



VILLE KUJALA

Human Pluripotent Stem Cell
Derived Cardiomyocytes

Differentiation, analysis and disease modeling



ACADEMIC DISSERTATION

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To Maria with love

Abstract

Stem cell technology is an area of research that will hopefully bring new treatment options to the currently available traditional medical therapies, drug therapies and surgeries. In particular, this field brings hope of new disease and drug testing models and the potential for human spare parts. New disease models would allow for the more accurate study of major illnesses at the cellular and tissue level using cells of human origin. Pharmaceutical testing models could, in turn, provide more detailed information on the pharmacodynamic effects of medicines that are not able to be investigated using current cellular and animal models. A particularly important application area of stem cell technology are the heart muscle cells cardiomyocytes, for which there has been no human cell model, as genetic heart diseases can be fatal and because a large number of drugs cause serious cardiac side effects. Pluripotent stem cell-derived cardiomyocytes bring hope to these medical challenges.

This thesis describes the differentiation of human pluripotent stem cells into cardiomyocytes. The molecular biology of these cardiomyocytes was analyzed using several methods. The electrophysiological properties of these cells were recorded with a microelectrode array (MEA) platform, in addition to other methods, and analyzed using in-house developed software. The responses of the cardiomyocytes to various drugs were also studied using the MEA platform. Using hiPSC technology, we investigated the properties of a genetic cardiac disease, the long QT syndrome, in human cells.

This research demonstrates that human pluripotent stem cells can differentiate towards multiple cell lineages, including functional cardiomyocytes, which have spontaneous beating activity. Moreover, these cells express cardiac-specific markers, exhibit the typical electrophysiological profiles of cardiomyocytes, and respond to different pharmacological substances in a way that is characteristic of cardiomyocytes. The electrophysiological properties that were recorded with the MEA platform were robustly analyzed for different cardiac parameters using software that we developed. Correct phenotypic characteristics of the long QT syndrome could also be recapitulated in the laboratory, paving way for more in-depth future studies.

Tiivistelmä

Kantasoluteknologia on tieteenala, jonka toivotaan tuovan uusia hoitomuotoja perinteisen lääkehoidon, kirurgian ja muiden lääketieteellisten hoitomuotojen rinnalle. Erityisesti alan toivotaan tuottavan tulevaisuudessa soluperäisiä tauti- ja lääketestausmalleja sekä ihmisen varaosia. Uudet tautimallit mahdollistaisivat vaikeiden sairauksien tutkimisen solu- ja kudostasolla entistä tarkemmin ihmisperäisissä soluissa. Lääketestausmallit puolestaan voivat mahdollisesti antaa tarkempaa farmakodynaamista tietoa lääkkeiden vaikutuksista ihmisen eri solutyyppeihin, mitä ei voida tutkia nykyisillä solu- ja eläinmalleilla. Erityisen tärkeä sovelluskohde kantasolutekniikalle on sydänlihassolut eli kardiomyosyytit, joille ei ole ollut ihmisperäistä solumallia, koska geneettiset sydänsairaudet ovat pahimmillaan kuolemaan johtavia ja suuri määrä eri lääkeaineita aiheuttaa vakavia sydänperäisiä sivuoireita. Kaikkiin näihin lääketieteellisiin haasteisiin pluripotentista kantasoluista erilaistetut kardiomyosyytit tuovat oman toivonsa.

Väitöskirjatutkimuksessani kuvataan ihmisen erittäin monikykyisten kantasolujen erilaistuminen sekä näiden rakenteiden ominaisuudet. Tutkimme myös sydänlihassolujen erilaistumista ihmisen alkion ja uudelleenohjelmoiduista kantasoluista sekä niiden molekyylibiologisia ominaisuuksia. Mikroelektrodi arraylla (MEA) nauhoitettujen kardiomyosyyttien elektrofysiologisten ominaisuuksien analysoimiseksi kehitimme oman tietokoneohjelman. Tutkimme myös kardiomyosyyttien vasteita eri lääkeaineille käyttäen hyödyksi MEA teknologiaa. Ihmisen uudelleenohjelmoituja kantasoluja käyttäen kykenimme myös tutkimaan geneettisen sydänsairauden, nimeltään pitkä QT oireyhtymä, ominaisuuksia ihmisperäisissä kardiomyosyyteissä.

Väitöskirjatutkimukseni osoittavat, että ihmisen erittäin monikykyiset kantasolut kykenevät erilaistumaan useiksi solutyypeiksi, mukaan lukien toimiviksi kardiomyosyyteiksi, jotka sykkivät spontaanisti, ilmentävät niille ominaisia merkkiaineita, omaavat niille tyypillisen elektrofysiologisen profiilin ja reagoivat niille ominaisella tavalla erilaisille lääkeaineille. Näiden kardiomyosyyttien elektrofysiologisia ominaisuuksia pystyttiin tarkaan analysoimaan hyödyntäen kehittämäämme tietokoneohjelmaa. Kykenimme myös laboratorio-oloissa onnistuneesti mallintamaan pitkä QT oireyhtymää uudelleenohjelmoiduista

kantasoluista erilaistamissamme kardiomyosyyteissä. Osoitimme, että oireyhtymän fenotyyppi on mahdollista tuottaa soluviljelyolosuhteissa.

Table of contents

Abstract.....	4
Tiivistelmä.....	5
List of abbreviations.....	10
List of original publications	13
1. Introduction	14
2. Review of literature.....	15
2.1 Stem cells.....	15
2.1.1 Definition, sources and potency of stem cells.....	15
2.1.2 Human embryonic stem cells	15
2.1.3 Human induced pluripotent stem cells	17
2.1.3.1 Challenges with induced pluripotent stem cells	19
2.2 Cardiomyogenesis and pluripotent stem cells	19
2.2.1 Heart development and regulation	19
2.2.2 Cardiomyocyte differentiation of human pluripotent stem cells.....	23
2.2.3 Properties of pluripotent stem cell-derived cardiomyocytes	26
2.2.4 Ways to improve cardiomyogenesis of pluripotent stem cells.....	27
2.3 Electrophysiology of cardiomyocytes	28
2.3.1 Electrophysiological aspects of human cardiomyocyte differentiation.....	28
2.3.2 Cardiac field potentials and the microelectrode array.....	32
2.3.2.1 Cardiac field potential parameters.....	34
2.3.2.2 Signal analysis	35
2.4 Pluripotent stem cell-derived cardiomyocytes and drug testing	35
2.5 Long QT syndrome.....	37
2.5.1 Acquired and congenital long QT syndrome	37
2.5.2 Modeling long QT syndrome with patient-specific induced pluripotent stem cell-derived cardiomyocytes	39
3. Aims of the study	41
4. Materials and methods	42
4.1 Ethical approval (I-IV)	42
4.2 Culture of primary and other cell types (III, IV)	42
4.3 Establishment of induced pluripotent stem cell lines (III, IV)	42

4.3.1	Karyotype and teratoma formation analysis (III, IV)	43
4.4	Stem cell culture (I-IV)	43
4.5	Cardiomyocyte differentiation (I-IV)	44
4.5.1	Embryoid body formation and cardiac differentiation (I)	44
4.5.2	Co-culture with mouse visceral endoderm-like cells (II-IV)	45
4.6	EB morphology and size analysis (I)	46
4.7	Immunocytochemical analysis (I, III, IV)	46
4.8	Protein expression studies by Western blot (I)	47
4.9	Gene expression studies (I, III, IV)	47
4.10	Electrophysiological characterization of cardiomyocytes (II-IV)	48
4.10.1	Patch clamp analysis (III)	48
4.10.2	Cardiac field potential recordings (II-IV)	49
4.11	Pharmacological tests (II-IV)	50
4.12	Cardiac field potential analysis	50
4.12.1	Field potential averaging (II)	50
4.12.2	Noise attenuation (II)	52
4.12.3	Mean field potential duration (II-IV)	52
4.13	Statistical analyses (I-IV)	52
5.	Results	54
5.1	Stem cell characteristics and pluripotency and cardiac marker expression (I, II, IV)	54
5.1.1	Human embryonic stem cells (I)	54
5.1.2	Human induced pluripotent stem cells (III, IV)	54
5.2	Embryoid body growth (I)	55
5.3	Marker expression in developing embryoid bodies and their progeny (I)	56
5.4	Efficiency of cardiac differentiation (I)	57
5.5	Cardiac field potential analysis (II)	58
5.5.1	Validity of the peak detection algorithms (II)	58
5.5.2	Noise attenuation in averaging (II)	58
5.5.3	Variation of cardiac field potential parameters (II)	59
5.5.3.1	Field potential duration and beating rate (II)	59
5.5.3.2	Amplitude and peak-to-peak interval (II)	59
5.5.4	Cardiac field potential durations (II-IV)	60
5.6	Pharmacological responses of pluripotent stem cell-derived cardiomyocytes (II-IV)	60

5.7	Action potential and potassium current properties of long QT syndrome 2-specific cardiomyocytes (III)	63
6.	Discussion	65
6.1	Embryoid body development (I)	65
6.1.1	Forced aggregation and embryoid body size (I)	65
6.1.2	Marker expression (I)	66
6.2	Cardiac field potential averaging (II)	67
6.3	Long QT syndrome modeling (III, IV)	69
6.3.1	Long QT syndrome type 1 (IV)	69
6.3.2	Long QT syndrome type 2 (III, IV)	70
6.3.2.1	Rapid delayed rectifier current properties (III)	71
6.3.2.2	Genotype-phenotype relationship (III)	71
6.3.2.3	Potential mitigating effects of cardiomyocyte coupling (III)	72
6.3.3	Drug testing with long QT syndrome cardiomyocytes (IV)	72
6.3.4	Outlook for patient-specific long QT syndrome cardiomyocytes in research (III, IV)	73
6.4	Future perspectives	75
7.	Conclusions	77
	Acknowledgements	78
	References	80

List of abbreviations

α -MHC	α -myosin heavy chain
β -MHC	β -myosin heavy chain
Δ cFPD	Relative rate-corrected field potential duration
AFP	Alphafetoprotein
ANP	Atrial natriuretic peptide
ALP	Alkaline phosphatase
AP	Action potential
APD	Action potential duration
bFGF	Basic fibroblast growth factor
BMP4	Bone morphogenetic protein 4
BR	Beating rate
BSA	Bovine serum albumin
CHD	Congenital heart defect
c-MYC	c-myc myelocytomatosis viral oncogene homolog
cTNT	Cardiac troponin T
dB	Decibel
DKK1	Dickkopf homolog 1
DMSO	Dimethyl sulphoxide
EB	Embryoid body
ECG	Electrocardiogram
eGFP	Enhanced green fluorescent protein
END-2	mouse visceral endoderm –like
END-2-CM	END2 –conditioned medium
ESC	Embryonic stem cell
ETCP _{unbound}	Unbound effective therapeutic plasma concentration
FA	Forced aggregation
FBS	Fetal bovine serum
FHF	First heart field
Flk	Fetal liver kinase
FP	Field potential
FPD	Field potential duration
GF	Growth factor
GMP	Good manufacturing practice
HCN	Hyperpolarization activated cyclic nucleotide-gated potassium channel
hERG	Human ether-a-go-go related gene
hESC	Human embryonic stem cell
HFH	Human fetal heart
hiPSC	Human embryonic stem cell
HOP	Homeobox-only protein
hPSC	Human pluripotent stem cell
I _{Ca,L}	Voltage-dependent Ca ²⁺ current

ICC	Immunocytochemistry
iCM	Induced cardiomyocyte
I _f	Funny current
ISH	In situ hybridization
I _{Kr}	Rapid delayed rectifier current
I _{Ks}	Slow delayed rectifier current
I _{K1}	Inward rectifying potassium current
Isl1	Islet-1
I _{to}	Transient outward potassium current
KLF4	Kruppel-like factor 4
LIN28	RNA-binding protein LIN-28
LQTS	Long QT syndrome
LQT1	Long QT syndrome type 1
LQT2	Long QT syndrome type 2
MDP	Maximum diastolic potential
MEA	Microelectrode array
MEF2	Myocyte enhancing factor 2
mESC	Mouse embryonic stem cell
MESP1	Mesoderm posterior 1 homolog
MI	Myocardial infarction
MINK	Misshapen-like kinase
miPSC	Mouse induced pluripotent stem cell
MiRP	MINK-related peptide
mRNA	Messenger ribonucleic acid
MSE	Mean square error
NANOG	Nanog homeobox
NCE	New chemical entity
NCX1	Sodium-calcium exchanger 1
NIH	United States National Institutes of Health
Nkx2.5	NK2 transcription factor related, locus 5
NRC	Neonatal rat cardiomyocyte
OCT4	Octamer-4
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
POU5F1	POU class 5 homeobox 1
PPI	Peak-to-peak interval
QTc	Heart rate –corrected QT interval
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative reverse-transcriptase polymerase chain reaction
REX1	RNA exonuclease 1
RiPSC	RNA-induced pluripotent stem cell
RNA	Ribonucleic acid
ROCK	Rho-associated, coiled-coil containing protein kinase
RT	Room temperature
RT-PCR	Reverse-transcriptase polymerase chain reaction
RyR	Ryanodine receptor
SERCA	Sarcoplasmic-endoplasmic reticulum Ca ²⁺ -ATPase
SSEA	Stage-specific embryonic antigen
SC	Stem cell
SCNT	Somatic cell nuclear transplantation

SD	Standard deviation
SHF	Second heart field
SNR	Signal-to-noise ratio
SNS	Secondary neurospheres
SOX2	SRY (sex determining region Y)-box 2
SRF	Serum response factor
Tbx	T-box
TdP	Torsade de pointes
TF	Transcription factor
TRA	Tumor-related antigen
VEGF	Vascular endothelial growth factor
WT	Wild-type

List of original publications

The present study is based on the following original publications, which are referred to in the text by their Roman numerals (I-IV):

- I Pekkanen-Mattila M, Peltö-Huikko M, **Kujala V**, Suuronen R, Skottman H, Aalto-Setälä K, Kerkelä E. Spatial and temporal expression pattern of germ layer markers during human embryonic stem cell differentiation in embryoid bodies. *Histochemistry and Cell Biology*. 2010. 133 (5): 595-606.
- II **Ville J. Kujala***, Zaida C. Jimenez*, Juho Väisänen, Jarno M.A. Tanskanen, Erja Kerkelä, Jari Hyttinen, Katriina Aalto-Setälä. Averaging in vitro cardiac field potential recordings obtained with microelectrode arrays. *Computer Methods and Programs in Biomedicine*. 2011. 104 (2): 199-205. *equal contribution
- III Anna L. Lahti*, **Ville J. Kujala***, Hugh Chapman, Ari-Pekka Koivisto, Mari Pekkanen-Mattila, Jari Hyttinen, Kimmo Kontula, Heikki Swan, Bruce R. Conclin, Shinya Yamanaka, Olli Silvennoinen, Katriina Aalto-Setälä. Human Disease Model for Long QT Syndrome Type 2 Using iPSC Cells Demonstrates Arrhythmogenic Characteristics in Cell Culture. *Disease Models and Mechanisms*. 2011. First posted online November 3, 2011, doi:10.1424/dmm.008409. *equal contribution
- IV **Ville J. Kujala**, Anna Lahti, Marisa Ojala, Heikki Swan, Kimmo Kontula, Katriina Aalto-Setälä. Effects of cardioactive drugs on human iPSC-derived long QT syndrome cardiomyocytes. *Submitted*.

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1. Introduction

Human embryonic stem cells (hESCs) (Thomson et al., 1998) and human-induced pluripotent stem cells (hiPSCs) (Takahashi et al., 2007), collectively known as human pluripotent stem cells (hPSCs), offer an abundant source of cells that can be differentiated into multiple cell types, including cardiac cell types such as cardiomyocytes (Kehat et al., 2001; Zhang et al., 2009). This has created much hope for new therapeutic possibilities, disease modeling, and drug-testing platforms in the cardiac context. These are important issues that the current field of biomedicine is focused on resolving. Therapeutic cardiac applications of pluripotent stem cells are currently at an experimental level, but some promising advancements have been made in cardiac disease modeling (Itzhaki et al., 2011; Moretti et al., 2010b; Yazawa et al., 2011) and drug testing (Braam et al., 2010; Caspi et al., 2009; Tanaka et al., 2009; Yokoo et al., 2009) with cardiomyocytes derived from pluripotent stem cells, including some results presented in this thesis. These hPSC-derived cardiomyocytes can also serve as a valuable tool to study the developmental stages of cardiomyocyte lineage commitment and maturation (Bu et al., 2009; Moretti et al., 2010a; Pekkanen-Mattila et al., 2010b; Yang et al., 2008).

Disease modeling using hPSC-derived cardiomyocytes is important because these cells provide, for the first time, a tool to study the mechanisms of different diseases in an appropriate cellular context. Currently, other cell and animal models fail to recapitulate the relevant physiology. Primary cells obtained from heart biopsies also dedifferentiate *ex vivo*. Drug testing of new chemical entities (NCEs) during pharmacological development and preclinical testing is usually performed in animal models and with cells using heterologous expression of ion channels (ICH, 2005b). hPSC-derived cardiomyocytes offer an advantage over these systems in that they provide a proper human cellular milieu and other possible regulatory mechanisms that may modulate the response to disease and drugs.

The objective of this thesis was to investigate the embryoid body and cardiac differentiation of human embryonic stem cells and the electrophysiological properties and field potential analysis of the derived cardiomyocytes. We also sought to develop an *in vitro* cell model for long QT syndrome. Additionally, we investigated long QT syndrome type 2 (LQT2)-specific cardiomyocytes in depth as well as the drug responses of LQT1- and 2-specific cardiomyocytes.

2. Review of literature

2.1 Stem cells

2.1.1 Definition, sources and potency of stem cells

By definition, stem cells are able to self-renew and differentiate into specialized cell types. Self-renewal means that a stem cell can produce a copy of itself in a process called symmetrical division. Stem cells are also able to produce daughter cells that begin to differentiate down a specific pathway towards a certain lineage or cell type. This is called asymmetrical division (Mountford, 2008).

Stem cells can be broadly divided into four different categories according to their differentiation potency: toti-, pluri-, multi-, and unipotent stem cells (SC). Totipotent SCs are able to form all the cell types of a developing organism, including extraembryonic (i.e., placental) tissue. Totipotent cells are found during mammalian development from the fertilized zygote stage up to the eight-cell stage (Yamanaka et al., 2008). Pluripotent SCs are able to form an entire living organism, but not extraembryonic tissue. In other words, these SCs can differentiate into the cells of the three germ layers: the ecto-, meso-, and endoderm. Pluripotent SCs (PSCs) can be derived from a 4-7-day-old blastocyst-stage embryo (Thomson et al., 1998), or they can be induced from somatic cells (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Yu et al., 2007). Multipotent SCs are also called tissue-specific stem cells, meaning that these SCs reside in specific tissues and can only differentiate into cells found in that particular tissue (Yamanaka et al., 2008). Unipotent SCs can only differentiate into a single cell type; for example, germline SCs can only differentiate into germline cells (Yuan and Yamashita, 2010).

2.1.2 Human embryonic stem cells

Mouse embryonic stem cell (mESC) lines were derived 30 years ago (Evans and Kaufman, 1981; Martin, 1981), and the inner cell mass of human blastocysts was first derived and cultured *in vitro* in 1994 (Bongso et al., 1994). However, the

derivation of the first stable hESC lines in 1998 brought real hope for clinical applications when Thomson and colleagues described the derivation of five human embryonic stem cell lines from 14 isolated inner cell masses from five separate donated embryos. These cells had high telomerase activity, expressed distinct cell surface markers and retained pluripotency after 4-5 months of continuous culture (Thomson et al., 1998). The derivation of hESCs from a human blastocyst is illustrated in figure 1. hESCs are characterized mainly by the following properties: the ability to proliferate extensively *in vitro*; their expression of the pluripotency markers POU class 5 homeobox 1 (POU5F1, also known as octamer-4 [OCT3/4]), RNA exonuclease 1 (REX1), Nanog homeobox (NANOG), stage-specific embryonic antigen 3 and 4 (SSEA3 and SSEA4), tumor-related antigen (TRA)-1-60, TRA-1-81, and alkaline phosphatase (ALP); the ability to differentiate into cell types of all three germ layers; and the ability to form teratomas (Klimanskaya et al., 2008; Thomson et al., 1998). hESCs also have a high nucleus-to-cytoplasm ratio, prominent nucleoli, and high telomerase activity (Thomson et al., 1998).

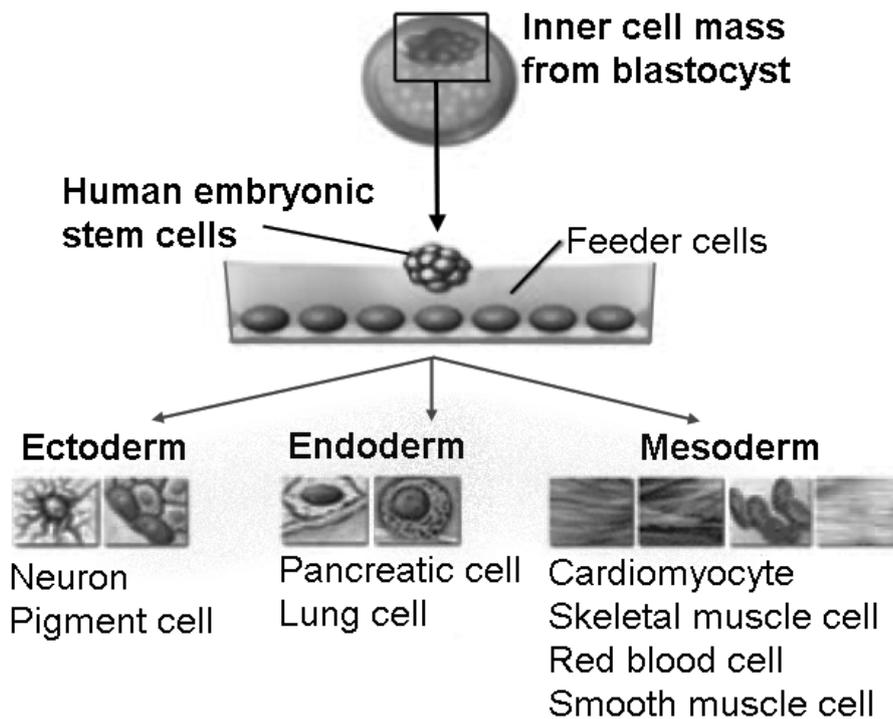


Figure 1. Derivation of pluripotent human embryonic stem cell (hESC) lines from the isolated inner cell mass (box on top). The derived hESC lines are able to differentiate into cells of all the three germ layers: the ectoderm, endoderm, and mesoderm. Figure modified from (Mountford, 2008).

2.1.3 Human induced pluripotent stem cells

The emergence of iPSC technology (figure 2) was preceded by many techniques and observations to which it owes, in part, its success. Some of the key steps include somatic cell nuclear transplantation (SCNT) (Briggs and King, 1952), the derivation of human embryonic stem cells (Thomson et al., 1998) and the observation that some transcription factors are able to change the identity of somatic cell types, which was first demonstrated by the conversion of fibroblast cells into myofibers by the retroviral expression of MyoD (Davis et al., 1987).

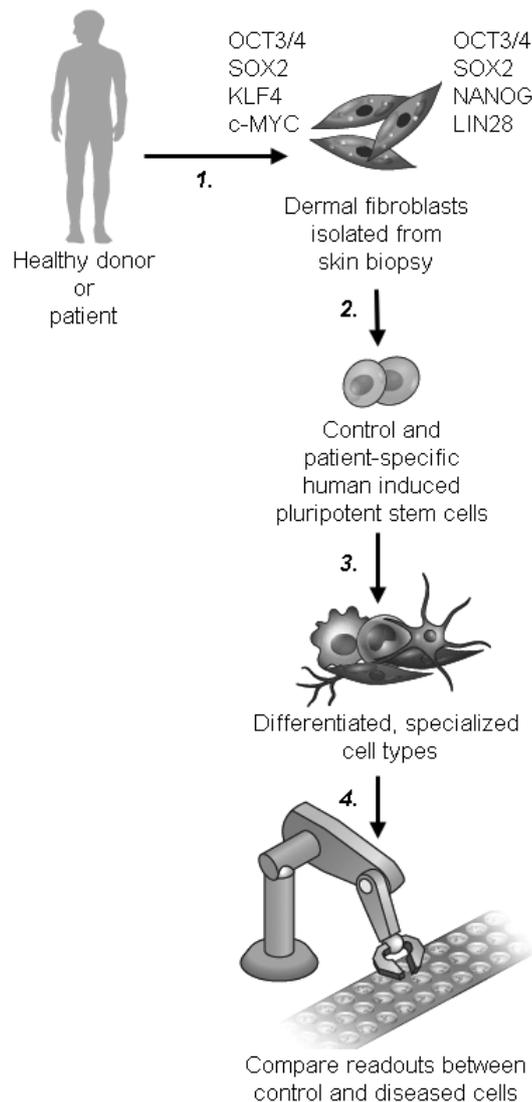


Figure 2. Somatic cell reprogramming into a pluripotent state. Two common sets of viral reprogramming factors are the “Yamanaka combination” (OCT3/4, SOX2, KLF4, and c-MYC) and the “Thomson combination” (OCT3/4, SOX2, NANOG, and LIN28), of which the former is probably more widely used. These transgenes convert differentiated somatic cells back into a pluripotent embryonic-like state. The human induced pluripotent stem cells can then differentiate into specialized cell types. This method enables the comparison of cell types between healthy individuals and patients carrying a monogenic disease, for example. Figure modified from (Passier et al., 2008).

The first cells to be successfully reprogrammed back into a pluripotent state were mouse embryonic and adult fibroblasts. This was achieved by retroviral transfection of four distinct transcription factors, namely, *Oct3/4*, SRY (sex determining region Y)-box 2 (*Sox2*), c-myc myelocytomatosis viral oncogene homolog (*c-Myc*), and Kruppel-like factor 4 (*Klf4*) (Takahashi and Yamanaka, 2006). Later, iPS cells were also generated from adult human fibroblasts using the same method (Takahashi et al., 2007) and from adult mouse liver and stomach cells. The liver and stomach cells do not appear to require retroviral integration to specific sites, which could lower the risk of tumorigenicity after transplantation to patients (Aoi et al., 2008). At the same time, human fibroblasts were also reprogrammed using another cocktail of transcription factors: *OCT3/4*, *SOX2*, *NANOG*, and the RNA-binding protein LIN-28 (*LIN28*) (Yu et al., 2007). These current iPS cell lines generated by retroviral transfection are not applicable for clinical use, however, due to potentially harmful genetic modifications. Reprogramming with excisable transposons, lentiviral vectors, transient adenoviral, episomal, plasmid vectors or recombinant proteins with cell-penetrating moieties all represent efforts to develop different reprogramming strategies (Chang et al., 2009; Kaji et al., 2009; Kim et al., 2009b; Okita et al., 2008; Stadtfeld et al., 2008; Woltjen et al., 2009; Yu et al., 2009; Zhou et al., 2009). The second major application for iPS cells after differentiation into the desired cell type is *in vitro* drug screening of drug candidates on healthy and diseased cells (Passier et al., 2008). These cells would be ideal to generate patient- and disease-specific stem cells, and the use of iPS cells circumvents the ethical issues that surround and hamper hESC research (Takahashi *et al.* 2007). The safety issues related to the reprogramming strategies do not hamper the *in vitro* disease model and drug testing development and the currently available lines can readily be used for such *in vitro* studies.

In addition, at least on one occasion, successful reprogramming has been reported without using *c-Myc*, which potentially has oncogenic properties (Nakagawa et al., 2008). However, another group has demonstrated the generation of iPS cells from adult mouse neural stem cells using only two exogenously introduced transcription factors (*OCT3/4* and either *Klf4* or *c-Myc*). This was possible because these neural stem cells already express higher endogenous levels of *Sox2* and *c-Myc* than embryonic stem cells (Kim et al., 2008). A new viral-free method of producing hiPSCs uses synthetic RNA, which can reprogram fibroblasts upon transfection (Warren et al., 2010; Yakubov et al., 2010). These reprogrammed cells are called RNA-iPSCs (RiPSCs) and can be generated using mRNAs for the *OCT3/4*, *SOX2*, *NANOG*, and *LIN28* reprogramming factors (Yakubov et al.,

2010) or for the KLF4, c-MYC, OCT3/4, and SOX2 transcription factors (Warren et al., 2010).

2.1.3.1 Challenges with induced pluripotent stem cells

Although hiPSCs are a potential tool for pharmacological and toxicological testing and for regenerative medicine in the future, currently there are still many obstacles to overcome. For instance, the question of how similar hiPSCs truly are to hESCs still remains unanswered. Although hiPSCs pass the standards required of hPSCs (Takahashi et al., 2007; Yu et al., 2007), a recent study suggests that these cells have aberrant epigenetic reprogramming in specific genomic hotspots (Lister et al., 2011). Reprogrammed hiPSCs appear to retain some somatic cell DNA methylation patterns along with hiPSC-specific methylation patterns (Lister et al., 2011). Another study found that hiPSCs can harbor aneuploidies, especially the duplication of chromosome 12 (Mayshar et al., 2010). Guenther and colleagues compared the chromatin structure and gene expression pattern of hESCs to hiPSCs and found some variations between them; however, they concluded that these variations were not enough to distinguish between the two pluripotent cell types (Guenther et al., 2010). Cardiomyocytes, that are differentiated from hiPSCs and hESCs, however, appear to possess similar global transcriptomes (Gupta et al., 2010).

2.2 Cardiomyogenesis and pluripotent stem cells

2.2.1 Heart development and regulation

In spite of intensive research on the molecular mechanisms of the developing heart, the chamber-specific gene expression underlying the spatiotemporal regulation within the heart remains largely unclear, although considerable success has been made recently to identify key cardiac commitment steps in heart formation (Chien et al., 2008; Musunuru et al., 2010; Nemer, 2008).

The heart is the first organ to develop in vertebrates (Buckingham et al., 2005). The mesodermal cardiac progenitors arise from two distinct fields: the first and second heart fields (FHF and SHF, respectively). Studies in mice have shown that the FHF contributes to both atria, the ventricles, and the atrioventricular canal (Meilhac et al., 2004). The SHF contributes to the outflow tract and the right ventricle (Domian et al., 2009; Meilhac et al., 2004) as well as the atrio-ventricular

canal and both atria (Meilhac et al., 2004); this includes all regions except the left ventricle. Table I summarizes some of the key proteins regulating cardiac differentiation, which are discussed below.

Table I. Key proteins regulating cardiac differentiation and the cell types in which they are expressed.

<i>Protein</i>	<i>Class</i>	<i>Expressed in</i>	<i>Reference</i>
Nkx2.5	Homeobox transcription factor	Cardiac precursor cells	(Anton et al., 2007)
Isl1	T-box transcription factor	Transiently in secondary heart field precursors	(Kattman et al., 2007)
GATA4	GATA family of zinc-finger transcription factor	Cardiac precursor cells	(Nemer, 2008)
Mef2c	MADS-box transcription factor	Cardiac precursor cells	(Lin et al., 1997)
Tbx5	T-box transcription factor	Heart and upper limbs	(Bruneau et al., 2001)
Flk1 (Kdr)	Receptor tyrosine kinase	Mesodermal cell populations	(Anton et al., 2007)

One of the earliest markers expressed in developing cardiac cells is NK2 transcription factor-related, locus 5 (Nkx2.5) (Abu-Issa and Kirby, 2007; Anton et al., 2007; Chen and Fishman, 2000; Kattman et al., 2007; Musunuru et al., 2010; Zhou et al., 2008). Nkx2.5 is an NK-class homeodomain-containing protein, which binds DNA through its homeodomain by forming a homodimer at its DNA binding sites. After Nkx2.5 is bound to DNA, it is phosphorylated by casein kinase II (CKII), thereby increasing its DNA binding affinity. Nkx2.5 interacts with many transcription factors (TFs), which include GATA-4, serum response factor (SRF), T-box 5, T-box 2, T-box 20, hand1/hand2, and Foxh1. Genes whose expression is regulated by Nkx2.5 include atrial natriuretic peptide (ANP), cardiac alpha actin, A1 adenosine receptor, calreticulin, connexin 40, sodium-calcium exchanger 1 (NCX1), endothelin-converting enzyme-1, homeobox-only protein (HOP), and myocardin (Abu-Issa and Kirby, 2007). Nkx2.5 is essential for appropriate heart formation (Kattman et al., 2007), and mutations in this gene can cause atrial and ventricular septal and outflow tract defects, cardiomyopathy, valvular abnormalities, tetralogy of Fallot, hypoplastic left heart, and arrhythmias (Musunuru et al., 2010). *Nkx2.5*-deficient mice die at embryonic day 9.5 (E9.5) due to an abnormal morphogenesis of the outflow and inflow components of the heart tube (Abu-Issa and Kirby, 2007). Heart formation in *Nkx2.5*-deficient mice is not necessarily compromised, however, because beating cardiomyocytes are still present and other NK2 homeobox

transcription factors may compensate for the lack of Nkx2.5 (Abu-Issa and Kirby, 2007; Nemer, 2008). Clearly, Nkx2.5 is important in establishing the ventricular gene expression program and septal morphogenesis and it works via combinatorial interactions with other cardiac regulators (Nemer, 2008). These target genes encode structural and transcriptional regulators that confer characteristic cardiomyocyte features (Abu-Issa and Kirby, 2007).

Islet-1 (Isl1) is a basic helix-loop-helix LIM-homeodomain T-box transcription factor that is expressed by the SHF. Cardiogenic progenitors transiently express Isl1 in addition to Nkx2.5. However, Isl1 is not expressed in terminally differentiated cardiomyocytes, unlike Nkx2.5 (Musunuru et al., 2010). Isl1⁺ progenitors develop into cardiomyocytes, smooth muscle and endothelial cells. *Isl1*-deficient mice lack SHF derivatives, resulting in the absence of the outflow tract, the right ventricle, and some atrial portions (Anton et al., 2007; Kattman et al., 2007; Srivastava, 2006). Zhou *et al.* (2008) identified a Wt1⁺ precursor cell population that resides in the epicardium. These Wt1⁺ proepicardial cells arise from progenitors that also express Isl1 and Nkx2.5, indicating that these cells share a developmental origin with these multipotent Nkx2.5⁺ and Isl1⁺ progenitors.

GATA-4, -5, and -6 belong to the GATA TF-family (which, in turn, belongs to the zinc finger superfamily) and bind the same DNA sequence, (A/G)GATA(A/T). These three TFs are expressed in the gut epithelium, the precardiac mesoderm, and heart regions overlapping with Nkx2.5 expression (Kattman et al., 2007; Peterkin et al., 2007). In addition, these factors may be involved in the initiation of Nkx2.5 expression (Abu-Issa and Kirby, 2007). GATA-5 is mainly expressed in endocardial cells, and GATA-6 is expressed in myocardial and vascular smooth muscle cells. GATA-4 is expressed in cardiac progenitor cells, regulates the expression of more than 30 cardiac promoters, and interacts with other TFs to regulate cardiac differentiation. The importance of GATA-4 is evident from the presence of congenital heart defects (CHDs) in mice and humans with GATA-4 haploinsufficiency (Nemer, 2008), which include atrial, septal and ventricular defects and tetralogy of Fallot (Musunuru et al., 2010). *Gata-4* null mouse embryos have fewer cardiomyocytes and their bilateral heart primordia fail to fuse, causing a condition known as cardiac bifida. *GATA-4*-negative ESCs, however, are able to form cardiomyocytes (Chen and Fishman, 2000).

The myocyte enhancing factor 2 (MEF2) family of proteins is also involved in cardiomyocyte and vascular cell differentiation (Ieda et al., 2010; Nemer, 2008). These proteins bind an A/T-rich regulatory sequence found on multiple skeletal muscle promoters (Lin et al., 1997). GATA TFs also recruit MEF2 proteins to their

target genes. MEF2 proteins are transcribed from four genes and the different isoforms are produced by alternative splicing (Nemer, 2008).

The T-box (Tbx) family of proteins comprises more than 20 members in mammals. These developmental regulators share a conserved 180 amino acid region responsible for the DNA binding T-domain. Holt-Oram syndrome, characterized by heart and upper limb abnormalities, is caused by a mutation in the *Tbx5* gene; a finding that implicated Tbx proteins as regulators of heart development. Dominant-negative Tbx proteins interfere with heart development in *Xenopus* as well. Tbx5 proteins work in cooperation with the Nkx2.5 transcription factor and mutations in either gene can lead to the same cardiac abnormalities (Nemer, 2008). *Tbx5* mutant mice also harbor defects in the inflow-regions of the heart and develop left ventricular hypoplasia, which leaves the left ventricle underdeveloped. Other T-box TF family members have also been implicated in vertebrate heart development, including Tbx1-4, -18, and -20. Tbx2 and -20 are thought to regulate chamber identity in the developing heart (Kattman et al., 2007). The transduction of mouse fibroblasts with Tbx5 and the aforementioned Gata4 and Mef2c converts these fibroblasts into cardiomyocytes, further illustrating their importance in the adoption of the cardiomyocyte fate in the transdifferentiation process (Ieda et al., 2010).

Fetal liver kinase (Flk)-1 is a marker for early cardiac progenitor cells and for smooth muscle, hematopoietic, and endothelial cells (Anton et al., 2007). This protein is expressed in multipotent cardiovascular progenitor cells of the SHF with *Isl1* and Nkx2.5 (Musunuru et al., 2010). Flk-1 encodes endothelial growth factor receptor-2 and is expressed in different mesodermal subpopulations (Kattman et al., 2007).

Notch signaling regulates the differentiation of cardiac muscle and conduction cells in ventricles (Chau et al., 2006). Humans have four Notch receptors: Notch-1 to -4. These receptors have five ligands: delta-like-1, -3, -4 and Jagged-1, and -2. Notch signaling suppresses cardiac differentiation, and the activation of Notch signaling in *Xenopus* decreases myocardial gene expression in early heart field crescent cells. Notch signaling also inhibits cardiac differentiation in the chick embryo. Conversely, the inhibition of Notch signaling promotes myogenic cell fate in *Drosophila* (Pedrazzini, 2007). Notch-1 mutations in humans are related to valvular abnormalities and tetralogy of Fallot (Musunuru et al., 2010). Notch1 activation in mice leads to abnormal cardiogenesis characterized by atrioventricular canal and ventricular deformities. Mutations in genes related to Notch signaling pathways can also cause cardiac abnormalities like pericardial edema, cardiac cushion and valve defects, as well as atrial and ventricular septal defects. These

genes include *Jagged-1*, *Hesr1/Hey1*, *Hesr2/Hey2*, and *mind bomb* (Chau et al., 2006).

With regard to these studies, it must be noted that the most common model organism used in research to understand the genetic regulation of heart formation is the mouse. Interpreting the results must, therefore, be performed with great care to avoid erroneous extrapolations to human conditions. For instance, one study found myosin light chain (MLC)-2a, -1v, and atrial natriuretic factor (ANF) expression to be essentially chamber-restricted in mice by mid-gestation; however, in humans, there was a broader distribution, and in addition, MLC-2v was more restricted to the ventricles in humans than in mice (Chuva de Sousa Lopes et al., 2006). This study highlights the importance of careful interpretation of results obtained from different species.

2.2.2 Cardiomyocyte differentiation of human pluripotent stem cells

Human PSCs (hPSCs) provide a renewable cell supply and can be differentiated into cardiomyocytes quite rapidly, usually in approximately 20 days or under. There are multiple cardiac differentiation methods, which all share common challenges, namely heterogeneity of the derivative cell population and low differentiation efficiency. The variation in the differentiation efficiency between hESC lines is in part due to their intrinsic cardiomyogenic propensities (Osafune et al., 2008; Pekkanen-Mattila et al., 2009). Figure 3 represents the cardiac differentiation of hPSCs.

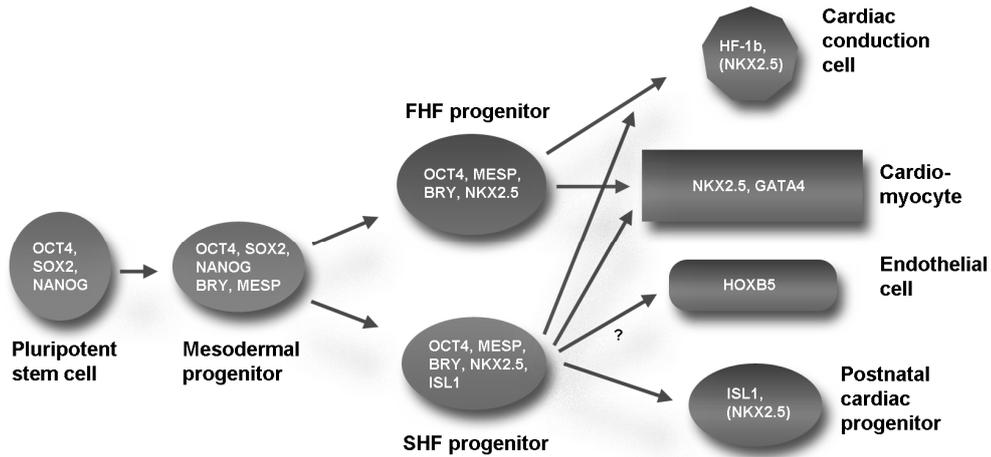


Figure 3. A cartoon of cardiac differentiation of pluripotent stem cells. Pluripotent stem cells express the pluripotency markers OCT3/4, SOX2, and NANOG. Differentiating cardiomyogenic cells express cardiac markers such as ISL1, NKX2.5, and GATA4. FHF: first heart field, SHF: second heart field. NKX2.5 in parentheses indicates that it is not expressed in all the cells. The gene expression data were partially adapted from (Srivastava and Ivey, 2006).

The embryoid body (EB) differentiation method is a conventional way to derive cells from hESCs (Passier and Mummery, 2005). In this method, EBs are allowed to form by dissociating the hESC colonies into smaller pieces and culturing them in suspension to initiate the differentiation process. These differentiating EBs are subsequently plated and allowed to grow in adherent culture. The first hESC-derived cardiomyocytes (hESC-CMs) were described a decade ago, three years after the establishment of the first stable hESC lines (Kehat et al., 2001). This method relied on the EB method to produce cells with phenotypic properties of cardiomyocytes. The authors reported that 8.1% of the EBs generated spontaneously beating areas, which expressed cardiac-specific marker genes and transcription factors, showed myofibrillar organization, stained positively for cardiac proteins, showed distinct electrograms, and showed the type of drug response expected of cardiac cells (Kehat et al., 2001). Other groups have also successfully used this method to derive cardiomyocytes from hESCs (Andersson et al., 2010; Kattman et al., 2011; Kim et al., 2009a; Norstrom et al., 2006; Synnergren et al., 2008) and hiPSCs (Kattman et al., 2011; Yokoo et al., 2009; Zhang et al., 2009; Zwi et al., 2009b). Forced aggregation (FA) is another approach to produce EBs that are more consistent in size. With this method, hESC colonies are dissociated into single cells and plated onto v-shaped bottom 96-well plates to facilitate cell aggregation with centrifugation (Burrige et al., 2007).

Due to the relative inconsistency of the EB method among different hESC lines, other techniques may be more desirable for cardiomyocyte production. The first effort to provide a more robust method of cardiac differentiation was to co-culture

hESCs with mouse visceral endoderm-like (END-2) cells. These END-2 cells guide hESCs towards the endoderm and mesoderm (Beqqali et al., 2006; Mummery et al., 2003; Passier et al., 2005). Cardiomyocytes originate from the mesoderm, but endodermal cues are critical in heart formation (Lough and Sugi, 2000). END-2 cells therefore induce hESCs to adopt a cardiomyocyte fate by providing these endodermal cues for differentiation (Mummery et al., 2003). This method relies on using a 20% fetal calf serum (FCS)-containing medium. However, the cardiomyogenic effectiveness can be increased 24-fold by omitting FCS from the culture medium and by an additional 40% by adding ascorbic acid (commonly known as vitamin C) to the culture medium (Passier et al., 2005). The addition of SB203580, a p38 MAP kinase-specific inhibitor, can also induce greater than 20% of the differentiating cells to adopt a cardiomyocyte fate in the END-2 co-cultures. Furthermore, SB203580 increases the total cell number, resulting in an overall yield of cardiomyocytes 2,5-fold higher than in serum-free END2 co-cultures alone (Graichen et al., 2007). Serum-free culture medium conditioned with the soluble factors secreted by END-2 cells can also be used for hESC-CM production (Braam et al., 2008). One likely mechanism of action for increased cardiomyogenesis with END2-conditioned medium (END2-CM) might be that the conditioning clears insulin, which can inhibit cardiomyogenesis, from the cell culture media (Xu et al., 2008). The same level of cardiac differentiation can be achieved with synthetic insulin-free medium supplemented with prostaglandin I₂, which is also an abundant compound in END2-CM (Xu et al., 2008). This synthetic medium represents a step towards a good manufacturing practice (GMP) level of cardiomyocyte production.

Cardiomyocyte differentiation of hPSCs can be enhanced with specific growth factors (GFs). Activin A and bone morphogenetic protein 4 (BMP4) were shown to induce greater than 30% cardiac differentiation of hESCs when activin A was used for the first 24 h to initiate differentiation and BMP4 exposure was then used over the next four days (Laflamme et al., 2007). Further optimization of the activin A and BMP4 conditions can yield highly enriched hESC-CM populations, as was noted in a recent study reporting that the differentiating EBs consisted of 58% cTNT⁺ cells at most (Kattman et al., 2011). Using a more intricate combination of multiple growth factors, including basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and dickkopf homolog 1 (DKK1), as well as activin A and BMP4, an approximately 50% cardiomyocyte differentiation efficiency can be achieved. In this case, growth factors were used for two weeks in a more complex sequence that more accurately mimicked embryonic heart development (Yang et al., 2008).

2.2.3 Properties of pluripotent stem cell-derived cardiomyocytes

One of the first signs of *in vitro* cardiomyocyte development is the appearance of spontaneously beating foci (Kehat et al., 2002; Mummery et al., 2003). The beating frequencies of the hESC-CMs differ among the foci (Asp et al., 2010). Beqqali and co-workers investigated the transcriptional regulation of developing hESC-CMs and found several genes that were upregulated during the first 12 days of development in END2 co-cultures. In this study, the group used DNA microarray analysis, quantitative polymerase chain reaction (qPCR), and *in situ* hybridization (ISH) to study gene expression in undifferentiated hESCs, hESC-CMs and the human fetal heart (HFH). During hESC-CM differentiation, several genes were up-regulated at the mRNA level, including many non-cardiac genes. The gene expression pattern reflected early embryonic events; the first genes to be up-regulated were primary mesodermal, followed by cardiac progenitor-associated genes and those expressed in fetal cardiomyocytes. The cardiomyocyte-enriched genes included troponin C (slow), actinin ($\alpha 2$), myosin light chain 2, and cardiac troponin T2 (Beqqali et al., 2006).

Synnergren and colleagues also investigated gene expression during the cardiomyocyte development of hESCs. In this study, microarray technology was used to investigate the global mRNA expression profile of hESC-CM clusters, and these profiles were compared with undifferentiated hESCs. The comparison revealed 520 up-regulated and 40 down-regulated genes in hESC-CMs. Up-regulated genes in cardiomyocytes were classified according to their Gene Ontology annotation. Several of the genes belonged to a “biological process” category. These genes were involved in mesoderm development, muscle development, striated muscle contraction, and the regulation of muscle contraction. Several genes also encoded cellular components, such as the troponin complex, striated muscle thin filament, the myosin complex, actin filament, and contractile fiber. The annotations for the up-regulated genes in the differentiated cardiomyocytes were associated with cardiomyocytes and cardiac development (Synnergren et al., 2008).

hESC-CM differentiation is characterized by the expression of specific genes and proteins. The expression of the mesodermal marker Brachyury T is upregulated during early phases of cardiogenesis and its expression peaks on the third day in END-2 co-cultures (Beqqali et al., 2006; Pekkanen-Mattila et al., 2009) and on the fourth day in differentiating EBs (Bettioli et al., 2007; Pekkanen-Mattila et al., 2010b). Cardiomyocyte differentiation is also characterized by the upregulated expression of cardiac TFs, including the mesoderm posterior 1 homolog (MESP1), ISL-1, GATA-4, and NKX2.5 (Graichen et al., 2008; Yang et al., 2008). The mRNA expression of *NKX2.5* and cardiac muscle actin is higher in hESC-CMs than in

human heart samples, according to one study (Asp et al., 2010). Over time, however, *NKX2.5* expression in developing hESC-CMs drops close to that of human heart samples. Cardiac troponin T (cTNT) is expressed at higher levels in hESC-CMs and ventricular heart samples compared to the atria. In hESC-CMs, cTNT mRNA expression ultimately declines as well (Asp et al., 2010). Phospholamban (a calcium [Ca^{2+}] ion pump) is expressed at lower levels in hESC-CMs and human atrial samples than in the ventricle. The cardiac structural protein α -myosin heavy chain (α -MHC) is more highly expressed in fast-beating hESC-CMs that have a beating rate of more than 50 beats per minute and atrial samples. The expression of this protein also increases in hESC-CMs as they age. In contrast, a related cardiac structural component, β -MHC, is expressed at higher levels in human ventricular samples. The mRNA expression of the potassium (K^+) ion channel coding human ether-a-go-go-related gene (*hERG*) in hESC-CMs resembles that of neonatal and adult atrial samples. According to that study, it seems the difference between hESC-CMs with slow or fast beating frequencies parallels the difference between human atrial and ventricular tissues, respectively (Asp et al., 2010).

With regard to structural features, developing hESC-CMs have an isotropic myofibrillar organization at early stages. Clear sarcomeric A, I, and Z bands develop later (Kehat et al., 2001; Pekkanen-Mattila et al., 2009; Snir et al., 2003), along with the development of structural proteins such as troponin I, T or C, myosins, and α -actinin (Kehat et al., 2001; Mummery et al., 2003). hESC-CMs also express desmosomes and gap junctions that facilitate coupling to neighboring cells (Kehat et al., 2001; Pekkanen-Mattila et al., 2009; Snir et al., 2003).

2.2.4 Ways to improve cardiomyogenesis of pluripotent stem cells

Proper cardiomyocyte differentiation and maturation may need more coaxing than just using END-2 co-culturing or GFs. Indeed, the oxygen concentration and mechanical stimulation have important roles in coaxing hESC-CMs. The oxygen tension of the cell cultures can guide hESC-CM differentiation. Under hypoxic conditions (i.e., 4% oxygen tension), the cardiomyocyte yield has been reported to be higher than in normoxic conditions (21% oxygen tension). Under hypoxia, the cell-fold expansion at day 16 was also reported to be significantly higher compared to under normoxia. These experiments were performed in dynamic hESC cultures with different kinds of bioreactors. Such conditions yielded at most 30% contracting EBs (Niebruegge et al., 2008). An essential point of these studies was that the cardiomyocyte yield and cell-fold expansion was greater under these conditions than

in traditional static cultures. Physical strain can also positively influence the generation of cardiomyocytes, at least from mouse ES cells (Gwak et al., 2008), cardiac progenitor cells harvested from mice and bone marrow stromal cells (Forte et al., 2008). Micrometer-scale surface topography in conjunction with biphasic electrical stimulation can also enhance cardiomyocyte morphology in neonatal rat cardiomyocytes (NRCs) (Heidi Au et al., 2009).

Another way to enrich the proportional number of cardiomyocytes in culture is through selection with fluorescence-activated cell sorting (FACS) after heterogenous differentiation. After sorting, the purified hESC-CM populations can be subcultured as a more homogenous population. Murine ESC-CMs have been successfully subcultured after CXCR4/FLK-1 double-positive selection with FACS. The cardiomyocyte properties of the differentiated ES cells were determined by immunocytochemical staining against cardiac nuclear TFs, cardiac alpha actinin and the presence of beating foci in approximately 40% of the double positive cells clusters (Nelson et al., 2008). Knocking in a reporter gene such as enhanced green fluorescent protein (eGFP) under a cardiac-specific promoter sequence is another way to select cardiomyocytes from hESC derivatives. Huber and colleagues created a transgenic hESC line by knocking in eGFP under the myosin light chain-2V promoter sequence. This method allowed eGFP-expressing cardiomyocytes to be selected and subcultured using positive selection with FACS. The sorted cardiac subpopulations retained greater than 95% purity and 85% viability. The cells also expressed cardiac-specific proteins in immunostaining studies, had electrophysiological properties characteristic of cardiomyocytes as detected by microelectrode array and patch clamp studies, and integrated into host rat myocardium (Huber et al., 2007). Lentiviral vector-based drug resistance selection of cardiomyocytes has also been successfully used to purify these cells from other hESC derivatives (Kita-Matsuo et al., 2009).

2.3 Electrophysiology of cardiomyocytes

2.3.1 Electrophysiological aspects of human cardiomyocyte differentiation

The electrical properties of cardiomyocytes have been investigated using either patch clamp analysis of single cells (Hamill et al., 1981; Sakmann and Neher, 1984; Zilberter et al., 1982) or a microelectrode array (MEA) platform with beating cellular aggregates (Kehat et al., 2002; Reppel et al., 2004; Reppel et al., 2005). The

MEA platform is discussed in greater detail later in this thesis. Using the patch clamp method, ion channels in excitable membranes can be studied by attaching a recording electrode pipette to the cell membrane. A so-called gigaohm seal is formed between the cell membrane and the recording electrode. The membrane located within the electrode tip is subsequently removed by suction or perforation, thereby establishing an electrical circuit allowing for the measurement of current and voltage changes across the cell membrane.

The main ions involved in the cardiac action potential are sodium (Na^+), Ca^{2+} , and K^+ cations. Some key cardiac ion channel types involved in the human ventricular action potential (AP) have been identified with patch clamp. These channels include (respective currents in brackets) $\text{Na}_{\text{V}1.5}$ (I_{Na}), $\text{K}_{\text{V}4.3}$ (I_{to}), $\text{Ca}_{\text{V}1.2}$ ($I_{\text{Ca,L}}$), $\text{K}_{\text{V}11.1}$ (I_{Kr}), $\text{K}_{\text{V}7.1}$ (I_{Ks}), and $\text{K}_{\text{ir}2.\text{X}}$ (I_{K1}) (Pollard et al., 2010). Ion channels mediate the complex interaction between the currents, resulting in a characteristic AP shape. The cardiac AP can be divided into five distinct phases (0-4) shown in figure 4. Phase 0 is a membrane potential depolarization from negative to positive, called the upstroke. Phase 1 is a short transient repolarization, which ends in phase 2, the plateau. The AP plateaus at a slightly less positive membrane potential than the peak of the upstroke and is more pronounced in ventricular cardiomyocytes. Phase 3 is the repolarization back to the resting membrane potential. The resting phase is phase 4 (Nerbonne and Kass, 2005). Na^+ , Ca^{2+} , and K^+ cations work in different phases of this cycle. Na^+ affects phase 0 depolarization. Ca^{2+} works in phase 2 and 3 to mediate cardiac contractions by binding to troponin C. K^+ mediates repolarization in phase 3.

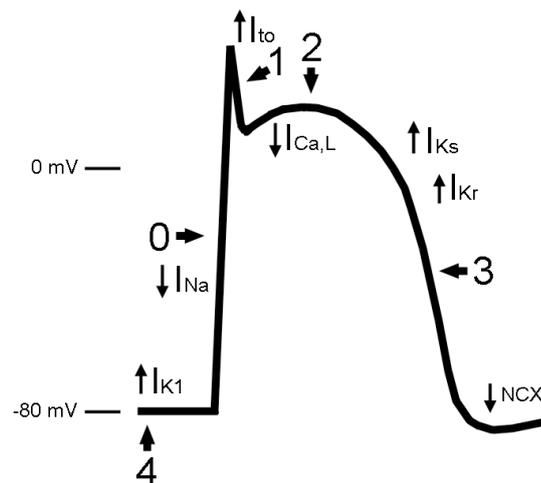


Figure 4. A schematic of a ventricular cardiac action potential. Some key currents (small arrows) during each phase (0-4) of the action potential are represented. Downward arrow: inward current, upward arrow: outward current. The different phases are indicated by the larger arrows: 0) upstroke, 1) transient repolarization, 2) plateau, 3) final repolarization, and 4) resting phase.

The cardiac action potentials in humans and rodents differ in the way they express ion currents and hence in their electrophysiological properties. To study the development of currents in human cardiomyocytes, Sartiani and colleagues investigated multiple ion channels and their respective current expression in hESCs and their early- (15 to 40 days old) and late-stage (50 to 110 days old) cardiomyocyte derivatives during their differentiation using the patch clamp technique (Sartiani et al., 2007). I_{Kr} , I_f , $I_{Ca,L}$ currents could be readily recorded from hESCs. During cardiomyocyte development, new currents (I_{to} and I_{K1}) were also detectable, indicating cardiac maturation (Sartiani et al., 2007).

mRNA levels of the two isoforms $K_{v1.4}$ and $K_{v4.3}$, mediating transient outward K^+ -current (I_{to}), were differentially expressed in developing cardiomyocytes. The longer splice variant ($K_{v1.4}$) was expressed in the early (25-day-old) cardiomyocytes, and the shorter variant ($K_{v4.3}$) was expressed in cardiomyocytes more than 57 days old. $K_{v4.3}$ mRNA was also expressed in hESCs, but no I_{to} current could be recorded. I_{to} current was detected on day 12 of differentiation in developing hESC-CMs, and the current density grew higher in later-stage cardiomyocytes (Sartiani et al., 2007).

An important repolarizing current in cardiomyocytes is the rapid component of the delayed rectifying K^+ current (I_{Kr}) mediated by the *hERG* channel. I_{Kr} is expressed in hESCs and developing cardiomyocytes, according to Sartiani and colleagues. The presence of I_{Kr} in undifferentiated hESCs was deduced from the fact that an outward K^+ -current sensitive to a compound called E-4031, which selectively blocks I_{Kr} current, could be recorded from the cells. The shorter splice variant mRNA (*HERG1b*), however, was expressed only in the hESC-CMs (Sartiani et al., 2007). The hERG current is a dominant factor in determining cardiac AP repolarization and hence its blockade by a multitude of drugs alters the K^+ efflux (Pollard et al., 2010).

Hyperpolarization-activated cyclic nucleotide-gated potassium channel (HCN) isoforms encode the depolarizing funny current (I_f). *HCN1* and *HCN4* are more strongly expressed in hESCs and early stage cardiomyocytes than in late-stage cardiomyocytes (Sartiani et al., 2007). *HCN4* is also expressed at higher levels in hESC-CMs that have fast beating rates (Asp et al., 2010). According to Sartiani and colleagues, the *HCN4* isoform is expressed in the adult heart, but *HCN1* is not. *HCN2* is expressed in all of these relevant systems: hESCs, early- and late-stage hESC-CMs, and the adult heart. I_f current was detected using the voltage clamp mode in hESCs and early- and late-stage developing cardiomyocytes. However, during maturation there is a reduction in the I_f current activation rate (Sartiani et al., 2007).

Inward rectifying K^+ -current (I_{K1}) is mediated by the $K_{ir2.1}$ channel. However, $K_{ir2.1}$ mRNA was already present in hESCs, while no current was present. The I_{K1} current could only be detected in developing cardiomyocytes (Sartiani et al., 2007).

Voltage-dependent Ca^{2+} -current ($I_{Ca,L}$) is mediated by the $\alpha 1C$ subunit of the Ca^{2+} -channel in many tissues and is encoded by the *CACNA1C* gene. *CACNA1C* mRNA is expressed in hESCs and hESC-CMs, and $I_{Ca,L}$ current is also present in both of these cells (Sartiani et al., 2007).

In terms of AP properties during hESC-CM development, the upstroke velocity (dV/dt) and AP duration (APD) increase markedly upon cardiac maturation. In contrast, the beating rate decreases during maturation and the diastolic depolarization rate flattens in late-stage cardiomyocytes. Pharmacological tests have demonstrated intact ion channel function: expected responses were achieved using E-4031, $BaCl_2$ (I_{K1} blocker), zatebradine (I_f blocker), and lacidipine ($I_{Ca,L}$ blocker). Challenging hESC-CMs with isoproterenol also demonstrated intact β -adrenergic signaling (Sartiani et al., 2007).

These in-depth results reported by Sartiani and colleagues and other groups indicate that hESC-CMs mature towards the cardiomyocyte phenotype *in vitro* and that the emergence of I_{to} and I_{K1} currents might serve as positive markers of cardiac differentiation (Sartiani et al., 2007). I_{to} current has also been shown to increase in postnatal rat cardiomyocytes (Guo et al., 1996; Shimoni et al., 1997). In contrast, I_{K1} stabilizes the myocytes diastolic potential (Silva and Rudy, 2003).

Compared to the END2 coculture method, the EB method produces cardiomyocytes that have a more consistent beating rate, ventricular type APs, and a significantly more hyperpolarized maximum diastolic potential (MDP) (Pekkanen-Mattila et al., 2010a). Low I_{K1} current seems to be responsible for the low MDP of developing hESC-CMs (Sartiani et al., 2007). The upstroke velocities (dV/dt) of the cardiomyocytes produced with either method, however, do not appear to differ (Pekkanen-Mattila et al., 2010a).

Dolnikov and colleagues studied the mechanical function of hESC-CMs. The authors found hESC-CMs to have a negative force-frequency relationship. In the mature human myocardium, this relationship is positive. Moreover, blockade of the ryanodine receptor (RyR) or the sarcoplasmic-endoplasmic reticulum Ca^{2+} -ATPase (SERCA) did not affect hESC-CM contraction, as it would in mature cardiomyocytes. Further, stimulation with caffeine did not increase the hESC-CMs intracellular Ca^{2+} concentration (Dolnikov et al., 2005). These results are indicative of immature sarcoplasmic reticulum (a critical structure in intracellular Ca^{2+} storage) development in hESC-CMs. Consequent studies have nonetheless documented the

caffeine-induced release of intracellular Ca^{2+} in hESC and hiPSC-derived cardiac cells (Itzhaki et al., 2011; Satin et al., 2008).

Overall, these results indicate that hPSC-derived cardiomyocytes are functional and provide optimism for their potential use in basic and translational cardiac research applications.

2.3.2 Cardiac field potentials and the microelectrode array

Cardiomyocytes can be studied in cell culture conditions using a microelectrode array (MEA) platform (Kehat et al., 2001; Meyer et al., 2004; Reppel et al., 2004). This platform consists of an MEA chip, which has a cell culture well and electrodes for recording electrical activity, an MEA amplifier, a filter amplifier, a heater controller and a data acquisition computer. A portable version of this setup is presented in figure 5.

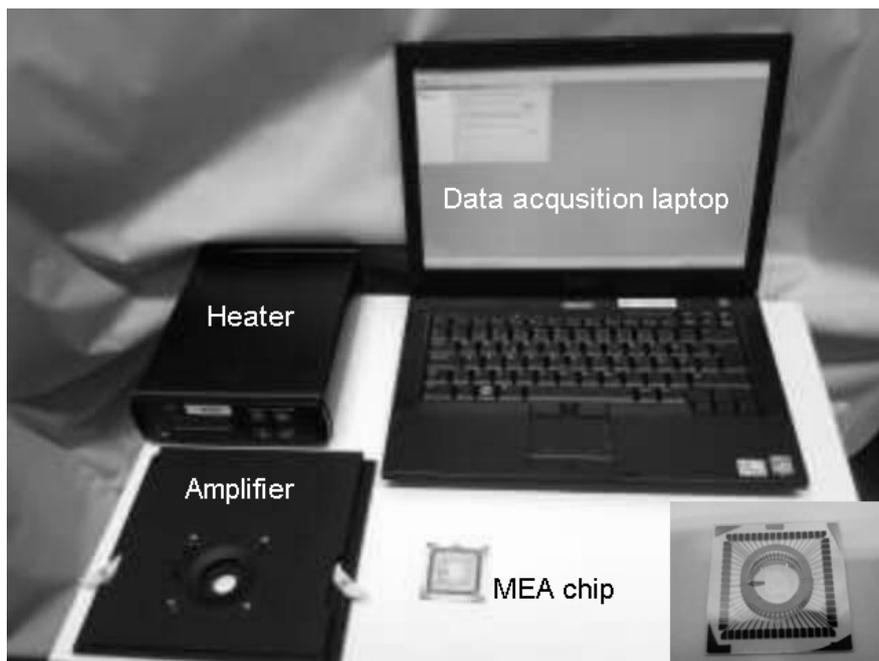


Figure 5. A portable USB-MEA setup depicting the MEA chip (see also inset lower right corner), the amplifier, the heater controller and the data acquisition laptop.

This system allows the changes in the field potential (FP) of cardiomyocytes to be recorded (Reppel et al., 2004). A characteristic field potential recording from hESC-CMs is shown in figure 6. The cardiomyocytes are plated on MEA chips in cell-culture wells. On the bottom of each cell-culture well, there are recording electrodes and, depending on the chip type, an internal reference electrode. Some of the MEA chips are fabricated without internal reference electrodes, in which case an external

reference is needed. MEA chips are manufactured with many electrode configurations, each suitable for a different application. The standard MEA (MEA 200/30iR-Ti-gr) has 59 recording electrodes in an eight-by-eight array (with four electrodes missing, one from each corner). This technique allows for high spatiotemporal resolution FP measurements of electrically active cells, including spontaneously beating cardiomyocytes derived from hPSCs. Temporal resolution is provided by fast sampling frequencies of up to 50 kHz, enough to capture the fast activating components of the cardiac cycle, namely, the depolarizing Na^+ peak. Another MEA chip type is the so-called 6-well MEA (6wellMEA200/30-iR-Ti-mr), in which the cell culture well is divided into six separate areas, each with its own reference electrode and nine individual recording electrodes. The main advantage of this type is that it can accommodate six different cell populations at once.



Figure 6. Field potential trace recorded from human embryonic stem cell-derived cardiomyocytes using a microelectrode array. This trace represents only one recording electrode.

Cardiomyocytes of various origins have been investigated with MEAs. Some of the first recordings were carried out with isolated chick cardiomyocytes (Meyer et al., 2004) and mESC-CMs (Reppel et al., 2007). These cardiomyocytes, however, are not a very relevant model for studying human heart development. Mouse models are more frequently used in the preclinical testing phase of drug development, but with respect to human cardiac electrophysiology, they still make for a suboptimal model. Neonatal rat cardiomyocytes (NRCs) have been used for MEA experiments as well (Berdondini et al., 2005). None of these cardiomyocyte types fully capture the intricacy of the human cardiomyocyte physiology. Human primary cardiomyocytes are difficult to obtain and culture; thus, to study human electrophysiology, researchers have relied on hESC-CMs. These cells provide the appropriate electrophysiological network to study human conditions. The identical genome of hESC-CMs affords a more reliable comparison of results to NRCs. In addition,

hiPSCs have been used to derive cardiomyocytes, which have been studied using MEA (Itzhaki et al., 2011; Mehta et al., 2011).

2.3.2.1 Cardiac field potential parameters

Cardiac FPs have a definitive morphology if the cardiomyocytes are plated as monolayers on the MEA. For example, isolated neonatal rat cardiomyocytes (NRCs) and chick cardiomyocytes can have defined interexperimental FP properties, and in some cases, hESC-CMs do as well, as shown in figure 7. In addition to the peak-to-peak interval (PPI, which is measured as the time between two individual FP peaks), from which the beating rate can also be calculated (see equation 1), the two most important FP parameters are the amplitude and the FP duration (FPD). *In vitro* FPD measurements can be used as a surrogate for the clinical QT interval and are usually measured as the time between the initial deflection and the return to baseline (i.e., the isoelectric point, see figure 7) (Caspi et al., 2009).

$$BR = \frac{60}{PPI} \quad (1)$$

where BR is the beating rate in beats per minute and PPI is the peak-to-peak interval in seconds.

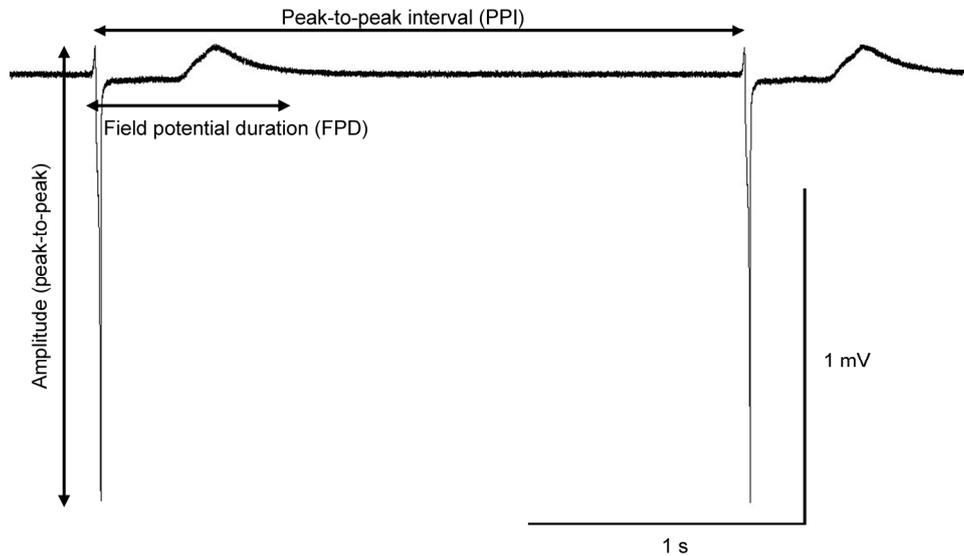


Figure 7. The cardiac field potential (FP) of human embryonic stem cell-derived cardiomyocytes and the nomenclature of FP components. The nomenclature data (with modifications) is from (Reppel et al., 2004).

2.3.2.2 *Signal analysis*

MEA FP recordings from the three-dimensional cardiomyocyte aggregates exhibit more variation in signal shape between recordings and also between different electrodes. This phenomenon is due to the three-dimensional structure of the cardiomyocyte aggregates. The orientation of these aggregates on the MEAs cannot be controlled, which presents a challenge for generating robust software algorithms for the analysis of cardiac FP properties.

High-quality FP recordings can also be difficult to acquire at times, particularly with hPSC-derived cardiomyocytes, as the signal quality can be limited by the low amount of differentiated cardiomyocytes in the beating hESC-CM foci. Irregular beating rhythms can present a challenge in analyzing the signals as well (Pekkanen-Mattila et al., 2010a). Acquiring clear high-quality signals is important in determining the cardiac FP components, particularly the return to baseline. A higher signal-to-noise ratio (SNR) indicates a higher signal quality.

Programs like MEA-tools have been developed for signal analysis. This program was developed by Ulrich Egert and colleagues for multielectrode data analysis but is a MATLAB (Mathworks, Inc.) toolbox that requires the software to run (Egert et al., 2002). Custom-made MATLAB toolboxes are also widely used for MEA analysis (Bussek et al., 2009; Halbach et al., 2006; Hannes et al., 2008; Liang et al., 2010; Reppel et al., 2005). FPD measurements have also been performed by an independent operator (Caspi et al., 2009).

2.4 Pluripotent stem cell–derived cardiomyocytes and drug testing

Over the years, many cardiac and non-cardiac drugs have been withdrawn from the market due to their adverse cardiac side effects. Although every new chemical entity (NCE) is tested according to set requirements, during the course of drug development, unexpected effects such as syncope (fainting), arrhythmia, a specific polymorphic ventricular tachycardia known as Torsade de pointes (TdP), and even sudden death are sometimes observed when a drug is already approved and on the market (Lexchin, 2005; Redfern et al., 2003a; Roden, 2004). Pharmaceutical regulatory authorities, namely the International Conference on Harmonisation (ICH), have specified and expanded the requirements for drug safety testing. This now includes tests to be performed using two different mammalian (a rodent and a

non-rodent) species. Required tests include electrocardiogram (ECG) recordings and histological examination of cardiac tissue (EMA, 2008; ICH, 2005a; ICH, 2005b).

The QT interval (figure 8), as seen on the ECG recordings, is the cornerstone for assessing the proarrhythmic potential of NCEs (ICH, 2005a; ICH, 2005b). The QT interval marks the length of the ventricular repolarization time in the heart. Prolongation of the QT interval can lead to life-threatening arrhythmias such as TdP (Redfern et al., 2003b). Many drugs can prolong the QT interval (Fenichel et al., 2004; Roden, 2004), making this the leading cause for drug use restrictions and withdrawal from the marketplace (Roden, 2004). ICH has a defined evaluation of this risk as a standard preclinical process for all NCEs (Bode and Olejniczak, 2002; Cavero and Crumb, 2005).

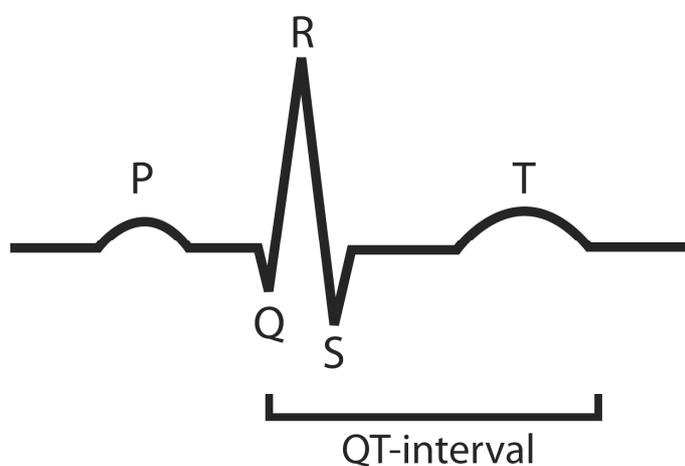


Figure 8. A cartoon of an electrocardiogram trace. The different peaks and the QT interval are depicted.

The inhibition of the hERG channel ($K_{V11.1}$) is the predominant basis for drug-induced QT-interval prolongation and TdP (Carlsson, 2006; Pollard et al., 2008). Pharmaceutical companies utilize many different preclinical assays and models to assess the risks of NCEs (Miyazaki et al., 2005). These assays include *in vivo* studies such as ECG telemetry with conscious dogs (Finlayson et al., 2004; Martin et al., 2004), *in vitro* assays such as a repolarization assay that detects APD changes in isolated animal Purkinje fibers, papillary muscles or cardiomyocytes, the hERG assay in which I_{Kr} current is expressed in heterologous expression systems like CHO or HEK293 cells, or the characterization of the native I_{Kr} current (Lu et al., 2008; Redfern et al., 2003a). These methods, however, are not necessarily sufficient (Braam et al., 2010; Mandenius et al., 2011) and can be very costly; in addition, the large number of required animal tests raises ethical concerns. Consequently, a human cardiomyocyte-based preclinical *in vitro* assay is needed for NCE testing. Cardiomyocytes derived from hPSCs can potentially serve the pharmaceutical industry for compound screening, target validation, and safety testing. These

cardiomyocytes can be produced from an unlimited cell source and could serve as a reproducible human model system. Further, cardiomyocytes can be used in conjunction with MEAs for a medium throughput readout of electrophysiological responses, such as the prolongation of repolarization (Caspi et al., 2009; Liang et al., 2010; Reppel et al., 2005).

Multicellular cardiac syncytia and their repolarization can be studied using MEAs. Thus, drug effects on cardiac repolarization can be investigated using this platform (Reppel et al., 2005). Caspi and colleagues studied drug effects on hESC-CMs using MEAs. The E-4031 compound blocks the I_{Kr} repolarizing current in cardiomyocytes, which was observed as cardiac FP prolongation in an *in vitro* electrocardiogram recorded with MEA. The authors also demonstrated a dose-dependent prolongation of FP duration (FPD) with escalating E-4031 concentrations. Sotalol is a class III antiarrhythmic agent, quinidine and procainamide are both class IA antiarrhythmic agents and cisapride is a gastrointestinal prokinetic drug that was withdrawn from market due to QT-interval prolongation. All of these substances caused FPD prolongation that was detected in MEA recordings (Caspi et al., 2009).

Braam and co-workers also investigated the FPD prolongation potential of multiple drugs using hESC-CMs in conjunction with MEA (Braam et al., 2010), examining 12 different drugs in total. Sodium, calcium and *HERG* potassium channel blockade with lidocaine, nifedipine, and E-4031, respectively, had effects on FPD, as expected. Quinidine and sotalol are both known to prolong cardiac repolarization in patients. These substances increased the FPD at concentrations slightly over the unbound effective therapeutic plasma concentration ($ETCP_{unbound}$) range. FPD prolongation with these drugs occurred at concentrations further from the $ETCP_{unbound}$ range. FPD could be reliably measured despite the variable FP shapes produced by hESC-CMs. (Braam et al., 2010). This study provided FPD data on many drugs over a high range of concentrations using hESC-CMs as a human cardiac model system.

2.5 Long QT syndrome

2.5.1 Acquired and congenital long QT syndrome

Long QT syndrome (LQTS) is mainly characterized by prolonged ventricular repolarization in individuals. The condition is observed as an abnormally long QT

interval on ECG, as discussed above, and is associated with an increased risk of the life-threatening arrhythmia known as TdP (see above). LQTS can be congenital or acquired. So far, 12 different congenital LQTS subtypes (LQT1-12) have been identified, and each one is caused by a mutation in a specific gene (Hedley et al., 2009). Acquired LQTS usually results from drug therapy, hypokalemia, or hypomagnesemia.

Some drugs have the undesired side effect of prolonging the QT interval as discussed above, thus resulting in acquired LQTS. This is caused by the ability of some drugs to block the hERG K⁺-channel trans-membrane pore. The hERG channel's S6 trans-membrane domain, which forms the K⁺ permeable pore in the tetrameric form, is pharmacologically vulnerable due to its atypical amino acid sequence in comparison to other ion channels. Although QT prolongation, caused by hERG pore blockade, might not be harmful, it is nonetheless associated with TdP. Naturally, this has an effect on the safety of drugs. Because of their tendency to prolong the QT interval, several drugs have been withdrawn from the market or their use has been restricted with warning labels, resulting in the loss of commercial value. The early drug discovery process aims to optimize compound efficacy, pharmacokinetics, and safety to patients (Pollard et al., 2010). In this context, hPSC-derived cardiomyocytes could be used in early preclinical compound safety assays to minimize this risk of QT prolongation.

The overall prevalence of the genetic form of LQTS in the general population is estimated to be as high as 1:2,000 (Hedley et al., 2009; van Noord et al., 2010). Of the different subtypes, LQT1 is caused by a mutation in the *KCNQ1* gene (Hedley et al., 2009) and LQT2 is caused by a mutation in the *hERG* gene (also known as *KCNH2*) (Curran et al., 1995; Hedley et al., 2009). Symptoms in LQT1 patients are triggered mainly by stress or exercise and in LQT2 patients by emotional stress or auditory stimuli accompanied by a reduced heart rate (van Noord et al., 2010). Specific types of mutations are enriched in both of these genes in Finland and these are known as founder mutations, causing approximately 73% of all LQTS cases in Finland. These founder mutations are named KCNQ1-FinA, KCNQ1-FinB, HERG-FinA, and HERG-FinB; all of these mutations impair the outward K⁺ efflux in cardiomyocytes during the cardiac repolarization phase (Fodstad et al., 2006; Zareba, 2007). LQT1 impairs the slow component of the delayed rectifying K⁺ current (I_{Ks}), whereas LQT2 impairs the I_{Kr} current (Fodstad et al., 2004). The KCNQ1-FinA founder mutation (studied in this thesis) is caused by a G589D missense mutation near the C-terminus of the *KCNQ1* gene, after the S6 domain. All the mutation carriers have mean QTc times of 457 ms and the KCNQ1-FinA carriers have corresponding mean times of 492 ms. On the other hand hERG-FinB (the other

founder mutation studied in this thesis) is caused by a R176W missense mutation in the proteins' N-terminus, close to the Per-Arnt-Sim (PAS) domain. Mutations in the PAS domain result in altered hERG channel-gating kinetics, which, in this case, is seen as an accelerated deactivation of the ion channel. This accelerated deactivation results in reduced K^+ efflux during repolarizations and hence prolongs the QT interval. hERG-FinB mutation carrier patients have QTc times with a mean of 448 ms (Fodstad et al., 2006). In the Finnish population, this mutation is estimated to be present in every one out of 400 individuals (Marjamaa et al., 2009b). The mean QTc of the se mutation carriers' unaffected relatives is 417 ms (Fodstad et al., 2004).

Having two recessive disease-causing alleles (also known as compound heterozygote) can cause severe cases of LQTS, where the patient has very long QTc times and is more likely to suffer cardiac events and cardiac arrest (Westenskow et al., 2004).

2.5.2 Modeling long QT syndrome with patient-specific induced pluripotent stem cell-derived cardiomyocytes

The potential to reprogram somatic cells into hiPSCs and subsequently differentiate them into cardiomyocytes creates a splendid *in vitro* opportunity for studying molecular interactions in LQTS. Modeling congenital LQTS is a pressing task for the biomedical field, and in addition to results presented in this dissertation, only three studies have reported LQTS modeling using hiPSC technology. The first report focused on LQT1 (Moretti et al., 2010b) and the second two on LQT2 (Itzhaki et al., 2011; Matsa et al., 2011).

Moretti and colleagues reprogrammed *KCNQ1* (R190Q) mutant pluripotent stem cells from fibroblasts of two family members with LQT1. The group subsequently differentiated cardiomyocytes from hiPSCs. These cardiomyocytes possessed the LQT1 genotype and had prolonged APD as seen in single-cell patch-clamp recordings. The AP prolongation was determined to be caused by an ion-channel trafficking defect resulting in a 70-80% I_{Ks} current density reduction. The group also noted altered activation and deactivation kinetics of the ion channel and demonstrated increased arrhythmogenicity with isoproterenol (a β -adrenergic agonist), which was rescued by β -blockade (Moretti et al., 2010b).

Itzhaki and colleagues had similar findings with LQT2, using *hERG* (A614V) missense-mutated hiPSC-CMs derived from a patient with severe LQT2. The patient had previously presented with episodes of TdP. The LQT2-cardiomyocytes derived from the patient's hiPSCs demonstrated increased arrhythmogenicity that was associated with early after depolarizations (EADs). These cells also had significant

APD prolongation due to a reduced I_{Kr} current density (Itzhaki et al., 2011). Matsa and colleagues also produced LQT2-cardiomyocytes from patient-specific hiPSCs with a *hERG* (G1681A) mutation. Exposing these LQT2-cardiomyocytes to E-4031 caused EADs and arrhythmia. In another study, isoproterenol treatment led to EADs that were reversed by β -blockade (Matsa et al., 2011).

Other cardiac-related diseases have been also modeled using hiPSC-CMs, including Timothy syndrome. Timothy syndrome is caused by a mutation in *CACNA1C*. This gene encodes the $Ca_v1.2$ Ca^{2+} channel; therefore, cardiomyocytes carrying a mutation in this gene have excessive Ca^{2+} influx and hence abnormal calcium transients and irregular contractions, and they exhibit AP prolongation (Yazawa et al., 2011).

These reports support the idea that disease modeling *in vitro* in the cardiac context is feasible. These human cell models allow thorough analysis of the cellular signaling pathways and molecular interactions that occur in the pathological state. Because these cells are also patient-specific, they provide the necessary human genome in which to study the diseased state.

3. Aims of the study

The aim of the first study (I) was to investigate the growth and propensity of cardiomyocyte differentiation among eight different hESC lines. More importantly, the aim was to study spatiotemporal germ layer marker expression in developing embryoid bodies at the protein level using novel tissue microarray technology, as most studies have relied on marker profiles at the mRNA level.

In the second study (II), our aim was to develop analysis software based on averaging the cardiac field potential signals that were recorded from neonatal rat cardiomyocytes and human embryonic stem cell-derived cardiomyocytes with microelectrode arrays. Using this new software, our aim was to investigate the effects and feasibility of averaging the recorded field potential signals for downstream analysis of cardiac FP parameters, such as field potential duration and peak-to-peak interval.

In the third study (III), we sought to develop a patient-specific cell model based on long QT syndrome type 2 (LQT2), using induced pluripotent stem cell technology. Using this cell model, our aim was to recapitulate the electrophysiological phenotype of LQT2 *in vitro* and to validate this method for further studies.

In the fourth (IV) study, we used the induced pluripotent stem cell technology developed in the third study (III) to expand the investigations to also include LQTS type 1. Using these patient-specific cardiomyocytes that have mutations for LQT1 and -2, we performed a series of drug tests to determine whether the responses of these LQTS cardiomyocytes differed from those of control cardiomyocytes.

4. Materials and methods

4.1 Ethical approval (I-IV)

The Institute of Biomedical Technology has permission (1426/32/300/05) to conduct research on hESCs from the National Authority for Medicolegal Affairs. The ethical committee of Pirkanmaa Hospital District has granted the Institute of Biomedical Technology permission for the derivation, culturing, characterization, and differentiation of hESC lines (R05116) and iPS cell lines (Aalto-Setälä R08070). Regarding the HS hESCs, the Karolinska Institute has approval for the derivation, characterization and differentiation of hESCs from the ethics committee of the Karolinska Institute. All of the embryos used were surplus embryos that were donated by couples after both partners provided informed consent. The couples received no monetary compensation.

4.2 Culture of primary and other cell types (III, IV)

Culture medium for the primary fibroblasts obtained from skin biopsies for hiPSC induction was composed of Dulbecco's Modified Eagle Medium (DMEM, Lonza, Switzerland) supplemented with 10 % fetal bovine serum (FBS, Lonza), 2 mM L-glutamine, and 50 U/mL penicillin/streptomycin. The medium for commercial 293FT cells (Invitrogen, Carlsbad, CA, USA) was additionally supplemented with 1% non-essential amino acids (NEAA, Cambrex, NJ, USA). Mouse embryonic fibroblasts (MEF, Millipore, MA, USA) and Plat-E cells were cultured without penicillin/streptomycin.

4.3 Establishment of induced pluripotent stem cell lines (III, IV)

The primary fibroblasts for hiPSC induction were obtained from skin biopsies donated by healthy volunteers and patients diagnosed with either LQT1 or LQT2.

Control (from healthy subjects) and patient-specific (from patients with a confirmed mutation predisposing them to LQTS) hiPSC lines were induced from primary fibroblasts using lentiviral and subsequent retroviral infections. This was achieved using 293FT and Plat-E cells as packaging cells along with the following plasmids and reagents: pLenti6/UbC/mSlc7a1-vector (Addgene, Cambridge, MA, USA), ViraPower packaging mix (Invitrogen), Lipofectamine 2000 (Invitrogen), pMX retroviral vector (with hOCT3/4/3, hSOX2, hKLF4, or hc-MYC, Addgene), and Eugene (Roche Diagnostics, Mannheim, Germany). The hiPSC lines were induced as described previously (Takahashi et al., 2007).

H7 hESCs and hiPSC lines UTA.01006.WT (hereafter called WTa), UTA.00112.hFF (WTb), and FiPS 6-14 (derived at University of Helsinki, kindly provided by Prof. Timo Otonkoski) were used as controls. UTA.00208.LQT1 (LQT1a) and UTA.00313.LQT1 (LQT1b) were used as patient-specific hiPSCs with a KCNQ1-FinA (G589D) mutation. UTA.00514.LQT2 (LQT2a) and UTA.00525.LQT2 (LQT2b) were used as patient-specific cells lines, which had a hERG-FinB (R176W) mutation. Both mutations were detected by PCR.

4.3.1 Karyotype and teratoma formation analysis (III, IV)

Karyotypes of the cell lines were defined using standard G-banding chromosome analysis by a commercial company (Medix laboratories, Espoo, Finland), according to standard procedures. For teratoma formation, hiPS cells were injected into nude mice testis capsules. Tumor samples were collected 8 weeks after injection and fixed with 4 % paraformaldehyde (PFA). The sections were stained with haematoxylin and eosin.

4.4 Stem cell culture (I-IV)

The human embryonic stem cell (hESC) lines Regea 06/015 and Regea 06/040 derived at the Regea Institute for Regenerative Medicine (University of Tampere, Finland) and HS181, HS293, HS346, HS360, HS362 and HS401 derived at Karolinska University Hospital, Huddinge (Karolinska Institute, Stockholm, Sweden) were cultured as follows: commercially available human foreskin fibroblast cells (CRL-2429, ATCC, Manassas, VA, USA) were used as feeder cells for hESCs. The hESCs were passaged mechanically. The hESC culture medium consisted of knockout DMEM (Invitrogen, Carlsbad, CA, USA), 20% serum

replacement (SR) (Invitrogen), 2 mM GlutaMax (Invitrogen), 1% NEAA (Cambrex Bio Science Inc., Walkersville, MD, USA), 50 U/mL penicillin/streptomycin (Cambrex Bio Science Inc.), 0.1 mM 2-mercaptoethanol (Invitrogen), and 8 ng/mL basic fibroblast growth factor (bFGF) (R&D Systems, Minneapolis, MN, USA). Colonies were passaged mechanically on a weekly basis. The undifferentiated state of the colonies was confirmed daily by morphologic analysis, and periodic testing for the expression of the stem cell markers NANOG, POU5F1, SSEA4, and TRA-1-60 was carried out. The differentiation ability of the lines has been tested in vivo and in vitro.

H7 hESCs (WiCell) and established control and patient-specific hiPSCs were cultured on mitomycin C-inactivated mouse embryonic fibroblasts (MEF) in hES medium, which consisted of DMEM/F-12 (Invitrogen) supplemented with 20% knockout serum replacement (KO-SR) (Invitrogen), 1% nonessential amino acids (Lonza), 2 mM L-glutamine (Glutamax, Invitrogen), 50 U/ml penicillin/streptomycin (Lonza), 0.1 mM beta mercaptoethanol (Invitrogen), and 7.8 or 4 ng/ml basic fibroblast growth factor (R&D Systems). The medium was refreshed daily, and the hESC colonies were passaged onto a new MEF layer once a week using 1 mg/ml collagenase IV (Invitrogen).

4.5 Cardiomyocyte differentiation (I-IV)

4.5.1 Embryoid body formation and cardiac differentiation (I)

EB formation was performed by mechanically excising undifferentiated hESC colonies into small pieces and placing them on a U-shaped low attachment 96-well plate (Nunc, Roskilde, Denmark) (one piece per well) in EB-medium consisting of Knockout DMEM (KO DMEM) (Gibco Invitrogen, USA) supplemented with 20% fetal bovine serum (FBS) (Gibco Invitrogen, USA), 1% non-essential amino acids (Cambrex BioSciences, Verviers, Belgium), 1% L-glutamine (Invitrogen, USA), and 50 U/ml penicillin/streptomycin (Cambrex BioSciences, Verviers, Belgium). The hESC colonies were cut and detached in the same way as with the normal passaging of hESCs; however, the pieces were cut larger (one cell colony into 2-4 pieces). For each hESC line, three individual 96-well plates were prepared: one for immunocytological analysis (2-12 days) and two for determining growth and differentiation. EBs were cultured on the 96-well plates for 7 days and were subsequently plated onto 0.1% gelatin type A-coated (Sigma-Aldrich, Germany)

Nunclon™ D surface 12-well plates (Nunc) in EB medium. A total of eight EBs were transferred from the 96-well plate to each 12-well plate well. The EBs were allowed to adhere. Medium was refreshed three times per week. Cultures were checked daily for formation of beating foci under a phase contrast microscope (Olympus, Tokyo, Japan). Cells were grown on 12-well plates for up to six weeks before they were further analyzed or discarded. Additional EBs were collected from the Regea 06/015 and Regea 06/040 lines for western blotting and qRT-PCR. Thirty-day-old beating foci were collected from some of the lines for immunocytochemistry. The cardiomyocyte differentiation efficiency was calculated as a percentage of beating areas per plated EB on each plate. Efficiency was calculated for each plate and the mean value was calculated from two individual plates for each cell line. The standard deviation for the differentiation efficiency of each cell line was calculated between the differentiation efficiency percentages of each plate. EB formation was also performed using forced aggregation (FA) (Burrige et al., 2007). In this method, undifferentiated hESC colonies were dissociated into single cells enzymatically with tryple and collagenase IV (Gibco) or by Accutase (Millipore), and 3,000–15,000 cells/well were plated in EB medium on untreated V-shaped 96-well plates (Nunc) and centrifuged for 5 min at 950 g. EBs formed on the bottom of the wells were transferred to a 0.1% gelatin type A-coated (Sigma-Aldrich) Nunclon™ D surface 12-well plate (Nunc) in EB-medium after 4–7 days of culturing.

The hiPSC EBs were cultured in EB-medium (KO-DMEM with 20 % FBS, NEAA, L-glutamine and penicillin/streptomycin) without bFGF for 5 weeks. The expressions of the ectodermal, endodermal, and mesodermal markers were determined using RT-PCR.

4.5.2 Co-culture with mouse visceral endoderm–like cells (II-IV)

Cardiomyocyte differentiation was carried out by co-culturing hESCs and hiPSCs with mouse visceral endodermal-like (END-2) cells, which were a kind gift from Professor Mummery (Humbrecht Institute, Utrecht, The Netherlands). END-2 cells were cultured as described previously (Mummery et al., 1991). Briefly, to initiate cardiomyocyte differentiation, undifferentiated hESC colonies were dissected mechanically into small aggregates and plated on mitomycin C-treated END-2 cells in hESC medium without serum, serum replacement, or bFGF (Mummery et al., 2003). Differentiating cell colonies were monitored with phase-contrast microscopy, and medium was changed on days 5, 8, and 12 onto the co-cultures. After two weeks, 10% SR was added to the medium.

4.6 EB morphology and size analysis (I)

The growth and morphology of plated hESCs were examined daily with phase contrast microscopy. Pictures were taken from ten plated EBs, and the sizes were determined from the pictures during the suspension phase (7 days from the start of the differentiation protocol). EBs diameters and cross-sectional areas were measured from the pictures with Cell^D imaging software (Olympus Soft Imaging Solutions GmbH, Japan). The overall EB size for each cell line and each day was determined from the mean value of ten EBs.

4.7 Immunocytochemical analysis (I, III, IV)

For immunocytochemical (ICC) staining, undifferentiated hESC colonies from the Regea 06/015 and Regea 06/040 lines were fixed with 4% paraformaldehyde for 20 min and stained as previously described (Pekkanen-Mattila et al., 2009). Antibodies included anti-OCT3/4 (1:300, R&D Systems, Minneapolis, MN, USA), anti-Brachyury T (1:100, Abcam, Cambridge, UK), anti-alpha-fetoprotein (AFP) and anti-SOX1 (both 1:100 and both from Santa Cruz Biotech, Santa Cruz, CA, USA). The time series of developing EBs for immunocytochemical staining was 2, 4, 6, 8, 10 and 12 days (n=8), and these cells were fixed with 4% PFA for 2 h. Older EBs with and without beating foci were prepared for immunocytochemistry as well. Samples were cryoprotected with 20% sucrose in phosphate-buffered saline (PBS) for several days. A tissue 10% agarose gel (BioWhittaker Molecular Applications, Rockland, ME, USA) multiarray was prepared with 1-mm holes as originally developed by Pelto-Huikko (Parvinen et al. 1992). 400 EBs were individually transferred into the wells filled with OCT compound. The multiarray was frozen on dry ice, and 6- μ m frozen sections were cut throughout the chuck and thaw mounted on Polysine glass slides (Menzel, Braunschweig, Germany). Sections were stored at -70°C until use. Immunocytochemistry was performed using the N-Histofine simple stain MAX PO staining method (Nichirei Biosciences Inc., Tokyo, Japan). Antibodies used were mouse anti-OCT3/4 (1:200), goat anti-SOX-1 (1:500), goat anti-AFP (1:500, all from Santa Cruz Biotech) and rabbit anti-Brachyury T (1:300, Abcam), NKX2.5 (1:200, R&D Systems), caspase-3 (1:500, Cell Signaling Tech., Danvers, MA, USA), anti-PAX6 (1:300, Developmental Studies Hybridoma Bank, The University of Iowa, Iowa City, IA, USA) and anti-cardiac troponin T (1:50, Abcam). Several sections (n=5-6) cut from different levels of the tissue multiarrays were labeled with each antibody. Sections were incubated overnight at +4°C with

primary antibodies followed with appropriate N-Histofine staining reagent for 30 min. ImmPACT™ (Vector Laboratories, Burlingame, CA, USA) diaminobenzidine solution was used as the chromogen. All antibodies were diluted in PBS containing 1% bovine serum albumin (BSA) and 0.3% Triton X-100. The sections were briefly counterstained with haematoxylin, dehydrated and then embedded in Entellan. Controls included omitting the primary antibodies or replacing them with non-immune sera. No staining was observed in these controls.

hiPSCs were fixed with 4 % PFA (Sigma-Aldrich) at passage 8 and stained with anti-Oct3/4 (1:400, R&D Systems), anti-TRA1-60 (1:200, Millipore), and anti-Sox2, anti-Nanog, anti-SSEA4, and anti-TRA1-81 (all 1:200, from Santa Cruz Biotechnology, Santa Cruz, CA, USA). Secondary antibodies (Invitrogen) included Alexa-Fluor-568-donkey-anti-goat-IgG, Alexa-Fluor-568-goat-anti-mouse-IgM and Alexa-Fluor-568-donkey-anti-mouse-IgG.

4.8 Protein expression studies by Western blot (I)

Regea 06/015 and Regea 06/040 EBs were collected at days 0, 3, 7, 10 and 20 for protein isolation. Proteins were isolated using the M-PER reagent (Pierce, Rockford, IL, USA), and protein concentrations were determined by the BCA method (Pierce). Proteins were separated on a 12% SDS-PAGE gel and transferred to a PVDF membrane (Hybond-P, GE-Healthcare). The membrane was blocked with 2% BSA (Sigma-Aldrich, St. Louis, USA) overnight at 4°C. Primary antibodies used were anti-OCT3/4 (1:100, Santa Cruz Biotech.), anti-Brachyury T (1:400, Abcam), anti-AFP (1:200, Santa Cruz Biotech) and anti-SOX1 (1:400, Abcam) and were diluted with TBS-Tween. Beta-actin (1:1,000, Santa Cruz) was used as an endogenous control. Primary antibodies were incubated overnight at +4°C. Peroxidase-conjugated antibodies (1:4,000, Zymed, Invitrogen) were used for 1 h at room temperature (RT), and the ECLplus kit (GE Healthcare) was used as the detection reagent. Exposure was performed using a CCD camera and Quantity One software (Biorad, Hercules, CA, USA).

4.9 Gene expression studies (I, III, IV)

Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) was performed according to the standard protocols using Abi Prism 7300 (Applied Biosystems, Foster City, CA, USA). Total RNA was isolated from Regea 06/015

and 06/040 EBs on days 0, 3, 7, 10, 20, 32 and 41 using the RNAeasy Plus Mini Kit (Qiagen, Valencia, CA, USA). The concentration and the quality of RNA were monitored spectroscopically with a nanodrop (Wilmington, DE, USA). 0.2 µg of total RNA was transcribed to cDNA in a total volume of 20 µl. Each PCR reaction consisted of 0.25 µl of cDNA, 7.5 µl of 2x SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA) and 200 nM of each primer. Brachyury T, AFP, and SOX1 mRNA expression was examined using previously described primer sequences (Pekkanen-Mattila et al., 2009), and peptidyl-prolyl isomerase G (PPIG) was used as a housekeeping control. OCT3/4 was also determined using the same cDNA, but detection was made with Taqman chemistry and using the primer-probe sets Hs99999905_m1 for GAPDH and Hs00999632_g1 for OCT3/4 (Applied Biosystems). Ct values were determined for every reaction, and the relative quantification was defined using the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001). Data were normalized to the expression of the housekeeping genes PPIG or GAPDH, and the 0-day sample (or 3-day sample for AFP) of the hESC line Regea 06/040 was used as the calibrator.

For markers of pluripotency, hiPSC total RNA was collected at passages 3, 6, and 11 and after cardiac differentiation. H7 hESC RNA was used as a positive control and EB RNA was used as a negative control. RNA was purified using the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany), and cDNA conversion was performed using a high-capacity cDNA reverse transcriptase kit (Applied Biosystems, Carlsbad, CA, USA). PCR was performed with Dynazyme II (Finnzymes Oy, Espoo, Finland), using 1 µl of cDNA as the template and 2 µM of the primers. As positive controls for the exogenous primers, PCR was also performed using the transfected plasmids (hOCT3/4, hSOX2, hKLF4, and hc-MYC) as templates. PCR primers for hiPSC characterization and detailed reaction conditions have been described previously (Takahashi et al., 2007). Housekeeping controls were *β-actin* and *GAPDH*.

4.10 Electrophysiological characterization of cardiomyocytes (II-IV)

4.10.1 Patch clamp analysis (III)

APs were recorded from spontaneously beating dissociated cells using amphotericin for the perforated patch configuration of the patch clamp technique. Data were

acquired with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA). A coverslip with adherent cardiomyocytes was placed in a recording chamber and perfused with an extracellular solution consisting of 143 mmol/L NaCl, 4 mmol/L KCl, 1.8 mmol/L CaCl₂, 1.2 mmol/L MgCl₂, 5 mmol/L glucose, and 10 mmol/L HEPES (pH 7.4 with NaOH; osmolarity of 301 ± 3 mOsm). Patch pipettes were pulled from borosilicate glass capillaries (Harvard Apparatus, Kent, UK) and had resistances of 1.5-3.5 MΩ when filled with a solution consisting of 122 mmol/L K-gluconate, 30 mmol/L KCl, 1 mmol/L MgCl₂, and 5 mmol/L HEPES (pH 7.2 with KOH; osmolarity adjusted to 290 ± 3 mOsm). The final concentration of amphotericin B (solubilized in dimethylsulfoxide) in the pipette was 0.24 mg/ml.

Data were filtered at 2 kHz and digitized (Digidata 1322A, Molecular Devices) at 10 kHz; data acquisition and analysis was performed with pClamp 9.2 software (Molecular Devices). Voltage-clamp experiments were also performed using some cardiomyocytes to record I_{Kr}. Experiments were conducted at 36 ± 1 °C.

4.10.2 Cardiac field potential recordings (II-IV)

Spontaneously beating hESC- and hiPSC-cardiomyocyte aggregates were mechanically excised from the cell cultures and plated onto FBS (30 minutes, Invitrogen) and 0.1% gelatin-coated (1 hour, Sigma-Aldrich) MEAs (Multi Channel Systems MCS GmbH) in EB medium. Medium was refreshed three times per week.

NRC were extracted from the hearts of newborn Sprague-Dawley rats as described elsewhere (Tokola et al., 1994). Neonatal rats were quickly decapitated, and their hearts were then harvested. Cardiomyocytes were extracted with multiple rounds of collagenase treatment, preplated for 1 hour at +37 °C, 5% CO₂ and plated on coated MEAs. 800,000 NRCs per MEA were plated, and culture medium was refreshed daily.

The FP recordings took place in room air with an MEA1060-Inv-BC or USB-MEA amplifier at a 20-kHz sampling rate (Multi Channel Systems MCS GmbH). MC_rack software (Multi Channel Systems MCS GmbH) was used for data acquisition. Standard 200/30iR-Ti-gr or 6-well-MEA200/30iR-Ti-mr MEAs were covered with a gas-permeable membrane (ALA Scientific) during recording to keep the cultures sterile. The temperature was kept at +37 °C using the heating element of the MEA amplifier during the recordings.

4.11 Pharmacological tests (II-IV)

Baseline conditions as well as drug effects were recorded for two minutes after a two-minute stabilization period. In study II, E-4031 (Alomone labs) was diluted in the cell culture medium to a final concentration of 600 nM. The FP signals were recorded from MEAs with NRCs (n=9) and from MEAs with hESC-CMs (n=4). In study III, baseline FPDs were measured from 43 control cardiomyocyte aggregates and 30 LQT2-cardiomyocyte aggregates. In study IV data were recorded from 11 hESC-CM, 6 WTa-CM, 11 WTb-CM, 9 LQT1a-CM, 5 LQT1b-CM, 6 LQT2a-CM, and 7 LQT2b-CM aggregates.

Cisapride monohydrate, quinidine, and D,L-sotalol (all from Sigma-Aldrich) were solubilized in dimethyl sulphoxide (DMSO, Sigma-Aldrich) at 10 mM. E-4031 (Alomone labs) and erythromycin (Abbotcin i.v., Amdipharm, Abbott, IL, USA) were solubilized in sterile H₂O at 1 mg/mL and 50 mg/mL, respectively. Isoprenaline (Isuprel, Hospira, Lake Forest, Illinois, USA) was supplied in ready-ampoules. The drug stock solutions were diluted in 5 % FBS-containing EB medium for drug tests. The tested concentrations of cisapride monohydrate, quinidine, (±)-sotalol hydrochloride and erythromycin were chosen accordingly: half the concentration of the lower limit for therapeutic blood serum concentration, the lower limit of therapeutic blood serum concentration range, the average therapeutic serum concentration, the upper limit of therapeutic range and twice the upper limit of therapeutic concentration (Schulz and Schmoldt, 2003). Isoprenaline concentrations were chosen according to previously published values and multiples thereof (Pekkanen-Mattila et al., 2009). Serial dilutions of the drugs were made in EB medium with 5% fetal bovine serum (FBS, see below).

4.12 Cardiac field potential analysis

4.12.1 Field potential averaging (II)

A MATLAB-based (Mathworks, Inc.) analysis program was programmed to obtain average FP complexes to calculate specific cardiac parameters from this average. Files were imported into the MATLAB-based, in-house programmed analysis program. The program had two peak detection algorithms from which the appropriate one can be selected to align individual FPs correctly for the calculation of the average FP. The files were analyzed with the algorithm that detected the

peaks correctly. Both algorithms have a tenth-order FIR low-pass filter with a cutoff frequency of 1 kHz.

The first peak detection algorithm is based on the QRS peak detection originally presented by Pan and Tompkins (Pan and Tompkins, 1985). The algorithm squares the signals after which the edges of the squared signal are detected. The peak is identified as the maximum of the original signal between the samples under these squares. The second peak detection algorithm applies first- and second-order derivatives of the signal to obtain the signals local maxima. The signals are divided into 0.05-second sections, and the local maxima within these sections are identified with the Matlab implementation, provided by Vargas Aguilera (<http://www.mathworks.com/matlabcentral/fileexchange/12275-extrema-mextrema2-m>). To be identified as a peak, the detected maxima must register above a threshold. This threshold is adaptive and is set to 90% of the maximum of the previous group.

A detection dead time option was provided to avoid detecting double peaks related to the same FP complex. Any peaks during the dead time were ignored. Detected peaks were later used to calculate an average complex as one activation cycle produced from the continuous FP signals. This was achieved by aligning the cardiac cycles by the detected peaks and normalizing them for averaging. This representative average was used to calculate the FPD. BR was calculated from the detected peaks of the original imported signal. To validate the algorithms for detecting the correct peaks in the imported recordings, we performed manual, user-operated peak detection and generated an FP average based on the detected points. We then compared the averages generated by the automatic peak detection algorithms to the averages based on manual user-operated peak detection. The mean square error (MSE, equation 2) was used to calculate the errors between the results found manually and those found automatically.

$$MSE = \sqrt{\frac{\sum_{i=0}^n (x'i - x''i)^2}{n}} \quad (2)$$

where x' is the automatically generated complex, x'' is the average complex based on the manual peak detection, and n is length of the complex. Analysis results from the program are saved automatically, and the options also permit creating a session for analyzing multiple files.

4.12.2 Noise attenuation (II)

Equation 3 presents the calculation of noise attenuation in decibels (dB), which served as the signal quality criterion.

$$A[dB] = 20 \log \left(\frac{V_{average}}{V_{signal}} \right) \quad (3)$$

Where $V_{average}$ is the root mean square (RMS) voltage of the averaged signal, and V_{signal} is the RMS voltage of the unaveraged signal. Noise attenuation (3) was calculated from periods of the averaged and unaveraged signals resulting from noise (i.e., without cardiac FP activation).

4.12.3 Mean field potential duration (II-IV)

In study II, the FPD was measured from three individual cardiac cycles of the original unaveraged recordings and the mean FPD and the standard deviation were calculated based on these values. In study III, cardiomyocytes from the two LQT2-hiPSC lines were performed similarly, so the data were combined. Data from cardiomyocytes from the three control hiPSC lines was combined for the same reason. In study IV we calculated the beating rate-corrected FPD (cFPD) using the Bazett formula (equation 4). The drug-induced prolongation of the cFPD for individual signals were calculated as relative changes from the baseline among the cell lines.

$$cFPD = \frac{FPD}{\sqrt{PPI}} \quad (4)$$

Where cFPD is the beating rate corrected FPD, FPD is the uncorrected field potential duration measured in milliseconds, and PPI is the peak-to-peak interval measured in seconds.

4.13 Statistical analyses (I-IV)

In study I, the statistical significance of the EB growth data was determined by paired samples t-test and the standard deviation (SD) for the differentiation

efficiency of each cell line was calculated between the differentiation efficiency percentages of each plate. In study II, data are presented as the mean \pm SD. In study III, data are given as the mean \pm SEM or SD. A comparison of patch clamp data between LQT2 cardiomyocytes and control cardiomyocytes was performed using Student's t-test for independent data. One-way analysis of variance followed by Tukey's test was used for comparison of multiple groups. The I_{K_r} data were assessed using Student's t-test for independent data. The difference in FPDs between different populations of spontaneously beating cardiomyocyte aggregates was determined by nonlinear regression analysis using R software. In study IV, data are given as the mean \pm SD and the differences in basal beating rate and rate-corrected FPD (cFPD) were determined using the t-test.

5. Results

5.1 Stem cell characteristics and pluripotency and cardiac marker expression (I, II, IV)

5.1.1 Human embryonic stem cells (I)

The hESC colonies had defined edges and the hESCs themselves had a round shape and a high nuclear-to-cytoplasm ratio. When investigating the pluripotency markers, we detected robust expression of the OCT3/4 pluripotency marker in undifferentiated hESCs. We also observed some sporadic Brachyury T⁺ cells in the hESC colonies (figure 9).

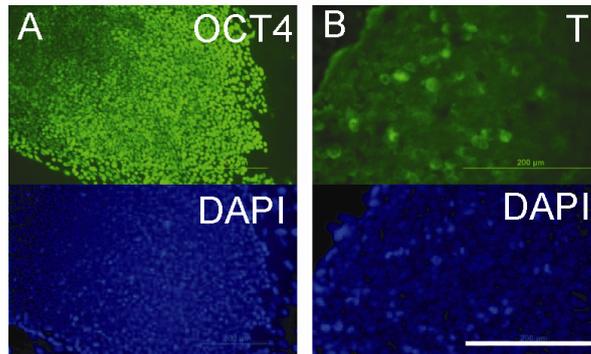


Figure 9. A) All of the human embryonic stem cell colonies had robust OCT3/4 protein expression (green). B) Infrequent brachyury T positive cells (green) were present in the human embryonic stem cell colonies. DAPI (blue) stained the DNA. Figure modified from study I (Pekkanen-Mattila et al., 2010b) supplementary figure 3. Scale bar: 200 μm .

5.1.2 Human induced pluripotent stem cells (III, IV)

The characteristics of our reprogrammed WT and LQT hiPSCs resembled those of normal hESC features. The hiPSCs had clear colony edges, a round shape and a high nuclear-to-cytoplasm ratio, as expected of *bona fide* pluripotent cells. A typical hiPSC colony is seen in figure 10A. The hiPSCs also expressed endogenous pluripotency marker mRNAs (studies III & IV), and their cardiac derivatives expressed the cardiac transcription factor NKX2.5 and the structural protein cTNT, as seen in figure 10B, as well as alpha-actinin and connexin 43 (Study III), as

normally expressed by differentiated cardiomyocytes. The retrovirally introduced transgenes were silenced in the hiPSCs by the sixth passage of the cell lines and these transgenes were not reactivated following cardiomyocyte differentiation (study III). All the pluripotency markers were also expressed at the protein level (studies III & IV). The karyotypes were normal in the hiPSCs as well, after the primary fibroblasts had been reprogrammed into the pluripotent state (study III).

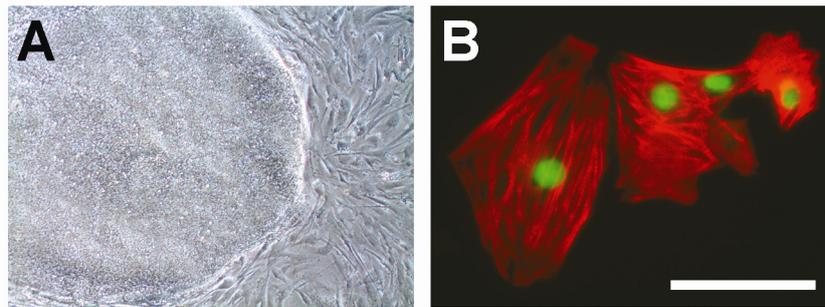


Figure 10. A) Phase contrast image of a colony of human induced pluripotent stem cells (hiPSCs) surrounded by mouse embryonic feeder cells. B) Dissociated single cardiomyocytes differentiated from hiPSCs expressed the NKX2.5 transcription factor (green in nuclei) and the structural cardiac troponin T protein (red), as detected by immunofluorescence. Scale bar: 100 μm for panel B.

With regard to their differentiation potential, the EBs from WT and LQT hiPSCs expressed the endodermal, ectodermal, and mesodermal germ layer markers that are expected of pluripotent cells (study III). The teratoma formation assay confirmed the pluripotency of the LQT2b-, WTa-, and WTb-hiPSC lines, as they resulted in the tissue formation of all germ layers (study III).

5.2 Embryoid body growth (I)

For differentiation of the hESCs, the FA method (Burrige et al., 2007) formed no viable EBs. Therefore, using the hESC Regea 06/015, Regea 06/040, HS181, HS293, HS346, HS360, HS362 and HS401 lines, we decided to initiate EB formation manually by cutting the hESC colonies with a scalpel. These manually cut EBs were allowed to form and differentiate on U-shaped, low attachment, 96-well plate wells for 7 days. In most cases, during this time, a thin layer of cellular outgrowth was observed in the cell culture wells. The initial EBs had a mean diameter of 444 μm (210 μm min., 847 μm max.) on day 1. One week later, the mean diameter had increased to 563 μm (139 μm min., 1,288 μm max.). Approximately one month after the EBs were transferred onto the 12-well plates, we noticed that the outgrowth from the EBs covered the bottom of the wells. The

expansion of the differentiating cells differed among the hESC lines. We also noticed differences in the densities of the outgrowths, and in some cases, cystic structures were evident in them as well. With regard to cardiomyocyte differentiation, only viable and well-growing EBs gave rise to spontaneously beating foci containing the differentiated cardiomyocytes.

5.3 Marker expression in developing embryoid bodies and their progeny (I)

To analyze how the different germ layer and cardiac markers were expressed in the developing EBs, we performed ICC staining to detect the expression of the following proteins: OCT3/4 for pluripotency, Brachyury T for the mesoderm layer, AFP for the endoderm layer, SOX1 for the ectoderm layer, NKX2.5 for cardiac cells, and Caspase-3 for apoptotic cells. We also performed additional western blot protein expression analyses for OCT3/4 and Brachyury T. Corresponding mRNA levels were assessed using qRT-PCR.

The pluripotent OCT3/4⁺ cells resided in the center of the EBs or on the edges, and their amounts reduced during differentiation. However, persistent OCT3/4⁺ cells were detected sporadically up to day 10 or 12. In general, all the hESC lines had similar expression patterns of the OCT3/4 protein. Our western blot experiments verified that OCT3/4 protein expression persisted up to day 20. However, on day 20, *OCT3/4* mRNA was not detectable.

Overall, we detected few Brachyury T positive cells in the EBs on days 4 to 12. Multiple positive cells were observed in only a few cases. Surprisingly, scarce Brachyury T⁺ mesodermal cells were detectable in the HS346 hESC line, which produced the most beating foci out the eight stem cell lines. The western blot experiments also verified the low expression of the Brachyury T protein. Brachyury T mRNA expression peaked in day 7 EBs and decreased steadily thereafter.

We detected AFP⁺ endodermal cells in 4-day-old and older EBs. These cells resided in the centers as well as the edges of the EBs. While the AFP proteins were irregularly expressed in some of the samples and more copiously in others, they were not systematically expressed in any of the hESC lines nor were they affected by the age of the EBs. AFP expression was non-overlapping with the endodermal SOX1 and mesodermal Brachyury T proteins. However, AFP and OCT3/4, which mark the pluripotent cells, overlapped in multiple EBs. In terms of mRNA expression, *AFP* was detectable on day 3 and peaked on day 20. In our own hESC

lines, the amount of *AFP* mRNA was higher in Regea 06/015 hESCs than in Regea 06/040 hESCs.

The earliest SOX1⁺ cells first appeared on day 4 in the center and on the edges of the EBs and most of the positive cells were detected from day 8 onward. The expression varied to some extent, however, depending on the hESC line. The strongest SOX1 expression was detected in HS362 and HS181 EBs. HS181 EBs had a few positive cells already on day 4. HS346 demonstrated a high cardiogenic potential, and SOX1⁺ cells were few in these EBs. SOX1⁺ expression did not overlap with any other germ layer markers or OCT3/4. The mRNA expression peaked at around day 20.

NKX2.5 transcription factor proteins, marking the developing cardiac cells, were detectable on day 4 in a few of the cells. The expression usually persisted until day 12, and all the hESC lines expressed NKX2.5 in a similar manner. NKX2.5 mRNA increased during the initial 7-day EB formation and peaked at around day 11.

Many cells in the developing EBs had fragmented nuclei, and we observed caspase-3 immunoreactivity throughout the experiment. In the beginning, the caspase-3⁺ apoptotic cells were located in the middle of the EBs, and in the later stages of EB development, caspase-3 reactivity diminished upon random cavity formation.

We also stained the excised beating foci with antibodies for the OCT3/4, Brachyury T, SOX1, AFP, cTNT, PAX6 and NKX2.5 proteins. We detected no OCT3/4 or SOX1 reactivity and only few Brachyury T⁺ cells in these older clusters. We observed discrete positive areas of AFP, cTNT and PAX6 in the beating foci and only spontaneously beating cells expressed the cardiac structural protein cTNT. AFP expression co-localized with cTNT in some of the EBs, and we observed AFP⁺ areas where cTNT was absent. PAX6⁺ ectodermal areas were non-overlapping with respect to AFP and cTNT. The cardiac NKX2.5⁺ areas were irregularly expressed and partly co-expressed cTNT. The number of the cells and the size of the excised beating foci differed considerably between the samples from tens to hundreds of cells. These spontaneously beating cells expressed striated patterns of the cardiac structural protein cTNT.

5.4 Efficiency of cardiac differentiation (I)

We initiated spontaneous EB differentiation by manually cutting the hESC colonies. To investigate approximately how well the hESC lines produced cardiac cells, we determined the efficiency of cardiac differentiation as the amount of formed beating

foci per plated EBs. These cardiac differentiation efficiencies varied from 0% to 12.5% across the hESC lines, with most lines having an efficiency of approximately 1 or 7%. The earliest spontaneously beating foci appeared on day 7. In most cases, we first detected the appearance of these foci at around day 14. The size and shape of the foci differed from 100 μm diameter circular areas to larger millimeter-scale areas in length with complex shapes and structures.

5.5 Cardiac field potential analysis (II)

5.5.1 Validity of the peak detection algorithms (II)

To analyze the electrophysiological cardiac MEA data, we developed automatic peak-detection algorithms that worked well in analyzing the cardiac FP traces. The averaged signals produced using the automatic peak detection were highly identical to those averaged signals that we obtained by manually selecting the correct peaks in the cardiac FP signals, and the root mean square (RMS) errors between the averages generated using the two methods were small. With regard to FPD, the errors were 0.13 ms using NRCs and 0.95 ms using hESC-CMs and with regard to amplitude they were 49 μV using NRCs and 79 μV using hEC-CMs. In addition to the small RMS error values, the proper functioning of the peak detection and averaging algorithms was demonstrated by the E-4031 drug tests, in which the alteration of the FP signal shape and FPD prolongation from the baseline was clearly detectable.

5.5.2 Noise attenuation in averaging (II)

Using our averaging method, we achieved approximately $-10^{1.2}$ dB noise attenuation in the cardiac FP signals when we applied 50 complexes for the average. This level of attenuation reduced the noise in the FP recordings to one-sixth of the initial level, reducing the noise sufficiently for the accurate analysis of the cardiac FP parameters.

5.5.3 Variation of cardiac field potential parameters (II)

5.5.3.1 Field potential duration and beating rate (II)

Overall, we noted greater FPD and BR parameter fluctuations with NRCs than with hESC-CMs, as seen in table II.

Table II. Relative fluctuations of field potential duration (FPD) and beating rate (BR) in neonatal rat cardiomyocytes (NRC) and human embryonic stem cell-derived cardiomyocytes (hESC-CMs, H7) during different parts of the microelectrode array recordings. σ : standard deviation.

<i>Population and parameter</i>	<i>Beginning (%)</i>	<i>σ (%)</i>	<i>Middle (%)</i>	<i>σ (%)</i>	<i>End (%)</i>	<i>σ (%)</i>
NRC FPD	0.6	16.2	-8.4	6.4	1.1	20.1
NRC BR	15.9	33.2	13.5	28.8	7.8	18.5
hESC-CM FPD	-3.9	8.3	-2.7	9.9	-7.1	10.1
hESC-CM BR	-6.5	0.6	-3.4	1.1	0.9	0.9

The BRs of different cardiomyocyte clusters differentiated from hESCs and hiPSCs had high deviations within some of the cell lines. The rate-corrected cFPDs also varied across and within all the CMs from the seven hPSC lines, but they did not differ significantly from each other between the different lines.

Table III. Beating rates (BR in beats per minute [bpm]) of cardiomyocytes differentiated from hESCs (H7) or from control or long QT syndrome (LQTS) type 1- or 2-specific human induced pluripotent stem cells (UTA lines) and their minimum and maximum values as measured from different cardiomyocyte clusters. σ : standard deviation.

<i>Stem cell line</i>	<i>BR (bpm)</i>	<i>σ (bpm)</i>	<i>Minimum (bpm)</i>	<i>Maximum (bpm)</i>
hESC-CM	74	20	48	101
WTa	95	81	13	199
WTb	91	33	59	153
LQT1a	61	47	29	177
LQT1b	86	67	23	160
LQT2a	57	20	28	78
LQT2b	79	38	47	155

5.5.3.2 Amplitude and peak-to-peak interval (II)

To further validate the averaging method used by our analysis program, we also investigated the relationship of the FP amplitude variation and PPI using scatter plots. Recordings with stable beating rhythms (i.e., consistent PPI) and consistent systolic amplitudes naturally had tightly grouped data points on the scatter plots, whereas those with a higher variability due to arrhythmic behavior had scattered

data points. Interestingly, even though the amplitude varied with PPI in the less stable recordings, the FPDs were still rather stable, allowing us to use, in some cases, the less stable FP recording for analysis.

5.5.4 Cardiac field potential durations (II-IV)

To test the reliability of the measured cardiac FPD values using our analysis program, we compared the mean NRC and hESC-CM FPD values measured from three individual cardiac FP cycles by hand to FPDs measured with the program. The FPDs measured using the program fell mostly within one SD and all within two SDs of the manually measured FPD values (study II).

To investigate the relationship between FPD and BR (as *in vitro* surrogates of QT interval and heart rate) in the control and LQTS2-specific cardiomyocytes (LQT2-CMs), we plotted these values on a scatter plot. We found that these two variables had an exponential negative correlation (study III) within the control CM and LQT2-CM populations. For the LQT2-CMs, however, this relationship was significantly more pronounced, particularly when the BR was low ($p=0.014$).

The mean cFPDs in study IV were 389 ms in hESC-CMs, 310 ms in WTa-CMs, 376 ms in WTb-CMs, 355 ms in LQT1a-CMs, 335 ms in LQT1b-CMs, 365 ms in LQT2a-CMs, and 374 ms in LQT2b-CMs.

5.6 Pharmacological responses of pluripotent stem cell-derived cardiomyocytes (II-IV)

To test the pharmacological responses of the hESC-CMs and control and LQTS patient-specific CMs we challenged them with multiple drugs while recording their electrophysiological responses with MEAs. From the recordings we calculated the cFPDs (i.e. beating rate corrected FPDs) as well as counted any incidences of adverse effects (EADs and pauses). The changes in cFPD prolongation were plotted in relation to the baseline values as Δ cFPD.

To investigate the β -adrenergic responses of the cardiomyocytes we exposed them to isoprenaline, which is a β -adrenergic agonist. All cardiomyocytes demonstrated increased chronotrophy upon an isoprenaline challenge which was seen as increased BR in FP recordings (studies III, IV). Isoprenaline increased the BR at 80 nM concentrations in all of the lines, but higher than 80 nM concentrations had no further BR increasing effect (study IV).

Erythromycin, a macrolide antibiotic, is known to prolong the QT interval in patients (Shaffer et al., 2002). Erythromycin prolonged the Δ cFPD in all of the cardiomyocytes. The magnitude of prolongation varied between ~10-40% among the lines. Controls had Δ cFPD prolongations of ~10-35% at maximal concentration. The highest prolongation (~40%) was seen with LQT2a-CMs at the lower therapeutic level. Between the LQT1-lines erythromycin prolonged Δ cFPD slightly more in the LQT1a-CMs, from the symptomatic patient, than in the LQT1b-CMs which were from the symptomatic patient. We did not observe any adverse effects in control or in LQT-CMs with erythromycin.

Cisapride is a serotonin 5-HT₄ receptor agonist that was used a gastrointestinal stimulant and an anti-emetic (Salvi et al., 2010). It was drawn from the market due to its tendency to induce TdP in patients (Darpö, 2001). We detected no adverse effects but among the lines cisapride caused Δ cFPD prolongations of 6-19% at the maximal test concentration. The controls had approximately 10% prolongation. The lowest Δ cFPD prolongation was seen with LQT2b-CMs and the highest with LQT1b-CMs.

Sotalol is both a Vaughan-Williams class III antiarrhythmic due to its tendency to block K⁺ channels and a β -adrenergic antagonist (Edvardsson et al., 1980). Sotalol caused Δ cFPD prolongation in all of the cardiomyocytes. Controls exhibited a 25-35% prolongation at the maximal concentration. The most pronounced effect was seen in LQT1b-CMs, which exhibited a 57% prolongation, whereas in the other lines the prolongations were more moderate. We also noted sotalol-induced EADs in some of the cardiomyocytes. These EADs were more numerous in the LQT2-CMs than in the LQT1-CMs. Notably, the LQT1b-CMs from the asymptomatic sibling did not present with any EADs even though they exhibited the highest Δ cFPD prolongation among the lines. In the controls we noted only transient EADs once in the WTb-CMs.

Quinidine, a class IA antiarrhythmic and multiple ion channel blocker that is known to trigger TdP (Bauman et al., 1984), caused Δ cFPD prolongation in all lines. Among controls the prolongation was approximately 25-40% at maximal concentration. The LQT-CM responses varied, but at maximal concentration the LQT2- and LQT1a-CMs had more prolonged Δ cFPD than the LQT1b-CMs from the asymptomatic patient. Quinidine also induced EADs and pauses in the cardiomyocytes. Figure 11 depicts a case of quinidine-induced EADs on LQT2-CMs. Pauses occurred in cardiomyocytes differentiated from all lines. The controls were more resistant to pauses than the LQT lines in that they exhibited pauses at higher concentrations. Quinidine-induced adverse effects were most prominent in the LQT1a-CMs from the symptomatic patient. These cardiomyocytes exhibited

EADs already at low concentrations (3 μM) and the number of EADs increased with escalating drug doses.

E-4031, the HERG K^+ channel blocker, prolonged the ΔcFPD in all cardiomyocytes. Especially in some hESC-CMs we saw massive prolongations. The WT-CM controls behaved similarly and had at most $\sim 40\%$ prolongation. The prolongation among LQT-CMs varied between $\sim 30\text{-}50\%$. Overall, the LQT1-CMs were more consistent between the two lines and had less prolonged ΔcFPD than the LQT2-CMs and the controls. E-4031 also caused adverse effects in LQT-CMs at lower concentrations than in control cardiomyocytes. Overall, the LQT2-CMs were more susceptible to adverse effects than the LQT1-CMs. Importantly, LQT1a-CMs from the symptomatic patient exhibited adverse effects at lower E-4031 concentrations than the LQT1b-CMs from the asymptomatic patient. E-4031-induced pauses and EADs were evident already at 10 nM and 300 nM concentrations, respectively, with the LQT1a-CMs, whereas in LQT1b-CMs these effects were recorded only at the highest (700 nM) concentration.

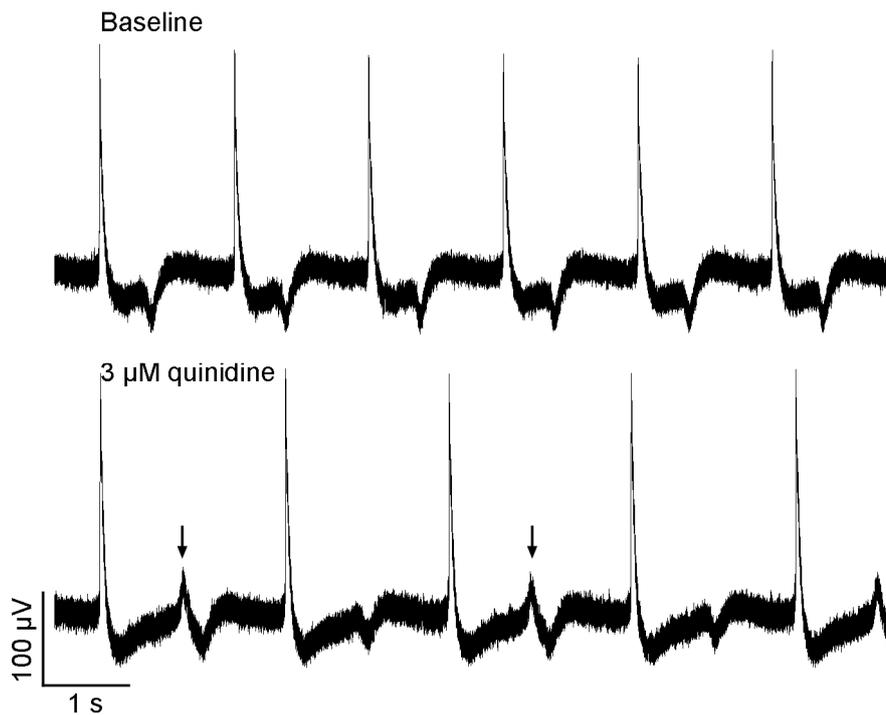


Figure 11. Quinidine-induced early after depolarizations (EADs) on long QT syndrome type 2–specific cardiomyocytes. The upper field potential trace recorded with a microelectrode array shows the baseline condition and the lower trace shows the effects of added 3 μM quinidine. The arrows mark the EADs.

5.7 Action potential and potassium current properties of long QT syndrome 2–specific cardiomyocytes (III)

To investigate the electrophysiological properties of single dissociated cardiomyocytes from the control and LQT2-specific hiPSC lines, we subjected them to patch clamp analysis. The differentiated and subsequently dissociated cardiomyocytes had either ventricular- or atrial-like cardiac AP properties according to their AP morphology. The ventricular-like APs had a clear phase 2 plateau, whereas the atrial-like APs were more triangular in shape. The majority of the investigated AP parameters had non-significant differences between control and LQT2 CMs ($p > 0.05$). Importantly, however, in a subset of beating rate-matched cardiomyocytes, we noted a significantly prolonged AP duration (APD) at 50% and 90% repolarization (APD₅₀ and APD₉₀, respectively) in the ventricular-like APs of LQT2-CMs versus control cardiomyocytes, as shown in figure 12 ($p < 0.001$). These values were non-significant between the atrial-like cardiomyocytes. APD₉₀ had a mean value of 516 ms in LQT2-CMs, 310 ms in control hiPSC-CMs, and 338 ms in hESC-CMs, and these values were non-significant between the different LQT2-CM groups as well as between all the control cardiomyocyte groups. Spontaneous EADs were observed in just one out of the 20 patch-clamped LQT-CMs and in none of the control cardiomyocytes.

To study the current properties of cardiomyocytes with ventricular-like APs using the voltage clamp mode of patch clamp, we isolated the I_{Kr} current, mediated by the hERG channels, by blocking it with E-4031. The isolation of the I_{Kr} current revealed that it was noticeably reduced in the LQT2-CMs compared to the control cardiomyocytes. We noted a significant decrease of 40-46% in the tail I_{Kr} density upon depolarization of the cells from 0 to +40 mV ($p < 0.01$) during I_{Kr} blockade by E-4031 as well as by isotonic cesium conditions (Zhang, 2006). A patch clamp ramp protocol also illustrated the role of reduced I_{Kr} in the LQT2-CMs.

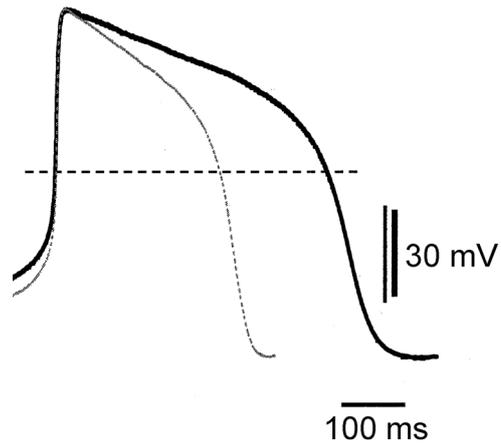


Figure 12. Action potential properties of a ventricular-like control cardiomyocyte (shorter dashed line) and a long QT syndrome type 2 (LQT2)-specific cardiomyocyte (longer solid line). The control cardiomyocytes were differentiated from induced pluripotent stem cells (hiPSCs) derived from fibroblasts obtained from a healthy donor and the long QT type 2 cardiomyocytes were derived from hiPSCs obtained from a LQT2 patient with a defined ion channel mutation.

6. Discussion

6.1 Embryoid body development (I)

We aimed to see if there were any differences between the eight hESC lines in germ layer marker organization or in the temporal sequence of expression during EB development and whether such patterns might help in predicting the cardiomyogenic potential of the hESC lines we studied.

6.1.1 Forced aggregation and embryoid body size (I)

The FA method has been reported to increase cardiac differentiation in the HUES-7 stem cell line by 13-fold when activin A and bFGF were also added (Burrige et al., 2007). Since in our study the FA method failed to produce any viable EBs, we focused our analysis to include only the mechanically excised EBs. The initial size of the EB is known to affect cardiomyocyte differentiation potential (Bauwens et al., 2008; Mohr et al., 2010), and hESCs are a rare cell type that undergo dissociation-induced apoptosis as single cells (Amit et al., 2000) (a phenomenon known as anoikis). This can be counteracted to some extent using Rho-associated, coiled-coil containing protein kinase (ROCK) inhibitor during dissociation (Watanabe et al., 2007), which protects the cells against dissociation-induced apoptosis. Other factors that may have affected the relative inefficiency of the FA method might have included the possible detrimental effects of the centrifugal forces on the cells during the hESC plating phase and the fact that these hESCs were not adapted to handle dissociation, as they were passaged mechanically as a cell line.

In the EB initiation phase, the size of the cut hESC colony pieces by mechanical excision was kept as constant as possible. The success of this strategy was corroborated by the fact that in the initial stage (i.e., on day 1), differences between EB sizes were non-significant. A week after the spontaneous EB differentiation was initiated, the average areas of the EBs from all of the hESC lines were significantly larger than on day one. The size and shape of the differentiating EBs varied widely, and no clear protein expression patterns occurred in early stage EBs. This might be, in part, due to differences in the diffusive transport of biochemicals between the

EBs, which was previously observed (Sachlos and Auguste, 2008). Nonetheless, we observed expression of every germ layer marker in the 12-day period.

6.1.2 Marker expression (I)

Compared to the first 12 days of differentiation, the month-old EB progeny had a more defined arrangement, with clear and separate PAX6⁺, AFP⁺, and cTNT⁺ areas. Interestingly, the mRNA levels of the pluripotency marker *OCT3/4* declined faster than the protein expression levels, which persisted up to 20 days. The *OCT3/4* gene expression results were consistent with previous observations (Adewumi et al., 2007), and our results suggest lower turnover rates for OCT3/4 protein than mRNA.

AFP mRNA expression in the EBs was also in agreement with earlier reports (Bettioli et al., 2007). The protein was expressed rather abundantly in the inner and outer parts of the EBs in the first 12 days. AFP⁺ areas have previously been reported in the peripheral sections of early EBs or in the inner EB elements in later stages (Conley et al., 2004; Itskovitz-Eldor et al., 2000; Kim et al., 2007). The co-localization of AFP and OCT3/4 suggested that some of the differentiated endodermal cells expressed OCT3/4 TF. Indeed, mouse primitive endodermal cells have been previously shown to express OCT4 protein (Palmieri et al., 1994). After a month of spontaneous EB differentiation, we detected co-localization of the endodermal AFP with the cardiac structural protein cTNT. Because endodermal cells are capable of inducing cardiac differentiation in hESCs (Mummery et al., 2003), this raises the possibility that endodermal areas might coax the formation of cardiomyocytes in the developing EBs as well.

Ectodermal SOX1 was differentially expressed in the developing EBs among different hESC lines. SOX1 was more abundantly expressed in lines that had poor cardiac differentiation. Interestingly, the hESC lines with higher SOX1 expression have previously been shown to have a good neurogenic capacity (Lappalainen et al., 2010).

Brachyury T is an early mesodermal marker. *Brachyury T* mRNA expression peaked on day 7 in our EBs but earlier reports have placed this peak around the third day of differentiation (Bettioli et al., 2007; Graichen et al., 2008; Pekkanen-Mattila et al., 2009). The protein expression was scant in all EBs, and there was no clear link with the hESC lines' cardiomyogenic potential and Brachyury T expression. The hESC line that produced the most cardiomyocytes (HS346) did not have many Brachyury T⁺ areas. This observation contrasts with some previous studies (Kim et al., 2007; Zhang et al., 2009). However, the spontaneous formation of beating foci was relatively low among all the hESC lines we studied (12% maximum),

suggesting that they have a naturally low occurrence of mesodermal derivatives. This possibility was further reinforced by a previous observation that prolonged *Brachyury T* expression in combination with *OCT3/4* might be indicative of an absence of endo- and mesodermal derivatives (Bettioli et al., 2007).

The expression profile of the early cardiac marker NKX2.5 in the EBs was in harmony with previous reports (Graichen et al., 2008; Hsiao et al., 2008), but previous observations on the cardiomyogenic propensity and *NKX2.5* mRNA expression (Osafune et al., 2008; Pekkanen-Mattila et al., 2009) did not translate to the protein level in our study. In early-phase (days 4-12) EBs, NKX2.5 protein expression was random and in month-old structures it overlapped with cTNT only partially. Previously it was shown that Nkx2.5 is expressed in mESC-derived non-cardiac cells as well (Hsiao et al., 2008), supporting the observation that not all cells expressing NKX2.5 are necessarily cardiomyocytes.

Caspase-3 is involved in apoptosis and is critical for normal development (Jacobson et al., 1997). In mESCs, this protein has also been shown to turn off Nanog expression (Fujita et al., 2008). We performed staining for this protein after noticing many fragmented nuclei in the histological sections of EBs. Overall, caspase-3-positive cells were observed from the second day onward, which was in accordance with previous observations with mESC EBs (Joza et al., 2001). Cavity formation was detectable on the fourth day and previous reports have also noted cavity formation in early-stage hESC EBs (Conley et al., 2004). Thus, our results recapitulated the previous observations on caspase-3 in developing EBs.

Overall we could not detect any major differences among the lines in their marker expression pattern. The EB development and the spatiotemporal expression of different markers were comparable across the eight hESC lines. All of the markers were detectable but had no conclusive expression trajectories. Older (month-old) structures had specialized regions and a distinct localization of the germ layers. However, predicting any cardiogenic potential of the hESC lines based on early phase marker localization during the first 12 days of development was inconclusive, most likely due to the time frame involved in the formation of distinct regions in the spontaneously differentiating EBs.

6.2 Cardiac field potential averaging (II)

Alongside the well-established patch clamp technique (Hamill et al., 1981; Sakmann and Neher, 1984), the MEA platform is gaining ground as a means to investigate cardiac electrophysiology *in vitro*. While patch clamp is an excellent method to

study specific ion current properties of single cardiomyocytes, for example, it requires a lot of training and is tedious to perform. Further, some experimental settings may require cardiomyocyte coupling via gap junction proteins such as connexin 43 to tease out a proper phenotype. MEAs may assist in determining cardiac parameters such as the duration of repolarization in three-dimensional cardiac syncytia. Therefore, one application could be to study drug effects with hPSC-CMs for possible adverse effects (Braam et al., 2010; Caspi et al., 2009).

For accurate analysis of the cardiac FP parameters recorded with MEAs, high-quality signals are needed because low SNR leads to reduced signal quality. Especially with hESC-CMs, the low SNR may be a consequence of the potentially low amount of cardiomyocytes in a given spontaneously beating cluster. Low cardiomyocyte amounts lead to weaker FP signals in MEA recordings. The quality of these lower quality signals can be improved by averaging multiple individual cardiac FP cycles from a single recording to provide a representative higher quality cardiac FP cycle. These averaged signals provide a more accurate basis for analysis of FP parameters.

Our averaging program, which was based on Matlab software, was able to detect the correct cardiac FP peaks, align these peaks on top of each other, and generate a representative average cardiac FP complex from a single MEA recording. We were able to accomplish this using both of the two algorithms that were available for peak detection in the program. Correct peak detection by the program was validated by comparing the averages generated using the algorithms to averages generated based on manual peak selection. Due to the diverse nature of the cardiac FP traces, different approaches had to be implemented for peak detection. Therefore, for each recording, the algorithm that detected the peaks correctly was selected.

Two NRC signals demonstrated arrhythmic behavior, which was seen as a variation in the PPI (or BR). The irregular beating rhythm obstructed accurate PPI calculations, but surprisingly the FPD remained quite stable and could subsequently be determined from the averaged signal.

Noise attenuation in the cardiac FP signals presents an interesting conundrum. On one hand, the more cardiac FP cycles that are included in the average, the less there is noise. On the other hand, when more of these cycles are used for the average, there is a greater potential for a misalignment of the peaks, thus resulting in a distorted average. In our experiments, we found that including approximately 50 cardiac FP cycles generates a high-quality average without compromising the integrity. Fewer cycles might suffice as well, however, because the noise reduction varies case by case. On the same note, we found that the NRCs showed greater a variation of FPD and BR in different parts of the recording than hESC-CMs. The

greater the variation of these parameters, the higher the chance of introducing alignment errors in the average, based on the whole recording period. Interestingly, we noted that the individual cardiac FP cycles retained rather stable duration (FPD), making it feasible to produce the averages. Overall, the FPD and BR values of the averages from the different parts of the recordings were sufficiently close to those of the averages from whole recordings.

When the amplitude of the signals was compared to the PPI values, we noticed that most of the signals had quite stable dynamics. There was some variation in a few of the signals, and when the PPI increased, the amplitude decreased. Comparing the cardiac FP cycles and their FPD in arrhythmic recordings showed little variation compared to the average FP complex. The first cardiac FP cycles in the arrhythmic “bursts” had a slightly prolonged FPD, but these first cycles are automatically in the minority and thus skew the average complex negligibly.

When the FPDs of averaged signals were compared to averages measured from three individual cycles by hand from the same MEA recording, we noticed that the values were close to each other (mostly within one SD, all within two SDs). Additionally, the sensitivity of averaging algorithms was proven by the clear detection of an altered FP signal shape upon exposure to a pharmacological agent, thus validating the peak detection and averaging algorithms of the program as a basis for proper cardiac FP analysis.

In summary, the averaging method can be reliably used in certain conditions to analyze cardiac FP properties of otherwise hard-to-interpret, low-SNR MEA signals. In particular, the comparison of different conditions (e.g., in drug tests) by signal averaging might present a useful opportunity. Creating algorithms for the automatic online detection and recording of FPD based on this technology might be beneficial, as the MEA platform has been previously used in hESC-CM drug tests (Braam et al., 2010; Liang et al., 2010). However, automatic FPD algorithms are harder to implement than amplitude or PPI measurements, as the signal shape with hESC-CMs varies and the FPD end-point therefore often depends on interpretation.

6.3 Long QT syndrome modeling (III, IV)

6.3.1 Long QT syndrome type 1 (IV)

The KCNQ1-FinA founder mutation lies in the C-terminal end of the K^+ channel and likely renders its assembly domain, which is needed to form the $K_{v7.1}$ channel,

dysfunctional (Piippo et al., 2001). This effect leads to LQTS by obstructing the K^+ flux in cardiomyocytes. Hence, this mutation may obstruct the K^+ channels' ability to form functional tetramers and thus mediate the I_{Ks} current. Patients with the KCNQ1-FinA mutation have a mean QTc of 462 ms (Marjamaa et al., 2009a). Our symptomatic patient had a QTc time of 456 ms and the asymptomatic patient had a QTc time of 426 ms. The mean basal cFPD times in the MEA recordings were 335 ms and 365 ms (for the symptomatic and asymptomatic, respectively). In the first LQT1 paper by Moretti and colleagues, the AP_{90} was increased by half in R190Q *KCNQ1*-mutated cardiomyocytes, whereas the ECG only revealed up to 15% QT prolongation (Moretti et al., 2010b). In our MEA drug tests with the KCNQ1-FinA mutant LQT1-CMs, we noted more adverse effects in the LQT1a-CMs derived from the only symptomatic patient in these studies. The LQT1b-CMs derived from the asymptomatic sibling had less EADs and pauses and at higher drug concentrations than the LQT1a-CMs.

6.3.2 Long QT syndrome type 2 (III, IV)

hERG-FinB is another Finnish LQTS founder mutation (Fodstad et al., 2004) that predisposes individual to LQT2. Most of these mutation carriers in the Finnish population are asymptomatic, but mutations in hERG have also been associated with sudden cardiac death (Tester and Ackerman, 2007; Tu et al., 2011). The LQT2-patients carrying hERG-FinB have QTc times of 448 ms on average, which have been documented to be as high as 596 ms. Non-carriers typically have average QTc times of 416 ms (Fodstad et al., 2006).

The patient from whom we obtained the skin biopsy had a latent LQT2 mutation. Immediate relatives carrying the same mutation had, however, suffered from palpitations, syncope, and even sudden cardiac death triggered by auditory stimuli during sleep, all of which are typical symptoms of LQT2 (Roden, 2008; Schwartz, 2001).

We aimed to recapitulate the LQTS2 phenotype *in vitro* for disease modeling purposes. Indeed, the cardiomyocytes differentiated from hiPSCs reprogrammed from fibroblasts of a LQTS2 patient exhibited prolonged cardiac repolarization. This was observed in both the patch clamp experiment and the MEA recordings. The MEA recordings also revealed that the prolongation was more pronounced at slow beating rates, which corresponds to the clinical observations of LQT2 where QT-interval prolongation is observed at slower heart rates (Swan et al., 1999). This prolongation effect was likely due to decreased I_{Kr} current caused by the R176W (hERG-FinB) missense mutation in *hERG*, as observed in voltage clamp recordings.

6.3.2.1 *Rapid delayed rectifier current properties (III)*

In heterologous expression systems, hERG-FinB reduces the tail current density by approximately 75%, and wild-type (WT) hERG co-expression blocks this effect (Fodstad et al., 2006). The I_{Kr} current density in hERG-FinB cardiomyocytes may be modified by an interaction with misshapen-like kinase (MINK)-related peptide (MiRP) subunits (Abbott et al., 1999) and the co-expression of endogenous hERG1a and hERG1b isoforms. We noted a 43% reduction in the hERG current in our LQTS2-CMs.

hERG-FinB does not have a dominant-negative effect like the *hERG* A614V missense mutation (Nakajima et al., 1998), which another reported case of patient-specific hiPSC-derived LQT2-CMs possessed (Itzhaki et al., 2011). The A614V LQTS2 hiPSCs reported in this study were reprogrammed from fibroblasts obtained from a highly symptomatic patient who had documented episodes of TdP. *In vitro* the A614V mutation caused a more severe decrease in I_{Kr} current density (-72% at 0 mV membrane potential and -64% at +20 mV) in the LQT2-CMs. The corresponding values in our study were -43% and -40%. These A614V cardiomyocytes also had more prolonged APD₉₀ (+200% compared to control cardiomyocytes) than our hERG-FinB cardiomyocytes (+166%) at a 1Hz (60 pbm) beating frequency. The patch clamp recordings also revealed the A614V cardiomyocytes to have EADs in 66% of cases as opposed to 5% (only one cell) in our study. These results, and the fact that the A614V *hERG* mutation has a dominant-negative effect, imply that the severity of the mutation is well represented by the *in vitro* LQT2 model. Matsa and colleagues have also recapitulated the LQT2 phenotype with G1681A-mutated cardiomyocytes using patient-specific hiPSCs. The patient in this study had a prolonged QTc interval of up to 571 ms and also suffered from arrhythmic episodes. Interestingly, the G1681A LQT2-CMs did not demonstrate any spontaneous arrhythmias. Such episodes were nonetheless triggered by β -adrenergic stimulation with isoproterenol. With the exception of APD, other AP properties were comparable to those reported for WT cardiomyocytes in other studies (Gai et al., 2009; Yokoo et al., 2009; Zwi et al., 2009a).

6.3.2.2 *Genotype-phenotype relationship (III)*

The concept of repolarization reserve (Roden, 1998) states that, in cardiomyocytes, there are redundant mechanisms that can bring about normal repolarization at times of stress, such as during a pharmacological challenge (Couderc et al., 2009;

Kannankeril et al., 2005). A full-scale LQT2 phenotype might, therefore, require compromising multiple ion channel targets. Interestingly, we were able to show that the LQT2-CMs generated more adverse effects in terms of EADs than controls when these cells were exposed to certain drugs. Sotalol caused arrhythmias only in LQT2-CMs at a therapeutic (ETCP_{unbound}) range. Sotalol has indeed been known to trigger TdP in *hERG* mutation carriers (Couderc et al., 2009; Lehtonen et al., 2007), most likely by intensifying the K⁺ efflux deficiency because it blocks the hERG WT and variant hERG channel alike (Männikkö et al., 2010). Blocking the I_{Kr} current with E-4031 caused EADs in both LQT2-CMs and controls.

6.3.2.3 *Potential mitigating effects of cardiomyocyte coupling (III)*

Interestingly, comparing the relationship between single-cell patch-clamp recordings, MEA recordings and clinical ECG recordings revealed some interesting discordances. The single-cell recordings indicated prolongative differences of up to 66% in APD₉₀ of LQT2-CMs versus the control cardiomyocytes. However, the FPDs in the MEA recordings differed by a maximum of only 20% between the groups and thus resembled more of the differences in clinical ECG data from LQT2 patients and healthy individuals (Fodstad et al., 2006). The two previous LQT2-CM studies also reported an agreement between ECG and MEA data (Itzhaki et al., 2011; Matsa et al., 2011) as well as more prolonged (up to 2.5 fold) repolarization in single cells. This led us to postulate that there may be a compensatory mechanism in the cardiac syncytia.

6.3.3 Drug testing with long QT syndrome cardiomyocytes (IV)

The hiPSC-CMs had more deviation in the BR than the hESC-CMs. Within all of the lines, however, isoprenaline increased the BR and showed a saturating effect after 80 nM. This observation was in accordance with previous studies from our group, as this concentration has previously been shown to increase the BR in hESC-CMs (Pekkanen-Mattila et al., 2009).

The LQT1-CMs had a more prolonged repolarization than controls when exposed to higher than clinically relevant concentrations of cisapride. This might suggest that LQT1-CMs are more vulnerable to hERG block than control CMs. Previous studies have reported approximately 20% relative cFPD prolongation in hESC-CMs at 100 nM, which is in the therapeutic range, (Caspi et al., 2009) and up to 200% at higher than therapeutic concentrations (Braam et al., 2010). The effects

of erythromycin on the prolongation of Δ CFPD varied among the cell lines in our study, but nonetheless caused prolongation in all of them, which was expected as it has led to QT prolongation (Shaffer et al., 2002). The 19-24% Δ CFPD prolongation in hESC-CMs at 6-10 μ M sotalol concentrations was in accordance with previous reports of 20% prolongation in hESC-CMs at 1.8-14 μ M (Braam et al., 2010) and up to 60% prolongations with concentrations up to 200-400 μ M (Caspi et al., 2009). Exposing the cardiomyocytes to quinidine resulted in bigger prolongations in all lines even at the therapeutically relevant concentrations. Previously published prolongation values for hESC-CMs vary between 20 and 50% (Braam et al., 2010; Caspi et al., 2009), which corresponds to the values observed in our study. Caspi and colleagues have previously reported Δ CFPD prolongation of approximately 30% for hESC-CMs with 8 μ M quinidine exposure, which is similar to our results. The hERG channel blocker E-4031 prolonged repolarization in all lines and also caused adverse effects. Braam and colleagues have also previously reported EADs at micromolar E-4031 concentrations (Braam et al., 2010), which is in line with our results from hESC-CMs, although we noted EADs and pauses at concentrations as low as 10 nM and 100 nM in LQT-CMs.

The difference in the beating rates of hPSC-CMs may reflect the differences related to atrial and ventricular CMs, with the fast-beating CMs being more atrial-like (Asp et al., 2010). We noted a high variability in the BR of the spontaneously beating control and LQT1 and -2-CMs. Taking into consideration that these CM subtypes have different ion channel compositions, the drug responses in our studies might reflect the atrial versus ventricular compositions in the beating foci and the responses of different cardiomyocyte subtypes to the same drug. The hESC-CMs had a more consistent and physiological beating rate and therefore were in agreement with previously published reports. These subtype- and mutation-dependent drug responses might be affected by the differences in their repolarization reserve (Roden, 1998), which might, along with the cardiomyocyte subtype variation, explain these different drug responses. The challenge in this system is to differentiate enough CMs from the patient-specific hiPSC lines to choose the beating foci with an appropriate BR.

6.3.4 Outlook for patient-specific long QT syndrome cardiomyocytes in research (III, IV)

As stated previously, most of the LQTS mutation carriers are unaware of their mutation (i.e., they have latent LQT2). The two previous LQT2 *in vitro* models were generated from highly symptomatic patients whereas in our study the patient had a

mild LQT2. While the studies by Itzhaki and colleagues and Matsa and colleagues highlight the successful creation of an *in vitro* model, it might be more practical to study *in vitro* models of mild or even latent LQT2; this might represent the general LQT2 patient population more accurately and, according to our results, this phenotype can also be teased out *in vitro*. Finland is in advantageous position in the sense that we can potentially collect a large amount of data on the effects of one mutation type in multiple patients because of the founder mutation effect. This should help in determining the putative modifiers of the LQTS phenotype. It will also be interesting to study drug effects on latent LQTS to see if particular drugs might have more deleterious effects in combination with the mutation.

In conclusion, hiPSC-derived cardiomyocytes from an LQT2 patient displayed the disease cardiac phenotype in cell culture conditions even though the patient was relatively asymptomatic. This model provides an additional platform to study the basic pathology of LQTS and to individualize drug treatment in a patient-specific manner. It also provides the means to explore the differences between clinical patients and mutation carriers and to scan the cardiac effects of different drugs on both.

Taken together, the results from our studies indicate that patient-specific LQT-CMs derived from LQT1 and -2 patients recapitulate the phenotype *in vitro*, namely, delayed cardiac repolarization, and that these patient-specific CMs can likely, upon refinement, be used to generate robust cellular human *in vitro* models for drug testing. Drug testing with LQTS-CMs generated from donors with mild or latent LQTS could be beneficial, especially in the Finnish population, as many mutation carriers are unaware that they are carriers for these mutations with a subclinical phenotype. Consequently, the patients may experience a severe adverse reaction only after being prescribed a drug that is potentially life-threatening for LQTS patients.

However, there are still challenges related to this hiPSC strategy. One challenge is that while we can generate patient-specific hiPSC lines rather easily, the validation of these lines and the pluripotency assays are still very time-consuming, thus limiting the time frame for downstream applications such as drug testing. Another challenge is providing proper controls. Ideally, the best control would have the same genetic background as the patient-specific hiPSc line. To this end, the genetic mutation in the patient-specific hiPSC line could be corrected using gene technology, or specific mutations could be introduced to WT hiPSC or hESC lines. This is however also very time-consuming and requires expertise and validation of the cells lines and their genetics.

6.4 Future perspectives

The ultimate goals for hPSC applications are in regenerative medicine, disease modeling and preclinical drug testing. All of these goals present crucial steps in bringing these fields to the next level for more accurate treatments and for safer, efficacious drugs. While academia can often provide the basic proof-of-concept studies for these applications, the full-scale realization of these goals often requires investing a large amount of capital. Close-knit, multidisciplinary academia-industry collaborations are likely to be important for success. These goals require plenty of hard work and large financial investments but promise to produce immense medical advancements and possibly relative economical savings.

While hiPSCs can be a reliable source of material for disease modeling and pharmacotoxicological applications, the currently widely used and successful reprogramming strategy developed by Dr. Yamanaka's research group (Takahashi et al., 2007) curb their use in applications requiring transplantation into a host due to retroviral integration into the recipient genome. This is of concern because, at least in mouse iPSC (miPSC), the differentiated secondary neurospheres (SNS) were found to cause teratomas upon transplantation into mice. The teratoma-forming propensity of SNS varied depending on the origin of tissue from which the miPSCs were derived, and the highest propensity was with miPSCs reprogrammed from mouse tail-tip fibroblasts (Miura et al., 2009). In addition, the time frame of deriving hiPSCs and their subsequent differentiation into functional, specialized cells might be too long for some acute conditions like myocardial infarction (MI). To this end, direct reprogramming of dermal fibroblasts into specialized subtypes, for example, might overcome this issue. Direct reprogramming of mouse fibroblasts into induced cardiomyocytes (iCMs) using *Gata4*, *Mef2c*, and *Tbx5* transgenes has already been reported (Ieda et al., 2010), and this work might lay the foundation for future work aimed for regenerative medicine applications in MI. Currently, however, in the MI treatment context, this strategy suffers from the same retroviral integration issues as the hiPSC approach.

All of these aforementioned examples of current hPSC research fall under the concepts of human spare parts or novel disease models and preclinical pharmaceutical testing. This early progress provides cautious optimism for new treatment options and improved discovery of novel drugs for patients suffering from currently difficult-to-treat diseases. Certainly there is great anticipation of progress in regenerative medicine taking place perhaps even within the next decade. Only time will tell whether the regenerative medicine applications become a reality, but,

at the least, important initial advances have been made in disease modeling and drug testing.

7. Conclusions

The aim of this thesis was to study the EB differentiation of hESCs and their cardiomyocyte derivatives and to study the electrophysiological properties and pharmacological responses of the hPSC-derived cardiomyocytes. Based on the four studies presented here the following conclusions can be made:

- hESCs were able to differentiate into functional, spontaneously beating cardiomyocytes, but did so with different propensities depending on the hESC line. During the cardiac differentiation they expressed endo-, meso-, and ectodermal as well as cardiac protein markers in specific regions of the embryoid bodies.
- Spontaneously beating hESC-CM aggregates had field potential properties that differed from those of NRC monolayers due to their three-dimensional nature. Using our custom-made analysis program, the hESC-CM and NRC field potentials could be averaged for more accurate analysis of their properties, such as field potential duration and peak-to-peak interval, by generating higher quality cardiac FP signals.
- Using hiPSC technology, we established LQT1- and LQT2-specific stem cell lines, and the cardiomyocytes derived from them harbored the genetic mutations affecting their electrophysiological properties. The LQT2-cardiomyocytes recapitulated the disease phenotype *in vitro*, and this was observed as prolonged cardiac repolarization.
- The cardioactive effects of different drugs could be investigated using the hiPSC-derived control and LQTS cardiomyocytes. The LQT1- and LQT2-cardiomyocytes had more of adverse drug effects than controls. Thus, these cells can be used as *in vitro* models of LQTS and they can perhaps be used to tailor medication for individual use.

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"Nothing in biology makes sense except in the light of evolution."
-Theodosius Dobzhansky

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