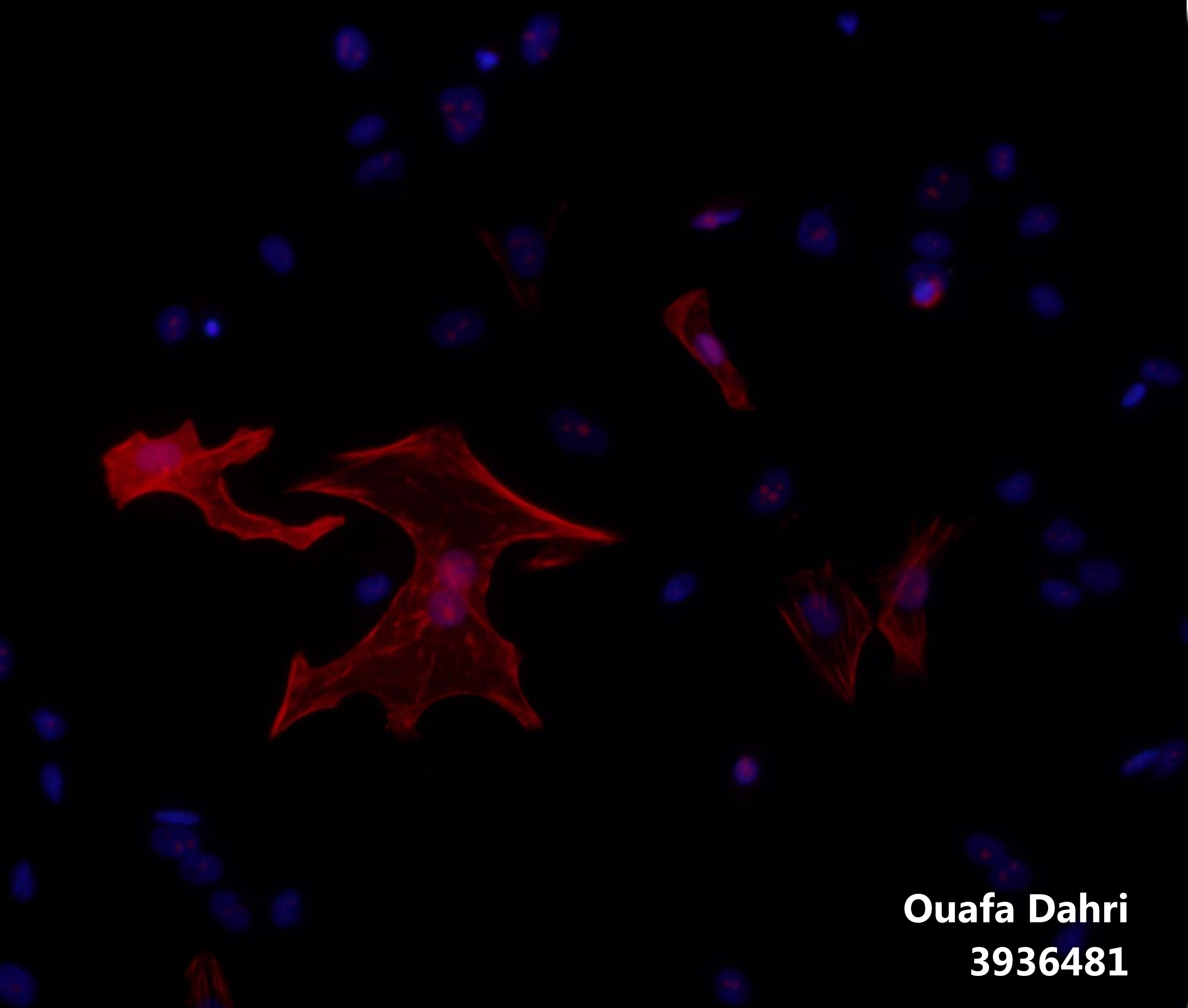
Engineering maturity of human induced pluripotent stem cell derived cardiomyocytes



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Engineering maturity of human induced pluripotent stem cell derived cardiomyocytes

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Preface

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Abstract
Cardiomyocytes are a fundamental cell type in cardiac research. But, obtaining working adult human cardiac cells is difficult. Alternative cell sources such as human pluripotent stem cells might be a feasible source to obtain cardiomyocytes from. Cardiac microtissues derived from hPSCs could provide a platform for high throughput screening to test drug safety. However, even with the best differentiation protocols hiPSC-CMs appear to be immature. The hiPSC-CMs do not show functional and morphological characteristics of an adult cardiomyocyte. Bioengineering contributes significantly to research on maturation of hPSC-derived cardiomyocytes. Maturity is preferably to mimic native human heart physiology. With the use of bioengineering, mechanical, electrical and biofabrication approached have been designed to mature hPSC-derived cardiomyocytes. Mechanical stimulation is critical for cardiac function since the heart undergoes stress continuously as a result of hemodynamic load, physical interaction with the ECM and shear stress. Electrical stimuli determine contraction of the cardiomyocytes and play a role in maturation of several contractile proteins. Biomaterials and supporting devices have been used in the attempt to mature hPSC-CMs. Significant progress has been accomplished in just a few. However, so far no conditions have been reported under which hPSC-CMs obtain full functionality in a similar way as their adult counterparts.

Acknowledgments

With great pleasure I introduce my bachelor thesis which represents the final test of my bachelor at Universiteit Utrecht. I really want to thank certain people that, since the summer of 2013, believed in me, supported me, motivated me and most of all challenged me to give nothing more than my best effort in order to get where I am.

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Ouafa Dahri

Introduction

Drug-induced cardiotoxicity accounts for a major part of drug recalls and regulatory approval delays in the current pharmaceutical industry[1]. These limitations can be attributed to current drug testing strategies. Most strategies rely on animal testing which differ fundamentally human cardiomyocytes on for example electrophysiological properties but also on morphological properties. These differences limit the significance of preclinical studies. In addition, clinical trials have their limitations because of small sample sizes and lack of genetic and phenotypic variability between test subjects. The most ideal cell source should be as close as possible to the human patient, for example cardiomyocytes from the patient's heart himself. However, human heart models are difficult to establish because heart cells or heart tissues from patients are difficult to obtain. Cardiomyocytes isolated directly from heart have been studied, but they do not seem useful since they do no divide in culture[2]. Furthermore, they can only be preserved for a relatively short time thus they do not survive in culture long-term[2,3]. Although electrophysiological characteristics can be analyzed, cultured cardiomyocytes cannot provide a feasible system to reduce drug-induced cardiotoxicity. Together with the fact that cardiovascular diseases remain the leading death cause in the western world[4] it is of significant interest to have relevant cardiomyocyte models to study diseases and to test drug safety.

Ever since the discovery of pluripotent stem cells (PSCs), the development of new strategies to overcome the earlier mentioned limitations seems very feasible. PSCs are cells that are self-replicating and are known to develop into cells and tissues of the three primary germ layers. PSCs include embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). Induced pluripotent stem cells are somatic cells that are reprogrammed to an embryonic-like state. A defined set of transcription factors is introduced to the somatic cells. The somatic cells with the introduced transcription factors are then cultured under embryonic stem cell conditions. This yields to pluripotent cells.[5]

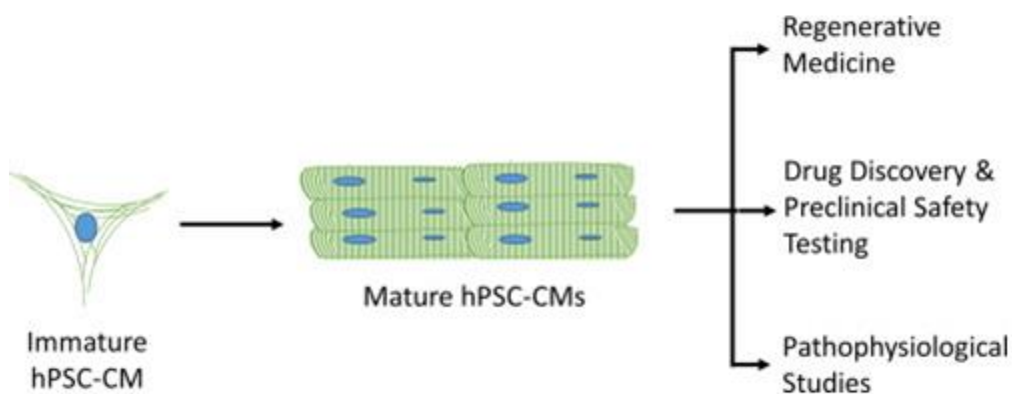

Figure 1 | Applications of hPSC-CMs *Adapted from Feric et al.* [6]
hPSC-CMs are now immature and therefore research attempt to improve maturity. When we are able to mature hPSC-CMs, these cells applied in several ways.

iPS-cells can be differentiated toward different cell types including cardiomyocytes. Human pluripotent stem cell-derived cardiomyocytes (hPSC-CM) are able to offer approaches to study cardiac disease and treatments. For example, patient-specific human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM) lines with monogenetic cardiac defects can now be generated and used as a model to study cardiac diseases and to gain insights into disease mechanisms. Most

importantly, the hiPSC-CMs can be used for testing drugs and for assessment of cardiac drug toxicity *(figure 1)*.[7] From hiPSC-CMs, cardiac microtissues (CMTs) between 3D printed poles can be created. When optimized, these CMTs can be used for high throughput screening to eventually prevent drug-induced cardiotoxicity.

To date, it is well known that independent of the cell line or the protocols used for reprogramming and cardiac differentiation, the obtained hiPSC-CMs have gene expression patterns, structural and functional properties that resemble the human fetal cardiomyocytes phenotype rather than adult cardiomyocytess.[3] The focus of current research is on the improvement of both ESC and iPSC derived cardiomyocytes after differentiation. Bioengineering strategies have been shown to improve the maturation processes significantly. This thesis focusses on bioengineering as a tool for hPSC-CM maturation.

1. Cardiomyocytes in the heart

There are mainly two types of muscle cells in the heart: the cardiomyocytes and the cardiac pacemaker cells. The cardiac muscle contracts by the "sliding filament mechanism", in a similar way to the skeletal muscle. Both anatomy and mechanisms of cardiac contraction are important in the study of *de novo* cardiomyocytes and are therefore important for maturation of pluripotent stem cell derived cardiomyocytes. The following section will focus on contraction and anatomy of cardiomyocytes.

1.1 Anatomy of cardiomyocytes

Cardiomyocytes are short, fat, branched, and interconnected. Each cardiac fiber contains one or at most two nuclei. After birth, cardiomyocytes lose their ability to divide. Because of the low proliferative capacity of adult cardiomyocytes, injured cells must be compensated by the remaining cardiomyocytes therefore their workload increases.[8]

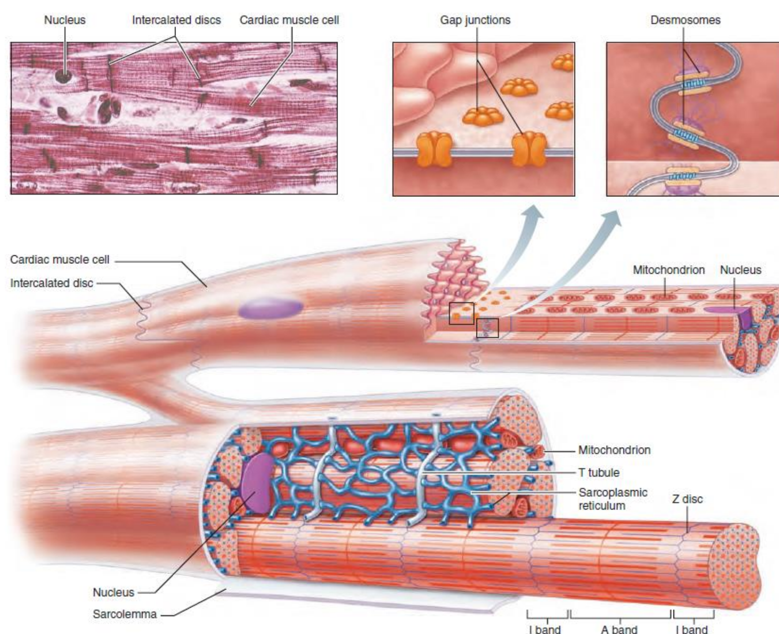

Figure 2 | Anatomy of cardiomyocytes *Marieb, E. N., & Hoehn, K.*[9]
Left a photomicrograph of cardiomyocytes is depicted (600x). The cardiomyocytes are short, branced and striated. Intercalated discs and junctions between adjacent cells are dark stained.

The adult cadiomyocytes is around 15-30 µm in diameter and 85-120 µm long. The cardiac muscle contains transverse lines, the intercalated discs that cross the fibers at places where the cardiomyocytes join. These intercalated discs form the border between adjacent cells and consist of complex junctions. Transverse regions of the intercalated discs consist of numerous desmosomes and fascia adherent junctions. Together these junctions provide strong intercellular adhesion during contraction of the cells. Longitudinal regions of the intercalated discs are full of gap junctions and provide ionic transfer between the cells. These regions are the electrical synapses of the heart and promote quick impulse conduction through many cardiomyocytes and allow the cells to act like a multinucleated syncytium.[10]

Contractile proteins are essentially the same as skeletal muscle proteins, both functionally and structurally. However, the T-tubules, which are invaginations of the sarcolemma are more abundant and larger. In contrast, the SR is less in number compared to skeletal muscles. About 40% of the

cell volume is occupied by mitochondria which is in line with the continuous need for aerobic metabolism.[10] Most of the remaining volume is occupied by myofibrils composed of sarcomeres. The sarcomeres consist of Z-discs, A-bands, and I-bands which consist of thick myosin and thin actin filaments. The myofibrils of cardiomyocytes vary in diameter and branch extensively.[9] Figure 2 provides a schematic overview of the anatomy of cardiomyocytes.

1.2 Contraction of cardiomyocytes

Cardiomyocytes are autorhytmic and can therefore initiate their own depolarization and through that the depolarization of the rest of the heart. Gap junctions connect all the cardiomyocytes together into one contractile unit. Cardiomyocytes act according to the "all or none" phenomena. This means that either all fibers in the heart contract as a unit or the heart does not contract at all. The depolarization wave travels across the heart from cell to cell via ion passage through the gap junctions.[9]

The cardiac action potential is divided into the phases 0 to 4 *(figure 3)*. Phase 4, the resting potential, is stable at ≈−90 mV in adult cardiomyocytes. Phase 4 is followed by phase 0, the phase of rapid depolarization. The membrane potential now becomes positive. The rapid repolarization phase, phase 1, sets the potential for the action potential. Phase 1 is followed by a plateau phase, the longest phase of the cardiac action potential. The last phase, phase 3, is the rapid repolarization phase that reinstates the membrane potential. A complete cardiac action potential lasts for 200 ms. The separate phases have different potentials which involve smaller currents directed to inward or outward. The inward currents are I_{NA}, I_{Ca}, and I_f. The outward currents are I_{KAch}, I_{K1}, I_{to}, I_{Kur}, I_{Kr}, and I_{Ks}. The sodium-calcium exchanger (NCX) is electrogenic and generates either inward or outward currents.[11]

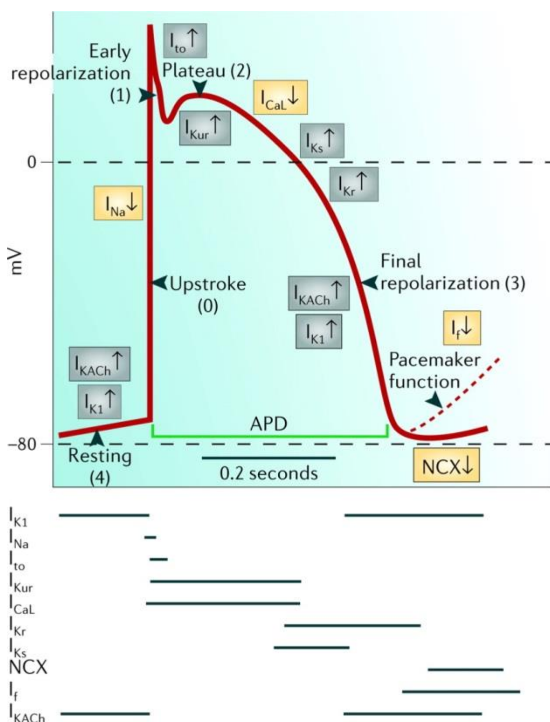

Figure 3 | Cardiac action potential *Adapted from Grant.*[11]

Cardiac action potential consists of 5 phases. Resting (4), Upstroke (0), Early repolarization (1), Plateau (2) and final repolarization (3). Abbriviations: APD; action potential duration, NCX; sodium-calcium exchanger. The duration of the currents that play a role during the cardiac action potential are shown with stripes under the cardiac action potential.

Contractility of individual cardiomyocytes is regulated by the contraction-relaxation cycle. The coordinated contraction is due to the intercalated discs. Intercalated discs distribute action potentials over the heart and cause synchronized contractions. Contraction occurs through a process known as excitation contraction coupling (ECC). During this process an electrical stimulus, the action potential, is converted to a mechanical response, the contraction. The ECC depends on calcium-induced calcium release, the conduction of calcium ions into the cell that triggers further release of ions into the cytoplasm. Ca^{2+} homeostasis in the cell is accurately controlled by ion channels and exchangers. Ca^{2+} release, and therefore contraction of the cardiomyocytes, is predominately achieved via the electrical activity of the sarcolemma. The cardiac action potential initiates with depolarization of the sarcolemma and sustains in the plateau phase when voltage-gated L-type Ca^{2+} channels ($I_{Ca,L}$) are activated as seen in figure 3. [8] Myosin filaments slide along actin filaments in order to contract the muscle. The sliding filaments shorten or lengthen the muscle fiber in order to reach contraction and relaxation.

Contraction of the heart can be summarized as follows. Pacemaker cells induce an action potential that is conducted through gap junctions in the intercalated discs. The action potential travels between sarcomeres and activates Ca^{2+} channels in the T-tubules. This results in an influx of Ca^{2+} into the cell. Ca^{2+} binds to cardiac troponin-C and moves the troponin complex away from the actin binding site where after actin bind to myosin in order to initiate contraction. The head of the myosin moves the actin filament in the direction of the centre of the sarcomere resulting in contraction of the muscle. After contraction, the intracellular calcium concentration drops by removal of Ca^{2+} trough the SR. Troponin-C returns to its position and the contraction is then ended.[9] Troponin-C is an important element in contraction and is, as we will see later on, subjected to many researches.

Cardiomyocytes are a fundamental cell type in cardiac research. But, obtaining working adult human cardiac cells is a difficult process. In addition, feasibility of the use of adult cardiomyocytes for pre-clinical drug discovery or cardiac drug toxicity screening is low since their limited proliferation capacity and their incapability to undergo long-term culture.[12] Alternative cell sources such as pluripotent stem cells might be a feasible source to obtain cardiomyocytes from.

2. Human induced pluripotent stem cells and their immaturity

Human embryonic stem cells (hESCs) isolated from the inner cell mass of blastocysts are pluripotent and have the capacity for unlimited proliferation. Although these hESCs seem to have potential to provide an unlimited supply of cells to investigate diseases or even to restore organ functions after damage, ethical questions arise.[13] The discovery of induced pluripotent stem cells may overcome these concerns.

2.1 Differentiation of hiPSC-CMs

Induced pluripotent stem cells are mature somatic cells which are reprogrammed with a set of transcription factors to embryonic pluripotent state.[14] In 2006, Takahashi and Yamanaka showed for the first time that mouse fibroblasts can be reprogrammed to embryonic stem-like pluripotent cells. This has been done by retroviral transduction with use of four transcription factors: OCT4, SOX2, KLF4 and MYC.[13] In 2007, Yu et al. generated human induced pluripotent stem cells. They used lentiviral transfer of *OCT4, SOX2, LIN28* and *NANOG*.[15] To date, to avoid permanent genomic integration of viral vectors, various non-viral vectors have been successfully employed to generate iPSCs.[13] These iPSCs can then be used to be differentiated towards different cell types including differentiation towards cardiomyocytes.

Differentiation of *in vitro* cells into cardiomyocytes mimics the steps of embryonic cardiac development including activation or inhibition of signalling pathways.[16] Studies with animal models have demonstrated signalling pathways that are key in the establishment of the cardiovascular system.[17] The first methods to differentiate pluripotent cells into cardiomyocytes were developed in mice. Later on, similar protocols for human cells were established.[16] Cardiomyocyte differentiation is arranged by expression of sets of genes in specific stages. *(table 1)*[13]

Stage	**Genes**
mesoderm formation	*BRY, MIXL1, FOXC1, DKK1*
cardiogenic mesoderm	*MESP1, ISL1, KDR*
specific progenitors	*NKX2.5, GATA4, TBX5, MEF2C, HAND1/2*
cardiomyocyte maturation	*ACTN1, MYH6, TNNT2*

Table 1 | Sequential expression of different sets of genes in specific stages.

For redirecting iPSC cells toward cardiac differentiation it is important to understand heart development. Growth factors have an important role in the establishment of the heart. Bone morphogenetic protein (BMP) signaling promotes cardiogenesis, wingless in Drosophila (Wnt) proteins play a role in cardiac specification. Fibroblast growth factors (FGF) contribute to myocardial differentiation of mesodermal cells. During gastrulation at day 5 of embryogenesis the mesoderm arises. The proximal epiblast will contain NODAL signalling and in the extra-embryonic ectoderm bone morphogenic protein 4 (BMP4) expression will be maintained. NODAL and Wnt signalling will be restricted in the posterior epiblast through expression of the genes *Dkk1*, *Cer1*, and *Lefty1*. Before the epithelial-mesenchymal transition of the anterior primitive ectoderm, *T(Brachyury)* and *Eomes*, two mesendodermal markers, will be expressed under influence of WNT. These two mesendodermal markers are involved in the expression of MESP1, the key regulator of cardiac specification. [18]

If DKK1 is present, MSEP activates the differentiation of cadiac progenitor cells. Terminal cardiomyocyte differentiation is coordinated by interaction of transcription factors such as Nkx2.5, Mef2C, Gata-4, and Tbx5. Wnt canonical signalling preserves the cardiac progenitor cell pool and

NOTCH signalling controls the differentiation. Further development of the complete myocardium involves the pathways of BMP, Wnt and fibroblast growth factor (FGF). Recent research identified BMP, FGF, NODAL, and Wnt signalling as the four key signalling mechanisms involved in the cardiac differentiation of iPSCs. This indicates an interesting correlation between *in vitro* cardiac differentiation and the native heart development.[18] Crucial for controlling the signalling pathways during directed differentiation of cardiomyocyte are timing and concentrations of these growth factors.

2.2 Immaturity of hiPSC-CMs

Applications of human derived iPSC-CMs look promising, however many lines of evidence indicate that under the conditions currently used, hiPSC-CMs resemble immature cardiomyocytes. Even with the best differentiation protocols we see that hiPSC-CMs show immaturity features.

Human pluripotent stem cell derived cardiomyocytes have been reported to have a size around ≈600 μm^2, which is significantly smaller than human adult cardiomyocytes. Cell size is an important characteristic of a cardiac cell since it influences impulse propagation, action potential depolarization, excitation-contraction-coupling and total contractile force. Also, membrane capacitance is proportional to cell surface area. An adult human cardiomyocyte has a membrane capacity around≈150 pF, compared to 17.5±7.6 pF in hPSC-CMs. In addition, the shape of hPSC-CMs is circular instead of rod shaped. Immature cadiomyocyte express fundamental proteins of contraction such as cardiac troponin T, cardiac troponin I, α-actinin, and β-myosin heavy chain (β-MHC), but expression levels are lower compared to adult cardiomyocytes. Also sarcomeres are shorter and disorganized whereas adult sarcomeres are more organized in that force generation can be facilitated.[20]

Myofibrillar protein isoforms undergo switching during development of cardiomyocytes. Titin switches from N2BA to the shorter and stiffer form N2B. Troponin I in the adult cardiomyocyte is present as cardiac troponin I whereas the developing cardiomyocyte contains slow skeletal troponin I. From the isoform of the MHC protein in human hearts, the β-MHC is higher expressed than α-MHC at all stages of life except in fetal hearts. hPSC-CMs appear to have the immature isoforms. Transverse tubules (T-tubules) are a hallmark of maturation since they play a big role in ECC. Studies in hPSC-CM showed that hPSC-CMs have a few or no T-tubles at al. Mitochondria in immature cell account for a small fraction of the cell while adult cardiomyocytes occupy on average ≈40%. As a result, the immature cells use glucose instead of fatty acid as a metabolic substrate. In summary, the hiPSC-CMs do not show the morphological characteristics of an adult cardiomyocyte *(figure 4)*.[19,20]

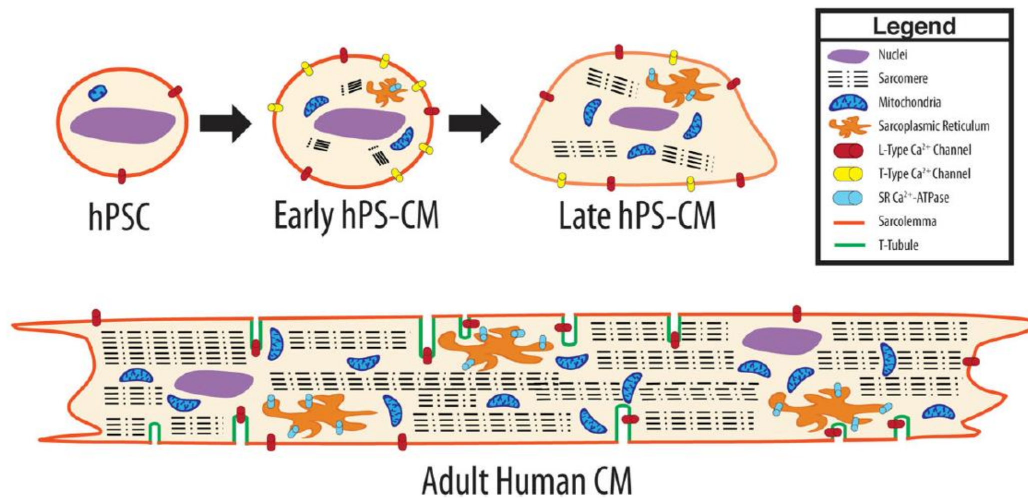

Figure 4 | Visualization of differences between hPSC-CMs and adult CM *Adapted from Robertson et al.*[20]
Late hPSc-CM are different from adult CMs regarding shape, nuclei, amount of mitochondria, sarcomeric areas and receptor expressions. Late hPSC-CMs lack T-tubules.

Furthermore, immature cardiomyocytes have lower levels of I_{K1} channels resulting in a higher membrane potential of ≈−60 mV. Contractile force in both immature and adult cardiomyocytes is the least studied parameter. The differences reported between hPSC-CMs and adult cardiomyocytes is enormous. The force in immature cells is in nN-range/ cell and in adult µN-range/cell. As for the excitation-contraction coupling, it is partly developed in immature cardiomyocytes. The gap junctions are distributed circumferential in hPSC-derived cardiomyocytes while adult cardiomyocytes display highly concentrated proteins in the intercalated disks at the ends of the cells. As a result, conduction velocities of hPSC-CMs are lower compared to adult cardiomyocytes. Upon stimulation of hPSC-CMs with the β-adrenergic agonist, isoproterenol (isoprenaline) a dose-dependent increase of the frequency of spontaneous beating can be induced. However, an inotropic response was not observed most likely due to the immaturity of the sarcoplasmatic reticulum.[19]

Table 2 provides an overview of the differences between the immature and adult cardiomyocytes. These immature cells should be directed towards more mature cells in order to mimic human physiological conditions of the heart in a better way. Immature features are not unique for cardiomyocytes derived from iPSC, also cardiomyocytes derived from ESC appear to have the same immaturities. Therefore, these two cell types have been used next to each other when investigating maturation of pluripotent stem cell derived cardiomyocytes.

	Parameters	Immature cardiomyocytes	Adult cardiomyocytes
Morphology	Cell shape	Circular	Rod shaped
	Membrane capacitance	17.5±7.6 pF	≈ 150 pF
Sarcomere	Structure	Disarrayed	Higly organized
	Length	≈ 1,6 μm	≈ 2,2 μm
Myofibrillar isoform	Titin	N2BA	N2B
	Troponin I	ssTnI	cTnI
	MHC	β>α	β>>α
T-tubules		Absent	Present
Mitochondria		Irregular reticular network in cytoplasm	About 40% of cytoplasm
Metabolic substrate		Glucose	Fatty acid
Shape nucleus		Mono-nucleated	≈25% multinucleated
Electrophysiological properties	Resting membrane potential	≈ -60 mV	≈ -90 mV
Contractile force		≈ nN-range/ cell	≈ μN-range/cell
Excitation-contraction coupling		Partly developed	Mature
Gap junction distribution		Circumferential	Polarized to intercalated disks
Responses to β-adrenergic stimulation		Chronotropic response present. Inotropic reaction absent.	Chronotropic response present. Inotropic reaction present.

Table 2 | Overview differences between immature and adult cardiomyocytes. *Adapted from Yang et al* [19]
These data refer to human pluripotent stem cell derivatives. Abbreviations: slow skeletal Troponin I (ssTnI); cardiac troponin I (cTnI); Myosin Heavy Chain (MHC);

3. Bioengineering methods for maturation of hPSC-CMs

Bioengineering is the application of engineering principles to biological systems. Bioengineering can include elements of electrical and mechanical engineering, computer science, materials, chemistry and biology. Bioengineering focusses on its practical application of scientific knowledge to solve problems in a cost-effective manner. [21] With the use of bioengineering, significant research on maturation of hPSC-derived cardiomyocytes has been conducted. As mentioned earlier, hPSC-CMs resemble cardiomyocytes of a human foetus. The hPSC-CMs show compared to adult cardiomyocytes disorganized sarcomeres, small action potentials and many morphological immaturities *(table 2)*. Maturing hPSC-CMs to a more adult state would increase their value for drug-toxicity studies significantly. Cardiogenesis is regulated by the interplay between biochemical, mechanical and electrical stimuli. All these stimuli should be taken into account when methods for *in vitro* engineering of cardiac tissues are being designed. In this section mechanical stimulation and stresses, electrical stimulation and biofabrication in the context of hPSC-CM maturation will be discussed.

3.1 Mechanical stimulations and stresses

Mechanical stimuli are a critical aspect of the cardiac function. The heart undergoes mechanical stress continuously as a result of hemodynamic load, physical interaction with the extra-cellular matrix (ECM) and shear stress. Mechanical stretches in the heart force cells to change shapes through transduction of mechanical forces via the cytoskeleton. The mechanical forces are followed by changes in gene expression pattern of the cardiomyocytes.[22] Cardiac tissues derived from hPSCs do not have proper force-frequency relationships leading to immature cardiac tissues making them less useful for drug-toxicity screening. As mentioned earlier, contraction of cardiomyocytes is caused by binding of calcium to cardiac troponin C (cTnT). It is therefore of interest to apply stimuli that up-regulates the expression and assembly of cTnT. Bioreactors with mechanical stimulation could contribute to the contractile function of the cardiac tissues derived from hPSCs[23]. The ECM and cytoskeletal organization are also known to respond to mechanical stimuli. Because mechanical stimulation is such a critical aspect of cardiac function much effort has been put into implementing mechanical stimuli *in vitro*.

Schaaf et al. placed two silicone posts in each well of a 24-wells plate filled with a matrix consisting of fibrinogen and thrombin together with hPSC-CMs to create human engineered heart tissues (hEHTs) and to analyze if the format used improves maturation of the derived cardiomyocytes. The two ends of the created microtissues were anchored to the silicone posts. They compared the cardiomyocytes from hEHTs to cardiomyocytes from embryoid bodies (EBs). Histology showed that cardiomyocytes in EHTs had better sarcomeric organization and alignment compared to those from EBs. This could be explained by the straight force lines imposed by spanning of the tissue between the two silicone posts. Furthermore, the hEHTs constantly perform contractile force against the elastic posts which could be an explanation for the better sarcomeric organization. Also, compared to EBs the cardiomyocytes from hEHTs had a significant increase of transcript concentrations of β-MHC, the adult isoform in humans. Thus, the microtissues display a highly organized cellular alignment and intracellular structures. However, the cardiomyocytes appear to be electrophysiologically immature.[24]

Similar to pre-defined physical conditions, external mechanical stimulations have been demonstrated to contribute to the development of mature 3D cardiac tissue from hPSC-CMs.

External direction stresses both static and cyclic were shown to improve hPSC-CMs.[25] Tulloch et al. found in rat neonatal cardiomyocytes (rNC) a significant 2-fold increase of cell alignment with either cyclic or static stress conditioning (alignment values of 5.30 and 5.41 respectively) compared to no stress conditions (alignment value of 2.68). However, there was no significant difference between static stress and cyclic stress. In addition, they looked into intercellular alignment. Binucleation and sarcomeric banding perpendicular to the direction of stress were observed in the cardiomyocytes that underwent static stress. Tulloch et al. also looked into hPSC-CMs and the ability of exogenous stress to promote cardiomyocyte self-organization. They did so by generating human cardiac constructs and subjected them to no stress, static stress, and 1 Hz cyclic stress conditioning for 4 days. Both static and cyclic stress did show a significant and strong increase in cell alignment. As with rNCs there was no significant difference between static and cyclic stresses. Thus stress, either cyclic or static improves cell alignment.[25]

Ruan et al. investigated mechanical stress on maturation of cardiovascular progenitors (CVP) derived from hiPSC-CMs. They found that mechanical stress conditioning in a 3D bioengineered tissue environment influences cardiac maturation of cell types derived from a single multipotent progenitor. Also, they demonstrated an increased force production and calcium handling of the cells that underwent cyclic stress in this engineered tissue in comparison to no stress or static stress conditions.[26]

Mihic et al. seeded absorbable gelatin sponges with hESC-CMs and divided the constructs in two groups: a static culture condition which was used as a control and a stretching culture condition in which constructs were uniaxially cycled between stretched and relaxed states. Cyclic stretching was performed with a frequency of 1.25 Hz. Stretched constructs were cultured under for 72 h, and control constructs remained under static but under the same culture conditions. They reported hallmarks of maturation, such as cell elongation, increased expression ion channels and gap junction proteins. Expression of L-type voltage- gated calcium channels increased 4.90-fold in stretched constructs compared to control constructs. Also expression of other ion channels such as voltage-gated sodium channels and inward-rectifier potassium channels were upregulated (5.25-fold and 3.23-fold respectively). Also, the stretched condition did show a significant increase of 2.00-fold in the expression of β-MHC. Furthermore, both histology and western blotts showed that stretched constructs expressed more TnT than control constructs. To confirm increased expression of gap junction proteins compared to the control group they immunostained for connexion 43 (Cnx43) and performed a Western blot [27].

Investigators have been assessing the impact of mechanical stimulations in order to mimic the intra and extracellular stresses that cardiomyocytes experience. We can conclude that mechanical stimulation and stresses contribute to maturation of pluripotent stem cell derived cardiomyocytes in a significant way. However, much effort should be put in optimizing systems and combining several conditions in order to make maturation of pluripotent stem cell derived cardiomyocytes more feasible.

3.2 Electrical stimulation

Cardiomyocytes derived from human embryonic or induced pluripotent stem cells are arrhythmogenic. As mentioned earlier, for *in vitro* modelling cardiomyocytes with proper electrophysiological conditions are preferred. The arrhythmogenicity of the cardiomyocytes could be explained by the natural automaticity of growing cardiomyocytes to beat spontaneous and

uncontrolled which can lead to areas with abnormal beating. Furthermore, gap junctions are circumferential distributed in immature cardiomyocytes but proper coupling of cardiomyocytes via connexins is needed for the cardiomyocytes to functional properly.[19,28] Electrical signals are critical to the heart and thus to the cardiomyocytes. However, not much research has been conducted yet on electrical stimulation as a way to structurally mature human pluripotent stem cell-derived cardiomyocytes but are beginning to be explored.

A system termed 'biological wire' or biowire has been designed to combine structural cues and electrical stimulation in order to promote maturation of hPSC-CMs. This system makes it possible to generate aligned cardiac tissue that afterwards can be exposed to electrical stimulation in order to promote cardiomyocyte maturation on a structural and electrophysiological level. The device *(figure 5a)* used has been designed using AutoCAD. The biowire mold has been made of PDMS. A suture was placed in the center of the channel, hESC-CM suspension in collegen type I gel was seeded around the suture. After 7 days, the gel matrix has been remodeled by the cells and they start to contract around the suture. The second step is to apply electrical stimulation for biowire cultivation. This is done by transferring the biowire to an electrical stimulation chamber *(figure 5b)* during the second week of cultivation. The electrical stimulator was set on a biphasic repeating pulse with a pulse duration of 1 ms, an electrical field of 3 V/cm, and a pacing frequency of 1 pulse per second (PPS). Every 24 hours, the PPS was increased as follows: 1.83, 2.66, 3.49, 4.82, 5.15, and 6. After 7 days, PPS was set to 1. After 2 weeks in culture, cells throughout the biowires showed parameters of mature cardiomyocytes including strong expression of cardiac contractile proteins sarcomeric α-actinin and actin. Also, sarcomeric banding of the contractile apparatus and myofibrillar alignment along the suture axis was comparable to the structure in adult hearts. Second, calcium handling and cardiomyocyte electrophysiology maturation were improved due to biowires. Maturity of these cells was also examined with caffeine. Mature cardiomyocytes respond to caffeine by inducing an abrupt release of Ca^{2+}. hESC-CMs under electrical stimulated circumstances were responsive to caffeine by induction an increase in cytosolic Ca^{2+}. These cells were compared to non-stimulated controlled cells. Quantification of Ca^{2+} transient amplitudes indicated that electrically stimulated cells have significantly higher amplitude intensity in response to caffeine than non-stimulated controls, in a stimulation frequency-dependent manner contributing to the evidence of maturity due to the biowire system. Also, compared to non-stimulated biowire cells, the stimulated cells showed an improvement of hERG current and inward rectifier current (I_{k1}) densities. Considered these results, it suggested that promotion of hPSC-CM maturation can be achieved through biowires and electrical stimulation. [29]

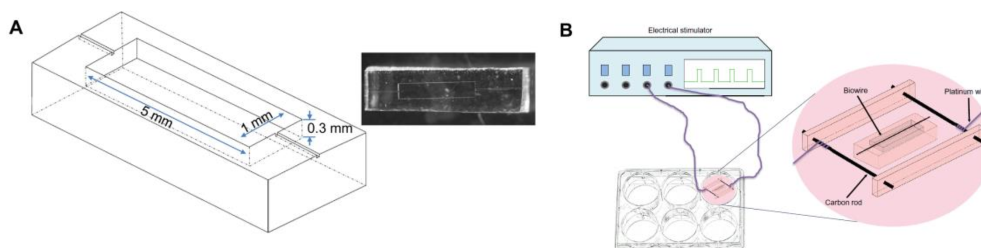

Figure 5 | Biowire system *Adapted from Sun et al.*
(A) Schematic (left) and actual (right) PDMS mold of biowire. (B) The biowire was transferred to electrical stimulation chamber during the second week of cultivation with the biowire placed vertical to the carbon electrodes.

Tan et al. attempted to improve hPSC-CMs organization in cardiac spheroids through incorporation of electrically conductive silicon nanowires (e-SiNWs) in order to mature these cells. SiNWs are biodegradable making them potential for *in vivo* application. The e-SiNWs create electrically conducting microenvironments and induce synchronized and enhanced contraction. These constructs showed promotion of structural and contractile maturation. They also examined if e-SiNWs alone were sufficient enough to derive matured hiPSC-CMs through long-term culture of 3 weeks. Improvements seen at day 7 such as contraction amplitude, expression level and assembly of contractile proteins maintain at day 21. However, long-term culturing did not result in additional improvements in the maturation of hiPSC-CMs. For future research, additional chemical and/or physical stimuli in combination with e-SiNWs-reinforced human cardiac spheroids could contribute to the production of fully mature hiPSC-CMs. [30]

Eng et al. hypothesized that electrical stimulation can structurally mature human stem cell-derived cardiomyocytes and alter their intrinsic beating properties. The cardiomyocytes used for this experiment were cultured as three-dimensional embryoid bodies (EBs) formed from either hESCs or iPSCs. They used a custom-designed microbioreactor that delivered electrical signals continuously for 7 days. The device could provide different stimulation frequencies. They stimulated the cells with 0.5, 1 or 2 Hz and compared these to unstimulated controls. They demonstrated that cardiomyocytes adapted the frequency which they were stimulated with. The 2-Hz stimulated group beated at this rate for over 1 week. The 1-Hz stimulated group showed spontaneous beating rates similar to the control. The 0.5-Hz-stimulated group had a lower beating rate than the control cells which shows that the presence of electrical stimulation alters beating of cardiomyocytes. Additionally, gap junction genes *GJA1* (Cx43) and *GJA5* (Cx40) were found to be upregulated. The higher the frequency which the cardiomyocytes were stimulated with, the higher the gene expression of gap junctions.[28]

In conclusion, electrical stimulation seems to enhance maturation of hPSC-CMs. Systems such as biowire and nanowire could be used on a larger scale when optimized. Electrical stimulation could be used to get cardiomyocytes right on the preferred beating frequency when testing drugs. Besides, preconditioned electrical stimulation contributes to structural maturation of hPSC-CMs. Further study on electrical stimulation is required to investigate the proper preconditions needed for clinical practices.

3.3 Biofabrication

Biofabrication can be defined as the ability of science organize living and nonliving biological products coming from sources like living cells, molecules, extracellular matrices and biomaterials.[31] From biomaterials scaffolds can be made to better mimic human *in vivo* conditions since 2D cultures, which are commonly used for *in vitro* studies, do not come near the real physiological situation. Biofabrication has been used on a wide scale in the attempt to mature hPSC-CMs. This section will discuss biomaterials and supporting devices used for hPSC-CM maturation.

3.3.1 Biomaterials

Feaster et al. investigated a new way to rapidly promote maturation of hiPSC-CMs. The new methodology allows contractile performance assessment at single-cell level. The hiPSC were cultured on a conventional cardiac differentiation media until day 30. After day 30 of cardiac differentiation the cells were dissociated and replated as single cells for 5-7 days on a thicker mattress of undiluted matrigel. The cells were then compared to those cultured on diluted matrigel

(1:60 dilution) and to isolated adult rabbit CMs. In comparison to the control hiPSC-CMs, the mattress-supported hiPSC-CMs showed a rod shape and had increased sarcomeric length *(figure 6)*. Also, the cardiomyocytes displayed spontaneous contractile activity compared to the smaller and circular-shaped control hiPSC cardiomyocytes. Compared to the isolated adult rabbit CMs, the mattress-supported hiPSC CMs showed similar contractile kinetics and excitation–contraction coupling, including the intracellular calcium release from sarcoplasmic reticulum. This new method allows single cell hiPSC-CMs to undergo fast physiological assessments which will be very valuable for high-throughput screening strategies of drugs, assessment of cardiotoxicity and disease modelling. [32]

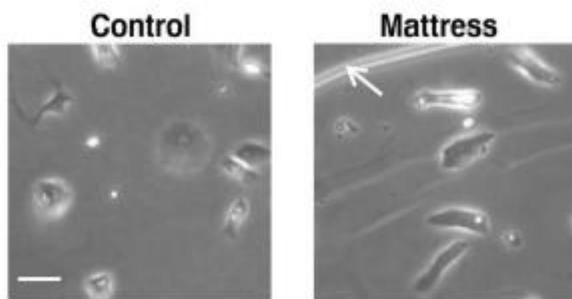

Figure 6 | hiPSC-CMs control vs. matress *Adapted from Feaster et al.*
hiPSC-CMs plated on control (left) and mattress (right). In comparison to the control hiPSC-CMs, the mattress-supported hiPSC-CMs showed a rod shape. Scale bar, 50µm. White arrows indicate the edge of mattress platform.

Matrigel is frequently used in as a biomaterial to mature hPSC-CMs. Similar to Feaster et al., Herron et al. used matrigel in their attempt to mature hPSC-CMs. The ECM plays an important role in differentiation of hPSCs. ECM provides both structural support and signaling molecules to the developing myocardium. With bioengineering approaches, the ECM can be mimicked using biocompatible synthetic materials close to native matrices. Herron et al. investigated the hypothesis that hPSC-CM monolayers can be matured rapidly by plating them on a soft silicone surface. They also investigated the role of integrin signaling in maturation. For this study they used iCell cardiac myocytes, cells which are highly purified (>98%) hPSC-derived cells that are cryopreserved after 30 to 31 days of cardiac-directed differentiation. Their research demonstrated that monolayers cultured on soft PDMS membrane coated with matrigel increases the impulse conduction velocities to ≤48 cm/s, which is 2 times faster than previously reported values for human iPSC-CM monolayers.[33,34]

In addition, they showed that the combination of PDMS with matrigel ECM promotes electrophysiological maturation of single cell hPSC-CM. These single cells showed an increased inward rectifier potassium and an increased sodium inward current densities which gave rise to a polarized maximal diastolic potential (MDP) and faster action potential (AP) upstroke velocity. The MDP is a property of the AP that provides a quantitative metrics for the point of myocyte maturation. The combination of PDMS with matrigel ECM also promoted formation of intercellular gap junctions and mechanical junctions, another sign of maturity. Furthermore, when hPSC-CMs plated on rigid glass coverslips compared to pliable PDMS, hypertrophy of the hPSC-CMs and mature myofilament isoform expression are induced. The remarkable results of this paper show that the electrophysiological and structural maturation of iCell cardiomyocytes plated on the

optimal biomatrix combination can be achieved after replating cryopreserved cardiomyocytes. Previous reports showed modest maturation over a longer period of time (more than 9 months). The short time needed to obtain this level of maturity makes this approach very interesting for further investigation.[34]

3.3.2 Supporting devices

A way to scaffold hPSC-CMs is with tissue-engineered cardiac patches. Zhang et al. investigated cardiac tissue patches using human embryonic stem cell-derived cardiomyocytes. The patches were made from 7 × 7 mm^2 polydimethylsiloxane (PDMS, Dow Corning) molds with staggered hexagonal posts (1.2 mm long). They showed that sheet-like aligned human heart tissues with high conductive velocities up to 25.1 cm/s, contractile forces of 3.0 mN and stresses around 11.8 mN/mm^2, can be engineered. They also showed that hESC-CM maturation in 3D patch environment is enhanced compared to 2D cultures. Maturation has also been confirmed through adrenergic stimulation demonstrating that the engineered cardiac tissue patches had significant adrenergic responsiveness. This study used non-cardiac cells, such as endothelial, smooth muscle, and fibroblastic cells derived from the same hESC source in order to support formation and maturation of functional human myocardium. The patches had high contractile stresses and conduction velocity values that are just 2-3 times lower than values for adult human myocardium.[35]

Electrospinning is a technique that produces anisotropic fibrous scaffolds which can be used to support maturation of several cell types including cardiomyocytes. These scaffolds possess fibrous structures that resemble the native ECM. The fibrous structures can be fabricated with different anisotropies. Han et al. examined whether aligned electrospun fibrous scaffolds could induce the anisotropic cell alignment and thereby improve maturation of hPSC-CMs. They attempt to do so with a couple of experiments. Aligned and isotropic polycaprolactone (PCL) fibrous scaffolds were prepared and they confirmed the anisotropic and isotropic alignment of hPSC-CMs which were cultured on substrates coated with matrigel. Tissue culture polystyrenes (TCPs) were used as a control. After 2 weeks culture the cells were evaluated on structural, molecular and functional properties. Maturation was measured by cardiac protein and cardiac gene expression, evaluation of pharmacological responses and by assessment of intracellular calcium transients. The overall conclusion of Han et al. is that aligned electrospun fibrous scaffolds can induce the anisotropic cell alignment of hPSC-CMs but do not improve the maturation of hPSC-CMs significantly. However, their work provided a proof-of-concept for the use of electrospun aligned fibrous scaffold for efficient hPSC-CMs alignment. Further research should be conducted on combining the cell alignment with other methods to create physiologically relevant microenvironment in order to improve hPSC-CMs maturation. [36]

In contrast to the use of a single polymer for scaffolds, Chun et al. researched combinatorial polymer scaffolds as a tool for *in vitro* maturation of iPSC-CMs. They used a copolymerization technique to synthesize copolymers of different mole percentages of three components. These components are PCL, PEG and cPCL and they are known to alter physicochemical properties that affect cardiac maturation. Every component has its own distinct material properties. PCL was used as the primary component because of biocompatibility, hydrophophilicity, and slow degradation rate. PEG was used to promote hydrophilicity and water adsorption and has a repellent effect on proteins and cells. cPCL was used to increase hydrophilicity and to expose a negative surface charge that was found to reduce the repellent effect of PEG. Their results suggested that 4%PEG-

96%PCL copolymer was the best combination and was shown to promote maturation. This was indicated by several phenotypic features such as organized sarcomeres, abundant mitochondria, increased contractility and higher expression of cardiac myosin light chain-2v, cardiac troponin I and integrin alpha-7. The exact mechanism however remains unknown. In conclusion, this research showed the effects of synthetic biomaterials on human pluripotent stem cell differentiation and the results will contribute to further ongoing research in tissue engineering and regenerative medicine. [37]

Rapidly evolving technology in material science has opened the door for more complex scaffold design. Zhang et al. recently published an article where they reported remarkable results of using their designed AngioChip to engineer heart tissue. The AngioChip is a biodegradable scaffold with a built-in branching microchannel network that supports the assembly of parenchymal cells on a mechanically tunable matrix surrounding a perfusable, branched, three-dimensional microchannel network coated with endothelial cells (ECs). The AngioChip was constructed of poly-octamethylene maleate (anhydride) citrate (POMaC). POMaC polymerizes with UV-light and is biodegradable via hydrolysis it is elastic and has a low thrombogenicity. [38]

Pre-patterned sheets of POMaC formed complex suspended microstructures and internal cavities through layer by layer alignment. A vascular bed within a fully interconnected lattice matrix has been mimicked through the technique of 3D stamping to support parenchymal cells. The AngioChip makes nutrient exchange possible through nanopores. The chip has been designed to function without pumps, allowing access to both the parenchymal space and the internal vasculature. Tissue remodelling is possible since parenchymal cells were cultured within the lattice with native ECMs and ECs were cultured within the internal network. The AngioChip has been used to create vascularized cardiac tissues showing promising results for further research.[38]

The cardiac tissues were engineered from hESC-derived CMs or neonatal rat CMs. To create a human AngioChip cardiac tissue, hESC-derived cardiomyocytes were seeded with 15 µl matrigel at 100–200 million cells/ml onto each AngioChip scaffold. They showed that condensed tissue has been formed within the same time frame as the previous discussed biowire experiment which was non-vascularized. The formed cardiac tissues showed macroscopic contraction. During contraction the scaffold compresses at each beat while being perfused through the internal vessel network. Overall, the AngioChips compared to Biowires showed similar improvement of parameters such expression of cardiac contractile proteins and striated cardiac bundles. However, conduct velocities were lower compared to Biowires. This may be due to the fact that AngioChips were cultivated for a shorter period of time and without electrical stimulation. The AngioChip has been used to create thick cardiac tissue with high density of elongated cells between 1.75-2.0 mm. Another advantage of the AngioChip is that the system contributes through perfusion to cell survival. However, when maturation of the hPSCs has to be taken into account, the Biowire system seems to be better than the AngioChip system. [38]

Furthermore, Rao et al. investigated if microgrooved structured culture substrates influence Ca^{2+} cycling of iPSC-CM and if they contribute to more mature cellular behavior. The lack of mature Ca^{2+} cycling properties in iPSC-CM is an important limitation that has been studied several times. Fabrication of the microgrooves was done with molds made of negative photoresist polymers (SU-8) followed by exposure to UV light. The SU-8 mold had patterned circular areas with a diameter of 14 mm, parallel lines etched into them 10 µm apart, 10 µm wide and 4 µm deep. Then PDMS was

poured over the molds resulting in PDMS scaffolds with microgrooves. *(figure 7)* These were removed from the molds and sterilized by emersion in 70% ethanol and 4 h expose to UV light. The day after the scaffolds were coated with fibronectin and seeded with iPSC-CMs in each well in the 12-well-pate. Cells from microgrooved PDMS substrates were compared to cells from unstructured substrates. iPSC-CM alignment was significantly improved by the microgrooved PDMS substrates.[39] Furthermore, they found that structured tissue culture substrates significantly changed the Ca^{2+} cycling properties of iPSC-CM by shortening the time to peak Ca^{2+} transient amplitude (tP). The transient amplitude controls Ca^{2+} fluxes across the sarcolemma and on SR content. The fast tP could be due to changes in Ca^{2+} entry and trigger for calcium-induced-calcium-release. Another explanation could be that changes in intracellular Ca^{2+} buffering may account for the changes in Ca^{2+} cycling induced by microgrooved PDMS substrates. In conclusion, the study showed that structured tissue culture substrates affect Ca^{2+} cycling and structural properties in cultured human iPSC-CM. However, further improvement of the constructed is needed to investigate Ca^{2+} cycling properties more properly.[39]

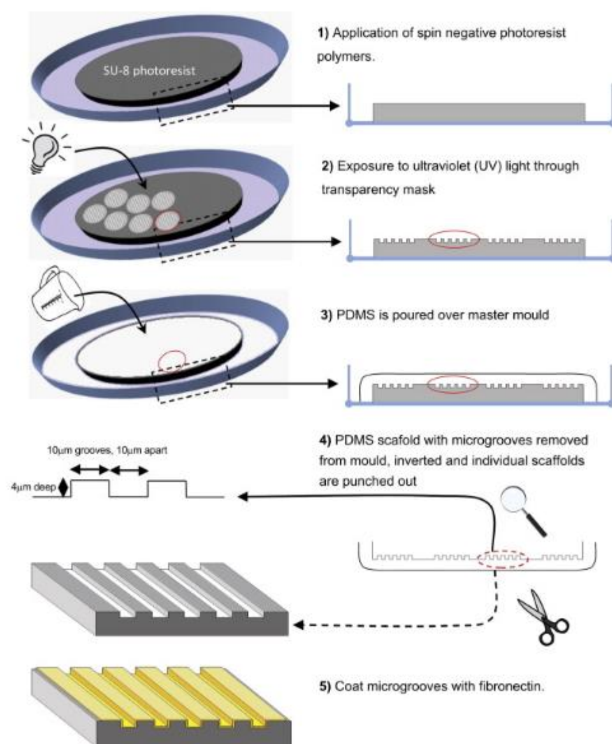

Figure 7 | Schematic overview of the fabrication of microgrooved tissue culture substrates. *Adapted from Rao et al.*[39]
First SU-8 is applied. Then it is exposed to UV light followed by pouring of PDMS over the master mold. The scaffold with the mcirogrooves are removed from the mold. Last, the microgrooved scaffolds are coated with fibronectin.

Much research to mature hPSC-CMs has been conducted and remains ongoing. We have seen that mechanical stimulations and stresses, electrical stimulation and biofabrication contribute in a significant way of hPSC-CM maturation. However, more research is required to optimize systems that already have been established and to discover additional ways to mature hPSC-CMs. As a means to discover new ways, bioengineering approaches should be combined to find the ideal conditions to properly reach the level of maturity that we desire.

As we have seen, from hPSC-CMs cardiac microtissues (CMTs) can be acquired[24]. We aim to miniaturize CMTs with the intention to design a model in which high throughput screening becomes feasible. The next section describes experiments that were performed order to achieve that goal.

4. hPSC-CMs for cardiac microtissues

Introduction

Pluripotent stem cells as mentioned earlier can be used for several applications. The rational of our research is to create a platform that is miniaturized for high throughput screening to test drugs and therefore prevent cardiac drug toxicity.

We tried to create cardiac microtissues with hPSCs to eventually use them in high throughput screening. The microtissues are created between 3D printed poles that are put in a 96-wells plate together with hPSCs and human fetal heart fibroblasts (HFCF) in a collagen gel *(figure 8)*. All these components together will result in a beating ring of tissue. Both hESC-CMs and hiPSC-CMs cells were used. The 3D printed poles provide mechanical stress and are flexible as well so that the microtissues can contract without being limited in their movements. Here we looked at different time points to compare ring formation and cardiomyocyte morphology. In addition, some CMTs underwent pharmalogical assessment as proof-of-principle.

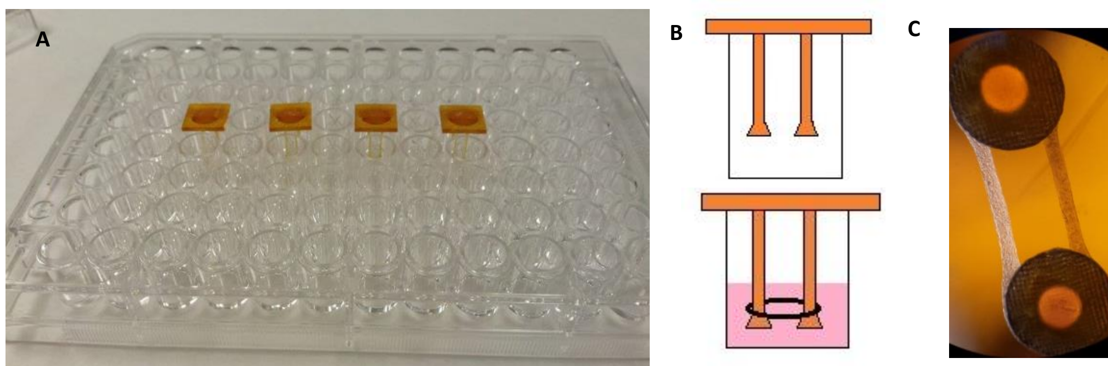

Figure 8 | Experimental set up
(A) Photograph of 96-wells plate with the poles. (B) Schematic overview of poles and poles with CMT and culture medium. (C) Light microscopic picture of cardiac microtissues on the poles.

Materials and methods

Production of cardiac microtissues

HFCFs and cardiomyocytes derived from PSCs were thawed (ES cells from San Diego, iPS cells from San Diego or iPS cells from Hubrecht Institute). A 96-wells was treated with 0.2% Pluronic F127 solution for 60 minutes and washed after with PBS. HCFCs were trypsinized and the hPSC-CMs were loosened with TryplE. The CMs were spinned down for 5 min at 300xg at room temperature. The pallet with CMs was resuspended in 1 mL of CM medium with ROCK inhibitor (Y27632, 1:1000). After cell counting of both HFCFs and CMs, the cells were put together in a 50 mL tube with ratio CMs:fibroblasts = 3:1 and spinned down for 5 min at 300xg at room temperature. The medium was aspirated and the cells were resuspended in a hydrogel (consisting of 165 µl 3 mg/ml collagen, 250 µl of 2x medium, 35 µl of PBS, 50 µl of geltracks and ~ 16.5 µl of NaOH). 15 µl was put into each well of the 96-wells plate which was treated with Pluronic F127 solution. Then the poles were inserted. The constructs were incubated for 30-40 min at 37 °C in a humidified incubator with 5% CO_2 to let the hydrogel solidify. Afterwards 100 µl prewarmed CM medium was added to the well so that the hydrogel was completely immersed. This was done carefully in order to not disrupt the rings. Every other day the medium was changed by adding 100 µl of new medium to the old medium and taking off 100 µl again.

Cryosections

The CMTs were washed in PBS and then fixed in 4% paraformaldehyde (PFA) for 15 minutes. After fixation the CMTs were incubated with 10x diluted food dye (Americolor Soft Gel Paste Food Color) for 3-5 minutes. Food dye ensures the visibility of the tissue in the cryoblocks. After the food dye the CMTs were washed in PBS and incubated overnight at 4 °C in Tissue Tek. After this incubation, the CMTs were transferred to a mold filled with Tissue Tek. The tissue was then frozen with liquid nitrogen and 2-methylbutane. After freezing sections of 7 μm thick were made with a cryostat.

Staining

For both embedding methods the same staining protocol was used. A line around the tissues on the slides was drawn with a Dako pen. The cells were permeabilized with 0.1% Triton X-100 1% BSA in PBS for 10 minutes and after permeabilization washed in PBS for 3x5 minutes. The cells were then blocked with 10% goat serum in PBS for 1 hour and afterwards rinsed shortly in PBS. The slides were then incubated over night at 4°C with primary antibodies* in 0.01% Triton X-100/0.1% BSA in PBS. The following day the slides were washed in PBS for 3x5 minutes and incubated for 1 hour with secondary antibodies* in 1% PBSA at room temperature. After incubation the slides were washed in PBS for 1x5 minutes and 2x10 minutes. Then the slides were incubated with Hoechst dye for 5 min at room temperature and washed in PBS for 1x5 minutes. Finally, the slides were dried and mounted with fluoromount.

*Used antibodies:

For Cryosections

- Primary: Troponin I sc-15368 (Rabbit, 1:50)
- Secondary: Goat anti-Rabbit Alexa-555 (Goat, 1:400).

Quantification of troponin levels

With ImageJ the amount of troponin within individual microtissues was calculated by measuring the percentage red area per tissue.

Assessment of beating rate

Rings were put in warmed medium with serum (37 °C) in a construct which allowed the rings to move freely. Isoprenaline (100 nM) was pipetted into the medium. The microscope used to measure was designed by dr. de Boer and produced by Cairn research. With ImageJ the edge region of the ring was selected and plotted in a z-axis profile to count the contractions (peaks). The beating rate was calculated as follows: 10 seconds (duration measurement) divided by N contractions. Stimulation rate was calculated as follows: $N_{isoprenaline}$-$N_{noisoprenaline}$/ $N_{isoprenaline}$ *100%.

Results

ESC-CMs display more mature cardiomyocytes compared to iPSC-CMs after mechanical strain. After three weeks in culture, the cardiomyocytes derived from ES cells showed more mature phenotypes. The amount of troponin in each cardiac microtissue on different time points was measured. The tissues obtained from ESC-CMs contained a higher percentage troponin *(figure 9.A)*. The iPSC-CMs based tissues were obtained from the Hubrecht Institute and were used a few days after differentiation. The ESC-CMs were from San Diego and were first frozen and then thawed before they were used for the experiments. Although the differences between iPSC-CM and ESC-CM were present, they were not significant. Both cell types formed actual rings *(figure 9.B-C)*.

However, we observed that ESC-CM based rings were contracting as a whole while iPSC-CM based rings were contracting locally *(Supplementary 1 and 2)*. Furthermore, on a microscopic scale *(figure 9D-I)*, ES cells showed more elongated cells compared to iPS cells, in line with the higher percentage troponin. *(Weeks defined in Supplementary 3).*

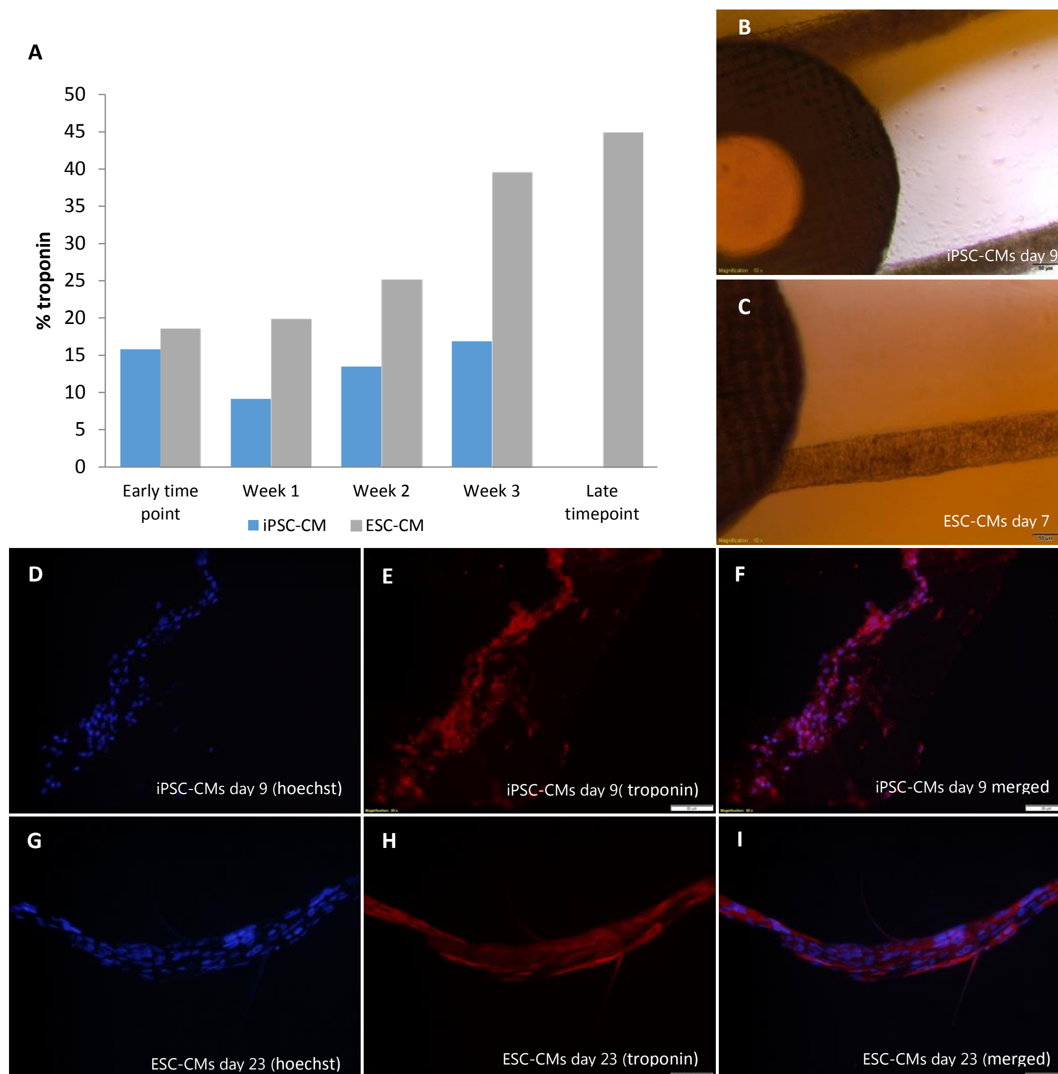

Figure 9 | iPSC-CM based rings compared to ES-CM based rings
(A) Percentage troponin of iPSC-CMs compared to ESC-CMs. iPSC-CMs: Early time point n=4, week 1 n=4, week 2 n=4, week 3 n=1. ESC-CMs: early time n=4, week 1 n=2, week 2 n=3, week 3 n=4, late time point n=1. (B) Light microscopic picture of iPSC-CMs on day 9. (C) Light microscopic picture of ES-CMs on day 7. (D-F) iPSC-CMs day 16 in culture. (G-I) ES-CMs day 23 in culture.

Previously frozen iPS cells show same capacity as ES cells. The differences between iPS cells and ES cells could be explained by the state of the cells before ring formation. Three groups of iPSC-CMs sources were compared to each other. The cell groups used were treated differently before using them for the experiments. The groups used were: iPSC-CMs used directly after differentiation, iPSC-CMs which were first frozen and then thawed and iPSC-CMs which were replated before using them for the experiments. The iPSC-CMs directly from differentiation *(figure 10A-D)* did show elongated cells but less compared to previously frozen iPSC-CMs *(figure 10E-H)* or iPSC-CMs that were replated *(figure 10I-K)*. When we look at the differences in progression of troponin levels over time, we see that iPSC-CM based rings from previously frozen iPSC-CMs show a strong relation between the days in culture and the amount troponin *(figure 10L)*. The progression of troponin on

"week 1" is relatively high compared to "early time point". The same accounts for rings made with iPSC-CMs that were replated. Unfortunately, for "week 3" the replated iPSC-CMs were infected and therefore died which reduced the cell number.

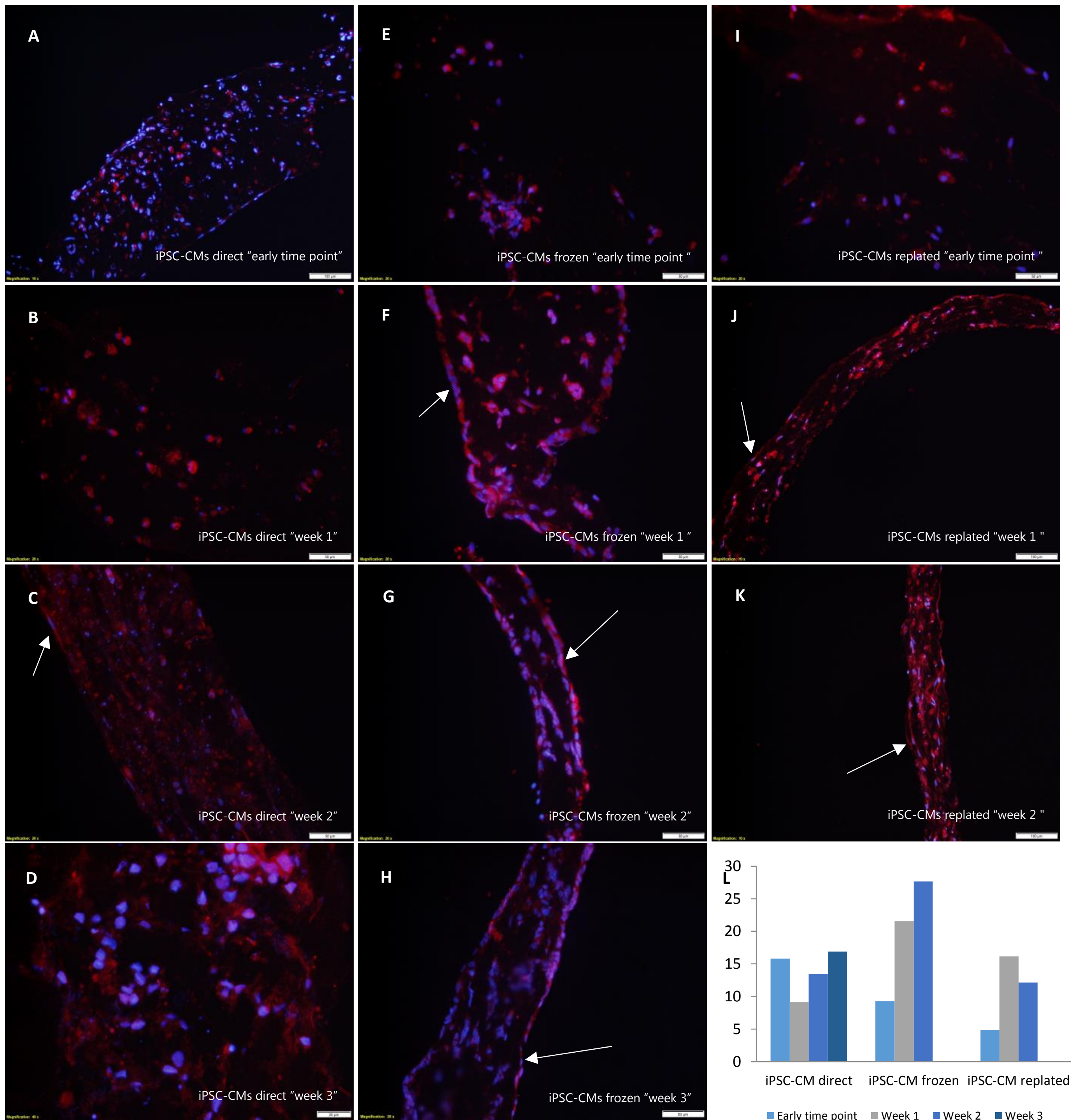

Figure 10 | Different iPSC cell sources White arrows indicate elongated cells (A-D) iPSC-CMs directly from differentation hoechst+troponin I staining. (E-H) Previously frozen iPSC-CMs hoechst+troponin I staining. (I-K) Replated iPSC-CMs hoechst+troponin I staining. (L) Percentage troponin. iPSC-CMs direct: Early time point n=4, week 1 n=4, week 2 n=4, week 3 n=1. iPSC-CM frozen early time point n=1, week 1 n=1, week 2 n=1. iPSC-CM replated: early time point n=1, week 1 n=1, week 2 n=1.

Mechanical strain contributes to mature phenotype of hPSC-CMs. After day 23 in culture one CMT made of ESC-CMs slipped up along the poles. As a result, the CMT did not receive the same amount of mechanical strain. Microscopically we saw that the ESC-CMs lost their elongation and became round cells *(figure 11A-B)*. Macroscopically we saw a more loose tissue around the poles (*figure 11C*). Also the amount of troponin I decreased after slipping up *(figure 11D)* in line with what was seen under the microscope.

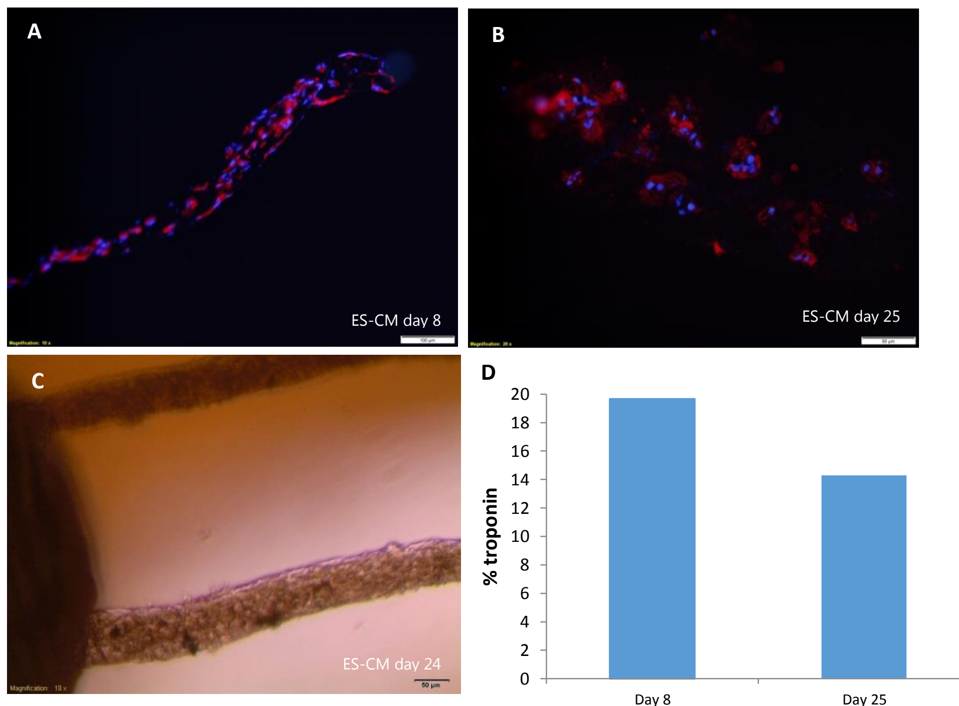

Figure 11 | The effect of mechanical strain on the morphology of hPSC-CMs
(A) Day 8 hoechst+troponin I staining, cells within the tissue are elongated. (B) Day 25 hoechst+troponin I staining, cells within tissue lost elongation. (C) Light microscopic picture of CMT on day 24 in culture. (D) Graph percentage troponin I of day 8 and day 25

Stimulation with isoprenaline increases and synchronizes beating frequency of hPSC-CMs. CMT rings made of ESC-CMs were stimulated with isoprenaline. The ring was placed on its side under a microscope to measure the contraction rate and to calculate beating speed. Before stimulation, the ring was contracting 4.3 times per second with a beating rate of 0.23. Upon stimulation with isoprenaline, the contraction increased to 7.5 times per second and a beating rate of 0.13. The stimulation rate was therefore 42%. In addition, we observed a synchronization of the contractions after stimulation with isoprenaline *(figure 12)*.

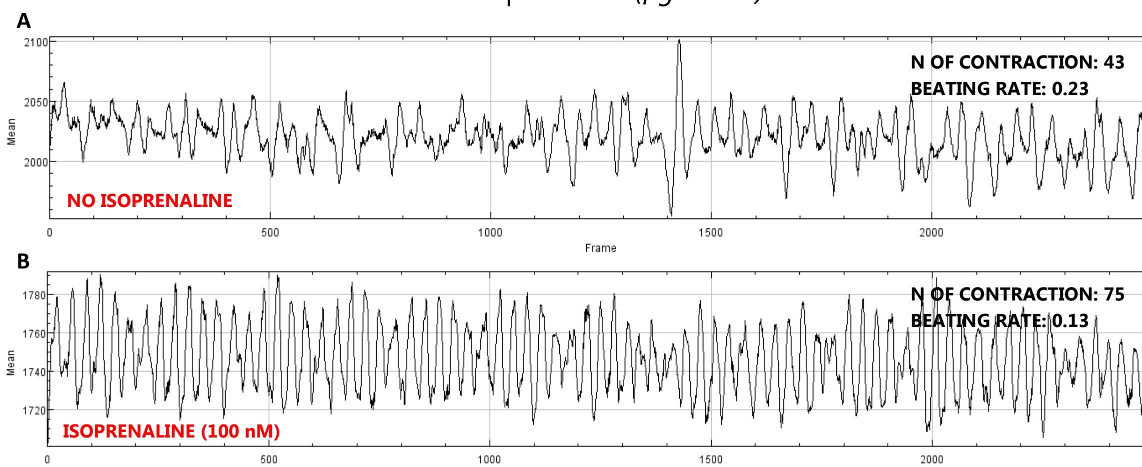

Figure 12 | Stimulation of ring with isoprenaline
(A) Ring before stimulation with isoprenaline. Contractions: 4.3/s. Beating rate: 0.23. (B)Ring upon stimulation with isoprenaline. Contractions: 7.5/s. Beating rate: 0.13.

Discussion

We demonstrated the capacity of hPSC derived cardiomyocytes to create cardiac microtissues in the form of rings. ESC-CM based rings appeared to have more mature phenotypes compared to iPSC-CM based rings. The levels of troponin I were higher. Furthermore, the rings themselves were beating more as a whole while the iPS cell based rings displayed local beating areas *(Supplementary 1 and 2)* .

Troponin levels of iPSC-CMs *(figure 8A)* decreased after "early time point". This could be due to cell death. After "week 1" the troponin I level increased but it was not nearly as high as the troponin I levels of ESC-CMs. The ESC-CMs were obtained from San Diego, therefore the cells were frozen and thawed before they were used. Conversely, the iPS cell-derived cardiomyocytes were obtained from Hubrecht Institute and could be used directly a few days after differentiation. We hypothesized that the differences seen between iPSC-CMs and ESC-CMs were not due to the differences between induced pluripotent stem cells and embryonic stem cells but due to the way the cells were treated before usage in the experiments.

For that reason, three groups of iPSC-CM were compared to each other. The iPS cells from the Hubrecht Institute directly used from differentiation, iPS cells from San Diego which were frozen and thawed and iPS cells from the Hubrecht Institute which were replated. Surprisingly, the frozen iPS cells showed a remarkable increase of troponin I levels over the course of 2 weeks. The levels troponin I of "week 2" were almost as high as the levels troponin of "week 2" of the ESC-CM based rings. The levels of troponin I of the replated iPSC-CMs at "early time point" are lower compared to the frozen group and to the direct group. However, the progression of troponin on "week 1" is relatively high compared to "early time point". Unfortunately, the replated iPSC-CMs were infected after 12 days in culture and therefore could not be compared properly to the other cell sources. Also, we could not infinitely make rings as the cell supply was limited. Hence our sample sizes were too small to calculate if there were any significant differences. However, it seems that freezing and thawing of hPS cells before using them for experiments had a positive effect on the maturation process since the ES cells displayed higher troponin levels. Nevertheless, this experiment should be repeated multiple times in order to get significance and with proper negative controls (ES and iPS cells directly from differentiation).

Our study confirmed that mechanical strain contributes to maturation of hPSC-CMs. Mechanical stimuli are a critical aspect of cardiac function, as mentioned in section "3.1 Mechanical stimulations and stresses". We saw that the cardiac microtissue clearly started to lose elongation after slipping up along the poles. Even though the observations showed maturation lost, additional experiments should be done in order to draw a reliable conclusion.

Similar to the slipping up of CMTs along the poles, several CMTs slipped off the poles. The slipping off limited our study since the poles are essential for the CMTs to keep the maturation process of the hPSC-derived cardiomyocytes going. After slipping off, the CMTs could not be put back on the poles as this would lead to tissue damage. A solution to this problem could be another design for the poles. Figure 8B shows the experimental set up used. As you can see, the poles are not

touching the bottom because the rings should be able to contract without being limited in their movements as contact with the bottom would provide a rigid environment. Slightly longer poles could prevent slipping off as long as the poles do not touch the bottom. Another design that could provide a solution for slipping up or off is a design with double bases (*figure 13)*. If the tissue forms in-between the bases, slipping up of the tissue could be prevented. An addition limitation of this experimental set up is that despite the use of the same amount of cells, the CMTs are shaped differently. We should design a system which combines poles with a mold that could be removed after the tissue obtains a proper shape so that it will not limit movement after formation. Since we miniaturize tissues, it is difficult to use molds that should be removed afterwards. Therefore, we should use a mold dissolves after a short period of time.

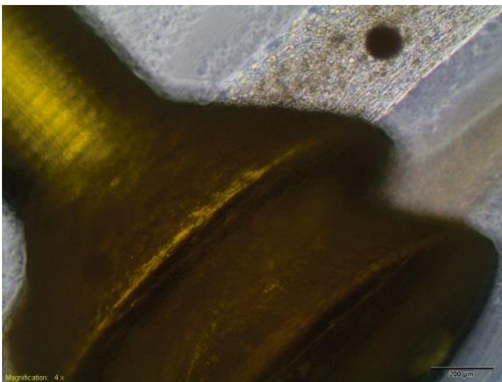

Figure 13 | Poles with double bases
3D printed poles with double bases. In between the bases cardiac microtissue should form to prevent slipping up of the tissue.

Isoprenaline is a nonselective β-receptor agonist. A positive response of cardiomyocytes to stimulation with isoprenaline indicates the presence of β-receptor. As we have seen in table 2, adult cardiomyocytes compared to immature cardiomyocytes contain higher levels of beta receptors. *In vivo* isoprenaline increases contraction rate and amplitude of calcium transient and decreases relaxation time. Literature shows that isoprenaline, unlike in adult cardiomyocytes, does not increase contraction force in hPSC-derived cardiomyocytes[40]. Here we demonstrated, as a-proof-of-principle, that ESC-CM based rings were able to be stimulated with isoprenaline resulting in an increased beating rate and synchronized contractions. However, we were not able to measure contraction force. For further investigation, an experimental set up in which this is possible would be beneficial to assess maturation of hPSC-derived cardiomyocytes.

Conclusion and future perspectives

Cardiomyocyte maturation *in vivo* is controlled by multiple factors and is a very complex mechanism with many pathways involved. Regulation of just one factor may have influence on a set of the overall processes. It is evident that exposure to multiple regulatory factors at the same time will contribute to the maturation of hPSC-CMs. The field of bioengineering continues to progress.

So far, no conditions have been reported under which hPSC-CMs become exactly like adult cardiomyocytes. Therefore, we have not been able to prove yet if it is achievable to reach this state. Nonetheless, not every application of hPSC-CMs needs a fully mature state. By now, hPSC-CMs have been used successfully for human heart disease models and for drug screening assays[41,42]. However, an optimal combination of mechanical, electrical and biochemical stimuli still should be defined to drive hPSC-CM towards adult state.

Cardiomyocytes derived from hPS cells, either from ES cells or iPS cells are able to form cardiac microtissue between 3D printed poles. Efficiency of ring formation and maturation of hPSC-derived cardiomyocytes can partly be attributed to the state of the cells before usage. When cells were frozen and then thawed (both ES and iPS cells) better ring formation and a more mature phenotype was displayed. Stimulation of CMTs with a drug, in our case isoprenaline, is possible. Creating a platform for high throughput screening to test drugs and prevent cardiac drug toxicity after optimizing the CMT formation and maturation seems therefore feasible.

In conclusion, significant progress has been accomplished in just a few years since the discovery and development of hESCs and hiPSCs. Bioengineering as a tool for hPSC-CM maturation is continuously contributing to that development. However, more research should be conducted to promote maturation of hPSC-derived cardiomyocytes since maturation remains essential to fulfill the potential hPSC- derived cardiomyocytes have in cardiovascular biology and medicine.

Supplementary

Supplementary 1: Video CMT from ESC-CMs Day 16 https://vimeo.com/172539565

Supplementary 2: Video CMT from replated iPS cells https://vimeo.com/172540465

Supplementary 3: Weeks defined

Name	**Definition**
Early time point	Day 2 - Day 5
Week 1	Day 6 - Day 7
Week 2	Day 8 - Day 15
Week 3	Day 16 - Day 25
Late time point	Day 33

Supplementary 4: Origin of hPSC-CMs

Cell type	**Origin**
ESC-CMs	San Diego
iPSC-CM direct	Hubrecht Institute
iPSC-CM replated	Hubrecht Institute
iPSC-CM frozen	San Diego

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