From cell to muscle: development of new ways to study inherited cardiomyopathies based on engineered heart tissue

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Thesis submitted for the degree of Doctor of Philosophy

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Abstract
The ACTC E99K mutation is associated with hypertrophic cardiomyopathy (HCM- predominantly apical) and left ventricular non-compaction in a large group of patients from Northwest Spain. The aim of this thesis is to set up a new way of characterising patient phenotypes combining the technologies of engineered heart tissue and iPSCs. Punch biopsies were taken from 18 individuals (14 mutation carriers and 4 related non-carriers), fibroblasts were expanded, and 6 samples (2 mutation carriers and one healthy relative each from two different families) were taken forward for Sendai virus reprogramming. Five fibroblast lines were successfully reprogrammed, which was confirmed by staining for pluripotency markers. To make isogenic pairs of cells CRISPR-Cas9 PiggyBac gene editing was used to correct the E99K mutation in two disease carrying lines, and the mutation was knocked into one wild type line. We also analysed sudden cardiac death in the ACTC1 E99K mouse model. Higher rates of SCD were seen in enriched CBA/Ca mice, but SCD was almost absent in pure black6 mice. Using two photon microscopy, pico-sirius red staining, and gene expression we showed up regulation of fibrosis in young SCD mice. In addition, pure black6 mice were also found to have the same number of calcium release events as wild type. Pluripotency was confirmed after targeting of iPSCs, and differentiation into cardiomyocytes was achieved in all six lines: Donor I E99K, Donor I WT, Donor II E99K, Donor II WT, Donor III WT, Donor III E99K. The presence of the mutation in iPSC-CM was confirmed with an antibody specific to the ACTC E99K mutation. Donor I WT line had significantly fewer cardiomyocytes which affected the subsequent experiments. Under stimulation Donor II E99K and Donor III E99K had significantly longer relaxation times than wild type isogenic pairs. Isogenic pairs were treated with increasing concentrations of calcium Donor II E99K and Donor I E99K
were hypercontractile compared to Donor III WT, however, this hypercontractility was not restored in the Donor II line when the mutation was corrected to wild type. Moreover, hypercontractility was not induced in the Donor III line with the E99K mutation knocked in compared to its wild type isogenic line. Lastly, under stimulation we also found a higher rate of abnormal arrhythmogenic like contractions in Donor II E99K and Donor III E99K compared to their wild type isogenic pairs. iPSC-CM with the E99K mutation are a useful model to understand HCM and have been shown to recapitulate some aspects of the human disease. Data suggest that poor relaxation and arrhythmic events are directly related to the mutation, while hypercontractility may be related to a co-inherited genotype or epigenetic change.
Acknowledgements

I would like to thank my supervisors Steve Marston and Sian Harding for giving me the opportunity to study and learn in their labs, for putting up with my questions, my sometimes pig-headed attitude, and allowing me to wear some truly awful outfits around the lab. I am grateful their support throughout and for their instruction and advise, and for their openness to talk to a PhD student at almost any time. I would also like to thank all the members of Sian and Steve’s labs for and helping with experiments. Thusharika was very helpful in making more and more cells every time I came to her to make EHTs. Nicola was very nice, useful in the lab and for talks about life as a postdoc, and allowed me to work on some of her interesting studies. Gabor may be as old as any postdoc but his humour made lab life enjoyable. O’Neal and Andrew made life fun in Steve’s lab and I was sad to see them leave they are true experts in heart muscle work.

I would also like to thank Chris Denning his Nottingham lab and specifically James Smith. James is an expert in cell culture and without his work the iPSC work in this thesis would not have happened. He made CRISPR lines and produced many millions of iPSC-CM, and is still working on this project at the time of writing.

I would like to thank Imperial College London for allowing me to take up this study and providing excellent if sometimes a little leaky labs. This work was funded by the British Heart foundation and without their excellent financial support this work could not have taken place.

To my friends in the lab who truly made work fun, who shared the hard and good times, I could not write down all the ways in which you’ve helped and the time we have shared. Lastly, I would like to thank my family and God for always being there for me.
Declaration of Originality and copyright declaration

The work presented is my own unless otherwise stated.

The original idea of the study was conceived and developed by Professors Sian Harding and Steven Marston who helped with experimental ideas and practical advice throughout the project.

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Publications
Abbreviations
2D – two dimensional
3D – three dimensional
ANP – atrial natriuretic peptide
APS – ammonium persulfate
ATP – adenosine triphosphate
BDM – 2,3-Butanedione monoxime
BNP – brain natriuretic peptide
Ca$^{2+}$ – calcium ions
CaAβ – calcineurin Aβ
CamKII - the Ca$^{2+}$/calmodulin-dependent protein kinase
Cas9 – CRISPR associated protein 9
CM – cardiomyocyte
Col – collagen
CRISPR – clustered regularly interspaced short palindromic repeats
CTD – calcium transient duration
cTnI – cardiac Troponin I
CV – contraction velocity
DADs – delayed after depolarisations
DAPI – 4’,6-diamidino-2-phenylindole
DBP – diastolic blood pressure
DCM – dilated cardiomyopathy
DMEM – Dulbecco’s modified eagle medium
DNA – deoxyribonucleic acid
DSB – double strand breaks
E99K – glutamine to lysine change at codon 99
EDP – end diastolic pressure
EDV – end diastolic volume
EMD 57033 – (+)-5-(1-(3,4-Dimethoxybenzoyl)-1,2,3,4-tetrahydroquinolin-6-yl)-6-methyl-3,6-dihydro-2H-1,3,4-thiadiazin-2-one
EHT – engineered heart tissue
Epac - Exchange Protein directly Activated by cAMP
ESPVR – end systolic pressure volume relationship
ESV – end systolic volume
FBS – fetal bovine serum
FCS – fetal calf serum
FDHM – full duration at half maximum
FFR – force frequency relationship
FWHM – full width at half maximum
G2 – gap phase one of the cell cycle
GAPDH – Glyceraldehyde 3-phosphate dehydrogenase
HCM – hypertrophic cardiomyopathy
HDR – homology directed repair
hEHT – human engineered heart tissue
HBSS – Hank’s balanced salt solution
HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
iPSCs – induced pluripotent stem cells
ITR – inverted terminal repeats
LAP – left atrial pressure
LOX – lysyl oxidase
LVNC – left ventricular non compaction
LVWT – left ventricular wall thickness
MAPK – mitogen activated protein kinases
MHC – myosin heavy chain
mm – millimetre
mN – millinewton
NHEJ – non homologous end joining
OCT – optimum cutting temperature
P – postnatal day
PDMS – polydimethylsiloxane
PES – end systolic pressure
PLN - phospholamban
PV loop – pressure volume loop
rEHT – rat engineered heart tissue
RNA – ribonucleic acid
RV – relaxation velocity
RyR – ryanodine receptor
S – synthesis phase of the cell cycle
SBP – systolic blood pressure
SCD – sudden cardiac death
SDS-PAGE – sodium dodecyle sulfate polyacrylamide gel electrophoresis
SERCA2a – sarco/endoplasmic reticulum Ca\(^{2+}\) ATPase
SL – sarcomere length
SMAD – portmanteau of Small body size (Caenorhabditis elegans) and Mothers Against Decapentaplegic (Drosophila)
SOCK – The Yamanaka’s factors: Sox2, Oct4, C-myc and Klf4
SR – sarcoplasmic reticulum
ssTnI – slow skeletal Troponin I
SV – stroke volume
T1 – contraction time
T2 – relaxation time
TEMED – N,N,N’,N’ tetramethylethylenediamine
TG – transgenic
TGF-β – transforming growth factor β
TID – a combination of T3, IGF-1 and Dexamethasone
TnI – Troponin I
TTP – time to peak
WGA – wheat germ agglutinin
WT – wild type
\(\Delta F/F_0\) – baseline fluorescence / peak fluorescence
<table>
<thead>
<tr>
<th>Contents</th>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Statement of originality and copyright declaration</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Publications and abstracts</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>Abbreviations</td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>Contents</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>List of figures</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>List of tables</td>
<td></td>
<td>18</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
<td>19</td>
</tr>
<tr>
<td>The sarcomere</td>
<td>1.1</td>
<td>19</td>
</tr>
<tr>
<td>Cardiac contraction</td>
<td>1.2</td>
<td>20</td>
</tr>
<tr>
<td>Comparison of muscle contraction</td>
<td>1.3</td>
<td>21</td>
</tr>
<tr>
<td>Interaction between the sarcomere and heart pressure</td>
<td>1.4</td>
<td>24</td>
</tr>
<tr>
<td>Isoform switching during development of the heart</td>
<td>1.5</td>
<td>26</td>
</tr>
<tr>
<td>Cardiomyopathies</td>
<td>1.6</td>
<td>27</td>
</tr>
<tr>
<td>Hypertrophic cardiomyopathy</td>
<td>1.7</td>
<td>28</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>1.8</td>
<td>32</td>
</tr>
<tr>
<td>Sudden cardiac death</td>
<td>1.9</td>
<td>33</td>
</tr>
<tr>
<td>The E99K mutation</td>
<td>1.10.1</td>
<td>34</td>
</tr>
<tr>
<td>E99K mutation in patients</td>
<td>1.10.2</td>
<td>34</td>
</tr>
<tr>
<td>The E99K mouse model</td>
<td>1.10.3</td>
<td>36</td>
</tr>
<tr>
<td>Induced pluripotent stem cells</td>
<td>1.11</td>
<td>38</td>
</tr>
<tr>
<td>CRISPR/Cas9 gene editing</td>
<td>1.12</td>
<td>41</td>
</tr>
<tr>
<td>Section</td>
<td>Section No.</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>-------------</td>
<td>------</td>
</tr>
<tr>
<td>Multicellular models</td>
<td>1.13</td>
<td>43</td>
</tr>
<tr>
<td>Engineered heart tissue</td>
<td>1.15</td>
<td>46</td>
</tr>
<tr>
<td>Aims and hypothesis of the current study</td>
<td>1.15</td>
<td>52</td>
</tr>
<tr>
<td>Specific hypothesis</td>
<td>1.15.1</td>
<td>52</td>
</tr>
<tr>
<td>Specific aims</td>
<td>1.15.2</td>
<td>53</td>
</tr>
<tr>
<td>Methods</td>
<td>2</td>
<td>54</td>
</tr>
<tr>
<td>Animal husbandry and tissue isolation</td>
<td>2.1</td>
<td>54</td>
</tr>
<tr>
<td>Cell maintenance and differentiation of hiPSC</td>
<td>2.2</td>
<td>54</td>
</tr>
<tr>
<td>Cardiomyocyte isolation</td>
<td>2.3</td>
<td>55</td>
</tr>
<tr>
<td>Thawing iPSC differentiated iCell Cardiomyocytes</td>
<td>2.4</td>
<td>55</td>
</tr>
<tr>
<td>Generation of EHTs</td>
<td>2.5</td>
<td>56</td>
</tr>
<tr>
<td>EHT solutions</td>
<td>2.6</td>
<td>58</td>
</tr>
<tr>
<td>Force calculation in EHTs</td>
<td>2.7</td>
<td>58</td>
</tr>
<tr>
<td>Extraction of myofibrils from tissue samples</td>
<td>2.8</td>
<td>61</td>
</tr>
<tr>
<td>Western Blots and phosphate affinity SDS PAGE</td>
<td>2.9</td>
<td>61</td>
</tr>
<tr>
<td>Fixing hearts for cryosectioning</td>
<td>2.10</td>
<td>62</td>
</tr>
<tr>
<td>Wheat germ agglutinin (WGA) staining for cell size</td>
<td>2.11</td>
<td>62</td>
</tr>
<tr>
<td>Picro-sirius red (PSR) staining for collagen</td>
<td>2.12</td>
<td>63</td>
</tr>
<tr>
<td>Immunocytochemistry</td>
<td>2.13</td>
<td>63</td>
</tr>
<tr>
<td>Two photon tissue preparation and staining</td>
<td>2.14</td>
<td>64</td>
</tr>
<tr>
<td>RNA Extraction (hearts)</td>
<td>2.15</td>
<td>65</td>
</tr>
<tr>
<td>RNA Extraction (EHTs)</td>
<td>2.16</td>
<td>65</td>
</tr>
<tr>
<td>Gene expression analysis</td>
<td>2.17</td>
<td>65</td>
</tr>
<tr>
<td>Ca$^{2+}$ Transients</td>
<td>2.18</td>
<td>66</td>
</tr>
<tr>
<td>Topic</td>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>--------------------------------------------</td>
<td>---------</td>
<td>------</td>
</tr>
<tr>
<td>Calcium spark measurement</td>
<td>2.19</td>
<td>66</td>
</tr>
<tr>
<td>Calcium spark analysis</td>
<td>2.20</td>
<td>67</td>
</tr>
<tr>
<td><em>Methods in the next section were conducted in Chris Denning’s lab in Nottingham</em></td>
<td>2.21</td>
<td>67</td>
</tr>
<tr>
<td>Derivation of fibroblasts</td>
<td>2.21.1</td>
<td>67</td>
</tr>
<tr>
<td>Sendai virus reprogramming</td>
<td>2.21.2</td>
<td>68</td>
</tr>
<tr>
<td>Fixing cells</td>
<td>2.21.3</td>
<td>69</td>
</tr>
<tr>
<td>Immunostaining</td>
<td>2.21.4</td>
<td>69</td>
</tr>
<tr>
<td>Cell Culture</td>
<td>2.21.5</td>
<td>70</td>
</tr>
<tr>
<td>Targeting vector construction</td>
<td>2.21.6</td>
<td>70</td>
</tr>
<tr>
<td>Transfection in hiPSCs</td>
<td>2.21.7</td>
<td>71</td>
</tr>
<tr>
<td>Cardiomyocyte differentiation</td>
<td>2.21.8</td>
<td>72</td>
</tr>
<tr>
<td>EHT transport</td>
<td>2.22</td>
<td>72</td>
</tr>
<tr>
<td>Calcium exposure experiment</td>
<td>2.23</td>
<td>72</td>
</tr>
<tr>
<td>Arrhythmia analysis</td>
<td>2.24</td>
<td>73</td>
</tr>
<tr>
<td>Statistics</td>
<td>2.25</td>
<td>73</td>
</tr>
<tr>
<td>Results chapter 1. The properties of rat and human engineered heart tissues</td>
<td>3</td>
<td>75</td>
</tr>
<tr>
<td>Basic characteristics of EHTs</td>
<td>3.1</td>
<td>75</td>
</tr>
<tr>
<td>Variations in different iPSC lines</td>
<td>3.2</td>
<td>78</td>
</tr>
<tr>
<td>Extended culture and Matrigel effect on contractility</td>
<td>3.3</td>
<td>80</td>
</tr>
<tr>
<td>T3, IGF-1 and Dexamethasone effect on contractility</td>
<td>3.4</td>
<td>82</td>
</tr>
<tr>
<td>Batch variability and increased numbers of cells in EHTs</td>
<td>3.5</td>
<td>84</td>
</tr>
<tr>
<td>Spontaneous beating activity in rEHTs</td>
<td>3.6</td>
<td>86</td>
</tr>
<tr>
<td>Positive ionotrophic responses in EHTs</td>
<td>3.7</td>
<td>88</td>
</tr>
</tbody>
</table>
3D vs 2D calcium transients 3.8 90
Troponin I isoform switching in EHTs 3.9 92
Discussion chapter 1. Engineered heart tissue as an experimental model 3.10.1 94
Problems with human tissue as a model 3.10.2 94
Advantages of the EHT platform 3.10.3 95
Maturation strategies effect on force 3.10.4 96
Cell density effect on EHT culture 3.10.5 98
Problems with white box measurements of force 3.10.6 99
iPSC-CM EHT is not a fully mature heart model 3.10.7 101
EHTs verses monolayer culture 3.10.8 103
Conclusion 3.10.9 104
Results chapter 2. Characterisation of the ACTC E99K mouse model 4 106
Sudden cardiac death in three different backgrounds 4.1 107
Black6 TG mice have the same level of sparks as WT 4.2 108
Characterising fibrosis in the E99K ACTC mouse model 4.3 110
Aging as a determining factor in fibrosis 4.4 112
Increased fibrosis confirmed by staining and qPCR 4.5 114
Isoform switching in SCD mice 4.6 116
Aberrant phosphorylation in SCD mice 4.7 118
Protein expression of RyR2, Epac and PLN 4.8 120
Discussion chapter 2. The mechanism of sudden cardiac death in E99K mice 4.9.1 122
E99K mice and sudden death window 4.9.2 122
Characterisation of fibrosis in E99K mice 4.9.3 123
<table>
<thead>
<tr>
<th>Topic</th>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrosis as a link to arrhythmias</td>
<td>4.9.4</td>
<td>123</td>
</tr>
<tr>
<td>Aberrant calcium handling and stain effects of E99K mice</td>
<td>4.9.5</td>
<td>125</td>
</tr>
<tr>
<td>Conclusion</td>
<td>4.9.6</td>
<td>129</td>
</tr>
<tr>
<td>Results chapter 3. The E99K mutation in iPSCs</td>
<td>5</td>
<td>131</td>
</tr>
<tr>
<td>Patient details in this study</td>
<td>5.1</td>
<td>131</td>
</tr>
<tr>
<td>Sendai virus reprogramming of patient lines</td>
<td>5.2</td>
<td>135</td>
</tr>
<tr>
<td>Preliminary characterisation of iPSC-CM EHT</td>
<td>5.3</td>
<td>137</td>
</tr>
<tr>
<td>CRISPR/Cas9 PiggBac gene editing of iPSC lines</td>
<td>5.4</td>
<td>139</td>
</tr>
<tr>
<td>Spontaneous contraction of CRISPR lines over time</td>
<td>5.5</td>
<td>141</td>
</tr>
<tr>
<td>Stimulated contraction parameters of CRISPR lines</td>
<td>5.6</td>
<td>143</td>
</tr>
<tr>
<td>Calcium sensitivity of CRISPR lines</td>
<td>5.7</td>
<td>146</td>
</tr>
<tr>
<td>Calcium sensitivity of CRISPR lines with absolute forces</td>
<td>5.8</td>
<td>149</td>
</tr>
<tr>
<td>Calcium handling properties of CRISPR lines</td>
<td>5.9</td>
<td>151</td>
</tr>
<tr>
<td>A novel method for analysing arrhythmogenic contractions in EHTs</td>
<td>5.10</td>
<td>153</td>
</tr>
<tr>
<td>Arrhythmogenic contractions in stimulated CRISPR lines</td>
<td>5.11</td>
<td>155</td>
</tr>
<tr>
<td>Arrhythmogenic contractions in CRISPR lines exposed to different calcium concentrations</td>
<td>5.12</td>
<td>157</td>
</tr>
<tr>
<td>Gene expression in CRISPR lines</td>
<td>5.13</td>
<td>159</td>
</tr>
<tr>
<td>Discussion chapter 3. HCM in diseased and corrected iPSC-CM EHT</td>
<td>5.14.1</td>
<td>161</td>
</tr>
<tr>
<td>Transport between Nottingham and Imperial</td>
<td>5.14.2</td>
<td>161</td>
</tr>
<tr>
<td>E99K expression in CRISPR lines</td>
<td>5.14.3</td>
<td>162</td>
</tr>
<tr>
<td>Contractile dysfunction in CRISPR lines</td>
<td>5.14.4</td>
<td>163</td>
</tr>
<tr>
<td>Evaluating calcium sensitivity comparing CRISPR and original lines</td>
<td>5.14.5</td>
<td>164</td>
</tr>
<tr>
<td>A novel method to analyse arrhythmic contractions in EHTs</td>
<td>5.14.6</td>
<td>166</td>
</tr>
<tr>
<td>Donor I contraction different to Donor II and III</td>
<td>5.14.7</td>
<td>167</td>
</tr>
<tr>
<td>Conclusion</td>
<td>5.14.7</td>
<td>168</td>
</tr>
<tr>
<td>Final Discussion. Is iPSC CM EHT a good model of the E99K mutation</td>
<td>6</td>
<td>170</td>
</tr>
<tr>
<td>Insights from the mouse model of E99K</td>
<td>6.1</td>
<td>170</td>
</tr>
<tr>
<td>Moving from animal to human experiments</td>
<td>6.2</td>
<td>173</td>
</tr>
<tr>
<td>Analysing contractility in iPSCs</td>
<td>6.3</td>
<td>175</td>
</tr>
<tr>
<td>Contractile dysfunction caused by the E99K mutation</td>
<td>6.4</td>
<td>176</td>
</tr>
<tr>
<td>Variable phenotypic presentation in E99K patients</td>
<td>6.5</td>
<td>177</td>
</tr>
<tr>
<td>The future</td>
<td>6.6</td>
<td>180</td>
</tr>
<tr>
<td>Conclusion</td>
<td>7</td>
<td>182</td>
</tr>
<tr>
<td>Aims and hypothesis</td>
<td>7.1</td>
<td>182</td>
</tr>
<tr>
<td>Clinical implications of the current work</td>
<td>7.2</td>
<td>184</td>
</tr>
<tr>
<td>Limitations of the current work</td>
<td>7.3</td>
<td>185</td>
</tr>
<tr>
<td>Future work</td>
<td>7.4</td>
<td>189</td>
</tr>
<tr>
<td>Concluding remarks</td>
<td>7.5</td>
<td>194</td>
</tr>
<tr>
<td>Bibliography</td>
<td>8</td>
<td>196</td>
</tr>
<tr>
<td>Appendix</td>
<td>9</td>
<td>235</td>
</tr>
<tr>
<td>Pictures of EHT components</td>
<td>9.1</td>
<td>235</td>
</tr>
<tr>
<td>Provisional protocol for generation of human engineered heart tissues</td>
<td>9.2</td>
<td>236</td>
</tr>
<tr>
<td>White Box Step By Step Guide</td>
<td>9.3</td>
<td>240</td>
</tr>
<tr>
<td>List of antibodies</td>
<td>9.4</td>
<td>246</td>
</tr>
<tr>
<td>List of Primers</td>
<td>9.5</td>
<td>247</td>
</tr>
<tr>
<td>Plasmids for CRISPR/Cas9 and piggybac gene editing</td>
<td>9.6</td>
<td>248</td>
</tr>
<tr>
<td>Step by step pictures for arrhythmia analysis</td>
<td>9.7</td>
<td>252</td>
</tr>
<tr>
<td>Nottingham quick media reference guide</td>
<td>9.8</td>
<td>257</td>
</tr>
<tr>
<td>List of figures</td>
<td>Number</td>
<td>Page</td>
</tr>
<tr>
<td>--------------------------------------------------------------------------------</td>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>Cartoon image of the sarcomere</td>
<td>1.1</td>
<td>20</td>
</tr>
<tr>
<td>The cardiac cycle plotted on pressure and volume axis</td>
<td>1.2</td>
<td>25</td>
</tr>
<tr>
<td>Diagram showing isoform switching in humans over development</td>
<td>1.3</td>
<td>27</td>
</tr>
<tr>
<td>The major signalling pathways and the major forms of HCM</td>
<td>1.4</td>
<td>30</td>
</tr>
<tr>
<td>Electron microscopy revealed disorganization rescue of myofibrils in young dTG hearts</td>
<td>1.5</td>
<td>37</td>
</tr>
<tr>
<td>Overview of CRISPR/Cas bacterial immune system</td>
<td>1.6</td>
<td>41</td>
</tr>
<tr>
<td>Different types of hydrogel approaches</td>
<td>1.7</td>
<td>46</td>
</tr>
<tr>
<td>EHTs and the white box set up</td>
<td>1.8</td>
<td>47</td>
</tr>
<tr>
<td>EHTs and the white box</td>
<td>2.1</td>
<td>57</td>
</tr>
<tr>
<td>Contraction measurements on traces from the white box</td>
<td>2.2</td>
<td>60</td>
</tr>
<tr>
<td>Immunohistochemistry images of rEHT</td>
<td>3.1</td>
<td>77</td>
</tr>
<tr>
<td>Contractile characteristics of iPSC-CM from different lines</td>
<td>3.2</td>
<td>79</td>
</tr>
<tr>
<td>Assaying different culture conditions and their effect on human EHTs</td>
<td>3.3</td>
<td>81</td>
</tr>
<tr>
<td>Culturing human EHT with T3, IGF, and Dexamethasone</td>
<td>3.4</td>
<td>83</td>
</tr>
<tr>
<td>The effect of different culturing conditions in rat EHTs</td>
<td>3.5</td>
<td>85</td>
</tr>
<tr>
<td>Loss of spontaneous beating activity in rat EHTs</td>
<td>3.6</td>
<td>87</td>
</tr>
<tr>
<td>Contractile properties of human and rat EHTs</td>
<td>3.7</td>
<td>89</td>
</tr>
<tr>
<td>Calcium transients from 2D and 3D cultures</td>
<td>3.8</td>
<td>91</td>
</tr>
<tr>
<td>Analysis of Troponin I isoforms and phosphorylation level</td>
<td>3.9</td>
<td>93</td>
</tr>
<tr>
<td>The white box not taking into account passive tension</td>
<td>3.10</td>
<td>100</td>
</tr>
<tr>
<td>Kaplan-Meier plot of different survival rates in C57Bl6 x CBA/Ca, pure C57BL/6 and enriched CBA/Ca background E99K mice</td>
<td>4.1</td>
<td>107</td>
</tr>
<tr>
<td>Spontaneous calcium spark parameters in Black6 E99K (TG) and wild type (WT) mice</td>
<td>4.2</td>
<td>109</td>
</tr>
<tr>
<td>Collagen expression in wild type (WT) transgenic (TG) and sudden death (SD) animals by two photon microscopy</td>
<td>4.3</td>
<td>111</td>
</tr>
<tr>
<td>Topic</td>
<td>Page</td>
<td>Section</td>
</tr>
<tr>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>------</td>
<td>---------</td>
</tr>
<tr>
<td>Collagen expression of E99K animals over time</td>
<td>4.4</td>
<td>113</td>
</tr>
<tr>
<td>The expression of Collagen I and II in wild type (WT), transgenic (TG), and sudden death (SCD) animals</td>
<td>4.5</td>
<td>115</td>
</tr>
<tr>
<td>Isoform switching in E99K animals</td>
<td>4.6</td>
<td>117</td>
</tr>
<tr>
<td>Phosphorylation levels in E99K hearts</td>
<td>4.7</td>
<td>119</td>
</tr>
<tr>
<td>Ryanadine Receptor (RyR2), Epac2 and Phospholamban protein levels in wild type (WT), transgenic (TG) and sudden death (SCD) E99K mice</td>
<td>4.8</td>
<td>121</td>
</tr>
<tr>
<td>Patient information from 18 punch biopsies</td>
<td>5.1</td>
<td>132</td>
</tr>
<tr>
<td>Characteristics from 14 patients with the E99K mutation</td>
<td>5.2</td>
<td>134</td>
</tr>
<tr>
<td>Production of hiPSC-cardiomyocytes with or without c.301G&gt;A E99K mutations</td>
<td>5.3</td>
<td>136</td>
</tr>
<tr>
<td>Contractile responses of E99K iPSC-CM Engineered Heart Tissue</td>
<td>5.4</td>
<td>138</td>
</tr>
<tr>
<td>Generation of isogenic E99K hiPSC-cardiomyocytes</td>
<td>5.5</td>
<td>140</td>
</tr>
<tr>
<td>Spontaneous contraction of CRISPR lines over time</td>
<td>5.6</td>
<td>142</td>
</tr>
<tr>
<td>Stimulated contraction of CRISPR lines</td>
<td>5.7</td>
<td>144</td>
</tr>
<tr>
<td>Normalised force curves of Crispr lines</td>
<td>5.8</td>
<td>145</td>
</tr>
<tr>
<td>Calcium sensitivity of CRISPR lines</td>
<td>5.9</td>
<td>147</td>
</tr>
<tr>
<td>Calcium sensitivity in CRISPR lines</td>
<td>5.10</td>
<td>148</td>
</tr>
<tr>
<td>Calcium sensitivity of CRISPR lines with absolute forces</td>
<td>5.11</td>
<td>150</td>
</tr>
<tr>
<td>Calcium handling properties of CRISPR lines</td>
<td>5.12</td>
<td>152</td>
</tr>
<tr>
<td>A novel method for analysing arrhythmogenic contractions in EHTs</td>
<td>5.13</td>
<td>154</td>
</tr>
<tr>
<td>Arrhythmogenic contractions in stimulated CRISPR lines</td>
<td>5.14</td>
<td>156</td>
</tr>
<tr>
<td>Arrhythmogenic contractions in stimulated Crispr lines during exposure to increasing calcium concentrations</td>
<td>5.15</td>
<td>158</td>
</tr>
<tr>
<td>qPCR gene expression of Myosin Heavy Chain 7 (MHC7), Transforming Growth Factor β1 (TGF-β1), and Sarco/Endoplasmic Reticulum ATPase (Serca2a) in six lines</td>
<td>5.16</td>
<td>160</td>
</tr>
</tbody>
</table>
**List of tables**

<table>
<thead>
<tr>
<th>Table Description</th>
<th>Number</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Publications of human heart tissue and contraction parameters</td>
<td>1.1</td>
<td>22</td>
</tr>
<tr>
<td>Patient characteristics of the E99K population</td>
<td>1.2</td>
<td>35</td>
</tr>
<tr>
<td>References of papers with a HCM phenotype being modelled in iPSCs</td>
<td>1.3</td>
<td>40</td>
</tr>
<tr>
<td>Table of primers for clones</td>
<td>2.1</td>
<td>71</td>
</tr>
<tr>
<td>Patient information from 18 punch biopsies</td>
<td>Fig.5.1</td>
<td>132</td>
</tr>
<tr>
<td>Characteristics from 14 patients with the E99K mutation</td>
<td>Fig.5.2</td>
<td>133</td>
</tr>
</tbody>
</table>
1 Introduction

Heart failure is a major killer of people in the western world and can be caused by a variety of factors including: hypertension, myocardial ischemia, and cardiomyopathies (Francis 2001). The most common inherited cardiac disease is hypertrophic cardiomyopathy which affects around 1 in 500 of the population (Maron 1995), however, this disease is complex with sufferers having an age related phenotype, and a mixed penetrance of underlying genetic mutations (Cahill 2013). Increasing our understanding of hypertrophic cardiomyopathy may help us unravel the mechanism behind this common disease, and could be applied to discover new treatment options. Engineered heart tissue is a relatively new technique which could be taken advantage of to learn more about disease progression, mechanism, and possibly cell replacement therapy. Therefore, this current study focuses on studying a hypertrophic cardiomyopathy disease causing mutation, and setting up the engineered heart tissue platform to model this mutation using induced pluripotent stem cells. We shall also take advantage of CRISPR technology to create isogenic controls of our mutant lines and produce a E99K mutant line from control. Using our knowledge from the mouse model and iPSCs with isogenic controls will allow us to study the mutation in unprecedented detail.

1.1 The sarcomere

The basic functional unit for force in cardiomyocytes is the sarcomere (figure 1.1). It is given its characteristic striated appearance by repeating units of thick and thin filaments made principally of myosin and actin respectively. Many sarcomeres lined into a series make up myofibrils which combine to make a muscle fibre. Muscle fibres in the heart then work together to produce a force to contract and expel the
contents of the atria/ventricles. The sarcomere also has been divided into distinct areas and one sarcomere is defined as being located between two Z lines. The thin filament consists of: Actin, α Tropomyosin, Troponin C and Troponin I and along the Actin protein is the location for docking of myosin heads. The thick filament is made mostly of myosin which forms long fibres but also myosin heads, and these myosin heads will bind to the thin filament and create the force necessary for filaments to slide past each other.

Figure 1.1. Cartoon image of the sarcomere. The actin containing thin filament is shown in red with the myosin containing thick filament in grey which slide past each other during a contraction. Taken from Redwood 1999.

1.2 Cardiac Contraction

Although hypertrophic cardiomyopathy has many contributing factors around 60% of patients have disease causing mutations in the genes of the sarcomere (Cahill 2013). The cardiac sarcomere is a highly specialized repeating unit in heart muscle which carries out the work to produce a contraction (Frey 2011). To produce a
contraction calcium ions (Ca\textsuperscript{2+}) enter into a cardiomyocyte through L-type Ca\textsuperscript{2+} channels which bind to clusters of Ryanodine receptors to induce release of more Ca\textsuperscript{2+} from the sarcoplasmic reticulum. Ca\textsuperscript{2+} will then bind to Troponin C in the sarcomere activating the thin filament. Thus, a contraction is produced when Actin thin filaments slide past myosin thick filaments powered by myosin heads in an ATP dependent fashion. Cardiac Troponin I, T and C bind to cardiac Actin in the thin filament and are critical in switching on a contraction. Upon Ca\textsuperscript{2+} entry into the cardiomyocyte these proteins undergo a conformational change exposing binding sites on Actin for docking of myosin heads. Actin-myosin cross bridges initiate a force in the sarcomere and the summation of many of these interactions will cause a contraction in cardiac muscle. Relaxation of the contraction, and thus diastole, is caused by taking up of Ca\textsuperscript{2+} into the sarcoplasmic reticulum by the ATP dependent pump SERCA2a and by the action of the Na\textsuperscript{+}/Ca\textsuperscript{2+} pump. Cardiomyopathies develop in this system when a break down occurs at any of these steps which adds to the beautiful complexity of this disease.

1.3 Comparison of muscle contraction

There is a lack of normal human tissue for control experiments, since donated hearts which are not able to be used for transplant (and yet are still classified as normal) are rare in Europe. Although rare, some groups have studied the contractile responses of donor heart preparations and compared them with various forms of heart failure (table 1.1). What is clear from these findings is the variability in the force produced by these differing methods and preparations. Moreover, the term heart failure is not exact and reports use multiple cardiomyopathies with differing severities of the diseases. The calcium sensitivity of these samples is more consistent, with
diseased myocardium being more sensitive to calcium than donor hearts. These studies can also be seen in the light of the contraction of human foetal tissue which progressively gets stronger with gestation, but does not reach the strength of contraction of adult preparations (Racca 2016). By comparison to these preparations the force of stem cell derived cardiomyocytes can be evaluated properly with a range of force kinetics under differing conditions. Therefore, Pioner et al. carried out late stage force measurements on iPSC-CM myofibril contraction and found $18.6 \pm 2.5 \text{ mN/mm}^2$ was produced, which is similar to foetal myofibrils at the same age (Pioner 2016).
<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Force</th>
<th>Contraction Time (ms)</th>
<th>EC50 (Calcium)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>End stage heart failure papillary muscle</td>
<td>19.39±688 mN (maximum Ca2+)</td>
<td>310</td>
<td>475</td>
<td>4.22±0.74mM</td>
</tr>
<tr>
<td>End stage heart failure myocytes</td>
<td>7.93±0.87 (% shortening maximum Ca2+)</td>
<td>400</td>
<td>470</td>
<td>3.65±0.13mM</td>
</tr>
<tr>
<td>Normal transplanted endomyocardial biopsies</td>
<td>67±1.3mN (2mM Ca2+)</td>
<td>182±6</td>
<td>140+4 (50%)</td>
<td></td>
</tr>
<tr>
<td>Non failing left ventricle</td>
<td></td>
<td>168±10</td>
<td>303±20 (95%)</td>
<td>~5.6mM</td>
</tr>
<tr>
<td>Ischemic cardiomyopathy left ventricle</td>
<td></td>
<td>180±9</td>
<td>270+11 (95%)</td>
<td>~4mM</td>
</tr>
<tr>
<td>Dilated cardiomyopathy left ventricle</td>
<td></td>
<td>146±5</td>
<td>268+20 (95%)</td>
<td>~4mM</td>
</tr>
<tr>
<td>Donor left ventricle</td>
<td>29.664.5 kN/m2</td>
<td></td>
<td>5.186±0.02 (pCa)</td>
<td></td>
</tr>
<tr>
<td>Failing NYHA IV left ventricle</td>
<td>29.662.9 kN/m2</td>
<td></td>
<td>5.356±0.03 (pCa)</td>
<td></td>
</tr>
<tr>
<td>End stage heart left or right ventricle</td>
<td>25.7±6.5mN/mm2 (2mM Ca2+)</td>
<td>233±18</td>
<td>345±31</td>
<td></td>
</tr>
<tr>
<td>Normal right atrium</td>
<td>24±18mN (2mM Ca2+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal right atrium</td>
<td>14mN (2mM Ca2+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTEPH</td>
<td>10.5mN (2mM ca2+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left ventricle donor</td>
<td>16.86±2.20mN/m m2 (2mM Ca2+)</td>
<td></td>
<td>5.89±0.04 (pCa)</td>
<td></td>
</tr>
<tr>
<td>Left ventricle heart failure</td>
<td>8.69±0.99mN/m m2 (2mM Ca 2+)</td>
<td></td>
<td>6.09±0.04 (pCa)</td>
<td></td>
</tr>
<tr>
<td>Normal atria</td>
<td>6.2±3.5mN (1.8mM Ca2+)</td>
<td>80±0.03ms (80%)</td>
<td>110±0.02ms (80%)</td>
<td>~3mM</td>
</tr>
</tbody>
</table>

Table 1.1. Publications of human heart tissue and contraction parameters. TTP = time to peak, R90 = relaxation at 90% of peak, NYHA = New York Heart Association function classification, CTEPH = Chronic Thromboembolic Pulmonary Hypertension.
1.4 Interaction between the sarcomere and intraventricular pressure

The Frank-Starling mechanism of the heart states that when a muscle is stretched increased amounts of force will be produced, this was first defined by using pressure volume loops in the heart showing increased filling and stretching of the ventricles produces more force (figure 1.2A de Tombe 2010). During a contraction the heart will under-go isovolumetric contraction building pressure in the ventricle. When pressure in the ventricle exceeds pressure in the aorta the aortic valve opens and an auxotonic contraction occurs while the ventricle is ejecting blood. Then relaxation occurs where the volume of the ventricle with some blood inside stays constant. When pressure in the ventricle falls below pressure in the atrium the mitral valve will open and filling of the ventricles will cause the ventricles to stretch. Indeed, when isolated muscle is stretched to normal lengths found in the heart the force increase is directly proportional to the amount the sarcomere is stretched. However, this relationship is also dependent on the level of activation of the sarcomere by external calcium, and, increasing levels of external calcium increased the level of force produced by the sarcomeres (figure 1.2B). This is a complicated relationship with many contributing proteins and components, however, within this model we can assess subsequent attempts to recreate heart muscle in the lab. Therefore, the heart cycle must under-go a filling or stretching phase, followed by an isometric force production phase, then an auxotonic contraction phase, followed lastly by relaxation.
Figure 1.2. The cardiac cycles plotted on pressure and volume axes. A the cardiac cycles begins with mitral closure (A) followed by isovolumetric contraction. When LV pressure exceeds aortic pressure the aortic valve opens and ejection begins (B). The point of maximal ventricular stiffness corresponds to the end of systole (C). This is followed by isovolumetric relaxation. When LV pressure drops below LA pressure the mitral opens and LV filling begins (D). Common physiologic parameters can be derived from the PV loop. On the volume axis: End systolic volume (ESV) and end diastolic volume (EDV), stroke volume (SV), and ejection fraction (SV/EDV). On the pressure axis: systolic blood pressure (SBP), end systolic pressure (PES), diastolic blood pressure (DBP), end diastolic pressure (EDP) and left atrial pressure (LAP). B The Frank–Starling mechanism and myofilament length dependent activation. The Frank–Starling Law of the Heart describes a fundamental property of the heart (figure on the right). That is, for a given contractile state there is a unique relationship between end-systolic pressure reached in the heart and end-systolic pressure, end systolic pressure volume relation (ESPVR); increased contractility results in an increased slope of the ESPVR (cf. blue arrow). Increased ventricular filling (pre-load; red PV loop) leads to an increase in ventricular pressure development at end-systole which allows for (i) increased stroke volume for a given systolic pressure (after-load) and (ii) sustained stroke volume at elevated systolic pressure. The Frank–Starling mechanism has, as its basis, a modulation of myofilament Ca2+ sensitivity upon a change in sarcomere length as illustrated in the left graphs. Myofilament force development is the result of activation by Ca2+ ions. The relationship between force development and activator [Ca2+] is shifted up and to the left at longer sarcomere length (short SL, green; long SL, red). For a given contractile state (and, thus, cytosolic [Ca2+]; dashed vertical line), more myofilament force is developed at the longer SL (red) leading to a higher ventricular pressure at higher end-systolic volume (red PV loop). Thus, the Frank–Starling Law of the Heart is a direct consequence of the myofilament length-dependent activation properties of the cardiac sarcomere. A From Green 2011, B from de Tombe 2010.
1.5 Isoform switching during the development of the heart

Mature cardiac tissue has different isoforms of proteins compared to skeletal tissue or even foetal and neonatal cardiac tissue. The different isoforms produce different contractile properties to allow the adult heart to meet the constant power demands and high stresses of the body (Marston 2003 figure 1.3). This process has been well established in cardiac tissue with both Troponin I and Myosin isoforms undergoing isoform switching. Troponin I slow isoform (herein TnI1) is predominantly expressed in the developing heart while the Troponin I cardiac isoform (TnI3) is the more dominant isoform in the mature heart (Bhavsar 1991). These two isoforms have different Ca$^{2+}$ handling properties with TnI1 having a higher Ca$^{2+}$ affinity than TnI3 (Liu 2012). Another two isoforms that indicate the maturity of cardiac tissue is the ratio of Myosin Heavy Chain (MHC) isoforms. MHCα is expressed in the foetal stages of human development and MHCβ is expressed later on. To compensate for the higher beating rate seen in smaller animals and our mouse model the reverse to the human expression pattern is true (Lompre 1984, Gupta 2007). These isoforms that have been shown to change throughout development, and in some cases have also been shown to change in disease and physiological hypertrophy (Malhotra 1984, Waspe 1990, Ng 1991, Huang 2001, Li 2013).
1.6 Cardiomyopathies

Cardiomyopathies can be split into two main groups: primary cardiomyopathies are caused by a disease directly affecting the heart, and secondary cardiomyopathies are caused by a systemic disorder (Alves 2010). Inherited primary cardiomyopathies can be broadly categorized into: dilated cardiomyopathy, hypertrophic cardiomyopathy, and arrhythmogenic right ventricular cardiomyopathy (Quarta 2017). Left ventricular non-compaction (LVNC) is characterised as an abnormal morphology of the ventricular wall where trabeculae are much more prominent, with a thin compacted layer. LVNC is also associated with the cardiomyopathies although there is some discussion whether it should be considered as a disease in its own
right (Arbustini 2014). LVNC is thought to occur during embryogenesis when the endomyocardial layer of the heart has been perturbed and is usually diagnosed by echocardiography. These diseases are complex and some presentations can be life threatening while others asymptomatic, however, the techniques for screening for disease are quite common in affected families which allows for diagnosis and treatment in advance. The emphasis of this piece of work is hypertrophic cardiomyopathy (HCM) and therefore will be focused on in the coming sections.

1.7 Hypertrophic cardiomyopathy

HCM is defined as a malign increase in heart muscle mass with no other explanation. HCM affects about 1 in 500 of the population and is also the most common cause of sudden cardiac death in young athletes (Maron 1996). HCM is a complicated disease because it can be asymptomatic and it also has a varied presentation with variable penetrance even within the same family (Baxi 2016). If an individual is suspected with having HCM then echocardiography and electrocardiograms are carried out to look for abnormal thickening and heart structure or abnormal conduction (Sen-Chowdhry 2016). Signs of fibrosis and myocardial disarray are also looked for and can be useful indicators of the progression of the disease. More than >1400 mutations have been identified as causing HCM in >11 genes, and these mutations are usually found in the contractile proteins of the sarcomere (Maron 2012). Interestingly, most disease causing mutations are found in Myosin Binding Protein Protein C and β Myosin Heavy Chain (Force 2010, Frey 2012). However, in the western world less than half of patients with HCM have mutations in the sarcomere (Van Driest 2005). HCM is a varied
Hypertrophy of the heart is a natural response to physiological exercise to increase cardiac output for extra oxygen and nutrient demands of the body. However, this mechanism can become pathological with increases in heart mass due to hemodynamic load, contractile dysfunction, and neurohumoral stimuli. Moreover, the mechanism for hypertrophy has not been fully elucidated and effective treatments are still lacking in this area. There have been many pathways implicated in hypertrophy which also interact, and there are compounding issues that in a disease state many of these pathways maybe activated (figure 1.4). Hypertrophic cardiomyopathy is the subject of this piece of work and is defined as a disease of malign hypertrophy where these pathways are activated, even though the heart does not need any increase in cardiac output. Moreover, as the disease continues these signaling mechanisms stay activated and the heart tissue enlarges in response to a functional impairment of the heart. Over time this leads to a decrease in cardiac output which can lead to many symptoms including: shortness of breath, reduced exercise ability, chest pain and palpitations (Sen-Chowdhury 2016).
Figure 1.4. A schematic of the major signaling pathways involved in cardiac hypertrophy, showing cross-talk and integration of various pathways (A). Due to the complex nature of signaling cascades and on-going discoveries it was not possible to illustrate all interactions. The three main forms of cardiomyopathy. The normal heart (i). Dilated cardiomyopathy (ii). Two examples of hypertrophic cardiomyopathy: classic asymmetric septal hypertrophy, most often limited to the basal portion (not shown), and apical hypertrophy (iii). Arrhythmogenic right ventricular cardiomyopathy, with right-sided involvement only (iv). A from Bernardo 2010 and B from Wilde 2013.
The first HCM associated gene Myosin Heavy Chain 7 and its causative mutation R403Q was published in 1990 by the Seidman and Seidman lab (Geister-Lowrance 1990). Early reports supported the conclusion of reduced function of the myofilaments and based on these results the hypothesis was formed that hypertrophy was compensatory for reduced contraction caused by the mutation, however, this has been challenged with reports showing increased contractile performance of the myofilaments in MYH 7 and others (Poggesi 2014). For example in the myc7 gene, mutations which cause HCM can be seen with increased and decreased force development compared to control (Belus 2008, Seebohm 2009, Sommese 2013, Kraft 2013). Moreover, the hypertrophic disease state can complicate investigations involving contractile force, and parameters like: reduced myofibril density, fibrosis, and myocyte disarray can be problematic in measurements.

When the amount of force developed in different levels of calcium (termed calcium sensitivity) is also investigated along with maximal force of contraction, reports which define the parameter calcium sensitivity are more consistent. HCM myofilaments whether from humans or animals show increased myofilament calcium sensitivity, and this finding is consistent across different mutations in different genes (Marston 2016). This may show an underlying phenotype of HCM and may be what triggers hypertrophic gene programs, although, investigations linking calcium sensitivity to gene induction are sparse. These conflicting reports on force development and the consistent observation of increased calcium sensitivity have led to a hypothesis
being formed in which increased amounts of energy are used by mutant myofibrils to conduct a contraction (Ashrafian 2003, Ferrantini 2009). This hypothesis has been supported by direct measurements of cardiac creatine to ATP levels by nuclear magnetic resonance, and by mutations in metabolism associated genes which also give rise to HCM (Balir 2001, Vakrou 2014). In a comprehensive study, 31 patients with varied levels of hypertrophy which had been diagnosed with HCM with mutations in different genes showed increased energy consumption (Crilley 2003). Therefore, these results suggest that increased calcium sensitivity and increased energy consumption is the primary effect of a HCM mutation, and this eventually leads to malignant hypertrophy and further adverse effects.

1.8 Fibrosis

Collagen makes up a normal component of the extra cellular matrix and is essential to the proper working of the heart. There are 18 types of collagen of which collagen type I and III are the most common in the heart, where type I is known to be the stiffer than type III which is more extensible and elastic (Jong 2011). Fibroblasts produce collagen and process the proteins into mature collagen fibrils, and the pathogenic build-up of this is called fibrosis. There are two types of fibrosis: one which occurs when cells die and the space is then filled by collagen and is termed replacement fibrosis, the second occurs when abnormal amounts of fibrosis are found in between myocytes which is called interstitial fibrosis.

Fibrosis is associated with the progression of HCM, however, its relationship to the disease is complicated. In humans fibrosis is a good predictor of the severity of the disease and in animal models fibrosis is common to almost all models of HCM.
Apoptosis of myocytes has been considered a trigger for fibrosis such that collagen fibrils replace areas of dead myocytes to ensure the functionality of the myocardium (Konno 2010). Moreover, fibrosis is linked to the action of fibroblasts and the levels of TGF-β (Khan 2006). Increased levels of TGF-β is found in HCM models and neutralising antibodies directed against TGF-β showed a reduction in the amount of fibrosis (Teekakirikul 2010). Mechanistically TGF-β has been shown to be downstream of Angiotensin II and has been shown to act through the SMAD pathway to initiate fibrosis (Kahn 2006).

Collagen type I is known to be a stiff component of the extra cellular matrix and that passive ventricular stiffness is correlated with collagen volume (Jalil 1989). Furthermore, because collagen deposition is linked to ventricular stiffness, relaxation is also affected by increased amounts of collagen. This causes impaired relaxation and problems with filling of the ventricles which can be modified by reducing the amount of fibrosis (MacKenna 1994). Fibrosis is upregulated in humans with HCM and more severe fibrosis is linked with more severe outcomes or cardiac events (O’Hanlon 2010, Bruder 2010). Moreover, Ho et al showed that the profibrotic biosynthesis can be initiated before hypertrophy has set in, therefore, it may act as an early marker for disease (Ho 2010).

1.9 Sudden cardiac death
SCD is defined by acute onset of symptoms and death within one hour, it usually presents with no antecedent symptoms and clinicians diagnose the cause post mortem (Hayashi 2015). In the US, SCD accounts for 15-20% of all deaths, however, these deaths have a range of causes. The leading cause of SCD is coronary artery
disease accounting for as much as 24% in Australia and New Zealand (Bagnall 2016). Here we are focusing on HCM which is the cause of much lower numbers compared to the overall number of SCDs, but sufferers of SCD due to HCM are usually less than 30 years. HCM is the most common cause of SCD in young athletes accounting for around 30% of deaths, it is hypothesized that the malformed architecture of the heart leads to a deadly ventricular arrhythmia (Maron 1996). Also, increased Ca\(^{2+}\) sensitivity seen in HCM is sufficient to increase the probability of potentially fatal arrhythmias (Huke and Knollman 2010). There has been a lack of mechanistic studies in this area due to the difficult nature of the condition, however, high incidents of SCD have been reported in our own ACTC E99K mutation.

1.10.1 The E99K Actin Mutation

1.10.2 E99K mutation in patients

The E99K mutation in Actin will be studied for this project. Actin, as previously mentioned, is a crucial component of the thin filament. Thus far, nine disease causing mutations have been discovered in the actc gene which cause HCM (Mogensen 1999, Mogensen 2004, Arad 2005, Monserrat 2007, Olivotto 2008, Kaski 2009). Taken together, the mutations in Actin account for around 1% of all HCM cases in the western world (Force 2010). However, higher numbers of actc mutations have been reported in smaller cohorts which may be particularly important in areas like Japan, which have higher incidents of apical hypertrophy (Arad 2005, Kitaoko 2003).

The E99K mutation has also been studied in a large patient population in Spain (Monserrat 2007). A screen was carried out on 247 families with HCM, DCM or left
ventricular non-compaction (LVNC) using echocardiography and genetic data from restriction fragment length polymorphism. The E99K mutation was found in 5 families and Monserrat et al reported 49% of patients positive for the mutation diagnosed with LVNC, and 49% with apical HCM. In addition, 2% were identified with restrictive cardiomyopathy with a further 9/48 patients found with septal defects. To date 76 mutation carriers (35 male, 41 female) from 10 families have been characterized presenting with mainly apical hypertrophy (table 1.2). The outflow tract of the left ventricle was never observed to be obstructed in any patients. Lastly, 5 of the 8 sudden cardiac deaths were observed in one family which suggests an important hereditary component to this phenotype.

<table>
<thead>
<tr>
<th>Property</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients studied</td>
<td>76 (35 males, 41 females)</td>
</tr>
<tr>
<td>Mean age</td>
<td>33±19 (range 5-80)</td>
</tr>
<tr>
<td>Maximal LVWT</td>
<td>18.6±5mm (range 9-30mm)</td>
</tr>
<tr>
<td>Area of hypertrophy</td>
<td>Apical 57/61</td>
</tr>
<tr>
<td></td>
<td>Asymmetric septal 2/61</td>
</tr>
<tr>
<td></td>
<td>Concentric 1/61</td>
</tr>
<tr>
<td></td>
<td>Atypical 1/61</td>
</tr>
<tr>
<td>Echocardiography</td>
<td>Diastolic dysfunction 14/55</td>
</tr>
<tr>
<td></td>
<td>Restrictive filling 5/55</td>
</tr>
<tr>
<td></td>
<td>Left atrial dilation 19/55</td>
</tr>
<tr>
<td></td>
<td>Ventricular dilation and systolic dysfunction 4/65</td>
</tr>
<tr>
<td>Electrocardiogram</td>
<td>Abnormalities 53/61</td>
</tr>
<tr>
<td></td>
<td>Atrial fibrillation/flutter 7/53</td>
</tr>
<tr>
<td></td>
<td>Conduction disease 5/53</td>
</tr>
<tr>
<td></td>
<td>Abnormal voltage or repolarisation 49/53</td>
</tr>
<tr>
<td></td>
<td>Negative T-waves 35/53</td>
</tr>
<tr>
<td></td>
<td>Abnormal Q waves 14/43</td>
</tr>
<tr>
<td>Adverse events</td>
<td>Sudden deaths 8 (4 with mutation 4 without genetic study)</td>
</tr>
<tr>
<td></td>
<td>Heart failure 1</td>
</tr>
<tr>
<td></td>
<td>Heart transplants 2</td>
</tr>
<tr>
<td></td>
<td>Cardiovascular intervention 2</td>
</tr>
<tr>
<td></td>
<td>Cerebrovascular accident 2</td>
</tr>
<tr>
<td></td>
<td>Other cardiovascular causes 2</td>
</tr>
<tr>
<td></td>
<td>Unknown 4</td>
</tr>
</tbody>
</table>

Table 1.2. Patient characteristics of the E99K population.
1.10.2 The E99K mouse model

To further characterize this mutation a transgenic mouse model harboring the E99K mutation was developed and the Marston lab have been studying this model for around 9 years (Song 2011). Ca^{2+} sensitivity of Actin filaments and papillary muscles were found to be increased which led to impairment in relaxation in these muscles (Song 2013). Therefore, the E99K mutation causes a hyper contractile phenotype causing mutant hearts to carry out more work than wild type hearts. Moreover, classical signs of hypertrophic cardiomyopathy have been shown with increased cell size, and increased fibrosis in E99K animals of 12-15 months of age (Abou 2016). This model reflected many of the same phenotypes found in man, however, it had a much higher rate of sudden cardiac death (SCD). The higher death rates were found to be between 25-45 days with 48% of females, and 22% of males dying early (Song 2011). In further experiments from our group we have crossed the ACTC E99K mutant mouse line with Calcineurin Aβ knock out (CaAβ^{-/-}), which was shown to rescue some effects of the Myosin Binding Protein C deleted mouse model (Abou 2016). Interestingly, all CaAβ^{-/-} and CaAβ^{-/+} mice with the E99K mutation died within the sudden death window, and had the same increase in fibrosis compared to controls. Electron microscopy showed that sarcomere disarray induced in the E99K model was corrected after Calcineurin Aβ ablation (figure 1.5). So, in CaAβ and ACTC E99K double transgenic mice SCD is increased, but myofibril disarray is normal which is an interesting observation. This shows the complexity of signaling mechanisms in HCM and that a multi-faceted approach may be needed to cure all the symptoms of HCM in our model.
Figure 1.5. Electron microscopy revealed disorganization rescue of myofibrils in young double transgenic hearts. Electron micrograph of WT ventricular tissue showing highly ordered myofibrils, in contrary to ACTC E99K TG that shows extensive myofibril disarray. Myofibril disarray in ACTC E99K TG was rescued after partial and complete removal of calcineurin. Scale bar=2µm. Reproduced from Abou 2016.
Further study into the mechanism of SCD was carried out by Christina Rowlands where the properties of calcium regulation were dissected (Rowlands 2014, Rowlands 2017). Young TG mice within the sudden death window were found to have larger calcium transients and increased sarcomere shortening. While adult TG mice were found to have the opposite phenotype and became known as survivors. SCD in these animals is thought to be caused by arrhythmias through β adrenergic stimulation, and when mice were challenged with isoprenaline one of six mice died.

Specifically, the mutation c.301G>A is modified post translation to produce E99K which is a change in amino acid 99 from glutamic acid to lysine, and has also been called E101K if referring to the unmodified genetic code. E99 is thought to act with E93, and E100 in the Actin protein in a secondary binding site, which interacts with Myosin heads to aid in ATP powered sliding force. The change from glutamic acid to lysine results in a charge reversal which impairs the binding affinity of Actin to Myosin which initially is ionic in nature (Olson 2000, Bookwalter 2006).

1.11 Induced pluripotent stem cells
Shinya Yamanaka first discovered that adult cells could be reverted to a pluripotent state in 2006 (Takahashi 2006). Since then this technology has been widely taken up and has become useful in the heart field. iPSCs have been used to model a number of diseases in the heart including: long QT, left ventricular non compaction, arrhythmogenic right ventricular cardiomyopathy, dilated cardiomyopathy, and hypertrophic cardiomyopathy (for review see Buikema 2017).
iPSC technology was taken up quickly by the heart field and early phenotyping showed that these cells could be useful to model diseases and work out molecular mechanisms. At the time of writing there were seven papers which modelled HCM using iPSCs which investigated various aspects (table 1.3). Although, what all these studies lack is proper controls as HCM lines were compared against control lines which were not related to the patient. iPSCs showed many aspects of the HCM phenotype including increased cell size and activation of hypertrophic genes. Studies also demonstrate the dysregulated calcium transients and action potentials which could lay the basis for delayed after depolarisations, and could cause deadly arrhythmias in the heart (Lan 2013, Han 2014). Moreover, studies using iPSC-CM have identified novel targets that could be helpful for future drug treatments (Lan 2013, Han 2014, Kodo 2016). Pharmaceutical treatment with drugs given to patients could be tested and was demonstrated by treating HCM iPSC-CM with verapamil which could ameliorate calcium handling abnormalities (Han 2014). In an elegant study from the Wu lab pharmacological intervention was taken advantage of to inhibit TGF-β to show that this is causative in a proliferation defect observed in LVNC (Kodo 2016). Lastly, iPSC-CM HCM models also served as the test bed for viral correction of disease traits when the mechanism of disease was investigated. Prodzynski et al showed that adeno associated viral transduction of Myosin Binding Protein C rescued hypertrophy caused by a truncated mutant form of the protein (Prodzynski 2017).
<table>
<thead>
<tr>
<th>Patient (Method)</th>
<th>Phenotype</th>
<th>Mutation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>53yrs female, African American (Lentiviral SOCK, dermal fibroblasts)</td>
<td>Palpatations, shortness of breath, exertional chest pain, concentric left ventricular hypertrophy</td>
<td>Hypertrophy, n-fat4c activation, patch clamp DADs, irregular calcium transients, increased [Ca2+]</td>
<td>β-MHC Arg663His</td>
</tr>
<tr>
<td>37yrs female (Retrovirus SOCK, dermal fibroblasts)</td>
<td>Hypertrophy</td>
<td>Hypertrophic genes, nuclear n-fat4c, hypertrophy, myofibrillar disarray, increased irregular AP, increased [Ca2+], increased calcium transient decay time</td>
<td>β-MHC Arg442Gly</td>
</tr>
<tr>
<td>3 patients (Retrovirus SOCK, dermal fibroblasts (1), sendai virus SOCK, T lymphocytes (2-3))</td>
<td>&quot;typical clinical manifestation&quot;</td>
<td>Hypertrophy, myofibrillar disarray</td>
<td>No mutation in 7 genes (1-2) MYBPC Gly999-Gln1004del (3)</td>
</tr>
<tr>
<td>1 patient (Sendai virus SOCK)</td>
<td>Late onset Pompe's disease</td>
<td>Increased glycogen accumulation</td>
<td>Compound mutation c.796C&gt;T and c.1316T&gt;A</td>
</tr>
<tr>
<td>4 patients: 49yrs male, 19yrs male, 16yrs female, 13yrs male (sendai virus, peripheral blood mononuclear cells)</td>
<td>Abnormal echo parameters including: fractional shortening, left ventricular end diastolic and systolic diameter, and degree of hypertrabeculation</td>
<td>Decreased proliferation in iPSC-CM, hypertrophic genes upregulated, TGF-β upregulated and downstream targets implicated in disease progression</td>
<td>TBX20 c. 951C &gt; A and c. 785C &gt; T</td>
</tr>
<tr>
<td>1 patient (Retrovirus SOCK, dermal fibroblast)</td>
<td>Intraventricular septal thickness 26mm, ejection fraction &gt;60%</td>
<td>Hypertrophy, MYBP-C haploinsufficiency, AAV gene therapy correction</td>
<td>MYBP-C c.1358_1359 insertion of C</td>
</tr>
<tr>
<td>30yrs Chinese (Sendai virus SOCK, peripheral blood mononuclear cells)</td>
<td>Fabry disease with: palpitations, exertion dyspnea, atrial fibrillation, left ventricular hypertrophy</td>
<td>Hypertrophy, hypertrophic genes, downregulation of galactosidase gene</td>
<td>GLA IVS4+919G&gt;A</td>
</tr>
</tbody>
</table>

Table 1.3. References of papers with a HCM phenotype being modelled in iPSCs. 
SOCK = Sox2, Oct4, C-myc and Klf4, DADs = delayed after depolarisations, MHC = Myosin Heavy Chain, MYBP-C = Myosin Binding Protein-C, TBX = T-box.
1.12 CRISPR/Cas9 gene editing

Clustered Regularly Interspaced Palindromic Repeats (CRISPR) and the CRISPR Associated Systems (Cas) was originally described in bacteria and archaea as an adaptive immunological defence system to recognise viruses and destroy them (Marraffini 2010). Immunity to a specific virus is acquired by cleavage of the virus and integration of the DNA sequence into the CRISPR locus in so called ‘spacers’ by the Cas1-Cas2 integrase (Wright 2017, figure 1.6). CRISPR spacers are then transcribed into CRISPR RNAs which act as a guide to Cas proteins to recognise invading DNA and create double strand breaks. Initially this mechanism was taken advantage of to produce bacteria with resistance to viruses, however, more recently CRISPR/Cas9 has been used to edit genes.

Figure 1.6. Overview of CRISPR/Cas bacterial immune system. (A) A typical structure of CRISPR locus; (B) illustration of new spacer acquisition and invading DNA cleavage. From Zhang 2014.
A guide RNA sequence can be easily designed, and therefore the system can be manipulated for gene editing whereby the Cas9 protein will cut the DNA, causing a blunt double strand break (DSB, Strong 2016). A break of this nature activates the endogenous DNA repair pathways: Non Homologous End Joining (NHEJ) or Homology directed repair (HDR). NHEJ is error prone because it is mostly active during the G1 phase of the cell cycle when there is no template DNA and can produce insertions or deletions. This can be taken advantage of to produce gene knockouts especially if the insertion or deletion causes a frameshift. In comparison HDR is mostly active in S and G2 phases of the cell cycle and uses a template DNA for high fidelity reconstruction of the DNA, and alternatively, an exogenous piece of DNA could be used for this template which could introduce point mutations. The specificity of Cas9 targeted to certain desired areas in the genome causing DSBs, and HDR with exogenous DNA as a template lies as the basis for genome editing, which has been taken advantage of in the heart field (Strong 2016).

The approach used in this report takes advantage of both Cas9 technology and PiggyBac technology. The PiggyBac transposon system works by the transposase recognising Inverted Terminal Repeats (ITRs) at the ends of the transposon and excises the transposon from the DNA and integrates it into another part of the DNA sequence next to TTAA sites (Zhao 2016). The PiggyBac transposase can excise and integrate DNA in between ITRs without altering the DNA in anyway and is referred to as footprint free. Moreover, recently this technology has been combined with specific endonucleases including Cas9 to produce seamless editing of the genome (Xie 2014, Wang 2016, Qing 2017). In the PiggyBac/Cas9 approaches
Cas9 is used to specifically cause a DSB and homologous recombination occurs with the PiggyBac sequence, this introduces the mutation, and a selection cassette like a puromycin/thymidine kinase fusion gene. Positive selection for cells containing the selection cassette is carried out assaying for puromycin resistance and then the PiggyBac transposase is transfected into the cells which would cut out the selection cassette leaving the gene modified in some way. Negative selection using Ganciclovir is then carried out and any cells where the selection cassette has not been removed or has been incorporated after treatment with the transposase are killed. This leaves genome modified and without any footprints of modification apart from the stress of undergoing the process. It is a very clever approach and can be taken advantage of to produce isogenic controls in iPSCs relatively quickly (Wang 2016).

1.13 Multicellular models

There have been a large number of varied attempts at 3D tissue engineering to produce an improved *in vitro* model for cardiac tissue regeneration, predictive drug toxicology, or for more accurate disease modelling. However, they all share important basic features in attempting to create heart tissue including a matrix for the cells and a number of different cell types. These constructs are then assessed for their similarity to heart tissue in a way that would test the pseudo tissue for connectivity including parameters which involve many cells like: force generation, and conduction from one point to another.

Advances have been made in the tissue engineering field using an engineering component in fabricating porous scaffolds. Researchers have become quite good at adjusting many properties of materials and can use: collagen, alginate, gelatin.
sponges, and polyglycolic acid derivatives (Radisic 2004, Leor 2000, Li 1999, Carrier 1999, Lesman 2010, Marsano 2010). These scaffolds are quite easy to make in large quantities to specific parameters, and some functional improvements to cells have been reported using these methods. Moreover, electrical stimulation can be applied to fabricated scaffolds using conductive material (Dvir 2011).

Another avenue of research being investigated is decellularising existing heart tissue to leave the extracellular matrix components, and then using this backbone to seed cells onto the remaining scaffold (Aubin 2013). This allows cells to be seeded onto a real extracellular matrix and will give seeded cells the same contours of a real heart. This approach works successfully in valves, however, as yet recellularised hearts do not contract strongly enough (Ott 2008). Importantly this approach leaves perfusable vascular networks which are severely lacking in most approaches (Sun 2016).

The ‘bottom up’ approach in tissue engineering is defined as starting with cells combined together in a dish, instead of seeding cells into a scaffold or into a hydrogel. This approach is colloquially called cell sheets and it has made large strides in the past two decades (Sakaguch 2015). Teruo Okano has mainly led this effort from Japan and they have shown that stackable cell sheets can have microvasculature if placed on top of a perfusable section of femoral tissue, or taking advantage of microchannels in a collagen gel (Sekine 2013, Sakaguchi 2013). These cell sheets can be stacked on top of each other in vitro making relatively thick constructs of around 100µm. In vivo cell sheets can be stacked on top of each other following multiple surgeries to produce thicker pseudo tissue, although this is highly stressful to the animal (Shimizu 2006).
The hydrogel technique has been around since 1988 and has been developed since then in multifaceted avenues (Hirt 2012). A range of different strategies have been employed using the hydrogel technique but they all have similar properties, where cardiomyocytes and non cardiomyocytes are combined with a matrix which is usually collagen or fibrin and left to solidify. Furthermore, the hydrogel is usually formed around a static or dynamic arm which gives the construct load, then various methods are used to improve the culture (figure 1.7). The development of Engineered Heart Tissue as a 3D experimental model has been driven by a few groups but this hydrogel technique was first published by Thomas Eschenhagen in 1997 (Eschenhagen 1997). Tissue engineering by Eschenhagen’s group has developed considerably in the last twenty years, going through an evolution from early constructs utilising glass tubes with Velcro, to a medium through put method using silicone posts and a fibrin extracellular matrix (Hansen 2010).
1.14 Engineered heart tissue

The engineered heart tissue technique was first carried out using isolated embryonic chick cardiomyocytes mixed with collagen to form a contracting three dimensional construct. The cardiomyocyte and collagen mix was named Engineered Heart Tissue (herein EHT) which resembled heart tissue in vivo. This seminal paper by Eschenhagen showed that EHTs have similar properties to heart muscle where increased force was seen by: increasing extracellular Ca$^{2+}$, increased frequency of beating, and also a positive Frank-Starling mechanism was observed (Eschenhagen, 1997). EHT was developed further by making circular engineered heart tissue using neonatal rat heart cells. Circular EHTs were created by combining neonatal cells with collagen I and including matrigel which gave improved cellular maturation, myofibre organisation, coupling, and force generation (Zimmerman, 2002a). The applications of EHT is in tissue regeneration and drug screening, both of which need large numbers of constructs that are reproducible. Hence, in 2010 a medium through put method of generating EHTs was developed taking advantage of the reaction of
fibrinogen and thrombin to create fibrin based EHTs (Hansen 2010). Fibrin EHTs are suspended between two silicone posts which give auxotonic load to the tissue under contraction. Heart cells undergo spontaneous rhythmic contraction that is detected by bending of posts, and the force of individual EHTs can be measured automatically using a custom made program (figure 1.8 and section 2.7 for more detail).

Figure 1.8. EHTs and the white box set up. A schematic of the cell culture incubator and overhead camera is shown in A. B shows 4 EHTs attached to one rack of silicone posts with a millimetre scale for reference. C shows custom made software recognises EHTs and silicone posts, the software detects the movement of the silicone posts and force can be calculated over time D. Adapted from Hansen 2010

To be practical and efficacious, any tissue engineering technology has to meet certain requirements. The first requirement is that the tissue must recapitulate the tissue it is attempting to mimic in the lab. In an EHT cells are aligned and can generate calculable force contractions which in early work measured around 0.3mN when measured in an organ bath (Eschenhagen 1997). Although this measures well below contraction forces seen in native human tissue others have improved on this method. Insulin and auxotonic load have been shown to have a positive effect on the
contraction of EHTs when using rat neonatal ventricular cardiomyocytes showing an increase in overall force production (Zimmerman 2006). Martin Ulrich’s group used a custom made bioreactor, vitamin C, fibroblasts, and increasing static stress to improve collagen and Matrigel based constructs (Kensah 2013). In addition to this, Kensha et al used a novel approach of selecting for stem cell derived cardiomyocytes in an embryoid body differentiation protocol and made constructs without disassociating cells. These factors combined gave force measurements of 4.4mN/mm² which is still lower than endogenous human muscle at around 20mN/mm². The contractile force of EHTs has been reported at adult levels if comparing force by unit area (Baar 2004, Jackman 2017). In these approaches the active level of force does not change massively but the width of the construct is thinner, therefore, force per unit area increased. A decrease in contract width is thought to be carried out by fibroblasts pulling cardiomyocytes closer together. This phenomenon has been investigated and it has been shown that ~10% fibroblasts in a hydrogel is important for construct performance and especially thinning (Kensah 2013). Moreover, at the time of peak force contraction fibroblasts which were added within EHTs at 30% proliferate to about a 1:1 ratio (cardiomyocytes to fibroblasts) which is similar to what is seen in the heart (Tiburcy 2017).

In vivo regeneration has always been one of the goals of tissue engineering especially in the cardiac field because heart failure is characterised by death of cardiomyocytes. Therefore, EHT technology could be used as a grafted beating 3D construct that could aid in the contraction of heart tissue in vivo (Eschenhagen 2005, Naito 2006). Zimmerman et al showed that fusing EHTs together to create large contracting constructs could be used to improve infarcted rat hearts (Zimmerman...
Fusing EHTs together formed constructs of 15mm in diameter and 1-4mm in length which were surgically grafted onto rat hearts. The fused multiple EHT construct showed structural integration and electrical coupling twenty eight days after implantation. Furthermore, grafting of EHTs induced thickening of the systolic wall and fractional area shortening was improved in animals. Recently, EHTs were placed onto guinea pig hearts with large cryo-injuries to act as a bigger animal model of myocardial infarction in steps to up-scale to human like conditions (Weinberger 2016). Curiously, the cardiomyocytes in the EHTs in this model proliferated massively, so the constructs were very large at the end of assessment. But this experiment showed proof of concept in a large animal model improving left ventricular function including returning fractional shortening to levels before injury. Larger hydrogels are necessary for a regenerative medicine approach in heart failure patients because of the large amount of cells lost during ischemia.

EHTs can be generated easily with minimal variation and they have similar characteristics to heart tissue which means that they are suitable for drug toxicology. Moreover, The Eschenhagen set up can be used to test multiple drugs and for abnormalities in contractions or cardiotoxic actions (Eder 2014, Eder 2016). Many of the drug responses of iPSC-CM EHT are similar to normal human trabeculae, although, there is still a maturity difference between iPSC-CM and adult cells (Mannhardt 2017). Therefore, iPSC-CM EHT allow for drug toxicology to be carried out with abundant material and could help large drug companies in rejection rates of drugs at phase one clinical trials.
As soon as pluripotent cells were differentiated into cardiomyocytes the drive in the field has always been to show how similar they are to adult cells. Although these cells are not adult cardiomyocytes a range of strategies have been used to try and make adult cells including: embryoid bodies, cell purification, ascorbic acid, Matrigel, electrical stimulation, noradrenaline, cell culture time, T3 IGF-1/Dexamethasone/VEGF/FGF-2/TGF-β in varying combinations and concentrations, secondary cell types (mesenchymal, fibroblast, endothelial), mechanical conditioning, insulin, and rotating tables (Zimmerman 2006, Tulloch 2011, Hirt 2012, Kensah 2013, Godier-Furnemont 2015, Birket 2015, Jackmen 2016, Tibucy 2017). Depending on what parameters are measured these approaches have varying levels of success although all would claim that the strategy has increased maturation.

In animal models, transverse aortic constriction increases the after load on the heart by placing a band around the aorta and it has been used to study cardiac hypertrophy for many years (Rockman 1991). Although this method causes hypertrophy, using animal models is expensive, time consuming, and there are limited possibilities of experimenting with human cells. 2D culture methods have been developed to try and reproduce hypertrophy which include: stimulation by the α-agonist phenylephrine, or through applying equibiaxial strain on an elastic membrane (Frank 2008, Lee 1996). The problem with these approaches is that cells are not typically isotropically aligned unlike endogenous heart tissue which is highly aligned, moreover, this alignment is integral to the function of the heart. The Eschenhagen group developed a method of producing after load in EHTs using braces placed into silicone posts (Hirt 2012). The addition of metal braces increases the load on the EHT by about 12 fold which induces the hypertrophic gene program
with Atrial Natriuretic Peptide (ANP) and Brain Natriuretic Peptide (BNP) being upregulated (Hirt 2012). In addition, when braces were removed EHTs had longer relaxation times and reduced contraction forces.

Disease modelling is used to investigate mechanisms behind diseases which would otherwise be extremely difficult or unethical in humans. Hypertrophic cardiomyopathies are difficult to model in conventional two dimensional systems because the contraction of a cell sheet does not faithfully recapitulate the contraction parameters of cells in an electrically coupled tissue. EHT has been shown to be able to model hypertrophic cardiomyopathy caused by Myosin Binding Protein-C (MyBP-C, de Lange 2011 and 2013, Stohr 2013). Work with this model showed that EHT with the MyBP-C mutation had altered contraction kinetics including shorter relaxation and contraction times. Moreover, mutated EHTs showed an increased Ca$$^{2+}$$ sensitivity and increased sensitivity to verapamil, isoprenaline and EMD 57033. Thus, taking advantage of the EHT system uses fewer animals while being able to model a complex disease like hypertrophic cardiomyopathy. This approach shows proof of concept that EHTs can be used as an experimental model of hypertrophy which is similar to the effects of mutations \textit{in vivo}.
1.15 Aims and hypothesis of the current study

Study of hypertrophic cardiomyopathy is three-fold: first, investigation into a specific mutation will uncover information about how that mutation causes HCM, second, the disease mechanism may be applicable to all HCM sufferers, and thirdly, a model of HCM can be used to investigate sudden cardiac death. The study of HCM in the current piece of work has two angles: the E99K mouse will be used to investigate HCM, and iPSCs from our E99K patient population will also be used. The study of HCM is also coupled with setting up the EHT platform, including optimizing construct fabrication and setting up the main method to non-invasively follow contractions. We hypothesize that the high level of SCD in the E99K mouse model will allow us to study some aspects of this disease. Moreover, SCD mice have been analyzed to understand if there are any differences between transgenic mice which die early or survive later into life. We hypothesize that iPSCs will have the same phenotype as the patients they were derived from and that we will be able to tease out the HCM phenotype using EHT and subsequent experiments.

1.15.1 Specific hypothesis

We hypothesize that EHT will allow for the phenotype of the E99K mutation to be modelled with iPSCs, and that this platform will allow us to carry out phenotyping experiments which are not possible in patients.

1.15.2 Specific aims

i. Set up the EHT platform including non-invasive measurements of contraction activity.
ii. Development of the EHT platform to optimize the contractile performance of constructs including maturation strategies which may increase contractility.

iii. Study the E99K mouse model including assaying mice which die suddenly.

iv. Ensure iPSCs are derived induced to pluripotency and differentiated. To make sure iPSCs are edited correctly by CRISPR/Cas9 PiggBac technology to produce isogenic controls. To optimize techniques for transporting cells between Nottingham and Imperial (all in collaboration with A Coruna and Chris Denning’s lab).

v. Study iPSC-CM EHT E99K CRISPR/Cas9 PiggBac isogenic lines to assess the action of the E99K mutation.
2 Methods

2.1 Animal husbandry and tissue isolation

All experiments and animal husbandry were carried out in accordance to Imperial College and Home office regulations. Neonatal rats, and mice of various ages including the ACTC E99K transgenic mouse developed in the Marston laboratory were used in this work. Mice and rats were anaesthetised using oxygen and isoflorane before cervical dislocation or beheading was carried out according to Schedule I of the Scientific Procedures Act 1986. Hearts were collected using macro dissection by opening the chest cavity and removing the heart from the major vessels. DNA was extracted using tails and ears which were collected post mortem using the DNeasy Blood and Tissue Kit (Qiagen 69504) as the manufacturers instructions. PCR was carried out on extracted DNA to genotype animals. Ventricular cardiomyocytes were isolated from rats 0-3 days of age by the Miltenyi Biotec Neonatal Heart Dissociation Kit (130-098-373) via the manufactures instructions. Where mice pups were used the whole heart was taken due to the small size of the organ.

2.2 Cell maintenance and differentiation of hiPSC

Cells differentiated by Thusharika Kodagoda.

Undifferentiated cells were maintained in mTeSR/TeSR-E8 (Stem Cell Technologies) and were split at a 1:15 ratio by washing one 6 well with Versene (Thermo Fisher Scientific) and incubating with 1ml of versene for 5 minutes. Versene was aspirated and 1ml fresh media was added to the cells and they were dislodged carefully by pipetting then cells were added to 29.5 ml of media with 10mM Rock Inhibitor (Stratech Scientific Ltd) and cells were plated 2ml per 6 well. Differentiation was
initiated when iPSCs were 80-90% confluent and CHIR 99021 was added at 6µM (Tocris Bioscience) which was called day 0, on day 2 cells were switched to RPMI-B27 minus insulin, on day 3 WNT-C59 2.5µM (Tocris Bioscience) is added, two days later RPMI-B27 minus insulin is added again and cells are maintained in this media until the onset of beating (around day 8-10). At day 11 cells under-go metabolic selection where they are cultured without glucose for 4 days and at day 15 cells are maintained in RPMI-B27 with insulin.

2.3 Cardiomyocyte isolation

Neonatal ventricular cardiomyocytes were isolated from new born rats in the first one to three days of life. The Neonatal Heart Dissociation Kit (Miltenyi Biotec 130-098-373) was used as the manufactures instructions to isolate ventricular cells. Neonatal rat hearts were removed by opening the chest cavity and dissecting away the heart, and the atria and any vascular structures were then removed from the ventricles. Dissected ventricles were placed into a gentleMACS C tube (Miltenyi Biotec) and incubated for 15 minutes at 37°C with an enzyme mix and run on a gentleMACS dissociator. This step was carried out for two repeats. Ventricular cells were suspended in 7.5ml M199 media (vitamin B12, glutamine, 0.5% penicillin/streptomycin, 10% horse serum, DMEM) and the mixture was run through a 70µm cell strainer (BD Falcon). The resulting cell mixture was centrifuged for 5 minutes at 1000 rpm and the pellet re-suspended in 20ml M199 media for counting.

2.4 Thawing iPSC differentiated iCell Cardiomyocytes

Cardiomyocytes from Cellular Dynamics (CMC-100-010-001) were used for making human iPSCs EHTs. Thawed iPSC cardiomyocytes (iPSC-CM) were removed from
1ml vials in a drop-wise fashion into a 50ml falcon tube with about 5 seconds between drops. 1ml of room temperature plating medium was used to rinse out the vial and this media was added to the 50ml falcon tube again in a slow drop-wise fashion. The 2ml mixture was suspended in 8ml plating medium and cells were counted using a haemocytometer.

2.5 Generation of EHTs

EHTs were generated as previously described (Hansen 2010, Breckwoldt 2017). Both rat neonatal ventricular cells and iPSC-CM EHTs were made using the same protocol as previously described (Hansen 2010). 2% agarose made with PBS was melted and 2ml was pipetted into wells of a 24-well plate. A Teflon spacer was used to create a casting mould by placement into the 24-well plate and agarose was left to set for 15 minutes (Appendix 9.1). A master mix was prepared where EHTs were made in a batch of 4 so 2x10^6 neonatal rat ventricular cardiomyocytes (NRVMs) or 4x10^6 iPSC-CM were re-suspended in (for a list of medias see below): 340µl NKM, 60µl 2x DMEM, 10µl bovine fibrinogen. 3µl bovine thrombin was not put into the master mix but pipetted into individual PCR tubes. Fibrinogen was added last to the master mix and mixed by pipetting about 10 times (it can clump together), before pipetting into PCR tubes containing Thrombin. Master mix plus thrombin was then mixed twice and pipetted quickly into agarose moulds with silicone posts (figure 2.1A), if there is a delay when fibrinogen and thrombin are mixed then the hydrogel will solidify within the pipette tip. EHTs were left to solidify for 90 minutes in a standard incubator (37°C, CO₂ 7%, O₂ 40%, humidity 100%) then 300µl EHT media was added to hydrate the gels and EHTs were left for >45 minutes. EHTs made with Matrigel (Sigma) were made by a similar protocol. For four EHTs (+10% extra)
4.4x106 iPSC-CM cells, 24.5µl 2xDMEM, 44µl Matrigel, 0.44µl Y-27632, 11.13µl fibrinogen, 3 µl thrombin in individual PCR tubes. When adding Matrigel to the master mix care must be taken to make sure it is defrosted and mixed into the media properly, fibrinogen is not added at this stage but the master mix is put to cool on ice for about 2 minutes (for more than four EHTs more time is needed). Step by step protocols can be found in the Appendix 9.2. Then fibrinogen was added as before. EHTs were transferred out of agarose moulds into a fresh cell culture plate with 1.5ml EHT media. EHTs were fed with 1.5ml EHT media Mondays, Wednesdays, and Fridays for maintenance. Teflon spacers and silicone racks were washed in de-mineralised water and autoclaved for re-use.

Figure 2.1. EHTs and the white box. A silicone rack is shown being placed into an agarose mould in A. Four EHTs attached to a silicone rack is shown out side of a well plate in B, and inside media in a 24 well plate in C. D is a 3D computer rending of EHTs within blue liquid (from Weinburger et al 2016). A contraction is recorded by the movement of the blue boxes in E which pick up the contrast difference between the EHT and the background. A picture of the outside of the white box is shown in F.
2.6 EHT solutions
Aprotinin - 33mg/ml in cell culture grade water, filter-sterilise, aliquot and store at 20°C.
Fibrinogen - 200mg/ml (sterile) in pre-warmed 0.9% NaCl solution (made up in sterilised cell culture water). Add 72.1 aprotinin (33mg/ml stock) to 25ml fibrinogen solution, mix, aliquot and store at -20°C.
Thrombin: 100U/ml made up in 60% sterile PBS and 40% sterile water. Aliquot and store -20°C.
10x DMEM solution - dissolve 670mg DMEM powder in 5ml cell culture grade water, sterile filter and store at 4°C.
2x DMEM - 10x DMEM solution 2ml, Horse serum 2ml, P/S – 0.2ml, cell culture grade water 5.8ml, sterile filter and store at 4°C.
EHT media - Horse serum 10%, insulin 0.1 (10µg/ml), Aprotinin 0.1% (33µg/ml), 1% penicillin/streptomycin, made in DMEM.
NKM media - Fetal calf serum inactive 10%, glutamine (200mM) 1%, 1% penicillin/streptomycin, made in DMEM.

2.7 Force calculation in EHTs
At the beginning of the current course of study EHTs measurements were taken from video recordings using a modified Labview program developed in our lab (developed by Petr Vikhorev). This was set up to carry out recognition of EHTs which had to be carried out by hand and was laborious. There were several problems with this early software as calibration, EHT size, and optical tracking was set up by hand. The movement of posts were tracked over 30 seconds and force was calculated by analysing the displacement of the posts.
The ‘white box’ (ingeniously called because the entire outside of the box is white, figure 2.1F) was developed in Hamburg specifically to automatically recognise the EHT construct and calculate frequency, fractional shortening, contraction and relaxation time parameters and the minimum, mean, maximum and standard deviation of these parameters (Hansen 2010, Consulting Team Machine Vision, figure 2.2). Contraction parameters are calculated by using edge detection software to pick up the extremities of the EHT where the EHT is formed around the posts (figure 2.1C), which allows the movement of the edges of the EHT to be picked up and tracked over time. The silicone posts which the EHTs are formed around are made to standard conditions (Young’s Modulus 1.7mPa, length 10mm, radius 0.5mm), and therefore, force can be calculated which is directly related to fractional shortening as Vandenbergh et al. 2008 given by:

\[ F = 3\pi ER^4\delta/4L^3 \]

Where:

- \( F \) = Force (N)
- \( \pi \) = the mathematical constant Pi
- \( E \) = Young’s Modulus (mPa)
- \( R \) = Radius (mm)
- \( L \) = Length of the posts (mm)
- \( \delta \) = Post deflection in mm which is calculated by: \( (L^{(EHT)}/FS)\times100 \) where:
  - \( L^{(EHT)} \) = Length of EHT (mm)
  - \( FS \) = Fractional Shortening in %

Velocity can also be calculated from the measurement by obtaining the derivative calculation of the contraction curve at each point. The temperature of the white box
automatically warms up to 37°, and composition of the ambient air can be controlled to set oxygen, carbon dioxide, and nitrogen levels to the same level as a cell culture incubator. A step by step guide to operating the white box is given in Appendix 9.3.

Figure 2.2. Contraction measurements on traces from the white box. An example of an EHT contracting for 2 seconds is shown indicating how different parameters are calculated from contractions (A). Peak contraction is taken at the green boxes and RR scatter as seconds is calculated as time between the two boxes. Time to contraction (T1) is calculated at 10%, 20%, and 50% of the peak from the midline to the edge of the curve, and relaxation time (T2) is calculated in the same way. Contraction Velocity and relaxation velocity are calculated as the derivative of the curve and is shown by the pink line. Each small box on the Red and Pink lines shows a frame taken by the white box camera. B shows a trace over 30 seconds, and C shows the parameters calculated from every trace.
2.8 Extraction of myofibrils from tissue samples

A percussion mortar was used to crush liquid nitrogen frozen heart samples chipped off of whole hearts (weighing around 50mg). The samples were hand homogenised using a micropestle in 1ml wash buffer (5mM NaH$_2$PO$_4$, 5mM Na$_2$HPO$_4$, 0.1 M NaCl, 5mM MgCl$_2$, 0.5mM MgCl$_2$, 0.5mM EGTA, pH 7.0, 5mM Dithiothreitol, 0.1% Triton-X (all Sigma), and 2µg/ml, with E64, chymostatin, leupeptin and pepstatyin (inhibitors from Peptide Institute Inc)) and centrifuged for 3 minutes at 14800rpm. Homogenising, and washing with centrifugation was repeated three times without Triton-X and the final pellet was dissolved in 2X Sodium Dodecyle Sulfate solution (20mM Tris-HCL (Fisher Scientific), 5% Sodium Dodecyle Sulfate (herein SDS, Fisher Scientific), 10% β-mercaptoethanol (Sigma), 10% glycerol (Sigma), 0.05% Bromophenol Blue (Sigma)) at 90°C for 5 minutes before being aliquoted.

2.9 Western Blots and phosphate affinity SDS PAGE

Western blots and phosphate affinity SDS PAGE were carried out using standard methods (Messer 2009). SDS-page mini gels (Biorad) were run for 1 hour at 200V and proteins were transferred using Trans Blot Turbo transfer system (Biorad) to a nitrocellulose membrane before staining using Pierce Reversible Protein Stain Kit (Thermo Scientific) as the manufactures instructions. Following washout of the protein stain membranes were put into blocking solution (20% Sea block (Thermo Scientific) 0.05% Tween-20 (Sigma in PBS (Sigma)) for one hour. Primary antibodies were incubated overnight at 4°C and secondary antibodies incubated for one hour at room temperature (antibodies in Appendix 9.4, both in blocking solution). After washing out of antibodies proteins were visualised using Amersham ECL prime western blotting reagent (GE Healthcare) for 5 minutes in a G Box (Syngene).
Phosphate affinity SDS PAGE was carried out exactly as the western blot protocol but with custom made gels: Resolving gel: de-ionised H$_2$O 3.78ml, 30% acrylamide 29:1 3.33ml (BioRad), 1.5M tris HCL pH 8.8 2.5ml, 10% SDS 100µl, 10mM MnCl$_2$ 100µl (Sigma), 5mM Phostag-acrylomide 100µl (Wako Chemicals), N,N,N',N' Tetramethylethlenediamine 28.6µl (herein TEMED, Sigma), 10% Ammonium Persulfate 143µl (herein APS, Sigma). Stacking gel: de-ionised H$_2$O 6ml, 30% acrylamide 29:1 1.32ml, 0.5M tris HCL pH 6.8 2.52ml, 10% Sodium Dodecyle Sulfate 100µl, TEMED 20µl (Sigma), APS 100µl. Gels were run starting at 25V until samples hit the resolving gel then the voltage was gradually increased over time to 100V until Bromophenol blue dye ran off the end of the gel. Densitometry of bands was calculated using G Box (Syngene) software and percentages were calculated compared to adult WT.

2.10 Fixing hearts for cryosectioning
Freshly dissected hearts were stored in liquid nitrogen. They were gradually thawed and then fixed in 4 % paraformaldehyde (PFA, Sigma) overnight at 4°C and cryo-protected with 30% sucrose (Sigma). The fixed hearts were frozen in optimum cutting temperature (OCT, Sigma) compound vertically, and transversal cryosections were cut at 7 µm intervals.

2.11 Wheat germ agglutinin (WGA) staining for cell size
For WGA staining, sections were rehydrated with PBS. Slides were blocked with 20% goat serum diluted in PBS for 30 minutes at room temperature. Then, slides were incubated with Alexa Fluor 488 conjugate of WGA (10 µg/ml, diluted in PBS, Invitrogen) for 30 minutes with protection from the light. Slides were washed twice
with PBS, mounted in Vectashield with DAPI (Vector Labs), and covered with 
coverslips. Images were recorded by fluorescence microscopy (Zeiss Axio Observer 
Inverted Widefield Microscope with LED illumination) at excitation and emission of 
495 nm and 519 nm, respectively. For each section, 4 images were captured using 
20x magnification and in each image 50-60 cells were analysed using ImageJ 
software.

2.12 Picro-sirius red staining for collagen
Sections were rehydrated with distilled water and stained in a solution of 0.1% Sirius 
red F3BA in saturated aqueous picric acid (Sigma) for 35 min at room temperature, 
followed by 4 washing steps in 100% methanol, as modified from other studies 
(Bauman 2014). Subsequently, the slides were rinsed twice in xylene, for 10 min 
each. Slides were mounted with Vectashield with DAPI (Vector Labs), covered with 
coverslips and left to dry overnight at room temperature. A series of images of one 
section were acquired at 10x magnification and were automatically stitched together 
using bright field mode of the Ziess LSM-780 inverted confocal laser scanning 
microscope. Collagen content was quantified in Image J software. Red colour was 
separated with following colour threshold: Hue 1-6, Saturation 10-255, and 
Brightness 20-255, as modified from Bauman 2014. Total collagen content was 
defined as the proportion of positive red pixels to total pixels of stained area.

2.13 Immunocytochemistry
EHTs were washed 2x 5 minutes with PBS on a rocker still attached to posts. EHTs 
were fixed with Histofix (Carl Roth GmbH, P087) overnight at 4 ºC still attached to 
posts. EHTs were washed 2x 5 minutes with PBS and removed from the silicone
posts by cutting the EHTs with scissors, at this point EHTs can be stored in PBS at 4 °C. EHTs were permeabilised/blocked for 24 h with blocking solution (Tris-buffered saline 0.05 M, pH 7.4, 10% FCS, 1% BSA, 0.5% Triton X-100 (all Sigma)) at 4 °C on a rocker in a 2ml tube. After permeabilisation, EHTs were stained overnight at 4 °C in antibody solution (blocking solution without FCS) with primary antibodies on a rocker in a 2ml tube. EHTs were washed 3x 5 minutes with PBS at room temperature and secondary antibodies were applied and incubated overnight at 4 °C in antibody solution on a rocker in a 2ml tube in the dark. EHTs were washed 3x 5 minutes with PBS in the dark at room temperature. If nuclei staining was needed Hoechst 33342 was used for 10 min at room temperature on a rocker in a 2ml tube in the dark. EHTs were washed 1x 5 minutes in the dark at room temperature and constructs were mounted in Vectashield mounting media with Dapi between a cover slip and an indented glass slide (Academy, single cavity). EHTs were visualised using a Zeiss Confocal Microscope.

2.14 Two photon tissue preparation and staining

Two photon microscopy and papillary muscle preparation was taught by Valentina Caorsi and the method published in Caorsi et al 2013. Papillary muscles were taken out of hearts by opening the left ventricle and carefully removing one end of the muscles and cutting along the ventricular wall while in Krebs solution (NaCl 118mM, KCl 4.75mM, MgSO₄ 1.18mM, CaCl₂ 2.5mM, KH₂PO₄ 1.18mM,NaHCO₃ 24.8mM and Glucose 10mM, BDM 30mM which were added freshly each day of dissection. Papillary muscles were placed in 2% Triton-X for 30 minutes for permeabilisation and then left overnight in glycerol. For consistency between images the same two photon settings were used: 900nm excitation wavelength, 12.5% filter wheel, averaging of 4
for all images, using 1µm steps in Z stacks and the photomultiplier tube was set at half the excitation wavelength to pick up second harmonic generation (440-460nm). Levels of collagen were determined by taking measurements of a clear area which counts as background and subtracting each score in each slice from the total score of the slice from second harmonic signals to background fluorescence. The scores were then added over a given slice range giving values of normalised fluorescence.

2.15 RNA Extraction (hearts)
Heart tissue (20mg) was homogenized and lysed in 350µl TRI-reagent (Sigma) Qiagen RNA Easy protocol (Sigma) following the manufactures instructions. RNA concentrations were normalised and cDNA was made by reverse transcription for qPCR by standard protocols (Applied Biosystems 1308188). Primers used are listed in Appendix 9.5.

2.16 RNA Extraction (EHTs)
RNA was extracted from two dimensional cultures by pooling four wells each with 90ul of TRI-reagent (Sigma) by titration and scraping with a pipette. One EHT was placed in 350ul tri-reagent and EHTs were homogenised by hand until cells were mostly in solution. RNA was then extracted from two dimensional and EHT samples via the Qiagen RNA Easy protocol following the manufactures instructions.

2.17 Gene expression analysis
RNA concentrations were normalised and cDNA was made by reverse transcription (Applied Biosystems 1308188) following the manufacturer’s protocol. Real time PCR was carried out via the manufacturer’s protocol to analyse gene expression. All
samples were run with and normalised to GAPDH. Delta cycle threshold was calculated by cycle threshold of the gene of interest minus GAPDH cycle threshold. Delta delta cycle threshold was calculated by delta cycle threshold minus control delta cycle threshold. Fold change was calculated by averaging minus delta delta cycle threshold values squared. Primers for rat, human and mouse are listed in Appendix 9.5.

2.18 Ca$^{2+}$ Transients

10µg Fluo4-AM (Invitrogen) was diluted in 50µl pluronic (Invitrogen). 6µl or 1µl was added to EHT or 2D cultures, respectively and cells were left for 30 minutes. Videos were taken under a Zeiss AxioObserver Microscope. Intensity profiles were plotted and transient kinetics were extracted and analysed by hand in Clampfit. Time to peak was calculated by reading off the time at baseline and subtracting this number from the time at peak voltage. Curve decay parameters were determined by calculating 50%, 75%, and 90% of the peak voltage, and then moving to the respective voltage and reading off the time in Clampfit.

2.19 Calcium spark measurement

Cell were loaded with 5µM Fluo-4AM (Sigma) and 0.16% Pluronic (Sigma) for 20 minutes on a rocker. Cells were then centrifuged at 400rpm for 1 minute. The supernatant was discarded and cells were left for another 20 minutes prior to being imaged. Cells were mounted on a line scan microscope and perfused with Tyrode’s solution (2mM Ca$^{2+}$, NaCl 140mM, KCl 6mM, Glucose 10mM, HEPES 10mM, MgCl$_2$ 1mM). Confocal laser scanning microscope was pre-set at 10% Argon laser power, using Lasersharp 2000 software. Cells were initially paced at a frequency of 0.5 Hz.
for 2 minutes, the laser shutter was opened initially at a rate of 1200 lines per second, to visualise the cell and nuclei. Laser shutter speed was then set to 500 lines/second in order to record Ca2+ sparks, and acquisition was carried out for 24 second (12,000 lines). The cell was then paced at 3Hz for 30 seconds, after which spark acquisition was carried out for 5 seconds.

2.20 Calcium spark analysis
Images were analysed using the spark master plug in for Fiji’s image J software. The Ca$^{2+}$ spark frequency, spark amplitude, full spark duration at half maximum (FDHM) and full spark width at half maximum (FWHM) were all automatically calculated, and from these spark mass and spark-mediated SR leak can be calculated from the formula below (Hollingworth et al 2001).

\[
\text{Spark mass} = \text{Amplitude} \times 1.206 \times \text{FWHM}^3
\]

\[
\text{Spark-mediated SR leak (Time)} = \text{Frequency} \times \text{FDHM}
\]

\[
\text{Spark-mediated SR leak (Mass)} = \text{Mass} \times \text{FDHM}
\]

2.21 Methods in the next section were conducted in Chris Denning’s lab in Nottingham

2.21.1 Derivation of fibroblasts

Skin punch biopsies were transported from the clinic into the laboratory in Transport media (1X HBSS (minus Ca and Mg), 1% Penicillin and Streptomycin, 1% Furazolidone) and washed three times on arrival in the same, after which they were transferred into bacterial-grade Petri dishes. Here, using sterile forceps and scalpels, the adipose and epidermal tissue was removed and the remaining sample cut into 1mm pieces. The cut tissue was digested, first with 2.5% trypsin for 20 minutes, and
then with 1mg/ml collagenase IV for 90 minutes, both at 37°C. The digested cells
were spun down at 200xg for 5 minutes, plated in FIB medium (20% FBS, 1% NEAA,
2mM GlutaMax, 100µM β-mercaptoethanol) and grown for 2-3 weeks until confluent,
with medium changes every 3-4 days. Once confluent, 1ml of spent medium was
analysed for mycoplasma contamination. When declared mycoplasma-negative,
cells were then expanded and cryopreserved.

2.21.2 Sendai virus reprogramming

Primary cells were plated in one well of a six well plate at a density of 2x10^6 per well.
The following day three tubes containing the viral vectors contained in the
CytoTune2.0 kit (Thermo Fisher) were taken from -80°C freezer and thawed briefly in
a water bath at 37°C. A calculated volume of virus from each tube was then added to
3ml of fibroblast medium which had been prewarmed to 37°C. Spent medium was
aspirated using plastic pipette and the new medium, containing virus was added to
the cells. This was incubated at 37°C with 5% CO₂ overnight. The following day
medium was changed to 3ml of fresh fibroblast medium and this was repeated until
the cells reached ~80% confluence. At this point medium was removed and cells
washed in 3ml PBS. This was then aspirated and 500µl 0.05% trypsin was added to
the well, this was incubated at 37°C for up to 2 minutes. 500µl FIB medium was then
added to the well and this 1ml was mixed and then added to 4ml of FIB medium in a
universal tube. The mixture was then centrifuged at 200g for 5 minutes. Supernatant
was removed and cells resuspended in 3ml of Essential 6 medium supplemented
with 100ng/ml bFGF. Three 35mm dishes were prepared with vitronectin-peptide and
the cells were added to these at a 1:3 dilution. Medium was changed daily with fresh
E6 + FGF added until the emergence of established colonies at which time the
medium was changed to Essential 8 medium. Following this medium was changed daily until colonies had reached a size ready for isolation. Isolation was performed using manual dissection.

2.21.3 Fixing cells
Cells were fixed using 4% paraformaldehyde. Medium was removed from cells which were then washed in PBS. Cells were predominately fixed whilst in 96 well plate format therefore 100μl PBS was added then aspirated from the wells using a multichannel pipette. 100μl of 4% paraformaldehyde was then added to the cells and this was incubated at room temperature for 15 minutes. After this the paraformaldehyde was then removed and the cells washed twice with 100μl PBS. Cells were then stored with 100μl PBS per well at 4°C for up to two weeks.

2.21.4 Immunostaining
Cells were plated in flat bottomed 96 well plates at a density of 10-20,000 cells per well. Medium was changed after 24 hours and cells were fixed at 48 hours. Details of the dilutions and antibodies used can be found in Appendix 9.4. Cells were first washed in PBS using 200μl per well. If cells were to be stained for nuclear markers then they were then incubated for 10 minutes at room temperature in 200μl of 0.1% Triton X-100, this was not carried out on cells which would be stained for surface markers. Cells were then washed in 0.1% PBS-Tween three times using 200μl per well each time. Cells were then blocked using 10% species block made up in PBS, species being that which the secondary was raised in. 200μl was added to each well and this was incubated for 1 hour at room temperature. This was then aspirated and cells washed twice using 200μl 0.1% PBS-Tween. After this washing step cells were
incubated in 50μl of primary antibody made up in 1% species block. Plates were wrapped in parafilm and left at 4°C overnight. The following day the primary antibody was aspirated and the cells washed three times using 200μl of PBS-Tween per well. This step included a five minute wash on a shaker. This was then aspirated and the cells treated with 50μl secondary antibody made up in 5% species block, this was incubated on a shaker at room temperature for 1 hour while plate was covered in foil. Secondary antibody was then removed and cells washed three times in 200μl 0.1% PBS-Tween, these washes also include one 5 minute was on a shaker at room temperature. Cells were then stained for DAPI by adding 50μl of DAPI in PBS to each well and incubating at 4°C for 15 minutes. This was then removed and cells washed twice with 200μl of 0.1% PBS-Tween then once in PBS. A list of antibodies used in this thesis is shown in Appendix 9.5.

2.21.5 Cell culture
All cell culture was at 37°C at 5% CO2 in a humidified atmosphere. Fibroblasts were derived under ethical consent from individual (Biomedical Institute of A Coruna, INIBIC). Reprogramming to hiPSCs was via CytoTune 2.0 (ThermoFisher), according to the manufacturer's instructions. Culture was in E8 medium on Matrigel, passaging approximately every 3 days with Accutase.

2.21.6 Targeting vector construction
The ACTC1 targeting vector was constructed through a four fragment Gibson assembly. Overlapping PCR fragments were produced for three DNA fragments; the dual drug selection cassette (Puro-ΔTK) flanked by PiggyBac recombination sites, and the two ACTC1 sequences (1kb upstream and 1kb downstream) homologous to
the endogenous target locus cut site. An EcoRV digested pBluescript backbone plasmid sequence was used as the fourth DNA fragment in the Gibson assembly. A 20µl reaction containing 0.05 pmol of each DNA fragment and 1X Gibson Assembly® Master Mix (NEB) was heated at 50°C for 60 minutes. Subsequent transformation into competent cells and colony sequencing identified correctly assembled plasmids.

2.21.7 Transfection in hiPSCs

One million hiPSCs were transfected with 3 μg of: PiggbyBac, guide RNA, and Cas9 plasmid components (Appendix 9.6) using Amaza 4D system with P3 solution, program CA-137. Transfected cells were maintained in stem cell maintenance E8. After 24h post-transfection, the medium was supplemented with Puromycin (0.25 µg/ml) for positive selection of clones up to two weeks. The puromycin-positive clones were then harvested using either Accutase or TryPle Selection following by seeding into Matrigel-coated 6 well plate at the seeding density of 3x10^5 cells/well. The collected cells were then cultured for 24h and subjected for Transposase plasmid transfection (3µg) using Fugene HD transfection reagent (Reagent:DNA was 4:1) and maintained in normal culture medium for 24h with/without ROCKi. On the next day, the cells were then exposed to medium containing Ganciclovir (2µg/ml) for negative selection of PiggyBac excision clones for up to 2 weeks. The clones were manually dissected and genotyped using analysis primers as shown in Table 2.1 for identification of corrected clones.

| NM_005159 | Primer a1 | Genotyping | Forward | GGTGATGAAGCCCAGAGCA | Product | GGTGATGAAGCCCAGAGCA | GGTGATGAAGCCCAGAGCA | GGTGATGAAGCCCAGAGCA |
| NM_005159 | Primer a2 | Genotyping | Reverse  | GTGGTGACAAAGGAGTAGCC |         | GTGGTGACAAAGGAGTAGCC | GTGGTGACAAAGGAGTAGCC | GTGGTGACAAAGGAGTAGCC |
| NM_005159 | gRNA     | Targeting  |          | GAGTTAACAGTAGTGCCC   |         | GAGTTAACAGTAGTGCCC   | GAGTTAACAGTAGTGCCC   | GAGTTAACAGTAGTGCCC   |

2.1 Table of primers for clones.
2.21.8 Cardiomyocyte differentiation:

Undifferentiated hPSCs were seeded onto Matrigel-coated dishes at a density of 4x10⁴ cells/cm², and allowing to expand for 48h until reaching 80% confluent. At this stage (d1 of differentiation), the culture was treated with medium comprising StemPro34 supplemented with 1:100 dilution Matrigel and 1 ng/ml BMP4 (R&D systems). After 24h (d2 of differentiation), medium comprising StemPro34 with 10 ng/ml BMP4 and 8ng/ml Activin A (Life Technologies). Medium exchange was performed on d4 of differentiation using medium comprising RPMI base supplemented with 1xB27 (Life Technologies) and a small molecule inhibitors, KY02111 (10 μM) and XAV939 (10 μM R&D systems). From d8 onwards, cells were maintained in RPMI medium supplemented with B27 only, with medium changes every 3 days. The cardiac differentiation efficiency was accessed by using immunocytochemistry. To count the number of cells, DAPI staining was used at 1:500 dilution and the immunofluorescence pictures were captured using Operetta High-content imaging system (Perkin Elmer) and analysed using Harmony high-content imaging analysis software.

These were done by James Smith

2.22 EHT transport

EHTs were transported in 24 well plates with 2ml of fresh EHT media and plates were sealed by parafilm EHTs were then placed into polystyrene boxes with gel pads heated to 37°C and transported by train to Imperial College.

2.23 Calcium exposure experiment
EHT were placed into Tyrode’s solution: NaCl 140, KCl 4.5, glucose 10, HEPES 10, MgCl₂ 1, CaCl₂ variable, pH set to 7.4 by NaOH (all mM concentration). EHTs were placed into increasing concentrations of CaCl₂ from 0, 0.1, 0.25, 0.5, 1, 1.5, 3, 5 (all in mM) for 40 minutes to one hour and measurements were taken in the white box while EHTs were stimulated at 1Hz.

2.24 Arrhythmia analysis

The contraction of EHTs is recorded frame by frame by the white box and these numbers are saved in an Excel file. Contractions were copied into another Excel file which acts as the template for arrhythmia analysis (Appendix 9.7.1). Averaging by ten was carried out to avoid the system picking up false peak in jagged traces. Contractions averaged by ten were copied into notepad and then opened in Clampfit where contraction traces were created, the sampling interval should be set at 10Hz (ten-fold higher because of averaging). A threshold search is then carried out with a ‘baseline’ and a ‘contraction count line’ used to identify events (contractions are named events by the software (Appendix 9.7.2). This produces a results page in Clampfit with the length of each contraction, which is copied into Excel (Appendix 9.7.3). To find out the length of a normal contraction 211, 113 and 218 contractions (without aftercontractions) were taken from Donor I-III WT lines. After the length of a WT contraction is known then each individual contraction can be compared against this number, and from this, contractions were considered abnormal if a contraction is longer or shorter than 25% of a normal contraction (Appendix 9.7.4). Using the same process the time in between contractions can also be calculated, and short or long lengths of time in between contractions also be identified.
2.25 Statistics

Experimental data was plotted in GraphPad Prism and all error bars indicate standard error of the mean. Comparisons with multiple samples with one variable were analysed by one-way ANOVA with Tukey’s correction. Comparisons with multiple samples with two variables were analysed by two-way ANOVA with Bonferroni’s correction. Student’s t-tests were carried to test for significance in two groups with one variable being assessed. A p value of less than 0.05 was considered significant and significance was indicated as: * p<0.05, ** p<0.01, *** p<0.001.
3 Results Chapter 1. The properties of rat and human engineered heart tissues

Culturing Engineered Heart Tissue (EHT) involves mixing a cell master mix with fibrinogen and combining this with thrombin. The addition of the thrombin enzyme causes the polymerisation of fibrinogen to fibrin, the mixture is then pipetted into a mould, and is left to set around two silicone posts. The critical aspect of the casting process is the concentrations of thrombin and fibrinogen. A range of concentrations was tried until an optimal concentration was found which allowed EHTs to spontaneously beat and to adhere to silicone posts. Although both human and bovine fibrinogen was used in generating EHT, bovine fibrinogen was produced at a higher and more accurate concentration, which made it easier to handle and led to more standardised conditions.

3.1 Basic characteristics of EHTs

Over time EHT would reduce in size in all dimensions, however this effect is most prominent in the x axis. Depending on the quality of the cells, single/clusters of cells can be seen beating inside the fibrin hydrogel at 1-3 days. After this point areas of cells would be seen beating at around 3-5 days and whole construct contractions at around 5-9 days. EHTs would progress in this manner as you expect from cells making progressively more cell to cell contacts in a hydrogel. Measurable post deflecting force would then increase over time with peak contractility to come at around 10-14 days. The length of EHTs reduces throughout time and because of the nature of the silicone posts this causes a preload on the constructs bending the posts towards each other slightly. Cells then align along force lines between posts throughout each EHT, which can be visualised in whole mount preparations by staining of Troponin T (figure 3.1A). Cells in rat EHTs (rEHTs) can be seen entering
the cell cycle with positive Ki67 staining (figure 3.1B). In rEHT preparations fibroblasts are present at around 50% of total cell number and can be stained with the marker Vimentin, which localises fibroblasts around the outside of the EHT (figure 3.1Ciii). Nuclei were stained for using Dapi and show cells were compacted together in the hydrogel (figure 3.1B and Ci), and moreover, nicely organised sarcomeres can also be seen in myofibrils (inset in figure 3.1Cii).
Figure 3.1. Immunohistochemistry images of rEHT. rEHT was stained for Dapi and Troponin T in A. B shows Ki67 positive cells present in EHT at day 14. In C a rEHT is stained for Dapi, Phalloidin and Vimentin (i-iv respectively). The inset in Cii is a zoomed in image of phalloidin staining showing myofibres.
3.2 Variations in different iPSC lines

Contractions of human EHTs (hEHTs) are constantly spontaneously rhythmic with little variation in peak height of contractions after around day 10. Contraction of different iPSC-CM lines of hEHTs is shown in figure 3.2, it must be noted that different lines of iPSC-CM produce varying amounts of force. The different way these lines are differentiated may explain some of the variation, however this is most likely multifactorial. Most iPSC-CM differentiations are pure with Axiogenesis and Cellular Dynamics reporting over 99% purity in their cell lines, and our differentiations at Imperial being >95% after metabolic selection. The Nottingham group get varied levels of cardiomyocytes in their preparations with JRSG WT line having around 70% cardiomyocytes, which probably accounts for its lower force (open circles and dotted line in 3.2A). The Axiogenesis cell line tended to have a higher beat rate compared to other lines which generally beat at just over 1Hz (figure 3.2B), and this faster beat rate was reflected in the relaxation and contraction times in that line (figure 3.2C and D respectively). Preparations within the same batch of hEHT from all lines showed little variation in force, frequency, or contraction and relaxation times (figure 3.2A, B, C, D respectively).
Figure 3.2. Contractile characteristics of iPSC-CM from different lines. 6 different iPSC-CM lines contractile performance is shown as force (A), beats per minute (B), contraction time at 20% of peak contraction (C), and relaxation time at 20% of peak contraction (D). Error bars represent standard error of the mean. N= 8 Axiogenesis, 4 Cellular Dynamics, 7 Imr90 (Imperial), 12 ASS WT (Nottingham), 7 JSS WT (Nottingham), 11 JRSG WT (Nottingham). Axiogenesis and Cellular Dynamics cells were bought in and defrosted, Imr90 (Imperial) were expanded and differentiated by T. Kodogoda, ASS WT, JSS WT and JRSG WT were expanded and differentiated by J. Smith.
3.3 Extended culture and Matrigel effect on contractility

Different culture conditions have been shown to increase the contractile performance of hydrogel constructs and other properties of two-dimensional iPSC-CM cultures. Therefore, we wanted to test some of these approaches in our hEHTs. Length of time in two-dimensional culture after differentiation was recently shown to increase force production in iPSC-CM hydrogels (Tiburcy 2017). Therefore, iPSC-CM cells were kept in culture after differentiation until day 18 or day 50 and EHTs were generated and shown in figure 3.3 (day 18 and day 50 were independent batches, Day 0 refers to the day EHTs were generated). Force was shown to be dramatically increased in cells left in culture for a longer time (figure 3.3A p<0.001 N=6,16).

Matrigel has been reported by multiple groups to increase the level of force in hEHTs and in some cases to be essential (Guo 2006, Naito 2006, Qin 2016). The addition of Matrigel needs the master mix solution to be cooled on ice and Y-27632 is added to attempt to stop cell death. Figure 3.3B shows force production between hEHTs cultured for 50 days and shows no significant differences between the two conditions. Kensah 2013 reported that the chronic addition of 10µm noradrenaline in culture medium could increase the level of force of their constructs. In our hEHTs a relative increase in force was seen in the noradrenaline group after the application of noradrenaline, however, this was not significant when compared to control by two-way ANOVA (figure 3.3C). To determine if Matrigel or longer culture was having an effect on the calcium cycling calcium transients were taken using fluo4-AM while stimulating the hEHTs at 1Hz. Time to peak, and calcium transient decline at 50%, 75% and 90% is shown in figure 3.3Di-iv respectively with a significantly longer difference only seen in the time to peak of day 18 hEHTs (figure 3.3Di N=3).
Figure 3.3. Assaying different culture conditions and their effect on human EHTs. Imr90 iPSC-CM were differentiated and kept in culture either 18 or 50 days before EHTs were generated and force was tracked over time (A, N= 16 day 18, 6 day 50). Culture day 50 cells were used to generate EHT constructs with and without Matrigel and force is shown in B (N= 10 Matrigel, 6 day 50). Cellular Dynamics EHTs that had been cultured for 35 days were treated with Noradrenaline 10µM on day 0. Relative force was calculated by normalising to day 0 and force was tracked over time compared to untreated controls (N=4 control,3 noradrenaline). Calcium transients were taken of hEHTs using fluo4-AM and stimulated at 1Hz in: day 50 Matrigel, day 50 and d18 hEHTs (D, N=3). Time to peak (TTP Di), calcium transient decline at 50% (Dii), 75% (Diii) and 90% (Div) of peak is shown. All error bars represent standard error of the mean. Significance testing was carried out by Two-way ANOVA in A,B,C or one way ANOVA in D. *=p<0.05, *** = p<0.001.
3.4 T3, IGF-1 and Dexamethasone effect on contractility in combination with stimulation

The Mummery group has published that the application of: 100nM T3, 100ng/ml Long form R3-IGF-1, and 1µm Dexamethasone (herein TID) can produce a more mature phenotype in iPSC-CM (Birket 2015), and this has also been shown to be effective for force development in hydrogels (Schwan 2016). We also tested the combination of TID in our hEHT platform in conjunction with the addition of chronic electrical stimulation (figure 3.4). TID significantly increased the beats per minute of our hEHT and this was reflected in faster contraction and relaxation times (figure 3.4A, C and D), however, the force development in these constructs did not increase (figure 3.4B). TID with the addition of chronic stimulation also did not increase the force production of these hEHTs either compared to TID alone or controls (figure 3.4B). Interestingly, the increase in frequency of the TID treatment was attenuated by chronic stimulation when stimulation was initiated from day 5 onwards (figure 3.4A). Moreover, when stimulation of hEHT was stopped at day 16 it took four days for beating frequency to increase (figure 3.4E). Furthermore, chronic stimulation of hEHTs requires changing of electrodes daily, but even with washing protocols, stimulated hEHTs contraction was seen deteriorated on day 14 compared to control (figure 3.4B).
Figure 3.4. Culturing human EHT with T3, IGF, and Dexamethasone (named TID for short). T3 (100nM), Long for R3-IGF-1 (100ng/ml), and Dexamethasone (1µm) was applied to Imr90 iPSC-CM EHTs from day 0 onwards (called T3 IGF Dex, N=6). EHTs cultured with T3, IGF-1 and Dexamethasone were also chronically stimulated (called Stim T3 IGF, Dex N=3) and were compared to control cells cultured under the same conditions (N=3), # marks where stimulation was initiated. EHTs were analysed over time and beats per minutes are shown in A, force in B, contraction time at 20% of peak in C, and relaxation time at 20% of peak in D. Stimulation was removed at day 16 and measurements were made at 18 and 22 days (E). All error bars represent standard error of the mean. Significance testing was carried out by Two-way ANOVA * = p<0.05. ** = p<0.01.
3.5 Batch variability, increased numbers of cells, chronic stimulation and increased ambient oxygen in EHTs

It has previously been showed that rEHTs contractile parameters vary by batch (Hansen 2010). In our lab cells are isolated from rat neonatal hearts and a different isolator is used each week. In figure 3.5A batch to batch variation is shown in rEHTs with 32 EHTs from six batches being shown with contractility between 0.15mN and 0.2mN. The best and worst performing groups show that the difference between batches can be significantly different, and this was taken into context for subsequent experiments. We have also tested differing culture conditions in our rEHTs. Increasing the number of cells from half a million to one million cells per construct significantly increased the level of force development over time, with $1 \times 10^6$ cell rEHTs contracting stronger earlier and producing more maximum force (figure 3.5B). Similarly to hEHTs, chronic stimulation did not increase the force of rEHTs (figure 3.5C). EHTs are under auxotonic mechanical stimulation from silicone posts which allows EHTs to contract against load caused by the elastic modulus of silicone. Hydrogels and trabeculae from hearts have maximal contraction when stretched a process defined by the Frank-Starling law. It was thought that extra load may allow EHTs to carry out more work and thus increase the force of contraction. Therefore, custom made stretchers, which gave rEHTs an extra pre-load by stretching the hydrogels, were compared against wild type in figure 3.5D. However, no significant differences were seen between the two groups. The Eschenhagen group carry out their EHT culture in 40% oxygen (Scaaf 2010), and so the ambient level of oxygen was increased and compared against normal levels of oxygen in rEHTs, showing no significant differences between the two conditions (figure 3.5E).
Figure 3.5. The effect of different culturing conditions in rat EHTs. 6 batches of neonatal rat ventricular heart preparations with fibroblasts not removed (rat) EHTs are shown and the worst and best batches are compared by force generation over time (A, N=32, 6 batches, 7 best batch, 7 worst batch). Rat EHTs were made with either 1x10^6 or 0.5x10^6 cells and were compared over time B. Electrical stimulation (C, N=3 control, 4 stimulation), increased mechanical load (D, N=8 control, 4 increased mechanical load) and increased ambient oxygen (E, N= 6 control, 6 high O2) were tested and force is shown over time. All error bars represent standard error of the mean. Significance testing was carried out by Two-way ANOVA * = p<0.05. ** = p<0.01, *** = p<0.001.
3.6 Spontaneous beating activity in rEHTs

Adult cardiomyocytes do not spontaneously beat and this is considered a hallmark of differentiation which neonatal rat cells and iPSC-CM do not have. In contrast to adult cells EHTs spontaneously beat and will continue beating spontaneously in culture. It was noticed that rEHTs made with higher numbers of rat neonatal cells would slow their beating rate in culture, and some would stop spontaneously beating. Thus, rEHTs were made from 0.5, 1, or 2 million cells and compared in figure 3.6A. The trend for frequency of beating would decrease the longer all rEHTs were kept in culture, and rEHTs made with $1\times10^6$ cells had an average of 14 beats per minute at day 14, while rEHTs with $2\times10^6$ cells had stopped beating altogether at day 11. rEHTs beat in a burst like fashion, and although it is unlikely that a healthy rEHT would not beat in a one minute measurement this is an inherent problem in these data. Therefore, $1\times10^6$ rEHTs were chronically stimulated for two weeks at 5Hz initiating stimulation on day 3 and compared against $1\times10^6$ rEHTs without stimulation from the same batch. At day 13 in a one minute measurement no spontaneous beating was recorded (figure 3.6Bi). Spontaneous and stimulated rEHTs were also measured for five minutes without stimulation to make sure contractions had not been missed, and only one contraction was observed from the spontaneous group (figure 3.6Bii, 0 b.p.m stimulated N=4, 0.07 b.p.m spontaneous N=3). Then to demonstrate that all these rEHTs were still able to contract they were stimulated and contractions were recorded (figure 3.6Bii N=4 stimulated, N=3 spontaneous).
Figure 3.6. Loss of spontaneous beating activity in rat EHTs. Neonatal rat ventricular heart preparations with fibroblasts not removed EHTs are shown generated with 2, 1 or 0.5 million cells per EHT. The beats per minute of EHTs were followed over time in A and the percentage of EHTs beating is shown in numbers above (N=8 $1 \times 10^6$, N=3 $2 \times 10^6$). EHTs were chronically stimulated for two weeks at 5Hz (Stimulation) and compared against control EHTs from the same batch. Beating activity on day 13 is shown in Bi with no spontaneous beating recorded (N=3 Control, N=4 Stimulation). A five minute trace of a non-stimulated $1 \times 10^6$ EHT is shown in Bi and the same EHT is shown with stimulated contractions in Bii.
3.7 Positive inotropic responses in EHTs

A positive response to increasing concentrations of external calcium is a property of all adult cardiomyocytes and this relationship has also been investigated in EHTs (Scaaf 2010, Pieske 1998). We also tested our iCell cardiomyocytes for their response to calcium in figure 3.7A where force and beat rate are shown. From these same data, we also plotted the contraction and relaxation times of hEHTs (figure 3.7B). A positive force frequency relationship (FFR) is found in adult cells and is usually lacking in iPSC-CM, however, some reports in EHTs have found this relationship (Godier-Fernemont 2015, Tiburcy 2017). We investigated FFR in our rat and human EHTs but never found a positive FFR (figure 3.7B and C), moreover, this parameter was difficult to quantify because of how EHTs respond to increased force and how the white box measures this force (figure 3.7D). A positive response to isoprenaline displays a fully differentiated β-adrenergic system and is indicative of adult cardiomyocytes. Earlier it was shown that there were meagre increases in force when noradrenaline was applied to hEHTs (figure 3.3C), and here, we also tested isoprenaline which targets all three β receptors in hEHTs (figure 3.7E). A small increase is shown with the addition of isoprenaline at 1.8mM Ca$^{2+}$, however, this was not significant by one way Anova. Lastly, force per unit area is shown together with absolute force generation in rEHTs, noteworthy here is that from the onset of beating force per unit area is linear even though absolute force is sigmoidal (figure 3.7F).
Figure 3.7. Contractile properties of human and rat EHTs. hEHTs were placed into increasing concentrations of calcium and force and beats per minute were plotted (A), the corresponding contraction and relaxation at 20% of peak contraction are also shown in Ai (N=6). rEHTs (B, N=8) and hEHTs (C, N=8) were exposed to increasing stimulation frequencies 0.5,1,2,3,3,5,10Hz in B and 0.5-5Hz in C. D shows individual measurements of rEHTs stimulated at different frequencies. 1µm isoprenaline was applied to hEHTs in E and the force is given as % change from baseline when stimulated at 1Hz. The absolute force in mN and force as a unit of area in mN/mm² is plotted from the same batch of rEHTs in F (N=12).
3.8 3D vs 2D calcium transients

Recently, Torsten Christ’s group has thoroughly investigated calcium transients in two dimensional cells and compared them to cells isolated from EHTs and adult cardiomyocytes (Uzun 2016). We have also investigated the effect of three dimensional culture in whole EHT constructs and compared them against monolayer culture. EHTs were generated from iPSCs that had been differentiated into cardiomyocytes (iCell, Cellular Dynamics) and calcium transients were recorded using Fluo4-AM. Flashes were taken in both two dimensional monolayers and three dimensional EHT cultures (figure 3.8A and B, representative traces are shown in Ai and Bi respectively) and comparisons of transient kinetics were recorded and shown in figure 3.8Ci-iv. Notably, EHT culture of iPSC-CM causes a significant shortening in the length of the transient, 75% decay of peak, 50% decay of peak and time to peak fluorescence (all n=4 p<0.05 figure 3.8C; three replicates of 2D culture were supplied by Dr. Hellen). This resulted in 39 and 69 transients per minute in 2D and 3D EHT, respectively.
Figure 3.8. Calcium transients from 2D and 3D cultures. When stained with Fluo4-AM 2D culture of cells increase in intensity when contracting (A), a representative trace of intensity is shown (Ai, 93 f.p.s). 3D EHT loaded with Fluo4-AM also shows an increase in intensity when contracting (B) and a representative intensity profile can be plotted (Bi, 45 f.p.s). Comparisons of 2D and 3D cell culture is shown by: transient length Ci, time to 50% transient decay Cii, time to 75% transient decay Ciii and time to transient peak Civ. Error bars represent standard error of the mean, a star indicates p<0.05 n=4 students t-test.

Three 2D Ca$^{2+}$ transient replicates were kindly donated from Dr. Hellen.
3.9 Troponin I isoform switching in EHTs

During development of the heart a switch from slow skeletal (TNNI1, herein ssTnI) to the cardiac (TNNI3, herein cTnI) Troponin I isoform occurs which is a marker of maturity of adult cardiomyocytes (Marston 2003). Therefore, TnI isoforms were examined using Western blots to compare the maturation level of rEHTs to neonatal and adult rat hearts (figure 3.9A). Probing individually for cTnI or ssTnI proved difficult because of the lack of protein that could be extracted from rEHT samples, and the inefficient way of normalising protein loading to a band on the nitrocellulose membrane. These blots led to the overall amount of protein in rEHTs to be underestimated. Thus, an antibody that recognised all isoforms of Troponin I was taken advantage of to analyse the maturity level of the rEHTs. The clear developmental switch of neonatal heart tissue to adult tissue is seen in the isoform switching from ssTnI to cTnI. cTnI is significantly up regulated in adult rat heart while ssTnI is significantly down regulated (figure 3.9B). Moreover, both cTnI and ssTnI levels were similar between neonatal and rEHT samples (cTnI: 34%, 36% ssTnI: 65%, 63% for neonates and EHTs respectively). Using the phosphate affinity SDS-PAGE technique, proteins can be separated out based on their phosphorylation level and quantification of the spread of phosphorylation can be calculated in a sample (Kinoshita 2006). Hence, this approach was used to measure the phosphorylation level of cTnI in rEHTs and compared against neonatal and adult hearts (figure 3.9). No significant differences were seen between adult and neonatal heart samples most likely due to the lack of power in this experiment (figure 3.9C). However, bis-phosphorylation was never seen in any rEHTs sample (N=5, 20 rEHTs) and strikingly, EHTs cultured without serum were never seen with any phosphorylation of cTnI (N=2, 6 rEHTs).
Figure 3.9. Analysis of Troponin I isoforms and phosphorylation level. Quantification of cTnI and ssTnI protein levels by western blots using antibodies against specific isoforms (A, representative blots of cTnI in Aii and ssTnI in Aiii), normalisation was carried out to adult cTnI or neonatal ssTnI respectively. An antibody which recognises all isoforms of Troponin I was used and the % intensity of cTnI and ssTnI was calculated (B, representative blots in Bii). Samples were run on custom made gels to separate out proteins based on phosphorylation level (C, representative blots in Cii). Bis phosphorylation is shown in black, mono phosphorylation in checked, and no phosphorylation in white. Replicates were as follows: adult rats (N=3), neonatal rats (N=3), rEHT serum (N=3, 14 rEHTs), rEHT serum free (N=2, 6 rEHTs).
3.10.1 Discussion Chapter 1. Engineered Heart Tissue as an experimental model
The heart is a complex machine whose design is still far from the reach of the
brightest minds in academia, and the goal of making a functional heart in the
laboratory is ambitious and has captivated people for decades. Currently, state of the
art tissue engineering in this field is held by three dimensional heart constructs that
come in different forms including: hydrogels, cell sheets and engineered polymers
(Hirt 2012). Although progress in this field has been slow, this search has produced
worthwhile progress, and knowledge about the heart.

3.10.2 Problems with human tissue as a model
The need for cardiac tissue engineering comes from the ever increasing burden of
people waiting on organ transplants. There is also a scarcity of normal human tissue
to carry out experiments, not only are human heart donors rare, but the amount of
tissue from elective surgeries is small. Although it is possible to acquire diseased
heart tissue at reasonable volumes and rates, this has the issue of not having a
proper control. Diseased human tissue also varies due to the amount of time
between isolation and experimentation, disease progression and disease type.
Moreover, normal or diseased adult cells cannot be cultured at present for long
periods of time and so experiments must be done whenever there is a surgery, and
long term conditions cannot be tested for example to assess responses to drugs. This
problem also extends to adult cells isolated from rodent models because methods for
long term culture of isolated myocytes are still lacking. Furthermore, rodent
cardiomyocytes are different to human heart cells in several different ways with beat
rate being the most obvious example (Cook 2009, Kaese 2012).
3.10.3 Advantages of the EHT platform

In comparison to these issues EHT has some advantages. Cell culture and differentiation has now progressed so that cardiomyocytes can be made with high purity and high volumes. This allows experimenters the convenience of planning and carrying out investigations in a controlled manner. Moreover, force of contraction can be measured in EHTs non-invasively over prolonged periods of time (months), along with important parameters of contractions like beat rate, force, contraction and relaxation times. This compares to two dimensional approaches of culturing cells where movement of pixels is sometime considered to be a surrogate measurement of force (Lan 2013, Tanaka 2014). Prolonged culture time allows for chronic effects on contractions to be investigated including long drug exposures or multiple exposures to different conditions, which is an advantage when considering heart disease patients receive multiple drugs to treat symptoms. EHTs combined with iPSC technology can also be used to model patient conditions (see chapter 5), where experiments can be conducted on the effect of the mutation on the contractile performance of cardiomyocytes with the same patient background. This last point is a massive advantage as it combines the human biology in iPSCs and the wealth of information that is gained from contractile analysis. Lastly, EHTs could be used for repairing damaged hearts. Although hydrogels are not currently large enough in the z dimension to replace segments of heart, they could be used as an aid of contraction to allow the heart to recover from a stress or ischemia. Some progress has been made in this area as hydrogels can be fused together to make large constructs (in the x and y axes) and serum free conditions have been developed (Zimmerman 2006, Hirt 2012, Tiburcy 2017). Moreover, EHTs have recently been
used to ameliorate the effects of heart disease in a medium sized animal model, this shows real promise in this field for the future (Weinburger 2016).

Although some progress has been made in cell therapy approaches using EHT, the maturity of these tissues cannot be overlooked. Here we tested some of these approaches including: T3 IGF and Dexamethasone, electrical stimulation, cell culture time, ascorbic acid (not shown), noradrenaline, mechanical load, increased oxygen, and Matrigel. The problem with our approach is that force was used as the main output and so these approaches may have changed other parameters to a more adult cell like phenotype but they were not investigated thoroughly. However, the main purpose of a heart cell is to contract and measurable contractions is the main advantage of EHTs, and all these maturation strategies showed increases in force.

3.10.4 Maturation strategies effect on force

Increased cell culture time to day 50 was the only approach which increased the force of human EHTs in this report. Increased numbers of cells in EHTs also increased the amount of force but this is probably due to more cells being in the hydrogel and not increased maturation. The increased cell culture time phenomenon was also published recently with a graded response taking place with increased force associated with increased time in culture (Tiburcy 2017). Moreover, the same cell line used in this study was well characterised comparing 20-40 days in culture vs 80-120 days in culture (Lundy 2013). The increased force seen in our hEHTs could be due to the increased maturation reported by Lundy et al where importantly, they saw increased multinucleation, elongated morphology, with higher alignment and density of sarcomeres. The increased properties reported, especially increased
density of sarcomeres, could have caused our increased force effect. In addition, similar to this study the authors also saw increased shortening and calcium cycling which would have affected force.

Although we did not see an increase in the force measured when our hEHTs were placed under hormonal stimulation (T3 IGF and Dex, and Noradrenaline), this approach should not be ignored in the future. The improved ‘force’ reported by this approach is based on the movement of cardiomyocytes changing in shape and is not a true force measurement (Birket 2015). Moreover, if there is a beneficial effect on force by this approach it is probably through the action of IGF which is well known to have similar gene targets to insulin, which is used at high levels in EHT culture (Zimmerman 2006, Boucher 2010). What is interesting in this approach is the effect of T3 IG and Dex on the calcium cycling of PSC-CM. T3 produces a quicker beat rate and a faster calcium transient, this is quite a convincing effect of T3 and makes PSC-CM more like adult cells (Yang 2014). When T3 IGF and Dex was applied an increase beat rate was also seen in our hEHTs and interestingly, the increase beat rate slowed down when stimulating hEHT at the same time. This suggests that hormonal stimulation and electrical stimulation approaches should be used together although more care would have to be used with this approach as it produces a lot of stress for EHTs.

Further arguments for the use of hormonal stimulation come from our phosphorylation measurements of cTnI. rEHTs always had reduced phosphorylation than neonatal or adult heart tissue with no bis-phorylation ever seen in rEHTs, and no cTnI phosphorylation seen at all in rEHTs cultured without serum. As failing
myocardium is known to have reduced levels of cTnI phosphorylation, this would imply that either during the generation or culture over two weeks EHTs resemble a heart failure phenotype (Messer 2014). This has significant implications for studies of EHT cell therapy, and certainly in studies which are considering moving to GMP compliance for cell therapy where serum free conditions are necessary.

3.10.5 Cell density effect on EHT culture

Another approach which had a increased effect on force in this study was increasing the cell density. rEHTs with one million cells carried out post deflecting contractions earlier, and produced more maximum force than rEHTs with half the number of cells. Contracting earlier is probably due more cell to cell contacts being made quicker, and thus, a more co-ordinated contraction could be carried out earlier. The maximum force output being higher is probably due to more cells surviving in the hydrogel during EHT generation. An unexpected side effect of a higher density of cells being used was that the spontaneous beating activity of these rEHTs decreased markedly. Decreased spontaneous beating activity has been seen previously by similar hydrogel culture techniques (Liau 2011). This phenomenon also occurred to control rEHTs when left in culture, however, reduced spontaneous beating would occur much more to higher density rEHTs. This effect was demonstrated in rEHTs where both spontaneous and stimulated hydrogels were left unstimulated for five minutes and only one spontaneous beat was recorded in the spontaneous group. This also adds credence to the argument of using stimulation in these hydrogels even though no beneficial effect was seen on force through stimulation. Moreover, it adds curious questions to cardiomyocyte culture in general and may be an avenue to explore if investigating the maturity of cells. Lastly, it must be noted that with one million cells
the density of the rEHT is still far from the density of adult myocardium, and if hydrogels are truly going to mimic adult cardiac muscle this factor must be taken into consideration. For example an average day 14 EHT has a volume of 0.14cm$^3$ and $1 \times 10^6$ cells, while myocardium has been estimated at a density of $0.5-1 \times 10^8$ cells/cm$^3$ (357-714 times smaller, Radisic 2002).

3.10.6 Problems with white box measurements of force

Both rEHTs and hEHTs were probed for a positive force frequency response and were never seen to have one. However, this response is hard to measure in the white box because when increasing frequencies are recorded in sequential measurements force is always reduced. This is in comparison to when force is measured with increasing frequency in one continuous measurement. This is due to the way that the white box measures force by shortening of the hydrogel between two points (ie fractional shortening), and under increasing levels of stimulation an EHT will shorten in length as well as under-go fractional shortening. An EHT which shortens is causing more force, however, this force is passive in nature and a passive contraction like this cannot be measured by the white box (figure 3.10). In addition to this problem, because of how the posts were developed, when an EHT has more passive tension it takes more force still to produce an active contraction. This is because when the posts are stretched more force is needed to stretch the posts even further. This is a shortfall of the posts and how EHTs work against this load, and the indirect measurement of the white box with force being a derivative of shortening and not an actual measurement of force itself. The way in which EHTs contract against the posts is auxotonic, which means there is a variable amount of tension depending on how much the EHT has shortened (in this case higher tension
with shorter EHTs). This is argued as being similar to heart contractions (Hansen 2010). However, this is not like a heart contraction. A contraction here is missing an isometric phase, which the left ventricle would achieve while building enough pressure to open the aortic valve. An isotonic contraction would then occur after the mitral valve has opened expelling the contents of the ventricle. Recreating this in the lab would be very difficult especially considering that an apparatus would need to be warm and sterile.

**Figure 3.10.** The white box not taking into account passive tension. A examples of EHTs with different lengths ranging from relaxed to extreme. A conceptual example is shown in B of EHTs with low and high passive tension. Another example is shown in C where EHTs shorten the same amount but start with different passive tensions. In set in C shows the actual way the white box measures force just taking into account the active movement of posts.
3.10.7 iPSC-CM EHT is not a fully mature heart model

hEHTs were also probed with increasing levels of calcium and the responses were not similar to adult tissue. The EC50 of hEHTs was around 0.65mM of calcium which is far from the EC50 of 3-5mM measured in normal adult heart tissue (see table 1.1). This is indicative of an immature calcium handling phenotype seen in all PSC-CMs and is probably related to the lack of T-tubules in these cells. hEHTs also do not have a strong inotropic response to isoprenaline when cultured in 1.8mM calcium. Previously published data with EHTs show a need for reduced calcium levels down to the EC50 to see this effect (Mannhardt 2017). This is probably due to the maximum amount of force also being generated from these tissues at 1.8mM and therefore, to see more contraction under β adrenergic stimulation this response must be reduced. The β receptor machinery is also perhaps not quite fully developed with some work in this area showing that adult myocardium has a dominance of the β1 receptor with iPSCs having both subtypes mediating responses (Wu 2015). However, when cells were isolated from hEHTs and the β adrenergic response was probed, sensitivity to noradrenaline was the same as adult cells but the maximum L-type calcium current was seen to be smaller (Uzun 2016). Authors here postulate that the smaller effect was related to insufficient cAMP generation. We have also investigated β receptor activity in iPSC-CM and have shown high basal activity of β receptors which could also reduce the effects of isoprenaline (Hellen 2017).

Another issue with this approach, and one that the field needs caution when interpreting, is giving force as a unit of area. With a good batch of EHTs average force can be 7.6mN/mm² (maximum recorded 14.16mN/mm²), which is in the range of neonatal rat trabeculae, and the maximum recorded is nearing adult myocardium.
The problem with this approach is that hydrogels will thin over time and keep on thinning after maximum force has been reached. This means that absolute force and force per unit area are not exactly related, moreover, the width of the construct has a large effect on this measurement (it is the denominator in the equation). So much so that in our study there was a two-fold difference between two constructs of the same force under the same culture conditions. Force per unit area is more quoted in the field because in general hydrogel constructs produce much lower absolute forces than adult myocardium, and width of constructs can be altered by several factors (Kensah 2013, Jackman 2016, Breckwoldt 2017). This reflects a general problem in the field to produce hydrogels that are densely packed enough with cardiomyocytes, a problem that may have been overcome by Jackman et al/ which used a rotating table culture approach. Although adult level forces have been reported as both absolute measurements in Kensah 2013, and as per unit area in Jackman 2016 et al, these were recorded in thinner constructs. However, this is an important step forward, and if these studies can be reproduced the focus must then move onto making larger constructs with mature vessels to overcome the oxygen diffusion gradient.

Even though force is a useful measurement of maturity, and EHTs can be used to measure this easily, many factors need to be considered when assessing what is a complicated issue. In a recent review as many as 32 factors have been considered when assessing cardiomyocyte maturity and this list is not exhaustive (Denning 2016). However, what is not largely considered is isoform switching, and here we have presented a paradigm to show a robust indication of maturity from neonatal to adult rat tissue. A clear switch from ssTnI to a dominance of the cTnI isoform is
demonstrated and we also show that rEHTs are similar to neonatal heart tissue. This has two important implications: one, that enzymatically isolating cells generating EHTs does not reduce the maturity of rEHTs, and two, standard two week culture does nothing to improve the maturity of rEHTs in respect to TnI isoforms. One report has shown that TnI switching can occur in hydrogels with neonatal rat cells and also iPSC-CMs re-expressed cTnI but only after engrafting into adult rats (Kadota 2016). This shows that cardiomyocytes are not experiencing the same cues as in vivo. Furthermore, that there are different environmental cues that change from neonatal heart to adult hearts, and that leaving cells in culture for a prolonged period of time will not improve some maturity factors. This idea was demonstrated when comparing calcium handling in hydrogels and comparing them against two week old isolated heart cells. Time to peak and relaxation times are still faster in heart cells even after two weeks of stimulation at physiological frequencies (Godier-Furnemont 2015).

3.10.8 EHTs verses monolayer culture

It seems clear that rEHTs are still at the neonatal time point, however, the question remains is three dimensional culture better than two dimensional culture. Direct comparison of two dimensional culture vs three dimensional culture shows that cells isolated from hEHTs have larger catecholamine responses than hiPSC-CM in a monolayer, however, the cell capacitance of both hEHT and monolayer single cells is much smaller than adults (Uzun 2016). EHTs also have 1.8-fold larger sodium current density than monolayer iPSC-CM, and also the up stroke time of the action potential in EHT was 219ms which was very near human myocardium at 253ms (Lemoine 2017). Therefore, some aspects of iPSC-CM maturity is improved by culturing cells in EHTs however fully mature cardiac tissue is still not possible.
We have tested the calcium handling properties of hEHTs and compared them against monolayers and found faster transients in hEHTs. This has also been shown by others using the same culture conditions when comparing monolayer and three dimensional culture (Liau 2011). Improvements have been well documented in two dimensional culture systems and arguments can be had over which culture condition produces more mature cells, but what is undeniable is that three dimensional culture produces measurable forceful contractions. If the field is going to advance and more is going to be learnt about the heart, larger constructs will have to be made under conditions that best mimic the heart which will cost a lot more cells, money, and need engineering skills as well.

3.10.9 Conclusion

Christine Mummery’s group has published a detailed transcriptome analysis of hPSC-CM and compared them against human foetal tissue showing that differentiated cells more resemble second trimester foetal heart (van den Berg 2015). Tiburcy et al also carried out similar transcriptional profiling, but compared: adult, foetal, hydrogel, and monolayer culture and found that the hydrogel system had more mature transcripts up regulated compared to monolayer culture. However, the estimate of the level of maturation was probably around the 13 week timepoint. These data need to be considered with the fact that one, positive force frequency responses have been shown in human hydrogels which are not present in human tissue until around the first year. Two, positive inotropic and lusotropic responses have been reported after β adrenergic stimulation. Three, ultra structural sarcomeres are intact showing M bands, and four as stated previously, similar levels of force to
the adult myocardium has been published (Kensah 2013, Jackman 2016). Taken together the field is advancing cell culture, however, the focus has always been on the functional properties of cardiomyocytes because that is how people have analysed the heart for decades.
4 Results Chapter 2. Characterisation of the ACTC E99K mouse model

4.1 Sudden Cardiac Death in three different backgrounds

The ACTC E99K mutation has been studied previously in our lab and a wealth of interesting information has been uncovered (see section 1.10). However, one aspect which has not been investigated is why transgenic mice die suddenly. Originally the E99K line was generated on a mixed background C57Bl6 x CBA/Ca (herein called mixed background) by Dominic Wells. A feature of this line was that a large proportion of transgenic mice would die suddenly. The amount of sudden cardiac death (SCD) in this line was variable from different animal houses and users, but it is still high compared to most reported HCM mutations. These mice were transported to Katja Gehmlich’s lab in Oxford and were back crossed onto a pure C57BL/6 (herein black6, more than 7 generations of back crossing) and then transported to Imperial where 6% of mice died suddenly, compared to 24% of mixed background (figure 4.1A). We also brought in pure CBA/Ca mothers and bred them, with transgenic mixed background males to produce an enriched CBA/Ca line which 35.7% of E99K mice died suddenly (figure 4.1A herein enriched CBA). Unfortunately, the high amount of sudden death in this line meant that regulators did not want us to work with this line anymore so we did not characterise this line in detail.

Furthermore, a second backcross was tried but this never yielded any transgenic pups. All three lines underwent sudden death in the same time frame between 25-40 days which was termed the sudden death window.

The Marston lab has previously generated a site specific antibody for the E99K mutation, which was used to probe wild type (WT), transgenic (TG) and SCD mice for expression of mutant protein (figure 4.1Bi-iii). The antibody was confirmed to be
mutation specific with very low or no expression in WT (figure 4.1Bi). Hence, E99K mutant expression was analysed from postnatal day 7 (P7) and normalised to adult transgenic animals. Mutant E99K expression was first seen at P7 at 38% of adult levels (figure 4.1Bii n=3), which was significantly increased when mutant protein expression reached adult levels at three weeks of age (figure 4.1Biii P21=108.71, n=3 p<0.001). Lastly, no differences were found between: P21, P28, SCD or adult TG animals (figure 4.1Biii).

Figure 4.1. Kaplan-Meier of survival rates in hybrid (C57Bl6 x CBA/Ca), BL6 (pure C57BL/6) and CBA (enriched CBA/Ca) background E99K mice and mutant protein expression. A large proportion E99K mice exhibit sudden cardiac death at 25-45 days old, which we termed the sudden cardiac death window (N=50,50,14 for C57BL/6 x CBA/Ca, C57BL/6, and CBA/Ca respectively, Ai). In Aii the distribution of only sudden deaths is shown. The expression of E99K mutant protein is shown in B using our mutation specific antibody Bi, and the corresponding MEMCODE is shown in Bii. These data are shown graphically in Biii given as a % of adult transgenic animals (N=2-4, P= postnatal day).
4.2 Black6 TG mice have the same level of calcium release events (sparks) as WT

In a recent study we have shown that young transgenic E99K mice (25-45 days) on a mixed background have a larger calcium transient and an increased propensity for spontaneous calcium release events, referred to as sparks (Rowlands 2017). In contrast to this, adult transgenic mice which survive this window are the same as WT (Rowlands 2017). We postulated a link between sparks and SCD assuming mice with high levels of sparks would have an arrhythmia, and because we had different backgrounds with different levels of SCD, we had a good model test this hypothesis. Therefore, we chose to test the Black6 background, which had very low SCD, for sparks (figure 4.2). Sparks were analysed using a set of conditions set out by Hollingworth et al 2001 and: amplitude, full duration at half maximum (FDHM), spark mass, spark frequency, sarcoplasmic reticulum leak by the length of time a spark occurs, and spark mediated sarcoplasmic reticulum leak by mass are depicted in figure 4.2 (A-F respectively). Amplitude and full duration at half maximum are taken as a direct measurement, however, all other parameters were calculated as detailed in the methods section (see section 2.10). No differences were found between WT and TG apart from FDHM which was significantly longer in TG (figure 4.2B). SR leak by the length of time a spark occurs was significantly shorter, however, this parameter is calculated by multiplying FDHM by the frequency of sparks and is lower in the TGs (figure 4.2E). These results demonstrate that E99K Black6 mice have spontaneous calcium release events similar to WT, which is very different to mixed background mice. Moreover, the data adds further support to our hypothesis showing that SCD is caused by aberrant calcium regulation in these mice, and abnormal calcium handling depends on the genetic background.
Figure 4.2. Spontaneous calcium spark parameters in Black6 E99K (TG) and wild type (WT) mice. Amplitude of sparks (A), full duration at half maximum (B), spark mass (C, ∆F/F₀*µm³) spark frequency (D, sparks/100µm/sec), spark mediated sarcoplasmic reticulum leak represented by how long sparks occur (E, FDHM*frequency), spark-mediated SR leak as mass (F, spark mass*frequency). Where error bars represent standard error of the mean and FDHM = full duration at half maximum, SR = sarcoplasmic reticulum. Significance was calculated using Students t-test where: * = p<0.05. WT N=64 cells from 7 WT animals and TG N=64 cells from 8 TG animals). Cell isolations were carried out by Hsiang Yu Yang and Saheed Lawal took confocal measurements.
4.3 Characterising fibrosis in the E99K ACTC mouse model

The upregulated expression of collagen in models of hypertrophic cardiomyopathy is well known (Khan 2006). Recently, two photon excitation microscopy using second harmonic generation has been used to study collagen levels in a rat model of myocardial infarction (Chen 2012, Caorsi 2013). Hence, to analyse E99K mice using this technique papillary muscles were taken out of SCD, TG and WT mice (figure 4.3A). No differences were seen in collagen expression based on total collagen measure in our set up (figure 4.3C, WT=314, TG=306, SCD=403 in arbitrary units N=7, 7, 6 animals respectively). Distinct collagen expression was seen between E99K mice throughout the thickness of papillary muscles, with SCD mice having more expression deeper into papillary muscles in contrast to WT animals (figure 4.3B). These results were presented graphically by splitting collagen expression into distinct areas (figure 4.3D). Comparing WT collagen expression through papillary muscles a significant reduction was seen deeper into the tissue at slices 121-200 compared to slices 1-50 at the surface (figure 4.3D 1-50=136, 121-200=56, n=7, p<0.0001). No differences were seen in TG animals, however, SCD mice had significantly higher collagen levels at slices 51-120 compared to both slices 1-50 and 121-200 (figure 4.3D n=6, p<0.01 and 0.05, respectively). No significant differences were found nearer the surface of papillary muscles (left ventricular lumen side) in WT, TG or SCD mice (figure 4.3D slices 1-50 WT=136, TG=78, SCD=72 n=7,7,6 respectively). However, deeper into papillary muscles, moving towards the left ventricular wall, significant differences were found between SCD and WT animals at slices 51-120 and 121-200 (figure 4.3D both p<0.001 n=6-7).
Figure 4.3. Collagen expression in wild type (WT) transgenic (TG) and sudden death (SD) animals by two photon microscopy. Papillary muscles were taken from WT, TG and SD animals and are shown with collagen signals taken from second harmonic generation shown in green, phalloidin labelled actin is shown in blue, and merge shown in the last panels (A). B shows collagen signals from WT, TG and SD animals in 1µm slice sections taken from z stacks. Total collagen levels were calculated by subtracting away background fluorescence and adding the summation of collagen signals from slices 51-150 (C). The distribution of collagen expression is shown in slices 1-50, 51-120, and 121-200 for WT, TG and SD animals in D. Error bars represent standard error of the mean. N=7,7,6 for WT TG and SD respectively. Significance was calculated using One-Way Anova where: ** = p<0.01 and is measurements taken between genotypes in the same slice area, and +++ = p<0.001, ++++ = p<0.0001 which are measurements taken between slice areas within the same genotype.
4.4 Aging as a determining factor in fibrosis

Collagen expression is known to be age dependent and is highly expressed in end stage heart failure patients (Biernacka 2011). Therefore, to understand disease progression over time in E99K mice, and how collagen expression interacts with aging, these parameters were investigated (figure 4.4A). Corroborating previously published results with E99K mice, SCD mice were found to die at the pre-identified death window (figure 4.4B, average age=37.75d, n=4 Song 2011). However, mice were also found to die much later; which is roughly middle age for the background strains, and correlates to when DCM was previously observed to develop in these mice (figure 4.4B, average age=233.25d n=3, Song 2011). There was no difference between collagen levels in SCD mice that die early or later (figure 4.4C early SCD=283, late SCD=265 n=4, 3 p=0.08). Lastly, a scatter plot of SCD animals was performed showing all SCD mice have roughly the same collagen levels (figure 4.4D n=7). There was one outlier where one animal did not reach the same collagen level in this analysis (figure 4.4D arrow). This mouse was later identified as a breeder, and we hypothesise that it had experienced high levels of β adrenergic stimulation during coitus.
Figure 4.4. Collagen expression of E99K animals over time. Individual scores of collagen expression are plotted against time in A with wild type in blue, transgenic in red, and sudden death animals in green. The arrow in A highlights one animal which did not reach the level of collagen seen in the others which died during coitus. The amount of collagen expression of sudden death animals from early (38-39 days n=3) and late (243-282 days n=3) was calculated between slices 51-150 and is shown in B. Students t-test was carried out in B with no significance difference p > 0.05.
4.5 Increased fibrosis confirmed by staining and qPCR

Two photon experiments showing an increase in collagen levels were extended by two methods: staining collagen in transverse sections, and investigating gene expression. Sections of WT, TG and SCD hearts were taken at 7µm and staining with Pico-sirius red was carried out in figure 4.5. SCD death animals had increased levels of fibrosis at 13.25%, compared to 5.4% in TGs and 3.3 in WTs (figure 4.5A-D). Phenotypically collagen types differ with type I being stiffer and type III being more extensible, and hence, type I is found upregulated in cardiomyopathies (Pauschinger 1999). SCD mice had an up regulation of collagen I over wild type and surviving transgenic animals (figure 4.5E WT=1.21, TG=3.9 and SCD=19.3, N=7,7,6), moreover, collagen III levels were found to be much less increased (figure 4.5F). The ratio of collagen I to III confirmed that SCD animals were expressing more of the stiffer type of collagen (figure 4.5G WT=1.17, TG=0.65, SCD=2.03). Lysyl Oxidase (LOX) is a collagen cross linking enzyme which has been found upregulated in heart failure and in SCD mice it was induced 34.2 fold compared to WT (figure 4.5H N=7,7,6 Lopez 2010). Mechanistically, it has been shown that TGF-β induces collagen biosynthesis by SMAD and MAPK signalling mechanisms (Teekakirikul 2001). Therefore, we also probed TGF-β levels and SCD animals were found to be increased 6.5 fold over WT while TG were only 2.9 (figure 4.5I, N=7,7,7).
Figure 4.5. The expression of Collagen I and II in wild type (WT), transgenic (TG), and sudden death (SCD) animals. Pico-sirius red staining of 7μm transversal frozen heart sections in WT (A) TG (B) and SCD (C) and the % covered by the stain is shown in D. Samples were also probed for Collagen biosynthesis associated genes by qPCR including Col1A1 (E) Col3A1 (F) and the ratio of Collagen I to III was calculated in G. Lysyl Oxidase (LOX) gene expression is shown in D and TGF-β expression is in E. Error bars represent standard error of the mean. Significance was calculated using One-Way ANOVA where: * = p<0.05, **=p<0.01, ***=p<0.001.
4.6 Isoform switching in SCD mice

As the neonatal heart matures, isoforms switch in the heart to accommodate for extra load placed upon it by the circulation and this mechanism has also been shown to occur in disease states (Marston 2003). Therefore, isoform switching was investigated in E99K mice using Troponin I and Myosin expression (figure 4.6). As expected, western blots for all isoforms of Troponin I showed higher ssTnI in P7 animals and almost no expression in adult (figure 4.6A P7=56% n=3 p<0.0001). ssTnI expression was barely detectable at P21 indicating that isoform switching of TnI occurs at around two weeks. When compared to WT, TG and SCD animals did not have significantly higher levels of ssTnI (figure 4.6A WT=6%, TG=5.5%, SCD=10% n=3). To investigate isoform switching further gene expression analysis was carried out. A general decrease in cardiac specific markers was seen in SCD mice which may be explained by increased cardiomyocyte cell death and more fibrosis, hence, this may affect all results with cardiac genes in these mice (figure 4.6B-G). Therefore, ratios were calculated on isoforms that under-go switching, the ratio of ssTnI/cTnI was significantly increased in SCD animals at 5.7 fold (figure 4.6 SCD=5.7, N=5,2,3). Atrial Natriuretic Protein (herein ANP) is well known to be upregulated in pathological hypertrophy (Bernardo 2010) and was shown markedly increased in both TG and SCD animals, there was also a difference seen between TG and SCD (figure 4.6H, N=4,3,3).
Figure 4.6. Isoform switching in E99K animals. Western blots were performed against all isoforms of Troponin I densitometry readings were taken and normalised for protein loading to α-actinin, and then all values were calculated as percentages of total Troponin I expression for wild type (WT), transgenic (TG), and sudden death (SCD) animals at postnatal day (P) 7, 28 and adult (A, N=3,4). The expression of Myosin 7β is shown in B and Myosin 6 in C (N=5,4,6 and 4,3,3 respectively). Gene expression of ssTnI and cTnI are shown in D and E(N=4,2,3 and 5,3,3) respectively, and ratios of ssTnI to cTnI is shown in F (4,2,3). Serca2a (G, N=3,5,4) and Atrial Natriuretic Peptide (H ANP, N=4,3,3) were also investigated. Error bars represent standard error of the mean. Significance was calculated using One-Way Anova with Tukey’s where: * = p<0.05, **=p<0.01, *** = p<0.001.
4.7 Aberrant phosphorylation in SCD mice

The phosphorylation status of key contractile and calcium handling proteins have been shown to be low in end stage heart failure patients (Messer 2014). Hence, to understand if SCD mice mimic phosphorylation states seen in human heart failure the phosphorylation status of cTnI was analysed by phosphate affinity SDS-PAGE (figure 4.7). WT and TG mice were not found to have any differences in phosphorylation level of cTnI at any of the ages studied (figure 4.7B n=3). Strikingly, SCD mice had much reduced phosphorylation levels and were never seen with any bis-phosphorylation in cTnI in any of the samples (figure 4.7B n=6). As another control wild type animals were left overnight after schedule 1 in the fridge and the phosphorylation levels were analysed showing no significant difference to WT (figure 4.7B n=3).
Figure 4.7. Phosphorylation levels in E99K hearts. Phosphorylation levels were determined by Phosphate Affinity SDS-PAGE and representative images are shown in A. Phosphorylation levels were calculated by determining the percentage of phosphorylation using densitometry readings and no phosphorylation, mono-phosphorylation and bis-phosphorylation is shown in B for all genotypes and ages assayed. N= 3, 3, 4, 4, 6, and 3 respectively left to right in B, P= postnatal day.
4.8 Protein expression of RyR2, Epac and PLN

We have conducted in depth characterisation of our mouse model in the sudden death window and showed increased calcium sparks at this time point (Rowlands 2017). Sparks are calcium release events at the ryanodine receptor (RyR), and therefore, we wanted to understand the molecular mechanisms that might be at the cause. RyR2 protein expression was analysed and significantly less expression was seen in TG and SCD animals compared to WT levels (figure 4.8A). RyR2 opening is controlled by phosphorylation, and so, phosphorylation was assayed using site specific antibodies. Although somewhat controversial in the field, serine 2814 is thought to be the Ca\(^{2+}\)/calmodulin-dependent protein kinase (CaMKII) site, while serine 2808 is thought to be phosphorylated by Exchange Protein directly Activated by cAMP (herein Epac, Lezoualc'h 2016). No phosphorylation was seen at serine 2814 in TG and SCD animals (figure 4.8B), while significantly reduced levels of serine 2808 phosphorylation were seen in TG and SCD animals (figure 4.8C). It is known that two Epac proteins can phosphorylate RyR2 in response to β adrenergic stimulation. No differences were found between WT, TG and SCD animals in Epac1 expression (data not shown). However, significantly more expression was seen of Epac2 in SCD animals compared to WT in mixed background mice (figure 4.8D), moreover, we did not see any difference between WT and TG mice on the black6 background (figure 4.8E). Phospholamban (herein PLN) has been implicated in heart failure and is critically important for the action of Serca2a and the uptake of Ca\(^{2+}\) back into the sarcoplasmic reticulum (Okumura 2014). The protein content of PLN was assayed by western blot in our three mouse backgrounds, and we found significantly reduced levels in only TG black6 mice (figure 4.8F). The phosphorylation at serine 16 of PLN was also assayed by site specific antibodies and again we found
reduced levels in TG black6 animals, however, we found no phosphorylation in SCD animals on the enriched CBA line even though there was a robust amount of protein (figure 4.8F and G).

Figure 4.8. Ryanodine Receptor (RyR2), Epac2 and Phospholamban protein levels in wild type (WT), transgenic (TG) and sudden death (SCD) E99K mice. Western blots were carried out for RyR2 protein and significantly reduced levels were seen in TG and SD compared to WT (A, N=10,3,14). Phosphorylated RyR2 serine 2814 (B, N=5,5,7) and serine 2808 (C, N=9,10,5) were also assayed using site specific antibodies. Western blots for Epac2 were also carried out in mixed (D, N=6,6,13) and pure black6 lines (E,N=6,8,3) showing increased expression in SD animals compared to WT. Phospholamban protein level (F) and site specific phosphorylation of s16 (G) were also assayed in three backgrounds. All error bars are standard error of the mean. All One Way ANOVA apart from CBA WT vs SCD which was carried out by students t-test. *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001. N=3,5.
4.9.1 Discussion Chapter 2. The mechanism of sudden cardiac death in E99K mice

The mechanism behind hypertrophic cardiomyopathy and sudden death has not been fully elucidated. We have furthered the understanding of the E99K mutation, and have looked at different backgrounds of mice to investigate the important role of genetics in SCD.

4.9.2 E99K mice and sudden death window

E99K mice have been studied in depth which makes them an invaluable resource for understanding hypertrophic cardiomyopathy as many aspects of the disease have been explored (Song 2011, Song 2013, Vikorev 2014, Papadaki 2015, Rowlands 2017). Nevertheless, SCD in E99K animals have not been previously studied which is important because these mice have a more severe phenotype than TG mice which survive. Therefore, studying these mice may have important insights into the more extreme phenotypic presentation of hypertrophic cardiomyopathy, and may be useful for studying sudden cardiac death.

E99K mice on the mixed background (C57Bl6xCBA/Ca) undergo SCD between 25-40 days which we have termed the death window (Song 2011). Between P10 and P35 (puberty in the mouse) mouse body weight increases dramatically and is accompanied by an increase in blood flow. To compensate for this the mouse heart increases its stroke volume 3.5 fold and weight 3.47 fold which is attributed to increased cardiomyocyte number (Naqvi 2014). In addition, mice in our facility are weaned at P21 and placed into separate cages which increases the stress on the animals (Nicholson 2009). Increased burden on the heart and increased stress could push our mice to have arrhythmias, especially if the excitation contraction coupling
has not yet adapted to the increased calcium sensitivity caused by the E99K mutation. Some TG mice die and others seem resistant to SCD during the death window and this important distinction has not been fully investigated in our model.

4.9.3 Characterisation of fibrosis in E99K mice
The link between hypertrophic cardiomyopathy and fibrosis has been well demonstrated and it is equally clear in E99K mice (Khan 2006). The higher levels of fibrosis that we see deeper into papillary muscles in SCD mice, and the lower levels in transgenic animals which survive is an interesting difference seen in our model. WT mice were constantly seen with high levels of collagen expression at the surface of papillary muscles which commonly projected at an angle perpendicular to myocyte fibres. However, levels of collagen quickly diminished further into the muscle. TG mice were unusual because they were never seen reaching high levels of collagen expression either early or later. This may reflect a compensatory mechanism in these mice or the differences in the hybrid background of these mice which will be discussed later. SCD mice had fibrosis of mature collagen fibres which appeared to occur in spaces where cardiomyocytes were lacking. Lastly, age analysis of Collagen in SCD mice showed that these mice have a similar level of fibrosis despite the age at which they died. This may suggest a point of no return where they are more likely to perpetuate aberrant calcium signalling to form arrhythmias.

4.9.4 Fibrosis as a link to arrhythmias
The evidence for arrhythmic sudden death in HCM models being caused by fibrosis is ambiguous, however, it is most certainly a part of a multifaceted story. This same paradigm has been seen in mutants of Troponin T and arrhythmogenic right
ventricular cardiomyopathy where humans carrying this disease suffer from sudden death (Lim 2001, Varnava 2001, Lodder 2012). Moreover, a more severe model of HCM, a double mutant mouse of TnI Gly203Ser and αMHC Arg403Gln, showed high interstitial fibrosis at day 14 before death at day 21 (Tsoustman 2008). These mice develop ventricular arrhythmias when challenged with adrenaline, and most likely died from arrhythmias. Death of cardiomyocytes is reported in these models and is most likely one of the causes leading to increased fibrosis. Moreover, it has been shown that the number of cells to propagate an arrhythmia is based on gap junction decoupling which is increased by fibrosis (Morita 2009). This has been modelled mathematically by decreasing amounts of myocyte-myocyte interactions (de Lange 2012, King 2013). Therefore, in a large study of patients, interstitial fibrosis has been used to improve detection of sudden cardiac death risk in patients suffering from non-ischemic dilated cardiomyopathy (Gulati 2013). However, fibrosis is not always associated with sudden cardiac death as aberrant calcium handling after β adrenergic stimulation can also be a cause, which has been seen in these data and others (figure 4.4D arrow, Maass 2004).

In our gene expression analysis, we found type I collagen upregulated over type III and increased Lox expression. Lox is found upregulated in cardiomyopathies where it serves to cross link collagen fibrils and further corroborates our findings of increased fibrosis in these mice. Moreover, Collagen I is more rigid than type III collagen and would increase the stiffness of the ventricle in SCD mice which may cause impaired relaxation of the ventricle, or increased preload which a contraction would have to overcome. We also found TGF- β1 increased in SCD mice which is known to be a mediator of fibrosis through the action of SMAD and MAPK pathways.
Moreover, TGF-β1 can drive fibroblasts to a myofibroblast phenotype and through interactions with cardiomyocytes they can enhance automaticity, and lead to a delayed after depolarisation (Askar 2011, Miragoli 2007). Fibrosis causes slowing of conduction speeds and interrupts organised electrical propagation in a myocardium, furthermore, increased numbers of myofibroblasts at border zones are especially dangerous for triggering an arrhythmia (Rohr 2009).

4.9.5 Aberrant calcium handling and strain effects of E99K mice

When the E99K mutation was bred onto a pure C57Bl6 background we found no differences between TG and WT spark frequency or severity in young mice. Black6 mice have been shown to be resistant to arrhythmia provocation when compared to three other strains of mice, and were more resistant to heart failure when exposed to Angiotensin II (Maguire 2003, Peng 2011). This corresponded with the absence of SCD in this background, therefore, it can be deduced that the E99K mutation does not directly cause calcium leakage from the sarcoplasmic reticulum. This is an important point because classical signs of hypertrophic cardiomyopathy are still seen in the pure Black6 background (Katja Gehmlich and Alice Sheehan unpublished data). Thus, SCD is caused by a genetically determined secondary response to the phenotypic change induced by the E99K mutation, and not by the mutation itself.

The question remains what is different between these two strains of mice which would cause a diverse phenotype. The factor in question must be related to calcium induced calcium release, and be responsive to contractile changes in the heart. Recently, Exchange protein activated by cAMP (Epac1) has been implicated in regulating RyR2 expression by phosphorylating sites serine-2808 and serine-2814 in
response to β adrenergic stimulation (Okumura 2014). In addition, Epac1 was shown to regulate Ca\(^{2+}\) handling by phosphorylating Phospholamban at serine-16. Epac1 knock out mice on the mixed C57Bl6xCBA/Ca background showed decreased susceptibility to β adrenergic arrhythmias and less cardiac apoptosis and fibrosis. Curiously, these results were strain specific, as when Epac1 was knocked out on a C57Bl6 background these mice developed normally (Pereira 2014). Thus, in Pereira’s model Epac was playing a more important role in mixed background of mice compared to the pure black6 background. These strain specific effects link to our mutation as it is known that when the E99K mutation is bred onto a pure C57Bl6 background the SCD phenotype is almost absent.

Calcium dysregulation in E99K mice has been shown previously with myofilaments being hypercontractile, and larger calcium transients seen in young mice (Song 2013, Rowlands 2017). Moreover, the load of the sarcoplasmic reticulum was tested in young E99K animals and no differences were found, implying a default in ryanodine receptor (RyR) regulation (Rowlands 2017). This is similar to other models of heart failure as an increase in RyR Ca\(^{2+}\) leakage is seen in rats, rabbits, and humans (Shannon 2003, Lyon 2009, Lyon 2011, Zhang 2014). Therefore, RyR2 regulation was tested and the phosphorylation of two sites that have been shown previously to be key in its operation were focused on. We hypothesised that over phosphorylation of the RyR2 may be causing increased spontaneous release events, however, we found the opposite - that much less phosphorylation was seen in TG and SCD animals. Interestingly, we never found any RyR2 phosphorylation at 2814 in TG or SCD mice and found robust phosphorylation at 2808 leading to the assumption that RyR2 regulation through phosphorylation may be largely directed
through this site in mutants. Although somewhat controversial in the field, serine 2814 is thought to be the Ca\textsuperscript{2+}/calmodulin-dependent protein kinase (CaMKII) site, while serine 2808 is thought to be phosphorylated by Exchange Protein directly Activated by cAMP (Epac, Lezoualc'h 2016). The TGs assayed here would be animals that survived the sudden death window and so, the lack of phosphorylation and the reduced levels of RyR2 can be explained as compensatory mechanisms which allow mice to deal with the hypercontractile effect of the E99K mutation.

A similar trend in RyR2 expression and phosphorylation was seen in SCD mice, although, the RyR2 protein was harder to blot in SCD mice than WT. Degradation of RyR2 is probably making it harder to detect, however, the stability of RNA and protein taken post-mortem has been shown to be longer than 24 hours (Finger 1987, Monique 2002, Swatton 2004, Javan 2015, Pozhitkov 2017), and our mouse line is checked every morning by technicians and any dead animals placed at 4\textdegree C when discovered. Here, we have found the abundance of RyR2 to be much reduced in SCD animals and it is known that animals without RyR2 die around E10 (Takeshima 1998). Inositol 1,4,5-trisphosphate receptors (InsP3R) are also involved in calcium release events, even though less abundant than RyR2, they are known to be upregulated in hypertrophy (Harzheim 2009). InsP3Rs cannot release enough calcium to replace RyR2s, but interestingly, they have been shown to be involved in calcium spark or wave events (Mackenzie 2002, Zime 2004, Kockskamper 2008), and they act to sensitisate RyR2s, and can even evoke extra calcium transients similar to our results in mixed background mice (Harzheim 2009, Rowlands 2017).

Based on strain related specific effects of the Epac proteins in the same background
which our mice are bred onto, the protein levels of Epac were investigated. Epac2 was found upregulated in SCD and was more highly expressed compared to wild type, or importantly, transgenic mice. We also found low levels of Epac2 in Black6 WT or TG animals. Epac2 is known to be activated by β adrenergic signalling, and in our model we could not breed E99K females because they would die from over stimulation during coitus. Moreover, Epac2 was shown not to be critical for normal contraction however, it is essential to RyR2 mediated leak, and over activation of Epac2 can cause arrhythmias (Lezoualc’h 2016). The exact mechanism of Epac induced arrhythmias is not known but Epac activation can elongate the action potential predisposing to early after depolarisations (Brette 2013).

In contrast to RyR2, phospholamban (PLN) and cardiac or slow skeletal Troponin protein expression was easily detectable in SCD animals. Robust expression of PLN was found in SCD animals although no phosphorylation was found in CBA SCD mice. However, there was phosphorylation of PLN at serine 16 in mixed background mice so these results are difficult to interpret, and show further evidence of differential expression patterns between strains. Black6 TG mice did have a reduced level of PLN and had less phosphorylation at serine 16 which may also explain a reduced propensity to undergo SCD. We did not find any phosphorylation of cTnI in SCD from the mixed background which mimics human end stage myocardium (Bodor 1997). The reduced phosphorylation is hypothesised to be adaptive to ameliorate the effects of a large calcium transient and hypercontractile myocytes at lower levels of calcium.

It has been known for some time that C57Bl6 mice are resistant to heart disease,
whilst other strains can be more susceptible (Huber and Loadge 1986, Liao 1993, Nishimura 2001, Liao 2005, Peng 2011). C57Bl6 mice have a preference for T-helper (CD4$^+$) type 1 lymphocytes (Th1), and therefore, have differing immune responses to insult (Wantanabe 2004, Wei 2011). In a pressure overload model C57Bl6 mice do not change the level of fibrosis after insult with L-NAME (Yu 2006). In addition, C57Bl6 SCID mice have significantly less fibrosis in this model, and BALC/c mice which are Th2 dominant, have significantly more fibrosis. CBA mice are Th2 dominant so it is possible that the immune system is acting together with the heart to produce phenotypic changes on a global scale. Further research into this area has shown that when Th1 cells are induced in C57Bl6 mice they have increased levels of fibrosis, and that the levels of Th1 to Th2 can directly remodel the myocardium (Yu 2005, Yu 2010). Hence, altered T-helper cell responses may be one mechanism acting in concert with altered Ca$^{2+}$ handling which contributes to the fibrosis phenotype in E99K mice.

4.9.6 Conclusion

An intriguong feature of the E99K model is the high incidence of sudden death and for the first time we could study these mice which die suddenly. E99K SCD were shown to have an interesting interstitial fibrosis phenotype which is mostly likely mediated through TGF-β. In addition, all SCD mice have a high level of interstitial fibrosis regardless of the age that they die. Moreover, the sudden death phenotype appears to be specifically absent in C57Bl6 mice and the frequency and severity of sparks in this background is the same as WT. SCD mice also had a high level of Epac2 which has been implicated in arrhythmogenic events which may be acting through phosphorylation of RyR2 at 2808. Using different backgrounds in our study
shows there is a secondary genetic component which is critical for SCD and our results suggest that Epac2 may be involved as a determining factor. Indeed, in our patient population of E99K carriers 8 SCDs were observed and 5 of these came from the same family indicating a strong genetic component for SCD. Therefore, this model allows us to test for variable penetrance in SCD and hypertrophic cardiomyopathy and could act as a staging ground for drug development.
5 Results Chapter 3. The E99K mutation in iPSCs

The University Hospital Complex A Coruna identified the E99K patient population in 2007 and currently there are 35 males and 41 females who are carriers of the disease, with a further 60 non carrier relatives without the mutation (see section 1.10, Monserrat 2007). This patient population offers a valuable resource and allows us to study the E99K mutation in the lab and have detailed patient histories to compare results against. We chose to compliment the patient information by taking advantage of iPSC technology and studying the mutation in EHTs. This would bring together the advantages of studying human tissue from patients without the disadvantages of being limited by ethical considerations etc (see section 3.11).

5.1 Patient details in this study

In collaboration with Chris Denning’s lab at the University of Nottingham punch biopsies were taken from 18 patients on our behalf by Dr Roberto Barriales: 14 mutation carriers, and 4 related non-carriers; from which fibroblasts were expanded and stored (figure 5.1). Patients specifically studied in this piece of work were characterised in A Coruna using echocardiography, MRI and ECG including Holter (figure 5.2). Patients were diagnosed with HCM if they had any section of the left ventricle with a wall thickness greater than 15mm, without dilation, and without any observed causes. Immediate family members were also characterised and average maximal wall thickness was 19.7±1.1mm, with the distribution of hypertrophy being mainly apical. Half of patients displayed a LVNC phenotype given by compact/non-compact ratio in both echocardiography and MRI. 12/14 patients also showed negative T waves in leads V3-V6 which is typical of apical hypertrophy.
<table>
<thead>
<tr>
<th>Sample</th>
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<td>47</td>
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<td>LVNC</td>
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<td>4</td>
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</tr>
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<td>A</td>
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<td>A</td>
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<td>LVNC</td>
</tr>
<tr>
<td>8</td>
<td>C</td>
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<td>Carrier</td>
<td>LVNC, ICD (NSVT, abnormal blood pressure response, family SD)</td>
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<tr>
<td>9</td>
<td>C</td>
<td>31</td>
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<td>LVNC/HCM, aborted SD, ICD, anoxic encephalopathy</td>
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<tr>
<td>10</td>
<td>C</td>
<td>70</td>
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<td>Normal (mother; in this family the disease comes from the father, who died in 2006 and also suffered from LVNC and ASD)</td>
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<td>11</td>
<td>C</td>
<td>37</td>
<td>Non Carrier</td>
<td>Normal</td>
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<tr>
<td>12</td>
<td>C</td>
<td>41</td>
<td>Carrier</td>
<td>LVNC</td>
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<tr>
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<td>B</td>
<td>57</td>
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<td>Normal</td>
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<td>Carrier</td>
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<tr>
<td>16</td>
<td>B</td>
<td>38</td>
<td>Carrier</td>
<td>LVNC + ASD (ostium secundum)</td>
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<td>B</td>
<td>65</td>
<td>Carrier</td>
<td>LVNC, atrial fibrillation</td>
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<tr>
<td>18</td>
<td>B</td>
<td>43</td>
<td>Carrier</td>
<td>LVNC</td>
</tr>
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</table>

Figure 5.1. Patient information from 18 punch biopsies. Punch biopsies were taken on our behalf by Roberto Barriales from 14 mutation carriers and 4 related non-carriers. Three families are shown in our punch biopsies with the age, genotype and phenotype.
Figure 5.2 Characteristics from 14 patients with the E99K mutation. Highlighted in yellow are the samples used in this thesis. Late gadolinium enhancement (LGE). WT = wall thickness, LV = left ventricle, LVH = left ventricular hypertrophy, NYHA = New York heart association, NC/C = ratio of noncompact/compact, NA = not available, IBBB = incomplete right bundle branch block, ICD = implantable cardioverter defibrillator.
Figure 5.2 (continued). Characteristics from 14 patients with the E99K mutation. Highlighted in yellow are the samples used in this thesis. Late gadolinium enhancement (LGE). WT = wall thickness, LV = left ventricle, LVH = left ventricular hypertrophy, NYHA = New York heart association, NC/C = ratio of noncompact/compact, NA = not available, IBBB = incomplete right bundle branch block, ICD = implantable cardioverter defibrillator.

<table>
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<tr>
<th>Sample No.</th>
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<th>Symptoms</th>
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<th>Holter</th>
<th>Treatment</th>
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<td></td>
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<td></td>
</tr>
<tr>
<td>6</td>
<td>Short PR in ECG</td>
<td>Sin us Yes No</td>
<td>Palpitations I</td>
<td>NSVT (8 beats), Paroxysmal atrial tachycardia</td>
<td>Nevirapin</td>
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<td>7</td>
<td>IRBBB</td>
<td>Sin us No No</td>
<td>No</td>
<td>I</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Sin us No No</td>
<td>ST depression inferior leads and V5-V6</td>
<td>Palpitations II</td>
<td>Paroxysmal atrial tachycardia, NSVT</td>
<td>Bisoprolol</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Short PR in ECG</td>
<td>Sin us No V1-V3 and ST depression in inferior leads and V3-V6</td>
<td>Palpitations I</td>
<td>Normal</td>
<td>No</td>
<td>Surgical repair of interatrial communication</td>
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<tr>
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<td>Short PR in ECG, interatrial communication ostium secundum</td>
<td>Sin us Yes No l, aVL, V3-V6</td>
<td>No</td>
<td>I</td>
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<tr>
<td>17</td>
<td>Short PR in ECG, Low voltage, paroxysmal atrial fibrillation, AS aneurism</td>
<td>Sin us No l, aVL</td>
<td>Palpitations, exertional fatigue</td>
<td>Paroxysmal AF</td>
<td>Atenolol, bisoprolol, HCTZ</td>
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<tr>
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<td>NA</td>
<td>Sin us No No</td>
<td>No</td>
<td>I</td>
<td>NA</td>
<td>No</td>
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<td>19</td>
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<tr>
<td>12</td>
<td>Overlapped phenotype (HCM-LVNC)</td>
<td>Sin us Yes No V3-V6</td>
<td>No Cardiac arrest at 22 yo with postanoxic encephalopathy</td>
<td>I</td>
<td>NSVT</td>
<td>ICD, Bisoprolol, hidrocoristolide</td>
</tr>
</tbody>
</table>

134
5.2 Sendai virus reprogramming of patient lines

Based on their patient characteristics we chose 6 samples to be taken forward for sendai virus reprogramming; two disease carriers and one healthy relative were taken from two different families (family A and B in figure 5.1). 5 of 6 samples were successfully reprogrammed which were confirmed by staining for pluripotency markers. Both mutation carriers and the healthy relative from family A were present so we took these three lines forward for experiments (figure 5.3A and B, herein named Donor I, II, III). Donor I had the mutation and was the parent of Donor II and III. Donor II carried the mutation and his brother a confirmed non-carrier (Donor III) are four years apart, therefore, we hypothesised they would be a good pair for minimising background genetic contribution to any phenotype which may be observed. Pluripotent cells from Donors I-III could be differentiated into cardiomyocytes showing strong staining of α-Actinin and showing spontaneous beating activity after 10 days (figure 5.3D). Therefore, the three iPSC lines I-III were expanded and differentiated into cardiomyocytes with 93.45 +-3.8%, 97.8+-0.83%, and 94.45+-3.35% purity respectively. Furthermore, we have an antibody shown to be specific for the E99K mutation (see 4.1) and could detect the presence of the mutation in Donor I and II (figure 5.3D). These two patients are known heterozygotes for the mutation and we found 56.86+-4.54%, and 57.89+-5.62% respective expression of the mutation using our antibody in iPSC-CM (figure 5.3D), suggesting allelic specific expression of either mutant or healthy allele in cardiomyocytes. The E99K mutation was also expressed in α-Actinin negative cells, which may point towards a phenotype in fibroblast cells.
Figure 5.3. Production of hiPSC-cardiomyocytes with or without c.301G>A E99K mutations. Shown in a) are 3 family members with (Donor I & II) or without (Donor III) the mutation who donated skin biopsies, which were reprogrammed (B) into hiPSC using non-integrating CytoTune 2.0 Sendai virus and E6/E8 culture medium (green is OCT4; blue is DAPI). In (C), sequencing confirmed the c.301G>A ACTC1 mutation that causes the damaging E99K polymorphic variant in diseased, but not healthy, lines. In (D), high efficiency differentiation of hiPSC yielded cardiomyocyte purities of >90% (red is α-actin staining; blue is DAPI). Counterstaining (green) with an antibody specific to the mutant E99K peptide showed that ~50% of the diseased cardiomyocytes were positive, suggesting biallelic expression of both mutant and healthy alleles (Di, Dii, E). In contrast, E99K reactivity was not detected in hiPSC-cardiomyocytes from the healthy individual (Diii, E). n=6. Scale bar b=100um, d=50um. *** P <0.001. Figure and work produced by James Smith in Chris Denning’s lab.
5.3 Preliminary characterisation of iPSC-CM EHT

We wanted to characterise iPSC-CM in EHT which was set up and optimised at Imperial College, however, the iPSCs were kept at Nottingham University which set up a logistical problem. iPSC-CM that were frozen were not found to beat in EHTs after thawing. However, freshly differentiated cardiomyocytes were also put into EHTs and the Donor I line was found to contract strongly, while Donor II and Donor III lines did not (data not shown). Therefore, the Donor I line which was positive for the E99K mutation was compared against wild type iPSC-CM from Cellular Dynamics which are used routinely in our lab. E99K iPSC-CM EHT showed higher forces of contraction from day 5 onwards compared to control constructs (figure 5.4A). Spontaneous contraction rates was variable with E99K constructs beating faster than controls from days 4-6, however E99K constructs beat slower than controls from day 12 onwards (figure 5.4B). We have shown previously that E99K mice have a blunted response to β-adrenergic stimulation (Song 2011), therefore, we tested E99K iPSC-CM EHT with 1μM isoprenaline (figure 5.4). Four out of five constructs stopped beating at one minute exposure (figure 5.4D), and three did not beat again till isoprenaline was washed off.
Figure 5.4. Contractile responses of E99K iPSC-CM Engineered Heart Tissue. iPSC-CM with the E99K mutation were used to generate EHT and compared against wild type iPSC-CM (Cellular Dynamics). E99K iPSC-CM EHT had higher levels of force (A, N=4,5) while beats per minute was variable (B). When isoprenaline was added (1μM) to E99K iPSC-CM EHT 4 out of five constructs stopped beating at 1 minute exposure and three constructs did not beat for ten minutes (C). The force of iPSC-CM EHT exposed to isoprenaline is shown in D with individual constructs labelled 1-5. Two-way ANOVA in A,B *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001. N=3,5.
5.4 CRISPR-Cas9 PiggyBac gene editing of iPSCs lines

These results were preliminary in nature although they gave us impetus to carry out CRISPR-Cas9 PiggyBac gene editing on the three donor lines to produce isogenic wild type controls for Donor I and II, and isogenic knock in E99K mutant for Donor III (figure 5.5 in collaboration with Chris Denning). The cartoon schematic strategy for CRISPR correction of the E99K mutation is shown in A. The correction of the mutation in lines I and II was confirmed, also the presence of the 301G>A ACTC1 mutation was identified in Donor III E99K line by sequencing in figure 5.5 Bi-iii. Thus, we now had 6 lines of three pairs with and without the mutation, which will be referred to by the donor number and WT or E99K for simplicity. Again, these lines were differentiated into cardiomyocytes as shown by α-Actinin staining and our site specific antibody for the E99K mutation demonstrated CRISPR corrected lines did not show the mutation, while Donor III E99K knock in line cardiomyocytes were positive for E99K (figure 5.5C and D)
Figure 5.5. Generation of isogenic E99K hiPSC-cardiomyocytes. (A) a novel footprint-free PiggyBac targeting strategy of the ACTC1 gene was used to correct or induce E99K mutation into hiPSC lines. In (B), sequencing confirmed the c.301G>A ACTC1 mutation had been corrected in Donor I & II and introduced in Donor III. In (C), high efficiency differentiation of hiPSC still yielded cardiomyocyte purities of >90% after gene editing (red is a-actinin staining; blue is DAPI). Counterstaining (green) with an antibody specific to the mutant E99K peptide confirmed protein expression was lost in diseased patients (Di, Dii, E). In contrast, E99K reactivity was now detected in hiPSC-cardiomyocytes from the healthy individual (Diii, E). n=6. Scale bar =50um. *** P <0.001. Figure and work produced by James Smith in Chris Denning’s lab.
5.5 Spontaneous contraction of CRISPR lines over time

We had previously observed Donor I E99K line to contract more strongly than EHTs generated from wild type EHT made with iPSC-CM from Cellular Dynamics (figure 5.4). However, as previously stated there is a wide variety of forces from different wild type lines from different differentiation protocols, and we also see batch to batch variation (see section 3.2). Therefore, isogenic lines from the same batch of differentiations would serve as the best controls for experiments. Spontaneously beating measurements start from the day EHTs first arrive in London and end at d14 (figure 5.6). Donor I E99K developed significantly more force than Donor I WT and beat rate was similar throughout measurements (figure 5.6Ai). Length of contractions was also similar between WT and E99K (figure 5.6Aii). Donor II forces were similar between WT and E99K until day 14 when E99K EHTs produced significantly less force (figure 5.6Bi). There were no differences between force, beat rate, or length of contraction in the Donor III line (figure 5.6Ci and Cii). Lastly, there was no differences between unstimulated contractions or stimulated (data not shown).
Figure 5.6. Spontaneous contraction of Crispr lines over time. Spontaneous force is shown for Donor I in Ai, Donor II in Bi and Donor III in Ci. The beats per minutes of Donors I-III is also shown in Aii-Cii. The length of contractions was calculated by adding together the time to peak and the relaxation to baseline and is shown in Aiii, Biii and Ciii. All error bars represent standard error of the mean. All significance was determined by two-way ANOVA with Bonferroni’s correction where: *** = p<0.001. N = 10 Donor I E99K, 11 Donor I WT, 5 Donor II E99K, 7 Donor II WT, 10 Donor III E99K, 12 Donor III WT.
5.6 Stimulated contraction parameters of CRISPR lines

Beating rate can affect relaxation and contraction parameters so on day 14 we stimulated iPSC-CM EHTs in EHT media, however not all constructs followed electrical impulses exactly probably due to there not being a conditioning period of stimulation. Donor I E99K had significantly stronger contractions than Donor I WT, however, Donor II E99K generated significantly less force than its WT control (figure 5.7Ai-iii). There was no significant difference between Donor III WT and E99K lines and so with all our lines there was no trend in force development. Although Donor I E99K line had significantly larger contractions the overall length of contraction time was the same as WT, however, both Donor II and III E99K lines had significantly longer contraction times than their WT isogenic controls (figure 5.7Bi-iii). When contraction velocity was analysed Donor I E99K line had significantly faster contraction speeds than its WT control, however, Donor II and III had slower contraction velocities with Donor II being significantly different (figure 5.7Ci-iii). Similar to contraction velocity, relaxation velocity was significantly faster in Donor I E99K but was significantly slower in Donor II and III. These data point towards Donor I being different from Donor II and III; where Donor I E99K contraction speeds were faster, taking less time to occur and producing more force than the wild type lines. The opposite was true for Donor II and III E99K lines whereby contractions speeds were slower taking longer to carry out a contraction and forces were less or equal to WT lines. Normalisation was carried out by calculating percentages from peak force generation for Donor I, Donor II, and Donor III (figure 5.8A-C respectively).
Figure 5.7. Stimulated contraction of CRISPR lines. Optical mapping of contractions were recorded at 100f.p.s and parameters are shown for all six lines which were stimulated at 1Hz. iPSC-CM EHT forces in mN are shown in Ai-iii. The length of contractions was calculated by adding together the time to peak and the relaxation to baseline and is shown in Bi-iii. Contraction velocity and relaxation velocity for the six lines are shown in Ci-iii and Di-iii respectively. All error bars represent standard error of the mean. Significance was determined by students t-test, where: * = p<0.05, and *** = p<0.001. N = 10 Donor I E99K, 10 Donor I WT, 5 Donor II E99K, 7 Donor II WT, 11 Donor III E99K, 11 Donor III WT.
Figure 5.8. Normalised force curves of Crispr lines. EHTs were stimulated at 1Hz and force was recorded, for normalisation percentages were calculated from peak force generation for Donor I (A), Donor II (B), and Donor III (C). The data is also plotted as original unedited lines in D and CRISPR edited lines in F. Errors bars represent standard error of the mean. N = 5 contractions for all lines. E99K is show in red wild type in black.
5.7 Calcium sensitivity of CRISPR lines

A classical phenotype of hypertrophic cardiomyopathy is hypercontractility at lower levels of calcium, termed calcium sensitivity. We have previously shown this phenotype in E99K mice, and in human samples from E99K hearts with different techniques (see section 1.10). We wanted to test this phenotype in iPSC-CM EHT using our patient derived and isogenic modified lines, hence EHTs were placed into increasing concentrations of calcium in Tyrode’s solution. The percentage of EHTs beating from each line was calculated as is shown in figure 3.9A, with E99K EHTs beating at a higher percentage in lower levels of calcium compared to their WT isogenic controls. On day 14 isogenic lines were producing different amounts of force, therefore, to determine if E99K EHTs were hypercontractile, force is shown as percentage of maximum contraction in individual EHTs over different calcium concentrations (figure 3.9B and C). From the original non CRISPR modified lines both Donor I and II E99K EHTs showed a significant sensitivity to calcium compared to Donor III WT line. Moreover, Donor II E99K force lowered after 1mM calcium due to some EHTs becoming arrhythmogenic and contracting poorly (figure 3.9D). However, CRISPR modified lines did not have the same trend, Donor II WT was found to be producing significantly more force, while there was no difference between Donor I WT and Donor III E99K (figure 3.9C). The individual isogenic pairs normalised contraction is shown in figure 3.10A-C. We also calculated the EC50 of all the isogenic lines which is shown for Donor I-III in figure 3.10Ei-iii. Therefore, CRISPR correction of the E99K mutation did not correct the calcium sensitivity phenotype seen in the original lines, and it did not induce calcium sensitivity when knocked into the WT Donor II line.
Figure 5.9. Calcium sensitivity of CRISPR lines. iPSC-CM EHT were exposed to increasing concentrations of calcium in Tyrode’s solution and contractions were recorded while being stimulated at 1Hz. Percentage of EHTs at the onset of beating was calculated for the six lines; mutants are shown in red and WTs in blue (A). The force of EHTs was calculated as a percentage of maximum for the original non-Crispr edited lines (B) and the same calculated is shown for Crispr edited lines (C). A Kaplan-Meier plot shows the survival of Donor II E99K and WT EHTs during the calcium concentration experiment where ‘death’ is an EHT having forces of contraction below the minimum parameter set (D). All error bars represent standard error of the mean. Significance was determined by two-way ANOVA with Bonferroni’s correction. In B for Donor I E99K vs Donor III WT ** = p<0.01 and *** = <0.001 and Donor II E99K vs Donor III WT ++ = p<0.01 and +++ = p<0.001. In C Donor I WT vs Donor III E99K * = p<0.05 and ** = p<0.01. N = 7 Donor I E99K, 6 Donor I WT, 5 Donor II E99K, 6 Donor II WT, 7 Donor III E99K, 7 Donor III WT.
Figure 5.10. Calcium sensitivity in Crispr lines. The comparison of normalised forces are shown for E99K (red) and WT (black) for Donor I (A), Donor II (B), Donor III (C). All error bars represent standard error of the mean. The dotted line in B is Donor II E99K EHTs with EHT that dropped below 0.03mN removed. Significance was determined by two-way ANOVA with Bonferroni’s correction. In B ** = p<0.01 and *** = <0.001 and +++ = p<0.001 Donor II E99K vs Donor III WT. In C * = p<0.05 and ** = p<0.01 Donor II WT vs Donor III E99K. N = 7 Donor I E99K, 6 Donor I WT, 5 Donor II E99K, 6 Donor II WT, 7 Donor III E99K, 7 Donor III WT.
5.8 Calcium sensitivity of CRISPR lines with absolute forces

The contraction of each EHT exposed to increases in calcium concentration is shown in figures 3.9 and 3.10, the results have also been given as absolute measures in force so that the differences between lines can be stated more clearly (figure 5). Similar to normalised forces original E99K lines produced more force at lower concentrations of calcium compared to WT, though this was only significant at 0.5mM (figure 5.11A). Donor I WT and Donor III E99K CRISPR corrected lines did not contract strongly, however Donor II WT did, so calcium sensitivity could not be seen segregating with the mutation in CRISPR modified lines (figure 5.11B). Donor I WT line did not contract well which was evident from the individual traces (figure 5.11Cii). However, Donor I E99K did contract strongly in comparison to both Donor II E99K and Donor III E99K which produced significantly less forces than their wild type isogenic controls (figure 5.11Ci, Di, and Ei respectively).
Figure 5.11. Calcium sensitivity of Crispr lines with absolute forces. iPSC-CM EHT were exposed to increasing concentrations of calcium in Tyrode’s solution and contractions were recorded while being stimulated at 1Hz. Absolute forces is shown for the original non-Crispr edited lines (A) and Crispr edited lines (C). Average absolute forces are plotted for each of the isogenic pairs Ci, Di, and Ei with individual traces shown for each EHT in WT (Cii, Dii, and Eii) or E99K lines (Ciii, Diii, and Eiii). All error bars represent standard error of the mean. All significance was determined by two-way ANOVA with Bonferroni’s correction. In A * = p<0.05 and *** = <0.001 and +++ = p<0.001 Donor II E99K vs Donor III WT. In C *** = p<0.001 Donor II WT vs Donor III E99K. In Ci, Di, and Ei E99K vs WT * = p<0.05, ** = p<0.01, and *** = <0.001. N = 5 Donor I E99K, 8 Donor I WT, 5 Donor II E99K, 7 Donor II WT, 7 Donor III E99K, 7 Donor III WT.
5.9 Calcium handling properties of CRISPR lines

It has been shown previously that iPSC-CM with HCM mutations have abnormal calcium handling properties (see section 1.11). We have also shown our E99K mice have abnormal calcium handling depending on mouse background and age (Rowlands 2017). Therefore, iPSC-CM EHT were taken from each of our six lines and loaded with fluo4-AM. Time to peak (TTP) was calculated from baseline to peak and calcium transient decline (CTD) was calculated as a percentage of the peak (figure 5.12). No differences were found in TTP between E99K and WT in any of the isogenic pairs (figure 5.12A and B). CTD at 90% was significantly longer in Donor I E99K compared to Donor I WT (figure 5.12Fi). No differences were found in any calcium handling properties in Donor II and III isogenic pairs (figure 5.12Eii-iii, Fii-iii).
Figure 5.12. Calcium handling properties of CRISPR lines. iPSC-CM EHT were loaded with Fluo4-AM and stimulated at 1Hz in Tyrode’s solution at 37 °C. Calcium transients were recorded and time to peak (TTP) was determined in each of the six lines with no differences found in original (A) or Crispr lines (B). Calcium transient decline (CTD) was measured in each of the six lines as a percentage of peak at 90% in original (C) and Crispr lines (D). TTP and CTD at 90% are also shown for the six lines comparing E99K and WT in their isogenic pairs (Ei-iii, Fi-iii). All error bars represent standard error of the mean. Significance was determined by students t-test, where: * = p<0.05, and ** = p<0.01. N = 3 Donor I E99K, 3 Donor I WT, 5 Donor II E99K, 3 Donor II WT, 3 Donor III E99K, 3 Donor III WT.
5.10 A novel method for analysing arrhythmogenic contractions in EHTs

When EHTs were contracting some were producing after contractions and fibrillating, the white box has measurements for length of contractions, but it does not specifically deal with abnormal contractions. The white box records the movement of EHTs and records how far the EHT moves at 100f.p.s, it records each position in each frame which can be extracted in a simple Excel file (see 2.24). These numbers were analysed in Clampfit and traces were created (figure 5.13A-ii). 211, 113 and 218 normal contractions were taken from Donor I-III WT lines, and each line was found to be a significantly different length, suggesting intrinsic differences between patient backgrounds (figure 5.13B). After the length of a WT contraction is known then each individual contraction can be compared against this number, and from this, abnormally short and long contractions can be determined (figure 5.13C see 2.24). Moreover, because the length of a normal contraction is known using the same thought process the time in between contractions can also be calculated, and short or long lengths of time in between contractions also be identified (figure 5.13C see 2.24 for details in full).
Figure 5.13. A novel method for analysing arrhythmogenic contractions in EHTs. Example traces of arrhythmogenic contractions are shown in Ai-iii. The amount of time for a contraction was calculated by pClamp software for 211, 113 and 218 normal contractions from Donor I-III WT lines and averaged in B. C shows an example trace of contractions analysed in pClamp with arrows pointing to abnormal beats. The contraction count line which touches all contractions and the baseline from where contractions relax to are also shown. All error bars represent standard error of the mean. Significance was determined by one-way ANOVA with Tukey’s correction, where: *** = p<0.001.
5.11 Arrhythmogenic contractions in stimulated CRISPR lines

HCM is associated with SCD and in our E99K patient population 8 sudden deaths were recorded (see 1.10). We hypothesise that SCD would be caused by abnormal contractions and using patient iPSC we can assess this in EHT. 31% of Donor I E99K contractions were found to be abnormal, however, this was not significant when compared to WT isogenic controls (figure 5.14Ai). Donor II E99K and Donor III E99K had significantly more abnormal contractions when compared to their WT isogenic controls, with 54% and 46% abnormal contractions respectively (figure 5.14Bi and Ci). All WT lines had roughly the same number of abnormal contractions with 18%, 14% and 17% abnormal contractions for Donor I-III WT lines respectively. Abnormal contractions were about double the length of normal contractions, and there was no difference in abnormal contraction length in Donor I and II (figure 5.14Bii and Cii).
Figure 5.14. Arrhythmogenic contractions in stimulated CRISPR lines. A novel method of arrhythmogenic contractions in EHTs was used to analyse the six iPSC-CM lines. Arrhythmogenic events were counted and expressed as a percentage for Donor I (Ai, N=10, 10), Donor II (Bi, N=8, 5), and Donor III (Ci, N=11, 11). The length of arrhythmogenic contractions was averaged and compared against normal contractions in Aii for Donor I (N=8, 10, 211), Bii for Donor II (5, 5, 113), and Cii for Donor III (N=10, 10, 218). All error bars represent standard error of the mean. Significance was determined by students t-test in Ai, Bi, Ci, where: * = p<0.05. In Aii, Bii, Cii significance was determined by one-way ANOVA, where: *** = p<0.001.
5.12 Arrhythmogenic contractions in CRISPR lines exposed to different calcium concentrations

It was noticed that some EHTs in higher levels of calcium also have more abnormal contractions, therefore, the data from the calcium exposure experiment was analysed for arrhythmias and is shown in figure 5.15. All E99K iPSC-CM had more arrhythmogenic contractions and this was significantly higher in Donor I E99K at 3mM calcium (figure 5.15A). Donor II E99K had significantly more abnormal contractions at 0.5mM and 1.5mM, and Donor II E99K had significantly more at 3mM and 5mM calcium compared to their WT isogenic controls (figure 5.15B and C). Lastly, an episode was defined as three times a normal contraction and was used to identify extremely irregular contractions, the percentage of these was then calculated and shown in 5.15D-F. Episodes were absent in Donor I WT and rare in Donor I E99K (figure 5.15D). Donor II E99K EHTs were more likely to be in an episode compared to WT, however this was never statistically significant (figure 5.15E). Donor III E99K had significantly more episodes than its WT (figure 5.15F).
Figure 5.15. Arrhythmogenic contractions in stimulated Crispr lines during exposure to increasing calcium concentrations. Arrhythmogenic events were counted and expressed as a percentage for Donor I (A), Donor II (B), and Donor III (C). An episode was defined as three times the length of an average contraction and episodic events are shown as a percentage for Donor I (D), Donor II (E), and Donor III (F). All error bars represent standard error of the mean. Significance was determined by students t-test in A, B, C, where: * = p<0.05. In Aii, Bii, Cii significance was determined by one-way ANOVA, where: *** = p<0.001. N = 7 Donor I E99K, 2 Donor I WT, 5 Donor II E99K, 7 Donor II WT, 7 Donor III E99K, 7 Donor III WT.
5.13 Gene expression in Crisr lines

RNA was extracted from iPSC-CM EHT and qPCR was carried out probing for Myosin Heavy Chain 7 (myh7), Transforming Growth Factor β1 (TGF-β1), and Sarco/Endoplasmic Reticulum ATPase (Serca2a). Both myh7 and TGF-β1 were found to be significantly upregulated in Donor I E99K compared to Donor I WT, however, Serca2a did not reach significance but was 5.5 fold upregulated compared to WT (figure 5.16Ai-iii). No significant differences was found between WT and E99K in Donor II EHTs in any of the three genes (figure 5.16Bi-iii). Myh7 was significantly reduced in Donor III E99K EHTs compared to WT, however, there was no difference in TGF-β1 or Serca2a (figure 5.16Ci-iii). Assessing gene expression in qPCR experiments is relative, therefore, both Donor I and Donor III WT and E99K lines were compared. Donor I WT had significantly less myh7 than Donor III WT and Donor III E99K, moreover, the increased expression seen in Donor I E99K myh7 compared to its WT was still lower than Donor III WT and similar to Donor III E99K (figure 5.16D). Significantly more TGF-β1 was seen in Donor I E99K compared to all other lines (figure 5.16E). Lastly, there were no significant differences seen when comparing Serca2a levels between all of the lines (figure 5.16F).
Figure 5.16. qPCR gene expression of Myosin Heavy Chain 7 (Myh7), Transforming Growth Factor β1 (TGF-β1), and Sarco/Endoplasmic Reticulum ATPase (Serca2a) in six lines. The fold change between WT and E99K is shown for Donor I (Ai-iii, N=4), Donor II (Bi-iii, N=4), and Donor III (Ci-iii, N=5,6). WT and E99K samples from both Donor I and Donor III were compared and are shown for myh7 (Di), TGF-β1 (Dii), Serca2a (Diii. All N=5,6,6,5). All error bars represent standard error of the mean. Significance was determined by students t-test in A-C or one-way ANOVA in D-F, where: * = p<0.05, ** = p<0.01, and *** = p<0.001.
5.14.1 Discussion Chapter 3. HCM in diseased and corrected iPSC-CM EHT

Here we have shown for the first time hypertrophic cardiomyopathy phenotype in three pairs of isogenic iPSC-CM EHT lines. We have taken advantage of CRISPR to correct the mutation and knock in the mutation to allow us to study the phenotypes of patients with HCM in the lab.

5.14.2 Transport between Nottingham and Imperial

iPSCs were expanded and differentiated at Nottingham and transported to Imperial, however, during the course of these experiments we experienced issues transporting cells. Initially we tried freezing cardiomyocytes and sending them on dry ice, this approach worked perfectly for cells from Cellular Dynamics which would make strong contracting EHTs from frozen cells. However, our defrosted iPSC-CM had far too many dead and non-beating cells. We then switched to sending live cells with warming packs transported by trains, this approach was much better at producing beating EHTs at Imperial, however, some lines still did not produce beating EHTs. Lastly, we found that if cells were transported without enzymatic re-plating, which was carried out in an attempt to count cells, then cells were much healthier after transport. Finally, Nottingham use a factor base protocol to differentiate iPSCs using both BMP4 and ActivinA, which are known to show batch to batch variation. Towards the end of these experiments iPSC-CM EHTs started to pull the silicone posts towards each other to such an extent that they could no longer produce contractions. This was a big problem and was seen before by our collaborators in Germany. The Eschenhagen group hypothesised that this was due to increased numbers of non-cardiomyocyte cell types like high numbers of fibroblasts or skeletal myoblasts.
5.14.3 E99K expression in CRISPR lines

The mutation specific antibody that was developed in the Marston laboratory is a unique tool and extremely useful. Using the antibody the presence of the mutation was confirmed in the original iPSC lines Donor I E99K and Donor II E99K, moreover, we also confirmed the presence of the mutation in our CRISPR knock in line and critically the absence of the mutation in our corrected lines. We found around 50% of α Actinin cells were positive for the E99K mutation which suggests repression of the mutant allele in half of cardiomyocytes. Epigenetic control of allele expression has been long known about due to X inactivation, and the mechanisms for random allelic expression in disease are being elucidated (Savova 2011, Gendrel 2014). Support for this mechanism has also been seen within β-myosin heavy chain mutations where the severity of the disease correlates with mutant protein expression (Tripathi 2011, Montag 2017). However, expression of E99K was similar in our mutant lines so this may not explain different phenotypic presentation in our lines but may be an important factor in different phenotypic expression in our patient cohort.

A small percentage of α Actinin negative cells also expressed the mutation. These cells were hypothesised to be fibroblasts which are known to express Actin, especially when in the activated myofibroblast state (Rohr 2009). There could be some unknown mechanism here which acts through fibroblasts in our model, however, studies to date about E99K show sliding filament phenotype based on improper binding of Actin to myosin heads (see section 1.10). It is possible that a perturbed Actin cytoskeleton or increased amount of Actin production produced due to deficient Actin trafficking could produce more myofibroblasts, this could hamper fibroblast response to stress and possibly contribute to the fibrosis phenotype.
However, this avenue was not explored here, and a primary phenotype of contractile dysfunction was observed.

5.14.4 Contractile dysfunction in CRISPR lines

In preliminary experiments Donor I E99K showed increased force production and a sensitivity to β adrenergic stimulation. This approach had a problem because the WT lines did not beat at this stage, so we compared Donor I E99K against our in house WT iPSC-CM line. There were a few differences between these two lines, not least of which was the genetic backgrounds were different. Moreover, in section 3.2 we showed a variation between batches of EHTs even if they are wild type. Therefore, we chose to make isogenic control lines, these preliminary results are not that reliable but are similar to previously published work in iPSC papers (see section 1.11). Isogenic lines allow experiments to be conducted on the same background, therefore, they should greatly reduce the variation and eliminate any unintended bias introduced.

Previously we have shown contractile dysfunction in mice and human tissue, including calcium sensitivity, and slower contractions (see section 1.10). Here forces were variable between isogenic lines with mutants having stronger, weaker, and the same amount of force produced between isogenic pairs. This is probably due the measurements being taken in EHT media which has 1.8mM calcium, enough to elicit a maximum contractile response. Moreover, the Donor I WT line had ~70% purity of cardiomyocytes compared to >95% for Donor I E99K, which may have affected force production, especially if non-cardiomyocyte cells were proliferating during the two week incubation period. Donor I E99K and Donor III E99K both had longer
contraction times and decreased velocity of contraction. This result is more reliable because both these isogenic pairs have the same phenotype even when one had the mutation knocked in and one with it corrected. Glutamic acid 99 is situated in a myosin binding domain and its change to lysine causes a charge reversal (see section 1.10). It is hypothesised that associations between Myosin and Actin are slower because of the perturbed interaction, thus, this could produce slower contractions seen in Donor II and III E99K. We also see the slower contractions by elongated relaxation times in our contraction data from papillary muscle, and from cells isolated from adult mice (Song 2013, Rowlands 2017). Moreover, isolated human E99K Actin shows decreased association, dissociation and slower velocities with Myosin than WT (Bookwalter 2006).

5.14.5 Evaluating calcium sensitivity comparing CRISPR and original lines
The calcium sensitivity of the original iPSC-CM EHT lines was increased in E99K compared to WT. The wild type used was son or brother to the two E99K Donors, and so is expected to be a close match genetically, thus, reducing the amount of background interaction in the experiment. Therefore, we were confident of showing the same calcium sensitivity using the isogenic control lines. When comparing CRISPR lines where the mutation had been corrected or knocked in we saw that calcium sensitivity was not present in Donor III E99K, or corrected in Donor II WT. Hence, CRISPR modification of the mutation did not modify this phenotype, and the calcium sensitivity was the same as the genotype of the original lines.

This has important implications, firstly, it shows that hypercontractility caused by calcium sensitivity is a secondary component and not directly caused by the
mutation itself. A build-up of stress or aging itself could cause this over time which would cause this phenotype to change with age, and may explain why we only see some hypertrophic cardiomyopathy phenotypes later in life. Donor I was 48 years old at the time of biopsy compared to his sons (Donor II and Donor III) who were 19 and 15, respectively. Indeed, contraction parameters have been shown to change with age, hormone levels, and an iPSC-CM HCM model has only shown phenotypes with stress (Tanaka 2014, MacDonald 2014, Feridooni 2015). Thus, iPSC-CM with the E99K mutation knocked into the Actc1 gene may need more time or extra stress to develop this phenotype. Secondly, it shows the need for isogenic controls when carrying out experiments and caution when reviewing experiments without these controls. Long standing phenotypes in HCM such as calcium sensitivity have been attributed directly to point mutations like ACTC E99K. Therefore, because isolated Actin has been shown to be sensitive to calcium these data point to some other modification that must be present which would be some direct modification of the thin filament itself.

These data could also be explained by the CRISPR lines not contracting as well. Recently it has been shown that CRISPR does not edit the genome with extremely high fidelity as previously thought, Schaefer et al showed by whole genome sequencing that many mutations can be found after CRISPR modification (Schaefer 2017). Moreover, we used a piggyback method which is longer and may introduce more genetic instability. The stress of going into 0mM calcium and slowly increasing levels could affect lines which had already experienced stress through CRISPR modification. Indeed, CRISPR modified lines Donor I WT and Donor III E99K did not contract strongly through the calcium exposure experiment, however, Donor I WT
was not contracting strongly under normal conditions. Though, this explanation seems unlikely because the CRISPR modified line Donor II WT contracted strongly and the calcium sensitivity phenotype was not corrected in this line.

5.14.6 A novel method to analyse arrhythmic contractions in EHTs

During contraction analysis, it was noticed that E99K iPSC-CM EHT had aftercontractions which the white box does not identify as abnormal. Therefore, we undertook a novel approach to analyse the length of each individual peak which was captured by the white box. Using this approach, we showed that WT contractions from the three different backgrounds were all different lengths. This shows that the background of each line has either responded differently to differentiation or that intrinsic differences of force are present in each background. This again reiterates the need for isogenic controls in experiments.

Donor II E99K and Donor III E99K had more arrhythmogenic events both under stimulated contraction and during the calcium exposure experiment. Most assays used in modelling arrhythmias in iPSCs to date are based off single cell autonomous phenotypes, like calcium handling and electrical dysregulation (Knollman 2013). These are important and add to our knowledge of arrhythmias, however, arrhythmias leading to sudden cardiac death are multi-cellular tissue level defects, and as such should be modelled on a multi-cellular level. Here, we show arrhythmias in engineered heart tissue with after contractions which progressively become more frequent and more severe, leading to fibrillation. This was most common in Donor II E99K which had 80% of EHTs drop below the minimum level for contraction detection due to fibrillation. In our patient population SCD was reported in 10.5% of
carriers which is considerable higher than reported for most HCMs (Sen-Chowdhry 2016). We found very few arrhythmogenic events in Donor I E99K, therefore, we show that patients can be screened and stratified for risk using iPSC-CM EHT technology. Moreover, we show that CRISPR technology can correct the mutation and abate arrhythmogenic contractions.

5.14.7 Donor I contraction different to Donor II and III
Donor I E99K had the opposite phenotype to Donor II E99K and Donor III E99K in stimulated contraction analysis, calcium transient length, and arrhythmogenic analysis. Donor I was Donor II and Donor III’s father, thus, Donor I would have different genetic background which could explain the differences seen. Moreover, Donor I was 48 years old, and may have developed coping mechanisms for the disease over time. Having a better contraction machinery that was somehow adapted to the E99K mutation could then reduce the incidence of arrhythmias. Donor II and III E99K lines had similar contraction phenotypes but the older Donor I E99K was markedly different. HCM is well known to be an age progressive disease and Donor II did have apical hypertrophy of 20mm but did not have any other symptoms. Therefore, its similarity in contraction phenotype to the E99K edited originally wild type line may be explained by the relative early stage of this disease compared to the much older Donor I. This gives two inferences: one, that the E99K mutation causes a contractile dysfunction in itself, and two, that age distinctly affects this phenotype. Moreover, the contractile dysfunction shown by the younger isogenic lines may be more dangerous as these lines had more arrhythmias.

TGF-β has been linked to cardiomyopathies through the action of myofibroblasts, and recently in an iPSC-CM model of left ventricular non-compaction (LVNC). Our
patient cohort has a predominantly apical hypertrophy, however, LVNC is also a common diagnosis. We saw a ten-fold increase in TGF-β levels in Donor I E99K, and it was also higher in Donor I E99K compared to Donor III WT and E99K. TGF-β which has been demonstrated to produce abnormal decrease in proliferation of progenitor cardiomyocytes, and arrested development of the heart (Shou 1998, Chen 2009, Kodo 2016). Unfortunately, we did not test proliferation or fibrosis in our iPSC-CM EHT model, though we did see increased levels of fibrosis in our mouse model. Therefore, it is possible that the abnormal contraction in E99K cardiomyocytes is causing increased fibrosis by myofibroblasts. Remarkably, the induction of TGF-β was fixed upon CRISPR correction of the mutation, therefore, TGF-β must respond to contractile dysfunction caused.

Donor I E99K may be able to better cope with stress as seen by its ability to produce a hyperbolic curve with strong contractions in the calcium experiment, it had an EC_{50} of 0.15mM of calcium but showed very few arrhythmogenic contractions. The upregulation of TGF-β causes higher levels of reactive oxygen species so it is possible that this line has developed adaptive changes for stress. Curiously, Donor I E99K and WT was the only pair to change its calcium sensitivity with CRISPR modification. This may be because Donor I WT force of contraction was 2.5 fold lower than Donor I E99K, so we never saw a strong enough effect. However, if the result holds true this would imply that the background of Donor I was truly different in some way and it would be fascinating to follow this in the future.
5.14.8 Conclusion

Importantly, here we show the power of CRISPR and how critical point mutations are specific places of essential contractile machinery. We have shown how differences can be found among patients with the same mutation with detailed analysis using three pairs of isogenic lines. CRISPR editing of iPSC-CM saw a reversal of phenotypes in contraction assays including reducing arrhythmias which would be deadly in humans. CRISPR allowed study of a mutation on the same genetic background which during the course of this study has been shown to be critical to a range of responses. Moreover, the phenotypes displayed on a ‘clean’ background should be more reliable.
6 Final discussion. Is iPSC CM EHT a good model of the E99K mutation?

HCM has been studied using the E99K mutation in actin for almost 10 years and our understanding of the disease has increased considerably in that time. Clinical characterisation has been carried out of a patient population in Spain with the E99K mutation with more than 70 persons in 11 families (Monserrat 2007). The patients do not present with a typical form of HCM since patients present with apical hypertrophy and have a higher proportion of SCDs. Therefore, because we have access to a large amount of patient data we wanted to study this mutation further which would help us understand the specific disease of the patients, and give insights into the general disease mechanism in HCM.

6.1 Insights from the mouse model of E99K

Traditionally mice have been used to study human diseases due to their quick reproduction rates and the ability to manipulate their genomes with little ethical qualms. We also studied the E99K mutation in mice and recapitulated many aspects of the disease (Song 2011, Song 2013, Rowlands 2017). There are two major factors likely to determine SCD, abnormal Ca2+-handling and increased fibrosis. Young TG animals likely to be destined for SCD had a hypercontractile phenotype, an increased Ca2+-transient, and enhanced spark mass that would predispose to fatal arrhythmias (Rowlands 2017). We also showed that different backgrounds of mice suddenly die at different rates, and importantly, we have a test bed for SCD which has been notoriously difficult to investigate.

Depending on which background the E99K mutation was bred onto changed the levels of calcium sparks and the incidence of sudden cardiac death. Our TG pure
black6 mice had the same number of sparks as WT, in stark contrast to mice on the mixed background where TGs had increased numbers of sparks (Rowlands 2017). SCD was also absent in black6 mice but occurred at around 24% in the mixed background. It was proposed that a sub population with a high spark mass in the mixed population corresponded to mice that undergo SCD while the survivors were able to compensate. Calcium release events at the ryanodine receptor can be large enough to initiate the inward mode of the sodium/calcium exchanger which is well known to lead to delayed after-depolarisations and arrhythmias (Fedida 1987, Zaugg 2001). We believe it is the calcium release events which trigger arrhythmias and lead to sudden death in our animals.

What is interesting is the regular timing of SCD which lies within a 20 day period. Animals within the death window of 25-45 days are experiencing puberty and mouse body weight increases dramatically during this period. Blood flow also increases and to compensate for this the mouse heart increases its stroke volume 3.5 fold and weight 3.47 fold which corresponds to an increase in cell number (Naqvi 2014). Furthermore, mice at Imperial are weaned at P21 and are placed into separate cages, which increases the stress on the animals as they assert dominance and get used to their new surroundings (Nicholson 2009). Increased burden on the heart and increased stress could push our mice to have arrhythmias, especially if the excitation-contraction coupling has not yet adapted to the increased calcium sensitivity caused by the E99K mutation.

Fibrosis has been clearly shown in HCM and has been demonstrated in mouse models of the disease (Shepard 2009). Here we showed that SCD animals have
higher levels of fibrosis than transgenic mice, which is similar to patients with higher levels of fibrosis that have more adverse outcomes (O’Hanlon 2010, Bruder 2010). These data are important because it shows that mice with the same mutation can have different clinical outcomes, and that our mixed background mice can have more adverse outcomes in the same way as humans. Therefore using our mice we investigated SCD and the mechanistic factors which trigger such a devastating outcome of HCM. We showed that E99K SCD mice have higher levels of fibrosis and this was also accompanied with increased amounts of TGF-β. This signalling molecule has long been implicated in fibrosis and has been shown up regulated in HCM (Khan 2006). Moreover, TGF-β has been shown to be causative of fibrosis and fibrosis can be attenuated by inhibiting TGF-β using antibodies (Teekakirikul 2010). Unfortunately, clinical trials following up targeting TGF-β have been unsuccessful because it is such an active signalling molecule involved in the immune system (Fang 2017).

More severe disease in mice is also seen when there are two known mutations in the same mouse which increases sudden cardiac death events and the amount of hypertrophy (Tsoustman 2008a). This point is highlighted in the TnI Gly203Ser and αMHC Arg403Gln mutated mouse, which showed high interstitial fibrosis at day 14 before death at day 21 (Tsoustman 2008b). Within those experiments or with our data we cannot determine whether high levels of fibrosis caused SCD, or whether these mice had a more severe form of heart disease which caused fibrosis and SCD.
6.2 Moving from animal to human experiments

The animal work with the E99K mutation has increased our understanding of the disease, however, the mouse is beset with problems as a model system for studying the cardiovascular system since it is different from human (Cook 2009, Kaese 2012). Another way to study the mutation would be to use human tissue from the patients themselves, and we have worked with frozen samples (Song 2011). Fresh samples are more difficult to obtain, since the affected patients seldom come to transplant in the UK. There is also a lack of normal human tissue for control experiments, since donated hearts which are not able to be used for transplant (and yet are still classified as normal) are rare in Europe. Indeed, there is considerable debate about whether donor heart can be normal (Jeweied 2007). Moreover, samples from the ACTC E99K patients were rare due to patients with apical hypertrophy not needing septal myectomies (the samples studied by Song 2011 came from an operation for atrial septal defect). Therefore we wanted to develop a new way to study human myocyte contractility which did not involve an animal model and was more efficient than using human tissue samples.

We chose to take advantage of iPSC technology and combine this approach with contractility measurements using EHTs. Working with iPSCs gave us some advantages in that cells induced to pluripotency will have the same mutation as the patients, with the same genetic background (Chen 2016). Importantly, iPSCs can proliferate and large quantities of cells can be produced and cardiomyocytes can be made with protocols that have become widespread. Moreover iPSC-CM can be kept in culture for prolonged periods which means studies of long term drug responses are also possible. The drawback of this technology is that the cardiomyocytes
produced are not as mature as adult cells (Denning 2016). Therefore, the constant thorn in the side of stem cell researchers is the demonstrated phenotype could be an artefact of a less mature cell, or that a phenotype may be missed due to the immaturity of iPSC-CM. Taken together the advantages of iPSCs are more numerous than the disadvantages, and it gives researchers insight into the specific patient condition when the patient is still alive.

Some progress has already been made in modelling HCM with iPSCs and some interesting insights have been found. iPSCs showed many aspects of the HCM phenotype including increased cell size and activation of hypertrophic genes (see table 1.3). Studies also demonstrate the dysregulated calcium transients and action potentials caused by mutations, which could lay the basis for delayed after depolarisations, and could cause deadly arrhythmias in the heart (Lan 2013, Han 2014). Pharmaceutical treatment with drugs given to patients could be tested and has been demonstrated, for example by treating HCM iPSC-CM with verapamil to ameliorate calcium handling abnormalities (Han 2014). Lastly, iPSC-CM HCM models also served as the test bed for genetic correction of disease traits when the mechanism of disease was investigated. Prodzynski et al showed that adeno-associated viral transduction of Myosin Binding Protein C rescued hypertrophy caused by a truncated mutant form of the protein (Prodzynski 2017). However, what all these studies lack is proper controls, as HCM lines were compared against control lines which were not related to the patient. These experiments could have been improved by using CRISPR to correct the mutation thus giving isogenic control lines. We have seen in our comparisons of different cell lines that differences exist in contractility and calcium handling properties, so, a control the same genetic
background would be ideal to distinguish the effect of the mutation itself instead of differences between cell lines.

We wanted to carry out a thorough examination of E99K iPSCs and therefore chose to make isogenic control lines using CRISPR and PiggyBac technology to produce footprint free isogenic lines (Wang 2016). We had produced one wild type and two E99K iPSC lines that were from direct relatives (a father and two sons), and produced isogenic lines with/without the mutation. This gave us two E99K lines originally with the mutation and corrected to wild type (Donor I and II), and one line originally wild type with the mutation induced (Donor III). This platform allowed us to investigate the E99K mutation on the same background circumventing problems with non-isogenic controls. Interestingly, because the donor of our E99K line (Donor I) was much older than the others we could also investigate if prolonged time with the mutation might have produced epigenetic changes which carried over into the iPSC-CMs.

6.3 Analysing contractility in iPSCs

There are a few methods now to analyse contractility using iPSCs including 2D and 3D approaches. 2D approaches use edge tracking to show the shortening of cell clusters using Ionoptics or similar apparatuses (Lundy 2013). iPSCs can also be measured using optical tracking without singling out clusters, by using either beads or outlines of cells (Lan 2013, Huebsch 2015, Ribeiro 2017). These methods do not measure force but analyse shortening, and they are limited by only measuring one cell in isolation which is not like the heart. 3D approaches are an improvement on 2D because they are a multicellular system with which cell-cell interactions can be
assayed and a pseudomuscle can be constructed to measure contractility. 3D systems for measuring contractility are either: edge detection software and using properties of posts to infer force, or a direct measurement of force using micro force transducers (Hansen 2010, Boudou 2012, Jackman 2016). We decided to use the edge detection platform invented by Thomas Eschenhagen because this allowed us to non-invasively measure force in a 3D multicellular environment over time. Moreover, this technique is easily multiplexed, and lends itself to producing high numbers of hydrogels with little variation within batches.

To provide valid controls, we have used iPSCs edited with CRISPR and PiggyBac to produce three pairs of isogenic lines, and have analysed them using the 3D contractility assay EHT. This approach gives us several advantages: the E99K mutation on three patient genetic backgrounds, both corrected and knocked in patient lines, isogenic controls for these three lines, and a three dimensional contractility platform to analyse the lines. These combined techniques give more exact experimental parameters than previous publications on HCM, because there are multiple lines with the same mutation to analyse and isogenic controls to compare mutants against. Furthermore, increased understanding about the E99K mutation can be gathered and correlated with our patient characterisation, and mouse data.

6.4 Contractile dysfunction caused by the E99K mutation

Previously, we have shown contractile dysfunction in mice and myofibrils from human tissue, including increased calcium sensitivity, and slower contractions (see section 1.10). Donor II E99K (original line) and Donor III E99K (edited line) both had
longer contraction times and decreased velocity of contraction compared to their isogenic controls. Both these isogenic pairs have the same phenotype even when one had the mutation knocked in and one had the mutation corrected. Glutamic acid 99 is situated in a myosin binding domain and its change to lysine causes a charge reversal (Olson 2000). The charge reversal was proposed to cause associations between Myosin and Actin to be slower because of the perturbed interaction at this critical residue, thus, this could produce the slower contractions and relaxations seen in Donor II and III E99K. In support of this hypothesis we see elongated relaxation times in our contraction data from papillary muscle, and from cells isolated from adult mice (Song 2013, Rowlands 2017). Moreover, isolated human E99K Actin also shows decreased association, dissociation, and slower velocities of interaction with Myosin than WT (Bookwalter 2006).

6.5 Variable phenotypic presentation in E99K patients

Echocardiography showed diastolic dysfunction in 14/55 patients with the E99K mutation, and similarly two out of three E99K lines had a relaxation defect, so there is variable phenotypic presentation of the mutation in EHTs and humans. The question of why there is a heterogeneous phenotype presented in HCM is interesting, and is directly highlighted by the results in this thesis. The E99K patient population all were diagnosed with HCM and/or LVNC, therefore, the difference seen in our iPSC-CM lines could be due to the difference in these two clinical presentations. There is some argument whether LVNC is a phenotype of HCM or a cardiomyopathy in its own right (Arbustini 2014). However, all our patients analysed by echocardiography had hypertrophy which was mostly apical (57/61 were apical), therefore it is safe to say our mutation produces apical hypertrophy characteristic of
HCM. Importantly, our assay is sensitive enough to pick up the difference between mutant lines and further investigation into the difference may uncover more information about disease presentation.

The heterogeneous phenotype presented in HCM which was showed by our patients, and by our isogenic lines, is also complicated by the patients being different ages. Donor I was 48 and his two sons 19 and 15 (Donor II and III respectively). Donor II and III E99K lines had similar contraction phenotypes but the older Donor I E99K was markedly different. HCM is well known to be an age progressive disease and Donor II did have apical hypertrophy of 20mm but did not have any other symptoms. Therefore, its similarity in contraction phenotype to the E99K edited originally wild type line may be explained by the relative less advanced stage of this disease compared to the much older Donor I. This gives two inferences: one, that the E99K mutation causes a contractile dysfunction in itself, and two, that age distinctly affects this phenotype. Moreover, the contractile dysfunction shown by the younger isogenic lines may be more dangerous as these lines had more arrhythmias.

We showed that Donor II and III E99K lines had more arrhythmogenic events than their isogenic controls, which is in contrast to Donor I E99K, where the increase was not significantly different to WT. Different backgrounds of mice had different levels of SCRD, therefore, this shows that the background of the mutation has a strong effect on how likely it is that an arrhythmia occurs. This adds further evidence that a phenotype such as arrhythmias is a multiple factor response to an underlying cause including: abnormal calcium handling, fibrosis, cell death, hypertrophy, and
congenital abnormalities (Klavdios 2015). Similar to Donor II and III E99K EHTs, the E99K mixed mouse population had a high level of arrhythmias which led to SCD at around 26% of the population. Likewise, the E99K patient population (and the E99K mouse) had a high level of abnormalities in ECGs, and increased arrhythmias which lead to a high rate of SCD compared to other HCMs (53/61 ECG abnormalities, E99K SCD = 10.5% compared to <2% Sen-Chowdhry 2016).

Results presented here are similar to a few isogenic studies conducted in DCM, and longQT which find differences between patients with the disease and even clones from the same patient (Bellin 2013, Wang 2014, Hinson 2015, Jehuda 2017). Hinson et al showed variable phenotypes were presented with different genetic backgrounds after correction of DCM Titin mutations using CRISPR (Hinson 2015). These results and ours show that genetic background is important to phenotypic presentation in the lab and may begin to help explain variable phenotypes in patients. The quality of a study relies upon the comparison between the experimental group and the control. Here, we show like others that genetic background is important and taking iPSCs from a healthy close relative where ~50% of the genome may be different could have a large effect on the result (Musunuru 2013). Furthermore, Vikhorev et al has shown a contrast in phenotypes seen in Titin mutated DCM samples from adult human heart tissue compared with iPSC-CM (Vikhorev 2017, Hinson 2015). Reduced contractility and haploinsufficiency was recorded in iPSC-CM however this was not seen in adult heart preparations which may be explained by the relative immaturity of iPSC-CM.
6.6 The future

The future of the field is shaping up to progress to more complexity in disease modelling using 3D multicellular models which recapitulate more of the environment of the heart compared to 2D culture alone. 3D multicellular models come with considerable challenges in producing multiple cell types and combining these cells with tissue engineering. Furthermore, standard 2D measurement techniques have to be modified for 3D models to glean as much information from this preparation as possible. The biggest obstacle to this aim will be creating large constructs with a workable perfusable network of vessels, although, it has been observed that microvasculature already exist in EHTs made with neonatal rats cells (Stoehr 2016).

The heart field is slowly recognising the importance of using isogenic controls in disease modelling and also drug screening. We have used CRISPR combined with PiggyBac due to the specificity of the Cas9 molecule and the protein’s ability to specifically cleave the genome. CRISPR was combined with PiggyBac because of the large pay load the PiggyBac system can deliver, however, TALENs and Zinc Finger Nucleases are also used effectively (Wang 2016, Ochiai 2015). Isogenic controls should allow more accurate assessment of disease phenotypes and importantly allow higher accuracy in understanding drug mechanics. This may help bridge the notorious gap between lab identified drug targets and toxicity or ineffective clinical trials.

What is interesting is that under close scrutiny in this thesis differences can be seen between patients/backgrounds which may go some way to explaining the variable phenotypic presentation in HCM. Moreover, further work into this area could help the
understanding of why people die suddenly and maybe set up better screening for this in the future, or a molecular understanding of the events.
7 Conclusion

7.1 Aims and hypothesis

The main aim of this thesis was to model the E99K mutation in EHT using iPSCs. This was based on about 10 years of work in the Marston laboratory into the E99K mutation in the mouse model. A significant challenge in this piece of work was that the EHT technique did not exist outside of Germany, and any techniques that were to be carried out on EHTs had to be set up at Imperial (section 1.1.5.2i).

Furthermore, the iPSCs did not exist until the final year of the PhD and the isogenic controls took longer still which meant not a lot of time was spent with these cells. EHT is now routinely made at Imperial and it is possible to carry out a range of techniques including: non-invasive contraction analysis (including any drug application), qPCR, western blot (including gel electrophoresis), sample preparation for proteomics, immunofluorescence, digestion of EHTs to recover cells, optical mapping with dyes at fast frame rates (fluo4-AM and fluovolt), and arrhythmia analysis.

Aim 1.1.5.2ii was to optimise the contractile performance of EHT using maturation strategies. Many approaches were trialled however using only force as the output for this aim hampered the results (discussed further in 3.14). It can be said that constructs are similar in their properties to the Eschenhagen group where the technique was learnt, and similar to published results (see 1.14). However, the approaches were largely unsuccessful with maybe the exception of longer time in culture, and increased numbers of cells in constructs the only two approaches creating notable differences in force. It must be said that an efficient technique and a
good batch of serum were the most important factors in EHT generation at least considering force as the main output.

Aim 1.15.2iii was to study the E99K mouse model and SCD in the model was focussed on mainly because this aspect had not been investigated in the Marston lab to date, and this may give insights into the mechanism of SCD. Interestingly, differences were found in fibrosis levels, gene expression, and protein expression in these mice. It was also fascinating to find that black6 mice did not have increased levels of spark events and had almost no SCD. Investigation of this aim led to a hypothesis being formed that impacted on the iPSC experiments in which different backgrounds of the mice had a large impact on the results.

Aim 1.15.2iv and v were addressed at the end of this project and it can be said that the Denning group made iPSCs and CRISPR/Cas9 PiggyBac lines. Unfortunately, the limited amount of time meant that aim 1.1.5.2v was not completed satisfactorily and much of the future work section is populated with further experiments to continue this aim. The limitations to this part are discussed in section 7.3, however, useful work was carried out that was interesting. Analysis of a point mutation with three isogenic pairs of iPSC lines allowed much more detailed experimentation than any other HCM publication to date, because background differences are accounted for with isogenic lines. Moreover, we found that one pair of isogenic iPSCs did not have the same phenotype as the two other pairs. This shows that comparing iPSCs to the correct control is critical, and that even father to son is not a close enough comparison.
7.2 Clinical implications of the current work

HCM affects 1 in 500 of the population and produces a critical burden on society and also results in a significant loss of quality of life. HCM is also the most frequent cause of SCD in the young and trained athletes. Although SCD caused by HCM is not very frequent in the general population (<100 deaths per year Maron 2009) it is considered an important public health problem due to the devastating effects and because this affects young otherwise healthy individuals.

The work presented here gives further mechanistic insight into HCM and SCD. We also show that it is possible to gain patient specific insights into the disease using iPSCs. This process was slowed down by getting ethics in place, however one could imagine in the future that iPSCs could be used to better phenotype individuals. In light of our results this becomes more important given that even with the same mutation HCM patients can and do present differently. Special significance should also be given to the differences between younger and older patients identified as having a disease causing mutation. It is becoming more frequent now to carry out genetic testing for known HCM causing mutations, however, these patients do not present the same as older sufferers with the disease.

Furthermore, our arrhythmogenic analysis showed that E99K iPSCs from younger patients were more likely to have arrhythmogenic events. Based on this finding it would be advisable for these patients to implement lifestyle changes especially not carrying out high intensity exercise. Moreover, implantable cardioverter/defibrillator devises have been shown effective in reducing mortality in patients with HCM at high
risk of SCD (Wordsworth 2010). Therefore, patients in our population should consider this treatment if possible.

Diastolic dysfunction has been shown in papillary muscles (mouse), myofibrils (mouse), single cells (mouse), and EHT (human). The phenotype was rarer in the patient population however this is probably due to the relaxation phenotype being masked by the whole heart and/or the progression of hypertrophy. Diastolic dysfunction can be alleviated by reducing hypertension or reducing left ventricular hypertrophy. ACE inhibitors and beta blockers are usually used for treatment, however, these drugs do not target the underlying cause of the disease but rather contributing exacerbating factors (Ha 2009).

Fibrosis was markedly increased in SCD animals and adds weight to an increasing body of evidence that fibrosis levels are critical compounding factor in SCD, which has also been shown clinically (O’Hanlon 2010, Bruder 2010). Moreover, we found increased levels of TGF-β which has been shown to be a driver of fibrosis. However, targeting TGF-β is problematic because it is a key molecule which negatively regulates the immune system and inhibition has negative cardiac effects in clinical trials (Fang 2017).

7.3 Limitations of the current work

This project does have some limitations which will be discussed in the following section. Induced pluripotent stem cells represent a remarkable feat that is fascinating, however, these cells cannot be considered mature. The hypothesis that iPSCs will recapitulate some of the adult phenotype is problematic. Firstly, even
within a multicellular three dimensional model like an EHT this is still not a perfect model of the heart. The heart is innervated and receives many neurohormonal signals. Although, attempts were made to stimulate constructs this still would not be anything like the impulses from a nerve. It will be interesting to see in the future if some attempt is made in combining nerve cells and heart constructs but to date nothing has been published. The heart is also under haemodynamic pressure which adds load to the heart, which is completely absent in EHTs. Moreover, this haemodynamic load in hypertrophic cardiomyopathy is constantly increased and a good model of this would allow for experimentation on the interplay between hypertension and E99K cells. The heart is also formed with thick muscle made possible by vascularisation which is absent in human EHTs. Although some vascular network is identifiable in rat EHTs, necrosis is still seen in the middle of constructs which are only ~100µm thick. The vascularisation of constructs is stopping progress onto larger constructs that would be similar to a ventricle.

The assumption is that a larger innervated construct, perfused, with correct neurohormonal signals would be better for modelling/regeneration proposes, however, there is still an inherit limitation with the maturity of iPSCs. It may be that if these obstacles can be overcome that iPSCs will become mature adult like cells. Some evidence for this comes from transplanting iPSC-CM into animal models, however, these cells are still not as mature as neonatal rat cells transplanted into an adult rat (Chong 2014, Kadota 2017). It is possible that iPSCs are an abnormal cell that cannot become adult cardiomyocytes, and the ‘maturation’ reported on the cell level is actually changing cell properties rather than truly maturing cells. Furthermore, an inherent problem with this is that if cells did become mature adult
cells using a maturation strategy in this manuscript, then this cell would most likely die within 24hrs because adult cells do not survive under current conditions. This also confounds the issue because dying cells would be trapped inside the construct. The problem is actually two fold, attempting to mature the cells and also maintain the cells in the matured state. It is possible that the same factors/approach could achieve both these aims, however, it is unlikely that signals to grow and mature the heart are the same as maintenance signals. Moreover, abusing signals of growth/maturation could cause a disease state.

We presented the EHT model and also tried to mature the construct using force as the only measure. This has one major problem in that a selection bias will be introduced and only approaches that increased force would have been considered maturation. Many approaches have shown an increase in maturation in calcium transients or action potentials, and so, any of these approaches would have been missed. Although, the action potential and calcium transient should be linked to force it is also possible that our system for measuring force has an inherent limit. If the cells are not connected enough through the hydrogel, or stretched to an optimal length then cell autonomous differences in transients could be missed on the large scale multicellular approach to measuring force.

Sudden cardiac death is a difficult phenomenon to study because by definition it is sudden. Therefore, you can never really know if mice you are observing when alive would be a part of the mice which would experience SCD. Using large numbers of mice and then selection of different phenotypes may circumvent this issue, however, this brings in human bias and because SCD occurs within two hours you still may
miss information. In this study mice which had already died were studied, this has the big problem of attempting to gain information from a dead animal. Certainly, cellular changes occur when an animal dies, and myocardial arrhythmia is a serious way to die. This is evidenced by examining the hearts of SCD mice post mortem, which have large atria with clots. Depending on the length of time that an animal was left at room temperature there would be degradation of tissues and proteins. This is mostly likely why it was difficult to gain RyR2 western blot results, since blotting receptors of large size is notoriously difficult anyway.

The mouse model of E99K could have been used to make EHTs and modelled in the same way as iPSCs. This was shown to be technically possible with the mouse model of HCM in the Carrier lab (De Lange 2013 A and B, Wijnkefr 2016). This approach has a problem because it is not possible to have homozygous pups from our mice (females die during coitus) and therefore pups would have been at best 50% positive for E99K. However EHTs could have been made with this caveat in mind which would have bypassed many of the problems with waiting for ethics, making iPSCs, and differentiating cells.

The patients used in this study are abnormal when compared to the overall HCM population. Mutations in actin only account for around 1% of total HCM cases and the E99K mutation would account for an even smaller part of the total population. Moreover, the type of hypertrophy produced by this mutation is predominantly apical which is quite common in Asia but not in Caucasian populations (25 vs 0.2% respectively Lakshmanadoss 2012). Moreover, because of the difficulty and time limitation of making isogenic iPSCs we only looked at three pairs. We had access to
more patient biopsies and although using isogenic controls is a significant improvement on HCM publications at this time, we have really compared one old mutant patient with one young mutant patient and one young control. It would really be interesting to compare the rest of the cell lines to see if the phenotypes were similar to Donor II or Donor I and to see if the phenotypes matched. It is possible that what has been recorded is specific to one generation compared to another or just an artefact of these cell lines.

Lastly, a major obstacle in this thesis was waiting for the ethics to be in place to allow us to conduct experiments on iPSCs. iPSCs were only really ready for experimentation in the last 12-6 months and so much more characterisation could have been achieved. Moreover, the transport of iPSC-CM from Nottingham to Imperial is largely unnecessary as we are completely capable of expanding and differentiating high quality iPSCs at Imperial. This problem was overcome, but it wasted time and resources and the stress of transporting the cells may have made poorer EHTs. Moreover, the end of the PhD EHTs started to spontaneously pull the posts together so that no contractions could be measured. We hypothesised this was due to Nottingham’s factor based differentiation protocol with higher levels of BMP4 pushing cells to a smooth muscle phenotype. Whatever the reason, because another differentiation protocol was not used no data was gained at a critical part of the PhD.

7.4 Future work
The main gap in the current body of research that this thesis highlights is the lack of understanding in HCM about variable phenotypic presentations of HCM, and how experiments are carried out without isogenic controls. We showed a big difference
with backgrounds of mice concerning SCD and contractility of iPSCs from different patients. It would be important for the field, and interesting to test this idea fully in the future. This would allow a better definition of a HCM phenotype in the lab, and importantly, give a better understanding of the molecular mechanisms of the disease instead of highlighting something that may be specific to a background or secondary cause. Experimentally this avenue of research could be explored using the iPSC lines already derived in this project and certain questions could be addressed. One, if there truly is a contractility difference between different lines of iPSCs. Two, the differences between E99K phenotypes with HCM can help determine the different presentation of this disease. Specifically, these could be addressed by making EHT from iPSCs with the mutation and comparing their contractility. These cells/EHTs could then be compared based on prior knowledge of important contractile proteins, or in a large scale approach using RNAseq or proteomics.

An important difference highlighted by this piece of work which is related to the variable phenotype expressed in our mice line is the higher expression of Epac2 in SCD mice. This represents a ‘smoking gun’ for variable phenotype approach because it was found upregulated in SCD mice and not in wild type or transgenic mice from either mixed or black6 backgrounds. The Epac proteins are useful targets because they are involved in arrhythmogenesis, they are a target of the β adrenergic pathway, and target RyR2. Epac proteins also have widely available inhibitors which could be taken advantage of to test Epac involvement in arrhythmogenesis in mixed background mice.
Further characterisation of the iPSC lines which were investigated in this project would be interesting. A blunted response to β adrenergic stimulation was seen in previous work with the mouse, and this response was tested in preliminary results with the E99K line. However, when this experiment was attempted with isogenic lines no clear result was found. Previous work in hydrogels have found positive ionotropic responses to isoprenaline and a refined technique would be needed to investigate this phenotype in iPSC-CM EHT. It is likely there is a change in the β adrenergic system in E99K HCM, however, Donor I was given a β blocker and this could affect the results in this line.

An improvement over the current EHT technique would be measuring force at an optimised length on a devise like an Aurora. Peak forces from hydrogels are always recorded at the optimal length for each construct and this is the only way force frequencies responses are recorded as positive. Optimal stretching of EHTs may uncover maximal force differences in disease lines or other phenotypes yet unknown. This approach can also be used to test the force frequency response in E99K EHTs and the response of β adrenergic agonists. Moreover, calcium sensitivity can be tested in this apparatus in a similar way to how the experiment was conducted in the white box.

Fibrosis was an important phenotype recognised in E99K mice and there was an interesting distinction between SCD and transgenic mice. Unfortunately, this was not tested in iPSC-CM EHTs. EHTs were made with fibroblasts from the patients and so it is possible that these cells could be producing a fibrosis like response. Fibrosis is complicated and it may not occur in a construct without neurohormonal stimulation.
and the fibroblasts may be in an immature state. However, if excess Collagen is being produced simple staining may have uncovered this phenotype. Moreover, in preliminary results an up regulation was seen in Collagen type I by qPCR in E99K EHTs, therefore, probing for Collagens and genes involved in fibrosis may be possible. It would also be interesting to study sparks in iPSCs and see if the level of calcium release events correlates to the numbers of arrhythmias seen in EHTs. This could quite easily be carried out by loading cells with fluo4 and following the sparks protocol. This experiment would add support to our hypothesis that there is uncontrolled release of calcium from RyR2.

The energy hypothesis involves mutations causing increased energy expenditure which becomes a causative factor in HCM. It would have been interesting to test this hypothesis and this could have been carried out using Sea Horse, which measures the metabolic activity of cells. Although, this would have had to be conducted on single cells not EHTs. Another method which has been used in EHTs is glucose depletion, but this does not give the same number of parameters of the Sea Horse machine.

This is the first time the black6 line has been characterised at Imperial. Some work has been done with this line in Oxford, however, it would be useful to test the key phenotypes seen in the mixed background mice. Calcium sparks were investigated but not calcium transients or contraction parameters from single cells. Moreover, the sensitivity to β adrenergic stimulation would be interesting since this line does not experience SCD. It would also be interesting to test contraction of papillary muscles and myofibrils to see if the same phenotype is observed as the mixed background.
Lastly, the level of fibrosis would also be interesting in this model to see if there is a high level of fibrosis in the sudden death window. If so these mice must either have a coping mechanism for this or fibrosis is not a determining factor in SCD. SCD mice were observed as having no phosphorylation of cTnl which was surprising as end stage heart failure patients have a reduced amount of phosphorylation but usually some is found. Confirming this result could be carried out using phosphoproteomics and a more in-depth analysis of the phosphorproteome of these mice could be carried out.

In the future, it would also be interesting to carry out further molecular characterisation of the E99K patient population. Heart tissue is sometimes collected in the form of myectomies or heart transplants and this would be an invaluable resource for studying the disease. Proteomics and transcriptomics could be carried out with minimal tissue, and targets involved in fibrosis or calcium induced calcium release could be investigated. Comparisons could also be made against iPSCs and the similarity of the iPSC model to the human model could be investigated more thoroughly.

Lastly, the differentiations for iPSCs at the end of the PhD lead to EHTs being produced which pulled the posts together to such an extent that contractions could not be taken. These differentiations could most likely be fixed by moving away from a factor based differentiation approach and using a small molecule approach.
7.5 Concluding remarks

During the course of this work the engineered heart tissue platform has been set up at Imperial and further information has been learnt about the ACTC E99K mutation. We have demonstrated contraction of EHTs from both rat neonatal cardiomyocytes and iPSC-CM. Furthermore we have set up automated optical tracking measurements using the White Box and tested multiple maturation approaches. We have further characterised the ACTC E99K mouse model and analysed animals that die suddenly. Lastly, in collaboration with Chris Denning we have analysed six iPSC lines by knocking in the mutation and correcting the E99K mutation in one wild type line and two transgenic lines, respectively. Thus, we have analysed a hypertrophic cardiomyopathy mutation in six iPSC lines which has given this work an increased level of detail not seen in previously published work.

Of the maturation strategies tested only increased cell number and increased time in culture were found to have an increase in force compared to controls. These approaches have subsequently been shown in recent publications with the same effect (Lundy 2013, Tiburcy 2017). The other maturation strategies may not have had an increase in force but may have increased maturation in other ways not tested in this work. It remains to be seen what factors are needed to make a construct populated with iPSC-CM similar to heart tissue. However, recent work from the Bursac group show almost adult levels of contraction in their constructs which are similar to the approach here (Shadrin 2017).

The work presented here with the ACTC E99K mouse model went into a recent publication from our group and added interesting observations on sudden cardiac
death caused by hypertrophic cardiomyopathy (Rowlands 2017). Although, SCD is difficult to study and results are hampered by the rapid onset of a SCD event, any added information about this tragic event will be helpful to finding effective treatments in the future. With our ACTC E99K mouse model a true cause and effect mechanism has not been found, hence, no specific effective drug has yet been developed. Therefore, we are currently working on modulating the calcium sensitivity of cells within this mouse line.

An effective drug may not need to be found if CRISPR gene editing can live up to its promise, which this is a particularly interesting time for as clinical trials are currently taking place with this new technology. We showed that contraction phenotypes and critically arrhythmia propensity can be reduced by CRISPR editing. Therefore, CRISPR correction by using AAV9 viral delivery could help patients suffering with this disease.


BIAN, W., LIAU, B., BADIE, N. & BURSAC, N. 2009. Mesoscopic hydrogel molding to control the 3D geometry of bioartificial muscle tissues. Nat Protoc, 4, 1522-34

BLAIR, E., REDWOOD, C., ASHRAFJAN, H., OLIVEIRA, M., BROXHOLME, J.,
Mutations in the subunit of AMP activated protein kinase cause familial
hypertrophic cardiomyopathy evidence for the central role of energy
compromise in disease pathogenesis. *Hum Mol Genet*.

BODOR, G. S., OAKELEY, A. E., ALLEN, P. D., CRIMMINS, D. L., LADENSON, J.

mutation in an expressed human alpha-cardiac actin at a site implicated in

BOSTROM, P., MANN, N., WU, J., QUINTERO, P. A., PLOVIE, E. R., PANAKOVA,
D., GUPTA, R. K., XIAO, C., MACRAE, C. A., ROSENZWEIG, A. &
SPIEGELMAN, B. M. 2010. C/EBPbeta controls exercise-induced cardiac
growth and protects against pathological cardiac remodeling. *Cell*, 143, 1072-
83.

BOUDOU, T., LEGANT, W. R., MU, A., BOROCHIN, M. A., THAVANDIRAN, N.,
RADISIC, M., ZANDSTRA, P. W., EPSTEIN, J. A., MARGULIES, K. B. &
CHEN, C. S. 2012. A microfabricated platform to measure and manipulate the

BRECKWOLDT, K., LETUFFE-BRENIERE, D., MANNHARDT, I., SCHULZE, T.,
ULMER, B., WERNER, T., BENZIN, A., KLAMPE, B., REINSCH, M. C.,
LAUFTER, S., SHIBAMIYA, A., PRONDZYSKI, M., MEARNINI, G., SCHADE,
D., FUCHS, S., NEUBER, C., KRAMER, E., SALEEM, U., SCHULZE, M. L.,


engineered heart muscle by electromechanical stimulation. *Biomaterials*, 60, 82-91.


LEOR, J., ABOULAFIA-ETZION, S., DAR, A., SHAPIRO, L., BARBASH, I. M.,
BATTLER, A., GRANOT, Y. & COHEN S. 2000. Bioengineered cardiac
graphs: A new approach to repair the infarcted myocardium. Circulation.

LESMAN, A., HABIB, M., CASPI, O., GEPSTEIN, A., ARBEL, G., LEVENBERG, S.
& GEPSTEIN, L. 2010. Transplantation of a tissue engineered human

LEVENBERG, S., ROUWKEMA, J., MACDONALD, M., GARFEIN, E. S., KOHANE,
D. S., DARLAND, D. C., MARINI, R., VAN BLITTERSWIJK, C. A.,
vascularized skeletal muscle tissue. Nat Biotechnol, 23, 879-84.

Sensor EPAC Proteins and Their Role in Cardiovascular Function and

Survival and function of bioengineered cardiac grafts. Circulation. 100(19

oxidant effect of transforming growth factor- beta1 mediates contractile

LIAN, X., HSIAO, C., WILSON, G., ZHU, K., HAZELTINE, L. B., AZARIN, S. M.,
RAVAL, K. K., ZHANG, J., KAMP, T. J. & PALECEK, S. P. 2012. Robust
cardiomyocyte differentiation from human pluripotent stem cells via temporal


engineered heart tissue format: Comparison with human atrial trabeculae. 

*Toxicol Sci.* 158(1):164-175.


LAFLAMME, M. A., NANTHAKUMAR, K., GROSS, G. J., BACKX, P. H.,
KELLER, G. & RADISIC, M. 2013. Biowire: a platform for maturation of
human pluripotent stem cell-derived cardiomyocytes. *Nat Methods, 10, 781-7.*

O'HANLON, R., GRASSO, A., ROUGHTON, M., MOON, J. C., CLARK, S., WAGE,
R., WEBB, J., KULKARNI, M., DAWSON, D., SULAIBEEKH, L.,
CHANDRASEKARAN, B., BUCCIARELLI-DUCCI, C., PASQUALE, F.,
COWIE, M. R., MCKENNA, W. J., SHEPPARD, M. N., ELLIOTT, P. M.,
PENNELL, D. J. & PRASAD, S. K. 2010. Prognostic significance of
myocardial fibrosis in hypertrophic cardiomyopathy. *J Am Coll Cardiol, 56,
867-74.*

OCHIAI, H. 2015. Single-Base Pair Genome Editing in Human Cells by Using Site-
Specific Endonucleases. *Int J Mol Sci, 16, 21128-37.*

ODDE, D. J. & RENN, M. J. 1999. laser guided direct writing for application in

OKUMURA, S., FUJITA, T., CAI, W., JIN, M., NAMEKATA, I., MOTOTANI, Y., JIN,
H., OHNUKI, Y., TSUNEOKA, Y., KUROTANI, R., SUITA, K., KAWAKAMI, Y.,
HAMAGUCHI, S., ABE, T., KIYONARI, H., TSUNEMATSU, T., BAI, Y.,
SUZUKI, S., HIDAKA, Y., UMEMURA, M., ICHIKAWA, Y., YOKOYAMA, U.,
SATO, M., ISHIKAWA, F., IZUMI-NAKASEKO, H., ADACHI-AKAHANE, S.,
TANAKA, H. & ISHIKAWA, Y. 2014. Epac1-dependent phospholamban
phosphorylation mediates the cardiac response to stresses. *J Clin Invest, 124,
2785-801.*

OLSON, T. M., DOAN, T. P., KISHIMOTO, N. Y., WHITBY, F. G., ACKERMAN, M. J.
& FANANAPAZIR, L. 2000. Inherited and de novo mutations in the cardiac


POZHITKOV, A. NEME, R., DOMAZET-LOZO, T., LEROUX, B. G., SONI, S.,
TAUTZ, D. & NOBLE, P. A. 2017. Open Biology Tracing the dynamics of gene
transcripts after organismal death. Open Biology. 7(1). pii: 160267.

PRONDZYNSKI, M., KRAMER, E., LAUFER, S. D., SHIBAMIYA, A., PLESS, O.,
FLENNER, F., MULLER, O. J., MUNCH, J., REDWOOD, C., HANSEN, A.,
Evaluation of MYBPC3 trans-Splicing and Gene Replacement as Therapeutic
Options in Human iPSC-Derived Cardiomyocytes. Mol Ther Nucleic Acids, 7,
475-486.

QIN, X., RIEGLER, J., TIBURCY, M., ZHAO, X., CHOUR, T., NODOYE, B.,
NGUYEN, M., ADAMS, J., AMEEN, M., DENNEY, T. S., YANG, P. C.,
Imaging of Cardiac Strain Pattern Following Transplantation of Human Tissue

QING, X., WALTER, J., JARAZO, J., ARIAS-FUENZALIDA, J., HILLJE, A. L. &
SCHWAMBORN, J. C. 2017. CRISPR/Cas9 and piggyBac-mediated footprint-
free LRRK2-G2019S knock-in reveals neuronal complexity phenotypes and
alpha-Synuclein modulation in dopaminergic neurons. Stem Cell Res, 24, 44-
50.

RACCA, A. W., BECK, A. E., RAO, V. S., FLINT, G. V., LUNDY, S. D., BORN, D. E.,
BAMSHAD, M. J. & REGNIER, M. 2013. Contractility and kinetics of human

RACCA, A. W., KLAIMAN, J. M., PIONER, J. M., CHENG, Y., BECK, A. E.,


SELLERS, R. S., CLIFFORD, C. B., TREUTING, P. M. & BRAYTON, C. 2012.
Immunological variation between inbred laboratory mouse strains: points to consider in phenotyping genetically immunomodified mice. *Vet Pathol*, 49, 32-43.

Update on hypertrophic cardiomyopathy and a guide to the guidelines. *Nat Rev Cardiol*, 13, 651-675.


SHIELS, H. A. & WHITE, E. 2008. The Frank-Starling mechanism in vertebrate

SHIMIZU, T. 2006. Polysurgery of cell sheet grafts overcomes diffusion limits to
produce thick vascularized myocardial tissues. *FASEB*.

SHOU, W., AGHDASI, B., ARMSTRONG, D. L., GUO, Q., BAO, S., CHARNG, M. J.,
MATHEWS, L. M., SCHEIDER, M. D., HAMILTON, S. L. & MATZUK, M. M.
1998. Cardiac defects and altered ryanodine receptor function in mice lacking

SIMPSON, J. LABUGGER, R., HESKETH, G. G., D'ARSlNY, C., O'DONNELL, D.,
Differential Detection of Skeletal Troponin I Isoforms in serum of patient with

SOMMESE, R. F., SUNG, J., NAG, S., SUTTON, S., DEACON, J. C., CHOE, E.,
consequences of the R453C hypertrophic cardiomyopathy mutation on human
beta-cardiac myosin motor function. *Proc Natl Acad Sci U S A*, 110, 12607-
12.

SONG, K., BACKS, J., MCANALLY, J., QI, X., GERARD, R. D., RICHARDSON, J.
coactivator CAMTA2 stimulates cardiac growth by opposing class II histone

SONG, W., DYER, E., STUCKEY, D. J., COPELAND, O., LEUNG, M. C., BAYLISS,
C., MESSER, A., WILKINSON, R., TREMOLEDA, J. L., SCHNEIDER, M. D.,
HARDING, S. E., REDWOOD, C. S., CLARKE, K., NOWAK, K.,


SUN, N., YAZAWA, M., LIU, J., HAN, L., SANCHEZ-FREIRE, V., ABILEZ, O. J.,
NAVARRETE, E. G., HU, S., WANG, L., LEE, A., PAVLOVIC, A., LIN, S.,
CHEN, R., HAJJAR, R. J., SNYDER, M. P., DOLMETSCH, R. E., BUTTE, M. J.,
Patient-specific induced pluripotent stem cells as a model for familial dilated
cardiomyopathy. Sci Transl Med, 4, 130ra47.

SUN, N. & ZHAO, H. 2014. Seamless correction of the sickle cell disease mutation
of the HBB gene in human induced pluripotent stem cells using TALENs.
Biotechnol Bioeng, 111, 1048-53.

SUN, X., ALTALHI, W. & NUNES, S. S. 2016. Vascularization strategies of
engineered tissues and their application in cardiac regeneration. Adv Drug
Deliv Rev, 96, 183-94.

Protein profiling of human postmortem brain using 2-dimensional fluorescence
difference gel electrophoresis (2-D DIGE). Mol Psychiatry, 9, 128-43.

TANAKA, A., YUASA, S., MEARINI, G., EGASHIRA, T., SEKI, T., KODAIRA, M.,
KUSUMOTO, D., KURODA, Y., OKATA, S., SUZUKI, T., INOHARA, T.,
ARIMURA, T., MAKINO, S., KIMURA, K., KIMURA, A., FURUKAWA, T.,
myofibrillar disarray and contractile vector variability in hypertrophic
cardiomyopathy-induced pluripotent stem cell-derived cardiomyocytes. J Am
Heart Assoc, 3, e001263.

TEEKAKIRIKUL, P., EMINAGA, S., TOKA, O., ALCALAI, R., WANG, L.,
WAKIMOTO, H., NAYOR, M., KONNO, T., GORHAM, J. M., WOLF, C. M.,
KIM, J. B., SCHMITT, J. P., MOLKENTIN, J. D., NORRIS, R. A., TAGER, A.
G. 2010. Cardiac fibrosis in mice with hypertrophic cardiomyopathy is
mediated by non-myocyte proliferation and requires Tgf-beta. J Clin Invest,
120, 3520-9.

TIBURCY, M., DIDIE, M., BOY, O., CHRISTALLA, P., DOKER, S., NAITO, H.,
KARIKKINETH, B. C., EL-ARMOUCHE, A., GRIMM, M., NOSE, M.,
ESCHENHAGEN, T., ZIESENISS, A., KATSCHINKSI, D. M., HAMDANI, N.,
differentiation, advanced organotypic maturation, and modeling of

TIBURCY, M., HUDSON, J. E., BALFANZ, P., SCHLICK, S., MEYER, T., CHANG
LIAO, M. L., LEVENT, E., RAAD, F., ZEIDLER, S., WINGENDER, E.,
RIEGLER, J., WANG, M., GOLD, J. D., KEHAT, I., WETTWER, E., RAVENS,
U., DIERICKX, P., VAN LAAKE, L. W., GOUMANS, M. J., KHADJEH, S.,
TOISCHER, K., HASENFUSS, G., COUTURE, L. A., UNGER, A., LINKE, W.
A., ARAKI, T., NEEL, B., KELLER, G., GEPSTEIN, L., WU, J. C. &
ZIMMERMANN, W. H. 2017. Defined Engineered Human Myocardium With
Advanced Maturation for Applications in Heart Failure Modeling and Repair.
Circulation, 135, 1832-1847.

TOHYAMA, S., HATTORI, F., SANO, M., HISHIKI, T., NAGAHATA, Y., MATSUURA,
T., HASHIMOTO, H., SUZUKI, T., YAMASHITA, H., SATOH, Y., EGASHIRA,
T., SEKI, T., MURAOKA, N., YAMAKAWA, H., OHGINO, Y., TANAKA, T.,

228


XU, X., TAY, Y., SIM, B., YOON, S. I., HUANG, Y., OOI, J., UTAMI, K. H., ZIAEI, A.,
NG, B., RADULESCU, C., LOW, D., NG, A. Y., LOH, M., VENKATESH, B.,
GINHOUX, F., AUGUSTINE, G. J. & POULADI, M. A. 2017. Reversal of
Phenotypic Abnormalities by CRISPR/Cas9-Mediated Gene Correction in
Huntington Disease Patient-Derived Induced Pluripotent Stem Cells. *Stem
Cell Reports*, 8, 619-633.

YANG, L., SOONPAA, M. H., ADLER, E. D., ROEPKE, T. K., KATTMAN, S. J.,
KENNEDY, M., HENCKAERTS, E., BONHAM, K., ABBOTT, G. W., LINDEN,
cells develop from a KDR+ embryonic-stem-cell-derived population. *Nature*,
453, 524-8.

YEH, Y. H., KUO, C. T., CHAN, T. H., CHANG, G. J., QI, X. Y., TSAI, F., NATTEL,
S. & CHEN, W. J. 2011. Transforming growth factor-beta and oxidative stress
mediate tachycardia-induced cellular remodelling in cultured atrial-derived

YUSA, K., RASHID, S. T., STRICK-MARCHAND, H., VARELA, I., LIU, P. Q.,
PASCHON, D. E., MIRANDA, E., ORDONEZ, A., HANNAN, N. R., ROUHANI,
F. J., DARCHE, S., ALEXANDER, G., MARCINIAK, S. J., FUSAKI, N.,
HASEGAWA, M., HOLMES, M. C., DI SANTO, J. P., LOMAS, D. A.,
BRADLEY, A. & VALLIER, L. 2011. Targeted gene correction of alpha1-

ZHANG, F., WEN, Y. & GUO, X. 2014a. CRISPR/Cas9 for genome editing:

ZHANG, M., D'ANIELLO, C., VERKERK, A. O., WROBEL, E., FRANK, S., WARD-
VAN OOSTWAARD, D., PICCINI, I., FREUND, C., RAO, J., SEEBOHM, G.,

232


ZIMMERMANN, W. H., MELNYCHENKO, I., WASMEIER, G., DIDIE, M., NAITO, H., NIXDORFF, U., HESS, A., BUDINSKY, L., BRUNE, K., MICHAELIS, B.,

9.1 Pictures of EHT components
9.2 Provisional protocol for generation of human engineered heart tissues (hEHT)

1. Prepare the following reagents:

a. 2% agarose in PBS, autoclave, store at 60°C (immediately!).

b. Aprotinin – 33mg/ml in cell culture grade water, filter-sterilise, aliquot and store at 20°C.

c. Fibrinogen – 200mg/ml (sterile)
   i. In pre-warmed 0.9% NaCl solution (made up in sterilised cell culture water).
   ii. Add 72.1 aprotinin (33mg/ml stock) to 25ml fibrinogen solution, mix, aliquot and store at -80°C.

d. Thrombin: 100U/ml. Make aliquots – 450ul stock; 3µl-per EHT

e. 10x DMEM solution: dissolve 670mg DMEM powder in 5ml cell culture grade water. Sterile filter and store at 4°C.

f. 2x DMEM
   i. 10x DMEM solution – 2ml
   ii. Horse serum (inactive) – 2ml
   iii. P/S – 0.2ml
   iv. Cell culture grade water – 5.8ml
Sterile filter (0.22µm) and store at 4°C.

g. EHT – medium
   i. Horse serum (inactive) – 10%
   ii. Insulin – 0.1% (10µg/ml)
   iii. Aprotinin – 0.1% (33µg/ml)
   In basal medium – DMEM (Biochrom F0415) + 1%P/S

h. NKM medium
   i. Fetal calf serum inactive – 10%
   ii. Glutamine (200mM) – 1%
   In basal medium – DMEM (Biochrom F0415) + 1%P/S

2. Add 1.6ml of 2% agarose solution (stored at 60°C) per well of 24 well plate. Place Teflon spacer on top of agarose solution.
   Note 1. Agarose solidifies very fast so pipette max 8 well before placing spacer and do NOT leave agarose solution at RT for longer than 5 minutes.

3. Prepare mastermix (per EHT) in universal tube, on ice – for 4 EHTs (+10% extra).
   a. Cells 4.4 million – concentration ~ 12M/ml is appropriate
   b. 2x DMEM - 24.5µl
   c. 10% Marigel (add to ice cold medium) - 44µl
   d. Y-27632 (1:1000) – 0.44µl
   e Fibrinogen – 11.13ul (5mg/ml)
f. Thrombin – 13.2µl (3U). Just for calculation purposes, do not add it to the mastermix

g. NKM up to 440

Note 2. Fibrinogen has to be luke-warm for pipetting. Slowly fill the tip of the pipet and do not touch the surface of the ice cold mastermix with the fibrinogen containing pipet – the viscosity of the fibrinogen would increase immediately.

4. When the agarose is solid (becomes opaque, approx. 10min) remove the spacer and place the silicone racks. Make sure the silicone posts are really in the agarose slot.

5. Triturate (stripette) the mastermix 10-15 times until the fibrinogen clot is dissolved (check the mastermix in the pipette for homogeneity).

6. For each EHT pipette 100µl mastermix in one thrombin aliquot (3µl), mix and pipet the mixture into the agarose slots quickly. Stripette mix the mastermix after 8 EHTs.

7. Place in the 37°C incubator for 2 hours.

8. Cover each well with a small amount of KNM (200-500µl). Shake the plate gently and incubate again at 37°C for 15 minutes.

9. Prepare a new 24-well plate with 1.5ml EHT medium per well.
10. Remove the silicone racks carefully from the agarose casting moulds and transfer them to the medium filled plate.

11. Place EHTs in the incubator, and feed every 2-3 days.
9.3 White Box Step By Step Guide

General guidelines – the software can be a bit slow, just be patient and allow it to carry out calculations in its own time. The software also doesn’t like any movement of boxes during a measurement this will cause a crash. Gloves must be worn with the box at all times to keep cell culture conditions as clean as possible. If for any reason there is a crash please turn it off and on again this usually fixes the problem, you may have to do this multiple times. Never rename any files or move any files for any reason whatsoever, files may be copied when you need to collect your data. A green highlighted box means the box has set up the axis positions for that well, yellow means no positions have been set, white means that is the well you have currently highlighted.

Taking a live cell measurement

1. Turn on hard switches for heater, 15V sensors, and voltage linear axis.
2. Turn on computer
3. Load white box software by double clicking on the CTMV physio icon
   CRITICAL – do not load more than one software window ie by clicking the icon a number of times. This will crash the system.
4. A window should load saying ‘Hardware activ?’ click ‘start position’ and wait
   Explanation – the software will move the axis to the zero position (over A6) so that it has a point of reference.
5. Click Protocol tab (righthand side) and enter in your details, here the length of a trace can be changed but these details will be unchangeable when a measurement is being taken. The remarks box can be filled up even during measurement and is useful for adding observations.
6. Put in your 24 well plate with EHTs.

7. Click Set Up tab (righthand side). This is where you ‘tell’ the computer where your EHTs are located the positions correspond to a standard 24 well plate. Double click on every well where an EHT is located, they should turn yellow.

8. Click Camera View (lefthand side). And under live view click ‘Start’, this will bring the camera online. Now when you click on wells under the Set up tab the camera will move to this location. If the camera is not near your EHT then move the camera towards it using the axis buttons, see trouble shooting. Check – if one of the axis has turned red it is not working and the camera will not move, you will need to re start the software and make sure the hardware is turned on, see trouble shooting. If you cannot click on ‘start’ then click on a well under the set up tab and it should allow you to click on it.

9. Click on manual mode (middle of screen) and position the blue boxes where the EHT meets the post and on the edge of the EHT to get the most contrast difference for your measurement.

Explanation – automatic mode works but sometimes gets a location wrong, it takes slightly longer to click the boxes in yourself but you should reanalyze less. CRITICAL – the movement of these boxes will determine the force, if the boxes do not move with the contraction of an EHT then an incorrect value will be recorded.

10. Once the boxes have been selected click ‘Position Ok’ (righthand side on Set up tab), this should turn the box green showing that the computer has remembered this position.

11. Click ‘Parameter’ tab (lefthand side) and click ‘Peak force’ and click ‘use default parameters’ this will load the standard set of parameters for a
recording, then click ‘use parameters on all wells’. These parameters are explained in the glossary and can be changed slightly for better recordings. CRITICAL – never change the force calculation parameters these are based on the standardized post characteristics.

12. Click ‘Automatic’ tab (righthand side) click ‘Start’ and then the box will automatically measure your EHTs.

13. If you want to view the measurements taking place click ‘Real time’ this is not essential but is advised if you think you will get a bad reading.

14. A pdf file will automatically be produced including the traces of your EHTs and the recorded parameters.

15. Your recording will be stored in the data folder on the desktop and stored by under sequential folders of year, month, day and then time. COPY your data onto an external drive. CRITICAL – never move or rename or delete any data for any reason!! Also put your EHTs back in the incubator….

The data recorded by the white box

The white box will produce a pdf file with traces pictures at the start and end of your trace and information on contractile parameters. T1 is contraction time, T2 is relaxation time. The 10, 20 or 50% refers to the percentage of the peak where the time is taken for point of contraction/relaxation. Sum FS is the sum of the fractional shortening of the EHT and is given in %.

The resting length is calculated between your blue boxes. RR Scatter is similar to RR interval on an ECG, this corresponds to the time between the peaks of contraction. Sum CV is
the sum of the contraction velocities and Sum RV is the sum of the relaxation velocities.

The white box will also produce: compressed video of the recording made for each EHT, a picture of your EHT at the beginning and the end of a recording, a graphic of the trace (poor quality), an excel file with the contraction parameters for all EHTs recorded, and the individual points of force/movement of each EHT for each frame recorded.

Offline mode
You can analyse your previous recordings offline, this mode is used mostly if there is a bad recording.

16. Click ‘Runs’ (lefthand side) and find your recording, click your recording and wait for it to load.

17. Click ‘Offline Mode’ (righthand side) and click on the well which you would like to adjust, blue boxes can be adjusted or how the box takes measurements under the ‘parameter peak force’ tab like in step 9 onwards.

18. If you want to change one well then click ‘Sample Review’ if want to reanalyse all the wells then click ‘Offline Analysis’.

19. This will produce another analysis which will be place into the same folder as the original measurement although will be labelled with the date and time which you carried out the new analysis.

CRITICAL – never move or rename or delete any data for any reason!!
Trouble shooting

The first thing to do is check there is not two versions of the software open. Next check the hardware is on, and was turned on in the correct order (hardware then software). Then restart the system, sometimes it turns on and just doesn’t want to work… Last is check is all the wires, the box is a little cramped and the area is busy so rarely this is the case, the axis also moves so the wires plugging in to each one occasionally move (there are small green lights to look for on each axis). If the camera is not on then you will have to restart.

There are a few ways the box will crash caused by the user, the most common is over clicking as the system is a little slow. Occasionally if you have made lots of measurements in 24 hours it may crash with the new update this just needs a restart of hardware and the computer and should fix the issue.

If you have tried all the trouble shooting then check is all the wires this is the first thing that the Hamburg group mentions when there is a problem, please tell them you have checked this in any correspondence…

Only once has the box actually malfunctioned seriously when being used correctly, this was a system error where all the measurements were lost. This was quickly fixed by the Hamburg group who can remote connect to the computer when its connected to the internet here, usually it is not. The Hamburg group are very nice and respond to emails, useful emails here are:

info@eht-technologies.com (the company mostly run by admin)
ar.hansen@uke.de (Arne professor – more hands off now)
a.eder@uke.de (Alex postdoc – set up the system very useful)

i.mannhardt@uke.de (Ingra postdoc – does more help with carrying out experiments)
### List of Antibodies

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<td>Alexa donkey anti mouse 546</td>
<td>Invitrogen</td>
<td>1:400</td>
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<tr>
<td></td>
<td>Alexa goat anti mouse 647</td>
<td>Invitrogen</td>
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## 9.5 List of Primers (all Applied Biosystems)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>Product code</th>
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<td></td>
<td></td>
<td>Hs00913333_m1</td>
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<td>Rat</td>
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</table>
9.6 Plasmids for CRISPR/Cas9 and piggybac gene editing

Selection cassette with homologous arms and the mutation plasmid:
Guide RNA for Cas9 plasmid:
Cas9 plasmid:
PiggyBac transposon plasmid:

![Diagram of the pCMV-hyPBase plasmid](image-url)
9.7.1 Step by step pictures for arrhythmia analysis

Copy numbers into Notepad and save, and open in Clampfit software.
Clicking ‘OK’ will only analyse one peak, then click on the arrows (below) to analyse the whole trace.
9.7.2 This produces a results table (minimise the trace to see it)

9.7.3 The results can then be copied into Excel where the formulas on the ‘Events’ tab will calculate the number of abnormal events.
9.7.4 Events tab calculates the number of abnormal contractions in a trace.

N.B. extra zeros after the last contraction must be deleted
9.8 Nottingham quick media reference guide

**MEF Medium:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Conc.</th>
<th>100ml</th>
<th>200ml</th>
<th>Bottle</th>
<th>Company</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBS</td>
<td>10%</td>
<td>10ml</td>
<td>20ml</td>
<td>57ml</td>
<td>Fisher</td>
<td>10664083</td>
</tr>
<tr>
<td>NEAA</td>
<td>1%</td>
<td>1ml</td>
<td>2ml</td>
<td>5.7ml</td>
<td>Fisher</td>
<td>10358342</td>
</tr>
<tr>
<td>Glutamine</td>
<td>2mM</td>
<td>1ml</td>
<td>2ml</td>
<td>5.7ml</td>
<td>Fisher</td>
<td>10104042</td>
</tr>
</tbody>
</table>

Mix the above together and filter sterilise in to DMEM.

| DMEM       |       |       |       | Fisher | 10749764|

Store at 4°C for up to 4 weeks (or make aliquots and store at -80°C)

**BGK Medium:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Conc.</th>
<th>100ml</th>
<th>200ml</th>
<th>Bottle</th>
<th>Company</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>KSR</td>
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<td>15ml</td>
<td>30ml</td>
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<td>NEAA</td>
<td>1%</td>
<td>1ml</td>
<td>2ml</td>
<td>6ml</td>
<td>Fisher</td>
<td>10358342</td>
</tr>
<tr>
<td>GlutaMAX</td>
<td>2mM</td>
<td>1ml</td>
<td>2ml</td>
<td>6ml</td>
<td>Fisher</td>
<td>10388582</td>
</tr>
<tr>
<td>β-mercapto</td>
<td>100μM</td>
<td>10μl</td>
<td>20μl</td>
<td>60μl</td>
<td>Sigma</td>
<td>63689</td>
</tr>
</tbody>
</table>

Mix the above together and filter sterilise in to DMEM-F12.

| DMEM-F12   |       |       |       | Fisher | 10770355|
| bFGF       | 8ng/ml| 100μl | 200μl | 602μl  | R & D   | 4114-TC-01M|

Store at 4°C for up 2 weeks.

**Conditioned Medium:**

Condition BGK medium for 24 hrs on MMC MEFs, filter sterilise and add 8ng/ml bFGF per ml (stock: 4μg/ml so use 1μl per ml of CM).

Use CM fresh, store for 24 hrs at 4°C or at -20°C for 3 months.
**Diff Medium:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Conc.</th>
<th>100ml</th>
<th>200ml</th>
<th>577ml</th>
<th>Company</th>
<th>Cat. No.</th>
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</thead>
<tbody>
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<td>40ml</td>
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<td>NEAA</td>
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<td>2ml</td>
<td>5.7ml</td>
<td>Fisher</td>
<td>10358342</td>
</tr>
<tr>
<td>GlutaMAX</td>
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<td>2ml</td>
<td>5.7ml</td>
<td>Fisher</td>
<td>10388582</td>
</tr>
<tr>
<td>β-mercapto</td>
<td>100μM</td>
<td>10μl</td>
<td>20μl</td>
<td>57.7μl</td>
<td>Sigma</td>
<td>63689</td>
</tr>
</tbody>
</table>

Mix the above together and filter sterilise in to DMEM.

| DMEM         |       | 78ml  | 156ml | 450ml | Fisher  | 10749764 |

Store at 4°C for up to 2 weeks.