Master's thesis

Novel Small Molecule in Differentiation of hiPSC Derived Cardiomyocytes and Single Cell Beating Analysis

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Cell and Molecule Biology

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PREFACE

This study was performed at BioMediTech in Tampere (Finland) with the heart group in the fall of 2015 and spring of 2016. I had the opportunity to work with human induced pluripotent stem cells to differentiate them into cardiomyocytes under the supervision of the experienced professionals working in the heart group.

I would like to thank my supervisor, Mari Pekkanen-Mattila, for her patience and guidance in the task of introducing me to the world of hiPSCs and cardiomyocyte research. I also want to thank our group leader Katriina Aalto-Setälä for accepting me into the group, Marisa Ojala for good advice, and all my fellow students for their support and friendship. Lastly, I thank Henna Lappi and Markus Haponen, for their expertise in cell culture, and BioMediTech for the coffee, facilities, and for making this thesis possible.

Thanks also to the department of biological and environmental science of the University of Jyväskylä and its excellent professors, who have enabled my 5-year-long studies, which are coming to a conclusion with the completion of this thesis. Thanks to your teachings I was well equipped to face the challenges of this project.

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Faculty of Mathematics and Science

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Title of Thesis: Novel Small Molecule in Differentiation of hiPSC Derived Cardiomyocytes and Single Cell Beating Analysis

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Abstract:

Cardiac disease remains the leading cause of death in the world. Therefore, the need for patient specific drug testing models, as well as tissue regeneration, are hot topics in medicine. The modern technology is still unable to efficiently produce pure cultures of human induced stem cell (hiPSC) derived cardiomyocytes, which are needed for several purposes including regenerative medicine and reliable drug testing for cardiac disease. Further development of hiPSC derived cardiomyocyte differentiation and characterization techniques is required. The objective of this experiment was to further characterize the differentiation of hiPSC derived cardiomyocytes obtained from patients and to test the effects of a novel putative cardiac differentiation enhancing small molecule (PCDE), on the efficiency of differentiation. The hypothesis was that the small molecule would increase the differentiation efficiency. The efficiency was tested with flow cytometry using a cardiac marker, Troponin T, and the cardiomyocytes were characterized by immunostaining and quantitative polymerase chain reaction (qPCR). Gene expression was analyzed from RNA samples collected from different time points of differentiation. During the experiment video-based beating analysis, a robust technique that utilizes a computer program to characterize beating patterns of single cardiomyocytes, was evaluated as a technique. Based on all results PCDE failed to enhance the differentiation efficiency of hiPSC derived cardiomyocytes and may have reduced the expression of cardiac markers. However, we found that the video based beating analysis could be used in combination with single cell PCR to better characterize cardiomyocytes.

Keywords: hiPSC, cardiomyocyte, stem cell, differentiation, video-based beating analysis, quantitative polymerase chain reaction, flow cytometry, single cell, characterization, small molecule

Jyväskylän Yliopisto **Pro Gradu –tutkielman tiivistelmä**

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Tiivistelmä:

Sydäntaudit ovat johtava kuolinsyy maailmanlaajuisesti, ja siksi potilasspesifisien lääketestausmallien sekä kudosregeneraation tutkimus on ajankohtainen aihe. Nykyinen teknologia ei pysty vielä tuottamaan suuria määriä puhtaita ihmisen indusoiduista pluripotenteista kantasoluista (hiPSC) erilaistettuja sydänsoluja tarpeeksi tehokkaasti, jotta niitä voitaisiin käyttää regeneratiivisessa lääketieteessä ja luotettavammassa lääketestauksessa. Sydänsolujen tuottamista ja karakterisointia täytyy kehittää. Tämän tutkimuksen tavoite oli jatkaa potilasspesifisten hiPSC sydänsolujen erilaistamisen karakterisoimista ja testata oletetun sydänsolujen erilaistusta tehostavan pienmolekyylin (PCDE) vaikutusta erilaistamisen tehokkuuteen. Hypoteesi oli, että kyseinen pienmolekyyli parantaisi erilaistustehokkuutta. Erilaistustehokkuutta mitattiin virtaussytometrisesti sydänmarkkerilla, Troponiin T:llä, ja kardiomyosiitit karakterisoitiin immunovärjäyksillä sekä kvantitatiivisen polymeraasiketjureaktion (qPCR) avulla. Geeniekspressiota analysoitiin RNA-näytteistä erilaistuksen eri vaiheista. Tutkimuksen aikana arvioitiin tekniikkana videopohjaista yksittäisten solujen sykeanalyysia, joka on tietokoneohjelmaan pohjautuva tukeva tutkimusmenetelmä yksittäisten solujen sykekuvion määrittämiseksi. Tuloksiin perustuen PCDE ei onnistunut tehostamaan hiPSC sydänsolujen erilaistamista ja saattoi vähentää sydänmarkkereiden ekspressiota. Totesimme kuitenkin, että videopohjaista sykeanalyysiä voitaisiin käyttää yhdessä yksittäisten solujen PCR-tutkimusten kanssa sydänsolujen paremman karakterisoinnin saavuttamiseksi.

Avainsanoja: hiPSC, sydänsolu, kantasolu, erilaistus, videopohjainen sykeanalyysi, kvantitatiivinen polymeraasiketjureaktio, virtaussytometria, yksittäinen solu, karakterisointi, pienmolekyyli

ABBREVIATIONS

BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
CMs	Cardiomyocytes
CPVT	Catecholaminergic polymorphic ventricular tachycardia
DMS	Differentially methylated sites
DMSO	Dimethyl Sulfoxide
EB	Embryonic body
ECM	Extracellular matrix
END-2	Mouse visceral endoderm-like cells
FGF	Fibroblast growth factor
FinA	KCNQ1-Fin (G589D) mutation
fps	Frames per second
hESC	Human embryonic stem cell
hiPSC	Human induced pluripotent stem cell
iCM	Induced cardiomyocyte
I_{Ks}	Delayed rectifier potassium current (slow)
IWP	Inhibitor of Wnt production
LQTS	Long QT syndrome
MEA	Multielectrode array
MEF	Mouse embryonic fibroblast
MLC	Myosin light chain
NDS	Sodium dodecyl sulphate
PCDE	Putative cardiac differentiation enhancing small molecule
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
qPCR	Quantitative polymerase chain reaction
RPLP0	Ribosomal protein large P0
Wnt	Wingless/INT protein

TABLE OF CONTENTS

1 INTRODUCTION

Currently, heart diseases are a leading cause of death with few treatment options available (for review see Yamakawa and Ieda, 2015). Mature cardiomyocytes have very limited regenerative abilities after injury. Instead, following cardiac injury cardiac fibroblasts proliferate causing fibrosis and heart failure. In the case of heart failure a heart transplant might be needed. However, the procedure is highly invasive and spare hearts are often not easy to find. Because of this, the option of regenerating cardiomyocytes or even entire hearts through the use of stem cells has become a hot topic. Human ESCs have been studied for the past two decades, but both ethical and legal concerns have hindered interest in ESC based treatment (for review see Okano et al., 2013). Therefore, possible therapies and drug testing involving the use of human induced pluripotent stem cells (hiPSCs) have gained interest. The in vitro hiPSC models have given insight into disease phenotypes and patient specific drug responses. Though hiPSCs have been successfully used for drug testing (Guo et al., 2013; Kuusela et al., 2016), and iPSCs have been successfully transplanted into animals (Kobayashi et al., 2012), issues with heterogenicity, predictability, culture purity, and cell maturity still require attention (for review see Chen et al., 2016; Yamakawa and Ieda, 2015).

1.1 hiPSCs

Originally hiPSCs were created by reprogramming fibroblasts with specific transcription factors: Oct3/4, Sox2, c-Myc, and Klf4 (Takahashi et al., 2007). Retrovirus-mediated transfection was used for deliver the factors into somatic cells, where they integrated the host cell genome and reprogrammed the cell. Stem cells created with this technique were similar to embryonic stem cells. Morphologically the iPSCs were indistinguishable from ESCs with large nuclei, the cells had similar doubling times, and they both expressed hESC specific surface antigens such as SSEA-3, SSEA-4, tumor related antigens TRA-1-60, TRA-1-80, TRA-2-49/6E, and NANOG protein. The gene expression levels were similar to hESC expression, though some variation was observed. Due to the gene integrations in the induction of hiPSCs, the cells were prone to tumorigenesis caused by reactivation of c-Myc, making them unsafe for cell therapy in tissue regenerative applications. The safety of iPSCs produced with retroviral induction is affected by the epigenetics of somatic cells (for review see Okano et al., 2013).

Recently, techniques which do not rely on gene integration have been investigated to find a solution to the tumor formation (for review see Okano et al., 2013). These techniques, designed to induce pluripotency in somatic cells, include the use of small molecules, adenovirus and sendai virus vectors, transposons, plasmids, episomal vectors, and modified RNA to alter gene expression of cells. One of the most efficient technique relies on the sendai virus vectors, which allow high level exogenous gene expression in cells without gene integration and disappears during cell growth (Fusaki et al., 2009).

In addition to the reprogramming technique, culture conditions and donor cell origin also affect the outcome of reprogramming (Nishino et al., 2011; Sanchez-Freire et al., 2014). The hiPSCs are known to have epigenetic memory, and the reprogramming process of somatic cells is recognized as significant epigenetic remodeling process that causes DNA methylation and histone modifications (Nishino et al., 2011). The epigenetic memory of cells is dependent on genetic variations and age of donor cells as well as on derivation methods, culture conditions, and number of passages. These inherited modifications, also known as differentially methylated sites (DMSs), cause iPSCs to steer differentiation towards specific lineages. For example, it's known that cardiac progenitor cells differentiate into cardiomyocytes more efficiently than fibroblasts from the skin (Sanchez-Freire et al., 2014). However, the more readily available fibroblasts share a common progenitor cell population with heart fibroblasts (Doppler et al., 2015). This makes skin fibroblasts a great target for differentiation.

1.2 Differentiation

The *in vitro* differentiation process of hiPSCs aims to mimic the natural differentiation of the cell to some extent. The onset of embryonic cardiogenesis is known to be strongly influenced by several growth factor families such as transforming growth factor subfamilies bone morphogenic proteins (BMPs) and activin, the wingless/INT proteins (Wnts), and fibroblast growth factors (FGFs) (Gaussin et al., 2002; Schneider and Mercola, 2001; Logan and Mohan, 1993). And like with all tissue types, the relative expression and timing of growth factors has to be right for the cells to differentiate successfully. Once the cells have received the necessary signals, transcription factors highly specific to cardiomyocytes are activated. Differentiation begins with the activation of Wnt/β-catenin pathway and the expression of Brachyury, a transcription factor of the T-box gene complex, and Flk, a type

3 receptor tyrosine kinase (Bondue et al., 2011; Hartogh et al., 2016). Brachury and Flk activate a Mesp1 expressing cell population, which downregulates the Wnt/β-catenin signaling pathway (Bondue et al., 2011). A subset of the Mesp1 expressing population begins to express Isl1, an insulin promoting transcription factor, which stimulates cardiovascular commitment. These steps lead to the activation of regulatory networks linked to the NKX2.5 gene (encoding a homeobox protein) and zinc finger transcription factor GATA4 especially (Hartogh et al., 2016). These and other networks of factors lead to the differentiation and maturation of cardiomyocytes, and therefore, expression of cardiac markers such as Troponin T (TNNT2), and atrial and ventricular isoforms of myosin regulatory light chain 2 (MLC2a and MLC2v) (Hartogh et al., 2016).

Three major culturing strategies exist for differentiation of cardiomyocytes from hiPSCs: 1) embryoid body (EB) (Aikawa et al., 2015), 2) two dimensional monolayer (Burridge et al., 2014), and 3) co-culture (Mummery et al., 2003; Passier et al., 2005). In EB cultures, cells are physically manipulated to form EBs, spheroid stem cell aggregates, in suspension (Aikawa et al., 2015). Differentiation is controlled by manipulation of aggregate size and with the use of growth factors, such as BMP4, and small molecules to achieve high efficiency (Aikawa et al., 2015; Burridge et al., 2006). In monolayer cultures cells are differentiated as a single cell layer on top of a gel matrix (Burridge et al., 2014). The method is highly efficient with a yield of >85% pre-enrichment. Chemically defined methods for monolayer differentiation offer scalability, reproducibility, and control over the cultures. This makes monolayer cultures a great tool for drug testing. In co-cultures mouse visceral endoderm-like cells (END-2) are used as feeders, which induce hiPSC differentiation into cardiac cells by activating the growth factor BMP2 and Wnt inhibitors (Bin et al., 2006; Mummery et al., 2003). END-2 cells are attached to culture dishes before the addition of hiPSCs, and their ability to proliferate is disabled chemically. Though the discovery of 0% FCS medium co-culture has increased the yield of cardiomyocytes 24-fold, the yield remains relatively low (5-20% post dissociation) (Passier et al., 2005).

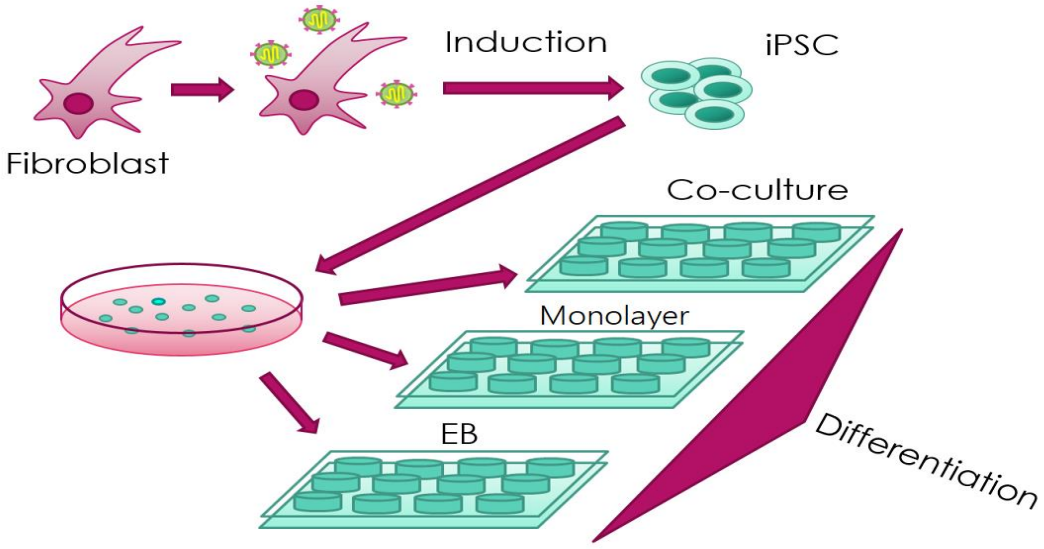

Figure 1 A figure describing the process of creating induced pluripotent stem cells and differentiating them into cardiomyocytes and many other cells types. Fibroblasts taken from a patient are first induced by viral vectors carrying pluripotency genes or by other means such as small molecules, plasmids, or RNA. The iPSCs then multiply until cells no longer carry transfected genes. The cells can then be differentiated with the preferred technique of co-culture, monolayer, or embryoid body.

1.2.1 Small molecules in differentiation

Small molecules can be used to chemically modulate the differentiation process of pluripotent stem cells and to manipulate cell fate and to increase the efficiency of differentiation in feeder free cultures (Fonoudi et al., 2015; Burridge et al., 2014). The chemical components work by manipulating differentiation events at a desired time points by activating or inhibiting key signaling pathways such as Wnt/β-catenin (Fonoudi et al., 2015). For example, small molecules such as CHIR99201 and IWPs can be used to initiate cardiomyocyte differentiation through the Wnt/β-catenin patway (Lian et al., 2013; Lian et al., 2012). CHIR99201 is a glycogen synthase kinase 3 inhibitor (Gsk3) activates the Wnt signaling pathway (Lian et al., 2013). Inhibition of Gsk3 causes β-catenin to accumulate in the nucleus and to activate gene expression, which initiates differentiation (Lian et al., 2012). Inhibitors of Wnt production (IWPs) can then be used for palmitylation of Wnt proteins to end Wnt secretion and activity, which is necessary for cardiomyocyte development.

Recently techniques that allow the bypassing of the pluripotency step when differentiating mouse fibroblasts into induced cardiomyocytes (iCMs), have been developed (Doppler et al., 2015). The so called process of transdifferentiation has been deemed relatively inefficient and risky with the use of viral induction (Doppler et al., 2015). Therefore, protocols that only utilize cocktails of small molecules and growth factors for direct cardiac differentiation from somatic cells have been invented (Cao et al., 2016). Small molecules are used to open the chromatin structures to allow cell type specific gene activation by growth factors, which induces direct differentiation without pluripotency. The resulting iCMs closely resemble cardiomyocytes differentiated from hiPSCs.

1.3 hiPSC derived cardiomyocytes

Human iPSCs can be differentiated into beating cardiomyocytes in 2-3 weeks (Ivashchenko et al., 2013). The timeframe corresponds to the natural embryonic development of heart cells. Cardiomyocytes differentiated from hiPSCs represent all of the three existing types of cardiac cells: nodal, atrial, and ventrical, and they possess similar actin-myosin structures as mature cardiomyocytes. The cells exhibit characteristic sarcomeric bands with cardiac specific proteins such as Troponin T. The presence of Troponin T and Connexin 43 (Cx43) is considered as an indication of the presence of cardiomyocytes in a culture as Cx43 functions as a mediator of electrical coupling between beating cardiomyocytes (Vozzi et al., 1999) The intracellular structures continue to develop towards a more mature phenotype with time (Kamakura et al., 2013). Cardiomyocytes have disorganized myofibrils at 14 days, and the developing of A-, H-, I-, Z-, and M-bands, as well as tightly packed organized myofibrils, may take even a year of culture. Despite the structural maturation, hiPSC derived cardiomyocytes express cardiac-specific genes less compared to adult cardiac cells even after a year of culture (Kamakura et al., 2013).

1.4 Video-based beating analysis

As the use of induced cardiomyocytes has become more routine, more emphasis is placed on the functionality of the cardiac cells. The functionality is assessed through the electrophysiology and mechanical beating behavior (Laurila et al., 2015). Electrophysiology is the traditional measure of cardiomyocyte functionality, and it can be analyzed by patch clamp, multielectrode arrays (MEAs), and fluorescent imaging, whereas the mechanical beating behavior of cardiomyocytes can be examined through traction force microscopy,

atomic force microscopy, impedance assays, and video microscopy (Plotnikov et al., 2014; Domke et al., 1999; Xi et al., 2011; Ahola et al., 2014; Laurila et al., 2015). Traction force microscopy measures the force of traction of cells attached to linearly elastic hydrogels with fluorescent beads as markers (Plotnikov et al., 2014). The location of the markers during cell contraction and relaxation are compared. Atomic force microscopy measures the movement of a cell with a small cantilever placed close to the cells surface, whereas impedence assays use microwells with electordes to measure impedance to detect beating (Domke et al., 1999; Xi et al., 2011). In addition to these methods, it has been shown that beating analysis from video recordings can provide a robust and reliable method for the identification of beating phases and time parameters in single cells (Ahola et al., 2014). With the use of digital image correlation (DIC), specifically the mum quadratic difference (MQD) method, radial and tangential signals can be obtained from moving areas of cells, which are divided into eight vectors. The signals from these vectors is interpreted through algorithmic analysis as seen in Figure 2. The method can even be used to recognize different mutations causing similar abnormalities in the beating behavior of cardiomyocytes (Ahola et al., 2014).

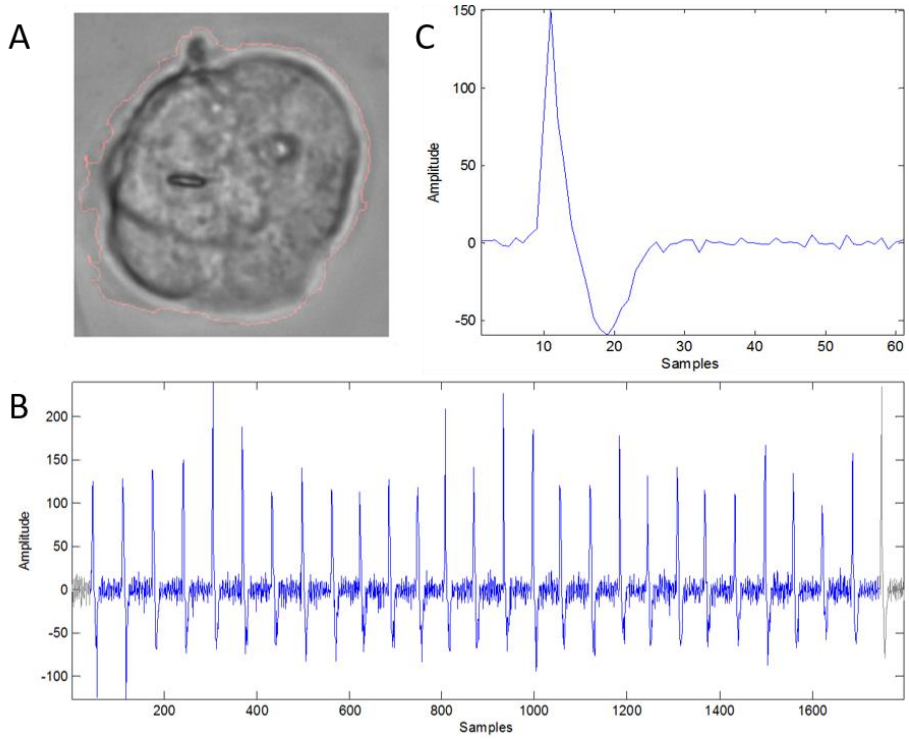

Figure 2 An example of the mechanical beating analysis results given by Beatview, which uses the mum quadratic difference (MQD) method. MQD method divides a cell into eight vectors and analyses the radial and tangential movement in each vector. Beatview allows the user to A) crop the target cell manually, B) view the rate, force, and direction of the beat, and C) creates an average beating pattern and allows user to choose the end and start points of phases.

1.5 Long QT syndrome

The ECG of the heart can be divided into phases P, Q, R, S, and T phases as shown in Figure 3 (for review see Katz, 2006). Atrial depolarization can be seen as the P wave. Following the P wave, the ECG returns to its baseline before ventricular depolarization begins. Ventricular depolarization is represented by the QRS complex. After depolarization repolarization can be seen as the T wave. The time between the onset of the QRS complex and the end of T wave is called the QT interval. The QT interval can be used to evaluate the time dependent properties of ion channels responsible for ventricular depolarization and repolarization. The duration of the QT interval is 0.35-0.40 seconds for a healthy person. Congenital LQTS is a hereditary disorder that causes the prolongation of the QT interval (Crotti et al., 2008). Symptoms of the disease include electrocardiographic abnormalities, sudden cardiac death, and syncopal spells, also known as fainting. LQT1 is caused by a missense mutation in the C-terminus of a gene called KCNQ1. The mutation studied in this thesis is known as G589D or as KCNQ1-Fin (FinA), and it accounts for 30% of LQTS cases in Finland. KCNQ1 encodes for α-subunit of the voltage gated ion channels responsible for the cardiac delayed rectifier current (I_{Ks}) in repolarization, during which K^+ ions flow out of the cell (Hedley et al., 2009). The ion channel is activated by increasing membrane depolarization. Mutations in KCNQ1, that cause loss-of-function or dysfunction in ion trafficking, lead to prolonged action potentials, which means the relaxation of the cardiomyocytes is delayed. It's one of the most prevalent types of LQTS (Piippo et al., 2001). Specifically, the mutation G589D is a recessive missense mutation that results in the substitution of aspartic acid with glycine at position 589.

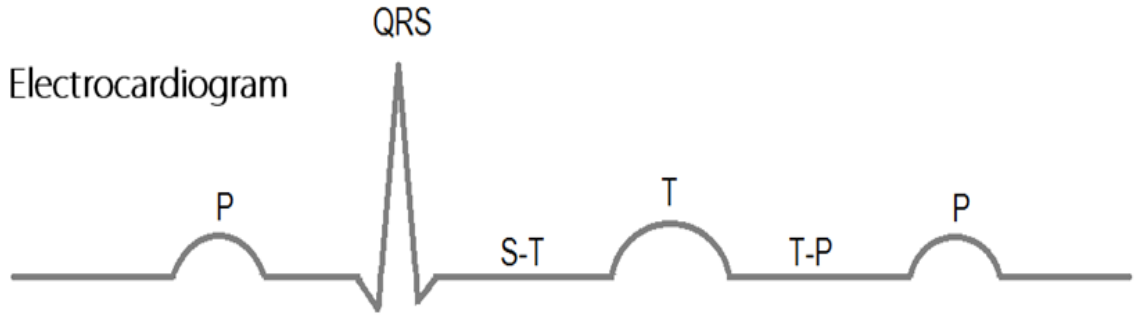

Figure 3 The electrocardiogram (ECG) of the heart has five phases: P, Q, R, S, and T. P wave represents the depolarization of the atria, the QRS complex represents the ventricular depolarization, and T wave represents the repolarization. The figure is based on a picture from the book, Kardiologia (Heikkilä et al., 2000)

2 AIMS OF THE STUDY

The aim of this study was to continue previous unpublished studies on the differentiation process of cardiomyocytes with the novel small molecule that has putative enhancing effects on cardiac specific transcription factors. The hypothesis was that the putative cardiac differentiation enhancing small molecule (PCDE) would enhance cardiac differentiation in iPS cells process compared to differentiation without the addition of the small molecule. In this project flow cytometry was used to define accurate cardiomyocyte percentages and qPCR was used to detect differentiation markers. The name and physiological qualities of PCDE are not known, and prior experiments have not yet been published. Another part of the research was concerning the single cell video-based beating analysis as a technique for functional analysis and disease modeling. The aim was to link video-based analysis to gene expression data from single cardiomyocytes and to evaluate mechanical beating analysis as a characterization technique.

3 MATERIALS AND METHODS

3.1 Ethical approval

The iPS cell lines used in this study were generated in BioMediTech at the University of Tampere with permission from the ethical committee of Pirkanmaa Hospital District (R08070). Written consent was received from all skin biopsy donors.

3.2 Thawing of cell lines

The following hiPS cell lines were used in this experiment: UTA.00303.LQT1, UTA.00313.LQT1, and UTA.04202.WT. The LQT1 biopsies were derived from a 28-year-old asymptomatic female, and the wild type cell line UTA.04202.WT was derived from a 55-year-old female with no heart condition (Ahola et al., 2014; Kuusela et al., 2016). Both cell lines were previously established by retroviral infection with lentivirus. The detailed protocol is described earlier elsewhere (Takahashi et al., 2007). The cryopreserved pluripotent stem cells were thawn in +37°C water bath and 10 ml of warm KSR medium was added into the tube drop by drop to reduce osmotic stress. The KSR medium consisted of knockout (KO)-DMEM (Invitrogen, Carlsbad, CA, USA) containing 20% KO-serum

replacement (KO-SR, Invitrogen), NEAA (Cambrex, NJ, USA), L-glutamine (Invitrogen), penicillin/streptomycin, 0.1 mmol/L 2-mercaptoethanol (Invitrogen), and 4 ng/ml basic fibroblast growth factor (bFGF, R & D Systems Inc., MN, USA). The cells were gently mixed, centrifuged at 200xg for 5 min, and re-suspended into 2.5 ml of KSR medium.

3.3 iPS cell culture

After mixing the cells gently by pipetting the cells were pipetted into a well with mouse embryonic fibroblast cells (MEF, Millipore, MA, USA) plated the day before in KSR medium (Kuusela et al., 2016). The medium was changed every other day. Once per week the cells were passaged onto new MEF cells. First the medium (1.5 ml/well) was changed for the MEFs an hour before the passage. The MEF layer was detached with a pipette tip and the old medium was removed. One ml of collagenase (1mg/ml) was added and the cells were incubated in +37°C for 5 min. The collagenase was replaced with 3 ml of KSR medium. Colonies of iPS cells were detached and broken by pipetting. An appropriate number of cells (~0.5-1.0 ml) was moved onto MEF plates.

3.4 Differentiation protocols

For the END-2 method MEFs were removed mechanically with a pipette tip from around the hiPSCs and the iPS cell colonies were carefully scraped off the bottom of culture wells and divided into approximately a few hundred cells per well on two 24-well plates. The culture method is described elsewhere (Kuusela et al., 2016; Mummery et al., 2003). The wells were previously coated with END-2 cells treated with mitomycin-C. The hiPSCs were cultured in 0% KSR medium supplemented with ascorbic acid (AA) for the first 14 days. After the two-week time point the culture medium was changed to 10 % KSR medium also supplemented with AA. 10 mM PCDE dissolved in dimethyl sulfoxide (DMSO) was added into medium on days 3 and 6 for a final concentration of 1µM, and removed on day 10. Co-cultured cells were collected for flow cytometry on day 17.

For monolayer cultures, the hiPSCs were enzymatically dissociated with versene at +37 ºC and counted as has been described earlier (Mummery et al., 2003). Approximately $0.7x10^6$ cells and 1 ml of mTeSR1 medium was added into GELTREX coated wells on two 24-well plates. Medium was changed every day. Once confluent (day 0), medium was changed to 2 ml of RPMI(-insulin) with 8 µM CHIR99021. After 24 hours (day 1) medium

was changed to 2 ml of RPMI(-insulin). On the third day 1ml of medium from each well was collected and mixed with an equal amount of fresh RPMI(-insulin) and small molecule IWP4 (5 µM). On days 5 and 7 medium was changed to fresh RPMI(-insulin). From day 10 on, half of the medium in each well was changed to RPMI(+insulin) every 2 to 3 days. 10 mM PCDE dissolved in DMSO was added into medium on day 3 for a final concentration of 1 µM and removed on day 5. The timeline for the differentiation protocols for both co-cultures and monolayer cultures are presented in Figure 4.

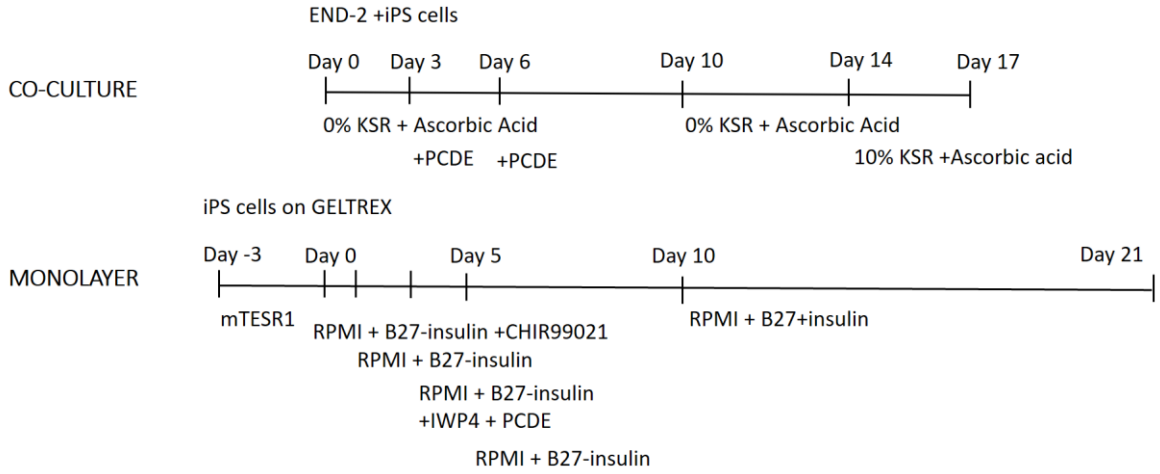

Figure 4 The basic differentiation protocols used in the experiment for differentiating cardiomyocytes from iPSCs are presented in timelines. Co-cultures with hiPSCs on END-2 coated plates were cultured in 0% KSR medium with ascorbic acid for 14 days until medium was switched to 10% KSR with ascorbic acid. PCDE was added on days 3 and 6 with fresh medium. Monolayer cultures with hiPSCs on Geltrex were first cultured in mTESR1 for 3 days until confluent. On day 0 of differentiation medium was changed to RPMI with CHIR99021 and B27 without insulin. On day 1 CHIR99021 was removed, and on day three small molecules IWP4 and PCDE were added with fresh medium. IWP4 and PCDE were removed on day 5, and medium was switched to RPMI with B27 with insulin on day 10.

3.4.1 Novel small molecule concentration

The most suitable concentration (1 µM) of the novel growth factor, referred to here as PCDE, was determined on both feeder free and END-2 cultures; Cells were grown according to protocol, but PCDE (in DMSO) was diluted with medium and carefully added into wells on appropriate days in final concentrations of 1µM, 3 µM, and 5 µM. Two wells with an appropriate plain medium and another two wells with 0.03% DMSO added into the medium were treated as controls. During additions of fresh medium the small molecule and DMSO were re-added until the date mentioned in the protocol (Figure 5). Beating areas were counted in every well since day 10 whenever the medium was changed. The concentrations were tested with replicates and with three technical replicates per concentration.

3.5 Flow cytometry

Flow cytometry was carried out with cardiomyocytes (≥14 days). Wells were washed with PBS 2x5 min at 37ºC and then incubated in 500 µl of Accutase® detachment solution (Sigma-Aldrich, Saint-Louis, Missouri, USA) for approximately 10 min at +37ºC. Cells were collected from two wells (2 cm^2) to warm EB medium (500 µl/well); one from a control culture and one from the test culture and pipetted gently to aid dissociation. The suspension was filtered through a 40 µm filter and cells were counted. Cells were divided into three sample tubes (200 000-300 000 cells per sample), and 1-2 ml of PBS was added. Samples were centrifuged 1900 rpm for 2 min, and supernatant was discarded. The cells were fixed with 500 µl of 4% paraformaldehyde (PFA, Sigma-Aldrich) for 10 minutes at 23ºC and centrifuged again. Then 500µl of permeabilization/wash solution (1% NDS, 0,1% TritonX-100, 1% BSA in PBS) was added, and the samples were mixed for 10 sec and incubated for 10 min on ice. Samples were then centrifuged again and the wash was repeated two more times. 500µl of R-phycoerythrin (PE) mouse anti-cardiac Troponin T (BD Biosciences, CA, USA) antibody, diluted 1:100 with permeabilization/wash solution, was then added to two of the three tubes per culture type (control/ PCDE sample) leaving negative controls without the antibody. The tubes were incubated in the dark at 23ºC for 40 min. The samples were washed two times as before and resuspended in 200 µl of perm/wash solution. The samples were kept on ice and analyzed with Accuri™ flow cytometry device (BD Biosciences, CA, USA).

3.6 Dissociation of beating cells

To create a cardiomyocyte culture with single cells, cardiomyocytes were enzymatically dissociated as described before (Mummery et al., 2003). Beating cell clusters were isolated with a micro scalpel on a heated platform (~37ºC), and collected into culture media. Collected cells were first washed with Low-Ca buffer (Appendix 1) for 30 min at 23ºC. The aggregates were then incubated in Enzyme medium (Appendix 1) for 45 min at 37ºC to loosen cell to cell connections. Finally the cells were pre-incubated in KB medium for 1h at 23ºC. The KB medium (Appendix 1) was supplemented with glucose right before use. During pre-incubation 24-well plates were coated with 0.1% gelatin for one hour to improve cell attachment. After the incubation cell aggregates were re-suspended in 20% embryonic body (EB) culture medium by carefully pipetting up and down (~2-5 times) against the

bottom of the plate. The cells were then moved to the previously prepared gelatin coated wells in EB medium and allowed to attach at 37ºC.

3.7 Characterization

3.7.1 RNA purification

Cell culture samples were collected at various time points of differentiation. Cells from two wells from both small molecule treated and control cultures were washed with 1 ml PBS, and incubated in 350-600 µl of RL-buffer (Norgen, Canada) for 5 min, scraped off the bottom of the well with a pipette tip, and collected into seperate 1.5 ml tubes. Unless RNA was purified immediately, the tubes were stored at -80ºC until purification. RNA was purified with Total RNA Purification Plus Kits (Norgen) according to the manufacturer's protocol. The RNA concentration and purity was measured with NanoDrop™ 1000 (Thermo Scientific, USA).

3.7.2 Polymerase Chain Reaction

The RNA concentration of each sample was measured with NanoDrop. The amount of 1 µg of RNA was transcribed into cDNA with the High Capacity Reverse Transcriptase kit (Applied Biosystems, USA) in 20 µl reactions according to the manufacturer's protocol. The cDNA was stored at -20ºC, and diluted 1:10 before use. Master mixes were prepared according to manufacturer's protocol. The qPCR was carried out with either SYBR Green (Brachyury T, BMP-4, NKX 2.5, RPLPO) or Taqman probes (TNNT1 and Nanog)(Thermo Fisher, USA). The nucleotide sequences of the primers are presented in Table 1.

Table 1 The primers and their sequences used in this study

Primers	Target	Forward	Reverse
SYBR Green	Brachyury	TGCTTCCCTGAGACCCAGTT	GATCACTTCTTTCCTTTGCATCAAG
	BMP-4	GTGAGGAGCTTCCACCACGA	ACTGGTCCCTGGGGATGTTCTC
	NKX 2.5	CAAGGACCCTAGAGCCGAAA	CAGCTCCACCGCCTTCTG
	RPLPO	AATCTCCAGGGGCACCATT	CGCTGGCTCCCACTTTGT
Taqman	TNNT2	Hs00165960_m1[1]	
	Nanog	Hs02387400_g1[2]	

[1] http://www.thermofisher.com/order/genome-database/browse/gene-expression/keyword/Hs00165960_m1
[2] https://www.thermofisher.com/order/genome-database/browse/gene-expression/keyword/Hs02387400_g1

The final concentration of each SYBR Green primer in reaction was 22,2 nM. Dilution series of cDNA (1, 1:10, 1:100, 1:1000, and 1:10 000) were created for each SYBR Green reaction plate for quality control. The 15 µl qPCR reactions with two biological replicates and three technical replicates per time point (2x3 replicates) were run on 96-well plates with Applied Biosystems 7500 equipment. Each plate contained three types of controls each with two replicates: 1) water, 2) cDNA mixed with water, and 3) sample and master mix without RNAse. The amplification program is shown in Table 2.

Table 2 The qPCR amplification program used.

Step	1	2	3	4	5	6	7
Temp (ºC)	50	95	95	60	95	60	95
Duration	1 min	10 min	15 sec	1 min	15 sec	1 min	15 sec
Repetitions X	-	-	40		-	-	-

3.7.3 Statistical analysis

Data was analyzed with Excel. A standard curve was created to test the SYBR Green primers by using the dilution series, and the quality of qPCR results was verified by determining the amplification efficiency (E). E was always within range 96-104%. Ct values were determined for all reactions and the $2^{-\Delta\Delta Ct}$ method was used to calculate the mean fold change for each RNA collection time point per gene (Livak and Schmittgen, 2002). The results were normalized to Ribosomal protein large P0 (RPLP0) and calibrated to day 0 ΔΔCt value, which gives day 0 the mean fold change value of 1.

3.7.4 Immunostaining

For immunostaining, beating areas of cells were dissociated onto coverslips as described before. Seven days after dissociation the cells were fixed with 4 % paraformaldehyde (PFA, Sigma-Aldrich) for 20 minutes and washed with phosphate buffer saline (PBS) 3x5 min. The cells were permeabilized and unspecific antibodies were blocked in a blocking solution (10% sodium dodecyl sulphate (NDS), 0,1% TritonX-100, 1% bovine serum albumin (BSA) in PBS) for 40 min RT. Primary antibodies were diluted with wash solution (1% NDS, 0,1% TritonX-100, 1% BSA in PBS). The coverslips were incubated in +4ºC over night with primary antibodies: 1:2000 goat anti-cardiac troponin T (ab64623, Abcam, Cambridge, MA,

USA), 1:100 mouse anti-KCNQ1 (ab84819, Abcam), rabbit anti-connexin 43 (MAB3067, 1:1000, Merck Millipore, Germany), 1:200 rabbit anti-MLC2v (310 003, Synaptic Systems, Goettingen, Germany) and 1:200 mouse anti-MLC2a (311 011, Synaptic Systems). The cells were washed with 1% BSA in PBS 3x5 min. Secondary antibodies were diluted 1:800 with 1% BSA in PBS. Depending on the origin of the primary antibodies, the cells were incubated for one hour at 23ºC in dilutions of secondary antibodies (Thermo Fisher, USA) Alexa fluor 568 donkey (A11057), and Alexa fluor 488 donkey (A21202 & A21206). The coverslips were washed 3x5 min in PBS and 2x5 min in 0,1 M phosphate buffer before they were dried, and mounted with mounting medium (Vectashield antifade mounting medium with DAPI, Vector laboratories, USA). Samples were stored at +4ºC protected from light.

3.8 Imaging and beating analysis

The individual dissociated beating cardiomyocytes were either recorded with a Nikon Eclipse TS100 (Nikon Corporation, Japan) wide field light microscope with a mounted camera (Optika DIGI-12, Optika Microscopes, Italy) or on a micromanipulator (Scientifica, United Kingdom) with a mounted camera and microscope (Olympus, Tokyo, Japan) on top of a heating disc. The disc was set to maintain a temperature of 37ºC degrees and it allowed the cardiomyocytes to be imaged for maximum of 30 minutes outside the incubator without visible effect on beating behavior. The videos recorded on the wide field light microscope were 30 seconds long with 60 frames per second (fps).

The video analysis was carried out with 31 videos of single cardiomyocytes with LQT1 syndrome from cell lines UTA.00303.LQT1 and UTA.00313.LQT1, and of 15 wild type cardiomyocytes, from cell line UTA.04202.WT, using a digital analysis program that creates vector field data from the contraction-relaxation dynamics of the cells. Moving areas from each cell were cropped and divided into eight vectors with the program, and movement was measured in radial and tangential directions from the focus point of beating, which was approximated by eye. From the data, starting and ending points of contraction, relaxation, and any additional activity were marked manually to obtain the beating rate and contraction/relaxation times of individual cells in Excel format.

3.8.1 Picking of single cells

Single cells were picked with the micromanipulator (Scientifica, UK) for further analysis. A glass micropipette connected to a syringe was used to detach single cells and to suction them into the pipette tip. The pipette tip was emptied and broken into 0,5 ml tube with buffer and stored in -80ºC. RNA sequencing was carried out in collaboration with FIMM (Helsinki, Finland).

4 RESULTS

4.1 Cardiac differentiation with PCDE

During differentiation PCDE appeared have little visible effects on the cells in 1 µM concentration and concentrations higher than 1µM had a negative effect on the cultures as seen in Figure 5. These cultures failed to survive past 14 days with negative correlation to the increase in PCDE concentration. Co-cultured DMSO control cells showed no significant proportional decrease in the amount of beating areas compared to the controls without DMSO, though the overall amount of beating areas was greater in control cells without DMSO. The average numbers of beating areas are listed in Table 3. The amount of beating cell aggregates approximately doubled between days 10 and 13 in all control wells and wells with 1 µM of PCDE. The two highest concentrations (3 µM and 5 µM) of molecule PCDE caused most cells to die by day 10. DMSO appeared to have a negative effect on the amount of beating cell aggregates, but the difference could be explained by differences in the number of cells in the beginning of differentiation. The survival of feeder free cultures was later tested with the same concentrations and these cells also died in 3 µM and 5 µM PCDE. Based on this, it was concluded that 1µM concentration of PCDE was the best concentration for further tests.

Table 3 The average number of beating cells during the differentiation of cardiomyocytes

PCDE	Day	DMSO	SD	Control	SD	1µM	SD	3µM	5µM
	10	3.5	2.1	5.5	3.7	3.7	2.9	0	0
	13	6.5	3.5	10.5	6.3	7	4.0	0	0
	15	12.5	12.0	14.5	9.4	9.7	6.0	0	0

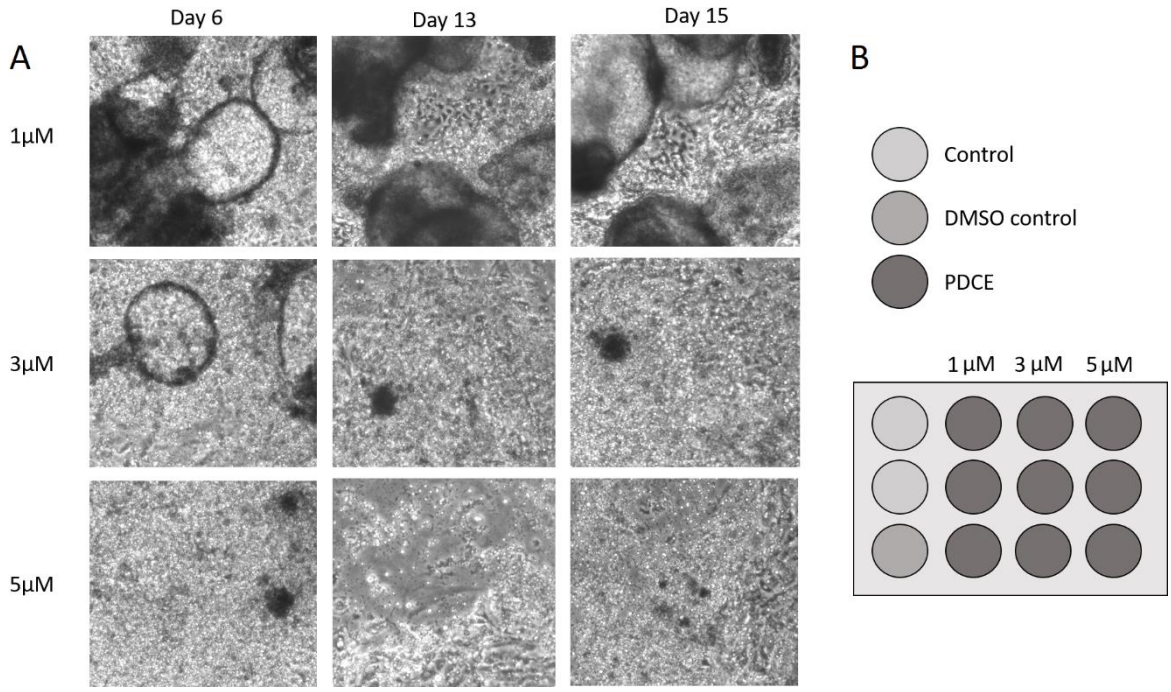

Figure 5 The concentration of PCDE used in the differentiation of hiPSCs into cardiomyocytes was defined by testing three different concentrations 1µM, 3µM, and 5µM. Controls contained no PCDE. DMSO controls contained the same amount of DMSO as was in the 5µM PCDE cultures. Microscope images from different phases of differentiation (A) as well as the experiment setup (B) are presented in the figure. PCDE appeared to cause cell death and lack of aggregates in concentrations higher than 1µM.

4.1.1 Flow cytometry

Flow cytometry results showed a higher percentage of cardiomyocytes in the PCDE treated END-2 cultures, 7.80 %, compared to controls, 4.65 %. PCDE had no effect on the feeder free monolayer differentiation efficiency. As expected, the monolayer culture was a more efficient culture technique achieving >50 % differentiated cardiomyocytes. The flow cytometry results are presented in Table 4.

Table 4 The differentiation efficiency of END-2 and feeder free cultured cardiomyocytes.

Differentation efficiency	Control (average %)	PCDE (average %)
END-2	4.65	7.80
Monolayer	51.90	51.45

4.1.2 Gene expression

The relative gene expression of NKX2.5, TNNT2, Brachuyry, Nanog, and BMP4 in control co-cultures and co-cultures with 1µM PCDE cultured until day 20 are presented in Figure 6. The expression of the homeobox protein NKX2.5 increased after day 4 with a statistically significant higher increase in the control cultures on day 12. Consecutively the expression of Troponin T (TNNT2) increased rapidly until day 12 in control cultures and until day 8 in PCDE cultures. Brachyury expressions peaked at day 2 in all cultures before to dropping drastically. Expression levels of Nanog dropped by day 4. Though there was some fluctuation between controls and PCDE cultures in BMP-4 expression, there was no significant difference between the cultures.

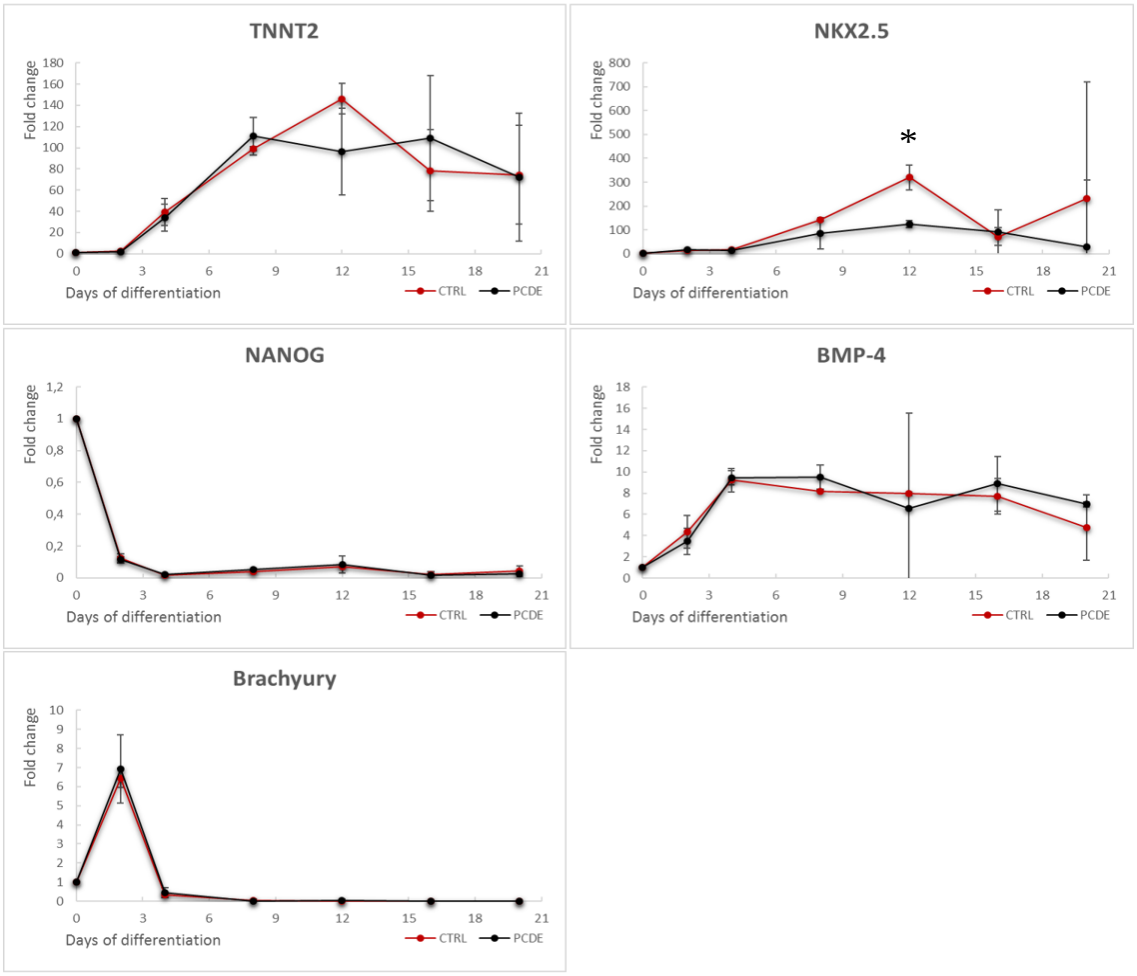

Figure 6 The figure presents the mean fold change of relative expression in genes NKX2.5, TNNT2, Brachury, NANOG, and BMP-4 in END-2 cardiomyocyte differentiation in co-cultures on days 2, 4, 8, 12, 16, and 20 from two biological replicates in both PCDE treated and control co-cultures. Scientifically significant difference for mean fold change is considered to be 2x fold change difference. Significant difference between controls and PCDE cultures is marked in the figure (*). The only scientifically significant difference was detected in the expression of NKX2.5, an early cardiac marker, on day 12.

The relative genetic expression of NKX2.5, TNNT2, Brachuyry, Nanog, and BMP4 in feeder free control and 1µM PCDE cultures are listed in Figure 7. The monolayer cultures grown on GELTREX showed a dramatic increase in the expression of both NKX2.5 and Troponin T between days 5 and 8 with a similar trend, though there was only a scientifically significant difference in the expression of NKX2.5 at day 17. The same way as in co-sultures, Brachyury expression peaked at the early stages of differentiation (day 3) and then dropped drastically. Nanog expression decreased from the start of differentiation. However, unlike in co-culture Nanog expression is higher in PCDE cultures, though there's no significant difference. BMP4 expression peaked earlier, on day 3, in PCDE cultures compared to control cultures in which the expression peaked on day 5. Otherwise BMP-4 expression followed the same pattern between PCDE and control cultures.

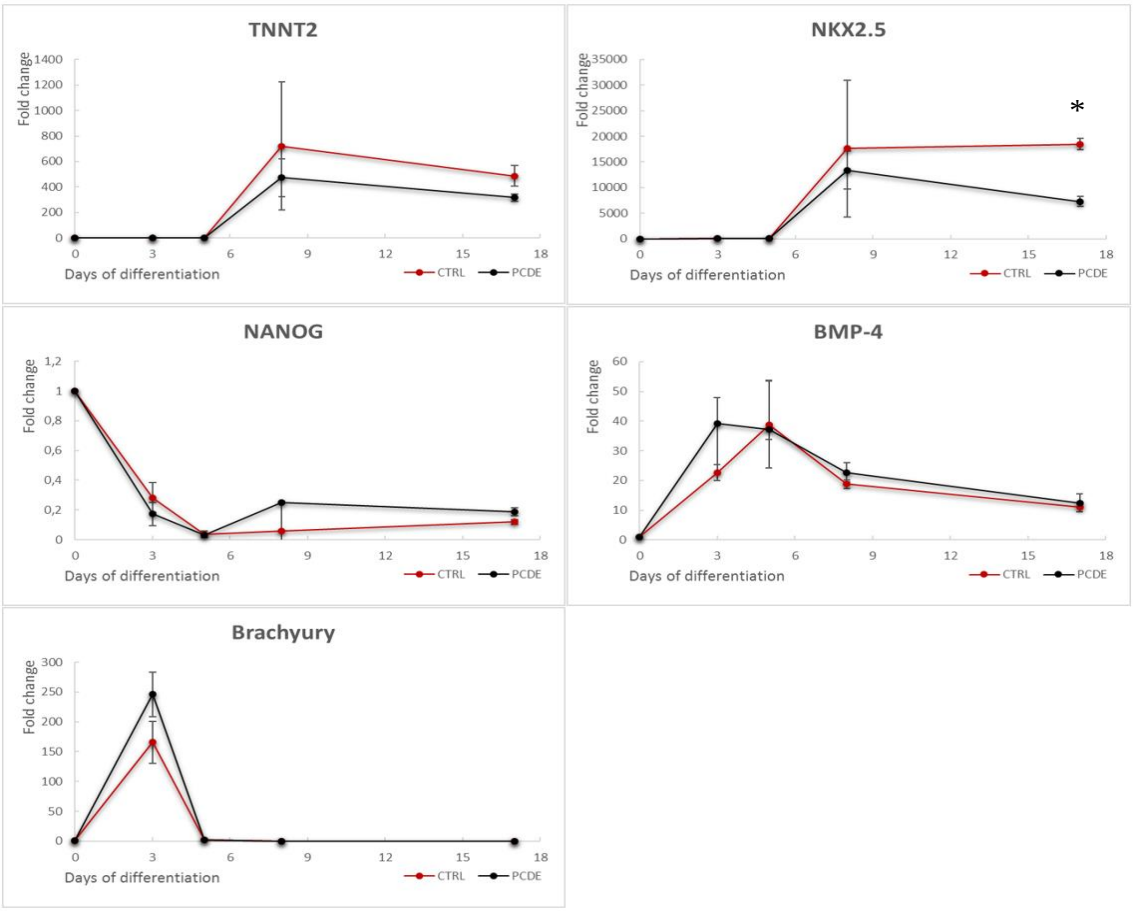

Figure 7 The figure presents the mean fold change of relative expression in genes NKX2.5, TNNT2, Brachyury, NANOG, and BMP4 in feeder free monolayer cardiomyocyte differentiation cultures on days 3, 5, 8, and 17 from two biological replicates. Scientifically significant difference for mean fold change is considered to be 2x fold change difference. Significant difference between controls and PCDE cultures is marked in the figure (*). Though minor differences can be seen in the expression of all genes under study, the only scientifically significant difference was detected in the expression of NKX2.5, an early cardiac marker, on day 17.

4.2 Cardiomyocyte characterization

The presence of cardiomyocytes in beating aggregates was confirmed by immulabeling as seen in Figure 8. In the figure, cardiac specific proteins Connexin 43 and Troponin T can be seen. When examining immunolabeled cells it could be seen the differentiated cells were presenting Troponin T, which is a specific for cardiomyocytes. As expected, cells with either atrial myosin light chain 2 (MLC-2A) or ventrical myosin light chain 2 (MLC-2V) were also labeled with Troponin T. The presence of both MLC-2A and MLC-2V proved the presence of both atrial and ventrical cardiomyocytes in the cultures. Both Troponin T and the MLC proteins were present throughout the cardiomyocytes as they are all connected to cardiac myosin. The gap junction protein, connexin 43, which plays a crucial role in the spread of action potentials between the cardiomyocytes, was localized mostly near the nucleus and at the cell membrane especially between cells. KCNQ1 channels were not visible regardless of labeling attempts.

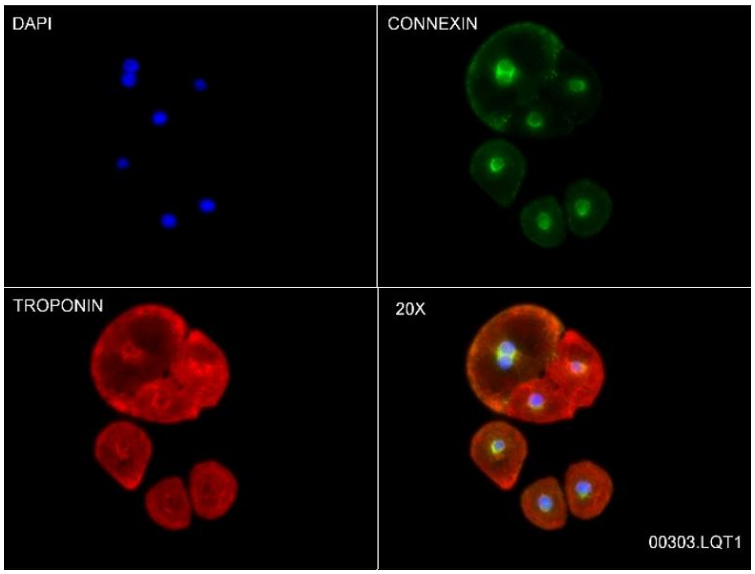

Figure 8 Example image taken of dissociated cells show that the cells differentiated into cardiomyocytes based on the presence of cardiomyocyte specific connexin 43 and Troponin T. Connexin 43 was seen mostly in the cells membranes and near the nucleus, whereas Troponin T can be seen throughout the cells. Thought beating cell aggregates indicate the presence of cardiomyocytes on their own, immunlabeling can be used to evaluate maturity and cell type. This image confirms the presence of cardiomyocytes in beating aggregates.

4.2.1 Beating analysis and single cell sequencing

The beating analysis revealed an average beating frequency of 73bpm for wild type cells (n=23) and 82bpm for LQT1 cells (n=25). There was no significant difference (p=0,6804) between contraction times of wild type cells (131±37 ms) compared to LQT1 cells (126±47 ms). Average relaxation time was also slightly longer for wild type cells, but the difference was not significant (p=0,6108). Abnormal beating was recorded in 30% of wild type cells and in 36% of LQT1 cells. Beating averages and abnormalities of both LQT1 and Wild type cells are presented in Figure 9. Single cell RNA sequencing was conducted succesfully at the collaborating institution on a few of the cells picked with a micromanipulator (data not shown).

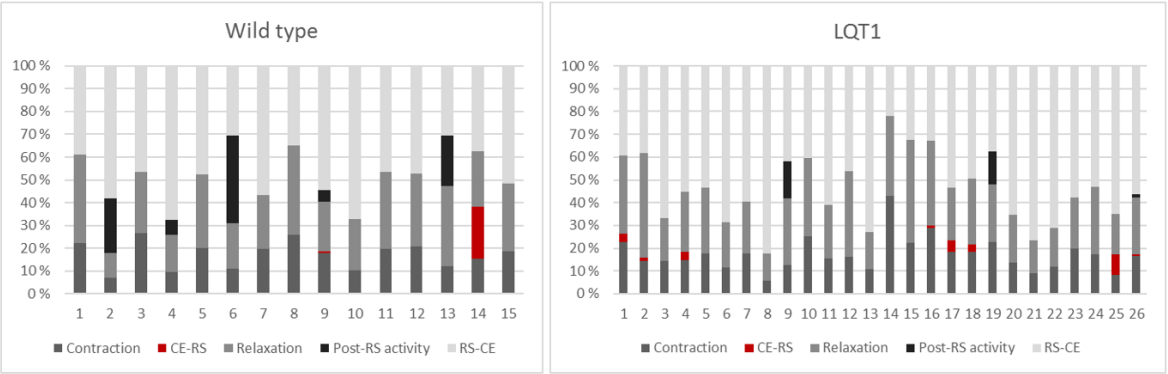

Figure 9 Videos of single beating cardiomyocytes were analysed with Beatview program and the average percentage of each phase of a beat was calculated for each single cell imaged from both LQT1 (00303.LQT1 and 00313.LQT1) and wild type cell lines. Abnormalities (red and black) were detected in all cell lines. The beating averages have no significant difference between LQT1 and wild type cell lines.

5 DISCUSSION

Heart disease is a leading cause of death in the world with little treatment options (Yamakawa and Ieda, 2015). The use of hiPSC derived cardiomyocytes could provide an ethical and economical tool for the treatment of heart disease in the future (for review see Okano et al., 2013; Giri and Bader, 2015). In this study the previously observed differentiation enhancing abilities of a novel small molecule, PCDE, were examined. Human iPSCs were differentiated into cardiomyocytes with two different methods and the number of beating areas was counted. The differentiation efficiency was mainly examined with flow cytometry and qPCR. The cell lines were characterized by immunolabeling and beating

behavior was studied with video-based beating analysis. The cardiomyocytes were found to be functional, though no significant differences were observed between LQT1 and wild type cell lines. PCFE did not seem to improve differentiation compared to controls.

5.1 The effects of PCDE on differentiation

The main interest of this study was the differentiation efficiency of hiPSC derived cardiomyocytes. The hypothesis was that a novel small molecule, PCDE, could improve the differentiation efficiency. Though there is no information available on PCDE, it was assumed that PCDE influences a signaling pathway connected to differentiation after Wnt-inhibition. The cardiomyocytes were exposed to the small molecule in both co-cultures with END-2 cells and in feeder free monolayer cultures at the time of Wnt-inhibition. The aim was to analyze the effects of PCDE on the two culture types separately as the differentiation efficiencies are not comparable between culture types. The gene expression studies revealed some statistically significant differences between control and PCDE cultures, which clarified the possible reasons behind the flow cytometry results and differences in differentiation efficiencies. To gain more reliable results the whole differentiation process should be repeated several times. However, due to the expensive and time consuming nature of hiPSC culture and differentiation, lack of information on PCDE, and discouraging results the differentiation was not repeated.

The concentration of PCDE that was used in the experiments was first defined by differentiating hiPSCs into cardiomyocytes in the presence of 1µM, 3µM, and 5µM of PCDE. The two higher concentrations of PCDE caused cell death and the cultures failed to produce beating aggregates. PCDE was dissolved in DMSO, which is known to improve cardiomyocyte differentiation in several cell types, including mouse embryonic stem cells (mESCs) (Edwards et al., 1983). However, hESCs differ from their mouse counterparts and respond to DMSO treatment negatively (Xu et al., 2002). Lower numbers of beating aggregates was reported for DMSO concentration of 0.5 % and toxicity was reported at 1.5% (Xu et al., 2002). In this experiment the final concentration of DMSO was 0.03 % for DMSO controls and 0.01 % for cultures with 1µM of PCDE. Though the DMSO concentrations in this experiment were low and did not seem to influence the number of beating aggregates, it’s not certain that the chemical had no effect at all on the differentiation ability of hiPSCs.

The qPCR results show that the expression patterns seen in this experiment closely follow patterns previously seen in hiPSC to cardiomyocyte differentiations (Ojala et al., 2012; Zwi et al., 2009). NANOG is a homeobox protein that has a crucial role in maintaining the undifferentiated state in human stem cells (Hyslop et al., 2005) A drop in the expression of the pluripotency marker, NANOG, in the beginning of differentiation initiates a sharp peak in the expression of Brachyury, a cardiomesoderm marker in the beginning of differentiation (Zwi et al., 2009). Previous studies show that Brachyury, a cardiomesoderm marker, peaks slightly earlier in END-2 co-cultures compared to monolayer cultures (Ojala et al., 2012). Another early marker, BMP-4, is known to initiate the expression of myofibrillar sarcomeric genes, such as NKX2.5, in mesodermal cells (Schultheiss et al., 1997). In hiPSC EB cultures the exponential expression of a cardiac-associated transcription factor, encoded by gene NKX2.5, and cardiac structure specific protein Troponin I began at the same time (Zwi et al., 2009). The qPCR results from different culture types are not directly comparable with each other, as the culture conditions impact the differentiation efficiency and gene expression of cardiomyocytes (Ojala et al., 2012). However, the sequential expression of NANOG, Brachyury, BMP-4, NKX2.5, and TNNT2 (cTnT, Troponin T) followed the typical pattern of expression for hiPSC to cardiomyocyte differentiation.

In this experiment, the expression of NKX2.5 peaked at the same time on day 12 of differentiation in both control and PCDE co-cultures. However, the NKX2.5 levels remained lower and more steady in PCDE co-cultures compared to control co-culture, which showed a significant peak in NKX2.5 expression. The expression of TNNT2 also peaked on day 12 in control co-cultures, but there was a non-significant drop in TNNT2 expression in PCDE treated co-cultures. The expression of NANOG, Brachyury, and BMP-4 were very similar in both control and PCDE co-cultures. These results were most likely both influenced by both the feeder cells and the novel small molecule, PCDE. In earlier studies, MEF cells have been shown to increase, and in some cases advance, the expression of Brachyury T and NKX2.5 (Pekkanen-Mattila et al., 2012; Ojala et al., 2012). PCDE did not appear how to have impact on the timing of the expression of any transcription factors under study in co-cultures.

Clearer expression differences between controls and PCDE cultures could be seen in the feeder free monolayer cultures. PCDE appeared to increase the expression of the markers Brachyury and NANOG associated with undifferentiated or immature cells, and speed up the expression of the growth and differentiation factor, BMP-4. PCDE treated monolayer cultures also expressed cardiomyocyte specific proteins such as homeobox protein NKX2.5 and cardiac marker Troponin T (cTnT, encoded by TNNT2) less than control cultures. Both NKX2.5 and TNNT2 should be expressed in developing cardiomyocytes (Hartogh et al., 2016). Based on the gene expression analysis alone it appeared that PCDE had a negative effect on the differentiation efficiency, and that it may have delayed or decreased cardiomyocyte differentiation. However, the flow cytometry results did not support this hypothesis.

Initially, it seemed as if the small molecule had increased differentiation efficiency by approximately 68% in PCDE co-culture compared to controls. However, the gene expression results showed similar Troponin T expression and significantly higher expression of NKX2.5 in the controls, on the day of flow cytometry measurements, on day 17. This might suggest that though there were less beating areas and cells altogether in PCDE co-cultures, the culture consisted of a higher percentage of cardiomyocytes. For more reliable results, cells should have been collected from at least two different cultures from three different wells. The lack of difference in cardiomyocyte specific gene expression could be explained by the genes' lowered expression in cells exposed to PCDE, despite the higher number of cardiac cells. This theory was also supported by the monolayer cultures, where there was no statistical difference in the percentage of cardiomyocytes per culture, but the gene expression studies showed lower Troponin T and NKX2.5 expression in PCDE monolayer cultures.

Against expectations, instead of enhancing differentiation efficiency, PCDE seemingly increased the expression of the early differentiation marker, Brachyury, and the early mesoderm marker, BMP4, but may have lowered the expression of cardiomyocyte specific proteins in cardiomyocytes. Though flow cytometry results showed a higher cardiomyocyte percentage in PCDE co-cultures, the combined flow cytometry and qPCR results did not support the hypothesis that PCDE enhances the differentiation efficiency of hiPSCs into cardiomyocytes.

5.2 Phenotype characterization

Differentiated cells were characterized by immunolabeling. The presence of cardiac troponin T and Connexin 43 in the cells proved that the hiPSCs had differentiated into cardiomyocytes and they were successfully dissociated (Vozzi et al., 1999; Ivashchenko et al., 2013). Cx43, which is partially responsible depolarization of cardiomyocytes, was clearly visible and characteristically located in cell junctions in some aggregates also positive for troponin T, but not in others. Some cells also showed clear sarcomeric bands (pictures not shown) characteristic for cardiomyocytes labeled with troponin T (Ivashchenko et al., 2013). The cardiomyocytes were also labeled for both atrial and ventricular myosin light chains. This showed that both atrial and ventricular phenotypes were present in cultures, but also that there were no double positive cells that would suggest the presence of immature phenotypes (Kamakura et al., 2013). Though some research claims proteins such as Cx43 and MLC-2A and MLC2V could be used as indicators of maturity, the current understanding of iPSC derived cardiomyocytes is that they do not reach completely mature status even in long term cultures spanning over a year. (Vozzi et al., 1999; Kamakura et al., 2013) Therefore it can only be concluded that the hiPSCs were successfully differentiated into cardiomyocytes. The attempts to immunolabel the protein products of the gene KCQN1 failed. This was most likely due to dysfunctional antibodies, but it could also be affected by the lack of the voltage gates ion channels as KCQN1 codes for their α-subunit and cells with G589D mutation in the KCQN1 gene may suffer from the loss of function of the voltage gated ion channels (Crotti et al., 2008; Hedley et al., 2009).

5.3 Single cell analysis and beating behavior

Single cell analysis with Beatview is a robust and easy to use technique for the analysis of beating frequency in single cells, which has previously been successfully used for the analysis of beating behavior between arrhythmic cell with LQT1 and healthy phenotypes using (Kiviaho et al., 2015). Differences in beating behavior between control and LQT1 cells from a symptomatic donor were clearly demonstrated in the study. However, no statistically significant difference in contraction and relaxation times between wild type cells and LQT cell lines was recorded. The results of our experiment were similar, although abnormal beating behavior was observed in both control and LQT1 cell lines with no statistically significant difference in the duration of contraction and relaxation phases. This could be

influenced by the fact that we used LQT1 cell lines from an asymptomatic patient, which are proven to have shorter, less abnormal, contraction times compared to cells from symptomatic carriers (Piippo et al., 2001). This is due to the recessive nature of the KCNQ1-fin or G568D mutation used in this experiment. Earlier studies have also revealed that heterozygous G568D mutation is only expressed in mRNA by 20 % of the cell population in a mutated cell line due to allelic imbalance (Kiviaho et al., 2015). Allelic imbalance could be a factor in the asymptomatic phenotype of LQT1 cardiomyocytes in this study.

Some issues should be taken into consideration when attempting video-based beating analysis. It's difficult to distinguish single cells and a cluster of cells from each other, and cell clusters are known to behave differently compared to single cells (for review see Laurila et al., 2015). The detection of abnormal beating behavior in single cells is also a challenge. The beating of monolayers have been previously analyzed with frame rates as low as 7 fps and as high as 125 fps, but in this experiment the frame rate for single cell analysis was limited to 60 fps, which may not be sufficient for the detection of some abnormalities. In addition, the background noise is worse when analyzing a recording taken with a high magnification (≥20X) objective compared to recording of 4X magnification. For the video to be sufficient for analysis the microscope needs to be located on a very steady surface.

Some of the cells analyzed with by video-based beating behavior were successfully sequenced with RNAseq by the FIMM in Helsinki, which proves that the cell picking technique is functional and in the future, it should be possible to combine genetic information of single cells to their beating behavior. The beating behavior can be analyzed from the video taken from before the picking of cells, as was done in this experiment. However, when recording video of beating cardiomyocytes for analysis, recording conditions should be considered. The beat rate of cardiomyocytes *in vitro* decreases in time while the cells are kept outside the incubator regardless of the use of heating plates. Previously the problem has been addressed with perfusion chambers which provide the cells with a constant flow of warm medium and CO_2 (Kiviaho et al., 2015). Perfusion chambers suitable for the picking and imaging of cells were not available for this experiment.

5.4 Future Prospects

Based on this experiment the use of PCDE as a cardiomyocyte differentiation enhancer is not possible. However, more information on the specific role of PCDE in the manipulation of signaling pathways would help understand the effects of the novel small molecule on cardiomyocyte differentiation. This in turn would make it possible to tune the differentiation protocol to possibly enable the enhancement of differentiation. In the current protocol PCDE was either used on its own on co-cultures with END-2 feeders or in combination with other small molecules on feeder free cultures, so interaction with other small molecules can't be ruled out in the case of monolayer cultures. Future experiment possibilities might include the exclusion of DMSO and a detailed study of the specific role of PCDE on hiPSCs specifically. The characterization of cardiomyocytes could also be improved. During characterization, the effects recording conditions should be considered and new protocols should be designed for the beating analysis, as changes in temperature and CO_2 concentration can create inconsistency. In addition, cover slips with grids could be utilized to determine the percentages of ventricular and atrial cardiomyocytes present in cultures. This might allow the identification of specific cells imaged for beating analysis and give more detailed information on their cell structure and type, which could lead to a better understanding of the cell characteristics effecting beating behavior. More precise characterization is important for the reliability of general experiments, and toxicity and drug screenings conducted on hiPSC derived cardiomyocytes as they become more commonly used in medicine.

Heart disease is the leading cause of death in western countries, and over 23 million people suffer heart failure annually worldwide (Bui et al., 2010). Congenital long QT syndrome is a contributor to the problem affecting 1:2500 people (Johnson et al., 2008). Because of the lethality of cardiac diseases and the risks of current treatment methods, new treatment options are highly desirable. Human iPSC and iCMs could provide an ethical and renewable tool for patient specific drug testing and transplantation without the risk of immune rejection (Takahashi et al., 2007). These prospects have encouraged many research groups to develop new applications for iPSCs derived cardiomyocytes such as engineered heart tissue for disease modeling and hydrogel matrixes for the creation of cardiac tissue grafts (Cashman et al., 2016; Wang et al., 2016). These inventions could provide a much-needed cure for many heart conditions. The production of hiPSCs and iCMs is currently too

inefficient for mass production but efforts are made to create protocols that could be better applied for the mass production of hiPSC derived cardiomyocytes (Weng et al., 2014; Wang et al., 2016). Though based on this experiment PCDE may not be increase differentiation efficiency, experiments that aim to improve differentiation are necessary for the ultimate goal of mass production of pure and mature cardiomyocyte cultures. In the future, PCDE could be examined further with altered exposure times and a larger sample group.

Today animal models remain a major source of information regarding to drug discovery and disease modeling, but differences in physiology often lead to failure when translating results to human disease (Wendler and Wehling, 2010). For example, in drug tests toxicity should be studied with both rodent and non-rodent models to most accurately predict results in humans because of the major physiological differences. Mouse hearts develop in a similar way as human hearts, but there are differences (Bedada et al., 2016). In both human and rodent fetal hearts cardiomyocytes proliferate as mono-nucleated diploid cells, but rodent cells are faster to withdraw from the cycle. In an adult human heart most cells are mono-nucleated and tetraploid, whereas rodent cells are mostly bi-nucleated and diploid. The differentiation process of human and mouse cells is also known to have differences. For example, C-Myc has been reported to have a positive effect on maintaining mouse ESC pluripotency, but it's also known to induce differentiation and apoptosis in hESCs (Sumi et al., 2007; Cartwright et al., 2005). This supports the notion that the use hiPSCs in drug testing would be a valuable addition to use alongside animal testing or even on its own.

As research moves towards the mass production and clinical applications of hiPSC derived CMs, attention should also be paid towards better characterization techniques to improve possibilities in disease modeling. Between 1994 and 2006 45 % of all drugs withdrawn from the market by US food and drug administration were found cardiotoxic (for review see Dykens and Will, 2007). The main reason behind drug withdraws is the fact that adverse effects are so rare that even the large phase III testing may not reveal them. However, once released to the market 0,1 % suffering from adverse effects could mean more than 10 000 people. Currently, drug testing based on hiPSC derived cells isn't approved as an investigative toxicological method, but in the future hiPSC derived cells could provide a cheap, reliable, and ethical disease model for drug tests. According to an estimation the price of drug discovery could be decreased by US$ 100 million per drug discovery with the use of iPSC technology (for review see Giri and Bader, 2015).

5.5 Conclusions

This experiment aimed to further evaluate the effects of a promising novel small molecule, PCDE, regarding the differentiation efficiency of hiPSCs to cardiomyocytes. The hypothesis was that the PCDE molecule would enhance the differentiation efficiency of hiPSCs into cardiomyocytes. We found that PCDE was fatal to cells in concentrations above 1µM and failed to enhance cardiac differentiation in hiPSCs feeder free monolayer cultures with the concentration of 1µM, though it seemed to increase the number of cardiomyocytes in co-culture. However, the expression of cardiomyocyte specific proteins was not increased in either culture type, which leads to the conclusion that PCDE may have reduced the expression of cardiomyocyte specific proteins. However, the differentiation should be repeated for more reliable results. Another objective of this experiment was to combine single cell video-based beating analysis and single cell genome sequencing for better disease modeling and characterization. We did not find any significant difference between the specific LQT1 and wild type cell lines based on their mechanical beating behavior, but proved that combining single cell sequencing and video-based beating analysis is possible, though we were unable to fully carry out the complete method in this experiment. With improvements to the production, characterization and safety of hiPSC derived cardiomyocytes, the cells could provide safer and more economical treatment, research, and drug development options against heart disease.

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7 Appendices

Appendix 1 The buffers used in the dissociation of differentiated cardio myocytes included the following reagents. Buffers were filtered for sterilization and stored in -20ºC and the pH was adjusted with NaOH. Glucose in KB medium was added after thawing prior to use.

Reagents	Low-Ca buffer (pH 6.9)	Enzyme medium (pH 6.9)	KB medium (pH 7.2)
NaCl	1 M	1 M	-
$CaCl_2$	-	1 M	-
K_2HPO_4	-	-	1 M
KCl	1 M	1 M	1 M
Na_2ATP	-	-	2 mmol/L
$MgSO_4$	1 M	1 M	1 M
EGTA	-	-	1 M
Na Pyruvate	1 M	1 M	1 M
Glucose	1 M	1 M	1 M
Creatine	-	-	0.1 M
Taurine	0.1 M	0.1 M	0.1 M
Collagenase A	-	1 mg/ml	-
HEPES	1 M	1 M	-