The Effect of Biomimetic Tissue Engineering Constructs on the Phenotype of Immature Cardiomyocytes

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Mum, Dad and Kathie
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Declaration of Originality

The work presented in this thesis is my own work, supported by my supervisors. Where the thoughts, ideas, and work of others are presented, every effort has been made to ensure that it is appropriately acknowledged and referenced.

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Abstract

Several studies have suggested that induced pluripotent stem cell-derived cardiomyocytes (iPSC-CM) and human embryonic stem cell-derived cardiomyocytes (hESC-CM) are sufficiently comparable to adult myocardium to facilitate application in a wide range of toxicology, drug development and disease modelling applications. Evidence from the literature is however, inconstant. We tested the hypothesis that iPSC-CM and hESC-CM have a predictable and consistent response to pharmacological manipulation, finding in many instances this was not true.

We then focused on methods to improve the maturity of iPSC-CM, in particular, on the effect of cell alignment on intra-cellular Ca\textsuperscript{2+} cycling. For much of the exploratory work and validation of the techniques we used neonatal rat ventricular myocytes (NRVM). They are more readily available than iPSC-CM and have similar properties. We hypothesized that cell alignment of immature cardiomyocytes, in a fashion analogous to the adult myocardium, would improve the speed of intra-cellular Ca\textsuperscript{2+} cycling. We found that structured culture modulated Ca\textsuperscript{2+} cycling in iPSC-CM, and that this was probably due to improvements in sarcoplasmic reticulum Ca\textsuperscript{2+} release mechanisms. In contrast to NRVM, structured culture did not appear to have a significant effect on Ca\textsuperscript{2+} extrusion mechanisms in iPSC-CM. Furthermore, we found that the physical properties of the constructs made it difficult to fully explore the mechanisms underlying these experimental findings. Consequently, we developed and validated novel constructs which would facilitate exploration of the mechanisms underlying the association between cell-alignment and the functional properties of immature cardiomyocytes.
These findings suggest that tissue engineering approaches are likely to be relevant to *in vitro* modelling with iPSC-CM. An important next step will be to conclusively demonstrate that these techniques overcome the limitations of iPSC-CM highlighted in the first part of this thesis, and improve their efficacy for myocardial disease modelling.
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<tbody>
<tr>
<td>2D</td>
<td>Two-dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ACTB</td>
<td>Gene Encoding β-actin</td>
</tr>
<tr>
<td>ACTN2</td>
<td>α-Actinin</td>
</tr>
<tr>
<td>AMPH2</td>
<td>Gene Encoding BIN1</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>APD90</td>
<td>Time to 90% Action Potential Repolarization</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>ATP2A2</td>
<td>Gene encoding SERC2a</td>
</tr>
<tr>
<td>AVN</td>
<td>Atrioventricular Node</td>
</tr>
<tr>
<td>BDM</td>
<td>2,3-Butanedione Monoxime</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic Fibroblast Growth Factor</td>
</tr>
<tr>
<td>BIN1</td>
<td>Bridging Integrator 1</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenetic Protein</td>
</tr>
<tr>
<td>Bry</td>
<td>Brachyury</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CACNA1C</td>
<td>Gene encoding Ca,1.2</td>
</tr>
<tr>
<td>CACNA1G</td>
<td>Gene encoding Ca,3.1</td>
</tr>
<tr>
<td>CALR</td>
<td>Gene encoding Calreticulin</td>
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cAMP  Cyclic Adenosine Monophosphate
CASQ2  Gene encoding Calsequestrin 2
Ca_{v}1.2  L-type calcium channel, alpha 1C subunit
Ca_{v}3.1  T-type calcium channel, alpha 1G subunit
CAV3  Gene encoding Caveolin 3
cDNA  Complementary DNA
CICR  Calcium Induced Calcium Release
CSC  Cardiac Stem Cells
CSC-CM  Cardiac Stem Cells derived Cardiomyocytes
c-kit  Mast/stem cell growth factor receptor (SCFR)
Cx43  Connexin 43
DAPI  4',6-diamidino-2-phenylindole
DCM  Dilated Cardiomyopathy
DHP Receptor  Dihydropyridine Receptor, Also known as L-type Calcium Channel
DKK 1  Dickkopf-related Protein 1
DMD  Duchenne Muscular Dystrophy
DMEM  Dulbecco’s Modified Eagle Medium
DMSO  Dimethyl Sulfoxide
DNA  Deoxyribonucleic Acid
dV/dt_{max}  Maximum change of voltage with respect to time, usually the maximal rate of cell depolarization
EB  Embryoid Body
ECM  Extracellular Matrix
EDTA  Ethylenediaminetetraacetic
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>EHT</td>
<td>Engineered Heart Tissue</td>
</tr>
<tr>
<td>END-2</td>
<td>Mouse Endoderm-like Cell Line</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic Stem Cell</td>
</tr>
<tr>
<td>ESC-CM</td>
<td>Embryonic Stem Cell-derived Cardiomyocytes</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>Flk1</td>
<td>Fetal Liver Kinase 1</td>
</tr>
<tr>
<td>FP</td>
<td>Field Potential</td>
</tr>
<tr>
<td>FPD</td>
<td>Field Potential Duration</td>
</tr>
<tr>
<td>G/P</td>
<td>Glass-Parylene-C Construct</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>GATA4</td>
<td>GATA binding protein 4</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GSK 3</td>
<td>Glycogen Synthase Kinase 3</td>
</tr>
<tr>
<td>H/H</td>
<td>Hydrophobic-Hydrophilic Parylene-C Construct</td>
</tr>
<tr>
<td>HCM</td>
<td>Hypertrophic Cardiomyopathy</td>
</tr>
<tr>
<td>HCN Channel</td>
<td>Hyperpolarization-activated Cyclic Nucleotide-gated Channel</td>
</tr>
<tr>
<td>HMDS</td>
<td>Hexamethyldisilazane</td>
</tr>
<tr>
<td>HEK 293</td>
<td>Human Embryonic Kidney 293 Cells</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid</td>
</tr>
<tr>
<td>hERG</td>
<td>Human Ether-à-go-go Related Gene (see KCNH2)</td>
</tr>
<tr>
<td>hESC</td>
<td>Human Embryonic Stem Cell</td>
</tr>
<tr>
<td>hESC-CM</td>
<td>Human Embryonic Stem Cell-derived Cardiomyocytes</td>
</tr>
<tr>
<td>HPPA</td>
<td>Atrial Natriuretic Factor</td>
</tr>
<tr>
<td>I_{Ca}</td>
<td>Calcium Current</td>
</tr>
<tr>
<td>I_{Ca,L}</td>
<td>L-Type Calcium Current</td>
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</table>
$I_{Ca,T}$ T-Type Calcium Current

$iCM$ Induced Cardiomyocytes

$I_f$ “Funny” Current

$I_{K1}$ Inwardly Rectifying Potassium Current

$I_{Kr}$ Rapid Delayed Rectifier Potassium Current

$I_{Ks}$ Slowly Activating Delayed Rectifier Potassium Current

$I_{Kur}$ Ultra-Rapid Delayed Rectifier Potassium Current

$I_{Na}$ Sodium Current

IP3R Inositol 1,4,5-Trisphosphate Receptor

iPSC Induced Pluripotent Stem Cell

iPSC-CM Induced Pluripotent Stem Cell-derived Cardiomyocytes

ISL-1 Insulin gene enhancer protein

ITPR2 Gene encoding IP3R

$I_{to}$ Transient Outward Potassium Current

JPH2 Gene Encoding Junctophilin 2

KCNQ1 Gene Encoding Potassium voltage-gated channel, KQT-like subfamily 1

KCNH Gene encoding $K_v11.1$ (see hERG)

KDR Kinase Insert Domain Receptor

KO-DMEM KnockOut Dulbecco’s Modified Eagle Medium

KOSR KnockOut Serum Replacement

$K_v11.1$ Alpha sub-unit of rapid delayed rectifier potassium channel

L-type “Long lasting”-type (Calcium Channel), Also known as DHP Receptor
LEOPARD Syndrome  Syndrome of Lentigines, Electrocardiographic conduction abnormalities, Ocular hypertelorism, Pulmonary stenosis, Abnormal genitalia, Retarded growth, and Deafness

LQTS1  Long QT Syndrome Type 1
LQTS2  Long QT Syndrome Type 2
LQTS2  Long QT Syndrome Type 3
LQTS8  Long QT Syndrome Type 8, Timothy Syndrome

MAP  Mitogen-Activated Protein

MDP  Maximum Diastolic Potential

MEA  Multi-Electrode Array

MEF  Mouse Embryonic Fibroblast

MEF-CM  Mouse Embryonic Fibroblast Conditioned Medium

MEF2c  Myocyte-specific enhancer factor 2C

MHC  Myosin heavy chain

MI  Myocardial Infarction

mRFP  Monomeric Red Fluorescent Protein

MSC  Mesenchymal Stem Cells

MYH6  Gene Encoding α-Myosin Heavy Chain

MYH7  Gene Encoding β-Myosin Heavy Chain

MYL2  Gene Encoding Myosin Light Chain 2 Ventricular Isoform

MYL7  Gene Encoding Myosin Light Chain 2 Atrial Isoform

NANOG  transcription factor important for self-renewal of undifferentiated stem cells

NCX  Na+/Ca2+ exchanger

NEAA  Non-Essential Amino Acids
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name and Definition</th>
</tr>
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<tbody>
<tr>
<td>NFATC4</td>
<td>Nuclear factor of activated T-cells, cytoplasmic 4 transcription factor</td>
</tr>
<tr>
<td>Nkx2.5</td>
<td>Homeodomain Transcription Factor 2.5</td>
</tr>
<tr>
<td>NKX2.5</td>
<td>Gene encoding Nkx2.5</td>
</tr>
<tr>
<td>NPPA</td>
<td>Gene Encoding Natriuretic Peptide A (ANF)</td>
</tr>
<tr>
<td>NRVM</td>
<td>Neonatal Rat Ventricular Myocytes</td>
</tr>
<tr>
<td>NT</td>
<td>Normal Tyrode’s Solution</td>
</tr>
<tr>
<td>Oct-4</td>
<td>Octamer-binding Transcription Factor 4</td>
</tr>
<tr>
<td>PCL</td>
<td>Poly(caprolactone)</td>
</tr>
<tr>
<td>PDMS</td>
<td>Poly(dimethylsiloxane)</td>
</tr>
<tr>
<td>PGA</td>
<td>Poly(glycolic acid)</td>
</tr>
<tr>
<td>PIPES</td>
<td>Piperazine-N,N'-bis(2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>PLA</td>
<td>Poly(L-lactic acid)</td>
</tr>
<tr>
<td>PLN</td>
<td>Phospholamban</td>
</tr>
<tr>
<td>PTPN11</td>
<td>Gene encoding the Tyrosine-protein phosphatase non-receptor type 11</td>
</tr>
<tr>
<td>PPIG</td>
<td>Gene encoding Peptidylprolyl isomerase G (Cyclophilin G)</td>
</tr>
<tr>
<td>PU</td>
<td>Poly(urethane)</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
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<tr>
<td>RN18S</td>
<td>Gene Encoding 18S ribosomal RNA</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RyR</td>
<td>Ryanodine Receptor</td>
</tr>
<tr>
<td>RYR2</td>
<td>Gene encoding RyR 2</td>
</tr>
<tr>
<td>SAN</td>
<td>Sinoatrial Node</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>SERCA</td>
<td>Sarco/Endoplasmic Reticulum Ca(^{2+})-ATPase</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
</tr>
<tr>
<td>SHG</td>
<td>Second Harmonic Generation</td>
</tr>
<tr>
<td>SLC8A1</td>
<td>Gene encoding NCX</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>SU-8</td>
<td>A commonly used epoxy-based negative photoresist.</td>
</tr>
<tr>
<td>T50</td>
<td>Time to 50% Decay</td>
</tr>
<tr>
<td>T90</td>
<td>Time to 90% Decay</td>
</tr>
<tr>
<td>TCAP</td>
<td>Gene Encoding Titin-cap (Telethonin)</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TNNT2</td>
<td>Gene encoding Troponin T type 2 (cardiac)</td>
</tr>
<tr>
<td>TRDN</td>
<td>Gene encoding Triadin</td>
</tr>
<tr>
<td>TTP</td>
<td>Time to Peak Transient or Action Potential Amplitude</td>
</tr>
<tr>
<td>T-tubule</td>
<td>Transverse Tubule</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra Violet</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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Publications

Original Research Supporting Thesis

- The Effect of Microgrooved Culture Substrates on Calcium Cycling of Cardiac Myocytes Derived from Human Induced Pluripotent Stem Cells
  

- Selective hydrophilic modification of Parylene C films: a new approach to cell micro-patterning for synthetic biology applications
  
  Biofabrication (Accepted BF-100012 5/0/2013)

Other Original Research

- A critical role for Telethonin in regulating t-tubule structure and function in the mammalian heart.
  

- Mechanical unloading reverses transverse tubule remodelling and normalizes local Ca2+-induced Ca2+-release in a rodent model of heart failure.
  

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Published Review Articles and Commentaries

- Tissue Engineering Techniques in Cardiac Repair and Disease Modelling.
  
  Rao C, Barratt H, Prodromakis T, Terracciano CM.
  
  Curr Pharm Des. 2013 Jun 18. [Epub ahead of print]

- Mechanical unloading and cell therapy have a synergistic role in the recovery and regeneration of the failing heart.
  
  Ibrahim M, Rao C, Athanasiou T, Yacoub MH, Terracciano C.
  

- Heterotopic abdominal heart transplantation in rats for functional studies of ventricular unloading.
  
  

Book Chapters

- Phenotype and Developmental Potential of Cardiomyocytes from Induced Pluripotent Stem Cells and Human Embryonic Stem Cell.
  
  Rao C, Terracciano C, Athanasiou T, Ali NA, Harding S.
  
Published Abstracts

- Pronounced stress-induced lethality in popdc1/2 null mutants.


- Sarcoplasmic reticulum calcium release in response to caffeine is more organised in cardiomyocytes differentiated from iPSC cultured on structured substrates suggesting a matured phenotype.


  Cardiovascular Research. 93:S68-S68. OXFORD UNIV PRESS (15 Mar 2012).

- Structured Culture Scaffolds Improve the Calcium Handling Properties of Cardiomyocytes Differentiated from Induced Pluripotent Stem Cells


  Biophysical Journal. Volume 102, Issue 3, Supplement 1, 31 January 2012, Pages 103a


Fellowships, Grants, Travel Awards and Prizes

Clinical PhD Fellowship
11/09  Wellcome Trust Foundation - £200,386

Grants
11/11  Cellular Dynamics - $5,000
Grant of i-cell cardiomyocytes
06/10  Wellcome Trust Foundation - £13,755
Animal models of cellular integration

Travel Awards
12/11/11 Council for Peripheral Vascular Disease Travel Scholarship
American Heart Association
02/05/10 Hong Kong RSD Travel Scholarship
University of Hong Kong – Imperial College London

Prizes
18/03/13 Best Cardiac Poster Prize
Society of Cardiothoracic Surgery of Great Britain and Ireland
Annual Meeting, Brighton
15/12/11 Best Poster Presentation, Departmental Research Day
Department of Surgery and Cancer, Imperial College London
Annual Meeting London
“The individuation field, then, is the agent which controls the growth of the different parts in a harmonious way so that a normal individual is formed. In later life, the individuation field splits up into smaller separate fields, such as leg fields, head fields, etc.”

(Waddington, 1935)
1 Introduction
1.1 Introducing the heart, pluripotent stem cells and tissue engineering

Existing therapies for end-stage heart failure are largely palliative. This is because the pathological adult myocardium has limited regenerative capacity. Cell therapies, using immature myocytes or undifferentiated stem cells, have been widely proposed as a means by which lost myocardial tissue could be replaced. It is also suggested that cell therapy can be used to improve the function of the endogenous myocardium (Nelson et al., 2009, Menasche et al., 2008, Abdel-Latif et al., 2007, Bolli et al., 2011, Makkar et al., 2012, van Laake et al., 2009).

In marked contrast to adult cardiomyocytes the immature cardiomyocytes proposed for cell therapy are readily accessible and easily maintained in culture without loss of phenotype. Many of these cell types have also demonstrated an impressive potential to proliferate, differentiate and form beating syncytia in vitro. As a consequence several authors advocate the use of immature cardiomyocytes as in vitro models of cardiac physiology and disease (Abdul Kadir et al., 2009, Carvajal-Vergara et al., 2010, Moretti et al., 2010, Tanaka et al., 2009, Brand et al., 2010, Sun et al., 2012, Lan et al., 2013).

The immature, heterogeneous phenotype of these cells, however, is arguably poorly representative of adult myocardium (Ma et al., 2011, Kehat et al., 2002, Itzhaki et al., 2011b, Matsa et al., 2011). It has been suggested that this may limit the utility of immature cardiomyocytes in cell therapy, disease modelling, drug development and toxicology screening (Liu et al., 2009, Oh et al., 2012). Interestingly, several strategies including tissue engineering approaches have been explored to modify the phenotype of immature cardiomyocytes in vitro (Liu et al., 2009, Shimazaki et al., 2008, Kraehenbuehl et al., 2008,
The following paragraphs discuss the characteristics of pluripotent stem cells (Section 1.2), how they are differentiated into cardiomyocytes (Section 1.3), and the functional and structural properties of cardiomyocytes derived from pluripotent stem cells (Section 1.4). The application of cardiomyocytes derived from pluripotent stem cells in myocardial cell therapy (Section 1.5) and disease modelling is discussed (Section 1.6). Tissue engineering technology currently available to manipulate the phenotype of immature cardiomyocytes is also briefly discussed (Section 1.7). Finally experimental plans are outlined to test the hypothesis that modification of the structural phenotype of immature cardiomyocytes using tissue engineering techniques can improve the degree to which immature cardiomyocytes could be considered to be representative of adult myocardium (Section 1.8). In particular, we focus on intra-cellular Ca$^{2+}$ cycling, which is fundamental to excitation contraction coupling, a central component of cardiomyocyte physiology.

1.2 Types of pluripotent stem cells

Stem cells are undifferentiated cells (Thomson et al., 1998, Takahashi et al., 2007). They can renew themselves indefinitely through mitotic cell divisions and have the capacity to differentiate into specialized cell-types (Thomson et al., 1998, Takahashi et al., 2007). Stem cells can be classified according to how many distinct cell types they can be differentiated into, a property often called “potency” or “differentiation potential”. Pluripotent stem cells such as embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC) are truly pluripotent and can be differentiated into any type of somatic cell. Multipotent stem cells
such as mesenchymal stem cells (MSC) can differentiate into cells from a number of related tissues. Oligopotent stem cells, often called progenitor cells, can differentiate into only a few cell types from a particular organ or organ system, such as cardiac stem cells (CSC). Finally, unipotent cells, whilst still maintaining the property of self-renewal, can only differentiate into one cell type, for example skeletal myoblasts (Figure 1.1) (Schöler, 2007, Hochedlinger and Plath, 2009). Whilst all of these stem cell populations have been applied in cardiac stem-cell therapy, the focus of this work will be on ESC and iPSC which have proven in vitro cardiogenic differentiation potential, and therefore the most relevance to myocardial disease modelling.

Figure 1.1. The developmental potential and epigenetic states of cells at different stages of development. A modification of C. H. Waddington's epigenetic landscape model, showing cell populations with different developmental potentials (left) and their respective epigenetic states (right). Developmental restrictions can be illustrated as marbles rolling down a landscape into one of several valleys (cell fates). Coloured marbles correspond to different differentiation states (purple, totipotent; blue, pluripotent; red, multipotent; green, unipotent). Examples of reprogramming processes are shown by dashed arrows. Adapted, with permission, from Hochedlinger K, et al. Development 2009;136:509-523 (Hochedlinger and Plath, 2009)
1.2.1 Human embryonic stem cells

ESC are extracted from the inner cell mass of pre-implantation blastocysts (Thomson et al., 1998). Human ESC (hESC) have many properties that would make them amenable to cell therapy; they are pluripotent and can be propagated in vitro for many months without loss of normal karyotype (Ying et al., 2003).

hESC have been applied to rodent myocardial infarction (MI) models. Graft survival has been demonstrated beyond 6 months after cell therapy, albeit without electrical integration into the host myocardium (van Laake et al., 2007). Encouraging data on global cardiac function has usually come from relatively short (4–8 week) studies (Caspi et al., 2007, Laflamme et al., 2007). However, assessment of cardiac function beyond 12 weeks does not demonstrate any significant functional improvement (van Laake et al., 2007). It has been suggested that the improvement observed with hESC-derived cardiomyocytes (hESC-CM) is due to paracrine actions (See Section 1.5), and that the paracrine effect is diminished beyond 12 weeks, resulting in a reduction in benefit. It has also been suggested that hESC-derived grafts often remain encapsulated in a thin layer of extracellular matrix components, and that failure to fully integrate into the host myocardium may also explain the absence of long-term benefit (van Laake et al., 2007). The use of rodent models has also limited assessment of the safety of hESC cell therapy as the tumorigenic potential of hESC-derivatives may be impaired even in immunocompromised rodent models (Erdo et al., 2003). Significant heart rate differences between humans (70 beats per minute) and rodents (600 beats per minute in mice) may also impair survival and electro-mechanical integration of hESC-CM in rodent models (Xiong et al., 2011). Furthermore, as hESC are derived from embryonic tissue (Thomson et al., 1998) they are associated with both ethical (Lo and Parham, 2009, Passier et al., 2008) and
potentially technical problems such as host immune rejection of transplanted cells when used in cell therapy (Boheler et al., 2002, Saric et al., 2008).

1.2.2 Induced pluripotent stem cells

iPSC were first described by Takahashi and Yamanaka in 2006 and share many properties with ESC (Takahashi and Yamanaka, 2006). iPSC are reprogrammed pluripotent cells that are created by forced expression of embryonic transcription factors in non-pluripotent cells, such as adult somatic cells (Takahashi et al., 2007, Yu et al., 2007). In addition to being pluripotent, like hESC, they can be readily cultured and transfected. iPSC, however do not require the ethically problematic destruction of embryos (Lo and Parham, 2009, Passier et al., 2008). Furthermore, as patient-specific iPSC-derived cardiomyocytes (iPSC-CM) can be generated, syngeneic transplantation is feasible, and host immune rejection can potentially be avoided (Boheler et al., 2002, Saric et al., 2008, Takahashi et al., 2007).

iPSC-CM demonstrate similar properties to hESC-CM (Zhang et al., 2009, Zwi et al., 2009). Early studies suggest that injection of iPSC into ischemic and non-ischemic rodent models of cardiomyopathy results in integration of iPSC-CM into the host myocardium with promising improvements in contractile performance, pathological remodelling and electrophysiological properties (Nelson et al., 2009, Mauritz et al., 2011). More recently, improvements in cardiac function and pathological remodelling have also been demonstrated using iPSC-CM cell sheets in a rodent model of ischemic cardiomyopathy (Miki et al., 2012).

Despite promising early results concerns remain about the safety of iPSC cell-therapy. It has been suggested that undifferentiated iPSC may elicit a significant host immune response
(Zhao et al., 2011). There are also concerns about the tumorigenicity of undifferentiated iPSC, both because of their pluripotent nature and because of the activation of oncogenes during reprogramming (Lee et al., 2013, Okano et al., 2013). Novel reprogramming that avoids viral-genome integration may attenuate safety concerns associated with activation of oncogenes, and injection of differentiated cells reduces both their immunogenicity and tumorigenicity (Araki et al., 2013, Guha et al., 2013, Lee et al., 2013). This, however, may reduce the efficiency of therapy, as terminally differentiated myocytes do not have the proliferative potential of pluripotent stem cells. Consequently, there would be limited expansion of transplanted cells following introduction into the host myocardium. The use of differentiated cardiomyocytes necessitates optimization of reprogramming, expansion, and differentiation technology to improve the efficiency of this process. Finally, purification technology would also need to be optimized to ensure that only a pure differentiated myocytes population was used for cell therapy, free of contamination with potentially oncogenic iPSC (Okano et al., 2013).

1.3 Differentiation of human embryonic stem cells and induced pluripotent stem cells into cardiomyocytes

Fundamental to the application of hESC and iPSC as cardiac disease models, and arguably in cell therapy, is the differentiation of pluripotent stem cells into cardiomyocytes. This can be achieved using a number of different methods.
1.3.1 The embryoid body method

The most commonly reported method of differentiating hESC and iPSC into cardiomyocytes remains the Embryoid Body (EB) System. First applied to hESC in 2001 by Kehat et al (Kehat et al., 2001), the EB method requires hESC or iPSC cultures to be dissociated into multi-cellular three-dimensional (3D) aggregates termed EB. EB are cultured in suspension and plated after 4–10 days. Within a few days the cells from the plated EB will differentiate into cells of all three germ-layer lineages, and cells will express cardiac markers (Kehat et al., 2001). Spontaneously contracting areas subsequently appear in the cells which grow out from the EB (Kehat et al., 2001, Xu et al., 2002, Habib et al., 2008). Functional and gene expression analysis suggests that these areas have a number of cardiomyocyte characteristics (Ameen et al., 2008).

The EB method for cardiac differentiation has several limitations. Its efficacy is highly variable due to the heterogeneous size and shape of aggregates which can differ significantly in size and morphology (Vidarsson et al., 2010). Forced-aggregation methods have been developed to standardize the size and shape of EB by centrifuging a defined number of hESC or iPSC in low-adherence 96-well plates (Burridge et al., 2007). The requirement for serum not only introduces further heterogeneity to the efficacy of the EB method but makes future therapeutic application of hESC and iPSC technology problematic (Habib et al., 2008, Vidarsson et al., 2010, Ludwig and J, 2007). Consequently, there is considerable interest in developing defined, animal-free differentiation protocols (Habib et al., 2008, Vidarsson et al., 2010).
One of the most significant limitations of the EB method, however, remains the relatively low cardiomyocyte yield (Habib et al., 2008, Vidarsson et al., 2010). Consequently, several directed differentiation protocols have been developed to improve the efficiency of iPSC and hESC cardiomyocyte differentiation either with (Tran et al., 2009) or without EB formation (Laflamme et al., 2007) (Figure 1.2).

### 1.3.2 Directed differentiation techniques

![Directed differentiation techniques](image.png)

Figure 1.2. Summary of methods for differentiating pluripotent stem cells into cardiomyocytes. Figure reproduced with permission of Rao et al. Phenotype and Developmental Potential of Cardiomyocytes from Induced Pluripotent Stem Cells and Human Embryonic Stem Cells. In: Ainscough J et al eds. Nuclear reprogramming and stem cells. Humana Press, 2011 (Rao et al., 2011).
As differentiation of hESC and iPSC into cardiomyocytes parallels many of the molecular and structural events seen during early *in vivo* cardiogenesis (Lev et al., 2005) lessons from developmental biology are readily applicable in stem cell research (Habib et al., 2008). For example, the endoderm that lies directly posterior to the embryonic cardiac crescent is considered essential to initiate cardiac differentiation (Olson, 2001, Graichen et al., 2008). *In vitro* experiments confirm that cardiac differentiation requires extracellular signals from the surrounding endoderm (Zaffran and Frasch, 2002, Olson, 2004). Co-culturing of undifferentiated hESC with a murine visceral endoderm cell line (END-2) has been shown to induce cardiac differentiation (Mummery et al., 2003). Even hESC lines, which ordinarily do not spontaneously differentiate into cardiomyocytes, have been shown to differentiate into cardiomyocytes when co-cultured with END-2 cells (Mummery et al., 2003). The co-culture differentiation system has since been further optimized by the removal of serum and insulin and the addition of prostaglandin I2 to the culture medium (Graichen et al., 2008, Xu et al., 2008, Passier et al., 2005). Although the END-2 culture system improves efficiency of differentiation compared to the EB method, and results in more homogeneous cells, with up to 85% of the hESC-CM displaying ventricular phenotype (Mummery et al., 2003), reliance on murine feeder cells limits the therapeutic applications of the method and represents an additional source of heterogeneity.

By focusing on factors expressed in the early endoderm known to regulate cardiac development directed differentiation protocols have been developed. These protocols use defined factors from the Wnt family or the Transforming Growth Factor β (TGF-β) superfamily, such as TGF-β itself, Activin and Bone Morphogenic Proteins (BMP) (Filipczyk et al., 2007).
TGF-β and BMP have been shown to promote cardiomyogenesis in all species (Olson, 2001, Schuldiner et al., 2000) and are thought to be important in promoting mesoderm formation and myocardial lineage commitment during hESC differentiation (Yang et al., 2008, Laflamme et al., 2007, Nostro et al., 2008, Pal and Khanna, 2007, Schuldiner et al., 2000, Yao et al., 2006, Zhang et al., 2008). Activin A has also been shown to promote hESC cardiac differentiation (Yang et al., 2008, Laflamme et al., 2007, Yao et al., 2006) and a differentiation system which combines Activin and BMP 4 has been reported to improve the efficiency of hESC cardiac differentiation to over 30% cardiomyocytes using serum-free defined medium (Laflamme et al., 2007).

The Wnt signaling pathway is important for cardiogenesis, however, it has not been widely utilized for in vitro directed cardiac differentiation as unlike BMP and Activin pathways, Wnt signaling pathway can both promote and inhibit differentiation into cardiomyocytes at different stages of cardiogenesis. It has been shown to be important in maintaining pluripotency (Sato et al., 2004). In early differentiation and prior to gastrulation it leads to enhanced cardiomyogenesis, probably by promoting formation of mesoderm, but subsequently will inhibit cardiomyogenesis (Ueno et al., 2007, Yang et al., 2008). Finally, whilst it has been shown to be important in promoting the formation of cardiac progenitor cells, it negatively affects differentiation of these cells into cardiomyocytes (Cohen et al., 2007, Qyang et al., 2007). By contrast, activation of non-canonical Wnt signaling has been shown to promote cardiac differentiation (Flaherty et al., 2008). Early treatment of iPSC-CM with BMP-4 followed by late treatment with small molecule Wnt inhibitors has been shown to increase the production of cardiomyocytes in vitro (Ren et al., 2011). More recently iPSC have been differentiated into functional cardiomyocytes using a serum-free system by sequential regulation of the canonical Wnt pathway using glycogen synthase kinase 3 (GSK3).
inhibitor, combined with the expression of β-catenin shRNA or a chemical Wnt inhibitor (Lian et al., 2013). This system is reported to yield 80-98% cardiomyocyte differentiation without cell sorting or selection (Lian et al., 2013).

Several other compounds, such as specific p38 Mitogen-activated protein (MAP) kinase inhibitors (Graichen et al., 2008), apelin (Wang et al., 2012), and trichostatin A, a histone deacetylase inhibitor (Lim et al., 2013), have been found to promote cardiac differentiation in vivo. Research is currently underway using novel high-throughput screening technology and bioinformatic approaches to identify novel molecules that could further promote cardiac differentiation of hESC and iPSC (Takahashi et al., 2003, Wu et al., 2004, Willems et al., 2009, Yoshida and Yamanaka, 2010, Vieira and Riley, 2013) and there are early reports of small molecules which improve the efficiency of cardiogenesis (Shen et al., 2012, Oh et al., 2013). Finally there are reports that cardiogenesis can be directed using culture conditions (Moon et al., 2013), and plasmid-based over-expression of cardiac transcription factors (Hartung et al., 2013).

1.3.3 Identification of cardiac progenitor cells derived from human embryonic stem cells and induced pluripotent stem cells

Despite improvements in the efficiency of cardiomyocytes differentiation from hESC and iPSC, current directed differentiation protocols do not yield a pure population of cardiomyocytes. This is suboptimal for some envisaged applications and has resulted in considerable interest in developing methods for purifying cardiomyocytes without genetic modification which may compromise their utility (Hattori et al., 2010). These include identification of novel cell surface markers to facilitate conventional cell sorting (Dubois et
al., 2011), sorting using microfluidic techniques based on physical cell characteristics (Zhang et al., 2012), and novel bioengineering techniques (Terazono et al., 2012). There is also considerable interest in identifying lineage-committed cardiac progenitor populations that could be expanded in vitro and differentiated efficiently into homogeneous cardiomyocytes (Yang et al., 2008, Kattman et al., 2006, Mauritz et al., 2011).

It is increasingly understood that cardiac tissue, containing cardiomyocytes, smooth muscle, endothelium and connective tissue; develops from common cardiovascular progenitor cells (Noseda et al., 2011). Consequently, there is considerable interest in identifying these cells (Vidarsson et al., 2010). In cells derived from murine ESC lines, a population of cells expressing both fetal liver kinase 1 (Flk1) and insulin gene enhancer protein (Isl1) have been identified which differentiate into cardiomyocytes, endothelial, and smooth muscle cell lineages (Kattman et al., 2006, Moretti et al., 2006). Similarly, using cells derived from murine ESC with a Green Fluorescent Protein (GFP) reporter targeting the brachyury (Bry) gene, a population of Flk1+/GFP-Bry+ cells which differentiate into cardiomyocytes, endothelial, and smooth muscle cells have been identified (Kattman et al., 2006). More recently it has been demonstrated that murine ESC derivatives expressing the mast/stem cell growth factor receptor (SCFR/cKit) and homeodomain transcription factor 2.5 (Nkx2.5) can be expanded and differentiated into cardiomyocytes and smooth muscle cells (Wu et al., 2006) (Figure 1.3).

Similar cardiovascular progenitor cell populations have been identified in cell populations derived from hESC (Yang et al., 2008). Using a novel stage-specific, serum-free, defined medium, differentiation protocol involving the combination of Activin A, BMP 4, Basic Fibroblast Growth Factor (bFGF), Vascular Endothelial Growth Factor (VEGF), and
Dickkopf-related protein 1 (DKK1) at different stages in the protocol, a population of cells with low Kinase Insert Domain Receptor (KDR) (Flk-1) and no c-kit expression (KDR low/c-kit-) were derived from hESC. Over 50% of these cells subsequently differentiated into cardiac myocytes (Yang et al., 2008).

Figure 1.3. Summary of gene expression during differentiation from pluripotent stem cells into cardiomyocytes. Figure reproduced with permission of Rao et al. Phenotype and Developmental Potential of Cardiomyocytes from...
1.3.4 Direct transdifferentiation of fibroblasts into cardiomyocytes

Concerns about the safety of iPSC, and the expense, inefficiency and cost of reprogramming, were the catalyst for development of techniques to directly transdifferentiate fibroblasts into cardiomyocytes. In 2010 Ieda et al, showed that adult murine fibroblasts could be transdifferentiated into cardiomyocytes, without an intermediate pluripotent state, by retroviral delivery of 3 cardiac transcription factors: Gata4, Mef2c, and Tbx5 (Ieda et al., 2010). Direct differentiation has been shown to be considerably quicker and more efficient than generation of iPSC-CM from fibroblasts in murine cells (Ieda et al., 2010). Direct transdifferentiation of fibroblasts into induced cardiomyocytes (iCM) was replicated using different factors by an independent group using mouse embryonic fibroblasts (MEF) (Efe et al., 2011). The therapeutic potential of direct lineage reprogramming was demonstrated by a study which showed that endogenous murine cardiac fibroblasts could be transdifferentiated into cardiomyocytes in vivo using Gata4, Mef2c, and Tbx5 (Qian et al., 2012). The iCM infiltrated to the infarct border zone, electrically matured and coupled with endogenous cardiomyocytes, resulting in decreased infarct size and modestly improved cardiac function up to 3 months after coronary ligation (Qian et al., 2012).

iCM technology represents an interesting development of iPSC technology, offering improved efficiency and safety by avoiding the tumorigenicity associated with pluripotent stem cells. However, its continued reliance on viral vectors means that many of the safety concerns that are associated with iPSC technology persist. Consequently, full characterization
of iCM needs to be undertaken. This includes comprehensive functional comparison with iPSC-CM, hESC-CM and adult cardiomyocytes.

1.4 Structure and functional phenotype of human embryonic stem cell and induced pluripotent stem cell-derived cardiomyocytes

The study of structure and function in cardiomyocytes derived from pluripotent stem cells is complicated by evidence that differentiation method (Pekkanen-Mattila et al., 2010a) and culture conditions (Otsuji et al., 2010) may strongly influence phenotype. It is also uncertain whether hESC-CM and iPSC-CM have different phenotypes. This may have significant implications both for their use in cell therapy and disease modelling.

1.4.1 Structural properties

The structure and function of cardiomyocytes are intimately related. Ultrastructural analysis shows that hESC-CM develop in vitro from spherical cells to elongated cells with a more organized sarcomeric pattern (Snir et al., 2003) (Figure 1.4). Transmission electron microscopy of the hESC-CM at varying developmental stages shows progressive ultrastructural maturation from an irregular myofilament distribution with parallel nascent z-bands containing myofibrils to a more mature sarcomeric organization containing well-defined sarcomeres with recognizable A, I, and M-bands in older hESC-CM (Otsuji et al., 2010, Snir et al., 2003, Baharvand et al., 2006). iPSC-CM also have functional, albeit immature, sarcomeric structures (Zwi et al., 2009) and the finding that time in culture results in progressive ultrastructural maturation has also been demonstrated in iPSC-CM (Kamakura
et al., 2013). Finally, it has been suggested by some authors that maturation of the ultrastructure is associated with withdrawal from the cell-cycle in hESC-CM (Snir et al., 2003).

Figure 1.4. Myosin heavy chain (MHC, green) and nuclear 4’,6-diamidino-2-phenylindole stain (DAPI, blue) staining of hESC-CM without (A) and with (B) characteristic sarcomeric striation patterns, compared with adult rat ventricular myocyte. Scale bar is 20 µm. Figure reproduced with permission of Rao et al. Phenotype and
Most comparative studies of hESC-CM and iPSC-CM have not shown any difference in ultrastructural phenotype (Zhang et al., 2009). Other studies, however, suggest that whilst iPSC-CM and hESC-CM have broadly similar ultrastructural phenotypes, the sarcoplasmic reticulum (SR) may be less developed in iPSC-CM of equivalent age (Gherghiceanu et al., 2011). Interestingly, Gherghiceanu et al suggest that there was a marked difference between the structural maturity of cardiomyocytes on the periphery of beating clusters and the cardiomyocytes in the centre of beating clusters, and speculate that this may underlie the functional heterogeneity that is described in the following section (Gherghiceanu et al., 2011) (Section 1.4.2).

Figure 1.5. Diagram of an idealized adult human ventricular action potential (AP). The phases of the AP are labeled (Phase 0–4). The predominant cardiac ion currents at each point in the AP are labeled (I_{\text{Na}}=Na^+\text{ Current}, I_{\text{to}}=\text{Transient Outward K}^+\text{ Current}, I_{\text{Ca}}=\text{Ca}^{2+}\text{ Current}, I_{\text{Kr}}=\text{Rapidly Activating Delayed Rectifier K}^+\text{ Current}, I_{\text{Ks}}=\text{Slowly Activating Delayed Rectifier K}^+\text{ Current}, I_{\text{K1}}=\text{Inward Rectifier K}^+\text{ Current}). Figure reproduced with
1.4.2 Functional properties

hESC-CM and iPSC-CM form functional syncytia like adult myocardial tissue (Zhang et al., 2009, Kong et al., 2010, Caspi et al., 2009), with evidence that cells which beat together in clusters are connected by gap junctions (Pekkanen-Mattila et al., 2010a, Caspi et al., 2009). Action Potential (AP) can be recorded either from clusters of beating cells using sharp pipettes (Zhang et al., 2009) or enzymatically isolated myocytes (Pekkanen-Mattila et al., 2010a, Sartiani et al., 2007). It is suggested that even enzymatically isolated cells from the same cluster can have different AP characteristics (Denning and Anderson, 2008, Xu et al., 2002, Zhang et al., 2009).

hESC-CM and iPSC-CM are more heterogeneous than adult cardiomyocytes and have key functional differences (Zhang et al., 2009, Kong et al., 2010, Pekkanen-Mattila et al., 2010a). With the exception of a small number of cells within the pacemaker and conduction system (nodal cells), adult cardiomyocytes normally maintain a stable negative membrane potential and are electrically inactive until they are depolarized (Kleber and Rudy, 2004). This usually occurs by the passage of ions from adjacent myocytes through connecting gap-junctions (Desplantez et al., 2007). When cardiomyocytes are sufficiently depolarized an AP is initiated (Kleber and Rudy, 2004, Bers and Despa, 2009) (Figure 1.5). This consists of an initial influx of Na\textsuperscript{+} that further depolarizes the cell, followed by an influx of Ca\textsuperscript{2+} and efflux of K\textsuperscript{+} that maintain the cell in a depolarized state. The influx of intracellular Ca\textsuperscript{2+} causes the
SR to release stored Ca\(^{2+}\) and the cytoplasmic Ca\(^{2+}\) initiates contraction of the sarcomere (Bers, 2002) (Figure 1.6). Finally an efflux of K\(^{+}\) predominates which repolarizes the cell.

Figure 1.6. Excitation-Contraction coupling. The AP triggers L-type Ca\(^{2+}\) channels to open during the plateau phase of the cardiac AP, causing an influx of Ca\(^{2+}\) into the cardiomyocyte. The increase in cytosolic Ca\(^{2+}\) increases the open probability of the ryanodine receptor, causing Ca\(^{2+}\) stored in the sarcoplasmic reticulum to be released into the cytoplasm. Increased cytoplasmic Ca\(^{2+}\) binds to Troponin C, changing the configuration of the tropomyosin complex and exposing the actin binding site. This allows the myosin head to bind to the actin filament, facilitating cross-bridge cycling and cell shortening. Cytoplasmic Ca\(^{2+}\) is taken up by the sarco/endoplasmic reticulum Ca\(^{2+}\)-Adenosine-5'-triphosphate (ATP)ase 2a (SERCA2a) pump into the sarcoplasm, or ejected from the cell by the sodium-calcium exchanger. Intracellular Ca\(^{2+}\) concentration subsequently drops and the tropomyosin complex again covers the actin binding site, ending contraction. The cell then relaxes and lengthens. Reproduced with permission of Bers D, Nature 2002 (Bers, 2002).
A key difference between adult cardiomyocytes and hESC-CM or iPSC-CM is that although some are electrical inactive until stimulated, many exhibit a high degree of automaticity with spontaneous AP (Pekkanen-Mattila et al., 2010a, Kong et al., 2010, Zhang et al., 2009). In adult myocardium this is only seen in pacemaker cells (DiFrancesco, 2010). The mechanisms that underlie the automaticity in hESC-CM and iPSC-CM probably differ from the mechanisms that underlie automaticity in pacemaker cells. Hyperpolarization-activated Cyclic Nucleotide-gated (HCN) channels allow the passage of Na⁺/K⁺ into specialized pacemaker cells in the adult myocardium when the cell is repolarized in diastole. This slowly activating, inward “funny” current (I₉) (DiFrancesco, 2010) may play a role in the automaticity of hESC-CM; however, other mechanisms are probably more important (Satin et al., 2004). hESC-CM express a large Na⁺ current (I₉Na) density and their AP duration is shortened by L-type Ca²⁺ channel blockade (Habib et al., 2008, Satin et al., 2004). It has been suggested that, whilst a diverse set of K⁺ channels are expressed in older hESC-CM compared to early-differentiated hESC-CM (Sartiani et al., 2007), the expression of the I₉K₁ remains low (Satin et al., 2004). Consequently, the presence of comparatively large I₉Na and relatively small I₉K₁ probably explains the increased automaticity of hESC-CM (Satin et al., 2004). Fluctuations in intracellular Ca²⁺ play a critical part in the automaticity of developing murine myocardium (Viatchenko-Karpinski et al., 1999). Whilst they may also contribute to the automaticity of hESC-CM, it is not thought that they have a dominant role (Satin et al., 2004). This is supported by studies which show that co-culture of Human Embryonic Kidney 293 Cells (HEK-293) cells expressing I₉K₁ electrically coupled to rat neonatal cardiomyocytes reversibly inhibits their automaticity (de Boer et al., 2006), and emerging evidence which suggests that forced expression of the KCNJ2 gene in hESC which encodes the Kᵢ₂.1 channel, which mediates I₉K₁, results in a more mature electrophysiological phenotype (Kong et al., 2010, Liao et al., 2013).
The AP measured in hESC-CM and iPSC-CM are often categorized as “ventricular”, “atrial” or “nodal” (pacemaker) based on their resemblance to adult AP. “Ventricular” cells are said to be characterized by a fast and large upstroke, and plateau phase. “Atrial” cells are said to have more triangular AP. “Nodal” cells are said to have a slower and smaller upstroke, prominent phase four depolarization and to be relatively depolarized in diastole. In some cases these phenotypes are also quantified using parameters such as AP duration (APD) at 90% repolarization (APD90), maximum rise of the AP upstroke (dV/dtmax), AP amplitude (APA) and maximum diastolic potential (MDP) (He et al., 2003, Cao et al., 2008, Yoshida and Yamanaka, 2010, Zhang et al., 2009, Pekkanen-Mattila et al., 2010a, Mummery et al., 2003, Satin et al., 2004, Sartiani et al., 2007). Whether “ventricular”, “atrial” and “nodal” cells represent distinct populations of cells, or a continuous range of AP characteristics within one population is yet to be shown. Some studies suggest there is considerable heterogeneity in AP characteristics within these groups and considerable overlap in the characteristics between groups (Pekkanen-Mattila et al., 2010a). It is also suggested that “atrial” cells have a higher rate of spontaneous contraction (Zhang et al., 2009); however this is not supported by all studies (Pekkanen-Mattila et al., 2010a). In addition to using the electrophysiological characteristics, attempts have been made to employ specific reporters to delineate subtypes of hESC-CM (Muller et al., 2000, Kuratomi et al., 2009, Kolossov et al., 2005, Blazeski et al., 2012).

Neither direct nor indirect comparison of the electrophysiological properties of hESC-CM and iPSC-CM suggests that there is any difference in phenotype, with both cell types exhibiting similar AP morphology and response to pacing at different rates (Zhang et al., 2009). Finally, a number of studies have used either isolated cells (Pekkanen-Mattila et al., 2010a).
2010a, Otsuji et al., 2010, Caspi et al., 2009, Moretti et al., 2010) or beating clusters plated onto multi-electrode arrays (Zwi et al., 2009, Kehat et al., 2001, Otsuji et al., 2010, Tanaka et al., 2009, Caspi et al., 2009) to demonstrate that hESC-CM and iPSC-CM have an appropriate chronotropic response and change in AP morphology in response to several pharmacological agents including adrenergic and cholinergic agents (Zwi et al., 2009, Kehat et al., 2001, Pekkanen-Mattila et al., 2010a, Tanaka et al., 2009, Moretti et al., 2010, Xu et al., 2002, Mummery et al., 2003), $I_{Kr}$ channel blockade (Zwi et al., 2009, Pekkanen-Mattila et al., 2010a, Otsuji et al., 2010, Tanaka et al., 2009, Caspi et al., 2009, Moretti et al., 2010), $I_{Ks}$ channel blockade (Zwi et al., 2009), and $Ca^{2+}$ channel blockade (Zwi et al., 2009, Tanaka et al., 2009). These similarities to an authentic cardiac phenotype support the feasibility of using hESC-CM and iPSC-CM as *in vitro* model systems (Caspi et al., 2009, Moretti et al., 2010).

hESC-CM have intracellular $Ca^{2+}$ transients that correspond to their beating activity (Kehat et al., 2001, Mummery et al., 2003, Satin et al., 2008). Experiments with murine ESC derived cardiomyocytes (ESC-CM) demonstrated that a functioning SR was essential for regulating contraction (Fu et al., 2006). However, early experiments with hESC-CM suggested that they did not respond to caffeine, which depletes SR $Ca^{2+}$ stores, or ryanodine receptor (RyR) blockade (Dolnikov et al., 2006). This suggests that hESC-CM did not have functional SR and that their contractions result from influx of extracellular $Ca^{2+}$ into the cell (Dolnikov et al., 2006). A subsequent study however, suggested 40% of hESC-CM were responsive to caffeine, ryanodine and the Sarco/Endoplasmic Reticulum $Ca^{2+}$-ATPase pump (SERCA) inhibitor thapsigargin (Liu et al., 2007). Furthermore, in contrast to earlier work (Dolnikov et al., 2006), as in adult cardiomyocytes a positive force-frequency relationship was demonstrated in this population of cells (Liu et al., 2007). Other studies suggest that not only
is a functional SR present, demonstrated by caffeine and ryanodine in hESC-CM, but as in murine cells (Fu et al., 2006) this is present from as early as two days following the commencement of beating (Satin et al., 2008).

In adult human ventricular myocytes, the cell membrane has regular invaginations called t-tubules which are considered critical for normal Ca$^{2+}$ cycling (Brette and Orchard, 2003). Contraction is caused by a net flux of Ca$^{2+}$ ions into the cardiomyocyte through L-type Ca$^{2+}$ channels in t-tubules. The increase in cytoplasmic Ca$^{2+}$ causes the RyR in the membrane of the SR to release stored Ca$^{2+}$ into the cytoplasm. Ca$^{2+}$-induced Ca$^{2+}$-release (CICR) (Bers, 2002) accounts for between 70 and 90% of the raise in cytoplasmic Ca$^{2+}$ during contraction of the adult cardiomyocyte (Bers et al., 2006). Cytoplasmic Ca$^{2+}$ binds to Troponin C, displacing tropomyosin and initiating contraction of the sarcomere. Cytoplasmic Ca$^{2+}$ is taken up by the SERCA into the SR, or ejected from the cell by the sodium-calcium exchanger (NCX). Cytoplasmic Ca$^{2+}$ drops and tropomyosin complex returns over the active site of the actin filament, ending contraction. This beat-to-beat rise and fall in Ca$^{2+}$ is known as a Ca$^{2+}$ transient (Bers, 2002) (Figure 1.6). T-tubules ensure that the L-type Ca$^{2+}$ channels are in close proximity to the RyR, minimizing the diffusion distance between the two ion transporters (Brette and Orchard, 2003, Brette and Orchard, 2007). This ensures that Ca$^{2+}$ is released synchronously from RyR across the cell (Brette and Orchard, 2003), and that there is no delay in release of Ca$^{2+}$ from RyR in the centre of the cell. In pathological cells detubulation occurs, resulting in a time delay in release of Ca$^{2+}$ from the SR in the centre of the cell (Brette and Orchard, 2003, Brette and Orchard, 2007, Song et al., 2005). Some studies have been unable to detect t-tubules in hESC-CM (Lieu et al., 2009) while others find that they are present (Satin et al., 2008, Baharvand et al., 2006) but clearly less abundant and organized than in adult ventricular cardiomyocytes. This may explain why Ca$^{2+}$ handling of
hESC-CM resembles that of failing or atrial myocytes which have fewer and less organized t-tubules (Satin et al., 2008).

Finally, protein expression and functional data suggests that NCX expression is higher in hESC-CM compared to adult cardiomyocytes (Fu et al., 2010); by contrast expression of regulatory proteins such as junctin, triadin, and calsequestrin which are usually expressed in adult cardiomyocytes are completely absent in hESC-CM. Calsequestrin, in particular, plays a critical role in excitation contraction coupling, acting as a critically important Ca$^{2+}$ buffer in some intracellular compartments (Beard et al., 2004) and regulating CICR (Lukyanenko et al., 1996, Shannon et al., 2000, Bassani et al., 1995).

There is limited evidence to suggest that differentiation protocols may affect the electrophysiology of hESC-CM. In a study comparing the EB with the END-2 differentiation system, EB derived hESC-CM were found to be less likely to express an atrial phenotype, more likely to beat continuously, and to be significantly more polarized in diastole (Pekkanen-Mattila et al., 2010a). The effect of time in culture on the electrophysiological phenotype has not yet been fully characterized. Whilst it has been suggested that the AP maturity increases with time in culture (Sartiani et al., 2007), which is consistent with evidence from murine models (Hescheler et al., 1997), other studies show time in culture does not significantly affect the maturation of hESC-CM (Pekkanen-Mattila et al., 2010a). Whether or not hESC-CM and iPSC-CM mature in culture there is considerable interest in accelerating and homogenizing the maturation of cells derived from hESC and iPSC not only to facilitate representative disease models but also to ensure the safety of cell therapy. Promising techniques range from cyclical replanting and suspension of beating clusters in culture (Otsuji et al., 2010) to forced expression of the genes which encode the $I_{Kr}$ channel or
calsequestrin in order to improve the maturity of the AP or intracellular Ca$^{2+}$ handling respectively (Kong et al., 2010, Liao et al., 2013, Liu et al., 2009).

1.4.3 Differences in gene-expression and differentiation potential of induced pluripotent stem cells and human embryonic stem cells

During early cardiogenesis it is known that there is an initial reduction in the expression of pluripotency genes such as Oct-4 (Niwa et al., 2000) and NANOG (Mitsui et al., 2003, Chambers et al., 2003) following the initiation of differentiation, with a concomitant increase in the levels of mesodermal markers such as Bry (Marcellini et al., 2003). This is followed by expression of the cardiac transcription factors such as Nkx2.5 (Garg et al., 2003, Durocher et al., 1997), Myocyte-specific enhancer factor 2C (MEF2c) (Bi et al., 1999), and GATA4 (Garg et al., 2003, Durocher et al., 1997). Finally, genes encoding cardiac-specific structural proteins are expressed such as cardiac troponin T (TNNT2) (Townsend et al., 1994), α-myosin heavy chain (MYH6) (Brand et al., 1991), α-actinin (ACTN2) (Eldstrom et al., 2003), myosin light chain 2 atrial isoform (MYL7) (Kubalak et al., 1994) and myosin light chain 2 ventricular isoform (MYL2) (Macera et al., 1992); atrial natriuretic factor (HPPA) (Saito et al., 1989); and phospholamban (PLN) (Tada et al., 1974) (Figure 1.3). Whilst this is broadly consistent with gene expression patterns in the differentiation of hESC and iPSC into cardiac myocytes (Pekkanen-Mattila et al., 2009, Mummery et al., 2003, Zhang et al., 2009, Zwi et al., 2009, He et al., 2003, Xu et al., 2002, Kehat et al., 2001, Xue et al., 2005, Pekkanen-Mattila et al., 2010b, Habib et al., 2008) there are differences between gene expression patterns (Chin et al., 2009) and Deoxyribonucleic Acid (DNA) methylation patterns in hESC and iPSC (Deng et al., 2009). Furthermore, evidence has recently emerged that ESC may
differentiate into specific lineages more efficiently than iPSC (Deng et al., 2009, Hu et al., 2010).

Differences between iPSC and hESC in the expression of pluripotency genes have been reported, because of persistent transgene expression in iPSC during cardiogenesis (Zhang et al., 2009), however it is not clear if this affects either the potential of iPSC to differentiate into cardiomyocytes or the phenotype of the resulting iPSC-CM. Trends towards more efficient cardiac differentiation in hESC compared to iPSC have been reported (Zhang et al., 2009). However, this must be interpreted in the context of data which suggests that there is considerable variation between the efficiency with which different hESC lines differentiate into cardiomyocytes. Consequently, observed variation in the differentiation potential between iPSC and hESC may just reflect differences between pluripotent stem cell lines rather than inherent characteristics of either iPSC or hESC (Moore et al., 2008, Osafune et al., 2008, Adewumi et al., 2007, Pekkanen-Mattila et al., 2009). Whilst some studies suggest that there is no difference in the time taken for iPSC and hESC to differentiate into cardiomyocytes (Zhang et al., 2009, Narazaki et al., 2008), others report that iPSC take longer to differentiate into cardiomyocytes than hESC (Mauritz et al., 2008). It has been proposed by some authors that variation in the site of transgene integration may account for these differences (Zhang et al., 2009). This is not, however, consistent with reports that differences in gene expression between iPSC and hESC are independent of the method by which pluripotency is induced (Chin et al., 2009). As a consequence of concerns about continued expression of reprogramming factors, and the effect that this may have on the phenotype of iPSC-CM and hESC-CM, there is considerable interest in developing and refining methods to induce pluripotency without genetic manipulation (De Miguel et al., 2010, Selvaraj et al., 2010).
1.5 Cell therapy

The adult heart has limited regenerative capacity resulting in irreversible loss of myocardial tissue following injury (Bergmann et al., 2009). The resulting adaptive response of the myocardium to injury further worsens cardiac function resulting in permanent impairment of function (McMurray and Stewart, 2000). The associated clinical syndrome of heart failure is characterized by fatigue, oedema and dyspnea. The long-term prognosis is poor, with death often resulting from fatal arrhythmia in the pathological myocardium (McMurray and Stewart, 2000). Current therapies for heart failure are largely palliative, aiming to prevent progression of heart failure and to relieve symptoms (Yamada et al., 2008). In 2003, only 25% of patients survived 5 years after their first heart-failure-associated hospital admission (Stewart et al., 2001). The only treatment for end-stage heart failure with established long-term efficacy is cardiac transplantation (Taylor et al., 2007). However, the prevalence of heart failure is increasing and the supply of donor organs is insufficient to meet the current demand for transplant surgery (Gridelli and Remuzzi, 2000). Consequently, there is significant interest in developing novel regenerative strategies. Stem cell therapy, which aims to arrest further deterioration of cardiac function and induce recovery by replacing lost cardiomyocytes and improving the function of existing myocytes, is one of the more promising of these strategies (Bolli et al., 2011, Makkar et al., 2012). Cell therapy entails either mobilization of endogenous cardiac progenitor cells, or transplantation of exogenous stem cells; however, these therapies are not mutually exclusive and it has been widely suggested that cell transplantation promotes mobilization of endogenous stem cells (Mirotsou et al., 2011). The feasibility of cell therapy has been investigated using several stem cell populations (Nelson et al., 2009, Menasche et al., 2008, Abdel-Latif et al., 2007, Bolli et al.,
2011, Makkar et al., 2012, van Laake et al., 2009). A full review of the use of these cell populations in cardiac cell therapy, their properties, and their relative advantages lies outside the scope of this work. However, when considering the role of tissue engineering in cell therapy it is impossible to ignore some of the general principles about cell therapy that we have learnt over the last two decades.

Many cell types appear to induce a transient improvement in cardiac physiology in humans and animal models of cardiac disease (Menasche et al., 2001, Alaiti et al., 2010, Jiang et al., 2010a, Abdel-Latif et al., 2007, Bai and Alt, 2010). Often, however little effect is seen in studies of chronic heart failure (Fernandes et al., 2010), and the initial functional improvement generally diminishes over time in most studies (van Laake et al., 2007, Singla et al., 2011). It is now considered unlikely that significant myogenesis alone is responsible for these limited improvements in function (Alaiti et al., 2010). Improvements in function were often reported before significant myogenesis could have occurred (Gnecchi et al., 2008). Improvements in cardiac physiology were seen irrespective of cell type (Gersh and Simari, 2006) and delivery method, with over 6000-fold variations in the number of cells delivered (Murry et al., 2005). Despite promising early clinical reports (Makkar et al., 2012, Bolli et al., 2011), there has been a paucity of evidence demonstrating the presence of new cardiac myocytes significant enough to account fully for improvements in cardiac function (Nygren et al., 2004, Balsam et al., 2004, van Laake et al., 2009). Histological studies demonstrate widespread death of transplanted cells and an unpredictable distribution of cells, further suggesting that myogenesis alone does not account for improvements in function (Reinecke and Murry, 2000, Balsam et al., 2004, Zhang et al., 2009).
In the absence of significant evidence of myogenesis, it has been suggested that definite improvements in myocardial function are due to the secretion of soluble mediators by transplanted cells, termed “paracrine factors”. It has been suggested that these factors may improve survival of existing myocardium, promote neovascularization, improve cellular metabolism and contractile function, modulate remodelling of the extracellular matrix (ECM), and promote activation of native progenitor cells (Mirotsou et al., 2011). The mechanisms by which this is accomplished are not fully understood, are likely to be multiple, and may vary between stem cell populations (Mirotsou et al., 2011, Shintani et al., 2009, Doyle et al., 2008, Lee et al., 2008).

It has been suggested that the discrepancy between the impressive potential of stem cells to proliferate, differentiate and form beating syncytium in vitro, and disappointing in vivo studies could be explained by the environment of the pathological myocardium. Injected cells are exposed to significant mechanical forces in an often ischaemic, inflamed, biochemically hostile environment. Consequently, few cells survive, are retained at the site of injection, and proliferate (Dohmann et al., 2005, Nygren et al., 2004, Balsam et al., 2004, van Laake et al., 2009). It is hypothesized that tissue engineering techniques could be used to modify the environment to make cell retention, and survival more likely, and this may improve the efficacy of cell therapy (Li and Guan, 2011).

1.6 Disease modelling

In addition to applications in cell therapy there is also considerable interest in using hESC-CM (Abdul Kadir et al., 2009, Foldes et al., 2011), iPSC-CM (Carvajal-Vergara et al., 2010, Moretti et al., 2010, Caspi et al., 2009) and other immature cardiomyocytes such as neonatal
rat ventricular myocytes (NRVM) (Brand et al., 2010) as *in vitro* models of human physiology and disease (Tanaka et al., 2009).

Many of the properties that make stem cell-derived cardiomyocytes suitable for cell therapy also make them particularly useful as disease models. Pluripotent stem cells can be differentiated into all cell types in the body (Thomson et al., 1998). In specific conditions stem cells can be propagated *in vitro* for many months without loss of normal karyotype (Ying et al., 2003), manipulated and transfected, and stem cell-derived cardiomyocytes, unlike adult cardiomyocytes can be readily cultured (Yoshida and Yamanaka, 2010, Rao et al., 2011). The feasibility of human pluripotent-derived cardiomyocyte disease models was demonstrated when hESC-CM were used to investigate potential mechanisms for the arrhythmogenic effect of bile acids on the fetal heart (Abdul Kadir et al., 2009). Older hESC-CM were shown to be more resistant than early hESC-CM to bile acid-induced disruption of rhythm, depression of contraction and desynchronization of cell coupling, paralleling the difference between fetal and maternal susceptibilities (Abdul Kadir et al., 2009).

More recently described cell types such as iPSC-CM could be used to create patient-specific, disease-specific models (Carvajal-Vergara et al., 2010, Matsa et al., 2011), which could be used to elucidate the molecular mechanisms that underlie inherited disease phenotypes (Carvajal-Vergara et al., 2010), and suggest novel therapies (Matsa et al., 2011). Furthermore, as they can be easily obtained from any individual, they may also facilitate personalized medicine, the study of a genetic background on a disease process, and the study of pharmacogenetics at a cellular level (Inoue and Yamanaka, 2011).
1.6.1 Modelling inherited arrhythmias

The feasibility of using iPSC-CM to create disease-specific models for inherited myocardial disorders was first demonstrated when the prolonged AP duration seen in patients with inherited long-QT syndrome, type 1 (LQTS1), caused by mutation of the KCNQ1 gene encoding voltage-gated potassium channel, Kv7.1, was replicated in vitro using iPSC-CM (Moretti et al., 2010). The susceptibility to catecholamine-induced tachyarrhythmia which can be fatal in patients was also replicated in vitro (Moretti et al., 2010). Other groups have subsequently replicated the long-QT syndrome phenotype in vitro using iPSC-CM for LQTS1 (Egashira et al., 2011), LQTS2 caused by mutation of the KCNH2 gene (also known as the Human Ether-à-go-go Related Gene – hERG) encoding the alpha subunit of the voltage gated K⁺ channel, Kv11.1 (Itzhaki et al., 2011a, Matsa et al., 2011, Lahti et al., 2012), LQTS3 caused by mutation of the SCN5 gene encoding the voltage gated Na⁺ channel Nav1.5 (Terrenoire et al., 2013, Malan et al., 2011), and LQTS8 caused by mutation in the CACNA1C gene encoding the subunit Caᵥ1.2 of the voltage-gated Ca²⁺ channel (Timothy syndrome) (Yazawa et al., 2011). Similarly, disease phenotypes have been replicated in vitro using iPSC-CM for overlap syndromes caused by SCN5A mutation (Davis et al., 2012), catecholaminergic polymorphic ventricular tachycardia type 1 caused by mutation of the RYR2 gene encoding the RyR (Kujala et al., 2012, Jung et al., 2012, Itzhaki et al., 2012, Fatima et al., 2011), and catecholaminergic polymorphic ventricular tachycardia type 2 caused by mutation of the CASQ2 gene encoding calsequestrin (Novak et al., 2012).

Significantly many of these studies suggested novel mechanisms that may underlie the development of the disease phenotype (Itzhaki et al., 2011a, Yazawa et al., 2011, Egashira et al., 2011, Jung et al., 2012, Terrenoire et al., 2013, Kujala et al., 2012), and in one study this
was validated in an index patient (Kujala et al., 2012). In several studies the pathogenicity of a novel mutation was established (Moretti et al., 2010, Jung et al., 2012, Egashira et al., 2011). Many studies suggested novel therapeutic drug approaches (Itzhaki et al., 2011a, Matsa et al., 2011, Yazawa et al., 2011, Itzhaki et al., 2012, Jung et al., 2012). One study suggested a novel approach to myocardial pacing (Terrenoire et al., 2013). Finally, one study suggested that Ribonucleic Acid (RNA) interference may theoretically be an effective therapy in the clinic (Matsa et al., 2013).

1.6.2 Modelling inherited cardiomyopathies

Arguably, existing experimental tools are sufficient to model single ion channel disorders, and consequently the challenge is to leverage the potential of iPSC to model more complicated disease phenotypes. One of the earliest attempts to do this was for Syndrome of Lentigines, Electrocardiographic conduction abnormalities, Ocular hypertelorism, Pulmonary stenosis, Abnormal genitalia, Retarded growth, and Deafness (LEOPARD syndrome), an autosomal dominant multisystem disorder resulting from a missense mutation in the PTPN11 gene resulting in abnormalities of the skin, skeletal muscle and cardiovascular system (Carvajal-Vergara et al., 2010). The most commonly life-threatening cardiac anomaly associated with LEOPARD syndrome is hypertrophic cardiomyopathy (HCM). Carvajal-Vergara et al showed that compared to controls iPSC lines, iPSC derived CMs from a LEOPARD syndrome patient had a higher mean cell surface area, a greater degree of sarcomeric assembly and a nuclear localization of the nuclear factor of activated T-cells, cytoplasmic 4 (NFATC4) transcription factor. In addition, phospho-proteomic assays of these cardiomyocytes revealed a notable abundance or increased phosphorylation of proteins that could be involved in the cardiac hypertrophy observed in these patients. The observed
hypertrophic phenotype could not be fully characterized because of the heterogeneity of the iPSC-CM population. In a subsequent study, iPSC lines were created from a family with familial HCM who carried a missense mutation of the MYH7 gene. Despite mutations of genes encoding sarcomeric proteins being the classical cause for familial HCM, the mechanisms that lead to the development of the HCM phenotype is unclear. This study was able to replicate the HCM phenotype at the cellular level showing cellular enlargement, contractile and electrophysiological dysfunction (Lan et al., 2013). Unlike the previously mentioned study the authors were also able to demonstrate activation of a hypertrophic gene expression pattern; significantly this was achieved using single-cell gene expression analysis, negating the effect of population heterogeneity. Furthermore, not only were the authors able to demonstrate that deranged Ca\textsuperscript{2+} homeostasis was critical to the development of the HCM phenotype, but pharmacological normalization of Ca\textsuperscript{2+} homeostasis was able to prevent the development of the HCM phenotype, suggesting novel therapeutic mechanisms (Lan et al., 2013).

Similarly, iPSC cell lines have been generated from a family with familial dilated cardiomyopathy (DCM), caused by a mutation of the gene encoding cardiac troponin T (Sun et al., 2012). iPSC-CM differentiated from patients with DCM, exhibited a DCM phenotype with deranged sarcomeric organization, altered Ca\textsuperscript{2+} handling and an increased susceptibility to biomechanical stress and adrenergic stimulation (Sun et al., 2012). The authors found that β-adrenoceptor blockade and SERCA2a overexpression partially normalized the adverse phenotype observed in DCM iPSC-CM (Sun et al., 2012). There are even early results to suggest that it may be theoretically possible to modify the inherited cardiac gene expression profile associated with Duchenne muscular dystrophy \textit{in vitro} using iPSC-CM, supporting the efficacy of gene therapy for this condition (Dick et al., 2013).
A further study, which created iPSC-CM from patients with DCM demonstrated a different application in cardiovascular disease modelling (Tse et al., 2013). In this study recreation of the DCM cellular phenotype using iPSC derived cardiomyocytes from a patient with a novel mutation of the gene encoding desmin was used to support the assertion that this mutation was responsible for the development of the DCM phenotype (Tse et al., 2013). Interesting developing areas include work to replicate the cellular phenotype of diseases which have a complex anatomical basis such as arrhythmogenic right ventricular cardiomyopathy associated with plakophylin-2 mutation, in order to better understand disease mechanisms (Ma et al., 2013).

1.6.3 Drug development

The lack of suitable in vitro models makes drug development expensive (Gunaseeli et al., 2010, Dickson and Gagnon, 2004) and up to 80% of medications which enter clinical testing do not make it to the clinic (Dickson and Gagnon, 2004). The average cost of drug development is now estimated to exceed US $800 million (Dickson and Gagnon, 2004). In half of cases this is because of lack of efficacy, however in half of cases this is as a result of toxicity, most commonly cardiac or hepatic toxicity (Inoue and Yamanaka, 2011). Cardiac toxicity can lead to reactive oxygen species formation, altered contractility, arrhythmia, impaired gene expression, and cell death. Despite current best practice the toxicity of several medications is only discovered when they have made it to market or during phase 3 clinical trials, representing a significant monetary cost and associated morbidity (Gunaseeli et al., 2010, Tanaka et al., 2009). The considerable cost associated with drug development can be largely attributed to the “failures” (Dickson and Gagnon, 2004). Whilst pre-clinical
development costs probably only account for 2-3% of the cost of a drug’s development (Dickson and Gagnon, 2004), if the number of drugs which fail in late drug development could be reduced this would significantly reduce the average cost of drug development and the accessibility of novel therapeutic compounds.

The difficulty in obtaining and culturing human myocardial tissue makes studying pathophysiology and therapeutic mechanisms using standard techniques problematic. The current models used in the pharmaceutical industry for cardiac drug development and toxicity screening rely on animal cardiomyocytes, immortalized human cell lines, and animal models (Gunaseeli et al., 2010). Although these models provide useful information in evaluating the safety and efficacy of the drugs, they have a number of limitations. Firstly drug metabolism and mechanism of action can differ substantially between humans and even large animals. The effect of altered physiology seen in elderly and unwell patients cannot easily be modeled. Similarly, toxicity associated with combination pharmacotherapy can also not be studied. Consequently, a representative human in vitro myocardial model could have significant value, resulting in a more accurate description of the effects of novel drugs in a more cost-effective fashion (Gunaseeli et al., 2010, Dickson and Gagnon, 2004, Tanaka et al., 2009).

iPSC-CM from patients with a range of genetic backgrounds and disease phenotypes could potentially provide a high-throughput platform for toxicology screening and drug development (Zhang et al., 2009, Shiba et al., 2009), their utility is already being investigated by industry (Harris et al., 2013), and several studies have already demonstrated the utility of iPSC-CM for this application (Braam et al., 2013, Liang et al., 2013).
1.6.4 Limitations of immature cardiomyocytes as disease models

Despite promising evidence from these studies that complex cellular phenotypes can be modeled using iPSC-CM, and whilst they are an improvement on non-human cell types such as NRVM, there remain issues that need to be addressed. Firstly, all the cell types mentioned have the structural and functional properties of developing cardiomyocytes, including immature gene expression, immature electrophysiological properties, ultrastructural phenotype and altered Ca$^{2+}$ handling (Section 1.4). Furthermore, human iPSC-CM and hESC-CM have a heterogeneous phenotype (Matsa et al., 2011). This heterogeneous phenotype may make iPSC-CM unsuitable for disease modelling. Several of these studies suggest that there was heterogeneity in the population of iPSC-CM, or inclusion of a significant population of non-cardiomyocytes following the use of common differentiation techniques (Carvajal-Vergara et al., 2010). This limits the utility of iPSC-CM for multicellular assays which are the mainstay of molecular biological and cellular physiology, and consequently limited their utility as disease models. Some studies were able to recreate subcellular phenotypes (Hick et al., 2013, Ho et al., 2011) or gene expression patterns (Dick et al., 2013) in iPSC-CM whilst failing to recreate cellular phenotypes, suggesting that culture conditions, or cell-cell interaction may be critical in developing disease phenotypes (Hick et al., 2013, Ho et al., 2011).

Many of the caveats that apply to the use of iPSC-CM as disease models equally apply to their use in toxicology screening and drug development tools. As iPSC-CM have an immature and heterogeneous phenotype experimental findings will need to be triangulated with other experimental data. In reality this will mean that iPSC are likely to be used as an adjunct to animal models rather than as a replacement.
It has been suggested that a tissue engineering approach may be used to promote a more mature phenotype, which could not only improve the efficacy of cell therapy but make stem cell-derived cardiomyocytes more representative disease models, and thus improve their efficacy in drug development and toxicology screening.

1.7 *Myocardial tissue engineering*

Refinement of cell delivery may potentially improve the efficacy and durability of cell therapy (Singla et al., 2011). Novel tissue engineering approaches aims not only to optimize cell delivery by improving cell survival and retention, but also to promote beneficial reverse remodelling by manipulating the mechanical and chemical micro-environment of the heart. Additionally, a tissue engineering approach may be used to promote a more advantageous phenotype, which could not only improve the efficacy of cell therapy but make stem cell-derived cardiomyocytes more representative disease models (Rao et al., 2013). In this introductory chapter the field of myocardial tissue engineering is not comprehensively reviewed. This is huge field, a multitude of different approaches have been adopted and there are several existing reviews on this subject (Rao et al., 2013). However, in the following sections the technology and studies relevant to this work are briefly discussed.

1.7.1 *Synthetic tissue culture substrates*

Physical and chemical properties of synthetic polymers can easily be modified, altering characteristics of synthetic tissue culture substrates such as stiffness, water affinity,
degradation rate, geometry and porosity, making it possible to mimic myocardial characteristics in engineered heart tissue (EHT). However, cellular interaction with synthetic materials is limited, consequently substrates usually have to be coated in ECM components to promote cell adhesion (Li and Guan, 2011).

Electrospinning allows 3D synthetic tissue culture substrates to be fabricated from several synthetic biodegradable polymers including poly(urethane) (PU), poly(caprolactone) (PCL), poly(L-lactic acid) (PLA), poly(glycolic acid) (PGA). These substrates generally have a high surface area to volume ratio, form a porous network that mimics the fibrous architecture of ECM and promotes cell adhesion and support (Rockwood et al., 2008, Krupnick et al., 2002, Zong et al., 2005, Ishii et al., 2005, Papadaki et al., 2001, Xing et al., 2012, Shin et al., 2004). By altering the fabrication parameters the mechanical and physical properties can be manipulated, potentially effecting cellular or host organ function (Zong et al., 2005, Rockwood et al., 2008). Many of these materials have regulatory approval for in vitro use in humans (Zong et al., 2005), and there is evidence that electrospun 3D synthetic constructs, coated in ECM components can support NRVM in culture (Ishii et al., 2005, Rockwood et al., 2008), often for long periods of time, and promote greater cell maturation than standard culture techniques (Papadaki et al., 2001, Zong et al., 2005, Xing et al., 2012). Some studies suggest that electrospun substrates are flexible enough not to impede cardiomyocyte contractions (Shin et al., 2004, Ishii et al., 2005), and allow cardiomyocytes to generate force (Ishii et al., 2005), but are robust enough to survive surgical implantation (Shin et al., 2004) or use in bioreactors (Papadaki et al., 2001). In vivo characterization of electrospun polymers is limited, however PLA seeded with bone marrow derived stem cells was used in rats after ventriculotomy. PLA constructs contracted with the native myocardium and there was no evidence of arrhythmia development (Krupnick et al., 2002). There are however limitations
of electrospun substrates. Some persist *in vivo* for long periods of time whilst others elicit a significant host inflammatory response (Krupnick et al., 2002). Finally, several studies have suggested that thick constructs do not support cell culture in their core, with cell proliferation focused on the surface of the construct (Ishii et al., 2005).

### 1.7.2 Biological tissue culture substrates

Biological tissue culture substrates have historically been an integral part of almost all tissue culture. In order to facilitate cell therapy, 3D tissue culture substrates derived from extracellular matrix components such as collagen (Kutschka et al., 2006, Zimmermann et al., 2002), Matrigel (Kofidis et al., 2004b), gelatin (Li et al., 1999) and others have been used to fabricate constructs suitable for delivery and support of transplanted cells. Additionally, experimental studies have also been performed with other biological compounds such as fibrin (Birla et al., 2005), chitosan (Lu et al., 2010) and alginates (Dvir et al., 2009), which have been deemed to have properties suitable for the delivery and support of transplanted cells. The discussion of the relative advantages and efficacy of these constructs lies outside of the scope of this work, however several studies highlight key experimental findings. Firstly, whilst cell therapy appears to have at least transient efficacy (as previously discussed in Section 1.5), and modification of the mechanical properties of the diseased myocardium with an unseeded construct alone has been shown in several cases to be beneficial, the combination of cell therapy with tissue engineered constructs has been shown to be more effective in several studies (Kofidis et al., 2004b, Kutschka et al., 2006, Lu et al., 2010, Dvir et al., 2009). Secondly, several *in vitro* studies using tissue engineered biological constructs suggest that the cellular function (Zhao et al., 2005, Kofidis et al., 2004a, Zimmermann et al., 2002, Birla et al., 2005) or structure of immature cardiomyocytes (Birla et al., 2005, Yost et
al., 2004, Zhao et al., 2005) may be more representative of adult myocytes than immature cardiomyocytes cultured using conventional tissue culture methods (Yuan Ye et al., 2011, Zimmermann et al., 2002).

Since NRVM were used to recellularize a decellularized adult rat heart, there has been increasing interest in using decellularized ECM as a tissue engineering scaffold (Ott et al., 2008). Cells have been shown to infiltrate decellularized ECM and distribute evenly throughout the construct (Wang et al., 2010). Cells contract spontaneously and synchronously (Ott et al., 2008). Reseeded constructs generate more force comparable to that of artificial ECM constructs seeded with NRVM and a recellularized whole rat heart produces approximately 2% of the force produced by an adult rat heart (Ott et al., 2008). Decellularized porcine ECM has been used to support sheets of NRVM (Hata et al., 2010) and once added to the ECM the cells within the sheet align in the direction of the ECM fibers (Hata et al., 2010). Other tissue, including urinary bladder matrix has been used as an alternative to decellularized heart tissue. NRVM grow well on this substrate forming mature tissue with developed sarcomeres and striations (Shah et al., 2010b). Recently, a decellularized mouse heart has been successfully recellularized using iPSC-CM, raising the possibility that recellularized constructs may be well suited tissue culture substrates for in vitro disease modelling with iPSC-CM (Lu et al., 2013).

1.7.3 “Engineering” the phenotype of immature cardiomyocytes

Substrate stiffness (Shimazaki et al., 2008, Kraehenbuehl et al., 2008), mechanical (Tulloch et al., 2011, Fink et al., 2000) and electrical simulation (Chiu et al., 2011, Radisic et al., 2004) have all been shown to make aspects of immature cardiomyocyte function and
structure more representative of adult cardiomyocytes. Substrate geometry also has been shown to affect the phenotype of immature cardiomyocytes. The effect of substrate geometry is better characterized than stiffness, and has been suggested to have a more significant effect on cellular function (Wang et al., 2011b). Furthermore, unlike electrical and mechanical stimulation, specialist cell culture equipment are generally not required making experiments readily reproducible and experimental techniques readily applicable to high-throughput toxicology screening and drug development.

Synthetic substrates such as Poly(dimethylsiloxane) (PDMS) can be fabricated with micro or nanogrooves, using techniques such as photolithography. NRVM cultured on microgrooves are more elongated and aligned with well-organized sarcomeres (Yin et al., 2004, Bien et al., 2003). NRVM have more mature connexin 43 (Cx43) expression and the monolayer displays highly anisotropic conduction velocities. NRVM Ca\textsuperscript{2+} transient is altered with higher peak systolic levels and increased SR Ca\textsuperscript{2+} load (Yin et al., 2004, Bien et al., 2003).

Nanogrooved constructs have also been shown to elongate NRVM (Wang et al., 2011b). Anisotropic focal adhesion complexes form parallel to the grooves (Wang et al., 2011b) and cells develop anisotropic sarcomeres and actin cytoskeleton (Wang et al., 2011b, Kim et al., 2010b). Whilst cells form confluent monolayers, NRVM have a higher conduction velocity in the longitudinal direction and elliptical anisotropic propagation of the wavefront (Kim et al., 2010b). Synchronous beating rate is also higher in NRVM cultured on nanogrooves (Wang et al., 2011b).

The necessity to cover synthetic substrates with ECM components allows cell behavior to be manipulated by deposition of ECM proteins in a predetermined pattern on tissue culture
substrates producing an area of geometric control. Generally this technique is used to produce “lanes” of ECM proteins, commonly fibronectin and laminin, to produce anisotropic tissue. Cells cultured on lanes are spindle shaped (Kaji et al., 2003, Pong et al., 2011). Lanes induce myofibrillar alignment along the major axis of the cell (Pong et al., 2011), polarizing the contractile cytoskeleton of cardiac myocytes (Parker et al., 2008), increase binucleation, and promote more elliptical nuclei and higher nuclear eccentricity, similar to adult cardiomyocytes (Pong et al., 2011). Tissue alignment increases longitudinal conduction velocity and increases the maximum capture rate in response to electrical stimulation (Feinberg et al., 2012). More systolic stress is generated by anisotropically aligned cells (Feinberg et al., 2012). Cells cultured on lanes have lower diastolic Ca$^{2+}$ concentration, greater Ca$^{2+}$ transient amplitude, a faster Ca$^{2+}$ transient increase and faster Ca$^{2+}$ transient decay (Kaji et al., 2003, Feinberg et al., 2012). Lanes also alter the distribution of Cx43 on the cell membrane with increased Cx43 at bipolar ends of cells resembling the distribution on adult tissue (McDevitt et al., 2003).

Some studies have attempted to optimize lane micropatterning. One observation is that, when lane width exceeds 30μm, cells do not elongate and more than one cell attaches to the lane at each point (McDevitt et al., 2002). There is greater synchrony of beating between lanes with a smaller separation, 5-15μm, because of the formation of a monolayer as cells are close enough to interact (McDevitt et al., 2002). Diastolic Ca$^{2+}$ is altered more in NRVM seeded on lanes with 10μm-width than 20μm width (Kaji et al., 2003). Diffusion tensor magnetic resonance imaging patterns can be reproduced to form sheets of myocytes with representative tissue boundaries and fiber directions (Badie and Bursac, 2009, Badie et al., 2012). This technique uses lines with spacing of between 2-10μm (Badie et al., 2012). Cells have clear cross striations and abundant intercellular coupling with smooth transitions between the
subregions within the monolayer. Cell culture on these designs increases spatial nonuniformity of conduction (Badie and Bursac, 2009). Wavefronts have the pointed characteristic of elliptical anisotropic propagation (Badie and Bursac, 2009). This technique has been used to study ventricular conduction block \textit{in vitro}, suggesting that anatomical features can act as independent contributors to conduction failures and could precipitate arrhythmias which may be important when considering results from \textit{in vitro} disease modelling (Badie et al., 2012).

1.7.4 Limitations of current tissue engineering approaches

There is a clear clinical need for tissue engineering approaches in cell therapy to improve cell homing and survival. Tissue engineering approaches are also required for disease modelling, in order to improve both the homogeneity of the cellular phenotype, and the degree to which \textit{in vitro} disease models could be considered to be representative of adult human myocardium. Despite promising early results, the potential of tissue engineering approaches to contribute to myocardial disease modelling remains speculative and is yet to be definitively demonstrated. Evidence of the applicability of these techniques to iPSC-CM in the literature is limited. Finally, where improvements in the function of immature cardiomyocytes have been achieved, the mechanisms remain unclear.

1.8 Thesis aims

Although the potential of cell therapy has historically driven cardiac stem cell research, myocardial disease modelling is now an equally important, perhaps more significant
application for this technology. Consequently, the general aim of this thesis was to explore the feasibility of using iPSC-CM as in vitro models. In particular, we aimed to explore the limitations of current iPSC-CM technology and how they could be overcome.

As discussed previously, several studies suggest that iPSC-CM and hESC-CM are sufficiently comparable to adult myocardium to facilitate application in a wide range of toxicology, drug development and disease modelling applications (Sections 1.4.2 and 1.6.). However, the evidence highlighted in Section 1.4.1 and 1.4.2 suggests that the literature asserting that iPSC-CM and hESC-CM have a mature, representative phenotype is sometimes weak and inconsistent.

Consequently, we aimed to test the hypothesis that iPSC-CM and hESC-CM have a predictable and consistent response to pharmacological manipulation. We investigated whether differentiation protocols affect the response to pharmacological manipulation, and whether iPSC-CM and hESC-CM have different responses to pharmacological manipulation. We used currently-available high-throughput tools to undertake this work, in order to demonstrate the efficacy of iPSC-CM as drug development and toxicology screening tools.

Given the large body of literature (Section 1.4) which suggested that iPSC-CM have an immature phenotype we were keen to investigate if the phenotype of iPSC-CM could be manipulated to make iPSC-CM more representative of adult myocardium. Several techniques have been suggested to manipulate the phenotype of immature cardiomyocytes in culture (Section 1.7.3). We decided to focus on the effect of substrate geometry on the phenotype of immature cardiomyocytes as these techniques are readily replicated. Furthermore, we envisaged that these techniques could easily be applied to high-throughput drug development
or toxicology screening. Consequently, we investigated whether alignment using a range of methods could modulate the physiological properties of immature cardiomyocytes. In particular we focused on intra-cellular Ca\(^{2+}\) cycling, which is central to excitation contraction coupling, a fundamental component of cardiomyocyte physiology. Therefore, we tested the specific hypothesis that alignment of immature cardiomyocytes, in a fashion analogous to the adult myocardium, will improve intra-cellular Ca\(^{2+}\) cycling.

For much of the exploratory work and validation of the techniques we used NRVM as they are more readily available than iPSC-CM. The multitude of studies in the existing literature which demonstrate that cellular alignment has a positive effect on the Ca\(^{2+}\) cycling of NRVM, suggest that the positive effect of cell alignment on Ca\(^{2+}\) cycling may be generic to all methods of aligning the cells. We aimed to validate the reproducibility of these techniques in our laboratory and to conclusively establish that cell alignment, irrespective of the method used, has a positive effect on Ca\(^{2+}\) cycling in NRVM.

Although NRVM are readily accessible, and extremely cost-effective, they do not represent a human model, and are increasingly being superseded by iPSC-CM. This trend is likely to increase as iPSC technology becomes more cost-effective and widely accessible. Consequently, after validating the tissue engineering techniques required to promote cellular alignment, we aimed to test the hypothesis that cell alignment will also promote improved Ca\(^{2+}\) cycling properties of human iPSC-CM, making them more representative of adult myocardium. We also sought to elucidate any sub-cellular mechanisms that may underlie changes in cellular Ca\(^{2+}\) cycling brought about by cellular alignment of iPSC-CM.
Finally we aimed to explore how tissue engineering approaches could be further improved to optimize the Ca\(^{2+}\) cycling properties of immature cardiomyocytes, comparing the effect of cell alignment on Ca\(^{2+}\) cycling with the impact of 3D biological and synthetic constructs.
2 General Material and Methods
2.1 **Tissue culture methods**

2.1.1 **Isolation and culture of neonatal rat ventricular myocytes**

NRVM were isolated and cultured from Sprague Dawley neonatal rats 3 days after birth with complete compliance to United Kingdom (UK) Home Office regulations as determined by the Animals (Scientific Procedures) Act of 1986, as previously described (Brand et al., 2010).

Briefly, the rats were euthanized by cervical dislocation and the head was excised. A median sternotomy was performed taking care not to open abdominal viscous. The mediastinum was then exposed by applying simultaneous posterior traction to both forelimbs. The heart ventricles were excised by blunt traction with curved forceps to the great vessels. The ventricles were then cut in half with sterile forceps and placed in a 50ml Falcon tube containing ice cold 1x Ads buffer solution (2500ml 1x Ads solution containing 17g NaCl, 11.9g 4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid (HEPES), 0.3g NaH$_2$PO$_4$, 2.5g glucose, 1g KCl, 0.25g MgSO$_4$ and adjusted to pH 7.35 with NaOH (all from WVR)) for transfer to the tissue culture laboratory. The ventricular tissue was washed twice with 1x Ads buffer and minced into smaller pieces 2-3mm in diameter and then transferred into an upstanding culture flask. 10ml enzyme containing solution (containing 24mg collagenase type 2 (Worthington) and 38.4mg pancreatin (Sigma-Aldrich) dissolved in 50ml Ads buffer) was added to the flask, and the flask was agitated in a 37°C water bath for 5mins to remove blood cells and non-myocyte tissue. The supernatant was removed and replaced with a further 10ml enzyme containing solution and the flask was agitated for 20mins in a 37°C water bath. Enzymatic digestion was performed at intervals of 20mins, 25mins, 25mins, 15mins and 20mins. After each digestion the supernatant, containing detached cells, was transferred to a
50ml centrifuge tube (Corning) containing 2ml foetal bovine serum (FBS) and centrifuged at 800rpm for 5mins. The supernatant was then removed and the pellet of cells was re-suspended in 4ml FBS, before being incubated in a 5% CO₂ incubator at 37°C. Following collection of the final isolation, the FBS containing suspended isolated cells was centrifuged at 800rpm for 5mins. The FBS was removed and the pellet containing the cells was resuspended in Complete Medium (Dulbecco’s Modified Eagle Medium (DMEM) solution (Invitrogen), 25% medium 199 (Sigma-Aldrich), 100U/ml penicillin (Sigma-Aldrich), 50μg/ml streptomycin (Sigma-Aldrich), 15% horse serum (Invitrogen), 7% FBS (Invitrogen) and 3.4% HEPES (Sigma-Aldrich)) and pre-plated for 45 minutes in two 10cm petri-dishes. The myocytes were then swept with complete medium and counted using a Malassez hemocytometer (Fisher Scientific) to ensure sufficient numbers and adequate morphology before plating at a density according to the required application (Section 2.1). 48 hours following plating, non-adherent cells and debris were removed by banging the plate several times on the base of a tissue culture hood without spilling the medium and then gently washing the bottom of the plate twice with 37°C complete medium. Complete medium was then changed every other day after plating. Cardiomyocytes were cultured in a standard cell culture incubator in 5% CO₂ at 37°C.

2.1.2 Differentiation and maintenance of induced pluripotent stem cell-derived cardiomyocytes and human embryonic stem cell-derived cardiomyocytes

2.1.2.1 Maintenance of undifferentiated human embryonic stem cells

hESC from the ethically derived H7 line (Geron Corporation, USA) was imported with permission from the UK Stem Cell Bank. Undifferentiated hESC were cultured on plates
coated with Matrigel (BD Sciences) using MEF conditioned medium (MEF-CM) (supplemented with 8ng/ml recombinant bFGF (Invitrogen), 50U/ml penicillin and 50μg/ml streptomycin (both from Sigma-Aldrich)) which was changed daily, except for the first two days following passaging. The cells were passaged weekly. MEF-CM was made using MEF isolated from 13-day post coitum MF-1 strain mouse embryos which, after the fourth passage, were treated with 0.01mg/ml mitomycin C (Sigma-Aldrich) to mitotically inactivate them. hESC medium (containing 80% Knock Out Dulbecco’s Modified Eagle Medium (KODMEM), 20% Knock Out Serum replacement (KOSR), 1mM L-glutamine, 10mM non-essential amino acids (NEAA), 0.1mM β-mercaptoethanol, 4ng/ml bFGF (all from Invitrogen), 50U/ml penicillin and 50μg/ml streptomycin (both from Sigma-Aldrich)) was conditioned by adding 150ml to T225 flasks containing 1.88×10^7 mitotically inactivated MEF (Foldes et al., 2011, Xu et al., 2001).

2.1.2.2 Differentiation of human embryonic stem cell-derived cardiomyocytes by the embryoid body method

hESC-CM were differentiated from hESC line H7, using the embryoid body (EB) technique as previously described (Foldes et al., 2011, Xu et al., 2001, Brito-Martins et al., 2008). Briefly, colonies of hESC were mechanically disrupted after 3–10min of collagenase IV (Invitrogen) treatment to remove spontaneously differentiated cells. The resulting hESC EB were then cultured in suspension using low adherence plates for 4 days in differentiation medium (hESC medium in which KOSR had been replaced by non-heat-inactivated FBS). The EB were plated out onto 0.5% gelatin-coated plastic dishes. Spontaneously beating clusters of hESC-CM appeared from day-9 after EB formation in outgrowths of cells from EB (Foldes et al., 2011, Xu et al., 2001, Brito-Martins et al., 2008). Spontaneously beating
clusters of hESC-CM were micro-dissected and seeded onto gelatinized dishes overnight before being used in experiments (Foldes et al., 2011).

2.1.2.3 Directed differentiation techniques

hESC-CM were differentiated from dense mono-layers of hESC as previously described (Laflamme et al., 2007, Foldes et al., 2011). Briefly, hESC-CM were cultured in RMPI-B27 medium (Sigma-Aldrich), and treated with 100 ng/ml human recombinant Activin A (R&D Systems) for 24 hours, and 10 ng/ml BMP 4 (R&D Systems) for 4 days (Laflamme et al., 2007, Foldes et al., 2011). Spontaneously beating areas appeared after 1–2 weeks. Plates containing beating areas were enzymatically disassociated using 0.025% trypsin ethylenediaminetetraacetic (EDTA) (Sigma-Aldrich) and seeded onto gelatinized dishes overnight before being used in experiments (Foldes et al., 2011).

2.1.3 Thawing and maintenance of i-Cell cardiomyocytes

iCell cardiomyocytes (Cellular Dynamics, Wisconsin) are a commercially available population of iPSC-CM differentiated and purified using proprietary protocols. The cells express monomeric red fluorescent protein (mRFP) and blasticidin resistance, with an MYH6 promoter that facilitates cardiomyocyte purification and identification.

iCell cardiomyocytes were thawed and maintained according to manufacturer’s guidelines (Cellular Dynamics International., 2011). Briefly, vials containing 1ml cryopreserved single-cell suspensions of iCell Cardiomyocytes were stored in liquid nitrogen storage until
immediately before use. Prior to plating the frozen iCell Cardiomyocytes were removed from liquid nitrogen and placed stationary in a 37°C water bath for 4 minutes. The iCell cardiomyocytes were then gently transferred into a 50ml centrifuge tube (Corning). The empty iCell Cardiomyocytes vial was then washed with 1ml of room temperature iCell Plating Medium (Cellular Dynamics) to recover residual cells. This plating medium was then added to the 50ml centrifuge tube drop-wise over at least 90s while gently swirling the tube. This was then repeated. A further 7ml plating medium was added to the centrifuge tube drop-wise fashion over 30-60s while gently swirling the tube. The contents of the 50ml centrifuge tube were then mixed by gently inverting the tube 2-3 times. The thawed iCell cardiomyocytes were than counted with a Malassez hemocytometer (Fisher Scientific) using trypan blue (Sigma-Aldrich) to identify non-viable cells. Additional plating medium was then added to achieve the cell density required (Section 2.1) according to the following formula (Formula 2.1). If a higher cell density was required iCell Cardiomyocytes were pelleted by centrifuging at 180g for 5 minutes, removing the necessary volume of plating medium and then re-suspending the iCell Cardiomyocytes. 48 hours following plating, non-adherent cells and debris were removed by banging the plate several times on the base of a tissue culture hood without spilling the plating medium and then gently washing the bottom of the plate twice with 37°C iCell Maintenance Medium (Cellular Dynamics). Maintenance medium was then changed every other day after plating. iCell Cardiomyocytes were cultured in a standard cell culture incubator in 5% CO2 at 37°C.

\[
\frac{\text{Volume of iCell containing medium placed in each well (ml)}}{\text{Viable cell density after counting (cells/ml) x Plating efficiency}} = \frac{\text{Number of cells required in each well (cells)}}{\text{Plating efficiency}}
\]

**Formula 2.1.** Plating efficiency is a batch-specific quality metric defined by cellular dynamics representing the proportion of viable cells introduced into a well suspended in plating medium which will adhere to the bottom of each tissue culture well.
2.2 Tissue engineering methods

2.2.1 Fabrication of structured silicone culture substrates

Structured flexible microgrooved polydimethylsiloxane (PDMS) tissue culture substrates were fabricated by Miss Tatiana Trantidou and Dr Themistoklis Prodromakis at the Centre for Bio-Inspired Technology, Imperial College London. The fabrication of the PDMS constructs is described in Appendix A.

2.2.2 Fabrication of micro-patterned fibronectin lines

Micro-patterned fibronectin lines were fabricated on flat PDMS membranes (PDMS sheets 0.25mm in thickness; Specialty Manufacturing) (Figure 2.1) (Camelliti et al., 2006). A micro-grooved PDMS stamp was fabricated using standard photolithography rules as described in Appendix A. The stamp was 2cm x 3cm in dimension with parallel grooves 30µm in width and 10µm in depth, 300µm apart running lengthwise along the entire surface of the stamp. The stamp was sterilized by emersion in 70% alcohol and exposure to UV light overnight prior to use, and was washed several times with sterile water. Flat PDMS membrane was cut to an appropriate size to fit onto the bottom of a 60mm petri dish with a 1-2mm margin around the edge of the stamp when placed on top of the flat PDMS membrane. The flat PDMS membranes were pressed onto the bottom of 60mm petri dishes taking care to avoid bubbles and then were sterilized by immersion in 70% alcohol and exposure to UV light overnight.
Figure 2.1. Schematic diagram, not drawn to scale illustrating the fabrication of fibronectin lined constructs (With thanks to Umar Chaudhry for assistance in producing this figure)
The stamp was then pressed onto the PDMS membrane with the grooved surface placed downwards. Fibronectin lines were formed by microfluidic patterning whereby 50μg/ml fibronectin was flushed between the microgrooves of the stamp and the flat PDMS membrane by applying negative pressure using a standard tissue culture suction pump. Without removing the stamp the PDMS membrane was incubated at 37°C for 1 hour. The stamp was then removed, and the dish incubated at 37°C overnight prior to plating of the cells. 1 million NRVM were seeded onto each construct. Flat PDMS membrane coated with fibronectin of the same concentration were used as control constructs. Following use the stamps were sonicated at 37°C degree in collagenase (Worthington) for at least 15 minutes and were then rinsed thoroughly with distilled water before being cleaned as previously described.

Photolithography and PDMS molding was performed by Tatiana Trantidou and Themistoklis Prodromakis at the Centre for Bio-Inspired Technology, Imperial College London. This technique was optimized by Patrizia Camelliti, Laboratory of Cellular Electrophysiology, Imperial College London (Camelliti et al., 2006).

2.2.3 Fabrication of Parylene-C constructs

Oxygen plasma-treated structured Parylene-C constructs were fabricated by Tatiana Trantidou with the assistance of Themistoklis Prodromakis at the Centre for Bio-Inspired Technology, Imperial College London. The fabrication of the Parylene-C constructs is described in Appendix B.
2.2.4 Fabrication of three-dimensional polycaprolactone constructs

Three Dimensional Polycaprolactone (PCL) constructs were fabricated by Jerme Sohier, at the Harefield Heart Science Centre Tissue Engineering Group, Imperial College London. The fabrication of these constructs is described in Appendix C.

2.2.5 Decellularization of cardiac tissue slices

Heart slices were produced from canine ventricular tissue, obtained from control animals in toxicology trials performed at GlaxoSmithKline. All experiments strictly complied with UK Home Office regulations as determined by the Animals (Scientific Procedures) Act of 1986. Human heart slices were obtained either from the core of tissue removed from the apex of the left ventricle in patients with end-stage heart failure which is necessarily removed in order to insert a left-ventricular assist device, or from explanted hearts in patients with end stage heart failure who underwent heart transplant. In all cases appropriate consent was obtained and institutional ethical guidelines were adhered to (Royal Brompton and Harefield NHS Trust Ethics Committee permission number 02–039). Organotypic slices were cut as previously described (Camelliti et al., 2011). Briefly, tissue slices were cut parallel to the epicardial plane with a high precision vibrating microtome (7000smz, Campden Instruments Ltd., UK). 350μm slices were cut with a steel blade with vibration frequency 75Hz, amplitude 2mm and an advance speed 0.04mm/s in cold (4°C) oxygenated (100% O₂) Normal Tyrode (NT) solution (containing 140mM NaCl, 6mM KCl, 10mM glucose, 10mM HEPES, 1mM MgCl₂, 1.8mM CaCl₂, adjusted to pH7.4 with 2M NaOH ((all Sigma–Aldrich)) with 10mM of the excitation–contraction un-coupler 2,3-butanedione monoxime (BDM) added (Camelliti et al.,
2011). Slices were decellularized by placing them on a shaker (Platform Shaker STR6, Stuart Scientific, UK), agitating them at 70 rev/min, in 1% Sodium dodecyl sulfate (SDS) (Sigma-Aldrich) at room temperature for 24 hours then washing with PBS before being left on a shaker, agitating them at 70 rev/min, at room temperature for 4 hours in 1% Triton (Sigma-Aldrich) (Ott et al., 2008, Godier-Furnemont et al., 2011). These slices were then washed with PBS and frozen at -20°C in Freezing Medium (Containing 90% FBS (Invitrogen) and 10% Dimethyl sulfoxide (DMSO) (Sigma-Aldrich)) until required. Once defrosted the constructs were then stretched over a Scaffdex ‘cell crown’ (Scaffdex) and transferred to a new sterile plate. They were then sterilized by immersion in 100% ethanol for 10 minutes then washed with sterile PBS. 1 million NRVM were seeded onto each construct.

2.3 Electrophysiological techniques

2.3.1 Multi-electrode arrays

To characterize the electrophysiological properties of multi-cellular preparations of hESC-CM and iPSC-CM, a multi-electrode array (MEA) recording system (MEA1060, Multi-Channel Systems, Reutlingen, Germany) was used as previously described (Zwi et al., 2009). The MEA recording system allows simultaneous field potential recording with high temporal resolution, from 60 titanium nitride-coated electrodes, 30μm in diameter and 200μm apart (Nisch et al., 1994, Igelmund et al., 1999) embedded into the bottom a glass tissue culture dish (Figure 2.2).
Figure 2.2. A) A MEA dish showing the position of the electrode inserted into the bottom of the dish to facilitate superfusion of the cultured cells with physiological solutions or pharmacological agents. B) A beating cluster of iPSC-CM, differentiated using the EB method plated onto the electrodes of an MEA. C) The same cluster of iPSC-CM enlarged. D) hESC-CM differentiated using a directed differentiation monolayer technique plated onto a MEA at a similar magnification for comparison.

Clusters of contracting hESC-CM and iPSC-CM differentiated using the EB method were micro-dissected, plated on gelatin-coated MEA plates, and cultured for at least 8 hours in human differentiation medium before experiments were undertaken. hESC-CM differentiated using a directed differentiation mono-layer culture system were enzymatically digested with 0.025% trypsin EDTA (Sigma-Aldrich); after the trypsin was inactivated with FBS the suspension containing hESC-CM was centrifuged at 800rpm for 5mins and then re-suspended in human differentiation medium before being seeded onto the MEA. All hESC-CM and iPSC-CM were used between 40 and 80 days post differentiation. They were then maintained
in culture with human differentiation medium for a minimum 4 days until a beating mono-
layer of cells had been re-established. hESC-CM and iPSC-CM differentiated using both the
EB and directed differentiation method were cultured on the MEA dish for 5-7 days before
recordings were made. Field potentials recordings necessitate removing the MEA dish from
the incubator, in order to place the MEA dishes in the recording system. Whilst recordings
are made the MEA dishes are held on a temperature controlled plate. The hESC-CM and
iPSC-CM clusters are superfused with oxygenated NT solution at 37°C during recordings
(Figure 2.2).

![Figure 2.3. The field potential measured from a spontaneously beating cluster of iPSC-CM measured using a MEA.](image)

The recorded field potentials were used to determine the local field potential (FP) duration
(FPD). This parameter has been shown to correlate with the AP duration (APD) (Halbach et
al., 2003, Meiry et al., 2001). The estimated FPD was defined as the time interval between
the initial deflections of the FP to timing of the maximum of the local T wave (QT Interval) (
Figure 2.3). FPD measurements were normalized to the beating rate of the contracting areas
with the Bazett's correction formula which has been validated in patients (Yap and Camm,
2003), animal models (Farkas et al., 2002), and hESC-CM (Caspi et al., 2009). Custom
software was used to analyze field-potential recordings (MEA-Tools, version 2.8. University of Freiburg, Germany)(Egert et al., 2002).

For pharmacological studies, drugs were superfused dissolved in NT. The tested drugs included isoproterenol hydrochloride (or Isoprenaline), a non-selective beta-adrenergic agonist (3nM for 5mins, 30nM for 5mins, 300nM for 5mins) (Sigma-Aldrich); E-4031, a selective antagonist of the Kv11.1 or hERG channel which mediates Ikf (1nM for 5mins, 10nM for 5mins, 100nM for 5mins, 1µM for 5mins) (Sigma-Aldrich); chromanol 293B, a selective antagonist of the Kv7.1 channel which mediates Iks (10µM for 5mins, 20µM for 5mins) (Sigma-Aldrich); and ivabradine hydrochloride, a selective antagonist of If (300nM for 5mins, 3µM for 5mins, 30µM for 5mins, 300µM for 5mins) (Servier). Prior to superfusion with drugs, cardiomyocytes were superfused with NT for 10 minutes to obtain baseline readings, and an NT wash-out was performed afterwards until measured parameters returned towards baseline.

2.3.2 Action potential measurement

AP were measured using the perforated-patch technique, as previously described (Shah et al., 2010a). Briefly, adult ventricular myocytes were enzymatically isolated from Lewis rats and control-dogs sacrificed during drug development safety trials (Siedlecka et al., 2008). Ventricular myocytes were then attached to the top of a glass cover-slip freshly covered with laminin (Sigma-Aldrich) by incubating 1-2 drops of a suspension of cells in buffer solution on the cover slip for 5-10 minutes. hESC-CM were enzymatically digested either between 15-40 days or following 40 days following differentiation using the EB technique using 0.025%
trypsin EDTA (Sigma-Aldrich). Afterwards the trypsin was inactivated with FBS and the suspension containing hESC-CM was centrifuged at 800rpm for 5 minutes and then resuspended in human differentiation medium before being seeded onto laminin-covered 10mm glass-bottomed sections of 35mm MatTek dishes (MatTek corporation). Cells were then superfused with NT solution and studied using a Multiclamp 2B amplifier (Axon Instruments, CA, USA). The pipette resistance was less than 2MΩ. The pipette-filling solution contained 10mM NaCl, 20mM KCl, 125mM KCH$_3$O$_3$, 10mM HEPES, 5mM MgCl$_2$ (All VWR) 240μg/ml amphotericin-B (Sigma-Aldrich), and adjusted to pH 7.2 with 2M NaOH. AP were measured in current-clamp mode after stimulation at 1Hz, 3Hz and 5Hz using a 1ms, 0.6-1.8nA pulse. During recording the cells were superfused with NT at 37°C. Under direct vision the pipette tip was moved in close proximity to the target cell and then brought into contact using micro-manipulators (Burleigh Instruments), a seal was formed between the cell and the pipette, and after approximately 20s to allow the amphotericin to form micro-perforations without dialyzing the cell, recordings were commenced. The AP measured were analyzed using pCLAMP 10.3 software (Molecular Devices, Sunnyvale, CA). Traces were averaged with reference to the stimulation signal. The time-to-peak depolarization (TTP), time from peak depolarization to 50% repolarization (T50), time from peak depolarization to 90% repolarization (T90), and amplitude measured from resting membrane potential to peak depolarization in each group were compared (Peng et al., 2010, Shah et al., 2010a).

We were unable to patch clamp cells on opaque or translucent tissue culture constructs, consequently AP characteristics were measured by impalement with sharp pipettes, using floating electrodes, as previously described (Terracciano et al., 2002). Briefly, AP measurements were performed using an Axoclamp 2B system (Axon Instruments). High
resistance microelectrodes were used (15–25MΩ) (Harvard Apparatus). Cells were superfused with NT solution at 37°C and the microelectrode filling solution contained: 2M KCl, 0.1mM EGTA, 5mM HEPES adjusted to pH 7.2 with 2M NaOH (All Sigma-Aldrich). As the construct material was opaque, and the pipettes were exceedingly fine it was not possible to perform impalement under direct vision. After confirming the approximate location of the pipette tip it was lowered to the surface of the construct using micromanipulators whilst recording was performed. AP were recorded in current clamp mode and measured AP were analyzed using pCLAMP 10.3 software (Molecular Devices).

2.3.3 Measurement of calcium transients

NRVM and iCell cardiomyocytes (Cellular Dynamics) were seeded onto constructs described in (Section 2.1). At day 4 following seeding of NRVM, 1 week and 2 weeks following seeding for iCell cardiomyocytes the cells were loaded with the Ca²⁺ sensitive dyes Rhod-2 AM (10µM, Invitrogen) or Fluo-4 AM (10µM, Invitrogen) using 4µl (250nM) probenecid (Invitrogen) and 0.2% Pluronic acid (Invitrogen), in pre-warmed DMEM at 37°C for 30minutes. The myocytes were then washed and incubated with pre-warmed DMEM containing 2% FBS (Invitrogen) and 250nM probenecid for 30minutes to de-esterify.

The experimental dish was mounted on the stage of an upright Zeiss LSM510 confocal microscope (Carl Zeiss) and myocytes were observed through a x40 water-immersion magnification lens. Rhod-2 AM was excited at 552-nm of a HeNe1 laser, and the emitted fluorescence was collected through a 581-nm long-pass filter. Fluo-4 AM was excited using the 488-nm line of an argon laser, and the emitted fluorescence was collected through a 505-nm long-pass filter.
Line scanning was performed at suitable regions with the myocytes spontaneously beating or under field stimulation at 0.5Hz, 1Hz and 2Hz using an external pacing generator (IonOptix) (Figure 2.4). Charge-balanced biphasic pulses 10ms in duration typically of 1V amplitude were used for field stimulation. On the rare occasions when no electrical capture was achieved initially the amplitude of the pulses was increased incrementally to a maximum of 5V. During recording the cells were superfused with 37°C NT or low Na+ and Ca2+ solution (containing 140mM LiCl, 6mM KOH, 1mM MgCl2, 10mM glucose, 10mM HEPES, 0.1mM EGTA adjusted to pH7.4 with 2M NaOH (all Sigma–Aldrich)). RyR mediated SR Ca2+
release was elicited by spritzing the cells with solutions containing 50mM caffeine (Sigma–Aldrich).

Figure 2.5. Line scan images such as those shown in Figure 2.4 are quantified, to facilitate measurement of time to peak, time to 50% and 90% decay and amplitude.

Linear time-length images were converted into Ca\textsuperscript{2+} transients using ImageJ (National Institutes of Health, USA) and analyzed using pCLAMP10.3 (Molecular Devices, Sunnyvale, CA). Fluorescent values were normalized to baseline fluorescence (F/F0). TTP was taken as the time taken for the ratio signal to reach peak fluorescence from baseline fluorescence.
Similarly, T50 and T90 were taken as the time taken for the peak fluorescent transient to decline by 50% and 90% of the transient amplitude. The presence of spontaneous beating and the beating rate were also noted (Stagg et al., 2008) (Figure 2.5).

### 2.4 Molecular biology and cell imaging techniques

#### 2.4.1 Gene expression

Total RNA from iCell cardiomyocytes was isolated using the RNeasy Mini Kit (Qiagen) according to manufactures guidelines (Qiagen, 2010). Briefly, the structured constructs (Section 2.2.1) seeded with iCell cardiomyocytes were placed in new culture dishes 2 weeks following seeding of the myocytes, and remaining iCell maintenance medium (Cellular Dynamics) was removed by washing with PBS (Sigma-Aldrich).

After PBS was removed 350μl of lysis Buffer RLT (Qiagen) was added to each well to the cell-culture dish. The lysate was collected with a rubber policeman (Fisher Scientific) and pipetted into a micro-centrifuge tube (Eppendorf) and vortexed for one minute (ProScientific). The lysate was then pipetted into a QIAshredder spin column (Qiagen), placed in a 2ml collection tube, and centrifuged for 2min at full speed. 350μl of 70% ethanol was then added to the homogenized lysate, and the mixture was added to an RNeasy spin column (Qiagen) placed in a 2ml collection tube (supplied) and centrifuged for 15s at 8000g. 700μl of RW1 wash buffer (Qiagen) was added to the RNeasy spin column before centrifuging again for 15s at 8000g. Twice 500μl of RPE buffer (Qiagen) was then added to the RNeasy spin column before centrifuging for 15s at 8000g on the first occasion and 2min on the second occasion.
<table>
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<th>Gene</th>
<th>Encoding</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTB</td>
<td>Beta actin (β-actin)</td>
<td>(F)CAAACGCGGAGAAGATGA (R)CCAGGGGCTACGGGATAG</td>
</tr>
<tr>
<td>AMPH2</td>
<td>Bridging Integrator 1 (BIN1)</td>
<td>(F)ACGGGAGCACAACCTCTGA (R)GCGGCTAACAACAGTCTTCT</td>
</tr>
<tr>
<td>ATP2A2</td>
<td>SERCA2a</td>
<td>(F)AACGTCGGGAAGCTTCTA (R)GCTACGCACTGGCAGG</td>
</tr>
<tr>
<td>CACNA1C</td>
<td>L-type voltage-dependent Ca(^{2+}) channel (Ca(_{1.2}))</td>
<td>(F)TGACATCGGGAGAGAATACT (R)AGATTTGAGTAGGCTG</td>
</tr>
<tr>
<td>CACNA1G</td>
<td>T-type voltage-dependent Ca(^{2+}) channel (Ca(_{3.1}))</td>
<td>(F)TGCTTCATTGGCCTGTTCAT (R)TCTCCGGTCTGTGATTTC</td>
</tr>
<tr>
<td>CALR</td>
<td>Calreticulin</td>
<td>(F)CTATGATATCTTTGCGCTT (R)CATTGACGATGATCTCC</td>
</tr>
<tr>
<td>CASQ2</td>
<td>Calsequestrin 2</td>
<td>(F)GAGTGGGATGGCAGTGTGC (R)TTGTGCTGTGATGGATGA</td>
</tr>
<tr>
<td>CAV3</td>
<td>Caveolin 3</td>
<td>(F)GAAACATCTGACGTCAGG (R)CTTGGACGATGACGATGA</td>
</tr>
<tr>
<td>GADPH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)</td>
<td>(F)ACCATGTCGATAGTCTCACA (R)TCTCTGCTGGTCTGACCT</td>
</tr>
<tr>
<td>GATA4</td>
<td>GATA binding protein 4 (GATA4)</td>
<td>(F)GGAAGCCGCAAGACCTCAAG (R)CTCCTGCTTATTATACCA</td>
</tr>
<tr>
<td>ITPR2</td>
<td>Inositol 1,4,5-trisphosphate receptor, type 2 (IP3R)</td>
<td>(F)CCATCTCGAAACTCTCAAGG (R)GTCTGACATTGATATCC</td>
</tr>
<tr>
<td>JPH2</td>
<td>Junctophilin 2</td>
<td>(F)AAGCTTGTGCTGATGACGATG (R)CTTGGACGATGACGATGA</td>
</tr>
<tr>
<td>MYH6</td>
<td>Myosin Heavy Chain 6 (α-MHC)</td>
<td>(F)CTCAAGCTCATGGCCAC (R)GCCTCCTTTGCTTTTAC</td>
</tr>
<tr>
<td>MYH7</td>
<td>Myosin Heavy Chain 7 (β-MHC)</td>
<td>(F)ACACCCTGACTAAGGCCAAA (R)TCAGGGGATCCTTCCAG</td>
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<td>MYL2</td>
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<td>(F)ACAATGTCGATAGTCTCACA (R)TCTCTGCTGGTCTGACCT</td>
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<tr>
<td>N1KX2-5</td>
<td>Homeobox protein Nkx-2.5 (Nkx-2.5)</td>
<td>(F)ACCATGCACTTTACCGCAA (R)ATGGAACATGTCTGAGTA</td>
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<tr>
<td>NPPA</td>
<td>Natriuretic Peptide A (ANF)</td>
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</tr>
<tr>
<td>PLN</td>
<td>Phospholamban</td>
<td>(F)AGTGTGATACAGCTCGCAAG (R)TGAGGGAGGTAGTAT</td>
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<tr>
<td>PPIG</td>
<td>Peptidylprolyl isomerase G (Cycliphilin G)</td>
<td>(F)CTCTGTCATTGGCCTGCAAGG (R)GCCCATCTAAATGAGGAGGT</td>
</tr>
<tr>
<td>RN18S</td>
<td>18S ribosomal RNA (18S)</td>
<td>(F)GCAATTTACCCTTGGAGG (R)GGGACTTAAACCGAAGG</td>
</tr>
<tr>
<td>RYR2</td>
<td>RyR 2</td>
<td>(F)CTGCGCCATTCCTATAG (R)CTGACGGAAGGTAGTAT</td>
</tr>
<tr>
<td>SLCBA1</td>
<td>Solute carrier family 8 (Na(^+)/Ca(^{2+}) exchanger), member 1 (NCX)</td>
<td>(F)GGTGGGATCAACAGCTGGA (R)CCATCTCATCTCGTCA</td>
</tr>
<tr>
<td>TCAP</td>
<td>Titin-cap (Telethonin)</td>
<td>(F)GCGACAGATGGAGGATCTGA (R)TCATGTGCGTCTGAGTGGT</td>
</tr>
<tr>
<td>TNNT2</td>
<td>Troponin T type 2 (Cardiac) (cTnT)</td>
<td>(F)GTACCCATGCTGGAGTGCT (R)TTATACTGCTGAGTGGGG</td>
</tr>
<tr>
<td>TRDN</td>
<td>Triadin</td>
<td>(F)ACATATTGCTCATGGGGATT (R)TGGAAGCTTTCCTGCGGAT</td>
</tr>
</tbody>
</table>

Table 2.1. Summary of PCR primers used in gene expression analysis.
The collection tubes were then changed before the RNeasy spin column was centrifuged again at full speed for 1 min. The RNeasy spin column was then placed in a new 1.5ml collection tube and 30–50μl of Ribonuclease-free water (Qiagen) was twice added to the spin column before centrifuging for 1 min at 8000g to elute the RNA.

Genomic DNA was removed by DNase I (Invitrogen) treatment and total RNA (500ng) was reversed transcribed into Complementary DNA (cDNA). Quantitative Polymerase Chain Reaction (qPCR) was performed using 150ng of cDNA using SensiMix SYBR No-ROX Kit (Bioline, UK) on the Rotor-Gene™ 6000 (Corbett Research) according to manufacturer’s guidelines (Life Technologies, 2011). The primers used are summarized in Table 2.1. Total RNA from human adult and foetal heart tissue (Agilent) was used to evaluate gene expression levels in iCell cardiomyocytes. All values were normalized with respect to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression.

SYBR green primers were designed by Ljudmila Kolker, National Heart and Lung Institute, Imperial College London and National Institute for Biological Standards. Additionally, she assisted with all of the gene expression studies described in this thesis.

2.4.2 Immunocytochemistry

In order to evaluate cell structure on the surface of constructs, the constructs were fixed in 4% paraformaldehyde in 0.1M phosphate buffer (Agar Scientific) for 10 minutes, washed in PBS (Sigma-Aldrich) and permeabilized using 0.2% triton-X (Sigma-Aldrich) in PBS for 3 minutes followed by two PBS washes. The coverslips were then incubated with blocking solution containing 3% Bovine Serum Albumin (BSA) (Sigma-Aldrich) in PBS for 30
minutes. Various primary antibodies (Mouse α-Actinin IgG Ascites, 1:100, Sigma-Aldrich; Mouse RyR IgG 1mg/ml, 1:500, Abcam; Rabbit Cav1.2 IgG 0.8mg/ml, 1:100, Alomone Labs; Mouse PLN IgG 1mg/ml, 1:200, Badrilla; Rabbit Cx43 IgG 0.5mg/ml, 1:50, Millipore) were added for 1 hour at room temperature. The constructs were then washed at least 3 times in PBS for 3-5 minutes each. Secondary antibodies were then added (Alexa Fluor 488 anti-mouse, Alexa Fluor 488 anti-rabbit, Alexa Fluor 555 anti-mouse, and Alexa Fluor 555 anti-rabbit; all goat IgG, 2mg/ml, 1:800; Invitrogen) and incubated for 1hr at room temperature. The constructs were then washed again at least 3 times in PBS for 3-5 minutes each. This was repeated for each subsequent label. Finally constructs were washed twice with 300nM DAPI (Invitrogen) in PBS for 3-5 minutes each. Fluorescence imaging was performed using LSM510 confocal microscope using a x40 oil-immersion lens (Carl Zeiss).

Immunohistochemistry was performed with the assistance of Padmini Sarathchandra, Harefield Heart Science Centre and Ljudmila Kolker, National Heart and Lung Institute, Imperial College London and National Institute for Biological Standards.

2.4.2.1 Frozen Sections

Frozen sections were performed in order to evaluate cell structure throughout the entire thickness of 3D constructs. Briefly, the PCL constructs were coated with OCT (Sakura Finetek) and snap frozen by immersion in liquid nitrogen. 8μm thick section where cut using a cryostat at −20°C. The section was transferred to a room temperature microscope slide and immunohistochemistry was performed as described above.
2.4.2.2 Cell alignment

Figure 2.6. Figure illustrating the binary DAPI images used to calculated nuclear alignment. The images show DAPI stained nuclei in non-structured and microgrooved PDMS constructs.

Cell alignment was quantified using DAPI images which were converted into binary images using ImageJ (Figure 2.6). The long axis of each nucleus was measured relative to the horizontal axis of the image field using NIS-Elements AR3.2 software (Laboratory Imaging, Nikon Instruments, Melville, NY). Objects were gated according to size to exclude non-nucleus or composite structures. Alignment was defined as the lack of deviation in the axis of individual nucleus from the mean axis of all individual nuclei. In order to quantify cell alignment, the mean axis was first calculated and then the variance of the minimum angle between the long axis of each nucleus and the mean axis of all nuclei was compared using an F-test of equality of variances. The mean angle between the long axis of each nucleus and the mean axis of all nuclei was calculated. Analysis of colocalization was performed using the WCIF ImageJ plugin bundle (Wright Cell Imaging Facility, Toronto Research Institute). Alignment was defined as the lack of deviation in the axis of individual nucleus from the average axis. In order to quantify the alignment the average axis was first calculated, and then
the standard deviation of the difference in alignment between the average axis and the axis of each individual nucleus was calculated.

2.4.3 Second Harmonic Generation Imaging

Second harmonic generation (SHG) imaging was performed to visualize the extracellular matrix in myocardial tissue slices as previously described (Caorsi et al., 2013). Two-photon excitation and SHG imaging was performed using a Leica TCS SP5 upright laser scanning system (Leica Microsystem), coupled to a titanium-sapphire laser (Spectraphysics Mai Tai 690–1020nm, 90MHz; Spectra-Physics, Santa Clara, CA). All imaging was performed with an excitation wavelength of 900nm (except for the spectra collection) with an average power of 10–12mW using a long working distance immersion objective 25×0.9NA. The SHG was collected in the backward direction by selecting the 440–460nm range while auto-fluorescence light was collected in the 500–700nm range. All SHG imaging was undertaken with the assistance of Valentina Caorsi, National Heart and Lung Institute

2.4.4 Transmission electron microscopy

Constructs were fixed as previously described (Cloyd et al., 2012). Briefly, constructs were immersed in 4% glutaraldehyde (Agar Scientific) in 0.1M piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 7.4) at 4°C for at least two hours followed by 2 buffer washes. Specimens were post fixed with 1% osmium tetroxide (Agar Scientific) in 0.1M PIPES buffer for 1 hour at room temperature. After 2 buffer washes, specimens were incubated with 1% Tannic acid (Sigma-Aldrich) in 0.1M PIPES buffer for 1 hour. After 2
buffer washes specimens were dehydrated through ascending series of ethanol from 25% to 100% followed by drying with HMDS (Hexamethyldisilazane) (Agar Scientific). Specimens were then air-dried and mounted on Stubbs with Acheson Silver Dag (Agar Scientific). Transmission electron microscopy (TEM) was performed using the JEOL 2000 microscope operated at 120 kV (JEOL). Sample preparation was performed by Padmini Sarathchandra, Harefield Heart Science Centre.

2.4.5 Scanning electron microscopy

Constructs were fixed as previously described (Cloyd et al., 2012). Briefly, constructs were immersed in 4% formaldehyde diluted with PBS for 45 minutes at 4°C. After 2 buffer washes specimens were dehydrated through ascending series of ethanol from 25% to 100% followed by drying with HMDS (Hexamethyldisilazane) (Agar Scientific). Samples were sputter-coated with gold and Scanning Electron Microscopy (SEM) was performed using a Leo 1525 Gemini scanning electron microscope with an EDX detector operated at 15 kV (Carl Zeiss). Sample preparation was performed by Padmini Sarathchandra, Harefield Heart Science Centre.

2.5 Statistical analysis

Statistical analysis was performed using an unpaired Mann-Whitney U test, Student’s t-test, Fisher’s exact test, or 1-way Analysis of Variance (ANOVA) where appropriate. Data are expressed as mean ± standard error of the mean (SEM) unless specified otherwise. For Ca$^{2+}$ transient studies, AP measurements, and cell alignment, n represents the number of myocytes.
For experiments using MEA, n represents the number of spontaneously beating clusters. In the figures, * indicates p<0.05; ** p<0.01; and ***, p<0.001.
3 The Phenotype of Cardiomyocytes

Differentiated from Pluripotent Stem Cells
3.1 Introduction

Comparative characterization of the phenotype of hESC-CM and iPSC-CM is currently limited. Given, the emerging albeit limited evidence that there are differences between hESC and iPSC (Section 1.4.3), this must be further evaluated to ensure that knowledge about hESC-CM physiology can be readily applied to iPSC-CM.

Differentiation protocols also continue to evolve, in particular with emergence of directed differentiation protocols. Although the evidence suggesting that differentiation method may affect the phenotype of hESC-CM is weak (Section 1.4.2), the effect of differentiation method on the phenotype of iPSC-CM and hESC-CM needs to be characterized to ensure that they are robust and reproducible disease models.

In this chapter we aim to compare the electrophysiological properties of hESC-CM and iPSC-CM differentiated using an EB protocol and by directed differentiation protocols. We also aim to compare the electrophysiological properties of hESC-CM and iPSC-CM with adult myocardium. As human tissue is scarce and often represents end-pathological states, animal tissue will also be characterized and compared to hESC-CM and iPSC-CM.

The primary focus of this chapter is the response of hESC-CM and iPSC-CM to blockade of the $I_{Kr}$ with the selective antagonist E-4031. This was for several reasons; firstly there is considerable interest in using hESC-CM and iPSC-CM as models to study the pathogenesis and novel therapies for inherited cardiomyopathies (Wu and Hochedlinger, 2011, Inoue and Yamanaka, 2011). $I_{Kr}$ is an import starting point as mutation of the hERG channel is responsible for some forms of long-QT syndrome, a well-characterized inherited disorder that
predisposes patients to fatal arrhythmia (Prystowsky et al., 2012) (Section 1.6.1). Showing the presence of an I_{Kr} and response to pharmacological blockade in the hESC-CM and iPSC-CM is an important first step. Secondly, the cardiotoxicity of several potentially therapeutic compounds is because of arrhythmia caused by prolonged APD due to binding of the drug to the hERG channels (Ponte et al., 2010). Consequently if hESC-CM or iPSC-CM are to be used as toxicology screens to assist in drug development then it is important that it can be demonstrated that APD is sensitive to pharmacological agents known to prolong the APD. Finally, expression of K^{+} channels has been shown to be highly variable during development (Krishnamurthy et al., 2004, Nerbonne, 1998) and consequently differential response to I_{Kr} blockade between experimental groups may highlight differences in phenotype, in particular cardiac differentiation, development and maturation.

Supplementary experiments were also performed with chromanol 293B, a selective blocker of I_{Ks} which, like I_{Kr}, contributes to repolarization of cardiomyocytes (Strutz-Seebohm et al., 2011, Jespersen et al., 2005). I_{Ks}, like I_{Kr}, is differentially expressed during development (Krishnamurthy et al., 2004, Nerbonne, 1998) and is thought to be important in the pathogenesis of inherited arrhythmia such as the long QT-syndrome (Tsai et al., 2008, Lu and Kass, 2010). Whilst the potential for I_{Ks}-associated drug toxicity has historically been overlooked compared to I_{Kr}-associated toxicity (Curtis, 2004), there is evidence to suggest that impairment of I_{Ks} function may also be clinically important (Nakashima et al., 2004).

As we will discuss in Sections 3.3.1 and 3.3.2, blockade of repolarizing K^{+} channels can have a marked effect on the spontaneous beating rate of iPSC-CM and hESC-CM. Consequently further experiments with the selective antagonist of the I_{f}, ivabradine, were performed to investigate pacemaker mechanisms in iPSC-CM and hESC-CM. I_{f} is an inward current which
is highly expressed in cardiomyocytes in the pacemaker and conduction system of the heart (the sinoatrial node (SAN), the atrioventricular node (AVN) and the Purkinje fibers). The molecular determinant of the pacemaker is the HCN channel (Baruscotti et al., 2005). The current is called the “funny current” because of several unusual features. Firstly, it is a mixed Na\(^+\) and K\(^+\) current. Secondly, it becomes active when the cell repolarizes below the \(I_f\) threshold of approximately \(-40/-50\) mV, supplying an inward current which is responsible for starting depolarization of the myocyte in diastole. This mechanism is responsible for the spontaneous activity of myocytes within the SAN myocytes, and consequently the whole heart (Baruscotti et al., 2005, DiFrancesco and Ojeda, 1980). Finally, the “funny current” is activated by cyclic nucleotides in addition to voltage. The second messenger cyclic adenosine monophosphate (cAMP), which is produced in response to adrenaline and noradrenaline binding to \(\beta_1\) and \(\beta_2\) adrenoceptors, activates the HCN channels increasing their open probability (DiFrancesco and Tortora, 1991). Therefore, sympathetic stimulation by raising the level of cAMP molecules increases the \(I_f\) at diastolic voltages and consequently the speed of depolarization and beating rate. Ivabradine blocks \(I_f\) in a dose-dependent manner (Thollon et al., 1994) which is attenuated with reducing rate (Baruscotti et al., 2005). To investigate the maturity of pacemaker mechanisms in spontaneously beating iPSC-CM, they were superfused with increasing concentrations of ivabradine, isoproterenol (a non-selective beta-adrenergic agonist structurally similar to epinephrine), or a combination of both.

### 3.2 Materials and methods

hESC from the H7 line were differentiated into hESC-CM using directed differentiation or the EB technique and plated onto MEA or MatTek dishes as previously described (Section 2.1.2.). iPSC-CM fully differentiated using the EB method were obtained from ReproCELL.
(ReproCELL, Japan) and plated onto MEA over 40 days following differentiation. MEA and patch clamp experiments were performed as previously described (Sections 2.3.1 and 2.3.2).

For pharmacological studies briefly, spontaneously beating hESC-CM and iPSC-CM plated onto MEA dishes were superfused with NT alone, E-4031, chromanol 293B, ivabradine or isoproterenol dissolved in NT. In the case of E-4031, hESC-CM or iPSC-CM were superfused with NT for 10 minutes to obtain baseline readings. Cardiomyocytes were then superfused with NT containing 1nM E-4031 for 5mins, 10nM E-4031 for 5mins, 100nM E-4031 for 5mins, 1µM E-4031 for 5mins, before E-4031 was washed out with NT until a return to baseline readings were observed. For experiments with chromanol 293B, cardiomyocytes were superfused with NT for 10 minutes to obtain baseline readings, then superfused with NT containing 10µM chromanol 293B for 5mins, then 20µM chromanol 293B for 5mins, before the chromanol 293B was washed out with NT until a return to baseline readings were observed. For experiments with ivabradine, cardiomyocytes were superfused with NT for 10 minutes to obtain baseline readings, then superfused with NT containing 300nM ivabradine for 5mins, 3µM ivabradine for 5mins, 30µM ivabradine for 5mins, 300µM ivabradine for 5mins, before the ivabradine was washed out with NT until a return to baseline readings were observed. This experimental protocol was repeated with 30nM isoproterenol added to the ivabradine to investigate if the effect of ivabradine was attenuated by adrenergic stimulation (Section 2.3.1).
3.3 Characterization of human embryonic stem cell and induced pluripotent stem cell-derived cardiomyocytes using multi-electrode arrays

3.3.1 The effect of $I_{Kr}$ current blockade

Representative field potential recordings are shown in Figure 3.1. The predominant effect of E-4031 was to reduce the spontaneous beating rate in a dose-dependent fashion in hESC-CM
differentiated using both the EB and directed differentiation techniques. Similarly the spontaneous beating rate of iPSC-CM differentiated using the EB technique was also significantly reduced in response to $I_{Kr}$ blockade. Our data does not suggest that the effect of $I_{Kr}$ blockade on beating rate differed in any of the three groups (Figure 3.2). This is discussed further in Section 3.5.1, however the effect on beating rate is likely to be an effect of the cell being maintained below threshold potential for longer because of delayed repolarization.

Figure 3.2. Effect of $I_{Kr}$ blockade on beating rate of cardiomyocytes differentiated from hESC-CM differentiated using a directed differentiation protocol (Top Right), the EB method (Bottom Right), and iPSC-CM differentiated using the EB method (Bottom Left).
Figure 3.3. Effect of $I_{Kr}$ blockade on corrected FPD (Solid Bars) and uncorrected FPD (Striped Bars) of cardiomyocytes differentiated from hESC-CM and iPSC-CM (A). hESC-CM differentiated using a directed differentiation protocol (B) and the EB method (D). iPSC-CM differentiated using the EB method (C). EB
differentiated iPSC-CM and hESC-CM compared with hESC-CM differentiated using a directed differentiation protocol (E). Multi-cellular adult slice preparation demonstrating a mature response to I$_{Kr}$ blockade (F) (Camelli et al., 2011).

There was prolongation of the FPD in hESC-CM and iPSC-CM obtained using directed differentiation and the EB method in response to I$_{Kr}$ blockade at 100μm (Figure 3.3). However, in contrast to adult multi-cellular preparations treated with E-4031 (Figure 3.3F) there was no prolongation in FPD in hESC-CM or iPSC-CM obtained using directed differentiation or the EB method in response to I$_{Kr}$ blockade when the FPD was corrected for rate (Figure 3.3) (Section 2.3.1). In Figure 3.3 both the corrected and uncorrected beating rate are shown. No clear relationship was seen between spontaneous beating rate and field potential duration in spontaneously beating hESC-CM and iPSC-CM (Spearman correlation coefficient=0.2571) (Figure 3.4). As with the spontaneous beating rate, there was no significant difference in the effect of I$_{Kr}$ blockade on FPD between iPSC-CM and hESC-CM differentiated using different methods (Figure 3.3).

![Relationship between rate and Field Potential Duration (FPD)](image)

Figure 3.4. Plot of FPD against spontaneous beating rate in hESC-CM and iPSC-CM, showing no clear relationship
As many of the beating clusters did not have a discernible repolarization wave (contrast Figure 3.1 and Figure 3.5) there were fewer beating clusters in this dataset. The field potential recordings when a repolarization wave was not visible could be classified into three groups. In a few experiments the background noise was large relative to the typically recorded field potential amplitude (Figure 3.5A). In many experiments, particularly in directed differentiation monolayer cultures or when the beating cluster was small, the amplitude of the recorded field potential was much smaller than usually observed (Figure 3.5B). Finally in some experiments the repolarization wave was simply not visible (Figure 3.5C).

![Figure 3.5](image)

Figure 3.5. Field potential recordings of spontaneously beating hESC-CM showing examples of traces in which FPD could not be measured, either because the amplitude of the background noise was too large (A); the amplitude of the recorded trace was not sufficiently large (B); or simply because there was no discernible repolarization wave (C).
Figure 3.6. MEA traces showing the effect of $I_{Ks}$ blockade on field potential duration in hESC-CM

3.3.2 The effect of $I_{Ks}$ current blockade

Whilst repolarization waves were visible in some experiments (Figure 3.6) most recordings resembled those shown in Figure 3.5. The effect of $I_{Ks}$ current blockade on spontaneous beating rate was investigated in hESC-CM clusters differentiated using directed differentiation. Similar to $I_{Kr}$ blockade there was a reduction in the spontaneous beating rate, which was statistically significant at higher doses of chromanol 293B ($p=0.0121$) (Figure
The mechanisms underlying this reduction in spontaneous beating rate are likely to be similar to \( I_{Kr} \) blockade (Section 3.3.1) and are discussed in more detail in Section 3.5.1.

Unfortunately it was not possible to fully assess the effect of \( I_{Ks} \) blockade on FPD as only one of the clusters had a clear repolarization wave (Figure 3.6). Consequently, we were unable to assess if this was statistically significant. Furthermore, when adjusted for rate in this
experiment there was no clear relationship between increasing doses of chromanol 293B and FPD (Figure 3.8).

Figure 3.8. Effect of I_{Ks} blockade on the FPD of spontaneously beating hESC-CM
3.3.3 The effect of hyperpolarization-activated cyclic nucleotide-gated channel blockade

Ivabradine reduced the spontaneous beating rate of iPSC-CM in a dose dependent fashion and at higher concentrations spontaneous beating completely stopped (Figure 3.10A). The effect of ivabradine was also not attenuated with isoproterenol (Figure 3.10C), unlike in humans and large animals, despite isoproterenol having a dose-dependent effect on the spontaneous beating rate of iPSC-CM (Figure 3.10B and Figure 3.11).
Figure 3.10. Effect of ivabradine (A), isoproterenol (B) and combined administration on the spontaneous beating rate of iPSC-CM (C).
Figure 3.11. Representative MEA traces showing the effect of β-adrenergic stimulation on beating rate in iPSC-CM

3.4 Characterization of human embryonic stem cell and induced pluripotent stem cell-derived cardiomyocytes using the perforated patch-clamp technique

Representative AP recordings are shown in Figure 3.12. The absence of any effect of E-4031 on FPD measured with MEA, when adjusted for rate, was consistent with data from isolated hESC-CM (Figure 3.13). No change in hESC-CM APD was recorded using the perforated
patch technique. In contrast, both adult rat (p=0.0029) and dog (p=0.0124) ventricular cardiomyocytes had prolonged APD90 in response to E-4031. Adult dog ventricular cardiomyocytes also had prolonged APD50 because of their longer plateau phase (p=0.043).

Figure 3.12 Representative AP phenotypes recorded from hESC-CM (with Acknowledgment to Cesare Terracciano).

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3.5 Discussion

3.5.1 The effect of $I_{Ks}$ and $I_{Kr}$ current blockade on human embryonic stem cell and induced pluripotent stem cell-derived cardiomyocytes

There was significant heterogeneity in the response of hESC-CM and iPSC-CM to $I_{Kr}$ blockade even within experimental groups. This can be seen from the wide confidence intervals associated with estimates of the effect of E-4031 (Figure 3.2 and Figure 3.3).
E-4031 however, consistently reduced the spontaneous beating rate of iPSC-CM and hESC-CM (Figure 3.2). Whilst this may at first appear surprising as K\(^+\) channel blockade does not generally have this effect in patients and animal models, it is important to remember that these experiments occur in isolated cells without autonomic or electro-mechanical regulation of beating rate. The effect of E-4031 on spontaneous beating rate is supported by the literature where in isolated rabbit SAN myocytes E-4031 has been shown to significantly reduce the rate of spontaneous AP, in many cases completely stopping AP potentials at concentrations of 100nmol/L (Verheijck et al., 1995). The mechanisms underlying this are complex. E-4031 has been shown to have no direct effect at concentrations up to 10μmol/L on pacemaker currents such as I\(_f\) (Verheijck et al., 1995), no effect on the I\(_{Na}\) (Wettwer et al., 1991, Verheijck et al., 1995), and no effect on the long-lasting inward I\(_{Ca}\) (I\(_{Ca,L}\)) at concentrations up to 5μmol/L (well above the maximum concentration used), and only a partial effect on peak I\(_{Ca,L}\) thereafter (Verheijck et al., 1995, Wettwer et al., 1991). However, E-4031 probably has a marked indirect effect on these pacemaker currents by modifying repolarization, and the resting membrane potential, and consequently changing the size of several other key currents. At concentrations of 100nmol/L in isolated rabbit SAN myocytes, E-4031 increased the interval between spontaneous AP, increased APD, depolarized the MDP, depressed dV/dtmax, and reduced diastolic depolarization rate, stopping spontaneous AP in 50% of myocytes (Verheijck et al., 1995). By contrast in ventricular guinea pig myocytes the diastolic membrane potential was unchanged by E-4031 at concentrations of up to 10μmol/L, however the APD was markedly increased (Wettwer et al., 1991). Our experiments clearly suggest that prolongation of the APD occurs at least to some extent in some iPSC-CM and hESC-CM (Figure 3.3). Increasing the APD in itself could theoretically cause a reduction in the spontaneous beating rate by increasing the time each cell spends in a non-excitable depolarized state.
The hypothesis that the observed effect in beating rate can be entirely explained by APD prolongation in all cells was not supported by data from single cell perforated patch experiments (Figure 3.13) or the data from MEA experiments (Figure 3.3). AP recorded using perforated patch from isolated hESC-CM suggest that there was significant heterogeneity in APD in hESC-CM (Figure 3.12 and Figure 3.13), supported by similar studies from the literature conducted in both hESC-CM and iPSC-CM (Kong et al., 2010, Schenke-Layland and MacLellan, 2009, Sartiani et al., 2007, Zhang et al., 2009, Matsa et al., 2011, Pekkanen-Mattila et al., 2010a). This highlights the heterogeneous phenotype of cardiomyocytes differentiated from pluripotent stem cells and suggests that the mechanisms by which E-4031 reduces the beating rate of iPSC-CM and hESC-CM may be multiple and vary between populations of cells, or even between cells within populations. In some cells where \(I_{Kr}\) is the predominant repolarizing current, E-4031 may help to maintain them in a relatively un-excitible depolarized state for a prolonged period by significantly slowing repolarization through \(I_{Kr}\) blockade. In other cells, however, E-4031 may indirectly inhibit other pacemaker currents such as \(I_{f}\), \(I_{Ca,L}\) and \(I_{Na}\) by reducing the maximum diastolic potential and consequently reducing the excitability of hESC-CM and iPSC-CM in diastole, by a similar mechanism by which E-4031 reduces the excitability of isolated rabbit SAN cells (Verheijck et al., 1995). For this to occur however without significant prolongation of the APD, other repolarizing \(K^+\) currents must be present.

As E-4031 consistently reduced the beating rate, E-4031 also resulted in prolongation of the FPD of hESC-CM and iPSC-CM (Figure 3.3). Reduction in the beating rate of cardiomyocytes is a consequence of the refractory phase of the cardiac cycle. Consequently, it is expected that reduced beating rate will result in longer APD. To determine whether
prolongation in APD is as a consequence of the effect of E-4031 prolonging repolarization, or simply reducing the beating rate of iPSC-CM and hESC-CM, APD was corrected for the beating rate using Bazett’s formula (Yap and Camm, 2003)

When adjusted for the rate using Bazett’s formula most of the hESC-CM and iPSC-CM experiments did not show prolongation of the FPD. This is well illustrated in Figure 3.4 where there is no correlation between FPD and rate in either hESC-CM (Spearman's rank correlation coefficient (ρ) -0.2000, p=0.7833), iPSC-CM (ρ 0.2571, p=0.6583), cardiomyocytes differentiated using the EB technique (ρ 0.1667, p=0.7033), or all the experimental results pooled (ρ -0.1455, p=0.6731). Single cell perforated patch experiments with hESC-CM also suggest there is not a consistent effect of E-4031 on APD (Figure 3.13). This is in marked contrast to experiments conducted with multicellular preparations (Camelliti et al., 2011), isolated adult myocytes from animal models (Follmer and Colatsky, 1990, Sanguinetti et al., 1991) (Figure 3.13), large animal studies (Wallace et al., 1991), and from clinical studies (Isomoto et al., 1993) (Figure 3.3). It is also in marked contrast with several studies published following the start of this work which have demonstrated prolongation of APD in response to I_{Kr} blockade in single cell experiments (Liang et al., 2013, Braam et al., 2013) and in MEA experiments where FPD is adjusted for rate (Harris et al., 2013, Zwi et al., 2009).

A factor which made the investigation of the effect not only of E-4031, but the investigation of the effect of all pharmacological agents on FPD problematic was the absence in many MEA experiments of an obvious repolarization wave (contrast Figure 3.1 and Figure 3.5). The field potential recordings when a repolarization wave was not visible could be classified into three groups. In a few experiments the background noise was large relative to the
typically recorded field potential amplitude (Figure 3.5A). This was probably due to technical problems with the superfusion of the hESC-CM, and iPSC-CM. In these circumstances the recordings were only accepted after extensive attempts were made to eradicate or reduce the background noise. In many experiments, particularly in directed differentiation monolayer cultures or when the beating cluster was small, the amplitude of the recorded field potential was much smaller than usually observed (Figure 3.5B); in these circumstances not much could be done to optimize the recordings. Finally in some experiments the repolarization wave was simply not visible (Figure 3.5C). As the FP recording could be considered to be analogous to the differential of the AP, large deflections in membrane potential with respect to time would result in large waves in the FP recording. Many cells may have had phenotypes analogous to human atrial or nodal AP which are characterized by a limited plateau phase (Phase 2) or “triangulation” of the action potential (See Figure 1.5). This would result in an absence of repolarization wave distinct from the “QRS complex” or larger depolarization wave. Single cell perforated patch performed in hESC-CM and iPSC-CM confirm that many cells do have triangular AP morphologies (Figure 3.12) (Kong et al., 2010, Schenke-Layland and MacLellan, 2009, Zhang et al., 2009, Matsa et al., 2011, Pekkanen-Mattila et al., 2010a). Whilst some studies appear to consider the return to baseline following the depolarization wave as being analogous to the APD (Zwi et al., 2009, Banach et al., 2003), and thus make estimations of APD based on field potential records without discernible repolarization waves, in other studies this is not clear (Yamamoto et al., 1998) and is of debatable validity.

Characterization of the electrophysiological properties of isolated hESC-CM was also technically difficult. In particular, hESC-CM with specific and distinct morphological characteristics were far more amenable to patching than others. This raised the possibility that the hESC-CM patched represent only a sub-population in each cluster.
Finally, arguably at the concentrations of E-4031 used, a complete block of I_{Kr} was not achieved. Higher concentrations of E-4031 may have achieved greater prolongation of FPD. However, at 1\mu M/L concentration of E-4031 96% of the I_{Kr} is selectively blocked and above 1\mu M/L concentration E-4031 is no-longer a specific blocker of I_{Kr} (Verheijck et al., 1995). Furthermore, studying hESC-CM and iPSC-CM using MEA when they are no longer spontaneously beating is technically problematic as they cannot be readily point-stimulated.

The experiments with chromanol 293B were more limited because of a paucity of material, and as repolarization waves were not visible in most of the preparations. There appeared to be however, a reduction in beating rate at higher concentrations of chromanol 293B (Figure 3.7). This can be less easily understood than the effect of E-4031 on beating rate. It is unlikely that chromanol 293B was acting non-specifically on pace-maker currents such as I_{K1}, I_{Kr}, or I_{Ca,L} as several studies have shown that at concentrations well in excess of the concentrations used (20\mu M) chromanol 293B did not block these channels (Sun et al., 2001). It has been suggested that I_{Ks} does not significantly affect, even indirectly, the important pacemaker currents in SAN cells. For example, by modifying the resting membrane potential (Verheijck et al., 1995). Several studies however have shown that chromanol 293B can affect other repolarizing currents such as the Ultra-Rapid delayed rectifier K\(^+\) current (I_{Kur}) and the transient outward K\(^+\) current (I_{to1}) (Sun et al., 2001, Du et al., 2003) which are variably expressed in adult cardiomyocytes and may be expressed in hESC-CM and iPSC-CM depending on their phenotype and age (Sartiani et al., 2007, Du et al., 2003). The direct effect of chromanol 293B on prolonging repolarization through I_{Ks}, and potentially I_{Kur} and I_{to1}, is therefore probably the most likely explanation for the reduced beating rate by increasing the time that cells are in a non-excitable refractory depolarized state. Several studies which have
shown that chromanol 293B prolongs APD in hESC-CM or iPSC-CM appear to support this (Peng et al., 2010, Wang et al., 2011a, Zwi et al., 2009) although this is contradicted by other studies (Ma et al., 2011, Braam et al., 2013). Very limited data from one beating cluster suggested that chromanol 293B prolonged the FPD in hESC-CM (Figure 3.6 and Figure 3.8), with the important caveats that this is data only from one cluster, and secondly, like the experiments with $I_{Kr}$ blockade, when corrected for rate an effect on FPD was not observed.

Data from Braam et al suggest that $I_{Ks}$ is only important for repolarization in iPSC-CM with reduced repolarization reserve. In the case of their study this was iPSC-CM with an $I_{Kr}$ loss of function mutation resulting in long-QT syndrome (Braam et al., 2013). Reduced repolarization reserve compared to iPSC-CM in the literature highlighted by responsiveness to $I_{Ks}$ may highlight the relative immaturity of the cells we used in our experiments, which could perhaps explain differences between our data and that in the published literature.

The results of our experiments with E-4031 are contradicted by several reports in the literature, in particular the failure to demonstrate prolongation of the corrected FPD and APD in response to E-4031. Several studies have shown that hESC-CM (Caspi et al., 2009) and iPSC-CM (Zwi et al., 2009) have prolongation of single-cell APD recordings (Braam et al., 2013, Liang et al., 2013) or FPD when corrected for rate (Zwi et al., 2009, Harris et al., 2013), in response to $I_{Kr}$ channel blockade with E-4031. Furthermore, the effects on beating rates that were evident in our experiments are not reported in most studies, despite other studies reporting the effect on rate of other pharmacological agents (Zwi et al., 2009).

The differences between our data and the existing literature can only be explained by one of two reasons: either the properties of the cells used in our experiments were different, or the
differences reported are an experimental artifact. Several mechanisms are suggested to undelay rate-dependent APD prolongation. Homeostatic mechanisms regulating cardiac output at the level of the organism affect cAMP levels in cardiomyocytes, which in turn regulate K⁺ channel open probability and repolarization. At a cellular level accumulation of intracellular Ca²⁺ and extracellular K⁺ at higher rates may also play a role. Finally the direct effect of refractory time-dependent K⁺ channels are also important in APD shortening at faster beating rates (Boyett and Jewell, 1978). Of these mechanisms, probably only the direct properties of sarcolemmal K⁺ channels and intracellular Ca²⁺ are relevant in our experiments. There is no autonomic pressure to maintain cardiac output in cultured cardiomyocytes and as the cells were superfused with NT there would be no extracellular accumulation of K⁺.

The expression of K⁺ channels has been shown to increase with the age of hESC-CM (Sartiani et al., 2007). Whilst this suggests that the time from differentiation may be a significant confounding factor in experiments of this type, this is not supported by other studies which have not found an association between time since differentiation and phenotype (Pekkanen-Mattila et al., 2010a, Pekkanen-Mattila et al., 2009). Taken together these studies highlight heterogeneous K⁺ channel expression in stem cell-derived cardiomyocytes. Pekkanen-Mattila et al. even suggest that there is variation between cells differentiated using the same method in the same batch (Pekkanen-Mattila et al., 2009). Other authors suggest there may be variation between cells in the same cluster (Denning and Anderson, 2008, Xu et al., 2002). Similarly the debate about the extent to which in vitro stem cell-derived cardiomyocytes recapitulate adult cardiomyocyte Ca²⁺ cycling properties suggests that heterogeneity in Ca²⁺ handling properties could also feasibly explain differences between our experimental data and the existing literature (Dolnikov et al., 2006, Liu et al., 2007, Fu et al., 2006, Satin et al., 2008).
The differences between our experimental results and published data may also be due to differences in experimental protocols. Whilst some studies have corrected the FPD for rate (Caspi et al., 2009, Zwi et al., 2009), in many of these reports the FPD does not appear to have been corrected for rate (Matsa et al., 2011, Otsuji et al., 2010, Tanaka et al., 2009).

In summary, the cause for differences between our data and the published literature, and the differences between studies within the published literature with respect to the response of hESC-CM and iPSC-CM to E-4031 and chromanol 293 B are not clear. These differences could be explained by the heterogeneous phenotype of iPSC-CM and hESC-CM. It may be that differences in the cell lines used (Pekkanen-Mattila et al., 2009), contribution of non-cardiac cells (Kim et al., 2010a), differentiation protocol, culture conditions (Otsuji et al., 2010), age of the cardiomyocytes (Sartiani et al., 2007), or experimental conditions may also contribute to these findings. Consequently whilst pluripotent stem cells unquestionably have tremendous potential as in vitro myocardial models, if iPSC-CM and hESC-CM are to be widely applied to toxicology screening and drug development, experimental protocols will have to encompass methodology which will control for potential confounding factors.

3.5.2 The effect of hyperpolarization-activated cyclic nucleotide-gated channel blockade on induced pluripotent stem cell-derived cardiomyocytes

Automaticity is a characteristic feature of immature cardiomyocytes (Liang et al., 2010, Tuganowski and Tendera, 1973, Janse et al., 1976). Consequently, unlike in the healthy adult myocardium where usually only pacemaker cells in the SAN have autonomous AP
(DiFrancesco, 2010), most hESC-CM and iPSC-CM exhibit a high degree of automaticity (Pekkanen-Mattila et al., 2010a, Kong et al., 2010, Zhang et al., 2009).

Experiments designed to investigate the mechanisms underlying this automaticity demonstrated that the spontaneous beating rate of iPSC-CM was reduced in a dose-dependent manner by ivabradine. The effect of ivabradine on the spontaneous beating rate of iPSC-CM was not attenuated at low rates, in contrast with data from clinical studies (Borer and Le Heuzey, 2008, Savelieva and Camm, 2006). Furthermore, isoproterenol did not appear to attenuate the effect of ivabradine as one may expect from previous experimental studies (DiFrancesco and Tortora, 1991). These later findings are perhaps not surprising. Attenuation of the effect of ivabradine with reducing rate should probably not be expected when there is no intracellular raise in cAMP, caused by increased sympathetic activity in response to reduced cardiac output caused by reduced beating rate (Stieber et al., 2003). Furthermore, the dose of isoproterenol used may have been too small, and the heterogeneity and resulting confidence intervals too large to be sure that there was no attenuation of the effect of isoproterenol with ivabradine. The finding that spontaneous beating rate was reduced in a dose dependent manner, and in particular that at high concentrations beating ceased completely is more problematic as it appears to suggest that in the iPSC-CM the most critical determinant of their automaticity was I_f. As the mechanisms underlying the automaticity of human iPSC-CM have not currently been investigated in the literature, indeed there is even limited data from hESC-CM, we must look to experiments largely performed in vitro and using murine ESC-CM to explain these findings.

The mechanisms underlying automaticity in SAN cardiomyocytes is complex, and far from being completely understood (Lau et al., 2011, Lakatta et al., 2010). Much of the existing
literature appears to suggest that a complex interaction between spontaneous intracellular Ca\(^{2+}\) cycling associated with the SR, and fluctuations in sarcolemma membrane potential, due to the activity of several pacemaker currents including I\(_f\), I\(_{Kr}\), and I\(_{Ca,L}\), probably underlie spontaneous AP in the SAN (Lakatta et al., 2010, Verheijck et al., 1995). It is debatable to what extent this is the case in hESC-CM and iPSC-CM. It is likely, however, given their generally immature phenotype that the mechanisms underlying automaticity are more analogous to those seen in developing cardiomyocytes.

There is general consensus that in murine cardiomyocytes I\(_{K1}\) expression is a feature of terminal differentiation (Satin et al., 2004). Although the electrophysiology of developing human cardiomyocytes is very poorly characterized for obvious reasons this appears to be supported by the limited available data (Tuganowski and Tendera, 1973, Janse et al., 1976). Consequently, small Na\(^+\) or Ca\(^{2+}\) currents may potentially depolarize the cell (Satin et al., 2004). Evidence that extracellular Ca\(^{2+}\) cycling causing oscillations in membrane potential is the dominant pacemaker mechanism in immature cardiomyocytes is demonstrated by experiments in which blockade of I\(_{Ca,L}\), T-type Ca\(^{2+}\) current (I\(_{Ca,T}\)) and NCX all completely stop beating in murine embryonic cardiomyocytes at day 8.5. By contrast, blockade of the I\(_f\) current at day 8.5 does not stop beating in murine embryonic cardiomyocytes, suggesting that in less mature cells automaticity is completely related to extracellular Ca\(^{2+}\) cycling or “oscillations” (Liang et al., 2010). It has been suggested that I\(_f\) becomes critically important later in development, assuming a dominant role in pacemaker activity from day 9.5 in murine embryonic cardiomyocytes (Stieber et al., 2003). The HCN4 gene encodes the HCN channel predominantly expressed in the pacemaker regions of the heart. HCN4 Knockout mice have a reduced heart rate and death in utero occurs by day 11.5 (Stieber et al., 2003). However despite the significant reduction in HCN4 expression (75-90%) the hearts of HCN4 knockout
mice continued to beat after day 9.5, and mortality at day 11.5 may equally have been because of an indirect consequence of the reduced heart rate, rather than a direct absence of autonomic activity. Furthermore isolated cardiomyocytes with “atrial” and “ventricular” phenotypes from HCN4 knockout mice, rather like hESC-CM and iPSC-CM, had spontaneous AP. This suggests either other HCN isoforms are responsible for spontaneous membrane depolarization or other pacemaker mechanisms continue to be important (Stieber et al., 2003).

It has been suggested at least in cardiomyocytes differentiated from murine embryonic stem cells that normal, in utero development is recapitulated (Doevendans et al., 2000); however, there are several difficulties with this. Firstly, several mechanisms are suggested to be important in murine ESC-CM that have not been described in embryonic cardiomyocytes. For example, experiments performed on murine ESC-CM suggest that fluctuations in intracellular Ca$^{2+}$ cycling play a critical part in the automaticity of developing murine myocardium (Viatchenko-Karpinski et al., 1999, Zahanich et al.), or that in very early development I_{o1} is a critical pacemaker current (Hescheler et al., 1997). Explanations of this have focused on the relative maturity of the embryos (Viatchenko-Karpinski et al., 1999). However, it seems unlikely that intracellular Ca$^{2+}$ oscillations are important (Viatchenko-Karpinski et al., 1999), and then become less important as the cells mature (Liang et al., 2010), before then becoming important in adult cells again (Lau et al., 2011, Lakatta et al., 2010).

Some authors suggest that experimental artifacts may account for discrepant findings relating to intracellular Ca$^{2+}$ cycling in stem cell-derived cardiomyocytes (Satin et al., 2008), however if we take the published literature at face value then these findings could probably be
explained by the suggestion that ESC-CM are a heterogeneous cell population and that some of these cells may not recapitulate in utero development. Satin et al suggest hESC-CM have relatively immature expression of \( I_{K1} \) but mature expression of the voltage gated Na\(^+\) channel, Nav1.5 (Satin et al., 2004). Consequently the dominant pacemaker mechanism is the depolarizing effect of \( I_{Na} \), and blockade with tetrodotoxin (TTX) abolishes the automaticity of hESC-CM, unlike Ca\(^{2+}\) channel blockade. It is suggested that while the \( I_f \) may be present (they were unable to detect HCN-4 mRNA expression), it is unlikely to play a significant role in the automaticity of hESC-CM. These findings are in direct contradiction to studies in murine ESC-CM which suggest that \( I_f \) along with \( I_{Ca,T} \) is the dominant pacemaker mechanism at day 13.5, and that HCN expression in fact declined with time, although significantly FACS sorting was performed to purify the population of ESC-CM (Yanagi et al., 2007). The importance of \( I_f \) is supported by other studies performed with murine ESC-CM (Qu et al., 2008, Barbuti et al., 2009). A recent study suggests that three populations of hESC-CM exist with distinct pacemaker mechanisms (Weisbrod et al., 2013). Approximately 30% had \( I_f \) dependent automaticity, 41% had \( I_f \) independent automaticity (Ca\(^{2+}\) dependent) and the remainder of cells had mixed pacemaker mechanism (Weisbrod et al., 2013). In both \( I_f \)-dependent and independent cells suppression of pacemaker mechanisms resulted in depolarization of the maximal diastolic potential. In individual cells suppression of \( I_f \) in \( I_f \)-independent cells would not result in cessation of beating. However, as we performed our experiments on clusters of electrically coupled cells significant depolarization of the MDP in one third of the cells could potentially reduce the excitability of the whole cluster.

The complexity of the mechanisms underlying automaticity in iPSC-CM may not easily be resolved. If we assume iPSC-CM and hESC-CM to be functionally equivalent (Section 3.5.3), the finding that these mechanisms are not homogeneous is important. Furthermore,
this finding is supported by data from the existing literature. Variation in the mechanisms that underlie automaticity may potentially confound experiments in which hESC-CM or iPSC-CM are used as models of arrhythmia, or to investigate aspects of cardiomyocyte metabolism and function.

3.5.3 The effect of pluripotent stem cell type and method of differentiation on the functional properties of pluripotent stem cell-derived cardiomyocytes

There is limited evidence to suggest that differentiation protocols may affect the electrophysiology of hESC-CM (Pekkanen-Mattila et al., 2010a). There is evidence to suggest that there are differences between gene expression patterns (Chin et al., 2009) and DNA methylation in hESC and iPSC (Deng et al., 2009), and there are differences in the efficiency (Zhang et al., 2009) and time (Mauritz et al., 2008) it takes to differentiate iPSC-CM compared with hESC-CM.

For these reasons we were very keen to compare the response of hESC-CM and iPSC-CM to E-4031, as well as the response of hESC-CM differentiated using different methods. Our experiments did not suggest that there was a significant difference in the properties of hESC-CM and iPSC-CM measured. This is supported by most (Mandel et al., 2012, Gherghiceanu et al., 2011, Xi et al., 2010, Zhang et al., 2009), but not all studies in the literature (Jiang et al., 2010b).

We must however continue to be vigilant that the properties of hESC-CM and iPSC-CM could vary significantly. Our experiments were significantly limited by a restricted supply of hESC-CM and iPSC-CM, limiting both the number of times an experiment could be repeated.
and the number of different experiments that could be undertaken. More generally robust comparison between the properties of hESC-CM and iPSC-CM in the literature has been confounded by the significant heterogeneity within experimental groups and the number of potentially confounding factors (Section 3.5.1). This has precluded the use of historical data or pooling data from different groups.

### 3.6 Conclusion

In this chapter we have demonstrated that there is significant heterogeneity in the functional properties of iPSC-CM and hESC-CM. Our data appears to contradict the existing literature in some respects, such as the responsiveness of hESC-CM and iPSC-CM to \( I_{Kr} \) blockade. However, there is significant evidence within the literature to support the contention that hESC-CM and iPSC-CM have an immature and heterogeneous phenotype when the experimental data is examined more closely. This may make the application of iPSC-CM and hESC-CM unsuitable for some applications. Consequently, we would strongly argue that there is significant value in developing tools which could improve the homogeneity and maturity of the phenotype of cardiomyocytes differentiated \textit{in vitro} from pluripotent stem cells.
4 Structured Tissue Culture Constructs to Modulate the Calcium Cycling Properties of Immature Cardiomyocytes
4.1 Introduction

The immature, heterogeneous phenotype of iPSC-CM remains problematic despite the promise of iPSC-CM as in vitro models. Experimental results described in Sections 3.3 and 3.4, suggest that both iPSC-CM and hESC-CM have an immature, heterogeneous phenotype, unrepresentative of adult human myocardium. This is supported by evidence from the literature which suggests that iPSC-CM have gene expression characteristics of developing cardiomyocytes (Gai et al., 2009), immature ultrastructural phenotypes (Gherghiceanu et al., 2011, Itzhaki et al., 2011b), immature electrophysiological properties (Zhang et al., 2009, Ma et al., 2011) and abnormal Ca\(^{2+}\) cycling (Lee et al., 2011). As the practical value of hESC-CM, and iPSC-CM in particular, falls below their theoretical potential (Oh et al., 2012), we felt that there would be significant value in modifying the phenotype of iPSC-CM and hESC-CM in order to make them more representative of adult myocardium.

Approaches to modify the phenotype of iPSC-CM and hESC-CM can broadly be classified as methods that aim to modify the phenotype of cells through modification of gene expression (Kong et al., 2010), or those that use culture conditions to modify the expression of the immature phenotypes (Otsuji et al., 2010). We felt that for any tool which promotes a more mature phenotype in iPSC-CM or hESC-CM to be widely applied it must fulfill a number of criteria. Firstly, it must not further impair their application as disease models. Secondly, it must be readily reproducible without specialist techniques or equipment. Finally, it should be relatively economical in terms of laboratory time and other resources. For these reasons we chose not to investigate approaches in which genetic manipulation of the cells was a critical component. For iPSC-CM, in particular, where the patient-specific genetic background is a critical component of their value over other disease models, genetic manipulation would
significantly undermine their utility. Evidence of the effect of culture conditions on iPSC-CM and hESC-CM is limited. For other immature cardiomyocytes, in particular NRVM there is evidence that several aspects of cardiomyocyte culture could be modified to promote a more mature phenotype. Substrate geometry (Wang et al., 2011b, Yin et al., 2004, Bien et al., 2003, Kim et al., 2010b, Kaji et al., 2003, Pong et al., 2011, Parker et al., 2008, Feinberg et al., 2012, McDevitt et al., 2003, Badie and Bursac, 2009, Badie et al., 2012), substrate stiffness (Wang et al., 2011b, Shimazaki et al., 2008, Lieu et al., 2013), electrical (Chiu et al., 2011, Radisic et al., 2004) and mechanical stimulation (Tulloch et al., 2011, Fink et al., 2000, Gwak et al., 2008, Hecker et al., 2008, Brown et al., 2008), for example, have all been shown to modify the phenotype of NRVM. These methods can be thought of as representing a spectrum of complexity from relatively simple cell alignment (Yin et al., 2004, Bien et al., 2003), to cyclical mechanical or electrical stimulation which can be performed with proprietary systems (Fink et al., 2000, Radisic et al., 2004), to novel culture systems which modify several aspects of cardiomyocyte culture in order to promote a more mature phenotype (Boudou et al., 2012, Zimmermann et al., 2000, McCain et al., 2013). With increasing complexity culture systems require more expertise, resources, and time to develop and use. It also becomes increasingly difficult to apply many of the techniques widely used in cell culture, molecular biology, cell imaging and physiology to investigate the phenotype of the cultured cells as the complexity of the culture system increases. Ironically, this may mean that whilst a mature phenotype may be achieved the difficulty of interrogating the phenotype of the cultured cardiomyocytes becomes as complicated as evaluating cellular physiology in existing animal models. This obviously significantly limits the utility of such models. As there was existing evidence from NRVM that cell alignment was both easily achieved and had a positive effect on their phenotype of immature cardiomyocytes (Yin et al., 2004, Bien et al., 2003), we aimed to investigate whether iPSC-CM cultured in an aligned fashion,
analogous to adult myocardium, would exhibit a matured phenotype that could be considered more representative of adult myocardium.

The experimental results described in Chapter 3 (Section 3.3 and Section 3.4), suggest that iPSC-CM and hESC-CM had an immature phenotype. However, in many ways we felt that the differential response to K\(^+\) channel blockade was not necessarily the best experiment to demonstrate or quantify differences in cellular phenotype. Our concern was that despite significant “maturation” of the phenotype of iPSC-CM, their response to I\(_{Kr}\) blockade may not be significantly modified, and so the efficacy of any modification in culture technique would not be recognized. Conversely, modification of the culture condition of iPSC-CM may have only a marginal effect on the global phenotype of iPSC-CM, however if it modified I\(_{Kr}\) activity the effect of modification of culture conditions may be overestimated. As we need a more global indication of the functional maturity of iPSC-CM and the effect of changes in culture conditions on the phenotype of iPSC-CM, we chose to focus on the effect on the Ca\(^{2+}\) cycling properties of these cells. Intracellular Ca\(^{2+}\) cycling is central to excitation-contraction coupling, and is therefore a fundamental component of cardiomyocyte physiology. For these reasons, we felt that if cell alignment had any effect on cardiomyocyte physiology it would be evident from changes in cardiomyocyte Ca\(^{2+}\) cycling properties.

Several methods have been described to align cells, many of which appear to have a positive effect on the functional properties of immature cells such as NRVM. These include using substrates coated in micropatterned extracellular matrix components such as fibronectin (Kaji et al., 2003, Feinberg et al., 2012), microgrooved substrates (Yin et al., 2004) and nanogrooved culture substrates (Kim et al., 2010b), in addition to more sophisticated three-dimensional constructs (Dvir et al., 2011, Boudou et al., 2012). Structured tissue culture
substrates that bring about regular alignment and anisotropy of the cell culture have been previously used to improve Ca\textsuperscript{2+} cycling properties and sarcomeric organization in NRVM (Kaji et al., 2003, Feinberg et al., 2012, Yin et al., 2004, Kim et al., 2010b, Dvir et al., 2011, Boudou et al., 2012). There is no evidence in the literature either that these strategies can be used to align iPSC-CM, or modify their functional properties. With the ultimate aim of applying these techniques to iPSC-CM, in this chapter we aimed to test the validity and feasibility of these techniques in our laboratory. Pilot experiments to test the reproducibility of these techniques were performed using NRVM. NRVM are more readily available and less costly than iPSC-CM. However, as NRVM are also immature cardiomyocytes they have many similar properties.

The specific aims of the work presented in this chapter were to test the reproducibility of two methods to align NRVM in a fashion analogous to adult myocardium: the use of micropatterned fibronectin (Camelliti et al., 2006) and the use of microgrooved constructs (McDonald and Whitesides, 2002). We also aimed to determine if these methods would promote Ca\textsuperscript{2+} cycling properties which were more representative of adult myocardium. Therefore we aimed to test the hypotheses that structured tissue culture using micropatterned fibronectin and microgrooved substrates would have a positive effect on the Ca\textsuperscript{2+} cycling of NRVM.

4.2 Materials and methods

NRVM were used to validate methods used to promote cellular alignment and perform pilot Ca\textsuperscript{2+} cycling experiments. NRVM, like hESC-CM and iPSC-CM are immature cardiomyocytes and have many similar properties.
NRVM were isolated according to Home Office guidelines (Chapter 2.1.1) and aligned using micropatterned fibronectin lines (Chapter 2.2.2), or microgrooved tissue culture substrates (Chapter 2.2.1). Immunohistochemistry (Chapter 2.4.2), SEM (Chapter 2.4.5) and TEM (Chapter 2.4.4) were performed to confirm cell alignment, and to evaluate cell structure.

Ca\(^{2+}\) transient properties were measured in non-structured and structured NRVM cultures using confocal microscopy, after loading the cells with Rhod-2 AM, a fluorescent Ca\(^{2+}\) indicator. Recordings were performed when the cells were beating spontaneously and field stimulated at 0.5Hz, 1Hz and 2Hz (Chapter 2.3.3).

4.3 Results

4.3.1 Fibronectin lines

4.3.1.1 Effect of culture on fibronectin lines on neonatal rat ventricular myocyte structure

Micropatterned fibronectin lines were spaced 300\(\mu\)m apart and were 30\(\mu\)m wide. Immunofluorescence staining confirmed that NRVM exclusively attached to fibronectin lines. Consequently, although NRVM cultured on micropatterned fibronectin lines appeared to be electro-mechanically coupled (Section 4.3.2), NRVM in adjacent lines were not coupled. Therefore, unlike non-structured cells and cells cultured on microgrooved PDMS substrates (Section 4.3.2) NRVM cultured on fibronectin lines did not form a syncytial monolayer (Figure 4.1).
Figure 4.1. A) Bright field image showing geometry of micropatterned NRVM cultured on fibronectin lines. B) Immunohistochemistry of micropatterned NRVM. Green - Fibronectin, Red - Myosin Heavy Chain, Blue – DAPI (With Acknowledgment to Patrizia Camelliti). Bright field images contrasting the structured and non-structured constructs - Scale bar 30µm. (C and D)

NRVM cultured on micropatterned lines were more aligned than NRVM cultured on non-structured PDMS (Mann-Whitney U, p<0.0001, F=16.17, p<0.0001 – F-test) (Figure 4.2).
Figure 4.2. Quantification of cell alignment of NRVM on micropatterned fibronectin lines compared with NRVM cultured on non-structured PDMS. Alignment is quantified by measuring variation of the long-axis of each nucleus from the mean long-axis of all the nuclei. The graph on the left is a comparison in the variation in alignment (F-test), the graph on the right shows difference in average alignment (Mann-Whitney U).

4.3.1.2 Effect of culture on fibronectin lines on neonatal rat ventricular myocyte calcium cycling

Figure 4.3. Representative Ca\(^{2+}\) transient from NRVM cultured on micropatterned fibronectin lines (green) and non-structured constructs (grey) recorded with confocal microscopy in cells loaded with the Ca\(^{2+}\) indicator Rhod-2 AM and field stimulated at 0.5Hz. A Ca\(^{2+}\) transient recorded from an adult rat myocyte is shown for comparison (rust).
Micropatterned fibronectin lines did not have a clear beneficial effect on the Ca\(^{2+}\) cycling properties of the cells on the structured constructs compared to the cells on the non-structured constructs. In the micropatterned fibronectin lines there was no significant difference in Ca\(^{2+}\) transient parameters between NRVM cultured on flat PDMS membrane and those on fibronectin lines when the cells were beating at 1Hz or 0.5Hz (Figure 4.3 and Figure 4.4).

There was a significant increase in the TTP of the Ca\(^{2+}\) transient when the cells cultured on the fibronectin lines were stimulated at 2Hz compared to the non-structured constructs (p=0.0056), and there was a statistically significant reduction in Ca\(^{2+}\) transient amplitude in the micropatterned group at 0.5Hz (p=0.001), 1Hz (p=0.0004) and 2Hz (p=0.0008). When the NRVM were beating spontaneously the TTP (p=0.0135), T50 (p=0.0093), and T90 (p=0.0029) were all significantly shorter in the non-structured NRVM, however the amplitude was not significantly changed (p=0.1433).

This data must be interpreted in the context of the significantly reduced spontaneous beating rate on the micropatterned grooves compared to the non-structured constructs (p=0.0010). Fewer NRVM beat spontaneously on the fibronectin lines compared to NRVM seeded on non-structured PDMS (13% versus 37%) (Fisher’s Exact Test p= 0.0178) (Figure 4.4 and Figure 4.3).
Figure 4.4. The effect of micropatterned fibronectin lines (Mann-Whitney U) on Ca\textsuperscript{2+} transients. Time to Ca\textsuperscript{2+} transient peak, 50% decay, 90% decay, and amplitude of fluorescence (F/F0) of NRVM cultured on flat membrane compared with micropatterned fibronectin lines.
4.3.2 Microgrooved polydimethylsiloxane tissue culture substrates

4.3.2.1 Effect of grooved polydimethylsiloxane tissue culture substrates on neonatal rat ventricular myocyte structure

NRVM seeded onto fibronectin coated PDMS formed a confluent spontaneously beating syncytial monolayer unlike NRVM aligned using micropatterned fibronectin lines (Section...
4.3.1.1.) Cells within the microgrooves were longitudinally orientated (Figure 4.5). Whilst there was a suggestion that sarcomeres and intercalated disks were better organized in structured cultures when visualized using TEM, we were not able to quantify this (Figure 4.6), and overall no clear differences were found between the ultra-structural properties of structured and non-structured NRVM cultures. In particular, no evidence of t-tubule structure was seen in either the structured or the non-structured cultures stained with Di-8-ANEPPS (Figure 4.5) or visualized with SEM (Figure 4.7 and Figure 4.8), however given the highly irregular membrane (Figure 4.7 and Figure 4.8) and lack of sarcomeric organization even in the aligned cells this was probably not surprising (Figure 4.6).

![Image of TEM showing intercalated discs and sarcomeric proteins](image-url)

**Figure 4.6.** Representative TEM of NRVM grown on structured constructs (A) and non-structured constructs (B) highlighting improvement in organization of sarcomeric proteins and intercalated disks seen in the structured constructs.
Figure 4.7. SEM of NRVM cultured on non-structured PDMS tissue culture substrates. Cell membranes were irregular and no clear organized t-tubular structures were visible at higher magnification.
Figure 4.8. SEM of NRVM cultured on structured PDMS tissue culture substrates, showing alignment of cells in directed on substrate grooves (A and B). Cell membrane was very irregular and no clear organized t-tubular structures were seen on higher magnification despite improved sarcomeric organization (C and D).
NRVM were aligned in the direction of the PDMS grooves on both the 10µm (p<0.001 – Kurskal-Wallis, F=2.826 p<0.0001 – F-test) and 20µm (p<0.001 Kurskal-Wallis, F=2.115 p<0.0001 – F-test) microgrooved constructs (Figure 4.9, Figure 4.10 and Figure 4.11), resulting in more anisotropic cultures.

Cells that were seeded on top of the base layer of cells were still aligned in the direction of the grooves; however they were less strictly aligned than the cells which were directly in contact with the PDMS grooves (Figure 4.9 and Figure 4.10).

On those constructs in which the grooves were 20µm in width the cells were less strictly aligned than in the constructs in which the grooves where 10µm in width (F=1.336, p=0.0436 – F-test). Unsurprisingly, on both the 20µm and 10µm constructs the NRVM were more aligned than on the non-structured constructs (Figure 4.11).
Figure 4.9. Immunohistochemistry Z-stack of NRVM cultured on 10μm microgrooves stained green for myosin heavy chain. Each slice is 1μm thick; the lowest slice is shown in the bottom right, the highest slice in the top left. Scale bar 40μm.
Figure 4.10. Immunohistochemistry Z-stack of NRVM cultured on 20µm microgrooves stained green for myosin heavy chain. Each slice is 1µm thick; the lowest slice is shown in the bottom right, the highest slice in the top left. Scale bar 40µm.
4.3.2.2 Effect of grooved polydimethylsiloxane tissue culture substrates on neonatal rat ventricular myocyte calcium cycling

The TTP Ca$^{2+}$ transient was significantly shorter in the 10µm groove constructs compared with non-structured constructs (p<0.001), as was the T50 (p<0.01) and T90 (p<0.01) when...
the NRVM were paced at 1Hz. At 1Hz, the TTP was significantly shorter in the 20µm grooves (p<0.05), compared with the non-structured constructs, but there were no significant changes between the two groups in T50 or T90. At 0.5Hz the 10µm constructs also had shorter TTP and T50 than the non-structured constructs (p<0.01), whilst there was no difference between the 20µm constructs and the non-structured constructs. There were no significant changes in T90 between the three groups at 0.5Hz. Similar findings were seen when the cells beat spontaneously, with a shorter TTP, T50 and T90 in the 10µm group (p<0.05), despite no difference in the rate of spontaneous beating. There was no difference between the 20µm grooved constructs and the non-structured constructs (Figure 4.12 and Figure 4.13).

The NRVM cultured on the 10µm appeared to have better Ca\(^{2+}\) cycling properties than the NRVM cultured on the 20µm constructs. Specifically the T50 was significantly reduced at 1Hz, 0.5Hz and when beating spontaneously despite no difference in spontaneous beating rate (p<0.05). The T90 was also reduced at 1Hz and when beating spontaneously (p<0.05), however not significantly so at 0.5Hz (Figure 4.13).

Interestingly, there was no difference between the three groups when paced at 2Hz. This was because the cells did not return to a baseline diastolic Ca\(^{2+}\) level when paced at this frequency, suggesting immature cytosolic Ca\(^{2+}\) extrusion mechanisms. The amplitude (F/F0) was not significantly different between the three groups. It was noted that as the stimulation rate increased, the amplitude decreased in all three experimental groups, suggesting that these cells had a negative force frequency relationship at these beating rates (Figure 4.13).
Figure 4.12. Representative $\text{Ca}^{2+}$ transient from NRVM cultured on microgrooved tissue culture substrates with 10µm grooves, 10µm apart (blue); on microgrooved tissue culture substrates with 20µm grooves, 20µm apart (red); and non-structured constructs (grey) recorded with confocal microscopy in cells loaded with the $\text{Ca}^{2+}$ indicator Rhod-2 AM and field stimulated at 0.5Hz. A $\text{Ca}^{2+}$ transient recorded from an adult rat myocyte is shown for comparison (rust).
Figure 4.13. Effect of microgrooved tissue culture substrates on Ca$^{2+}$ transients. TTP, T50, T90, and F/F0 of NRVM cultured on non-structured PDMS, 10µm and 20µm grooved constructs field-stimulated at 2Hz, 1Hz, 0.5Hz, and beating spontaneously (Kruskal-Wallis test with Dunn’s Multiple Comparison test).
4.4 Discussion

The data presented in this chapter confirms that we can successfully fabricate a syncytial monolayer of beating cardiomyocytes on microgrooved PDMS culture substrates using NRVM. The NRVM cultured on microgrooved substrates were more strictly aligned in a longitudinal orientation than NRVM cultured on non-structured PDMS substrates (Figure 4.5 and Figure 4.11). NRVM were also successfully aligned using micropatterned fibronectin lines, although there were no cells between lines, and consequently adjacent lines were not electromechanically coupled. Consequently structured constructs fabricated using micropatterning could not really be considered to be a single beating syncytium (Figure 4.1). NRVM are more strictly longitudinally aligned in micropatterned fibronectin lines than when cultured on microgrooved tissue culture substrates, and more strictly aligned on 10µm microgrooves than on 20µm microgrooves (Figure 4.2 and Figure 4.11). The finding that structured tissue culture substrates promote alignment of NRVM is supported by several studies in the literature in which NRVM have been aligned in an anisotropic fashion using micro (Bien et al., 2003) and nanogrooved tissue culture substrates (Kim et al., 2010b), substrate stiffness (Wang et al., 2011b) and patterning of extracellular matrix components (Parker et al., 2008). All these methods appear to promote homogeneously aligned cells, with a major axis parallel to the direction of alignment (Bien et al., 2003, Kaji et al., 2003, Pong et al., 2011).

It has been suggested that anisotropic focal adhesion complexes form parallel to the grooves (Wang et al., 2011b), and this, together with evidence of the strain exerted on the substrate at sub-cellular level (Boudou et al., 2012), implies that the load that the cells exert on themselves may be an important factor in the development of elongated cells with aligned
myofibrillar, cytoskeletal and sarcomeric structures. There is also evidence to suggest that nuclear morphology is also altered. Cell alignment with external stimuli appears to promote binucleation, and higher nuclear eccentricity such as in adult cardiomyocytes (Pong et al., 2011, Bray et al., 2010). We did not find any evidence for an increase in binucleation; however, the nuclei in the structured group were more elliptical.

There is an overall shortening of Ca^{2+} transients in NRVM cultured on 10µm microgrooves compared to control experiments, with shortening of both TTP and Ca^{2+} extrusion that was not observed in NRVM cultured on the 20µm microgrooves or the micropatterned fibronectin lines (Figure 4.4, Figure 4.3, Figure 4.12 and Figure 4.13). The finding that structured constructs with specific geometries, and not others, affect the speed of Ca^{2+} transients is supported by evidence from the literature which suggest that culture conditions alter the Ca^{2+} handling properties of immature cardiomyocytes (Zimmermann et al., 2002). Structured substrates with specific geometry have been shown to reduce diastolic Ca^{2+} levels in several (Kaji et al., 2003, Feinberg et al., 2012, Pong et al., 2011) but not all studies (Bien et al., 2003). Microgrooved substrates in particular have been shown to elevate systolic Ca^{2+} (Bien et al., 2003). Cell elongation using aligned collagen constructs increases I_{Ca} and alters I_{Ca} regulatory properties (Walsh and Parks, 2002). NRVM cultured on structured constructs generally have faster TTP (Kaji et al., 2003, Feinberg et al., 2012, Pong et al., 2011), larger Ca^{2+} transient amplitude, and a trend towards faster Ca^{2+} removal (Yin et al., 2004, Bien et al., 2003, Pong et al., 2011). Many studies also report increased SR Ca^{2+} content (Yin et al., 2004, Bien et al., 2003). The changes in Ca^{2+} handling reported with NRVM culture on microcontact printed constructs are particularly interesting (Pong et al., 2011). Not only do the authors of this study also report similar findings to our results, confirming that structured constructs improve Ca^{2+} cycling of NRVM, they also have similar findings on the differential
effects of substrate geometry (Pong et al., 2011). Our finding that 10µm microgrooves and not 20µm microgrooves modify the Ca\(^{2+}\) handling is supported by the findings of Pong et al who suggest that constructs with 10µm geometry more successfully modify the Ca\(^{2+}\) cycling properties of NRVM than constructs with 20µm geometry (Pong et al., 2011).

The change in TTP in NRVM cells is likely to be because of alterations in SR Ca\(^{2+}\) regulation, given the relative importance of SR Ca\(^{2+}\) to CICR in rats and mice (Shattock and Bers, 1989). Increased SR Ca\(^{2+}\) load has been shown to increase the rate of SR Ca\(^{2+}\) release (Terracciano et al., 1995, Venetucci et al., 2007). Alternatively, improved RyR function itself may also increase the TTP. We cannot, however, exclude the possibility that these changes are not due to changes in SR function and are due to the contribution of extracellular Ca\(^{2+}\), in particular Ca\(^{2+}\) entry via the I\(_{Ca,L}\), and further experiments are required to demonstrate that improvements in Ca\(^{2+}\) cycling are a consequence of improved SR function.

The ability to shorten Ca\(^{2+}\) extrusion at higher frequencies, as the consequence of a shorter AP and other adaptations of the excitation-contraction coupling machinery, are fundamental properties of mature myocardium (Carmeliet, 2004). This phenomenon was clearly enhanced in the NRVM cultured on the 10µm microgrooved tissue culture substrates. A reduction in SERCA function has been shown to reduce the rate constant of Ca\(^{2+}\) transient decay (Stokke et al., 2010). It would be expected that a change in SERCA function, improving reuptake into the SR, would alter decay time at 50%. As the reduction was also significant for the 90% decay time at most frequencies, other Ca\(^{2+}\) extrusion mechanisms such as the NCX, mitochondrial Ca\(^{2+}\) handling or the sarcolemmal Ca\(^{2+}\) ATPase may also be implicated.
Studies in animal models (Montgomery et al., 1998, Tobita and Keller, 2000b, Tobita and Keller, 2000a, Voronov et al., 2004) and cultured cells (Tulloch et al., 2011, Kira et al., 1994) suggest that mechanical load plays a crucial role in the development of heart tissue. It is thought that, once cardiac tissue becomes longitudinally oriented, they form bundles, which are electrically and mechanically coupled. This creates a substrate for homogenous load (Zimmermann et al., 2002). Several studies suggest that aligned cells generate greater force (Bien et al., 2003, Feinberg et al., 2012), and we hypothesize that the load that cells exert on themselves (Boudou et al., 2012) may have a role in promoting cellular maturation. Mechanical stretch is known to enhance contraction force and velocity, and the expression of sarcomeric proteins of neonatal rat cardiomyocytes (Fink et al., 2000). It is this factor which could explain the greater efficacy of the 10µm microgrooves compared to 20µm microgrooves and non-structured tissue culture substrates. NRVM cultured on the 10µm microgrooves were more strictly aligned, perhaps resulting in more homogeneous load, and consequently a more mature phenotype. This does not really explain why improvements were not seen in the Ca$^{2+}$ cycling of NRVM cultured in micropatterned fibronectin lines. In fact, many of the parameters were actually worse in the micropatterned cultures than in the control cultures, despite the NRVM in fibronectin lines being more strictly aligned than the NRVM on 10µm microgrooves (Figure 4.2, Figure 4.4 and Figure 4.11). Perhaps, the absence of cells between the lines prevents the formation of a single beating syncytium, and this may cause the NRVM cultured in this fashion to experience less longitudinal load or lack some of the other electro-mechanical cues that prompt cellular maturation. Despite the evidence showing an association between cellular alignment and improved Ca$^{2+}$ cycling in NRVM it is not clear why this should occur. Cytoskeletal reorganization in response to external cues has previously been demonstrated in several studies (Bray et al., 2010, Geisse et al., 2009, Parker et al., 2008). It has been suggested that this occurs as a result of actin cytoskeletal
reorganization in response to mechanical load (Parker et al., 2008). It may be that this reorganization promotes better SR organization which influences Ca$^{2+}$ cycling, or that Ca$^{2+}$ cycling is modified by independent mechanisms. Further research needs to be undertaken to elucidate this. Whilst it is theoretically possible that closer opposition of the L-type Ca$^{2+}$ channel and the RyR may improve the efficacy of CICR, it is debatable whether this could fully explain the observed differences in Ca$^{2+}$ cycling. Furthermore, there is no evidence from the limited structural characterization that we undertook that cell alignment on the 10µm significantly improved ultrastructural properties of NRVM compared to NRVM cultured in other conditions.

The mechanisms underlying the experimental results observed in this chapter clearly need to be better elucidated, and experiments will be performed in subsequent chapters with this aim (Chapter 5). The pilot data presented here, however, is evidence that we have successfully fabricated constructs to align immature cardiomyocytes, and optimized the culture of immature cardiomyocytes in these conditions. We also optimized the experimental conditions to examine the Ca$^{2+}$ cycling of these cells, including the loading of Ca$^{2+}$ indicators and the use of confocal microscopy. The protocol described, using 10µm microgrooves represents a robust, reproducible and non-genetic strategy to improve the structure and function of immature cardiomyocytes. Moreover, the tissue engineering approach that we have adopted may be more amenable to further interrogation of the physiological mechanisms that underlie changes in the functional properties of cells than existing “more sophisticated” approaches (Tiburcy et al., 2011, Tulloch et al., 2011, Taylor, 2009, Hansen et al., 2010).

Whilst the finding that NRVM can be modified in this fashion is interesting, given the limitations of NRVM as a non-human disease model, the findings presented in this chapter
are only of secondary importance. More importantly, the experiments presented in this chapter are evidence that these techniques may be readily applied to iPSC-CM in our laboratory. It has not previously been demonstrated that the phenotype of iPSC-CM can be modified by structured culture. Consequently, the focus of the next chapter will be to demonstrate that the techniques characterized using NRVM can be applied to iPSC-CM.
5 Improved Calcium Cycling Properties of Induced Pluripotent Stem Cell-derived Cardiomyocytes Cultured on Structured Tissue Culture Constructs
5.1 Introduction

The data presented in Chapter 4.3.2, supported by the evidence form the literature (Kaji et al., 2003, Feinberg et al., 2012, Yin et al., 2004, Kim et al., 2010b, Dvir et al., 2011, Boudou et al., 2012) suggest that the Ca\(^{2+}\) cycling properties of immature cardiomyocytes can be enhanced by applying tissue culture techniques which encourage structured culture. There is, however, no evidence that these techniques will be effective for iPSC-CM.

Consequently, we aim to promote a matured phenotype in iPSC-CM using structured tissue culture substrates, and hypothesize that Ca\(^{2+}\) cycling of iPSC-CM is influenced by the 10\(\mu m\) microgrooved tissue culture substrates, described in Chapter 4.3.2 and Chapter 2.2.1. Whilst the data presented in Chapter 4.3.2 represents very clear evidence that in NRVM structured tissue culture substrates of a particular geometry improve their global Ca\(^{2+}\) cycling properties, we did not elucidate any of the underlying mechanisms. Consequently, in addition to studying the effect of microgrooved tissue culture substrates on iPSC-CM Ca\(^{2+}\) cycling, we also aim to examine SR Ca\(^{2+}\) regulation, AP properties, immunohistochemistry and gene expression, to investigate the mechanisms by which structured culture substrates may modify Ca\(^{2+}\) cycling in iPSC-CM.

5.2 Materials and methods

Structured microgrooved culture substrates with 10\(\mu m\) grooves, 10\(\mu m\) apart were fabricated from PDMS, as described previously (Chapter 2.2.1). iCell Cardiomyocytes, a commercially available frozen iPSC-CM line were seeded and maintained according to manufactures
guidelines (Chapter 2.1.3). 1/3 million iPSC-CM, were seeded in each well in the 12 well-plate. 1/6 million iPSC-CM were seeded in each well in the 24 well-plate (Chapter 2.2.1). As a consequence of the pilot data presented in Section 5.3.2.1, all subsequent experiments were performed 2 weeks following the recovery and seeding of iPSC-CM onto the PDMS constructs.

Immunohistochemistry (Chapter 2.4.2), assessment of cell alignment (Chapter 2.4.2.2), gene expression analysis (Chapter 2.4.1), and recording of AP characteristics was performed as previously described (Chapter 2.3.2). iPSC-CM Ca\(^{2+}\) transients were recorded according to the optimized protocols previously described (Chapter 2.3.2 and Chapter 4.3.2). Addition experiments were performed to investigate SR Ca\(^{2+}\) release mechanisms using high concentration caffeine dissolved in NT or low Na\(^+\) and Ca\(^{2+}\) buffered solutions. Apart from the pilot experiments described in Section 5.3.2.1, in which Rhod-2 AM was used, Fluo-4 AM was used in all subsequent Ca\(^{2+}\) studies. Rhod-2 AM fluoresces in the red spectrum, making it suboptimal for iCell cardiomyocytes which have an mRFP tag (Chapter 2.1.3) (Takahashi et al., 1999). Furthermore, Rhod-2 AM it is taken up and remains within intracellular organelles, in particular mitochondria, making it suboptimal for the measurement of cytosolic Ca\(^{2+}\) (Takahashi et al., 1999). Finally, Fluo-4 AM was adopted to better facilitate comparison with the large body of published literature in this area which have used this indicator.
5.3 Results

5.3.1 Cell alignment and sarcomere structure

Figure 5.1. Representative immunofluorescence of iPSC-CM cultured on non-structured PDMS (left) and microgrooved PDMS (right), Red– Sarcomeric α-actin, Blue– DAPI, scale bar 20µm.

Figure 5.2. Quantification of cell alignment iPSC-CM on structured and non-structured constructs. The graph on the left is a comparison in the variation in alignment (F-test), the graph on the right shows difference in average alignment (Mann-Whitney U).
Microgrooved PDMS substrates significantly improved iPSC-CM alignment compared to the non-structured substrates (p<0.0001 – Mann Whitney U, SD of non-structured 50.11° n=115, Structured 35.60° n=596; F=1.982, p<0.0001 – F-test). This resulted in more organized sarcomeric structures as seen in the aligned α-actinin striation pattern of the myofibrils (Figure 5.1 and Figure 5.2).

5.3.2 Calcium cycling

5.3.2.1 Effect of time in culture on the calcium cycling of induced pluripotent stem cell-derived cardiomyocytes

iCell cardiomyocytes when defrosted do not spontaneously beat until approximately day two, and do not form beating syncytia until day 4-5. Therefore it was unsurprising to find that in pilot experiments to determine if there was any effect of microgrooves on the Ca^{2+} cycling of iPSC-CM, structured constructs were not found to have any benefit at one week. The TTP was significantly less in spontaneously beating iPSC-CM cultured on microgrooved tissue culture constructs (p=0.0251). At this time point, however, iPSC-CM in both groups had very slow Ca^{2+} transients (Figure 5.3 and Figure 5.4).

Figure 5.3. Representative Ca^{2+} transients from spontaneously beating iPSC-CM, cultured on microgrooved PDMS substrates (blue) or non-structured PDMS substrates (grey) for 1 week. Recordings were made with confocal microscopy in cells loaded with the Ca^{2+} indicator Rhod-2 AM. A Ca^{2+} transient recorded from an adult rat myocyte is shown for comparison (rust).
At two weeks, in spontaneously beating iPSC-CM cultured on microgrooved tissue culture substrates there was a significantly reduced TTP (p=0.0124), and larger amplitude of peak Ca²⁺ transient (P<0.0001). There was no significant difference in spontaneous beating rate (Figure 5.5 and Figure 5.6). All subsequent experiments with iPSC-CM were performed two weeks following recovery from liquid nitrogen freezing.
microscopy in cells loaded with the Ca\textsuperscript{2+} indicator Rhod-2 AM. A Ca\textsuperscript{2+} transient recorded from an adult rat myocyte is shown for comparison (rust).

### 2 Week, No Field Stimulation

![Graphs showing changes in TTP, T50, T90, F/F0, and beating rate of iPSC-CM beating spontaneously, cultured on non-structured PDMS and microgrooved constructs for 2 weeks (Mann-Whitney U).]

Figure 5.6. TTP, T50, T90, F/F0 and beating rate of iPSC-CM beating spontaneously, cultured on non-structured PDMS and microgrooved constructs for 2 weeks (Mann-Whitney U).

#### 5.3.2.2 Effect of microgrooved constructs on calcium cycling in induced pluripotent stem cell-derived cardiomyocytes

iPSC-CM cultured on structured substrates had a shorter TTP (p=0.0002), and T50 (p=0.0065) when stimulated at 1Hz. There was no change in the T90. At 0.5Hz there was a shorter TTP (p=0.0073) but no changes in T50 or T90. Similarly while iPSC-CM were beating spontaneously, there was a reduced TTP (p=0.0012) in structured cells but no change in the T50 or T90. At 1Hz (p=0.0004) and 0.5Hz (p=0.0023) the amplitude was significantly reduced in the iPSC-CM cultured on microgrooved PDMS substrates, however not when beating spontaneously. There was no significant difference in the rate of spontaneous Ca\textsuperscript{2+} transients (Structured: 11.67 beats per minute +/-1.495, n=18; Non-structured: 12.43 beats...
per minute +/-1.432, n=37; p=0.8859). Similarly the proportion of iPSC-CM with spontaneous Ca^{2+} transients did not differ significantly between groups (Structured: 18/37 (48.6%); Non-structured: 37/64 (57.8%); p=0.73) (Figure 5.7)

Figure 5.7. TTP, T50, T90, F/F0 of iPSC-CM cultured on non-structured PDMS and microgrooved constructs field-stimulated at 1Hz, 0.5Hz, and beating spontaneously (Mann-Whitney U).
Figure 5.8. TTP, T50, T90, and F/F0 of spontaneously beating and non-spontaneously beating iPSC-CM cultured on structured and control substrates field-stimulated at 0.5Hz (Mann-Whitney U).
iPSC-CM spontaneously beating on structured tissue culture substrates had significantly reduced TTP, T50 and T90 when field stimulated at 0.5Hz compared with cells without spontaneous activity in culture, however this difference was not seen in non-structured constructs (Figure 5.8).

### 5.3.2.3 Contribution of the sarcoplasmic reticulum to changes in calcium cycling in induced pluripotent stem cell-derived cardiomyocyte cultured on microgrooved tissue culture substrates

In order to investigate whether differences in TTP between structured and non-structured cells was due to differences in SR Ca$^{2+}$ release, iPSC-CM were spritzed with solutions containing high concentrations of caffeine, as previously described (Lee et al., 2011, Itzhaki et al., 2011b). A “synchronous” SR Ca$^{2+}$ release, analogous to the response of an adult cardiomyocyte (Figure 5.9 A) and consisting of a single large transient, was elicited in response to caffeine-containing NT in 77% of the structured iPSC-CM (Figure 5.9 C and Figure 5.9 E). However, with iPSC-CM cultured on non-structured constructs we observed multiple peaks of the caffeine-transient indicating irregular, asynchronous release from the SR (p<0.0001) (Figure 5.9 D) analogous to the response observed in immature cardiomyocytes (NRVM) (Figure 5.9 B). The experiments were repeated in Na$^+$ and Ca$^{2+}$ free solution to exclude extracellular Ca$^{2+}$ cycling by preventing Ca$^{2+}$ extrusion via the NCX, or L-type Ca$^{2+}$ current-mediated Ca$^{2+}$ induced Ca$^{2+}$ release. Again, “synchronous” SR Ca$^{2+}$ release was observed in 70% of structured constructs but in only 21% of non-structured constructs (p<0.0001) suggesting that this effect was independent of sarcolemmal fluxes (Figure 5.9 F). Interestingly these changes occurred despite reduced SR Ca$^{2+}$ content,
assessed by the reduced F/F0 of caffeine-induced transient (Figure 5.9 G). It is important to note however that, as such quantification can only be undertaken when there is synchronous Ca\textsuperscript{2+} release from the SR, this only represents a fifth of the Ca\textsuperscript{2+} induced transients recorded from non-structured cells.

Figure 5.9. Representative traces showing response to the application of 50mM caffeine solution of isolated adult rat ventricular cells illustrating “mature caffeine response“ (A), NRVM illustrating “immature caffeine response“ (B),
iPSC-CM cultured on structured PDMS (C), and iPSC-CM cultured on non-structured PDMS (D). Proportion of experiments that elicited an organized response to caffeine when cells were superfused in NT (Fisher’s exact test) (E). Proportion of experiments that elicited an organized response to caffeine when cells were superfused in Na\(^+\) and Ca\(^{2+}\) free solution (Fisher’s exact test) (F). Amplitude of caffeine induced Ca\(^{2+}\) transient when cells were superfused in Na\(^+\) and Ca\(^{2+}\) free solution (Mann-Whitney U) (G).

### 5.3.3 Action potential duration

There was no significant difference in the spontaneous AP rate in either group (p=0.16) (Figure 5.10). Both the uncorrected (p=0.8904) and Bazett’s corrected APD (p=0.46) were not significantly different, however in both groups the rate-APD relationship was not well described by the Bazett’s formula (Figure 5.10).

Figure 5.10. Spontaneous APD measured using sharp microelectrodes. Time to peak (A), time to 90% decay (B), and APD corrected for spontaneous beating rate (C) (Mann-Whitney U). Panels D and E suggest that Bazett’s correction (curved line) does not adequately describe the relationship between APD and beating rate.
5.3.4 Protein localization

We did not find evidence that ultrastructural properties were affected by alignment of iPSC-CM on microgrooved PDMS substrates. For example, Cx43 did not appear to be preferentially expressed along the short axis of aligned cells, as in adult cardiomyocytes, and RyR and PLN expression did not suggest that SR organization was improved in structured iPSC-CM (Figure 5.11, Figure 5.12, Figure 5.13, and Figure 5.14).

Notably, the cells showed only week staining for RyR suggesting a low expression of the receptor, which is confirmed by qPCR (Section 5.3.5). Colocalization of RyR and Ca\(_{\text{v}}\)1.2 was increased in the structured group (Structured: Pearson’s coefficient (r) = 0.028, n = 6 images; Non-structured: r =-0.183, n = 4 images; p<0.001). However, this must be interpreted with caution given the minimal area colocalized in both groups (0.08% of all image pixels in the structured group compared to 0.24% in the non-structured group).
Figure 5.11. Immunohistochemistry of iPSC-CM cultured on non-structured PDMS, Red- PLN, Green– Cx-43, Blue– DAPI, scale bar 20µm.
Figure 5.12. Immunohistochemistry of iPSC-CM cultured on non-structured PDMS, Red- Cav1.2 channel, Green–RyR, Blue– DAPI, scale bar 20µm.
Figure 5.13. Immunohistochemistry of iPSC-CM cultured on structured PDMS, Red- PLN, Green- Cx43, Blue- DAPI, scale bar 20µm.
5.3.5 Gene expression

Gene expression was normalized according to expression of GAPDH. The use of alternative house-keeping genes for normalization, such as 18s ribosomal RNA, Cyclophilin G, and β-actin did not significantly change the results obtained (Figure 5.15).
Figure 5.15. Comparison of IP3R, RyR and SERCA2a expression when normalized to GAPDH, 18s ribosomal RNA, Cyclophilin G, and β-actin, and expressed relative to adult heart tissue in iPSC-CM cultured on structured and non-structured PDMS, fibroblasts, adult heart and foetal heart tissue (Kruskal-Wallis test with Dunn’s Multiple Comparison test).
Gene expression of early cardiac transcription factors and genes associated with pluripotency was higher in iPSC-CM than in adult and foetal tissue; however, there was no difference between structured and non-structured constructs (Figure 5.16).

Figure 5.16. Comparison of genetic markers of differentiation (GATA4, NKX2.5, and ANF) when normalized to GAPDH and expressed relative to adult heart tissue in iPSC-CM cultured on structured and non-structured PDMS, fibroblasts, adult heart and foetal heart tissue (Kruskal-Wallis test with Dunn’s Multiple Comparison test).

Figure 5.17. Comparison of expression of genes encoding ultrastructural proteins in cardiomyocytes (β-MHC, α-MHC, MLC2v, cTNT, K,4.3, CAV3) when normalized to GAPDH and expressed relative to adult heart tissue in
iPSC-CM cultured on structured and control substrates, fibroblasts, adult heart and foetal heart tissue (Kruskal-Wallis test with Dunn’s Multiple Comparison test).

Figure 5.18. Comparison of expression of genes encoding proteins important for Ca\(^{2+}\) cycling in cardiomyocytes (IP3R, RyR, SERCA2a, CASQ2, CALR, JPH2, PLN, Ca\(_{\text{v}}\)3.1, Ca\(_{\text{v}}\)1.2, NCX and TRDN) when normalized to GAPDH and expressed relative to adult heart tissue in iPSC-CM cultured on structured and control substrate, fibroblasts, adult heart and foetal heart tissue (Kruskal-Wallis test with Dunn’s Multiple Comparison test).
The expression patterns of genes encoding structural proteins such as alpha-myosin heavy chain (α-MHC), beta-myosin heavy chain (β-MHC), myosin light chain 2v (MLC2V), cardiac troponin T (cTNT), caveolin 3 (CAV3) (Figure 5.17) and those important for Ca\(^{2+}\) cycling (inositol trisphosphate receptor (IP3R), RyR, SERCA2a, calsequestrin 2 (CASQ2), calreticulin (CALR), junctophilin 2 (JPH2), PLN, T-type Ca\(^{2+}\) channel (Cav3.1), L-type Ca\(^{2+}\) channel (Cav1.2), NCX and triadin (TRDN)) (Figure 5.18) were similar in structured and non-structured iPSC-CM, and equally different from adult myocardium with gene expression levels generally close or below foetal heart controls. There was no significant difference in the expression of any gene in structured and non-structured iPSC-CM except triadin (p=0.0250).

### 5.4 Discussion

iPSC-CM cultured on microgrooved PDMS substrates adopted structural properties such as cellular alignment and sarcomeric organization which resembled adult cardiomyocytes (Section 5.3.1). At one week there was no benefit of structured tissue culture substrates on the Ca\(^{2+}\) handling properties of iPSC-CM, however at two weeks some of the changes observed in NRVM cultured on microgrooved PDMS (Chapter 4.3.2.2) were apparent in iPSC-CM (Section 5.3.2.1). This is probably because at one week iPSC-CM had not fully recovered from liquid nitrogen freezing, and had not yet formed beating syncytia. iPSC-CM cultured on microgrooved PDMS substrates for two weeks had shorter TTP and T50 when stimulated at 1Hz. When stimulated at 0.5Hz, and when spontaneously beating, structured iPSC-CM also had a shorter TTP (Section 5.3.2.2). The spontaneous beating rate and APD was unchanged.
between groups (Section 5.3.3), More organized SR Ca$^{2+}$ release was elicited in response to caffeine in structured iPSC-CM (5.3.2.3).

Patterning of iPSC-CM using structured PDMS substrates has previously been reported in the literature, however the structure and function of iPSC-CM was not characterized (Paik et al., 2012). The finding that structured tissue culture substrates promote alignment of iPSC-CM and improve sarcomeric organization is consistent with our experiments with NRVM (Chapter 4.3.2) and is supported by several NRVM studies in the literature (Bien et al., 2003, Kim et al., 2010b, Wang et al., 2011b, Parker et al., 2008). Alignment of myofibrillar, cytoskeletal and sarcomeric structures is widely reported in the literature and is consistent with our findings in human iPSC-CM (Pong et al., 2011, Parker et al., 2008, Wang et al., 2011b, Kim et al., 2010b).

Several studies report that aligned NRVM express more Cx43 in clusters (Bien et al., 2003) localized in a bipolar fashion analogous to adult cells (McDevitt et al., 2003), and have higher conduction velocities in the longitudinal direction (Bien et al., 2003, Kim et al., 2010b). We did not find a marked difference in the distribution of Cx43 (Section 5.3.4). Whilst, we did not investigate conduction velocity anisotropy other investigators have subsequently found that iPSC-CM aligned using structured tissue culture substrates do have anisotropic conduction velocity (Wang et al., 2013). Furthermore, syncytia fabricated from iPSC-CM cultured on aligned tissue culture substrates were shown to have significantly fewer re-entry arrhythmias than control constructs (Wang et al., 2013). Like other groups who report the results of NRVM (Feinberg et al., 2012) and iPSC-CM experiments (Wang et al., 2013) we did not find a difference between the AP morphology of structured and non-structured cells. Whilst higher synchronous beating rates have been reported in anisotropic NRVM cultures
(Wang et al., 2011b) and increased maximum capture rate in response to electrical stimulation in structured NRVM culture have also been described (Feinberg et al., 2012), we did not find any statistically significant difference in the spontaneous beating rate of the structured and non-structured iPSC-CM cultures.

The finding that structured tissue culture substrates significantly changed the Ca\(^{2+}\) cycling properties of iPSC-CM, reducing the TTP could be due to changes in entry of extracellular Ca\(^{2+}\) (\(I_{\text{Ca,L}}\)) and trigger for CICR. Immunohistochemistry suggests that Ca\(_{\text{v}}\)1.2 and RyR were poorly colocalized and the slight improvement in the structured cultures is unlikely to explain the faster TTP. Although expression of Ca\(_{\text{v}}\)1.2 was not significantly different between groups, it is possible that post translational modification may result in differential expression of Ca\(_{\text{v}}\)1.2 at the sarcolemma and extracellular Ca\(^{2+}\) influx may explain the faster TTP. Similarly, changes in intracellular Ca\(^{2+}\) buffering may also account for the changes in Ca\(^{2+}\) cycling induced by microgrooved PDMS substrates, however this is not supported by the gene expression data which suggest that the genes encoding calsequestrin and calreticulin were not differentially expressed (Figure 5.18).

The difference between the responses of iPSC-CM to caffeine suggests that the observed differences may also be partly due to regulation of Ca\(^{2+}\) by the SR. Ca\(^{2+}\) release from the SR is predominantly mediated by the RyR in adults, although in immature cardiomyocytes the IP3R plays a more significant role (Janowski et al., 2010). As IP3R mediated SR Ca\(^{2+}\) release is less efficient that RyR CICR mediated release (Kentish et al., 1990); a difference in the ratio of functional RyR and IP3R receptors, or difference in the absolute number of either receptor may explain our findings. This is supported by the morphology of the caffeine-induced Ca\(^{2+}\) transients (Janowski et al., 2010) (Figure 5.9). The gene expression data
presented here does not suggest that there was a marked difference in IP3R, RyR, or the ratio of IP3R to RyR between the structured and non-structured group (Figure 5.18). Several post-translational factors, however, could explain reduced RyR function including post-translational modification in any of the components of the quaternary structure of the RyR (including calsequestrin, junction, triadin and the RyR itself), failure of the RyR quaternary structures to assemble, phosphorylation of the RyR, or other factors including SR Ca\textsuperscript{2+} content (Terracciano et al., 1995, Lanner, 2012, Lanner et al., 2010, Van Petegem, 2012). Increased SR Ca\textsuperscript{2+} content is known to increase the open probability of the RyR, therefore SR Ca\textsuperscript{2+} content could explain these findings (Shannon et al., 2005). Whilst our data suggest that SR Ca\textsuperscript{2+} content was actually higher in the non-structured group, as most of the caffeine induced transients in the non-structured represented non-synchronous Ca\textsuperscript{2+} release and consequently could not be quantified, this data was from an extremely small and non-representative sub-group of the non-structured iPSC-CM and must be interpreted with caution.

The finding that triadin was more highly expressed in non-structured cells compared to structured cells is interesting as triadin overexpression has been shown to block excitation-contraction coupling in myotubes and cardiomyocytes in the absence of extracellular Ca\textsuperscript{2+} (Allen, 2009). However, the marginal raise in triadin, alone, is unlikely to explain the differences in Ca\textsuperscript{2+} cycling that we observed; firstly, despite being significantly raised in iPSC-CM cultured on non-structured tissue culture substrates it falls well below the levels seen in adult cardiomyocytes. Secondly, this small change would not be expected to reduce caffeine-induced Ca\textsuperscript{2+} transients, and might reasonably be expected to improve the efficacy of CICR. Finally, it is unclear from the literature what effect a small increase in triadin would
have in the presence of extracellular Ca\(^{2+}\), especially given the multiple isoforms, all with potentially different functions (Allen, 2009, Marty et al., 2009).

Whilst the role of structured tissue culture substrates has not previously been studied in iPSC-CM, their effect on Ca\(^{2+}\) cycling has been studied in NRVM (Kaji et al., 2003, Feinberg et al., 2012, Pong et al., 2011, Bien et al., 2003, Walsh and Parks, 2002). In contrast to our findings several studies report an increase in the amplitude of Ca\(^{2+}\) transients (Yin et al., 2004, Bien et al., 2003, Pong et al., 2011), similarly many studies report increased SR Ca\(^{2+}\) content (Yin et al., 2004, Bien et al., 2003). Whilst our experiments did, like several studies, show faster Ca\(^{2+}\) transient peak, in contrast to our own data (Chapter 4.3.2.2), and several other NRVM studies, we did not see any effect on Ca\(^{2+}\) extrusion (Kaji et al., 2003, Feinberg et al., 2012, Pong et al., 2011). The implication is that whilst Ca\(^{2+}\) release mechanisms from the SR have become more representative of adult myocardium, Ca\(^{2+}\) uptake mechanisms have not undergone a similar change. This is supported by the T50 and T90 which were not generally longer. The T50 at 1 Hz was reduced, but this result must be seen in the context of the markedly different properties between sub-populations of iPSC-CM, in particular between spontaneously beating and non-spontaneously beating cells which on structured constructs have significantly longer TTP, T50 and T90 (Figure 5.7).

The effect of structured tissue culture substrates on cytosolic Ca\(^{2+}\) extrusion is evident from our provisional experiments with NRVM (Chapter 4.3.2), which are concordant with the published literature showing reduced TTP, T50 and T90 at most frequencies. This effect is less evident at 2Hz where the Ca\(^{2+}\) extrusion was not sufficiently developed for it to return to baseline between transients (Chapter 4.3.2.2). The differences between our findings in human iPSC-CM and experimental data on NRVM may be due to differences in the maturity of
neonatal and “embryonic like” cells or due to interspecies differences. A critical difference in the physiology of rat and human cardiomyocytes is the relative contribution to normal Ca\(^{2+}\) cycling of SR Ca\(^{2+}\) compared to extracellular in Ca\(^{2+}\) (Bers, 2001). We could not quantify the effect of structured tissue culture substrates on the I\(_{Ca,L}\) in either NRVM or iPSC-CM. However, if we assume that the primary effect of cell alignment is to improve SR function, which is entirely constant with the data that we present, then the relatively modest effect of structured PDMS substrates on iPSC-CM compared to NRVM could be explained. Donald Bers in his comprehensive review of all the literature exploring the relative contribution of extracellular and SR Ca\(^{2+}\) to CICR suggests a hierarchy of SR Ca\(^{2+}\) dependence in several types of cardiomyocytes. The literature suggests that whilst NRVM are less dependent on SR Ca\(^{2+}\) than adult rat ventricular cells, they are more dependent on SR Ca\(^{2+}\) than adult human ventricular cells, and significantly more dependent on SR Ca\(^{2+}\) than human foetal cells (Bers, 2001). As the effect of SR Ca\(^{2+}\) in normal Ca\(^{2+}\) cycling is more modest in human foetal cardiomyocytes compared to NRVM, perhaps maturation of SR function in human iPSC-CM will have a more modest effect than equivalent maturation of SR function in NRVM.

Gene expression data did not show difference between structured and non-structured cultures. Even on microgrooved PDMS substrates iPSC-CM continue to express a globally immature phenotype. This suggests that other mechanisms which were not screened here or post translation modifications may be involved in the effects observed. An important caveat is that the summation of gene expression in all cells in a dish may not be representative of the gene expression of individual iPSC-CM in which Ca\(^{2+}\) were measured. Perhaps more sophisticated single-cell gene expression techniques should be employed to address this point (Buganim et al., 2012). It is telling that many of the studies employing iPSC-CM as disease models which have successfully demonstrated differences in gene expression have used single-cell gene
expression assays (Lan et al., 2013), and this may reflect the heterogeneity inherent in immature cardiomyocytes.

As we discuss in Section 4.4, it is not clear from existing evidence in the literature whether ultrastructural reorganization influences Ca\textsuperscript{2+} handling or whether changes in Ca\textsuperscript{2+} handling are independent. Several studies suggest that aligned cells generate greater force (Bien et al., 2003, Feinberg et al., 2012), and we hypothesized that the mechanical load that cells exert on themselves may have a role in promoting cellular maturation. This may be supported by our finding that iPSC-CM beating in culture on structured tissue culture substrates had significantly reduced TTP, T50 and T90, but cells beating on non-structured constructs did not. Spontaneously beating and non-spontaneously beating iPSC-CM were aligned on structured constructs; however, the fact that spontaneously beating iPSC-CM on structured constructs had reduced TTP, T50 and T90 suggests that load may be important. Although spontaneously beating cells may have different physiological properties for other reasons (e.g. a different sub-population with different properties), the fact that spontaneously beating cells on non-structured constructs were not different from non-beating cells suggests that anisotropic load in particular may have an important effect on Ca\textsuperscript{2+} cycling (Figure 5.7). This is supported by the rapid changes in cardiomyocyte morphology, Ca\textsuperscript{2+} cycling, and electrophysiology following birth (Zhang et al., 2013, Novak et al., 2009, Mahmoud and Porrello, 2012, Bers, 2001), and evidence from experimental models in immature cardiomyocytes in which stretch has been shown to have a direct effect on Cx43 expression, cell coupling, ion channel activity, and APD (Jacot et al., 2010b, Tulloch et al., 2011, Fink et al., 2000, Gwak et al., 2008, Brown et al., 2008).
Several critical experiments would have significantly improved our understanding of the mechanisms that underlie the changes in Ca\(^{2+}\) cycling that we observed in this chapter and in Chapter 4. In particular, we did not investigate if anisotropic conduction velocity was present in the structured constructs, as described by other groups (Wang et al., 2013). AP measurements were undertaken using sharp electrode measurement in current clamp mode, a technique which has a number of limitations including inaccurate measurements of small changes in voltage because of the high resistance associated with the thin pipette tip and ionic leak associated with membrane tearing (Waters et al., 2005). The I\(_{\text{Ca,L}}\), a critical component of CICR was not measured. Also, experiments were not undertaken to investigate our hypothesis that anisotropic self-loading of aligned cells was the critical determinant of the changes we observed. These experiments were not undertaken, despite being relatively simple, because of the technical problems associated with performing these experiments on cells cultured on the PDMS tissue culture substrates.

As a consequence of the thickness of the constructs we were unable to visualize the target cells using an inverted microscope. This meant that standard patch clamp, optical mapping and even serial microscopy of live cultures were technically problematic. The thick PDMS substrate would also insulate the cells from the underlying electrodes if field potential recordings were attempted with an MEA system. Whilst some of these problems could be overcome by adapting experimental apparatus to image the cells using an upright microscope, the difficulty in fully evaluating the physiology of iPSC-CM cultured on structured tissue culture substrates limits the utility of microgrooved PDMS substrates.
5.4.1 Conclusion

The data presented in this chapter, to the best of our knowledge, is the first demonstration, and at present the only demonstration in the literature, that structured tissue culture substrates affect Ca\textsuperscript{2+} cycling and structural properties in human iPSC-CM. This is an important first step in promoting a cellular phenotype in iPSC-CM that is more representative of adult myocardium. We did not investigate whether this may increase the utility of iPSC-CM as disease models; however other studies subsequently published suggest that this may be the case (Wang et al., 2013). Further construct development may be useful to facilitate investigation of the incremental value of structured constructs in myocardial disease modelling with iPSC-CM. Construct development may also make it easier to fully interrogate the complex interaction between structure, function and environment in developing cardiomyocytes.
6 The Effects of Further Development of Tissue Culture Constructs on the Calcium Cycling Properties of Immature Cardiomyocytes
6.1 Introduction

The data presented in Chapter 5.3.2 confirms that culture conditions can be used to modify the Ca\textsuperscript{2+} cycling of iPSC-CM in order to promote a phenotype which is more representative of adult myocardium. Despite being readily reproducible and cost-effective, our results suggest that the utility of microgrooved tissue culture substrates may be limited. This is primarily for two reasons. Firstly, despite their simplicity many commonly used techniques in cell physiology are difficult to apply. The thickness of the constructs makes the use of MEA impossible. It is also highly problematic to image the constructs using an inverted microscope. Whilst live cell imaging, patch clamping, confocal microscopy and optical mapping can be performed by adapting experimental apparatus to use an upright microscope this is not without problems. As most objectives are required to be immersed in the culture medium, serial evaluation is difficult because of difficulties in maintaining sterility. Whilst these problems can be overcome, a construct that could be used with an inverted microscope would be more versatile. The second limitation of microgrooved tissue culture substrates for the maturation of iPSC-CM highlighted by the data presented in Chapter 5 is the relatively modest effect microgrooved substrates had on the maturity of the structural and functional phenotype of iPSC-CM. Whilst the demonstration that the phenotype of iPSC-CM can be modified using structured tissue culture substrates is an important first step, several publications have highlighted the striking effect that constructs which facilitate 3D cell-cell interaction can have on the phenotype of NRVM (Boudou et al., 2012, Eschenhagen et al., 2002, Zimmermann et al., 2000). Recently, similar techniques have been shown to be also applicable to iPSC-CM (Christoforou et al., 2013).
The limitations with the microgrooved tissue culture substrates described in Chapter 5 therefore suggest two distinct strands of research. Firstly a 2D tissue culture substrate that is compatible with the tools currently used in cell physiology which would allow investigation of the mechanisms that link structural organization and improved function to be more readily investigated. In particular it is important to determine exactly why Ca\(^{2+}\) cycling is improved in aligned cells. A tissue culture substrate on which high-throughput techniques could be readily applied would also facilitate investigation of the value of structured 2D tissue culture techniques in drug development and toxicology screening. The second strand of research suggested by the limitation of PDMS microgrooved constructs highlighted in Chapter 5 is the development of a construct which permits 3D cell-cell interaction but which can be readily fabricated and examined without specialist equipment or techniques. Such a construct may have a more profound effect on the phenotype of cultured cells than existing 2D tissue culture systems (Wang et al., 2011b, Yin et al., 2004, Bien et al., 2003, Kim et al., 2010b, Kaji et al., 2003, Pong et al., 2011) whilst still being easier to interrogate than 3D tissue culture systems previously described in the literature (Boudou et al., 2012, Tulloch et al., 2011, Zimmermann et al., 2000, Eschenhagen et al., 2002).

### 6.1.1 Two-dimensional Parylene-C constructs

We determined to fabricate a novel 2D tissue culture substrate using Parylene-C. Parylene-C is widely used to coat implantable medical devices such as guide-wires, intra-arterial stents, implantable defibrillators and pacemakers (Khan et al., 2008, Loeb et al., 1977, Fontaine et al., 1996). It is insoluble at room temperature, has excellent barrier properties and is pinhole-free above 100nm. It is biologically inert, inexpensive, and easily processed (Khan et al.,
Furthermore, its mechanical properties mean it can be used in flexible devices, for example, implantable-flexible chemical sensing arrays (Zoumpoulidis et al., 2009), or pressure sensors for measuring intraocular pressure in glaucoma patients (Chen et al., 2008). These properties may make Parylene-C suitable as a thin tissue culture substrate, which would facilitate live-cell microscopy, and in which electrical and chemical sensors can readily be integrated. It is theoretically possible that thin Parylene-C layers could be added to existing proprietorial MEA dishes. The flexible properties of Parylene-C may also potentially allow the application of continuous or cyclical mechanical strain to cultures, which has been shown to promote maturation of immature myocytes in some studies (Fink et al., 2000, Tulloch et al., 2011, Gwak et al., 2008). Parylene-C has already been used to fabricate thin films, that can be peeled off tissue culture substrates after seeding or protein coating to facilitate cell and protein patterning applications (Tan et al., 2010, Wright et al., 2008). However, as with other existing technology to micropattern cells on thin tissue culture substrates, or around embedded electrodes this technique is technically difficult and time-consuming (Michel et al., 2002, Fan et al., 2008).

Despite the ideal chemical and biological properties of Parylene-C, a major obstacle to its application as a tissue culture substrate is its hydrophobic surface properties. Several approaches have been adopted to modify its surface properties. In particular, several groups have sought to exploit the poor oxidative resistance of Parylene-C (~115 °C) (Fortin and Lu, 2001), which mean that its surface properties can be readily altered using techniques such as controlled oxygen plasma exposure. Oxygen plasma treatment has been previously employed to effectively modify the surface properties of Parylene-C, in order to facilitate its use as a tissue culture substrate (Chang et al., 2007, Hoshino et al., 2012), and the surface properties
of Parylene-C have previously been successfully modified in order to fabricate neural cell cultures with specific patterns (Delivopoulos et al., 2010).

6.1.2 Three-dimensional biomimetic tissue engineered constructs

In order to investigate whether tissue culture constructs which facilitate 3D interaction between immature cardiomyocytes more effectively improve Ca$^{2+}$ cycling of immature cardiomyocytes we used a synthetic PCL construct and decellularized myocardial tissue. As discussed in Chapter 1.7.1, synthetic polymers such as PCL have the theoretical advantage that their physical and chemical properties can easily be modified in order to mimic myocardial characteristics. Unfortunately, cellular interaction with synthetic materials is limited, and consequently substrates usually have to be coated in ECM components to promote cell adhesion (Li and Guan, 2011). Electrospinning was performed to fabricate a construct from PCL with a high surface-area to volume ratio, which mimics the fibrous architecture of ECM and promotes cell adhesion (Rockwood et al., 2008, Krupnick et al., 2002, Zong et al., 2005, Ishii et al., 2005, Papadaki et al., 2001, Xing et al., 2012, Shin et al., 2004) (Chapter 1.7.1). There is already evidence in the literature that electrospun 3D synthetic constructs, coated in ECM components can support NRVM in culture (Ishii et al., 2005, Rockwood et al., 2008), often for long periods of time, and promote greater structural maturation than standard culture techniques (Papadaki et al., 2001, Zong et al., 2005, Xing et al., 2012). There is a paucity of evidence on what effect electrospun networks have on Ca$^{2+}$ cycling in NRVM compared to 2D tissue cultures. Consequently, in this chapter we aim to test the feasibility of using electrospun fiber networks for Ca$^{2+}$ cycling studies and to test the
hypothesis that NRVM cultured in these networks will have enhanced Ca\textsuperscript{2+} cycling properties compared to NRVM cultured on 2D constructs.

Since NRVM were used to recellularize a decellularized adult rat heart there has been increasing interest in using decellularized ECM as a tissue engineering scaffold (Ott et al., 2008). Cells have been shown to infiltrate decellularized ECM and distribute evenly throughout the construct (Wang et al., 2010). Cells contract spontaneously and synchronously (Ott et al., 2008). Reseeded constructs generate more force comparable to that of artificial ECM constructs seeded with NRVM (Ott et al., 2008). Significantly, a decellularized mouse heart has recently been recellularized with iPSC (Lu et al., 2013). For these reasons decellularized ECM appear to be an ideal tissue culture substrate for disease modelling. The use of whole organ recellularized preparations, however, is expensive and has limited utility as a cellular disease model. For this reason we determined to investigate the efficacy of using thin recellularized myocardial tissue slices for Ca\textsuperscript{2+} cycling studies. We aimed to investigate whether culture of immature cardiomyocytes in decellularized slices has a beneficial effect on their Ca\textsuperscript{2+} cycling, testing the hypothesis that NRVM cultured in decellularized myocardial tissue slices will have improve Ca\textsuperscript{2+} cycling properties compared to NRVM culture in 2D cultures.

6.2 Materials and methods

Two Parylene-C constructs were fabricated using oxygen plasma treatment as previously described (Trantidou et al., 2012) (Chapter 2.2.3). In summary, both constructs were fabricated from glass coverslips which were coated in Parylene-C films by chemical vapour
deposition. An HDMS contact mask was then spin-coated onto the Parylene-C-coated coverslips, followed by a 1.4μm thick positive photoresist. The samples were then exposed to UV light to remove the exposed photoresist. Oxygen plasma was then used to achieve selective hydrophobic patterning, with most of the Parylene-C was protected from the oxidative effects of the oxygen plasma by the contact masks. The geometry of the constructs (parallel lines 10μm wide and 10μm apart) was chosen as previous experiments (Chapter 4.3.2.2) and other groups (Pong et al., 2011) had shown this geometry effectively altered the physiological properties of immature cardiomyocytes. After plasma treatment, the remaining photoresist was removed. Short exposure of the 7μm thick Parylene-C films to oxygen plasma at 50W for 1 min altered the surface properties of the polymer without significant etching or altering its geometry, resulting in alternating hydrophobic and hydrophilic areas (H/H construct). Exposure of the 1μm thick Parylene-C constructs at 400W for 15 min resulted in the exposed areas of Parylene-C being completely removed. This resulted in alternating glass and Parylene-C areas (G/H Construct) and a construct with similar geometry in the case of the 4μm Parylene-C film to the previously described PDMS construct (Chapter 2.2.1 and Chapters 4.3.2.2). 2 million NRVM were seeded on each construct.

PCL constructs were fabricated as previously described (Chapter 2.2.4) (Zong et al., 2002, Jeun et al., 2005). Briefly, PCL granules were dissolved in chloroform and the solution was injected through a fine needle under pressure onto a collecting drum rotating at 3000 rpm. The resulting structure was formed from fibers 300-1100nm in diameter, 100-2000nm apart. The solvent was left to evaporate from the fabricated constructs. They were then washed several times to ensure all of the solvent was removed. The constructs were then stretched
over a Scaffdex ‘cell crown’. 2 million NRVM were seeded onto each construct after coating with fibronectin.

Decellularized heart slices were produced as described in Chapter 2.2.5. In summary, 350µm thick tissue slices were cut from ventricular tissue with a high precision vibrating microtome in cold solution (Camelliti et al., 2011). Slices were decellularized by placing them on a shaker in 1% SDS at room temperature for 24 hours then washing with PBS before being left on a shaker, at room temperature for 4 hours in 1% Triton (Ott et al., 2008, Godier-Furnemont et al., 2011). These slices were then washed with PBS and frozen until required. Once defrosted the constructs were then stretched over a Scaffdex ‘cell crown’. 1 million NRVM were seeded onto each construct (Camelliti et al., 2011).

NRVM on both 3D and 2D constructs were loaded with Fluo-4AM and Ca\(^{2+}\) transients were recorded according to the optimized protocols previously described (Chapter 2.3.3, Chapter 4.2, and Chapter 4.3). All experiments were performed at day 4 post-seeding.
Figure 6.1. Bright field images, scale bar 50μm (above) and immunohistochemistry (Green - Myosin heavy chain antibody, Blue – DAPI, scale bar 50μm) (below) images of NRVM cultured on non-structured hydrophilic (left), H/H (middle) and G/H Parylene-C constructs.

6.3 Results

6.3.1 Parylene-C constructs

6.3.1.1 Effect of Parylene-C constructs with modified hydrophobicity on neonatal rat ventricular myocyte structure

NRVM seeded onto non-structured glass or hydrophilic areas of the constructs were aligned in the direction of the construct (Figure 6.1). Consequently, NRVM cultured on both the H/H (p<0.001) and G/H (p<0.001) constructs showed greater anisotropy than NRVM cultured on non-structured hydrophilic substrates (Figure 6.2). G/H constructs were more strictly aligned
than H/H constructs (F=1.409, p=0.0034 – F-test). Both structured constructs were more strictly aligned than the non-structured controls (F=2.064, p<0.0001 – F-test, H/H) (F=2.907, p<0.0001 – F-test, G/H).

Figure 6.2. Quantification of cell alignment of NRVM cultured on Parylene-C with altered surface chemistry following exposure to oxygen plasma. Alignment is quantified by measuring variation of the long-axis of each nucleus from the mean long-axis of all the nuclei. The graph on the left is a comparison in the variation in alignment (F-test), the graph on the right shows difference in average alignment (Kruskal-Wallis test with Dunn’s Multiple Comparison test).

The constructs appeared to be robust and durable. Experiments were performed within one week of fabrication of the constructs, however NRVM seeded 4 weeks following fabrication of G/H and H/H were also strictly aligned in the direction of the construct (Figure 6.3).
Figure 6.3. Bright field images of NRVM on Parylene-C constructs at x10 (left) and x40 magnification (right). Images A and B show NRVM cultured on non-structured hydrophilic Parylene-C seeded 1 week after production. Images C and D show structured H/H constructs seeded with NRVM 1 week after production. Images E and F show structured H/H constructs seeded with NRVM 4 weeks after production, suggesting that modification of the surface properties of Parylene-C with oxygen plasma is durable. Images G and H show structured G/H constructs seeded with NRVM 1
week after production. Images I and J show structured G/H constructs seeded with NRVM 4 weeks after production, also suggesting that modification of the surface properties of Parylene-C with oxygen plasma is durable.

6.3.1.2 Effect of Parylene-C constructs with modified hydrophobicity on neonatal rat ventricular myocyte calcium cycling

At 0.5Hz, 1Hz and 2Hz field stimulation, the F/F0 of the Ca$^{2+}$ transients were significantly larger in NRVM cultured on both the G/H and the H/H constructs when compared to NRVM on non-structured constructs (p<0.001 in all cases) (Figure 6.4 and Figure 6.5). There was no statistically significant difference in transient amplitude in NRVM cultured on H/H and G/H constructs at any frequency. TTP was significantly shorter in NRVM cultured on H/H constructs when compared to those cultured on non-structured (p<0.01) and G/H constructs (p<0.001) at 0.5Hz. Similarly TTP was significantly shorter in NRVM cultured on HH constructs compared to non-structured constructs at both 1Hz (p<0.05) and 2Hz (p<0.001) and compared to G/H constructs at both 1Hz (p<0.001) and 2Hz (p<0.001). The T90 was significantly less in NRVM cultured on the G/H constructs compared to non-structured controls at both 0.5Hz (p<0.001), 1Hz (p<0.001) and 2Hz (p<0.001). Similarly, the T90 was significantly less in NRVM cultured on the H/H constructs compared to non-structured controls at both 0.5Hz (p<0.001), 1Hz (p<0.001) and 2Hz (p<0.001). There was no difference between NRVM cultured on G/H and H/H constructs in T90 at any frequency. Interestingly although there was no difference in T50 between the three groups at 0.5Hz and 1Hz, both G/H (p<0.001) and H/H (p<0.001) had shorter T50s at 2Hz. There was no difference in T50 between the two structured constructs at any frequency (Figure 6.4 and Figure 6.5).
Figure 6.4. Representative Ca$^{2+}$ transients for NRVM beating at 0.5Hz, cultured on H/H Parylene-C substrates (orange), G/H Parylene-C substrates (yellow), or non-structured Parylene-C substrates (grey), for 4 days. Recordings were made with confocal microscopy in cells loaded with the Ca$^{2+}$ indicator Fluo-4 AM. A Ca$^{2+}$ transient recorded from an adult rat myocyte is shown for comparison (rust).
Figure 6.5. Effect of Parylene-C with altered surface chemistry following exposure to oxygen plasma on TTP, T50, T90, and F/F0 (Kruskal-Wallis test with Dunn’s Multiple Comparison test).
NRVM cultured on none of the three constructs showed any obvious amplitude-frequency relationship or decay-frequency relationship, suggesting that whilst structured cultures on Parylene-C with altered surface chemistry did have improved Ca$^{2+}$ cycling properties, the NRVM in the structured constructs did maintain an immature phenotype (Figure 6.6).

6.3.2 Three-dimensional constructs

6.3.2.1 The structure of neonatal rat ventricular myocytes cultured on three-dimensional polycaprolactone constructs

Evaluation of the structural characteristics of NRVM cultured on 3D PCL constructs was problematic because of the significant auto-fluorescence of the PCL fibers and a tendency of the monoclonal antibodies to adhere non-specifically to the PCL fibers (Figure 6.7). Despite their poor quality DAPI staining of sectioned constructs suggested that cells migrated
throughout the full depth of the construct (Figure 6.7). SEM suggested that cells became aligned in the direction of the PCL fibers (Figure 6.8).

**Figure 6.7.** Immunohistochemistry of micro-patterned NRVM. Red - Myosin Heavy Chain, Blue – DAPI, Scale Bar 100μm. There is significant autofluorescence from the PCL fibres, consequently it is difficult to discern the cell bodies.

**Figure 6.8.** SEM of NRVM seeded onto 3D PCL constructs, showing cardiomyocytes aligned in the direction of the PCL fibers. The cells have not formed a confluent monolayer.
6.3.2.2 The structure of neonatal rat ventricular myocytes cultured on decellularized myocardial slices

Figure 6.9. Second harmonic imaging microscopy showing a myocardial tissue slice. Green – autofluorescence from cardiomyocyte sarcomeric proteins. Purple – second harmonic signal from extracellular matrix collagen.
Figure 6.10. Second harmonic imaging microscopy showing decellularized myocardial tissue slice. Green – autofluorescence from cardiomyocyte sarcomeric proteins. Purple – second harmonic signal from extracellular matrix collagen.
Figure 6.11. Immunohistochemistry Z-stack of NRVM seeded onto a decellularized myocardial slice. Red – MHC, Green – α-SMA, Blue – DAPI. Each slice is 1µm thick. Scale Bar 100µm.
SHG was performed to characterize the structure of myocardial slices before and after decellularization (Figure 6.9 and Figure 6.10). It is apparent that whilst decellularization removed cellular membranes, quite a significant amount, if not most of the myocyte cytoskeletal proteins remained (Figure 6.9 and Figure 6.10). It was not possible to remove these proteins as prolongation of the decellularization protocol resulted in complete loss of structural integrity in the myocardial slices making it impossible to use them as tissue culture substrates as they could not be sterilized, manipulated, seeded or placed on Scafdex frames to perform experiments using the slices.

The failure of decellularization protocols was confirmed by immunohistochemistry which suggest that sarcomeric proteins were not removed. As a consequence, whilst NRVM were successfully cultured on the decellularized myocardial slices, they did not infiltrate beyond the surface of the myocardial slice (Figure 6.11). Immunofluorescence demonstrated no obvious alignment of the NRVM on the decellularized slices (Figure 6.11 and Figure 6.12).

Figure 6.12. Immunohistochemistry of NRVM seeded onto a decellularized myocardial slice. Green – α-SMA, Blue – DAPI.

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6.3.2.3 Effect of three-dimensional cultures on neonatal rat ventricular myocyte calcium cycling

The amplitude of Ca\(^{2+}\) transients were significantly increased in NRVM cultured on decellularized myocardial slices compared to non-structured PDMS when field stimulated at 0.5Hz, 1Hz and 2Hz (p<0.001 in all cases). They were also significantly larger than the amplitude of Ca\(^{2+}\) transients in NRVM cultured in 3D PCL constructs at 0.5Hz (p<0.001), 1Hz (p<0.001), and 2Hz (p<0.05). Although NRVM cultured in 3D PCL constructs had larger amplitudes than NRVM cultured in 2D at 2Hz (p<0.001), this was not significant at 0.5Hz and 1Hz (Figure 6.14). The TTP of NRVM cultured on decellularized myocardial slices were significantly reduced compared to non-structured PDMS at 0.5Hz (p<0.001), 1Hz (p<0.01), however not 2Hz. Similarly the TTP of NRVM cultured on decellularized myocardial slices were significantly reduced compared to NRVM cultured in 3D PCL constructs at 0.5Hz, 1Hz, and 2Hz (p<0.001 in all cases. The T50 and T90 of NRVM cultured on decellularized myocardial slices were significantly reduced compared to NRVM cultured on non-structured PDMS at all frequencies (p<0.001 in all cases). The T50 of NRVM cultured on decellularized myocardial slices were significantly reduced compared to NRVM cultured in 3D PCL constructs at 0.5Hz (p<0.05), 1Hz (p<0.01), and 2Hz (p<0.001). Similarly, the T90 of NRVM cultured on decellularized myocardial slices were significantly reduced compared to NRVM cultured in 3D PCL constructs at 2Hz (p<0.001). The T50 of NRVM cultured in 3D PCL constructs were significantly reduced compared to NRVM cultured on non-structured PDMS at all frequencies, and the T90 was significantly reduced when field stimulated at 0.5Hz and 1Hz (p<0.001 in all cases) (Figure 6.14 and Figure 6.15).
Figure 6.13. Representative Ca\textsuperscript{2+} transients from NRVM beating at 0.5Hz, cultured in a PCL fiber network (purple), on decellularized myocardial slices (pink), or non-structured 2D PDMS substrates (grey), for 4 days. Recordings were made with confocal microscopy in cells loaded with the Ca\textsuperscript{2+} indicator Fluo-4 AM. A Ca\textsuperscript{2+} transient recorded from an adult rat myocyte is shown for comparison (rust).
Figure 6.14. Effect of 3D constructs on TTP, T50, T90 and F/F0 (Kruskal-Wallis test with Dunn’s Multiple Comparison test).
NRVM cultured on non-structured PDMS and 3D PCL constructs showed no change in Ca$^{2+}$ transient amplitude with increasing frequency of field stimulation. Interestingly, NRVM cultured on decellularized slices demonstrated a positive amplitude-frequency relationship (p<0.001) (Figure 6.15). NRVM cultured on non-structured PDMS, PCL constructs and decellularized slices showed no obvious T50 decay-frequency relationship (Figure 6.15).

![Amplitude-Frequency Graph](image1) ![Decay-Frequency Graph](image2)

**Figure 6.15.** Amplitude-frequency and decay frequency of NRVM cultured on 3D constructs (Kruskal-Wallis test with Dunn’s Multiple Comparison test).

### 6.4 Discussion

#### 6.4.1 Parylene-C constructs

The data presented in this chapter suggests that plasma-treated Parylene-C constructs are durable, influence cell alignment and modify Ca$^{2+}$ handling. Interestingly the effect of structured Parylene-C constructs on the Ca$^{2+}$ cycling properties of NRVM was more marked than that of the microgrooved PDMS tissue culture substrates (Chapter 4.3.2.2), with the H/H construct in particular having comparable Ca$^{2+}$ cycling parameters to rat adult cardiomyocytes at some frequencies (Figure 6.4 and Figure 6.5). Whilst this is impressive, in
some respects the Ca\textsuperscript{2+} cycling parameters of NRVM remained immature, for example lacking the characteristic negative amplitude-frequency relationship which is observed in adult rat myocytes (Shattock and Bers, 1989) (Figure 6.6).

The reasons why the H/H Parylene-C constructs should more effectively modify the phenotype of NRVM than microgrooved tissue culture substrates is unclear. There was not a marked difference in cellular alignment between the constructs (compare Figure 6.2 with Figure 4.5). Substrate stiffness has been shown to have a marked effect on cellular phenotype (Engler et al., 2006, Palchesko et al., 2012, Tzvetkova-Chevolleau et al., 2008, Chou et al., 2009, Wang et al., 2011b, Shimazaki et al., 2008), and whilst it is possible that this could explain differences in the effectiveness of the H/H and the microgrooved tissue culture substrate this is unclear. If the observed differences were because of substrate stiffness it would be expected that the control group cultured on the Parylene-C construct would have better Ca\textsuperscript{2+} cycling properties than the control group cultured on non-structured PDMS. Comparison of the Ca\textsuperscript{2+} cycling properties of the control constructs in both experiments do appear to suggest that Ca\textsuperscript{2+} cycling was faster on the Parylene-C constructs (compare Figure 6.4 and Figure 6.5, with Figure 4.12 and Figure 4.13). However, the G/H construct which will have comparable stiffness to the H/H construct (and did in fact enforce stricter alignment on the NRVM than the H/H construct) was not markedly more effective than the microgrooved construct in terms of amplitude and TTP (although Ca\textsuperscript{2+} extrusion was probably faster). As the H/H construct appears to be more effective than both the microgrooved G/H construct and the microgrooved PDMS construct perhaps the grooved substrate, whilst beneficial in promoting cellular alignment, is in other respects detrimental. It could for example result in the cells sitting in the groves having less exposed sarcolemma,
and therefore a reduced $I_{\text{Ca,L}}$ and impaired CICR. Alternatively it may inhibit coupling of cells in the adjacent direction to the grooves. Ultimately measurement of the $I_{\text{Ca,L}}$ and conduction velocity would be required to resolving this question, both these measurements are feasible using the Parylene-C constructs.

The strength of the plasma-treated Parylene-C constructs that we describe is that they have the possibility of being generated on MEA dishes potentially making them a tool for high-throughput toxicology screening and drug development. It would then be easy to evaluate whether structured tissue culture substrates have an incremental benefit for these applications over existing non-structured cultures used in Chapter 3 (Section 3.2 and Section 3.3). Perhaps more interesting is the possibility that MEA technology in conjunction with other tools of cell physiology such as patch clamping and optical mapping can now be applied to these constructs to elucidate the mechanisms by which structure modifies the function of immature cardiomyocytes. Not only is this important in understanding myocardial development prenatally and in early life, this may have a role in understanding myocardial pathological mechanisms and possible mechanisms implicated in recovery. The loss of structure in the failing myocardium is a well-documented phenomenon (Spinale et al., 2013, Goldsmith et al., 2013), and there is evidence that adult myocytes have phenotypic plasticity (Ibrahim et al., 2012, Mann et al., 2012). Finally, whilst some studies have been successful in recapitulating aspects of the molecular phenotype associated with inherited cardiomyopathies (Carvajal-Vergara et al., 2010, Lan et al., 2013, Sun et al., 2012, Tse et al., 2013, Hick et al., 2013, Dick et al., 2013), many such studies have failed to demonstrate physiological changes associated with the molecular phenotype at a cellular level (Dick et al., 2013, Hick et al., 2013). This has worrying implications for the application of iPSC-CM as disease models. Pathological mechanisms elucidated and novel therapies suggested in the absence of a
cellular phenotype may be irrelevant when patients eventually become symptomatic. It is important to note that patients in NYHA Class I failure are asymptomatic (New York Heart Association. Criteria Committee, and New York Heart Association., 1979) despite having a clear physiological phenotype (Rostagno et al., 2000). The substrate that we have developed theoretically represents a good balance between a construct that promotes maturity, but also one in which physiological mechanisms can be comprehensively interrogated. Therefore, it would be interesting to investigate whether differences in cell physiology could be elucidated in diseases such as DMD using iPSC-CM where only a molecular phenotype has previously been described (Dick et al., 2013).

The H/H construct theoretically has the possibility to be used in stretch-induced maturation systems. Uniaxial stretch improves cardiomyocyte organization, increases cell length, increases density and length of myofilaments, increases mitochondrial density and increases contractile force (Fink et al., 2000, Tulloch et al., 2011). It is feasible that the addition of mechanical stimulation to the structural alignment seen in the H/H constructs would have an incremental benefit on cell maturity. Undoubtedly this would add a degree of complexity that would have to be justified by the incremental utility of this system. Uniaxial stretch has been combined with structured tissue culture substrates in order to fabricate models of mechanical overload (McCain et al., 2013). This study showed that cellular structure and function were adversely affected by cyclical stretch. They did not, however, investigate if reduced load in combination with structured tissue culture substrates would have a beneficial effect on cellular phenotype (McCain et al., 2013).
6.4.2 Three-dimensional constructs

6.4.2.1 Fabrication of electrospun constructs

The evaluation of the structural properties of NRVM cultured in the 3D PCL construct was problematic, because of the auto-fluorescence of PCL and the tendency of antibodies to adhere to it. Although not explicitly discussed this appears to be a problem in other studies describing PCL constructs in the literature based on the representative images that they report (Fleischer et al., 2013). Immunofluorescence, however, suggests that cells were distributed throughout the construct. SEM culture of the PCL constructs suggested that NRVM were aligned in the direction in the fibers. Despite the significant number of NRVM seeded (PCL constructs were seeded at double the density compared to myocardial slices, and four times the density of the non-structured 2D PDMS controls), the PCL constructs were not densely populated with NRVM to the extent that formation of a single beating syncytium would be unlikely (Figure 6.7 and Figure 6.8).

There was significant variability in the density with which electrospun constructs were seeded. Whilst in some studies the seeding density was significantly greater than in our experiments (Xing et al., 2012), in others however it was significantly less (Zong et al., 2005, Fleischer et al., 2013). Despite efforts to increase the seeding density until a confluent syncytium was fabricated, the use of more cells of any type (including NRVM) would not be cost-effective.

It is possible that a significant number of cells was lost through the construct, and whilst some constructs had reduced porosity (Zong et al., 2005), this was not the case when
comparing our constructs to electrospun constructs described in the literature (Xing et al., 2012, Papadaki et al., 2001). It is interesting, however that the constructs where fewest cells were used had the lowest porosity (Zong et al., 2005). Further development of PCL constructs is probably required to ensure that fibers are smaller and closer together to recapitulate the structure of endogenous myocardium (Stevens and George, 2005). Whether this can be achieved using these fabrication techniques without critically compromising the mechanical properties of the substrate or fabricating a very thick construct is uncertain.

6.4.2.2 Decellularized myocardial slices as scaffold.

Myocardial slices were only partially decellularized. Consequently NRVM did not penetrate throughout the construct, but formed a beating syncytium on the surface of the myocardial slice. NRVM seeded onto the myocardial slices did not appear to adopt any aspects of the structure of the underlying myocardium (Figure 6.11 and Figure 6.12).

There are several examples in the literature where tissue has been decellularized prior to re-seeding (Lu et al., 2013), however we were not able to successfully apply this technique to myocardial slices. Attempts were made to optimize the protocol, but this resulted in a non-structured gel. A critical difference between reports in the literature and the techniques that we adopted are that many of the studies in the literature perfused the tissue through a vascular arcade (Lu et al., 2013, Song and Ott, 2011). Perhaps this facilitates better delivery of decellularizing detergents in a homogenous fashion throughout the tissue. By contrast exposing myocardial slices to these agents in a shaker may result in sub-optimal delivery to the centre of the tissue, whilst the outside including extracellular matrix components are
completely degraded. Other investigators have successfully decellularized myocardial slices, however they are usually much thicker, and thus maintain their structural integrity despite being decellularized (Wang et al., 2010).

6.4.2.3 Functional properties of three dimensional constructs

The Ca\textsuperscript{2+} cycling properties of NRVM cultured on the partially decellularized slices were undoubtedly modified compared to the NRVM cultured on the control constructs. At most frequencies the TTP, T50 and T90 were faster compared to the controls and the F/F0 was larger. Whilst the NRVM cultured in the PCL fibers generally had shorter T50 and T90 than control NRVM, there was difference in the F/F0 and the TTP at 0.5Hz and 1Hz. At 2Hz NRVM in the PCL constructs had a significantly larger F/F0 but longer TTP (Figure 6.13 and Figure 6.14). None of the constructs had a mature decay-frequency or amplitude-frequency relationships.

NRVM cultured on decellularized slices had the Ca\textsuperscript{2+} handling properties most representative of adult myocardium. As is often the case with complex culture systems the factors underlying this change in phenotype are probably multi-factorial. The degree to which any of these constructs facilitate 3D cell-cell interaction is debatable with the small cell density in the PCL constructs and the NRVM essentially seeded on the surface of the myocardial slices. Consequently, the critical differences between the three constructs are the substrate stiffness and the ECM the NRVM were exposed to. Although the failure to fabricate a 3D PCL construct fully populated with cardiomyocytes is disappointing, it was important to demonstrate the feasibility and ease with which Ca\textsuperscript{2+} cycling studies can be performed using
PCL given the paucity of reports in the literature of functional characterization of cardiomyocytes cultured in these constructs.

Substrate stiffness has been shown to alter cardiomyocyte maturation (Forte et al., 2012) (Jacot et al., 2010a). Culture on stiff substrates disrupts the contractile apparatus (Jacot et al., 2010a). Cardiomyocytes cultured on softer substrates are more elongated, have a predictable connexin-43 distribution and increased expression of cell adhesion and ECM remodelling genes (Forte et al., 2012). When cardiomyocytes have been cultured on substrates mimicking the stiffness of the myocardium they have better aligned sarcomeres and generate more force (Forte et al., 2012). The design of our experiments was not really optimal to test the effect of substrate stiffness in a controlled fashion. This is probably most easily done using 2D constructs with varying stiffness (Palchesko et al., 2012), however spiral electrospun PCL have recently been described that could be adopted for this purpose (Fleischer et al., 2013). It is possible that the improved phenotype of NRVM cultured on the partially decellularized slices compared to PCL substrates and the flat PDMS substrates could at least in part be attributed to the substrate stiffness. Studies which suggest that modification of PCL substrates to reduce their stiffness may have a beneficial effect on cellular phenotype partially support this hypothesis (Fleischer et al., 2013).

ECM protein signaling is known to influence the function of cultured cardiomyocytes. All the PCL and PDMS constructs were coated with fibronectin before seeding. The NRVM seeded on the PCL constructs were, however, probably not exposed to endogenous myocardial ECM proteins to the same extent that NRVM cultured on the myocardial slices were. Laminin and collagen proteins in hydrogel preparations increase cardiomyocyte cell adhesion, cell spreading, maturity of the contractile apparatus and SERCA expression (LaNasa and Bryant,
An increase in ECM content of hydrogel has been shown to increase the differentiation of cardiomyocytes when compared to hydrogel with low ECM content or collagen alone (Duan et al., 2011). High ECM content also increased cardiac striations, up-regulation of connexin-43 expression and improved contractile function. As these studies suggest that many endogenous ECM proteins have a positive effect on the cellular phenotype of NRVM, it would be reasonable to assume that a partially decellularized native ECM may have a positive effect on the Ca\(^{2+}\) handling properties of cultured NRVM. Future studies will aim to characterize the ECM components of the partially decellularized slices, and their effects on cell structure and function.

In contrast with the previous experiments where alignment has been the predominant factor in modifying the phenotype of immature cardiomyocytes, these experiments demonstrate that ECM proteins and substrate mechanical properties are also significant, however unlike the results presented in Section 6.3.1 further work is required to quantify these effects before they can be applied to myocardial disease modelling, and experiments need to be designed which will specifically investigate the mechanisms underlying the observed changes in Ca\(^{2+}\) cycling properties. It may even be possible to apply substrate stiffness and ECM after further characterization to aligned constructs in order to incrementally enhance the phenotype of immature cardiomyocytes.
7 Discussion
7.1 “All models are wrong, but some are useful.”

The words of the great statistician George Edward Pelham Box are very resonant in every branch of science. His advice that, “….the practical question is how wrong do they [models] have to be to not be useful?” underlies the interpretation of almost every scientific experiment, study, or body of literature (Box and Draper, 1987).

When this work was started three years ago there was significant interest in utilizing iPSC-CM as human, patient-specific disease models. Several instances were subsequently published in which cellular disease phenotypes were replicated using iPSC-CM (Chapter 1.6.1 and Chapter 1.6.2). In some studies novel disease mechanisms were elucidated and novel therapies suggested (Chapter 1.6.1 and Chapter 1.6.2). The other major application that was suggested was the use of iPSC-CM as a high throughput drug development or toxicology screening tools. It is with these applications in mind that we undertook a focused comparative characterization of iPSC-CM with respect to hESC-CM and established disease models.

Even the much focused characterization undertaken in Chapter 3 demonstrates that with respect to the function of K⁺ channels and pacemaker mechanisms these cells exhibit an immature and heterogeneous phenotype. The immature heterogeneous phenotype of iPSC-CM is now widely accepted in the literature. Several studies, however, have demonstrated that, despite this phenotype, it is feasible to use iPSC-CM as toxicology screening tools (Harris et al., 2013, Liang et al., 2013, Braam et al., 2013). The heterogeneous phenotype of iPSC-CM was controlled in one study by classifying cells according to their APD and performing single-cell studies (Liang et al., 2013), other studies have controlled for this heterogeneity by using a highly purified population of commercially differentiated cells.
(Harris et al., 2013). Despite their immature phenotype, however, iPSC-CM have already arguably yielded insights into the mechanisms underlying hypertrophic cardiomyopathy (Lan et al., 2013), and have been used to suggest novel therapy for cardiac arrhythmias (Matsa et al., 2011).

Does then in vitro modelling with iPSC-CM pass the “Box” test? Are iPSC-CM a useful tool despite the limitations that make them unrepresentative of adult myocardium in many respects? The answer is a cautious and qualified yes; iPSC-CM are a useful addition to the toolbox of scientific techniques that can be used to investigate the mechanisms underlying cardiovascular disease and therapies. Their immature, heterogeneous phenotype means that in many instances they are not representative of adult myocardium. However, as the multitude of experimental studies which show their feasibility as disease models or toxicology screening tools demonstrate these problems can be overcome. As a consequence of the limitations of in vitro iPSC-CM modelling, experimental results need to be triangulated with data from other cellular and animal models. In this respect however, iPSC-CM are no different from many of the most widely used tools in cardiovascular research, such as transgenic rodent models which also need to be triangulated with human or large-animal data.

### 7.2 Structured Tissue Culture Constructs

Whilst iPSC-CM are unquestionably useful tools in cardiovascular research there is still scope to further improve their utility. In several instances only a limited cellular phenotype or none at all has been elucidated using iPSC-CM derived from patients with inherited cardiomyopathies (Hick et al., 2013, Dick et al., 2013). The single-cell genetic (Lan et al., 2013) and electrophysiological assays (Liang et al., 2013) used to control for the
heterogeneous immature phenotype of iPSC-CM are arguably time consuming and poorly suited for high-throughput screening. Furthermore, there may also be some circumstances when iPSC-CM cannot be used to model myocardial disease because of differences between the structure of iPSC-CM cultures and adult myocardium (Wang et al., 2013).

These limitations with iPSC-CM, which stem from their immature heterogeneous phenotype, became evident to us during the experiments that we describe in Chapter 3. Consequently, we embarked on experiments to modify the phenotype of iPSC-CM. After validation of the methodology required to align immature cardiomyocytes in Chapter 4, we demonstrated in Chapter 5 that the phenotype of iPSC-CM could be modified using structured PDMS tissue culture substrates. In particular the speed of Ca\textsuperscript{2+} cycling was modulated by cellular alignment. Experiments to characterize the mechanisms underlying these changes suggest that the modulation of SR function may be an important consequence of structured tissue culture. The finding that the cellular phenotype of iPSC-CM was modulated using structured culture was a novel finding in the literature (Rao et al., 2012). Other investigators have subsequently shown that different aspects of the cellular phenotype are also be modulated in structured iPSC-CM cultures (Wang et al., 2013).

Interestingly, we also found that the response of NRVM and iPSC-CM to structured culture differed. The different response of NRVM and iPSC-CM to culture on structured tissue culture substrates highlights firstly, the necessity for a human myocardial cellular model. Secondly, it represents a note of caution in how the large body of literature on tissue engineering using NRVM should be interpreted. There are a paucity of studies in the literature which directly compare iPSC-CM and NRVM for tissue engineering applications,
and the different response of NRVM and iPSC-CM Ca\textsuperscript{2+} cycling to structured culture is to our knowledge a novel finding in the literature (Rao et al., 2012).

### 7.3 Development of Tissue Engineering Constructs and Suggestions for Further Work

A limitation of PDMS tissue culture substrates was the difficulty of investigating the cellular phenotype of cultured iPSC-CM to the extent that the mechanisms causing changes in Ca\textsuperscript{2+} cycling in iPSC-CM could not be interrogated to our satisfaction using the techniques available to us. This was the catalyst to develop a novel Parylene-C construct that could be fabricated on commercially available MEA dishes or glass coverslips suitable for optical mapping and cell electrophysiology using an inverted microscope. It was also hoped that such Parylene-C constructs would be suitable for experiments in which uniaxial stretch could be combined with cell-alignment.

We were also interested to develop a construct that would be suitable for optical mapping and MEA analysis that would also facilitate 3D cell-cell interaction. Consequently, in addition to electrospun 3D PCL constructs, two novel tissue engineering constructs were characterized in Chapter 6. The data presented in Chapter 6 demonstrates that Parylene-C tissue culture substrate successfully aligned immature myocytes and effectively modulated their Ca\textsuperscript{2+} cycling properties. Neither of the 3D constructs were suitable for assessment of whether 3D cell interaction had an effect on function. This was because the NRVM cultured in the PCL constructs did not form a syncytium and we were unable fully decellularize the tissue slices, and consequently the NRVM were cultured as a monolayer on the surface.
An obvious next step would be to use the novel Parylene-C constructs described in Chapter 6 with both NRVM and iPSC-CM to explore both the mechanisms underlying modulation of cellular phenotype caused by cell alignment, and the mechanisms underlying differences in the response of iPSC-CM and NRVM to structured tissue culture.

It has recently been shown that structured iPSC-CM cultures possibly have a role in modelling re-entry arrhythmias (Wang et al., 2013). A critical next step, however, will be to demonstrate that tissue engineering approaches, such as the ones we describe in Chapters 4-6, have a wider role in myocardial in vitro modelling. In particular it would be interesting to investigate if structured culture results in the demonstration of a cellular phenotype in pathological iPSC-CM cell lines in which no clear cellular phenotype has previously been demonstrated (Hick et al., 2013, Dick et al., 2013). It would also be interesting to investigate if structured culture improves the utility of multi-cellular toxicology screening assays.

It is not clear whether the 3D constructs that we attempted to optimize in Chapter 6 will ever realize their theoretical potential. They are not currently suitable for fabrication without specialist skills and knowledge. It is important that tissue engineering systems continue to be developed and optimized which promote the degree to which iPSC-CM are representative of adult myocardium, whilst also allowing the cellular function of cultured cells to be readily interrogated. Ultimately the techniques for fabrication of 3D constructs that we describe in Chapter 6 may not be the most effective way to achieve this and it is possible that they may be superseded by more sophisticated multi-modal tissue engineering approaches that combine electrical, mechanical and structural cues with integrated mechanisms for electromechanical interrogation (Rao et al., 2013, Boudou et al., 2012).
Finally, it can be anticipated that experimental results from *in vitro* disease modelling using iPSC-CM will be increasingly applied to clinical problems. As tissue engineering approaches become increasingly sophisticated, and more widely applied to disease modelling, it is important to establish fabrication standards to ensure robust and reproducible experimental results (Nawroth and Parker, 2013). It may also be useful, in the context of the rapid proliferation of tissue engineering approaches for industry, bio-medical and engineering stakeholders to discuss consensus guidance on critical cellular assays that tissue engineering approaches must facilitate.

### 7.4 Conclusions

In this thesis we highlight the limitations of iPSC-CM and hESC-CM as *in vitro* disease models. This work suggests that existing efforts to modify the phenotype of NRVM by systematic modification of culture conditions using tissue engineering approaches are likely to be relevant to *in vitro* modelling with iPSC-CM. This work also suggests that the effect that these techniques will have on iPSC-CM may differ subtly from NRVM experimental results. An important next step will be to demonstrate that these techniques improve the efficacy of iPSC-CM for myocardial disease modelling. Finally, tissue engineering techniques must continue to be developed that increase the degree to which iPSC-CM are representative of adult myocardium, whilst also allowing the cellular phenotype to be readily interrogated.
Appendices

Appendix A - Fabrication of structured silicone culture substrates

Structured flexible microgrooved silicone culture substrates were fabricated using PDMS, a biologically inert non-toxic silicon-based organic polymer (McDonald and Whitesides, 2002). PDMS is used extensively in contact lenses, medical devices, cosmetics, foods, and is often used as scaffold material for tissue engineering as its mechanical properties can be manipulated to resemble physiological values of human tissue. This is important as substrate elasticity has been shown to affect cellular functions such as adhesion, proliferation, migration and gene expression (Brown et al., 2005, Gray et al., 2003, Forte et al., 2012).

Micro-grooved PDMS scaffolds were produced using standard photolithography rules, as previously described (Figure A.1) (Isenberg et al., 2008). Briefly, these were generated by spinning SU-8, an epoxy-based negative photoresist polymer onto monocrystalline silicon wafers. The photoresist was then exposed to ultraviolet (UV) light (365nm) through a patterned high-resolution transparency glass mask containing areas with parallel grooves. They were then developed using 100% ethyl lactate. The result was SU-8 moulds with 22 patterned circular areas 14mm in diameter, with parallel lines etched into them. The SU-8 moulds contained four different patterned areas. Two patterned areas consisted of grooves 20µm apart, 20µm wide and either 3.75µm or 4.5µm deep. Similarly, two patterned areas were fabricated with grooves 10µm apart, 10µm wide and either 3.75µm or 4.5µm deep.

PDMS was prepared by mixing a pre-polymer and a curing agent (Sylgard 184 Kit, Dow Corning), and then a degassing process was applied to remove any gas bubbles. PDMS was casted onto the SU-8 mould. PDMS was then cured at 70°C for 1 hour before each PDMS
construct was carefully cut from the master mould. The individual circular patterned areas on the constructs were then removed using a 3/4 inch carpentry punch to fit into the bottom of a 12-well plate and a 9/16 inch carpentry punch to fit into the bottom of a 24-well plate. The larger constructs (3/4 inch) were more suitable for electrophysiological applications as the unpatterned area around the structured area reduced the volume of the bath, and consequently also reduced the volume of solution required to superfuse the constructed, facilitating faster changes of solution, and more accurate regulation of experimental temperature. The smaller constructs (9/16 inch) were used for imaging and molecular biological applications as they required fewer cells to be seeded and there was no unstructured area surrounding the structured area, thus preventing confounding of gene and protein expression studies by pooling cells from structured and unstructured areas. Unstructured PDMS tissue culture substrates were constructed by simply inverting the structured PDMS culture substrates fabricated as previously described. This ensured that the structured and unstructured constructs had the same stiffness. The constructs were then sterilized by immersion in 70% ethanol and then exposure to UV light overnight. The following day the constructs were rinsed three times with sterile water, coated with 50μg/ml human-plasma fibronectin (Sigma-Aldrich) and then left for at least four hours prior to plating of the cells. Excess fibronectin was removed immediately prior to plating of the cells. To ensure similar plating densities, 2/3 million NRVM were seeded into each well in the 12-well plate and 1/3 million i-cell cardiomyocytes were seeded in each well in the 12-well plate as i-cell cardiomyocytes had greater plating efficiency and contained a far greater proportion of cardiomyocytes (over 95% Purity). Similarly 1/3 million NRVM were seeded into each well in the 24-well plate and 1/6 million i-cell cardiomyocytes were seeded in each well in the 24 well plate.
Photolithography and PDMS molding was performed by Miss Tatiana Trantidou and Dr Themistoklis Prodromakis at the Centre for Bio-Inspired Technology, Imperial College London.
Appendix B - Fabrication of Parylene-C constructs

Parylene-C is the trade name for a group of poly(chloro-para-xylylene) polymers formed by chemical vapour deposition (Tan and Craighead, 2010). Parylene-C in particular is widely used as a coating for printed circuit boards and medical devices such as guide-wires, intra-arterial stents, implantable defibrillators and pacemakers (Khan et al., 2008, Loeb et al., 1977, Fontaine et al., 1996). It is insoluble at room temperature, has excellent barrier properties and is pinhole-free above 100nm, is biologically inert, inexpensive, and easily processed (Khan et al., 2008, Loeb et al., 1977, Fontaine et al., 1996, Tan and Craighead, 2010). Furthermore, its mechanical properties mean it can be used in flexible devices, for example, implantable-flexible chemical sensing arrays (Zoumpoulidis et al., 2009) or pressure sensors for measuring intraocular pressure in glaucoma patients (Chen et al., 2008). These properties may make Parylene-C suitable as a thin tissue culture substrate, which would facilitate live-cell microscopy, and in which electrical and chemical sensors can readily be integrated. The flexible properties of Parylene-C may also potentially allow the application of continuous or cyclical mechanical strain to cultures which has also been shown to facilitate promote cell maturation in some studies (Fink et al., 2000, Tulloch et al., 2011, Gwak et al., 2008).

Despite the ideal chemical and biological properties of Parylene-C, a major obstacle to its application as a tissue culture substrate is its hydrophobic surface properties. Several approaches have been adopted to modify its surface properties. In particular, several groups have sought to exploit the poor oxidative resistance of Parylene-C (~115 °C) (Fortin and Lu, 2001), which mean that its properties can be readily altered by oxygen plasma fabrication.
techniques. Oxygen plasma treatment has been previously employed to effectively modify the surface properties of Parylene-C, in order to facilitate its use as a tissue culture substrate (Chang et al., 2007, Hoshino et al., 2012), and the surface properties of Parylene-C have previously been successfully modified in order to fabricate neural cell cultures with specific patterns (Delivopoulos et al., 2010).

Two Parylene-C constructs were fabricated using oxygen plasma treatment using techniques previously described (Trantidou et al., 2012). In both cases glass coverslips 25mm in diameter were thoroughly degreased in acetone, isopropanol and deionized water, and dehydrated for 60s at 90ºC. They were then coated in Parylene-C films by chemical vapour deposition, using a commercially available coater (PDS2010, Specialty Coating Systems) by vaporizing (150ºC) and then pyrolising Parylene-C dimer (690ºC). Three runs were carried out, facilitating different thicknesses of 1µm and 7µm. The film thickness was determined through calibration samples where Parylene-C was selectively removed to create a step that was measured by a Veeco Dektak Stylus profiler (Figure B.1).

Parylene-C is ordinarily hydrophobic, and cells and tissues do not readily adhere to it; however, we sought to modify the surface properties using oxygen plasma (Tan and Craighead, 2010) (Figure B.1). A contact mask was fabricated using hexamethyldisilazane (HMDS) (Sigma-Aldrich) which was spin-coated on the Parylene-C-coated coverslips, succeeded by a 1.4µm thick positive photoresist (AZ5214, Clariant SE). The samples were then soft baked on a hotplate at 90ºC for 60s, exposed to UV light for 60s, and developed in AZ400K (Clariant SE) Developer and H₂O (1:4) to remove the exposed photoresist. The Parylene-C constructs were then treated with oxygen plasma to achieve selective hydrophobic patterning, with most of the Parylene-C protected from the oxidative effects of
the oxygen plasma by the contact masks. An ultra-high purity plasma etcher (Nano UHP) was used at a working pressure of 0.8mbar. Before exposure to oxygen plasma, samples were prebaked on a hotplate at 110°C for 60s to harden the protective photoresist mask. Several different patterns of exposed areas were tried; however, we focused on parallel lines 10µm wide and 10µm apart as previous experiments (See Chapter 4) and other investigators (Boudou et al., 2012) had shown this geometry effectively altered the physiological properties of immature cardiomyocytes (Figure B.1).

Figure B.1. Schematic diagram, not drawn to scale, illustrating the fabrication of Parylene-C constructs
After plasma treatment, the remaining photoresist was removed by immersing the constructs into acetone, isopropanol and deionized water. Short exposure of the 7μm thick Parylene-C films to oxygen plasma at 50W for 1min altered the surface properties of the Parylene-C without significant etching or altering its geometry, resulting in alternating hydrophobic and hydrophilic areas which will subsequently be referred to as the Hydrophobic-Hydrophilic (H/H) construct. Exposure of the 1μm thick Parylene-C constructs at 400W for 15min resulted in the exposed areas of Parylene-C being completely removed. This resulted in alternating glass and Parylene-C areas and a construct with similar geometry in the case of
the 4µm Parylene-C film to the previously described PDMS construct (see Section 2.2.1). This construct will subsequently be referred to as the Glass- Parylene-C (G/H) construct. Unstructured glass and Parylene-C coated areas were used as controls (Figure B.2).

Figure B.3. Scale Bar 20µm, GFP tagged fibronectin in green, dashed line indicates cross-section shown above. A) Hydrophilic glass (10µm) - hydrophobic Parylene-C (10µm), Groves depth ~1.1µm, Treated with plasma at 400W for 16min. B) Hydrophilic Parylene-C (10µm) - hydrophobic Parylene-C (10µm), Groves depth ~0.08µm, Treated with
plasma at 400W for 1min. C) Hydrophilic Parylene-C (10μm) - hydrophobic Parylene-C (10μm), Groves depth <0.01μm, Treated with plasma at 50W for 1min. D) Hydrophilic Parylene-C (10μm) - hydrophobic Parylene-C (10μm), Groves depth ~0.08μm, Treated with plasma at 400W for 1min, 8 weeks previously.

To assess the stability of oxygen plasma fabricated Parylene-C constructs we seeded them at 1 and 4 weeks after fabrication. Parylene-C has poor UV stability and yellows with regular exposure to sunlight (Fortin and Lu, 2001), consequently the constructs were not sterilized as previously described with UV light (Section 2.2.1). Prior to seeding these constructs were sterilized by emersion in 100% ethanol. The ethanol was left for at least 1 hour and then removed. The constructs were then washed with sterile phosphate buffered solution (PBS). They were then coated in 5μg/ml fibronectin (Sigma-Aldrich) and stored in an incubator (37ºC, 5%) until evaporated. Immunohistochemistry suggests that the geometry of the Parylene-C constructs did not exactly replicate the geometry of the contact photoresist, with the hydrophilic areas being slightly less than 10µm (Figure B.3). 2x10^6 cells were seeded on each construct. All seeded constructs were then incubated. Complete medium was changed every other day. All experiments were performed at day 4 post-seeding.

Oxygen plasma-treated structured Parylene-C constructs were fabricated by Tatiana Trantidou with the assistance of Themistoklis Prodromakis at the Centre for Bio-Inspired Technology, Imperial College London.
Appendix C - Fabrication of three-dimensional polycaprolactone constructs

PCL is biodegradable polyester. It is degraded by hydrolysis of its ester linkages in physiological conditions without changing the pH or releasing toxins into surrounding tissues. Consequently, it has been approved by the Food and Drug Administration for use in suture material, adhesion barriers and drug delivery devices (Zong et al., 2005, Labet and Thielemans, 2009).

A detailed description of the fabrication of PCL tissue culture scaffolds has previously been published (Zong et al., 2002, Jeun et al., 2005). Briefly however, PCL granules (PCL MW 40000, Solvay) were dissolved in chloroform at a concentration 0.1mg/ml. Chloroform and the solution was injected through a fine needle (diameter 0.56mm) under pressure (6.5bars) onto a collecting drum rotating at 3000rpm. The distance between the pump and the rotating

Figure C.1. Schematic diagram, not drawn to scale illustrating the fabrication of 3D PCL constructs
drum was 30 cm (Figure C.1). The resulting structure was formed from fibers 300-1100 nm in diameter, 100-2000 nm apart, with greater than 95% porosity. The solvent was left to evaporate from the fabricated constructs. They were then washed several times to ensure all the solvent was removed. The constructs were then stretched over a Scaffdex ‘cell crown’ (Scaffdex) and stored in 70% ethanol until required. Prior to seeding they were washed with sterile PBS several times, placed under ultraviolet light for 8 hours and then inserted into a 24-well plate (Figure C.2). 1 million NRVM were seeded onto each construct.

Three Dimensional Polycaprolactone (PCL) constructs were fabricated by Jermone Sohier, at the Harefield Heart Science Centre Tissue Engineering Group, Imperial College London.


FORTE, G., PAGLIARI, S., EBARA, M., UTO, K., TAM, J. K., ROMANAZZO, S.,
ESCOBEDO-LUCEA, C., ROMANO, E., DI NARDO, P., TRAVERSA, E. &
AOYAGI, T. 2012. Substrate Stiffness Modulates Gene Expression and Phenotype in
Neonatal Cardiomyocytes In Vitro. Tissue Eng Part A.

FORTIN, J. B. & LU, T. M. 2001. Ultraviolet radiation induced degradation of poly-para-

Na+/Ca2+ exchanger is a determinant of excitation-contraction coupling in human
embryonic stem cell-derived ventricular cardiomyocytes. Stem Cells Dev, 19, 773-82.

FU, J. D., LI, J., TWEEDIE, D., YU, H. M., CHEN, L., WANG, R., RIORDON, D. R.,
of the sarcoplasmic reticulum in the developmental regulation of Ca2+ transients and
contraction in cardiomyocytes derived from embryonic stem cells. FASEB J, 20, 181-3.

GAI, H., LEUNG, E. L., COSTANTINO, P. D., AGUILA, J. R., NGUYEN, D. M., FINK, L.
cardiomyocytes using induced pluripotent stem cells derived from human fibroblasts.
Cell Biol Int, 33, 1184-93.

GARG, V., KATHIRIYA, I. S., BARNES, R., SCHLUTERMAN, M. K., KING, I. N.,
BUTLER, C. A., ROTHROCK, C. R., EAPEN, R. S., HIRAYAMA-YAMADA, K.,
JOO, K., MATSUOKA, R., COHEN, J. C. & SRIVASTAVA, D. 2003. GATA4
mutations cause human congenital heart defects and reveal an interaction with TBX5. 


HATTORI, F., CHEN, H., YAMASHITA, H., TOHYAMA, S., SATOH, Y. S., YUASA, S., LI, W., YAMAKAWA, H., TANAKA, T., ONITSUKA, T., SHIMOJI, K., OHNO, Y., EGASHIRA, T., KANEDA, R., MURATA, M., HIDAKA, K., MORISAKI, T.,


IGELMUND, P., FLEISCHMANN, B. K., FISCHER, I. R., SOEST, J., GRYSHCHENKO,
potential propagation failures in long-term recordings from embryonic stem cell-
derived cardiomyocytes in tissue culture. Pflugers Arch, 437, 669-79.

INOUE, H. & YAMANAKA, S. 2011. The use of induced pluripotent stem cells in drug

ISENBERG, B. C., TSUDA, Y., WILLIAMS, C., SHIMIZU, T., YAMATO, M., OKANO,
engineering with defined structural organization. Biomaterials, 29, 2565-72.

cardiac graft using a degradable scaffold with an extracellular matrix-like topography.
J Thorac Cardiovasc Surg, 130, 1358-63.

ISOMOTO, S., SHIMIZU, A., KONOE, A., KAIBARA, M., CENTURION, O. A.,
class III antiarrhythmic agent, in patients with supraventricular tachyarrhythmias. Am
J Cardiol, 71, 1464-7.

ITZHAKI, I., MAIZELS, L., HUBER, I., GEPSTEIN, A., ARBEL, G., CASPI, O., MILLER,
catecholaminergic polymorphic ventricular tachycardia with patient-specific human-
induced pluripotent stem cells. J Am Coll Cardiol, 60, 990-1000.


KAMAKURA, T., MAKIYAMA, T., SASAKI, K., YOSHIDA, Y., WURIYANGHAI, Y., CHEN, J., HATTORI, T., OHNO, S., KITA, T., HORIE, M., YAMANAKA, S. &


KOLOSOV, E., LU, Z., DROBINSKAYA, I., GASSANOV, N., DUAN, Y., SAUER, H.,
MANZKE, O., BLOCH, W., BOHLEN, H., HESCHELER, J. & FLEISCHMANN, B.
K. 2005. Identification and characterization of embryonic stem cell-derived

and induced pluripotent stem cells for myocardial repair: insights from experimental

KRAEHENBUEHL, T. P., ZAMMARETTI, P., VAN DER VLIES, A. J.,
SCHOENMAKERS, R. G., LUTOLF, M. P., JACONI, M. E. & HUBBELL, J. A.
2008. Three-dimensional extracellular matrix-directed cardioprogenitor
differentiation: systematic modulation of a synthetic cell-responsive PEG-hydrogel.
Biomaterials, 29, 2757-66.

KRISHNAMURTHY, G., PATBERG, K. W., OBREZTCHIKOVA, M. N., RYBIN, A. V. &
ROSEN, M. R. 2004. Developmental evolution of the delayed rectifier current IKs in
canine heart appears dependent on the beta subunit minK. Heart Rhythm, 1, 704-11.

KRUPNICK, A. S., KREISEL, D., ENGELS, F. H., SZETO, W. Y., PLAPPERT, T.,
model of left ventricular tissue engineering. J Heart Lung Transplant, 21, 233-43.

KUBALAK, S. W., MILLER-HANCE, W. C., O'BRIEN, T. X., DYSON, E. & CHIEN, K.


NEW YORK HEART ASSOCIATION. CRITERIA COMMITTEE. & NEW YORK HEART ASSOCIATION. 1979. *Nomenclature and criteria for diagnosis of diseases of the heart and great vessels*, Boston, Little, Brown.


UENO, S., WEIDINGER, G., OSUGI, T., KOHN, A. D., GOLOB, J. L., PABON, L.,
REINECKE, H., MOON, R. T. & MURRY, C. E. 2007. Biphasic role for Wnt/beta-
catenin signaling in cardiac specification in zebrafish and embryonic stem cells. *Proc
Natl Acad Sci U S A*, 104, 9685-90.

VAN LAAKE, L. W., PASSIER, R., DEN OUDEN, K., SCHREURS, C., MONSHOUWER-
KLOOTS, J., WARD-VAN OOSTWAARD, D., VAN ECHTELD, C. J.,
DOEVENDANS, P. A. & MUMMERY, C. L. 2009. Improvement of mouse cardiac
function by hESC-derived cardiomyocytes correlates with vascularity but not graft

VAN LAAKE, L. W., PASSIER, R., MONSHOUWER-KLOOTS, J., VERKLEIJ, A. J.,
LIPS, D. J., FREUND, C., DEN OUDEN, K., WARD-VAN OOSTWAARD, D.,
KORVING, J., TERTOOLEN, L. G., VAN ECHTELD, C. J., DOEVENDANS, P. A.
& MUMMERY, C. L. 2007. Human embryonic stem cell-derived cardiomyocytes
survive and mature in the mouse heart and transiently improve function after

VAN PETEGEM, F. 2012. Ryanodine receptors: structure and function. *Journal of
Biological Chemistry*, 287, 31624-32.

receptor open probability alone does not produce arrhythmogenic calcium waves:
threshold sarcoplasmic reticulum calcium content is required. *Circ Res*, 100, 105-11.


ZOUMPOULIDIS, T., PRODROMAKIS, T., VAN ZEIJL, H., MICHELAKISY, K., BARTEK, M., TOUMAZOU, C. & DEKKER, R. Stretchable array of ISFET devices

“I am all for stem cell research”

Stevie Wonder at the BBC

30th January 1990