lineage in hPSC differentiation cultures (Figure 5).
Figure 5: Flow cytometric analyses plotting the frequencies of several important markers used in the optimization and validation of the differential protocols employed. The plot on the left demonstrates an optimal profile on day 4 with a high frequency of CD56+ and PDfR-α+ cells. The middle plot is representative of a good differentiation protocol which generates a high frequency of SIRPα+ cells. The frequency of CD90+ cells is also routinely assessed, and employed to optimize purification of cardiomyocytes, to quantify the frequency of CD90+ cells in the cell sheet culture, or to sort out CD90+ cells and then incorporate them into the cell sheet at controlled frequencies. The final plot on the right demonstrates an efficient differentiation protocol generating 91.9% cTNT+ cells.

4.2 Molecular markers of “atrial” and “ventricular” cardiomyocytes

Differentiation cultures were analyzed for atrial and ventricular specific markers with and without the addition of RA using qRT-PCR (Figure 6). Cultures that were exposed to RA compared to cultures that were not exposed to RA were enriched in the atrial specific markers ANF (148.3 ± 9.4 vs. 16.4 ± 3.9, p < 0.0001, n = 4), KCNJ3 (0.58 ± 0.16 vs. 0.046 ± 0.01, p = 0.01, n = 4), Cx-40 (1.6 ± 0.4 vs. 0.019 ± 0.01, p < 0.01, n = 5), and CaCNA1d (13.0 ± 1.5 vs. 5.6 ± 1.0, p < 0.01, n = 5). Cultures that were exposed to RA expressed significantly less ventricular specific markers compared to cultures that were not exposed to RA including MLC2V (0.63 ± 0.3, p < 0.01, n = 5), and IRX4 (0.037 ± 0.02 vs. 0.46 ± 0.1, p < 0.01, n = 5).
Figure 6: qRT-PCR-based expression analyses of a control protocol (10B/6A) generating “ventricular” cardiomyocytes and the RA protocol (3B/2A+RA) incorporating lower levels of BMP4 and Activin as well as RA. Cell populations were analyzed at T20 of the differentiation protocol. The RA protocol was able to generate cells that are enriched in atrial specific markers (NPPA, KCNJ3, and GJA5) and lack ventricular markers (MYL, IRX4). Values are shown relative the housekeeping gene TBP. Error bars represent standard deviation of the mean from the values of independent experiments (N≥4); *P≤0.05, **P≤0.01, ***P≤0.001 as analyzed by Student's T-test. Unpublished data reproduced with the permission of Jeehoon Lee and Dr. Stephanie Protze.

Immunostaining confirmed the qRT-PCR findings and demonstrated that RA treated cells expressed cTNT, but did not express MLC2v, whereas control cells expressed both MLC2v and cTNT (Figure 7).
Figure 7: Fluorescent immunostaining for the presence of MLC2v and cTNT in day 20 populations. DAPI staining shows cell nuclei. On the left is the control (10B/6A) protocol generating cells that express both cTNT and the ventricular specific marker MLC2v. On the right, the atrial protocol (3B/2A+RA), that includes the addition of retinoic acid, generating cells that express cTNT but do not express MLC2v. Unpublished data reproduced with the permission of Jeehoon Lee and Dr. Stephanie Protze.

4.3 Single Cell Electrophysiology

Cardiomyocytes derived from hESCs that received RA during their differentiation protocol ("atrial" cardiomyocytes) had predominantly atrial action potential (AP) morphologies, whereas cardiomyocytes that were not exposed to RA ("ventricular" or control cardiomyocytes) had predominantly ventricular action potential morphologies (Figure 8). The “atrial” differentiation protocol, that included the addition of RA, generated 90% atrial like APs, 5% nodal like APs and 5% ventricular like APs (n = 20 cells) (Figure 9). The control, or “ventricular” differentiation protocol, that did not include the addition of RA, generated 85% ventricular like APs, 10% atrial like APs and 5% nodal like APs (n = 20 cells) (Figure 10). The electrophysiologic characteristics of the recorded APs can be seen in Table 1.
Figure 8: Single cell patch recordings demonstrating typical APs of RA (3B/2A+RA) compare to the control protocol (10B/6A without RA). Cells are carried through to day 20, to allow for maturation and the genesis of contracting cardiomyocytes, where they were then plated as single cells and patched in Tyrode’s solution to record single cardiomyocyte APs. RA treated cells demonstrate atrial AP morphologies whereas control cells that were not exposed to RA demonstrate ventricular AP morphologies.

Figure 9: Showing the presence of cardiomyocyte types with representative AP profiles and their prevalence (n = 20 cells) in the atrial directed differentiation protocol (3B/2A + RA) used to generate atrial cardiomyocytes from hESCs. Cells are carried through to day 20, to allow for maturation and the genesis of contracting cardiomyocytes, where they were then plated as single cells and patched in Tyrode’s solution to record single CM APs. The majority of APs carried an atrial phenotype (90%).
Figure 10: Showing the presence of cardiomyocyte types with representative AP profiles and their prevalence (n = 20 cells) in the standard directed differentiation protocol (control, 10B/6A) used to generate cardiomyocytes from hESCs. Cells are carried through to day 20, to allow for maturation and the genesis of contracting cardiomyocytes, where they were then plated as single cells and patched in Tyrode’s solution to record single CM APs. The majority of APs carried a ventricular phenotype (85%).

<table>
<thead>
<tr>
<th></th>
<th>N (cells)</th>
<th>dv/dt&lt;sub&gt;max&lt;/sub&gt; (V/s)</th>
<th>DMP (mV)</th>
<th>APA (mV)</th>
<th>APD50 (ms)</th>
<th>APD90 (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control “Ventricular”</td>
<td>20</td>
<td>54 ± 3</td>
<td>-53 ± 1</td>
<td>101 ± 2</td>
<td>479 ± 59</td>
<td>547.2 ± 59</td>
</tr>
<tr>
<td>RA treated “Atrial”</td>
<td>20</td>
<td>44 ± 5</td>
<td>-50 ± 1</td>
<td>78 ± 3**</td>
<td>33 ± 6**</td>
<td>187 ± 13**</td>
</tr>
</tbody>
</table>

Table 1: Characteristics of APs recorded from the protocol generating atrial cardiomyocytes (3B/2A + RA) compared to the protocol used to generate primarily ventricular cardiomyocytes (10B/6A). The majority of cells demonstrated features of electrophysiologic immaturity with more positive membrane potentials, and lower upstroke velocities and action potential amplitudes. APA, action potential amplitude; APD50, action potential duration at 50% of repolarization; APD90, action potential duration at 90% of repolarization; DMP, diastolic membrane potential; dv/dt<sub>max</sub>, maximum action potential upstroke velocity; N, cell number; **P≤0.0001 as analyzed by Student's T-test.

Comparing the cardiomyocytes generated from the “atrial” differentiation protocol to the cardiomyocytes generated from the “ventricular” or control protocol, as would be expected, the “atrial” cardiomyocytes had a shorter APD50 (33 ± 6 ms vs. 479 ± 59, p < 0.0001) (Figure 11) There was no significant difference between differentiation protocols with respect to the maximum upstroke velocity, or the maximum diastolic potential. Of
note, both of these values are considerably more positive than that expected of adult tissue. In addition, “atrial” cells had a lower action potential amplitude (78 ± 2 mV vs. 101 ± 2 mV, p <0.0001), and a dramatically shorter APD90 (187 ± 13 ms vs. 547 ± 59 ms, p <0.0001).

Figure 11: Comparing the AP characteristics of cells generated from the “Ventricular” (10B/6A) differentiation protocol compared to the “Atrial” (3B/2A + RA) differentiation protocol. n = 60 APs representing 20 cells (3APs/cell). Error bars represent 95% standard deviation from the mean. **P≤0.0001 as analyzed by Student's T-test.

There was a significant correlation between the action potential amplitude and the maximum diastolic potential ($R^2 = 0.373$, p<0.001), which was also seen when considering the maximum upstroke velocity and the maximum diastolic potential ($R^2 = 0.176$, $p = 0.001$). Using a technique called an anode break, negative current was injected into the cells in order to achieve a more negative resting membrane potential. This was done repeatedly until a resting membrane potential of -70 mV was achieved, and then the current was extinguished allowing the cell to fire a spontaneous action potential. Using this technique, a higher action potential amplitude and maximum upstroke velocity were achieved (Figure 12). Finally, “atrial” cardiomyocytes had a much faster spontaneous beating rate, as demonstrated by a shorter cycle length, compared to the “ventricular” cells (680 ± 5 ms vs. 1670 ± 12 ms, $p < 0.0001$).
Figure 12: Patch clamp recording in Tyrode’s solution of a cardiomyocyte generated from the atrial protocol (3B/2A + RA) during an anode break. The injection of negative current into the cell drove the resting membrane potential to a more negative value and then released. The subsequent action potential amplitude is clearly higher than the subsequent APs starting from a less negative resting membrane potential in the image on the left. On the right, the same cell with multiple time sequences overlayed to demonstrate the reproducibility of this maneuver in achieving higher action potential amplitudes.

4.4 MEA Electrophysiology

“Atrial” and “ventricular” cell sheets were plated on MEA chips as described. External field potentials were successfully recorded from the MEAs of both atrial and ventricular cell sheets (Figure 13). These are akin to an electrocardiogram measurement on patients and have clearly demonstrable depolarization and repolarization phases facilitating measurements of field potential durations (FPDs) which are directly linked to the action potential duration. As expected from the single cell analyses, the FPDs of “atrial” cell sheetss were much shorter than that of “ventricular” cell sheets. Pacing was performed through the Cardio2D system in a unipolar configuration. Capture was reliably demonstrated by direct visualization of the cell sheet, the demonstration of capture at the tail end of the pacing artefact, and through routine electrophysiologic principles including
the perturbation of cycle length as demonstrated by the pacing cycle length, the spontaneous beating rate, and the post-pacing interval (Figure 14).

![Figure 13: MEA recording of a “ventricular” cell sheet on the left generated from the 10B/6A differentiation protocol compared to an “atrial” cell sheet on the right generated from the 3B/2A + RA protocol. Cell sheets were allowed to beat spontaneously. FPDs recorded and labeled for both cell sheets. The “atrial” cell sheet displays a shorter FPD as would be predicted by the single cell data in which atrial cells had significantly faster repolarization times. FPD = field potential duration.]

![Figure 14: MEA recording of “atrial” cell sheet (generated from the 3B/2A + RA differentiation protocol). Capture is demonstrated at the pacing rate and thus pacing has successfully overdriven (either suppressed in the case of automatic activity or accelerated in the setting of triggered activity) the spontaneous pacemaker. The post pacing cycle length (CL), commonly referred to as the post pacing interval, is the time required for the spontaneous activity to resume, which then returns to its spontaneous rate. The clear demonstration of these three intervals, in addition to the capture signal distinct from the pacing artefact, in this tracing strongly supports our ability to pace and capture the cell sheets at this pacing rate. Fibrillatory activity, appreciated by direct visualization of the cell sheets, was achieved in “atrial” (n=3), but not “ventricular” (n=3) cell sheets despite aggressive burst pacing.]

58
protocols down to the shortest cycle length of 50 ms. Similarly, 2 point stimulation using the S1-S2 protocol previously described was not able to induce fibrillatory activity in the ventricular cell sheets despite numerous and aggressive pacing protocols and field generating configurations (data not shown). Unfortunately, fibrillatory activity in the atrial cell sheets reduced the signal to noise ratio on the recorded field potentials, and signals were no longer distinguishable. The inability to monitor and thus map AF on the MEA system available led us to abandon this methodology in favour of optical mapping.

4.5 Optical mapping electrophysiology

The optical mapping configuration was optimized such that activation wavefronts could be visualized with minimal processing. Advanced processing techniques were utilized for noise reduction and signal augmentation, but were not used for analyses. Fifty percent of “atrial” cell sheets had a propensity to develop spontaneous fibrillatory activity within the first week of plating, with the prevalence of fibrillatory activity decreasing over time. At the time of optical mapping, 1 in 4 cell sheets would continue to exhibit fibrillatory activity. This occurred for the most part in cell sheets that had areas of non-confluency, where “holes” in the cell sheets acted as anchors for re-entry (not shown). Non-confluent cell sheets were discarded.

In confluent cell sheets, spontaneous fibrillatory activity at the time of optical mapping was infrequent. “Atrial” cell sheets demonstrated spontaneous beating rates of $78 \pm 14$ bpm, with the majority developing spontaneous activity typically at the edges of the cell sheets where a favourable source-to-sink relationship exists for cardiomyocyte clusters with the fastest intrinsic firing rate (Figure 15). Once optimized, optical action potentials (OAPs) could be recorded in a majority of cell sheets (Figure 16).
Figure 15: A series of sequential still images taken from the optical recording of an atrial cell sheet (3B/2A + RA) of 1 cm diameter using 10 μM Di-4-ANEPPS and 10 μM blebbistatin and captured on an EMCCD. Electrical activity appears to originate from the top right corner of the cell sheet and propagate more rapidly along the edge of the cell sheet than towards its centre. This generates an activation wave front that ultimately circumnavigates the cell sheet and then moves relatively uniformly towards its centre, where the greatest sink to source mismatch occurs. Ultimately the wavefront extinguishes at the centre of the cell sheet, and a new wavefront is generated from the same origin and propagates in the same manner.

Figure 16: Typical optically recorded APs of an “atrial” cell sheet (3B/2A + RA) in “AF” using 10 μM Di-4-ANEPPS and 10 μM blebbistatin and captured on an EMCCD. The OAPs carry the typical morphology of atrial APs. The amplitude relates to the amount of fluorescent change of the voltage sensitive dye that occurs during a depolarization and repolarization event.

The bipolar electrodes were able to pace and generate point stimulation which propagated uniformly through the cell sheet. Increase of the pacing rate resulted in shortening of the “atrial” APD (Figure 17). Burst on and off pacing at a cycle length of 50 ms for 1-2 minutes consistently generated “AF” as demonstrated by an increase in beating rate, the development of continuous electrical activity in the cell sheet, and the generation of
rotors (Figure 18). On one occasion, electrical alternans was recorded during rapid pacing (cycle length of 50 ms), and was followed by rotor initiation (Figure 17).

Figure 17: On the left, optical action potential duration (OAPD) measurements on the Y axis plotted as a function of rate on the X axis demonstrating the APD restitution curve of a typical “atrial” cell sheet. At faster rates, the APD restitution curve becomes increasingly steep. On the right, electrical alternans is induced in the same cell sheet at a pacing rate of 1200 bpm demonstrating cyclic, beat-to-beat variations in AP amplitude and action potential duration at a constant stimulation frequency. The cell sheet cannot be captured 1:1 at this pacing rate. After this recording, “AF” was recorded in the cell sheet upon cessation of pacing.

Figure 18: A series of sequential still images taken from the optical recording of an “atrial” cell sheet (3B/2A + RA) of 1 cm diameter using 10 μM Di-4-ANEPPS and 10 μM blebbistatin and captured on an EMCCD. A rotor continuously generates an electrical wavefront, anchored by the phase singularity.

Once initiated, the rotors were extremely stable, persisting in culture for as long as 8 weeks. When comparing the APD maps of a cell sheet in sinus rhythm compared to a cell sheet which had been induced into rotor formation and remained persistently so chronically (for 6 weeks), there is a clear difference in the APD heterogeneity (Figure
This heterogeneity is one manifestation of the consequences of electrical remodelling taking place in the cell sheet after rotor induction that can be identified both acutely and chronically in this model.

Figure 19: Action potential duration (APD) maps generated from the “atrial” cell sheets using DI4-ANEPPS and an EMCCD. The figure legend represents APDs in msec. The image on the left was generated from a cell sheet prior to arrhythmia induction, while the APD map on the right represents a stable rotor that was induced with burst pacing. Note the increased APD heterogeneity in the APD map of the rotor compared to the APD map on the left. This heterogeneity is one manifestation of the nearly immediate electrical remodelling that is taking place in the cell sheet that can be identified both acutely and chronically in our system.

As discussed previously, the inability of the excitation wavefront to depolarize the PS of an ongoing rotor, and the tendency of the wavefront to curve around the PS create the basis for rotor initiation and maintenance. It has been previously demonstrated that the wavefront curvature is highest around the tip resulting in slowing of the conduction velocity near the PS, or centre of the rotor. Further from the centre, the curvature is reduced and conduction velocity increases. Several mapping techniques have evolved to assist in the understanding and analysis of rotor dynamics. Phase mapping was first able to identify the PS by giving it an arbitrary phase, and its surrounding element a continuous progression of phases equal to $\pm 2\pi$. More recently, the Hillbert transform
has facilitated the computing of the instantaneous phase $^{217}$. Another important developments was the analysis of the time-dependent behaviour of rotors using a Fast Fourier Transform Analysis and generating a frequency map of the signals of interest. Selecting the maximum frequency in the Fourier spectrum allowed for the construction of dominant frequency maps $^{218}$.

Cardiac sheets, predominantly using rat neonatal rat and embryonic chick ventricular cardiomyocytes, have demonstrated their utility in generating and mapping 2-D rotors and re-entry $^{219}\,^{220}$. In these cases, owing to the stability of rotors in this model, activation maps were able to capture the same data content generated by phase maps and DF maps, required of more complex and three dimensional structures. We successfully generated activation maps (Figure 20) and conduction velocity maps (Figure 21) at both baseline, when a single nodal source acts as a pacemaker (henceforth referred to as “sinus rhythm” or “SR”) and after rotor induction (henceforth referred to as “AF”). Activation maps appropriately identified the PS where depolarization and repolarization wavefronts collided, and captured the property of increasing curvature as the excitation wavefront propagated away from the PS. Conduction velocity maps were able to demonstrate the inverse relationship between conduction velocity and distance from the PS.
Figure 20: Activation maps generated from recordings of “atrial” cell sheets using DI-4ANEPPS and an EMCCD. Optical APs are obtained on each pixel of the EMCCD and the accrued points are assigned to an isochronal color scale based on their respective activation times. On the left, a cell sheet before the induction of an arrhythmia. The cell sheet spontaneously generated a concentric ring of triggered activity which starts on the periphery of the culture and propagates uniformly to the centre (see Figure 15). This is likely a result of the aforementioned source sink relationship which generates edge effects in the culture. On the right, an activation map recorded after the induction of a rotor using burst pacing (see Figure 18). Note the increase in frequency after the induction of continuous rotor activity in the dish simulating the induction of a major driver of clinical AF. Isochronal lines are recorded in ms and labeled on the map.

Figure 21: Conduction Velocity (CV) maps generated from the activation maps in Figure 20. Again, the map on the left demonstrating baseline activity before rotor induction with uniform progression of electrical signal from the periphery of the dish to the centre. The arrows represent the vector of the electrical propagation at each point, and the size of the arrows represents the magnitude of the velocity. On the right, a CV map generated after rotor induction. Note the smaller magnitude of the arrows at the core of the rotor. This facilitates rotor stability and in fact anchors the rotor around an area of a phase singularity. Here, the convex curvature reaches a critical value preventing electrical activity to invade the core and thus maintaining the unexcitable obstacle around which the re-entrant rhythm circulates.
4.5.1 Optical mapping the effects of AADs

To test the appropriateness and applicability of our model system to the tasks of drug screening and drug discovery, we first sought out to demonstrate the known effects of commonly used AADs. Indeed in our preliminary studies we observed predictable effects of flecainide and dofetilide on the OAP morphology (Figure 22). The major and important limitation to these studies was the rapid photobleaching of voltage sensitive dyes thus limiting the exposure time and therefore the dose range of each drug trialed.

Figure 22: Optical action potentials (OAPs) from cell sheets of “atrial” cardiomyocytes derived from hESCs are recorded using the voltage sensitive dye DI4-ANEPPS on an EMCCD. Representative OAPs from an “atrial” cell sheet after induction of a rotor using burst pacing are demonstrated in Figure 5A. In Figure 5B, the baseline recording (in black) is compared to a second recording after the addition of 1 μM dofetilide (in blue), a specific IKr blocker. There is prolongation of the OAP as predicted by the effects of dofetilide on the major repolarizing K+ current in these cells. Figure 5C again demonstrates another “atrial” cell sheet in which a rotor is induced and OAPs are recorded (in black), and a second OAP recorded after the addition of 10 μM flecainide (in red). Slowing of the upstroke of the cardiac action potential can be appreciated, as would be predicted by the sodium channel blocking properties of the drug. Interestingly, there is also OAP prolongation, which may be related to the additional K+ blocking effects of flecainide that are predicted to extend the atrial wavelength and induce rotor termination.

Dofetilide is a class III anti-arrhythmic which selectively inhibits the rapid component of the time-dependent outward potassium current (I_{Kr}) thus causing APD prolongation without influencing the rate of depolarization or conduction velocity. As predicted however, dofetilide is not specific to the atria and thus causes APD prolongation and
resultant QT prolongation in the ventricles, predisposing patient to torsades-de-pointes and sudden death. Cardiomyocytes derived from hESCs and hiPSCs have demonstrated exquisite sensitivity to $I_{kr}$ blockade exceeding that of animal tissue assays. Atrial cardiomyocytes derived from hPSCs have not yet been tested for their response to $I_{kr}$ blockade, and so we chose to test dofetilide as our first AAD screen on our model. Importantly, we chose to apply AADs after induction of “AF” to generate a clinically relevant tissue model that would have greater translational meaning and potential. “AF” was induced in rotors during “SR” with burst pacing as described. The induction of “AF” in these cultures did not have an effect on the mean OAPDs across the cell sheet (Figure 23). As would be predicted, dofetilide at a concentration of 1 μM dramatically prolonged the OAPD of “atrial” cardiomyocytes during “AF”. When comparing cell sheets during “AF” and then after exposure of dofetilide, there was a significant prolongation of the OAPD ($529 \pm 137$ vs. $809 \pm 432$, $p = 0.02$) (n =3) without a significant effect on cycle length ($687 \pm 170$ ms vs. $809 \pm 432$ ms, ns) (n=3).

Figure 23: Plotting the sequential effects of rotor induction and 1 μM dofetilide on the optically mapped APDs and cycle length of “atrial” cell sheets. The induction of a rotor decreased the cycle length (increased the rate) without a significant effect on the APD.
Dofetilide significantly prolonged the APD without a significant effect on cycle length. Error bars represent 95% standard deviation generated from three independent experiments (N=3); *P≤0.05 as analyzed by Student's t-test.

Dofetilide alone was not able to convert “AF” to “SR” acutely (within 4 hours). The introduction of overdrive pacing (pacing at a cycle length exceeding that of the ongoing rotor) to the cultures that were exposed to dofetilide however was able to convert “AF” to “SR” as demonstrated by the return of a nodal pacemaker driving the rate of the system, and the elimination of reentry and rotor dynamics (n=2). Interestingly, on one occasion, overdrive pacing extinguished one rotor immediately transitioning to another rotor with a different PS that was geographically distinct from the first. This second rotor was then eliminated by overdrive pacing leading to “SR”. Dofetilide did not have an effect on conduction velocity across the cell sheets (n = 3)(Figure 24).

![Figure 24: Demonstrating the effects of rotor induction, 100 nM dofetilide and 1 μM dofetilide on conduction velocity in “atrial” cell sheets. When considering the conduction velocity across the cell sheet, dofetilide does not impact on conduction velocity. Error bars represent 95% standard deviation generated from three independent experiments (N=3).](image)

The next anti-arrhythmic trialed was flecainide, a class Ic AAD which blocks the Nav1.5 sodium channel in the heart, slowing the rapid upstroke component of the cardiac AP.
Flecainide has been shown to reduce excitability and slow conduction velocity. Flecainide also has effects on the repolarizing potassium currents which are proposed to increase wavelength thus explaining the clinical observation of decreased AF susceptibility for patients on this drug. Addition of flecainide to cell sheets in “AF” appeared to have an effect on the cycle length on two cell sheets trialed (840 ± 396 ms vs 1766 ± 278 ms), with less of an impact appreciable on OAPD prolongation in both cell sheets trialed (523 ± 119 vs to 789 ± 160 ms) (Figure 25).

Figure 25: Plotting the sequential effects of 5 μM flecainide and 10 μM flecainide on the optically mapped APDs and cycle length of a single “atrial” cell sheet after the induction of a rotor. Flecainide appears to slow cycle length in a dose dependent manner without exerting a significant independent effect on action potential duration.

Also as expected, the addition of flecainide appeared to decrease conduction velocity across the cell sheets in “AF” (3.5 ± 2.4 cm/s vs 2.0 ± 1.8 cm/s)(n=2). The addition of flecainide did not convert the cell sheets from “AF” to “SR” (n=3) (Figure 26).
Figure 26: Demonstrating the effects of 5 μM flecainide and 10 μM flecainide on the conduction velocity of an “atrial” cell sheet after the induction of a rotor. Flecainide appears to have a dose dependent effect on conduction velocity as seen on both cell sheets trialed. A mean and 95% standard deviation are represented for all conduction velocities recorded from the conduction velocity map of one cell sheet.

To further dissect the effects of AADs on our human model of SR and AF, we analyzed the conduction velocity maps in tertiles according to the geographic distance from the focal source, either the nodal pacemaker in the setting of “SR” or the PS in the setting of “AF”. This was based on our observation that our model had recapitulated the principles components of curvature and conduction velocity essential to rotor initiation and maintenance, and our hypothesis that AADs may have a differential effect depending on the location on the rotor. The induction of a rotor indeed had a dramatic effect on slowing conduction velocity at the source of the electrical wavefront (3.8 ± 2.1 cm/s vs. 0.5 ± 0.2, p = 0.04)(n=3) with a trend towards the inverse effect on the outer tertile of the cell sheet.
that did not reach statistical significance. Dofetilide, as would be predicted, did not have an effect on conduction velocity at any site along the rotor (Figure 27). Flecainide however appeared to have a differential effect on conduction velocity slowing that was dependent on the distance from the PS, unexpectedly slowing the CV disproportionately at the site of the broadest curvature on the rotor (from $5.2 \pm 4.9$ cm/s to $2.2 \pm 2.0$ cm/s) ($n=2$) (Figure 28).

Figure 27: Demonstrating the effect of dofetilide on conduction velocity as a function of distance from the origin of the electrical wavefront. The cell sheet was divided into thirds according to the distance from the origin. At baseline, the cell sheet has a spontaneous pacemaker which drives the cell sheet in “SR”. The origin is therefore at the site of the earliest activation in this case. After “AF” induction, the origin is defined as the phase singularity (PS), or centre of the rotor. Regions of interest are drawn over these regions of the conduction velocity maps generating means and standard deviations. There is a significant drop in the conduction velocity near the source after rotor induction ($p = 0.04$) which persists during the sequential uptitration of dofetilide, from $100$ nM to $1 \mu$M. Dofetilide does not appear to have any effect on conduction velocity, regardless of the distance from the PS. Error bars represent $95\%$ standard deviation generated from three independent experiments ($N=3$).
Figure 28: Demonstrating the effect of flecainide on conduction velocity as a function of distance from the origin of the electrical wavefront. Two independent “atrial” cell sheets are displayed. The cell sheets were divided into thirds according to the distance from the origin. Displaying only data obtained after the induction of a rotor with the centre of the rotor defined as the phase singularity (PS). Regions of interest are drawn over these regions of the conduction velocity maps generating means and standard deviations. After rotor induction, as before, the conduction velocity is slowest at the centre of the rotor and increases with increasing distance from the core. The addition of flecainide appears to have a dose dependent effect on slowing conduction velocity at the middle and outer third of the cell sheet, yet does not appear to exert any influence on the rotor’s core.
Chapter 5

Discussion

Atrial fibrillation is an important contributor to the morbidity and mortality of Canadians, and is a major and growing burden on our health care system and our economy\(^1\)\(^\text{--}^9\). While the pace of discovery and innovation in the understanding and treatment of AF has accelerated rapidly over the last two decades, the initial description of the disease nearly a century ago has withstood the test of time and remained remarkably accurate despite the relatively primitive tools of the age. A series of critical reviews in 1924 outlined the clinical principles of AF, and the general hypotheses regarding AF initiation and maintenance that continue to be hotly debated today\(^{222}\)\(^\text{--}\)\(^{223}\). Perhaps stemming from this confusion regarding the mechanistic basis of the disease, clinicians have limited tools with which to target and treat the substrate that drives recurrence and facilitates the remodeling process involved in the disease’s propensity for self-promotion. Rotor theory has become a pre-eminent component of AF mechanistic study and treatment owing to technologic innovations that have facilitated a greater appreciation of its importance, and a more detailed understanding of its components. Over a parallel, yet only recently overlapping time course, there has been a rapid advancement of hPSC technology in the generation of pure lineages of well characterized cardiomyocytes in vitro through the application of the fundamentals of developmental biology.

A major step forward in the application of hPSC derived cardiomyocytes to disease modeling has been the careful dissection of embryonic cues which drive cell specific fates in the embryo, and mimicking them in vitro to differentiate hPSCs into pure lineages of cardiomyocyte subpopulations. While the ability of hPSC derived
cardiomyocytes to model electrical cardiac disorders at a single cell level, predominantly in ventricular like cells, has been exploited in a number of disease phenotypes and drug screens, to our knowledge this is the first study to examine atrial tissue in the setting of a tissue based arrhythmia. This has important implications for the study of AF and the search for novel therapies, as well as significant potential to translate our growing understanding of the genetic contributors of AF directly to the application of personalized medicine.

5.1 Molecular markers of “atrial” and “ventricular” cardiomyocytes

Our group has developed a differentiation protocol that has generated an enriched population of atrial like cells through the manipulation of BMP and Activin signaling, and the addition of retinoic acid. The cells have undergone thorough molecular characterization which has demonstrated their similarities to atrial cardiomyocytes and their distinct signature compared to adult ventricular cardiomyocytes, and ventricular like cardiomyocytes generated from hESCs. Specifically, the atrial like cell population is enriched with the atrial markers ANF, KCNJ3, Cx40 and Cav1.3, whereas the ventricular like cell population is enriched in MLC-2v and IRX4. Functional analyses of these cells through patch clamping has demonstrated their clear and distinct electrophysiologic properties which again mimic atrial cardiomyocytes.

5.2 Single cell electrophysiology

We have shown that the atrial like cell population consists of an overwhelming majority of cells displaying an atrial AP morphology (90%), whereas the ventricular like cell population equally exhibits a majority of cells that display a ventricular AP morphology (85%). These cell populations importantly differ in their APD50 profiles, with atrial like
cells displaying dramatically shorter APD50s. As previously mentioned, the single cell electrophysiologic properties recorded suggest that these cells are immature compared to their adult counterparts. This finding is not unexpected given the fact that there are known differences between adult and fetal cardiomyocytes in terms of their complement of ion channels and the resultant AP profile\textsuperscript{224}.

Adult ventricular and atrial cardiomyocytes exhibit a resting phase (phase 4) in which the resting membrane potential (RMP) does not change. This is primarily due to the rectifying potassium current ($I_{k_1}$) which stabilized the RMP at $\sim$85mV, which is the reversal potential of K+. In hPSC derived cardiomyocytes, the RMP is much less negative, a property which has been attributed to the lower expression of $I_{k_1}$\textsuperscript{156,225}. We, and others, have indirectly shown that the relatively depolarized state of the hPSC cardiomyocytes leads to a decrease in the functional availability of Na+ channels by driving the membrane potential to more negative values and demonstrating an increase the action potential amplitude and upstroke velocity\textsuperscript{65,156}. An additional explanation for the relatively low upstroke velocity and action potential amplitude seen in hPSC derived cardiomyocytes compared to published values for adult cardiomyocytes may be that cells were patched at room temperature since the principal component of these measures is sodium current. Sodium channels are temperature dependent, and thus cells analyzed at 37 °C may demonstrate more sodium current, and thus higher values for upstroke velocity and action potential amplitude. Cardiomyocytes derived from hPSCs also demonstrate large pacemaker currents (funny current, $I_f$), which is very low in adult cardiomyocytes, causing diastolic depolarization and spontaneous contraction of hPSCs in culture as seen in our study.
There are many other features of immaturity not directly addressed in our study including morphologic parameters, sarcomeric organization, calcium handling and excitation-contraction coupling, and metabolism. There have been many proposed methods to promote maturation of hPSC derived cardiomyocytes including increasing time in culture, electrical stimulation, application of mechanical strain, chemically inducing maturation, and the promotion of maturation by incorporating other cells types or extracellular substrates into culture or engineered 3-D structures. In spite of all of these efforts, many of the features of maturation have only been seen in isolation, and certain features of the adult phenotype have never been reproduced in culture such as the present of T-tubules.

5.3 Modeling atrial diseases using hPSCs

Despite the known limitations related to the immaturity of hPSC derived cardiomyocytes, a fully mature state is not felt to be a necessary pre-requisite to the use of these cells to model disease. The FDA in fact has recently proposed a directive that all new drugs should be tested for their effects on all ion channels in human cardiomyocytes, and have suggested that hPSC derived cardiomyocytes were an ideal model and may be appropriate for this purpose. While the weight of evidence to support this notion in ventricular like hPSC derived cardiomyocytes has already been reviewed in the background section, there has been a recent study demonstrating the utility of atrial like cells in drug screening and drug discovery. By employing the same HES3 NKX2-5 cell line as we have employed for our studies, Devalla et. al, generated atrial like cells through the manipulation of BMP4, Activin and Wnt signaling, as well as the addition of RA. Similar to our findings, they showed that the expression of atrial specific ion
channel genes was confined to their atrial like cells compared to their ventricular like controls. Using patch clamping, they went on to show that in response to multiple atrial specific ion channel blockers; vernakalant and Kv1.5 blocker XEN-D0101, hESC derived atrial but not ventricular like cells demonstrated AP prolongation at the single cell level. Additionally, and as would be expected of atrial cells, a novel Kir3.1/3.4 blocker restored the AP shortening caused by carbachol in the hESC derived atrial cardiomyocytes. Taken together, these results convincingly demonstrate that the cell line employed in our study, when differentiated in cardiomyocytes using a very similar protocol to the one our group has independently developed, generates a cell type that is appropriate, at least at the single cell level, for the pre-clinical testing of atrial specific antiarrhythmics. We have taken this concept a step further by demonstrating reliable and predictable drug responses of atrial tissue in the setting of a clinically relevant arrhythmia model.

The novelty and strength of our study is the tissue based model of disease which encompasses many aspects of clinical arrhythmology that cannot be studied at the single cell level. In particular, we have focused on the determinants of the atrial wavelength, conduction velocity and action potential duration, as well as the fundamental properties of rotor dynamics. Optical mapping is a well known method to monitor and analyze the propagation of cell excitation in whole cardiac tissues, and thus we set out to employ this tool to study the functional properties of our multicellular network of “atrial” cells at baseline and with the induction of complex phenomena such as reentry and spiral wave propagation.

In our first efforts to validate our model, we have shown that “atrial” cell sheets can demonstrate properties of electrical wavefront activation and propagation which can be
captured on our optical mapping setup. We have successfully designed bipolar electrodes capable of capturing the cell sheets reliably, and generated point source stimulation with which to control and test our cell sheets. We have gone further to demonstrate the normal physiologic property of APD restitution in our “atrial” cell sheets, and taken advantage of this principle to generate “AF” by driving the cell sheets to the steepest part of their APD restitution curves to facilitate self-sustaining oscillations. We have for the first time, observed electrical alternans at rapid pacing rates preceding the onset of “AF” in human “atrial” tissue. Electrical alternans is a recognized clinical risk factor for cardiac arrhythmias including AF.

“AF” in our model system was defined by spiral wave activity that carried several signatures of rotor dynamics including the ability to self-sustain a PS, the generation of a curved wavefront with its maximal curvature and slowest conduction velocity centered on the PS, the ability to overdrive the system with the addition of AADs that extend wavelength, the spontaneous initiation of a distinct PS after overdrive pacing of the original PS, and the ability of overdrive pacing and AADs to convert the reentrant arrhythmia. We have successfully optically mapped the induced arrhythmia to generate activation maps, measure optical APs in the setting of an arrhythmia, and generated vector and conduction velocity maps giving us the unique ability to measure local conduction velocity.
5.3.1 APD restitution and heterogeneity

We have shown that OAPs across a cell sheet in “AF” display greater heterogeneity than OAPs in “SR” (Figure 18). Dispersion of APDs in the atria occur under normal physiologic conditions in humans, both in newborns and adults, and has been documented in a number of other animal species. The APD and effective refractory period shorten progressively with increased distance form the SAN, a finding which stems from the initial observation that the atrial AP shape changes as a function of distance and independent from the excitation sequence. This has been proposed to protect the atria from anatomic reentry because of the resistance to unidirectional block in the setting of an ectopic focus, and ascribed to changes in regional ionic properties.

In computer modeling studies, APD heterogeneity has indeed demonstrated a protective effect on the development of anatomic reentry, but in contrast, did not afford protection from functional reentry as would be seen in rotor formation. Wavebreak was in fact more notable in areas of increased APD gradient leading way to stable rotor formation. It has been proposed that such gradients contribute to the establishment of acute AF in the structurally normal heart. In human AF, the atrium can achieve exceedingly high frequencies, and thus extreme shortening of the APD would be required for APD maintenance. Mechanisms that increased APD heterogeneity and regional abbreviation in areas that achieve the highest frequencies, such as rotors driving AF, would lead to increased stability of microreentrant sources by reducing interaction between the front of the wavefront and its tail, and allowing for rapid curling of the wavefront around the PS. The APD maps generated in “SR” and “AF” indeed demonstrate a significant difference in APD heterogeneity (ΔAPD 38 in SR vs. ΔAPD 191 in AF). At the core,
there is a clear boundary where the wavefront, with a very short APD, meets the wavetail with a longer APD. It is not surprising however that we did not observe a dramatic change in the minimum APD observed because the AF model did not induce the dramatic increases in frequency that are observed in humans (up to 16-18 Hz), likely relating to the conduction velocity as the rate limiting component.

5.3.2 Conduction velocity

The conduction velocities observed in our model system are much slower than that seen in cardiac tissue in vivo. There are several possible explanations for this finding, which has been recapitulated by many other groups using hPSCs in a cell sheet format. As discussed, the RMP of the cells is less negative than that seen in vivo, leading to reduced availability of sodium channels which are a major contributor to the propagation velocity. This was demonstrated in our single cell patch clamping studies, and has also been observed in micropipette recordings of our cell sheets (Appendix Figure 1).

Another major contributor to conduction velocity is the density and composition of gap junctions. Conduction velocity however is determined by the structural organization of gap junctions as well which co-localize with sodium channels at the intercalated disks. Intercalated disks are found on the shortest edge of two neighbouring cardiomyocytes thus facilitating more rapid conduction of electrical signals in the longitudinal direction. In cell sheets of hPSCs, gap junctions are in contrast found on all sides of the membrane. A recent study has however demonstrated that over time and up to one month in culture, hPSC derived cardiomyocytes display increasingly developed intercalated disks, including attachment zones and gap junctions, compared to at baseline. This increasing degree of ultrastructural organization was associated with increasing
conduction velocity in the cell sheets over time. We hypothesize that this is the underlying mechanism for our observation that spontaneous rotors were less prevalent in our “atrial” cell sheets over time. Finally, the non-cardiomyocyte population in our cell sheet cultures could significantly impact on conduction velocity.

Most important for the applicability of our model to serve as a drug screen is the response of our system to AADs with known mechanisms of action. The first AAD that we chose to test in our model system was Dofetilide, a class III anti-arrhythmic that selectively inhibits the rapid component of the time-dependent outward potassium current ($I_{kr}$)\textsuperscript{75}. Dofetilide is a potent AAD which has demonstrated clear utility in the management of AF\textsuperscript{78}, however its use has been restricted by its lack of chamber specificity, causing significant APD prolongation of the ventricles thus putting patients at risk of sudden cardiac death. As previously mentioned, hPSC derived cardiomyocytes have previously been shown to be more sensitive to the effects of $I_{kr}$ blockade than other animal models, implicating $I_{kr}$ as a principal component of hPSC cardiomyocyte repolarization\textsuperscript{221}. Our study is the first to demonstrate the impact of dofetilide on APD prolongation in hESC derived atrial like cardiomyocytes, the first to demonstrate OAPD prolongation in a tissue model of hPSC derived cardiomyocytes, and the first to interrogate the effects of dofetilide on an ongoing rotor in an hESC derived tissue model of AF. As we would expect, dofetilide had no effect on conduction velocity over the cell sheets, and did not disturb conduction velocity at any site of the ongoing spiral wave.

### 5.3.3 Effects of dofetilide on “atrial” cell sheets

Dofetilide alone did not convert “AF” in our dish to “SR”. Previous studies have suggested that atrial wavelength plays a critical role in the antifibrillatory action of AADs
as wavelength prolongation was associated with atrial refractory period, and mapping of AF on AADs showed a reduction in the number of wavelets observed. Other groups have proposed that the antifibrillatory effects are indirectly associated with wavelength prolongation, and directly linked to prolongation of the excitable gap during periods of AF. According to the multiple-wavelet hypothesis, the stability of AF is determined by the number of wavelets. Widening of the excitable gap would lower the chance that fibrillation waves encounter areas of partially refractory tissue and thus slowing, and fractionation of wavelets will occur less frequently. Furthermore, widening of the excitable gap would promote fusion, and on balance, the generation and extinction of fibrillatory waves would be expected to be reduced. We have demonstrated our ability to overdrive pace ongoing rotors easily when treated with dofetilide, which was not the case prior to the application of the drug. This indirectly points to dofetilide’s action on extending the excitable gap, and supports the notion that fusion and extinction of rotors are important components of the conversion of AF to SR. Further in support of this theory are the mechanisms of action of flecainide, the second antiarrhythmic trialed in our model.

5.3.4 Effects of flecainide on “atrial” cell sheets

We chose to study flecainide as it is a commonly used AAD for the treatment of AF. Flecainide is a class I antiarrhythmic agent known to depress the maximum upstroke velocity of APs in atrial and ventricular tissue relating to a block of the sodium current (\( I_{Na} \)). Flecainide has been shown in a number of models to decrease conduction velocity significantly in the setting of AF without a significant effect on ERP. During functional reentry, the electrical wavefront is circulating around an area of functional
conduction block, often making sharp turns at pivot points. This would predict that the wavefront would be forced to increase its curvature and thus set up a source-sink mismatch resulting in conduction delay at the pivot points. This mismatch could theoretically be preferentially aggravated by flecainide’s effects on slowing of conduction velocity, and has been proposed as a mechanism explaining flecainide’s clinical efficacy. Since AF relies upon the random reentry effects of wavelets entering an area previously activated by another wavelet, drugs that increase the size of the functional circuit, and decrease the number of wavelets available, would in effect increase the excitable gap. The dogma that flecainide has no effect on ERP has been questioned, and may in fact be specific to the AF model in question. Flecainide has been shown to terminate AF in the vagotonic model of canine AF by causing tachycardia dependent increases in atrial refractoriness and wavelength. This has been attributed to the drug’s effect on decreasing atrial APD accommodation during periods of increased heart rate, possibly related to an effect on repolarizing potassium current, as well as decreasing the heterogeneity of atrial activation. Rate-dependent sodium channel block however could also explain the observed prolongation in refractoriness independently of changes in repolarization by depressing atrial excitability. Complicating the issue further is the observation that flecainide has shown differential effects on potassium currents in animal models and humans. As an example, the Kv1.5-based human Ikur has been proposed as a potentially interesting target for novel therapies of AF, with numerous compounds now undergoing clinical testing. The human channel is however resistant to flecainide, whereas the dog counterpart has been shown to be quite sensitive, highlighting the importance of using human tissue for drug screening.
We have shown that flecainide appears to have an effect on conduction velocity slowing in our model of AF. We also noted flecainide’s effect on cycle length without a clear independent effect of APD prolongation. While this data is clearly preliminary owing to the limited number of repeated experiments performed, we plan on further testing to determine the reproducibility of our findings. The following discussion is based on our preliminary observations which will form the basis for hypotheses that have yet to be tested.

As previously discussed, the known clinically efficacy of class Ic drugs is seemingly paradoxical in light of our understanding of wavelength dependent reentry when considering only the effects of conduction velocity. Anything that slows conduction velocity without altering refractoriness would be expected to decrease the wavelength and thus increase the propensity of tissue to develop reentrant arrhythmias. We have in fact clearly demonstrated this principal in our model with our unique ability to optically map rotors, and their response to drug effects. On one occasion, the addition of flecainide to a stable rotor induced by burst pacing had a dramatic effect on conduction velocity slowing that was not accompanied by a substantial increase in APD. This slowed the cycle length of the rotor and facilitated the initiation of two separate rotors in an “atrial” cell sheet (Appendix Figure 2). This finding, although predictable, would argue against the notion that flecainide decreases the number of wavelets in AF and thus would require an alternate explanation for its observed anti-fibrillatory effects. We propose an alternate mechanism, based on early preliminary findings that relate to the regional variation of drug effects along the curvature of an ongoing rotor.
Flecainide has been shown to produce rate-dependent reductions in atrial conduction velocity as well as reducing the heterogeneity of wavelengths across the atria. We have observed regional variation in the effects of conduction velocity slowing with the addition of flecainide to “AF” in our model. There was an effect on conduction velocity slowing at points along the spiral wave that had the broadest curvature, where the conduction velocity was greatest at baseline, while there was seemingly no effect on conduction velocity at the centre of the spiral where the wavefront curled tightly around the PS, corresponding to the area with the slowest conduction velocity at baseline.

Flecainide is known to cause potent voltage-and frequency-dependent inhibition of cardiac Na+ channels. Voltage gated sodium channels can exist in three distinct states; deactivated (closed), activated (open) or inactivated (closed). With the membrane at its resting potential, the Na+ channels are in their deactivated states. Activation gates subsequently open in response to an electrical current, thus allowing Na+ ions to flow into the cell and causing the membrane potential to increase and thus depolarize. At a specific membrane potential, the Na+ channels become inactivated until the membrane potential becomes negative enough and the cell returns to its deactivated state, ready to participate in the subsequent depolarization event.

Flecainide has a strong preference for binding to the activated states of the sodium channel (ie open and inactivated) and its effect is enhanced by rapid repetitive depolarizations, and increases over the range of voltages where the channels activate. This provides one potential explanation for our preliminary observation in that the centre of the rotor may be in a relatively deactivated state, while cells along the broader
curvature that demonstrate a more rapid conduction velocity before treatment with flecainide, are in relatively activated states and thus preferentially effected by flecainide. This would certainly be supported by the observation that conduction velocity in the rotor is lowest at its center and increases as a function of distance from the centre if the observed conduction velocity reflects the active and inactivated states of the sodium channel. It however directly contradicts the observations made by Allessie in a commonly used and referenced goat model of AF. Allessie described a preferential effect of class I drugs at areas where the rotor curvature was steepest, preferentially depressing conduction velocity of wavelets a their pivot points 236.

An alternative explanation for our preliminary finding is that it is in fact calcium currents and not sodium currents that predominate and drive the wavelets surrounding the rotor’s core in our model. It has been well established that patients who have had prolonged durations of AF show less of a response to both Na+ and K+ blocking agents 241. This has been a proposed consequence of changes in ion channel function during atrial remodelling in response to tachycardia and AF, a phenomenon known as “AF begets AF”. In light of our observation, it is interesting to reflect on the fact that T-type Ca+ channel blockers appear to suppress atrial remodelling whereas L-type Ca+ channels blockers are ineffective. In addition, Bepridil which acts on both L-type and T-type Ca+ channels has an unusual ability to convert long-standing AF242. It is possible therefore that this hypothesis holds true in human AF. Our enthusiasm for this observation is tempered by questions relating to the fundamental translational capacity of our model, specifically when interrogating ion channels that have a signature in our model that is quite distinct from the adult phenotype.
A feature of electrophysiologic immaturity, not previously discussed in this work, is the expression of T-type calcium channels. These channels help to confer automaticity in differentiating cardiomyocytes. In embryonic and neonatal cardiomyocytes, T-type calcium channels have been observed at higher densities and their expression nearly disappears in the working myocardium of the adult heart. Although difficult to quantify in terms of current because of the lack of specific blockers, greater than 50% of hiPSC derived cardiomyocytes display functional $I_{\text{Ca,T}}$. If we are therefore able to go on to conclusively demonstrate that the regional variation in flecainide’s effect on our model system is dependent on the function of T-type Ca$^+$ channels near the rotor, we would have to go on to demonstrate the applicability of this finding to the human system. This is of course a limitation of all model systems, emphasized here to contrast cell types, highlight limitations, and provide a cautionary note on the translation of this novel model.
Chapter 6

Future Directions

The immediate path forward is clear, and our experience to date has taught us important lessons regarding the limitations related to our current model system. Our future directions are divided into three main themes: 1) the validation of findings to date 2) the continued interrogation, refinement and improvement of our model system to study atrial remodeling 3) adapting our model to answer new fundamental and clinically relevant questions related to the identification, prevention and treatment of AF.

6.1 Validation of current findings

The principle outcomes under study have been the components of the atrial wavelength, namely the action potential duration and the conduction velocity. In order to validate our findings, we have incorporated micropipette recordings into our optical mapping setup. We have successfully recorded intracellular potentials using high resistance micropipettes, and will use these to quantify the effects of AADs on the AP morphology and APD measurements as a standard with which to judge the accuracy of our OAP recordings. Using this technique we have also begun to validate our conduction velocity measurements, and generate restitution curves for conduction velocity as a function of rate, using our stimulation probes and micropipette recordings. By measuring the time taken from stimulation on one end of the cell sheet to the depolarization of a cell on the opposite side of the cell sheet, and measuring the distance travelled, we have begun to generate accurate measurements of conduction velocity while simultaneously optical mapping to ensure point source stimulation and uniform unidirectional propagation. Finally, we are generating “ventricular” cell sheets in order to contrast our findings with a
distinct cell type which should display differences in the determinants of wavelength at baseline as well as different responses to AADs. We have already observed a significant decrease in the propensity of “ventricular” cell sheets to generate rotors using our MEA protocol, and expect to demonstrate the same findings in our optically mapped cell sheets. To further validate our findings, we plan on testing AADs that have atrial specific effects and demonstrating the predictable nature of responses in our “atrial” cell sheets compared to “ventricular” cell sheets. This would include more recently proposed agents for the treatment of AF: Tertiapin-Q, an I_{K,ach} blocking agent, and Ca-activated K+ channels blockers. In addition, we propose to analyze the effects of vernakalant, which preferentially blocks atrial-specific Kv1.5-based I_{Kur} in many models including atrial like hESC derived cardiomyocytes. Vernakalant is used exclusively for cardioversion with high efficacy, and thus is of interest to be tested in our model that has not yet demonstrated purely pharmacologic cardioversion.

In order to further interrogate our model, we plan on examining the structural components of our cell sheets in greater detail. Our first step will be the identification and quantification of Cx-40, a gap junction that is selectively expressed in atria. Another principle component of conduction velocity and its heterogeneity across cardiac tissue that we have not yet addressed is anisotropy, which will be interrogated by analyzing the gross arrangement of cells in culture through immunostaining and/or electron microscopy. Once quantified, it would be ideal to incorporate these structural measures, as well as the ionic determinants of wave propagation, into a mathematical model that could complement findings from the in vitro model as well as help direct future experimentation. There are 5 models of human atrial electrophysiology that have been
published\textsuperscript{248}. Our in vitro model could help to evaluate and refine these models. Collectively these tools could serve to provide an in vitro and in silico assessment of known and novel therapeutics.

The major limitation of our study has been our reliance on currently available voltage sensitive dyes. We have trialed 3 such dyes (Di-4-ANEPPS, Di-8-ANEPPS, and RH237) in our model system, and ultimately chose to optimize and utilize Di-4-ANEPPS as it displayed the best signal to noise (S:N) ratio on our optical mapping setup. These dyes all have the limitation of generating poor S:N ratios in the cell sheet format, and rapidly photobleaching thus limiting the recording time available when using a high intensity light source. For future studies we will use hESCs expressing Arclight\textsuperscript{249}, with better (~2-fold) S:N, and virtually no photobleaching compared to Di-4-ANEPPS\textsuperscript{249}. ArcLight is a novel genetically encoded voltage probe, developed by fusing the voltage sensor domain of the Ciona intestinalis voltage-sensitive phosphatase to a super ecliptic pHlurion carrying the point mutation A227D\textsuperscript{250}. Arclight has already demonstrated its utility in measuring APs noninvasively using optical mapping of hESC derived cardiomyocytes which quantitatively and qualitatively track with patch clamp recordings\textsuperscript{249}. Arclight is typically introduced into cells using transient transfection or lentiviral transduction. We have already acquired an hiPSC line that stably expresses Arclight from our collaborator. This line has successfully been differentiated using the EB protocol previously described. The derivative cardiomyocytes generated OAPs with excellent S:N that do not exhibit perceptible signs of photobleaching (Appendix Figure 3). Arclight has a relatively sluggish response time compared to Di-4-ANEPPS which will significantly affect estimates of AP upstroke (underestimated by ~30%), however will impact immeasurably
on key mapping results (activation/phase, CV and APD). We will overcome this limitation by recording APs using micropipettes, as previously described, which will be used to "calibrate" optical data.

Extracellular field recordings (ALA multi-electrode array or MEA system) will be used to: a) monitor electrical activity thereby (crudely) validating our activation/CV plots, b) assess the P-wave and local electrogram “fractionation”, which links to arrhythmia susceptibility\textsuperscript{251-253}, and 3) induce and terminate arrhythmias with appropriate pacing. This last point will be a necessary component of future studies, removing the necessity of introducing an unsterile pacing electrode into the tissue culture medium and thus preserving it for repeated studies. The incorporation of a stable and sterile pacing platform as well as the utility of Arclight in repeated measures over time, will allow us to interrogate a question that remains fundamental to the understanding and management of AF and has been alluded to repeatedly in this document, that is “AF begets AF”.

\textbf{6.2 Study AF remodeling}

Regardless of its ultimate cause, AF invariably begins as isolated short-lived episodes called "paroxysmal AF". Each paroxysm accelerates atrial remodeling thereby promoting "persistent AF"\textsuperscript{42,43}. The functional, structural, electrical and biochemical changes in atria leading to increased AF vulnerability, providing the substrate for AF maintenance, and making cardioversion increasingly difficult, are called remodeling\textsuperscript{14,15,254}. Although some clinical predictors of the transition from paroxysmal to persistent AF have been identified, the molecular, biochemical and electrophysiologic mechanisms for this remodeling remain largely unknown\textsuperscript{255,256}. We propose to utilize our novel model of AF to study the molecular, cellular and electrical changes underlying AF remodeling. As AF-
begets-AF, we hypothesize that rotor induction will drive time-dependent remodeling. Indeed, APD and CV heterogeneity have been shown to develop in our cell sheets with rotors. Moreover, we observe spontaneous rotors in a small subset of our cell sheets which implies the presence of triggers. Thus, our cultures display dynamic changes in wavelength (APD and CV) as well as triggers, the two key components of the AF syndrome, which is unlike mouse/goat/dog/sheep animal models in which triggering is externally induced.

To look at the effects of AF on remodelling in our “atrial” cardiomyocyte cultures, we propose to make measurements at 3 time points after the induction of stable rotors: after 1 day, representing early clinical AF, after 7 days, representing the clinical time point used to diagnose persistent AF, and after 30 days, representing chronic persistent AF. Control cultures will be maintained in regular “sinus rhythm” (“SR”) for equivalent periods. We will also study the effects of reverse remodelling; if possible, “SR” will be restored in the chronic persistent “AF” group and then studied 1 week later along with a time matched control. Since AF is ultimately an electrical phenomenon, we will use our optical mapping rig to interrogate the electrical remodelling process as previously described. Of course with the ability to make repeated optical mapping recordings over time, multiple timepoints can be chosen in each cell sheet until they reach a “terminal” endpoint, such as enzymatic digestion for single cell analysis. In addition we will be able to interrogate other important measures of electrical remodelling that require exposure times that exceed the capabilities of Di-4-ANEPPS because of the photobleaching and toxicity previously described.
Using the Arclight line we will be able to routinely measure the effective refractory period (ERP) with programmed external stimulation, as is routinely done in human subjects. This is achieved using trains of 8 field pulses at 500 msec (S1) followed by variable shorter cycle pulses (S2) until capture does not occur. S1 is also varied (400, 300, 200) allowing ERP to be determined at multiple cycle lengths. We expect that the longer cell sheets are kept in "AF", the easier it will be to generate rotors, and these differences will correlate systematically with differences in (inter-related) rotor/electrical properties such as shorter APD, shorter ERP, faster rotor frequencies, greater rotor curvatures, slower CV, and more complex activation patterns or wave breaks. Resting membrane potentials may become more negative (more background I_{K1}). These hypotheses will be tested by performing single cell cardiac electrophysiology studies using patch clamping in order to dissect the observed alterations caused by electrical remodelling into the functions and interactions of single channels and currents. To complement these single cell studies, RNA will be isolated for targeted qRT-PCR analysis. A future consideration could be the inclusion of RNA seq analysis.

To this point we have focused on the rapid pacing model of AF. We propose however to test multiple acquired models of AF in the future using the aforementioned study design. We currently are focused on three potential acquired stressors that we propose to include in future studies; elevated parasympathetic activity, stretch, and fibrosis.

6.3 Model other types and features of acquired AF

Previous studies have established the involvement of elevated parasympathetic activity and related I_{K,ACH} activity in AF which has resulted in efforts to develop blockers of these channels. We will look for changes in the expression pattern of I_{K,ACH}, GIRK1 and
GRK4, M2- muscarinic receptors, and RGS regulator proteins, as well as constitutive activity of GRK-related channels\textsuperscript{264}. We will also perform studies and analyses in the presence of low levels of the stable muscarinic agonist, carbachol. We predict that low levels of carbachol will be associated with electrophysiologic markers of maturation, and that higher levels of carbachol will facilitate wavebreak and multiple wavelets degenerating into chaotic activity.

Elevated venous/atrial pressure and atrial stretch are major factors in AF strongly stimulating cardiac remodeling via signaling pathways leading to increased oxidative stress\textsuperscript{18,265-267}. Atrial stretch shortens atrial ERP through increased $I_{\text{K,Acch}}$, $I_{\text{cl}}$, and $I_{\text{k1}}$\textsuperscript{268,269} as well as $I_{\text{C,L}}$ inactivation\textsuperscript{270} and reduced gap junctions\textsuperscript{271}. We plan on investigating the effects of cyclical stretch on hESC derived cardiomyocytes and looking at measures of maturation and remodeling.

Finally, since many acquired forms of AF demonstrate increased interstitial atrial fibrosis, which correlates inversely with treatment success\textsuperscript{272-275}, we will combine cardiac fibroblasts with our atrial CMs. Our initial experiments either used a cell sorting strategy to purify the population to contain only cardiomyocytes, or incorporated ill defined CD90+ population. We propose to repeat the same studies in cultures containing various percentages of cardiac fibroblasts generated from hESCs using a published protocol\textsuperscript{276}. It has already been established that CD90+ cells improve maturation of cardiac gene expression patterns, and various structural/functional measures\textsuperscript{277}. In addition, fibroblasts secrete endothelin-1 which promotes the generation and persistence of myofibroblasts, and induce the expression of a wide variety of ECM components, including collagen type I.
In contrast to other fibroblast sources, generating cardiac fibroblasts from hESCs allows for the production of pure unlimited numbers of well characterized cardiac fibroblasts that do not proliferate uncontrollably in our backbone culture medium. These are novel studies with uncertain outcomes; nevertheless, we do expect that the cell sheets in which “AF” has been induced will have increased accumulation of fibroblasts and connective tissue since AF induces atrial fibroblasts (myofibroblasts) to proliferate and deposit extracellular matrix. This is expected to alter tissue architecture and myocyte-to-myocyte electrical coupling as well as to create additional capacitive loads on CMs. We anticipate that the end result will be collective interference with electrical conduction and increased propensity to arrhythmia initiation and maintenance. All of these properties can be readily determined using the approaches detailed already. This novel model system could be a powerful platform for developing new comprehensive therapies for treating AF that specifically target fibrosis.

6.4 Introduce genetic variation as a determinant and model of AF

Building on the lessons learned from our model of acquired AF, we propose to study heritable forms of AF using hiPSCs and/or genomic editing. We have learned from the Framingham studies that 58% of AF patients have no obvious cardiac disease or risk factors. AF incidence shows a strong heritability, and a genetic predisposition has been shown to contribute to AF risk. Recently numerous AF associated mutations, candidate genes and risk loci have been identified, yet few functional studies have deciphered their mechanisms of action or validated their role in disease. We propose to employ our model of human “atrial” cardiomyocytes derived from hESCs to study the underlying mechanism for AF vulnerability in these patients with the potential to identify novel
therapies. As a first test of our hypothesis, we will use the CRISPR/Cas9 system to introduce genetic changes into a background of normal hESCs. As a test case, we propose to study the effects of GATA6 (heterozygous and homozygous) mutations in our system. Autosomal dominant mutations in GATA6 have been identified in cases of loneAF. GATA6 was chosen for several reasons; firstly, it has recently been linked to AF in rare families with mendelian inheritance patterns and strong penetrance and expressivity. Second, it is a transcription factor expressed in the early cardiac mesoderm. Third, although GATA6 has been implicated in normal gap junction expression during periods of stress or injury in the atria, GATA6 is likely (by virtue of being a transcriptional factor) to affect the expression of many genes and could provide some interesting clues into multiple functional genes impacting on AF, which could yield greater insights into AF mechanisms more broadly. To complement this data set, we propose to differentiate pure populations of nodal, atrial, and ventricular cell types from the genomically edited hESCs in order to study the effects of these mutations on the different cardiac lineages. By studying the impact of various drug treatments, we can determine whether and how the atrial remodeling and AF induced by GATA6 mutations relate to the remodeling induced by AF itself. If the mechanisms are quite different, these studies could be used to identify personalized optimal drug therapies for these patients carrying these mutations, as well as numerous other patients who may have as yet unidentified mutations. Most importantly, this type of investigation has the potential to generate and test new hypotheses regarding propensity to and mechanism of disease that may be potential preventative and/or therapeutic targets.
Other gene mutations will also be studied such as the TBX5 gene which causes Holt-Oram syndrome and has been linked to lone AF\textsuperscript{290,291}. TBX5 likely lies upstream of ANF and Cx-40, and may contribute to Nav1.5 expression\textsuperscript{290,291}. TBX5 mutations have been associated with sinus node disease, AV node disease as well as QT prolongation\textsuperscript{292}. Most recently, TBX5 has been identified as a novel locus for AF in a population of over 7000 European and Japanese patients with lone AF\textsuperscript{293}. Although informative, the analysis of isolated mutations in the backbone of an otherwise genetically normal stem cell line will require complementary studies using hiPSCs. The strength in the hiPSC model lies in the fact that it encompasses a host of unknown complementary and confounding genetic variations which may play an indispensable role in the expression of the disease experienced by the patients.

The final future direction to be discussed is the incorporation of bioengineering into our AF model, that will use engineered heart tissue for analyses. Although the 2D geometry of atrial cell sheets mimic in many respects that of atrial tissue, which is very thin and unlikely to support 3D rotors (scroll waves), there are unwanted edge effects which impact on source-sink matching, and have no parallels in human atria. Our atrial cell sheets also show characteristics consistent with immaturity such as spontaneous beating. Although it is conceivable that our cell sheets will mature with extended time in culture, we have found limitations to time dependent maturation, consistent with other similar studies\textsuperscript{153,244,294}. In fact, studies of heart development show us that cardiac cells require many environmental cues to drive adult patterning. Other efforts to promote maturity have focused on electrical stimulation, mechanical strain, biochemical agents, co-cultures, 3D culture, extracellular substrates, and genetic manipulations\textsuperscript{224}. We plan to
study the impact of geometry and immaturity simultaneously using co-cultures of cardiac fibroblasts (as previously described), as well as alternative sources of human cardiac fibroblasts and CD90+ cells mixed with cardiomyocytes and incorporated into 3D biowires\textsuperscript{295}. Biowires can be mechanically loaded, electrically stimulated, and exposed to selected chemical inducers of maturation. We will study biowires in detail using the same approaches previously outlined including arrhythmia induction (i.e. complete electrical datasets, beating rates, various membrane currents). We know that atrial cell sheets support sustained rotors much more easily than ventricular cell sheets, and expect that sustained rotors will be readily induced in atrial biowires. We will screen using markers of structural maturation such as sarcomeric banding and myofilament density on immunofluorescence and electron microscopy, physiologic maturation by examining the amplitude of calcium transients and the shape and resting membrane potentials of optically mapped and micropipette recorded action potentials, and finally looking at effects on cell cycle activity as well as monitoring mRNA and protein levels \textsuperscript{296, 297, 296}.

Numerous studies have shown that cell responses to drugs in 3D culture better predict in vivo tissue functionality compared to 2D high throughput screens. On the other hand, high throughput drug screening technologies relying on single cells or looking at single biochemical or gene expression assays have been enormously successful in testing and developing novel compounds cheaply and quickly. Most recently there has been a movement towards tissue based assays, or organ-on-chip platforms for high throughput assays to improve the translational relevance of these screens by approximating more closely the disease or the organ of interest. We would propose that our model is well
suited for the application of high throughput screening technologies once we can generate tissue efficiently and reproducibly that has predictable performance parameters.

The strength in our model is the extreme nature of control which can ultimately be exerted over countless variables, from the genetic make-up of the cell to the cell’s environment. The challenge henceforth will be to understand each of these variables under a defined set of extreme conditions such that the model’s behavior can be understood and evaluated. A further challenge will be the scalability of the readout analysis which must be interpreted in quantifiable patterns, and with the assistance of computer learning, rather than the current form of qualitative observations and semi-quantitative recordings that are hypothesis generating. The experiments described in our future directions will define the most reliable measures, and help to focus our attention on the outputs with the greatest signal. Once identified, and before a major investment into scalability, these variables must be strictly correlated with important clinical endpoints to ensure that our model has the capacity to exert a positive impact on patients for whom these studies have been devised.
References


Guedon-Moreau, L. *et al.* Impact of the control of symptomatic paroxysmal atrial fibrillation on health-related quality of life. *Europace: European pacing,
arrhythmias, and cardiac electrophysiology: journal of the working groups on cardiac pacing, arrhythmias, and cardiac cellular electrophysiology of the European Society of Cardiology 12, 634-642, doi:10.1093/eurpae/euq007 (2010).


Campbell, T. J. Kinetics of onset of rate-dependent effects of Class I antiarrhythmic drugs are important in determining their effects on refractoriness in guinea-pig ventricle, and provide a theoretical basis for their subclassification. *Cardiovascular research* **17**, 344-352 (1983).


172 Ackerman, M. J. *et al.* HRS/EHRA expert consensus statement on the state of genetic testing for the channelopathies and cardiomyopathies: this document was developed as a partnership between the Heart Rhythm Society (HRS) and the European Heart Rhythm Association (EHRA). *Europace : European pacing, arrhythmias, and cardiac electrophysiology : journal of the working groups on cardiac pacing, arrhythmias, and cardiac cellular electrophysiology of the European Society of Cardiology* **13**, 1077-1109, doi:10.1093/europace/eur245 (2011).


175 Schwartz, P. J. *et al.* Who are the long-QT syndrome patients who receive an implantable cardioverter-defibrillator and what happens to them?: data from the European Long-QT Syndrome Implantable Cardioverter-Defibrillator

Schwartz, P. J. et al. Left cardiac sympathetic denervation in the management of high-risk patients affected by the long-QT syndrome. *Circulation* 109, 1826-1833, doi:10.1161/01.CIR.0000125523.14403.1E


Ackerman, M. J. et al. HRS/EHRA expert consensus statement on the state of genetic testing for the channelopathies and cardiomyopathies: this document was developed as a partnership between the Heart Rhythm Society (HRS) and the European Heart Rhythm Association (EHRA). *Europace* 13, 1077-1109, doi:eur245 [pii] 10.1093/europace/eur245 (2011).


Nygren, A., Lomax, A. E. & Giles, W. R. Heterogeneity of action potential durations in isolated mouse left and right atria recorded using voltage-


Hanna, N., Cardin, S., Leung, T. K. & Nattel, S. Differences in atrial versus ventricular remodeling in dogs with ventricular tachypacing-induced


297 Yang, X. *et al.* Tri-iodo-l-thyronine promotes the maturation of human cardiomyocytes-derived from induced pluripotent stem cells. *Journal of
Appendix Figure 1: Intracellular recordings of a “ventricular” cell sheet performed with a high resistance (~30 MΩ) thin walled glass micropipette. Sharp spikes represent pacing artifact. The cell sheet was paced from the edge of one side of the cell sheet, and a cell on the opposite side was impaled for this recording. The conduction velocity was calculated to be ~4 cm/s for this recording. The resting membrane potential, upstroke velocity and action potential amplitude mimic those recorded from single cell patch clamping studies. In particular, the resting membrane potential sits at ~-55 mV, essentially the same as seen in single cell studies.
Appendix Figure 2: Activation maps generated from recordings of an “atrial” cell sheet and the sequential uptitration of flecainide dosing using DI-4ANEPPS and an EMCCD. Activation maps demonstrate the dose dependent effects of flecainide on the cycle length and the number of excitation wavelets in the cell sheet. Initially a stable rotor is induced on the left. After the addition of 5 μM flecainide, the cycle length is substantially reduced and the curvature of the rotor is decreased without affecting the location of the phase singularity (PS)(labeled as a white star on figure). After the addition of 10 μM flecainide, two distinct rotors appear, each rotating around their own PS independently and causing wave collision along sites of wavefront interaction. Wave collision did not generate more wavelets in this cell sheet, and instead the activation wavefront extinguished at the site of collision.
Appendix Figure 3: Optical Action Potentials (OAPs) recorded using the Arclight hiPSC cell line differentiated into cardiomyocytes and recorded on an EMCCD. Cells contain a genetically encoded voltage sensor attached to eGFP which reports a change in membrane voltage as changes in fluorescence. Note the 20% change in fluorescent signal intensity (Y axis) demarcating depolarization of the hiPSC derived cardiomyocytes. Note also the absence of photobleaching over 20 seconds of recording time, overcoming a major limitation of the voltage sensitive dyes. This was repeated on many occasions on the same day and on sequential days for up to 1 week with preservation of signal.