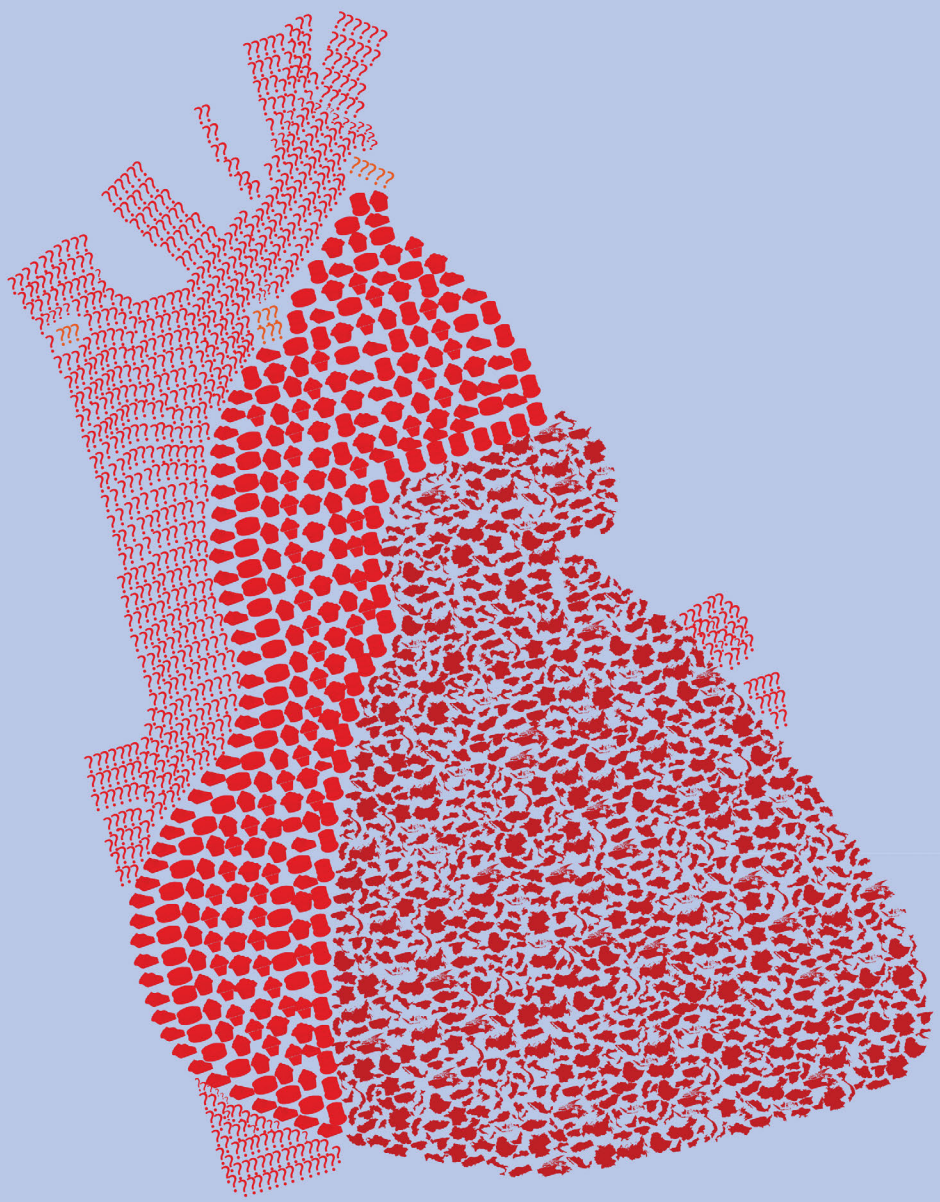
From Teeth, Skin or Blood to Heart:

Induced Pluripotent Stem Cells as an in vitro Model for Cardiac Disease



Cheryl S. Dambrot

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Colophon

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Cover illustraton:
Outline of human heart composed of question marks, baked goods and countries of the world

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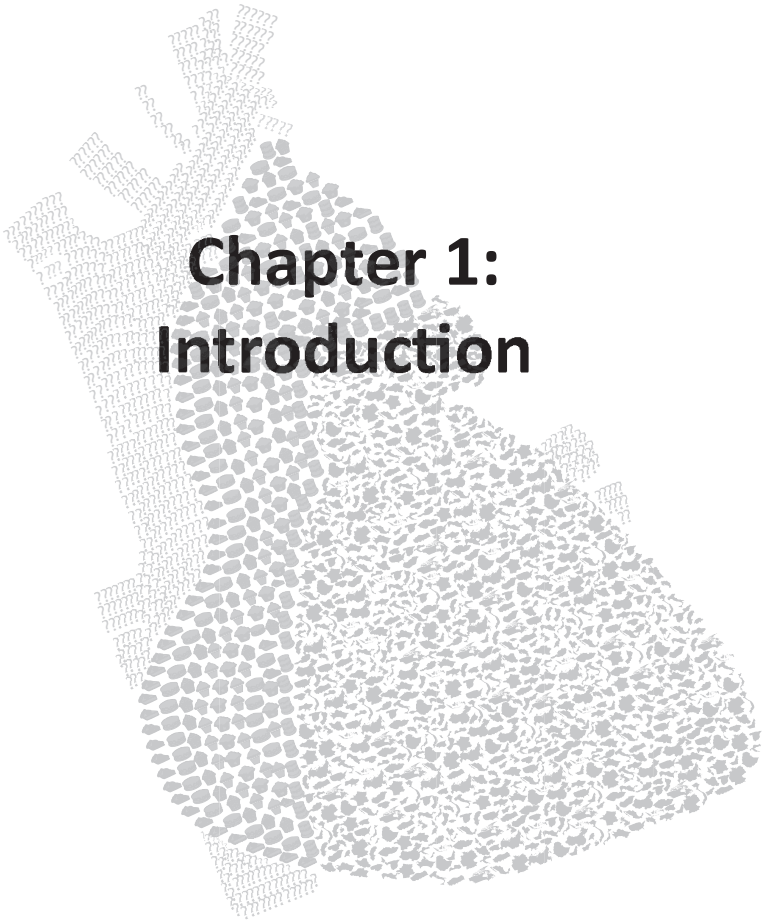

Chapter 1: Introduction

Embryonic Stem Cells

Mouse embryonic stem cells (mESC) have been a key tool in understanding early differentiation events in development since their first isolation in 1981[1]. These cells have the remarkable ability to continuously self-renew in culture and are pluripotent: they have the ability to differentiate into derivatives of the three germ layers that make up an embryo. In addition, mESC can be genetically modified and contribute to all somatic tissues and the germ line in chimeric mice, making them an ideal tool for deleting specific genes to study their function in development and creating disease models. Even though mESC research quickly became a robust technology and the potential for regenerative medicine became clear, it unfortunately it took another two decades before human embryonic stem cells (hESC) were derived [2]. One of the challenges in isolating hESCs was the fundamental difference in their biological characteristics, culturing techniques and the growth factors needed to maintain their pluripotent state compared to mESCs. For example, although both mESC and hESC were first isolated on mouse embryonic fibroblasts (MEFs) [1] mESC needed leukemia inhibitory factor (LIF) to maintain a pluripotent state[3] , whilst activin/nodal signaling was required to maintain hESCs [4]. While hESCs and mESCs do share some surface markers, such as E-cadherin and c-KIT, they have several distinct markers such as SSEA1 being expressed in mESC and not in hESC, whereas SSEA3, SSEA4, TRA-1-81 and TRA-1-60 are expressed in hESC and not in mESC. Even though hESC differ from mESC they are not completely unique as they are rather similar to human embryonal carcinoma (EC) cells (the undifferentiated cells in teratocarcinomas) with respect to alkaline phosphatase (AP) activity, and SSEA3 and SSEA4 expression [5;6] rather than mouse EC cells which also have high AP activity but differ in surface marker expression. This has been hypothesized as being due to differences in the 'ground state' of these cells. hESC are more closely related to mouse Epiblast Stem Cells (mEpiSC) [7;8]. mEpiSC have similar morphology to hESC and are maintained in culture in a similar manner. The derivation of mEpiSCs from the mouse epiblast, which develops soon after the blastocyst forms, brings into question the different ground states of pluripotent stem cells. mESC appear to be in a more naïve pluripotent state than mEpiSC or hESC [9]. Furthermore, it now appears that hESC are not as closely related to the inner cell mass (ICM) of the embryo as mESC [10]. There have been several attempts to transform hPSC into a more naïve pluripotent state but in the few successful reports, the cells appear to be only stable for a limited number of passages [11;12].

Induced pluripotent stem cells: Growth and Consideration

In 2006, Takahashi and Yamanaka from Kyoto University in Japan made a ground-breaking discovery for stem cell research. By introducing only four transgenes into mouse somatic cells, they were reprogrammed to a pluripotent, embryonic stem cell-like state [13]. In 2007 this was also done with human somatic cells [14;15]. This discovery changed the stem cell field in a multitude of ways: ethically because no embryos were required, for cell therapy since genetically matched pluripotent cells could be derived for transplantation, for disease modeling since the patient genome could be captured and understanding of human embryo and disease development. These new induced pluripotent stem cells (iPSC) have helped open the field to many for whom national regulation laws have limited the use of traditional human embryonic stem cells[16] because of ethical and moral concerns [17;18], over the requirement for destruction of an embryo. Furthermore, in regenerative medicine, these cells can be derived from the patient awaiting stem cell therapy, so in contrast to (unmatched) donor hESC derivatives, it has been predicted that they would not undergo immunorejection In addition patient-specific iPSC contain any (disease-causing) mutations affecting the patient allowing the disease in principle to be modeled. Although introduction of a disease mutation into hESC would also create a disease model, the severity of the phenotype would never been known.

Thus iPSCs have created a new impulse to researchers worldwide to study and create cures for many diseases as well as move towards the personalization of stem cell medicine. While this hope verges on hype among the general public and among many patient groups, researchers in

the field soon realized that while this discovery was a great leap forward in stem cell research, the use of human iPSC (hiPSC) in the clinic is still decades away for many diseases and their use as personalized medicine is, for the moment, economically prohibitive [19;20].

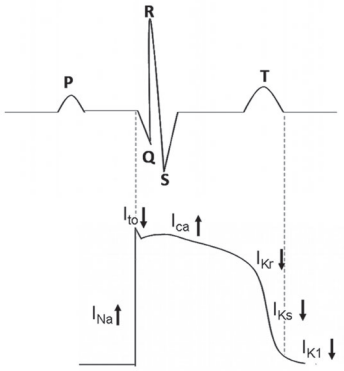

Figure 1: Schematic of an electrocardiogram (ECG) and its relationship to a cellular action potential. An ECG consists of a P wave (the electrical impulse of the atrium), the ORS interval (the depolarization of the heart's ventricles), and the T wave (ventricle repolarization). The activation, depolarization and repolarization of the heart are controlled by the fluxing are sodium, calcium and potassium as displayed by the ventricular action potential. I_{Na} soduim current; I_{to} outward potassium Current ; I_{Ca}: calcium current I_{Kr} : Delayed rectifier potassium current (rapid) I_{Ks} : Delayed rectifier potassium current (slow); I_{K1}: inwardly rectifying potassium current.

The first hiPSCs were generated using an "integrating" method in which genes were introduced into the somatic cell genome using, retroviral vectors. Four "reprogramming genes", OCT4, SOX2, KLF4, and c-MYC (more commonly known as the Yamanaka factors), [14] or OCT4, SOX2, NANOG and LIN28 (more commonly known as the Thomson factors [15]) were sufficient to return cells to pluripotency. Later reports also showed fewer transgenes could be used in some cells types. For example, if high levels of these genes were endogenously expressed [21-23]. Additionally, other studies showed that the combination of Yamanaka factors plus LIN28 and NANOG was even more efficient than just the original four [24;25]. Furthermore other myc family members has been demonstrated to be as effective with less chance of malignancies [26]. Nevertheless integrating methods, using retroviral or lentiviral vectors, permanently introduce new information into the DNA; the effects of permanently modifying the genome are unclear. Since the integration site(s) are often unknown there is a chance that normal gene expression patterns are altered, perhaps affecting maintenance and/or differentiation of the iPSC. Furthermore the possibility of re-expression of the exogenous reprogramming genes would always be a concern. In addition cells with ectopic gene expression may represent a major safety concern for clinical use. The use of excisable viral vectors and transposons which remove the reprogramming genes, has reduced these concerns but most excision methods still leave a genetic footprint such as a loxP site in the cells. The use of non-integrating methods (adenoviruses[27], miRNA[28], and Sendai virus[29;30] was another step closer to making iPSCs more closely resembling hESC , since they lacked the permanent integration of the exogenous factors and would therefore be safer in clinical use. Unfortunately, with the exception of the new Sendai virus method [29] and newer episomal methods [31] these methods are in general less efficient and relatively labor intensive (miRNA), which could lead to additional mutations in the newly derived iPSC lines or the preferential reprogramming of already mutated somatic cells. (A more detailed explanation of iPSC generation can be found in Chapter 2).

Not only should the method of reprogramming be a consideration but also the choice of somatic cell. While mouse iPSC (miPSC) have been generated from most cell types [32], the generation of hiPSC has been more challenging. Not only are some human somatic cell types more difficult to reprogram [33] but they are not all as easily accessible as others, especially (for ethical reasons) from children. While dermal fibroblasts were first used in reprogramming, collection of a skin biopsy is considered mildly invasive and so may be difficult to obtain from all patients of interest or from children. Confounding this issue is the possibility of epigenetic memory, the notion that iPSC 'remember' from which somatic cell they are generated from and thus directed differentiation may be effected. Epigenetic memory was first evident in miPSC but appeared to only be present during the early passages, just after reprogramming [32;34]. However, there

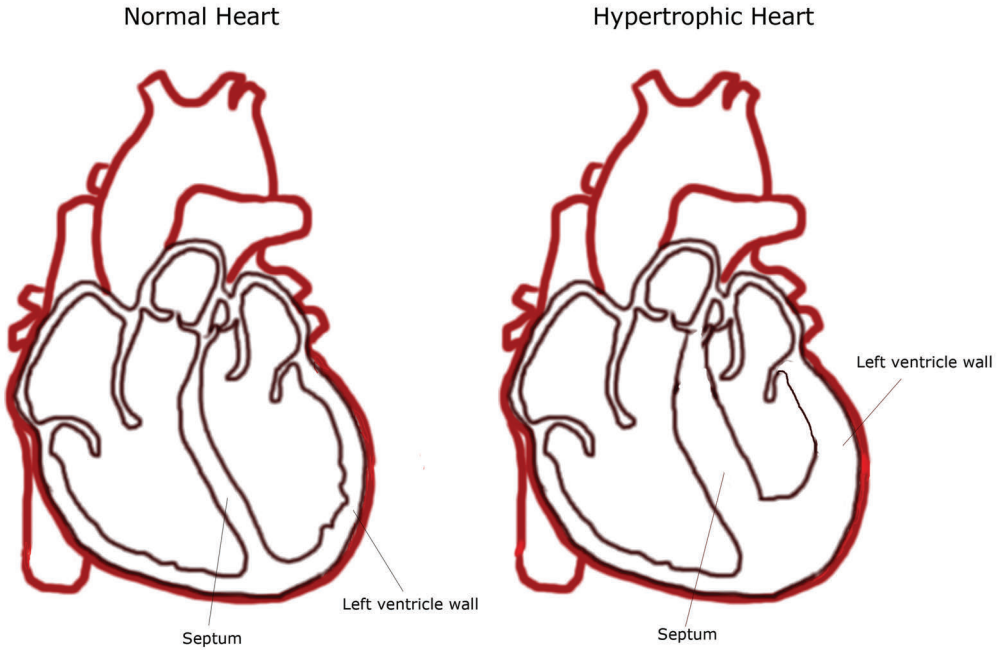

Figure 2: Schematic representation of a normal and hypertrophic heart. Hypertrophic heart are characterized by an increase wall thickening which can be seen in the interventricular septum and left ventricular wall.

are some reports which claim the epigenetic memory of hiPSC is retained even after long-term culturing [35] but these need to be confirmed by more laboratories.

Since the hiPSC can be derived from a patient's own cells, it has been assumed from the outset that immune rejection would be of no concern. However, a 2011 report from Zhao et al apparently demonstrated immunogenicity of miPSC [36] re-introduced into the same inbred mouse strain from which they were derived compared to mESC from the same strain. While this study only examined undifferentiated miPSC and mESC and not their differentiated derivatives, which would be more clinically relevant than the introduction of non-differentiated pluripotent stem cells into a patient, it does raise the question if these 'autologous cells' are really immunologically matched after going through reprogramming. However a newer report using several iPSC and ESC lines have found no difference in regards to teratoma formation or in grafts of differentiated cells [37].

Are hiPSC and hESC equivalent?

While hiPSC are very similar to hESC in terms of morphology, pluripotent gene expression and their ability to form teratomas [38], a number of differences between them have been reported particularly in their epigenetic state and transcriptome profiles [38;39]. Some early studies comparing iPSC and ESC found differences in the DNA methylation patterns [40-42] as well as persistent expression of donor cell genes [34;43;44]. Some of the differences found included X-inactivation. During development, female embryos inactivate one of their X genes and while this process is still not clearly understood, it has been found that some hESC have two active X genes. This re-activation of the X gene is sometimes but not always seen in hiPSC [39;45;46]. In addition the expression of the imprinted gene Dlk-Dio3 is often found to be differentially expressed in various iPSC lines [39]. Furthermore gene expression arrays have also found a separate set of genes, which are only expressed in hiPSC and not in hESC or the original somatic cells [34;39;41]. Still, later studies using increased numbers of independent ESC and iPSC lines (>50 as opposed to ±10) failed to find differences between hESC and hiPSC [47-49]. Nonetheless,

there have been reports of reduced differentiation potential of hiPSCs compared to hESCs [50;51] but this may be due to the increased heterogeneity (e.g. insert location, differentiation ability, single nucleotide variants [52], etc.) of hiPSC clones [38], or incomplete reprogramming[41] and not a fundamental difference.

hiPSC and disease modeling

While the clinical applications of hiPSC as cell therapy may be decades away for many conditions, their use for modeling human diseases is a more immediate possibility [53-55]. Using hESCs, which were difficult to modify genetically and could only be cloned after improvements in single cell culture conditions and recombination techniques, it has, until recently, been difficult to create targeted and disease lines [56;57]. However, if generated from a patient with a hereditary disease, hiPSC contain the disease mutation and they are genetically identical to the individual from whom they are derived. In addition any cellular phenotypes could be matched with the patient's clinical phenotype. hiPSCs can differentiate to multiple cell types much like hESCs using very similar protocols [58;59], thus their potential use for genetic disease modeling is evident. While many types of somatic cells, like fibroblasts or skeletal muscle cells, are already used in disease modeling, they are limited by their relatively short life spans in culture and/or sites in the body that make isolation difficult (e.g. the brain or heart). With the exception of cancer cells or immortalized cell lines, cells in culture are limited in proliferation by Hayflick's factor [60], the maximum number doublings before becoming senescent due to the shorting of the telomeres. hiPSC, like hESC, have telomerase activity [14;61;62], the ability to self-renew and thus provide a limitless supply of starting material to create the somatic cell type of choice. Unfortunately most of the somatic derivatives of pluripotent stem cells are in an immature state. This presents challenges on the extent to which they can recapitulate a disease phenotype, which often manifests postnatally, and often only in aged adults. Nevertheless there have been reports describing disease phenotypes in hiPSC derivatives as well as some examples of (partial) rescue of the phenotype (cardiac diseases reviewed in [53] ; see Table 1 for a summary of cardiac patient specific hiPSC).

The use of hiPSC is proving useful in studying some types of cardiac disease. The isolation of cardiomyocytes from a human patient is difficult and once isolated these cells can only be maintained in culture for several days if not hours [63]. Whilst there are many animals models for cardiac disease the differences between species can make them less than ideal [53]. For example the speed of the mouse heart makes studying human arrhythmias difficult as well as the differences in ion channel and their contribution to the action potential puts channelopathy studies in question. In addition, as previously stated above, the manipulation of hESC is complicated and so there are limited hESC cardiac disease models [56;57]. However, using cues from development, a number of cardiac differentiation methods have been established, first for hESCs and later hiPSC (see chapter 2 for a more details) facilitating their potential use in cardiac disease modeling. Even though the cells are in an immature state they express many of the ion channels and form sarcomeric structures. Nevertheless these cells also have their limitations such as a lack of organization (in regards to sarcomeres) and heterogeneity of cell types and impurity.

The Heart and Cardiac Disease

The human heart is a complex organ composed of excitable (e.g. cardiomyocytes, pacemaker cells, Purkinje fibers and smooth muscle cells) and non-excitable cells (e.g. fibroblasts, endothelial cells, and adipocytes). Cardiomyocytes are striated muscle cells, which can be characterized by a specific set of transcription factors (e.g. Nkx 2.5, GATA4 and Mef2a) and sarcomeric proteins (e.g. myosin heavy chain, troponin and α-actinin). Cardiac function is modulated by the flux of sodium, potassium and calcium ion channels. Alterations in these currents in the individual heart chambers can be visualized as an action potentials which taken together produce the PQRST intervals on an electrocardiogram (ECG) (Figure 1).

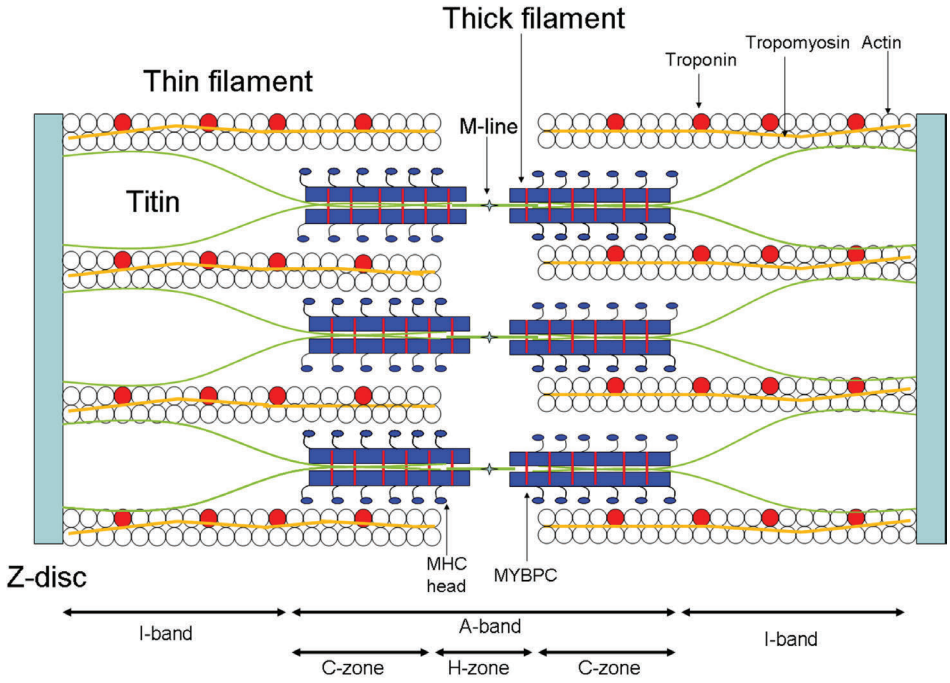

Figure 3: Schematic of a cardiac sarcomere. Cardiac sarcomeres consist of alternating segments of thick and thin filaments. The sarcomere contains numerous proteins including actin, troponin, titin, myosin heavy chain (MHC), Myosin Binding Protein C (MYBPC), and tropomyosin.

Cardiac disease is the one of the major causes of mortality in the Western world [56;64] and while many cardiac associated deaths are related to environmental factors (e.g. diet and exercise) or other diseases (e.g. cancer), there are a large number of channelopathies and cardiomyopathies which are linked to a genetic mutation. Channelopathies are cardiac diseases associated with mutations in the ion channel subunits [65]. For example, long QT syndrome (LQT), characterized by an increased QT interval is associated with various mutations found in the potassium and sodium channels [66]. On the other hand, cardiomyopathies are cardiac diseases, which more directly affect the heart muscle with mutations found in various genes, such as sarcomeric genes or transcription factors, depending on the myopathy [67;68].

One of the many cardiomyopathies is hypertrophic cardiomyopathy (HCM). HCM is a genetic cardiac disease characterized by increased heart size and wall thickening in the absence of other cardiac or systemic diseases (e.g. aortic valve stenosis or hypertension, figure 2) [68]. It affects 1:500 individuals and is the leading cause of sudden cardiac death in young adults[69]. Clinically, it is most often diagnosed by 2D echocardiography, presenting with a left ventricle wall thickness of greater than 15mm[70]. From the cellular perspective hypertrophy is characterized by an increase in cell size, increase in protein/DNA ratio and the re-expression of fetal genes, such as atrial natriuretic factor (ANF), brain natriuretic peptide (BNP) and fetal isoforms of contractile proteins[71]. In addition increased susceptibility to apoptosis, inefficient energy utilization and abnormal calcium signaling have been observed [71;72].

Mutations in eleven sarcomeric genes are associated with the familiar HCM (figure 3). Mutations are most prevalent in β- myosin heavy chain (MYH7, 40%), troponin T (TNNT2, <15%) and myosin binding protein C3 (MYBPC3, >30%) [73]. MYBPC3 is a cardiac specific sarcomeric protein involved in the modulation of force contraction through intracellular calcium regulated phosphorylation [69;74;75]. In addition MYBPC3 is important to sarcomere formation though not essential as evident from viable MYBPC3 knockout mice [76;77]. It is transversely arranged in the sarcomere

A-bands and binds to myosin heavy chain in the thick filaments and titin in the elastic filaments. It consists of eight Ig-like domains and three FN3 domains. The N-terminus contains myosin (s2) and actin binding sites as well as phosphorylation sites, while a titin-binding region and myosin (LMN) site as well as A-band incorporation are found in the protein's C-terminus [76;78].

In the Dutch population, three founder mutations in the MYBPC3 have been identified. These mutations account for more than 25% of HCM cases in the Netherlands. The most common of these mutation is 2373insG [70], a mutation on exon 25. This mutation is associated with a late onset of symptoms (after 30 years of age) and patients present with various clinical phenotypes [70]. The mutation is predicted to produce a truncated MYBPC3 protein. However studies of patient biopsies have never been able to detect the predicted protein thus the disease phenotype is thought to result from decreased protein levels (haploinsufficiency) [70;78]. Unfortunately there is a still a great deal of missing information regarding how this mutation causes an HCM phenotype in patients.

Table 1: Cardiac Disease-Specific hiPSC previously described in literature

Disease	Disease Category	Associated Genes	Rescue and drug testing	References
Arrhythmogenic Right Ventricular Cardiomyopathy	Cardiovascular	PKP2	N/A	[79;80]
Catecholaminergic Polymorphic Ventricular Tachycardia 1 & 2	Cardiovascular	RYR2, CASQ2	Dantrolene	[81-83]
Dilated Cardiomyopathy	Cardiovascular	TNNT2	SERCA2 overexpression, metoprolol	[84]
HCM	Cardiovascular	TNNT2, MYBPC3	Verapamil	[85]
LQT1	Cardiovascular	KCNQ1	Propranolol	[66;86]
LQT2	Cardiovascular	KCNH2	Nifedipine, pinacidil, ranolazine, propranolol, nadolol, nicorandil, PD-118057, sotalol, erythromycin, cisapride	[87-89]
LQT3, Brugada Syndrome	Cardiovascular	SCN5A	N/A	[58]
Down's syndrome	Multiorgan dysfunction	TRISOMY 21	N/A	[90]
Hurler syndrome	Multiorgan dysfunction	IDUA	Lentiviral transgene expression	[91]
LEOPARD syndrome	Multiorgan dysfunction	PTPN11	N/A	[92]
Pompe's disease	Multiorgan dysfunction	GAA	GAA transgene, rhGAA, 3-MA, L-carnitine	[93]
Becker's muscular dystrophy	Muscular & Cardiovascular	DMD	N/A	[90;94]
Duchenne's muscular dystrophy	Muscular & Cardiovascular	DMD	Genetic Rescue	[90;94]
Timothy syndrome	Neurological & Cardiovascular	CACNA1C	Roscovitine	[95;96]

Aim and outline of this Thesis

There is no single approach for using hiPSC as models for cardiac disease and development. There are a number of aspects, which must be carefully considering before generating and using hiPSC technology. Some are related to the hiPSC themselves, such as cell source, method of transgene introduction and the quality of the derivatives produced during differentiation. Others are related to the assays developed to assess cardiac phenotype at the cellular level and are perhaps the more challenging since they may be disease specific and ideally carried out on intact cardiac tissue or even the whole heart rather than on individual or groups of cells. Chapter 2 is a more in depth look into the history and development of hESC/hiPSC technology and its potential as a model for cardiac diseases. Chapter 3 investigates different somatic cell types for use in generating hiPSC lines, their pros and cons in regards to transduction and reprogramming efficiency using a polycistronic lentiviral vector as well as the ease of collection and isolation of samples from patients. Chapter 4 discusses the selection of hiPSC clones based on insert number and location along with cardiac differentiation potential. During the generation of hiPSC hundreds of clones are produced. Currently the clones used for further studies are randomly selected without any information regarding the insert number or location or if this clone can efficiently differentiate to the lineage of interest. One of the main reasons for this is a lack of rapid, high or mid-through put methods to determine this In chapter 4 an ultra-rapid method for identification of insert number and location as well as cardiac differentiation is described. Chapter 5 describes a cardiac differentiation method particularly designed to produce a large number of cardiomyocytes in a uniform manner. One of the interest areas of PSC is their use in drug testing and disease modeling. However if the lines differ in the efficiency and quality of differentiations, their usefulness is somewhat decreased. Thus a standard method for producing cardiomyocytes and selecting cardiomyocytes from mixed populations of PSC derivatives is ideal. Chapter 6 investigates the use of hiPSC as a model for HCM in patients with a mutation in MYBPC3. One of the major advantages of iPSC technology is to model a genetic disease and gain new insights into disease development. As a first step we have generated and confirm a phenotype in HCM-iPSC derived cardiomyocytes, which with further investigation will teach us something new about this disease and/or possible treatments. Chapter 7 studies the effects of serum on cardiomyocytes after differentiation from hPSC. In many studies cardiac differentiation of PSC is carried out in serum-containing medium. At the same time, there are several studies that have examined how serum affects rat cardiomyocytes; there is currently limited information on the effects of serum on PSC derived cardiomyocytes. Serum contains many unknown components which vary from batch to batch and thus can unknowingly alter the characteristics or responses of cardiomyocytes. Chapter 8 investigates the use of adeno-associated viral vectors (AAVs) as a method for purifying hPSC derived cardiomyocytes. As mentioned above one of the problems with PSC derived cardiomyocytes is that they are only a subpopulation among the mixture of cell types produced during differentiation. Currently there are limited methods to select out cardiomyocytes from non-genetically modified lines. A universal tool for selecting cardiomyocytes derived from any PSC line is needed. AAVs are small non-integrating viral vectors, which make them well suited for transient expression of a selection cassette. Finally, chapter 9 is a general discussion.

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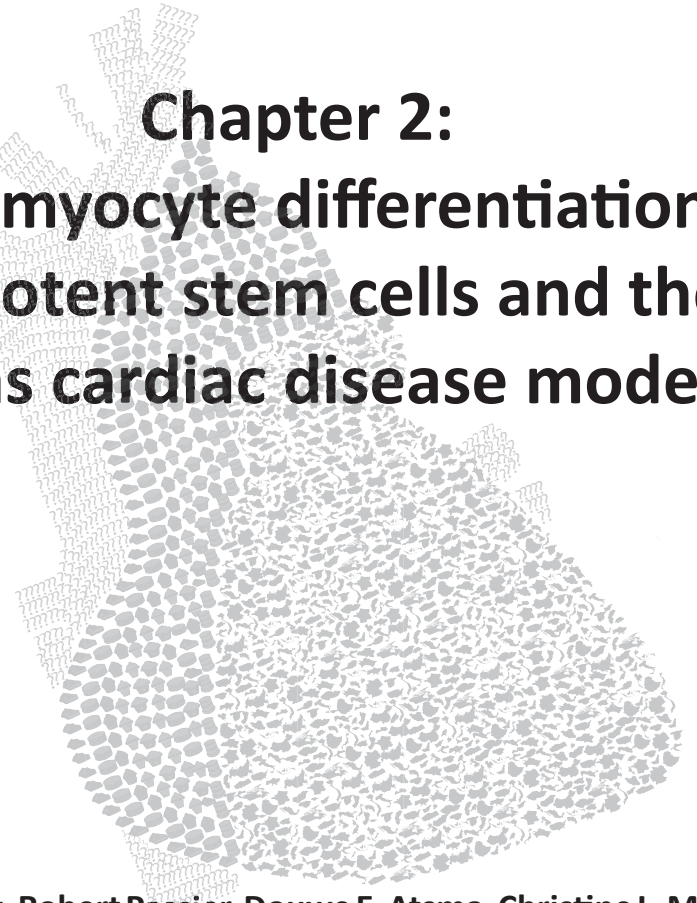
Chapter 2: Cardiomyocyte differentiation of pluripotent stem cells and their use as cardiac disease models



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Abstract

More than 10 year after their first isolation, human embryonic stem cells are finally "coming of age" in research and biotechnology applications as protocols for their undifferentiated expansion in culture and differentiation protocols become robust and scalable and validated commercial reagents become available. Production of human cardiomyocytes on a daily basis is now feasible for many laboratories with tissue culture expertise. An additional recent surge of interest resulting from the first production of human induced pluripotent stem cells (iPSC) from somatic cells of patients now makes these technologies of even greater importance since it is likely that (genetic) cardiac disease phenotypes can be captured in the cardiac derivatives of these cells. Whilst cell therapy based on replacing cardiomyocytes, lost or dysfunctional as a result of cardiac disease, are likely as far away as ever, biotechnology and pharmaceutical applications in safety pharmacology and drug discovery will likely impact this clinical area in the very near future. Here, we review the state-of-the-art in this exciting area of translational research.

Embryonic Stem Cells and Cardiomyocyte Development

Embryonic stem cells are derived from early embryos before they implant in the uterus. They are pluripotent that is they have the unique ability to differentiate into derivatives of the three germ layers of the body while retaining the capacity to self-renew indefinitely. This makes them important scientific tools in biomedical and cellular research since they can, in principle, form ~220 different cell types present in the adult body and do this repeatedly over many years.

After the isolation of the first mouse embryonic stem cells (mESC) in 1981 [1], the potential of these cells in regenerative medicine became evident. Researchers began attempts to isolate human embryonic stem cells (hESC) from surplus embryos used in assisted reproduction procedures (*in vitro* fertilization). However, due to differences in growth requirements, the limited availability of surplus human embryos and the nature of hESC themselves, it took another 17 years before the first hESC lines were isolated [2].

Aside from potential clinical applications in cell therapy for the heart, interest in deriving cardiomyocytes from hESC has been driven by curiosity on the differences in the physiology, metabolism and expression of protein markers in the mouse and human heart and the question of whether these differences would be reflected in cardiac derivatives of stem cells in culture in the absence of a true heart structure. The most obvious difference between the mouse (or any small rodent) and human heart is the rate of beating. The mouse heart beats at 500 beats per minute (b/m) while the human heart beats at 70 b/m. Another difference is the expression of myosin heavy chain (MHC) isoforms. βMHC is the isoform predominantly expressed in fetal mouse hearts whilst αMHC is predominant in the adult; the reverse is seen in humans [3]. Despite these differences, mice are often used as models in cardiac research because it is relatively easy to introduce specific mutations or targeted deletions in mice through mESCs. Some of these mutations result in cardiac developmental defects or adult disease, providing models that allow molecular and cellular dissection of the underlying mechanisms causing the abnormality. For studies on isolated cardiomyocytes though, stem cells can provide human alternatives as well as disease models, particularly now that methods for targeting specific genes in hESC have been developed [4-11] and cardiomyocytes can be derived from disease-bearing human induced pluripotent stem cells (iPSC). iPSC are a new kind of pluripotent stem cell that can be generated by reprogramming somatic cells directly rather than using embryos [12,13]. This is particularly attractive since it is difficult to obtain adult or even fetal primary human cardiomyocytes [14] and they cannot be kept in culture for more than a few hours or days.

In this review we describe the current state of hESC and human iPSC research with respect to their differentiation into cardiomyocytes and contribution to understanding normal and aberrant cardiomyocyte biology. In addition, we briefly consider the hurdles of using these cells for cell replacement therapy for the heart but refer the reader to specialized reviews on this subject for more detailed discussion [15,16].

Embryonic Stem Cells

Embryonic stem cells are derived from the inner cell mass of blastocyst stage embryos, in humans on day 5 post fertilization [2,17] in mice on day 3.5 [1] Most of our prior knowledge on the characteristics of these cells are derived from studies in the 1960s on embryonal carcinoma cells, the tumorigenic counterpart derived as spontaneous or induced testis tumors in mice [18] and as spontaneous germ cell tumors in humans [19]. Whilst expression of the principle pluripotency transcription factors, like OCT4, SOX2 and NANOG, is conserved between mouse and human ESC, these cells differ in their expression of surface markers and culture requirements [18]. This has recently been attributed to mESCs being in a more naïve state than hESC: mouse epiblast stem cells (EpiSC), isolated from mouse embryos later in development, appeared more like hESC than mESC [20]. For example, mESC require leukemia inhibitory factor (LIF) in the culture medium to remain undifferentiated and its removal will cause spontaneous differentiation. hESC and mEpiSC do not require LIF but bFGF and activin signaling to remain undifferentiated in

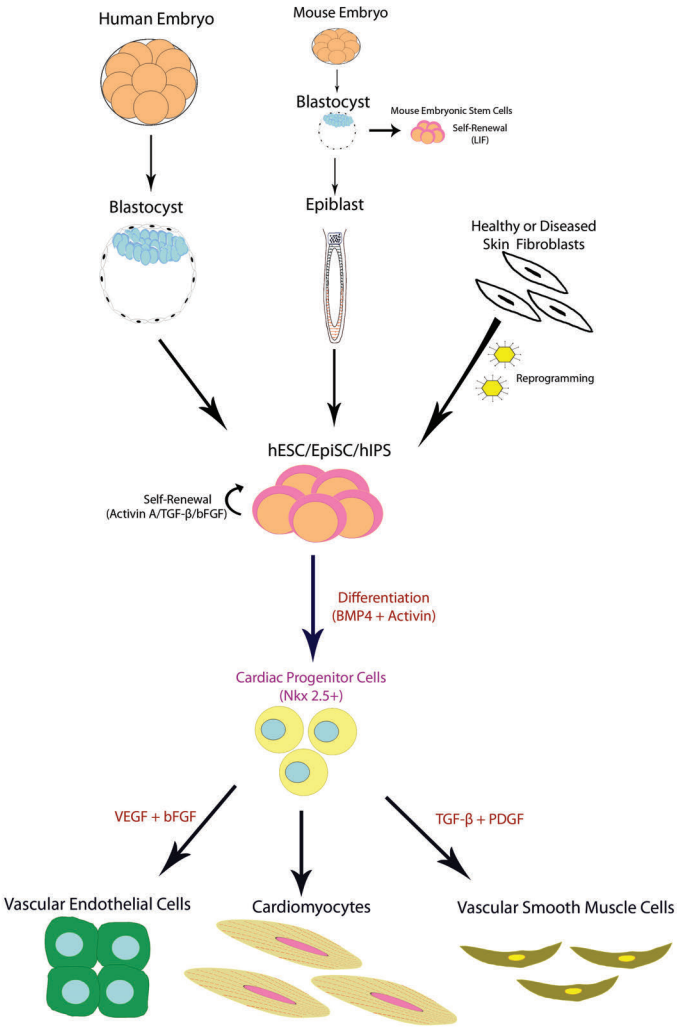

Figure 1: Pluripotent stem cells and cardiomyocytes. There are now multiple sources of pluripotent cells in humans. The most well known are embryonic stem cells isolated from the inner cell mass of human blastocyst stage embryos on day 5 after fertilization. Amongst the newest are induced pluripotent stem cells (iPSC) generated by introducing key pluripotency–associated transcription factors into somatic cells. In mice there are additionally (EpiSC) isolated from later stages of (postimplantation) embryo development which are similar to hESC in terms of conditions supporting maintenance, growth factor responses and gene expression, although not identical. These three pluripotent cell types are dependant on activin/TGF-β signaling for self-renew but have the ability differentiate into all three germ layers when these growth factors are removed. Upon directed differentiation towards cardiomyocytes, pluripotent cells will first pass through a progenitor stage which can be identified by the expression of specific transcription factors, for example Nkx2.5. The differentiation can continue further and produce various types of cardiomyocytes.

culture [21,22]. Typically though, hESC and mESC are maintained undifferentiated by co-culture on fibroblast "feeder" cells where they retain the ability to self-renew indefinitely. Removing the cells from feeders and/or growing them in suspension as aggregates (called embryoid bodies or EBs) causes differentiation to derivatives of the three germ layers, endoderm (pancreas, liver), ectoderm (neurons) and mesoderm (cardiomyocytes, vascular endothelial cells, muscle, blood). Much research effort has gone into simplifying these complex growth and differentiation protocols. In 2001 Xu et al. showed that hESC could be maintained in an undifferentiated state in the absence of feeder cells on Matrigel™ (an extracellular matrix derived from mouse sarcoma) or on natural laminin for at least 130 population doublings [23], although feeder-cell conditioned medium was required. Human recombinant extracellular matrixes, such as laminin-511 [24] or vitronectin [25] in combination with appropriate culture media, have more recently also been shown to maintain hESC in an undifferentiated state and Villa-Diaz et al have derived a synthetic polymer which reportedly sustains long term expansion [26].

Elimination of feeder cell requirements is not the only improvement that has been made to hESC culture conditions. The first fully defined culture medium was described in 2006 [27] and later marketed as mTeSR™1. This was shown to be effective in combination with a matrix containing either a mixture of human collagen IV, fibronectin, laminin and vitronectin [27] or Matrigel™. Further development has led to a xenofree version called TeSR™2 in which bovine serum albumin has been replaced by human serum albumin, making it in principle clinically compliant. Various groups have now described defined culture conditions using different basal media and supplements [27-31], although for most practical purposes, the commercial media are highly reliable for most cell lines used in most laboratories. Furthermore, the addition of a selective inhibitor of p160-Rho-associated coiled-coil kinase (ROCK) to the culture medium has been found to increase the survival of hESC cells during cryopreservation [32-34], after dissociation [35], passaging under serum free conditions [36,37] and during various differentiation procedures [38]. This has been most helpful when cloning from a single cell, often required during gene targeting or the generation of transgenic hESC lines.

In addition, improvement of hESC culture conditions continues with respect to optimizing methods for scale-up and maintenance of karyotypic stability either through enzymatic passaging [39] or suspension cultures [40,41]. Olmer et al. for example recently published a method for growing hESC and hiPSC in suspension cultures. While their procedure is not designed for continuous maintenance, they were successful scaling up cultures by several orders of magnitude [40], as would be required for future clinical application as well as biotechnology.

Embryonic Stem Cells and Induced Pluripotent Stem Cells

2006 was a landmark in the history of stem cell biology. The introduction of just four transcription factors into mouse somatic cells turned out to "reprogram" cells into a pluripotent, embryonic stem cell-like state [42] (fig 1). A year later, the procedure was repeated with human cells [43,44]. Apart from circumventing the ethical issues surrounding hESC derivation from human embryos, the procedure presented opportunities for deriving pluripotent stem cell lines from specific living individuals without the use of somatic cell nuclear transfer (SCNT or "therapeutic cloning")[45]. iPSC appear overall to be very similar to ESC in terms of morphology, global gene and protein expression and differentiation potential, but epigenetic variations between iPSC and ESC have been described [46-48].

The first iPSC lines were generated from fibroblasts using gamma-retroviral vectors to introduce OCT4, SOX2, KLF4, and c-MYC [42,43] or OCT4, SOX2, NANOG, and LIN28 [44]. Over a period of several weeks, these transcription factors induce expression of endogenous pluripotency genes like OCT4 and SOX2, and then become silenced once the endogenous genes take over control of pluripotency. Since the first description of reprogramming by ectopic gene expression in this way, reprogramming of many other somatic cell types, using different methods of transcription factor introduction have been published in quick succession. There are now reports describing miPSC

from almost every cell type in the mouse body, although creating hiPSC from the equivalent human cell sources is still more difficult. Nevertheless, in addition to adult and fetal dermal fibroblast, iPSC have been created from multiple human tissues including lung fibroblasts, fibroblast-like synoviocytes [43], keratinocytes [49], cord blood [50,51], peripheral blood [52,53], mesenchymal stromal cells [54] and more recently oral mucosa fibroblasts [55] and T-cells [56,57]. The use of retroviral vectors to create iPSC has a number of limitations and thus much effort has gone into finding alternatives. Among these are lentiviral vectors which, in contrast to retroviruses, can infect nondividing as well as dividing cells and also have a larger insert capacity than retroviral vectors. In principle this allows all of the reprogramming factors to be included in a single vector rather than requiring each to be incorporated into a separate vector. Secondly, other viral vectors which do not integrate into the genome, such as adenoviral vectors, have also been used although they reprogram cells with extremely low efficiency [58,59]. Thirdly, viral-free methods are under development. These include the use of transposons [60], protein [61] and mRNA [62] introduction methods. The first two methods also have low efficiency but the removal or absence of the transgenes has a number of advantages and more efforts into transgene-free or removable transgene methods are ongoing. The mRNA approach however, has efficiencies similar to viral integration so will likely become a preferred method in the future. A fourth area of research investigates the use of small molecules to improve or replace the use of transgenes in reprogramming. For example, the addition of valproic acid (VPA) has been found to improve the overall efficiency of reprogramming [63].

iPSC have not only expanded the ethical acceptability of pluripotent stem cell research but have also allowed the derivation of cell lines from individual patients, facilitating the creation of genetic disease models in culture [64-66]. Moreover, in the context of clinical application in cell therapy, once alternatives to viral integration methods for generating iPSC are fully developed, it is possible that tissue matching to the recipient may no longer be an issue although there will still be the same kinds of hESC associated risks, such as teratoma formation from any residual undifferentiated cells in the transplanted cell populations. Risks aside, the costs of cell production for transplantation would nevertheless need to be greatly reduced for any individualized treatment.

Human Embryonic Stem Cells and Cardiac Differentiation

While spontaneous differentiation of ESCs is easily achieved simply by omitting differentiation repressing factors or growing cells as EBs, directed differentiation, particularly of hESC, is more challenging. Using lessons from the embryogenesis, three main approaches have been developed to direct hESC towards the cardiac lineage *in vitro*. The first approach is based on EB formation but includes multiple inducers, usually growth factors, and repressors known to influence heart development in the embryo (fig. 2). This spontaneous form of differentiation can occur in regular growth medium containing fetal calf serum and is first evidenced by contractile areas of rhythmically beating cardiomyocytes [67]. The addition of growth factors and/or small molecules can further enhance EBs to a more directed differentiation down the cardiac lineage [68-72]. A method in which EBs were placed in “hanging drops” on a Petri dish lid had limited success with hESC compared with mESC [73]. However, the efficiency of cardiomyocyte differentiation in hESC, not normally more than 5% of all cells, was greatly increased using defined growth factor condition and “spin EBs” (EBs created from precise cell numbers - ~3000/100µl differentiation medium - and centrifugation in V-shaped wells of a 96-well plate) [74,75]. Crucial additions described in the literature include: transforming growth β-family members (bone morphogenetic proteins or BMPs and activin), members of the Wnt family and fibroblast growth factor (FGF) family, all major regulators in cardiac development [76]. Additonally, vascular endothelial cells growth factor (VEGF), p38 inhibitor, stem cell factor (SCF), the wnt inhibitor Dkk1 (added late) and ascorbic acid [77-79] have been shown to be important and more recently, sulfonyl-hydrazone [80], a GSK-3 inhibitor (BIO) [81] and dorsomorphin [82] have been added to the list as possible candidates to improve cardiac differentiation.

Table 1 Summary of mutations associated with two cardiac diseases

I_{Ks}, slowly activating delayed rectifier K+ current; I_{Kr}, rapidly activating delayed rectifier K+ current; I_{Va}, voltage-gated Na+ current; I_{K1}, inward rectifying K+ current; I_{CaL}, L-type Ca+ current.

Disease	Subtype	Gene	Protein	Ion current changed	Frequency (%)
HCM	–	MYH7	β-Myosin heavy chain	–	15–25
	–	MYBPC3	Myosin-binding protein C	–	15–25
	–	TNNT2	Troponin T	–	<5
	–	TNNI3	Troponin I	–	<5
	–	TPM1	α-Tropomyosin	–	<5
	–	MYL2	Myosin light chain 2	–	<2
	–	ACTC	α-Cardiac actin	–	<1
	–	MYH6	α-Myosin heavy chain	–	<1
	–	MYL3	Myosin light chain 2	–	<1
	–	TNNC1	Troponin C	–	<1
	–	TTN	Titin	–	<1
LQTS	LQT1	KCNQ1	KvLQT1	I_{Ks}	30–50
	LQT2	KCNH2	HERG	I_{Kr}	25–40
	LQT3	SCN5A	Nav1.5	I_{Na}	5–10
	LQT4	ANK2	Ankyrin-B	Multiple	1–2
	LQT5	KCNE1	MinK	I_{Kr}	2–3
	LQT6	KCNE2	MiRP1	I_{Ks}	<1
	LQT7	KCNJ2	Kir2.1	I_{K1}	<1
	LQT8	CACNA1C	Cav1.2	I_{CaL}	<1
	LQT9	CAV3	Caveolin-3	I_{Na}	<1
	LQT10	SCN4B	Navβ.4	I_{Na}	<1
	LQT11	AKAP9	α-Kinase anchor protein 9	I_{Ks}	<1
	LQT12	ANTA1	α-Syntrophin	I_{Na}	<1

A second approach is based of stromal cell co-culture. This exploits the instructive influence of endoderm for cardiac differentiation during embryogenesis and uses co-culture with endoderm-like cell lines or their conditioned medium. The use of a mouse visceral endoderm-like (END-2) cells without serum and insulin has been used to produce cardiomyocytes with a human fetal ventricular cell-like phenotype from various hESC lines provided they are “mechanically” rather than enzymatically passaged as undifferentiated cells [83-85].

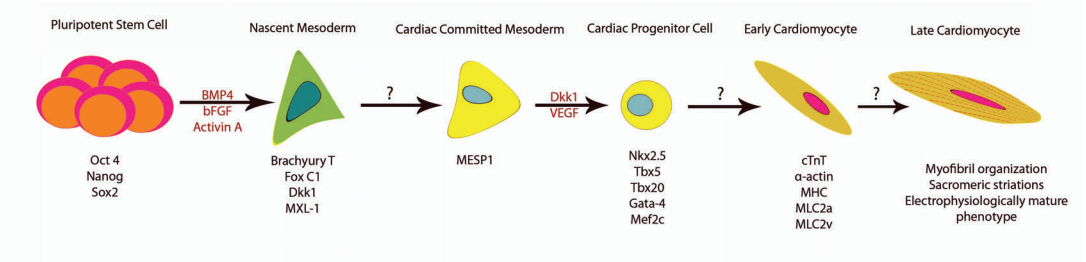

Figure 2: Cardiac transcription factors and exogenous control of pluripotent stem cell differentiation The differentiation of pluripotent stem cell to cardiomyocytes is sequential with steps being distinguished by the expression of various (combinations of) transcription factors. There are a number of different differentiation methods but a specific set of exogenous factors are still needed to force pluripotent stem cells down the cardiac lineage. While all the steps in the process are not clear, the time of introduction of these factors is very important. In general, initial introduction of BMP4, activin A and bFGF will start the directed differentiation and later introduction of a Wnt signal inhibitor (Dkk1) and VEGF will help to push the cells further to form cardiomyocytes.

A third method utilizes a high-density monolayer model supplemented with BPM4 and activin A in a defined serum-free medium. Laflamme et al. reported this method to be much more efficient than EB-based methods [86], although to date the number of publications describing its use is limited and its reproducibility across different hESC lines is still unclear.

Ongoing improvements are being made to these methods, often involving the introduction of different growth factors or small molecules at various phases in the differentiation process. In addition, many groups are looking into methods to produce more homogenous cardiac cell populations (i.e. specific atrial, ventricular, conduction system type cell populations rather than mixtures) whilst others are addressing the issue of cell maturity. Cardiac cells derived from stem cells generally show immature phenotypes. Recently, Otsuji et al. published a method for inducing maturation involving replating of cardiomyocytes derived using the END2 co-culture method onto fresh END2 cells followed by a short 3D culturing step [87]. These cardiomyocytes could be maintained for one year during which time they were found to become more mature in terms of their electrophysiological properties, and to contain an increased number of pacemaker cells [87]. Alternatively, allowing the cells to undergo cyclic stretch or forcing alignment using tissue engineering approaches [88] may enhance maturity.

Purification of hESC derived Cardiomyocytes

The use of embryonic stem cell derivatives in research as well as possible clinical therapies is plagued with many problems including insufficient purity. The inadvertent introduction of any undifferentiated ESCs into host tissue will result in teratomas, benign tumors containing derivatives of all three germ layers. In addition, many differentiation techniques produce heterogeneous cell populations with cardiomyocyte numbers varying from 1- ~50% of the cell total. In addition, in the END2 co-culture system, approximately 85% of the cardiomyocytes produce are ventricular [84], whilst other EB-based methods produce a mixture of cardiac atrial and ventricular cell types. Probably the most efficient cardiomyocyte differentiation protocols at the present time are based on adaption of undifferentiated hESC to feeder-dependent enzymatic passage in KOSR (knock out serum replacement) followed by spin EBs containing ~3000 cells and the addition of BMP4 and activin A at concentrations in the range of 20ng/ml [75]. Replating of these EBs after 7 days in regular culture medium can yield up to 50% cardiomyocytes. In attempts to enhance the differentiation of hESC to particular cardiac subtypes, Zhu et al found that inhibition of the NRG-1β/ErbB signaling pathway increased the proportion of nodal-like cells, whereas its activation produced the reverse effect[89].

While many researchers are working on improving the efficiencies of differentiation and producing more homogenous populations of cells, others are investigating how best to purify the mixture of cells and select the required cell type. There are a number of different approaches

being tested to select cardiomyocytes from mixed cell populations. In some cases, fluorescent reporters have been attached to cardiomyocyte-associated promoters and used to produce transgenic reporter lines [90-92]. The yield of cardiomyocytes after selection can be >90% but requires genetic manipulation which has not been successful in all hESC lines [90] and might not feasible for routine use in hiPSC. Another approach uses Percoll gradient centrifugation to isolate the cardiomyocytes physically, based on their larger size compared with most other cells [93]. A third method selects cardiomyocyte progenitors using the cell surface protein Flk1 (also called KDR or VEGF receptor) [77]. However, these cells are also progenitors for endothelial and smooth muscle cells so that mixed populations of cells might still result. Furthermore, Flk1 is expressed on undifferentiated cells of some lines. A fourth approach was recently reported by Hattori and colleagues [94]. The researchers used a nontoxic dye tetramethylrhodamine methyl ester perchlorate (TMRM) which reversibly labeled the mitochondria. Labeling a mixed culture of cells produced three fractions, a high fluorescent fraction with >99% cardiomyocytes, an intermediate fraction with other viable cells and a low fraction which contained dead or blood cells. The ease and reversibility of this method may prove extremely useful in the future although at present it only works on cardiomyocytes maintained for long periods in culture (>50days).

Potential Uses of hESC derived Cardiomyocytes

There are a number of uses envisaged for hESC derived cardiomyocytes both as research tools and for clinical applications. These include drug screening and pharmacosafety analysis, disease modeling and gene targeting, as well as regenerative medicine.

In vitro drug screening

Many steps are required before a drug is approved for clinical application and one of these is evidence for low toxic risk on the heart. Drugs may be recalled if significant side effects occur and the heart is one of the most sensitive organs for this. Even after numerous tests in animals these side effects may remain undetected [95]. This may not be surprising since the difference between animal and human physiology is significant, as mentioned earlier, and the way ion transport is handled differs between species. There is an increasing need for human models of healthy as well as diseased hearts. We have recently shown that hESC derived cardiomyocytes respond to specific drugs in much the way that the human heart responds, as assessed using readouts from microelectrode arrays [95]. Furthermore the ability of hiPSC to form various cardiac lineages may make them good candidates for some of the drug screening presently carried out using animals.

Gene targeting in hESC to create reporter lines and disease models

While there are now multiple descriptions of genetic modification of hESC in the literature, they have been considerably more challenging to modify than mESC. Substantial progress over the last several years has led to multiple cell lines becoming available that have undergone targeted mutation. Genetic modifications can now be introduced through non-homologous, homologous, site-specific or transpositional recombination. Non-homologous recombination is a straightforward and easy method of introducing exogenous DNA but has extremely low efficiency. In classical homologous recombination, exogenous DNA is introduced in the genome through the natural potential of cells to incorporate identical or similar DNA sequences. The efficiency of this process varies between species and cells types. The efficiency of homologous recombination has also been found to be extremely low. Among the hurdles that have confounded this technology are the difficulty in cloning hESC from single cells [96], and the low efficiency of DNA introduction using conditions which work well with mESC for example through electroporation [97]. The use of zinc finger nucleases in homologous recombination has been reported to increase efficiency signifcantly. Zinc finger nucleases introduce double strand breaks at specific sites in the genome using novel DNA binding proteins. Zinc finger nucleases have been used in targeting a number of human genes including VEGF-A, HoxB13 and CFTR [98]. They have been used in combination with defective lentivirus, electroporation and nucleofection in hESC [9-11].

Site-specific recombination allows for more controlled introduction of exogenous DNA sequences. Two types of site-specific recombination are Cre-loxP and phiC31. In the Cre-loxP system a pair of recognition sequences promotes a reciprocal recombination reaction, while phiC31 is an irreversible recombination using bacteriophages which attach to attP sequences in the genome. Although Cre-loxP has been successfully used with hESC [99], to date there are no reports of genetic modification of hESC using phiC31 [100]. Another technique uses transposons to modify hESC, employing three different systems, Sleeping Beauty [101], PiggyBac [102] and Mu [103]. All of these systems allow the integration of non-targeted DNA along chromosomes. They have different efficiencies, cargo sizes, and excisability but all offer an alternative to viral vector integration of DNA into the genome (reviewed in [104]).

The modification of hESC by introduction of ectopic reporters or targeting developmentally important loci is becoming a very useful tool in understanding the differentiation of cardiomyocytes. It is of significant interest in the study of heart disease, since it represents an opportunity to introduce different mutations against a single genetic background if using just one hESC line. In this respect genetic modification of hESC lines can compliment studies with human iPSC cells since deriving lines from different individual patients automatically implies a different genetic background.

Which cardiac diseases will be useful to model using human pluripotent stem cells?

The most important task of the heart is to pump blood throughout the body. For this highly orchestrated temporal and spatial activities consisting of electrical activation and subsequent mechanical contraction of cardiomyocytes are essential. Disturbances in these processes may lead to serious or even life-threatening conditions. Over the past two decades, our knowledge of the genetic basis of cardiac diseases has increased enormously. In that same period, many different animal models have been generated for studying cardiac diseases, and although these have shed light on our understanding of the onset and progression of cardiac diseases, the emergence of new drugs and improved therapies have lagged behind. Several major breakthroughs in the stem cell field in recent years, as described above, have made the academic and industrial biotechnology communities realize that development of human *in vitro* models for studying disease and drug discovery are within reach. In particular, development of more efficient and robust protocols for the production of specialized cell types, like cardiomyocytes, from hESC has strengthened this awareness.

In order to develop predictive human *in vitro* cardiac disease models, a rational choice would be to avoid complex, multi-gene cardiac diseases involving multiple cell types or secondary aberrations in morphology and/or function. Instead, initial preference should be in cardiac diseases associated with mutations in single genes which most likely have an impact at the single cell level (e.g. autologously on cardiomyocytes). In this regard, many mutations in genes encoding cardiac ion channels and sarcomeric proteins, associated, for instance, with disturbed ion channel function (cardiac channelopathies) or impaired contractility (cardiomyopathies), have been identified. For both channelopathies as well as cardiomyopathies, the majority of mutations associated with the disease are present in a relatively small set of genes (Table 1). Along with further characterization, of the hESC and hiPSC models, their value in understanding disease mechanisms needs demonstrating and their use as models for drug discovery remains to be shown. Nevertheless, proof of principle that this approach may be successful is beginning to emerge (see below).

The imbalance in ion currents across the sarcolemma in patients diagnosed with cardiac channelopathies is the cause of a change in the action potential morphology. It manifests as an abnormal electrocardiogram (ECG) with abnormal peaks and intervals indicating cardiac arrhythmias (fig.3). Heritable cardiac channelopathies include long-QT syndrome (LQTS), Brugada syndrome, catecholaminergic polymorphic ventricular tachycardia, cardiac conduction disease and sinus node dysfunction [105]. LQTS, has a prevalence of 1 in 5000 individuals and is

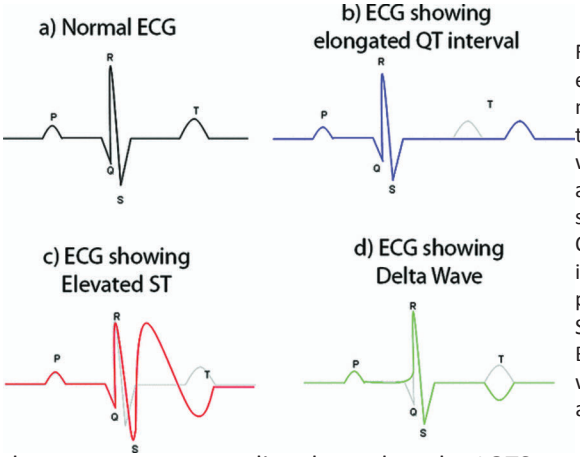

Figure 3: Schematic diagrams of normal and abnormal electrocardiograms (ECG). An electrocardiogram measures the electrical activity of the heart across the chest wall. It is collections of peaks and interval which help diagnose diseases. a) An example of a normal ECG. b,c,d) Examples of abnormal ECGs superimposed over a normal ECG. An elongated QT interval (b) is seen in a number of diseases including Long QT syndromes and catecholaminergic polymorphic ventricular tachycardia. The elevated ST (seen in ECG 'c') is often seen with a form of Brugada syndrome and a delta wave (d) is associated with Wolff–Parkinson–White syndrome which accompanies numerous heart defects.

the most common cardiac channelopathy. LQTS, can be subdivided into multiple types (LQT1 to LQT12) depending on which gene is mutated, but all prolong the duration of the action potential and the QT-interval on an ECG.

Cardiomyopathy is a heart disease that may result in inadequate pumping of the heart leading to congestive heart failure. Cardiomyopathy can be further classified into different forms, including hypertrophic (HCM), dilated (DCM) and restrictive (RCM) cardiomyopathy and arrhythmogenic right ventricular dysplasia (ARVD). Of these HCM is the most frequent with a reported prevalence of as high as 1 in 500 individuals [106]. It is also the most common cause of sudden cardiac death among young athletes and in people under 30 years of age. HCM is characterized by an increase in left ventricular wall thickness in the absence of increased afterload, combined with disarray of cardiomyocytes and cardiac interstitial fibrosis. It was the first cardiac disease for which a genetic basis was identified. To date, more than 450 mutations, distributed over 13 sarcomere and myofilament-related genes, have been classified (Table 1). Moreover, 50% of the total number of mutations appear in only two genes, MYH7 (β myosin heavy chain) and MYBPC3 (myosin binding protein C).

The challenge for the coming years is to generate *in vitro* models using human stem cell -derived cardiomyocytes harboring disease-associated mutations, which faithfully recapitulate the cardiac disease phenotype as it manifests in patients. This, in combination with development of robust predictive assay readouts (scalable for high throughput screening) will provide novel platforms for cardiac safety pharmacology, but, more importantly, also provide opportunities for novel drug discovery.

Disease modeling in human iPSC cells

The development of iPSC technology and the possibility of making patient specific embryonic stem cell-like cells, may complement the (rather slow) development of cardiac disease models based on hESC thus far. If hiPSC prove to be amenable to cardiac differentiation, which many researchers are beginning to show [107,108], disease specific human cardiomyocytes will become a very useful tool for studying and perhaps reversing cardiac disease. However, research into hiPSC characterization and the effect of reprogramming and (epi)genetic memory on these cells is still ongoing. The uniformity and reliability of differentiation of several well studied hESC lines may make these the most useful near-term research tool, thus research in targeting and genetic modification of hESC is still vital.

Nevertheless, recent successes using hiPSC as models for human cardiac disease have been reported. Two groups have generated hiPSC lines from patients with heart disease which show a disease phenotype in culture. Lemischka and colleagues produced cardiomyocytes from two

hiPSC lines derived from two patients with a heterozygous T468M substitution in PTPN11 which leads to LEOPARD syndrome [12]. LEOPARD syndrome affects various parts of the body but hypertrophic cardiomyopathy (HCM) is one of the most common life-threatening symptoms of the disease. The authors estimated hypertrophy based on cell size, sarcomeric organization and nuclear localization of NFATC4, which was comparable to the patient phenotype. Furthermore the use of hiPSC in this research has also lead to several new insights into signaling pathways related to the disease, something which may have eluded researchers if not for hiPSC technology. A second report by Moretti et al investigated an ion channel mutation in patients with LQT1 [13]. These patients have a mutation in the KCNQ1 gene which results in an elongated QT interval. The KCNQ1 gene encodes I_{Ks}, the ion channel controlling the slow component of the delayed rectifier potassium current which is partially responsible for the repolarizing phase of the action potential. The authors were able to recapitulate this electrophysiological phenotype in culture using whole-cell patch-clamp electrophysiology after the creation hiPSC lines from two related patients from a family with a R190Q missense mutation in KCNQ1.

These first two ground breaking articles on cardiac hiPSC disease modeling are only the first steps in an area of research with great potential. Both articles bring to light one very important aspect of iPSC technology: the need for proper assays to determine a cellular phenotype. The heart is not composed of just one cell type but several and therefore recapitulating a phenotype can be difficult. These two examples of hiPSC as cardiac disease models for basic characterization of the disease itself are of interest but the opportunities they present for drug discovery will, in the long run, be of greater interest.

Direct reprogramming of somatic cells to cardiomyocytes

In a recent advance in direct reprogramming, Ieda et al (2010) showed the differentiation of mouse fibroblasts directly into cardiomyocytes using just three cardiac transcription factors [109]. Mouse cardiac or tail tip fibroblasts were treated with a cocktail of genes which induced direct cardiac differentiation. After wading through 14 genes, the researchers found three 'master regulator genes', Gata4, Mef2c and Tbx5, which were essential for direct reprogramming of fibroblast to cardiomyocytes. The efficiency of this direct reprogramming was higher than reprogramming to iPSC (20% compared to <0.1%) and it was also more rapid. Using an inducible system, they were also able to show the stability of their procedure. Whilst this finding remains to be confirmed by others and has not yet been extended into human cells, it could represent another route to studying patient derived cardiomyocytes for certain purposes. This new development may help eliminate some of the obstacles when using traditional ESC or iPSC, including teratoma formation due to residual undifferentiated cells and lower the cost and delivery time to patients due to higher yields and faster production. It has the disadvantage however, that the emergent cardiomyocytes cannot be expanded in culture. The method thus is likely best seen as a supplement to research with iPSC cells rather than an alternative. It may however, represent an opportunity to investigate whether direct reprogramming to cardiomyocytes is feasible in the heart *in vivo*, for example after myocardial infarction.

Regenerative Medicine

The use of cell therapy to repair the heart is not a new concept. Its foremost aim is to introduce new cardiomyocytes into the heart to replace those that are damaged or dead for example after an infarct. However in practice this is rather complex with many aspects of how this could work still unclear. The first attempts at cell therapy for the heart were actually based on non-cardiomyocyte cell population like bone marrow derived cells (BMC). While initially BMC were reported to be able to transdifferentiate into cardiomyocytes thereby improving heart function, it was later found that this was not correct [110-112]. Nevertheless the use of BMC delivery to the heart continued, although with limited long term benefits in terms of cardiac function. Any short-term improvements, most likely involved enhanced local angiogenesis and thus blood supply in the border zone of the damaged heart tissue, or paracrine factors of an

unknown identity from the transplanted cells. This is also likely the reason for reduced chest pain symptoms and improved cardiac contractile and relaxation performance [15,16,113].

The inconclusive data from adult stem cell therapy trials to date and the limited success of sustaining non-cardiomyocyte presence in the heart, may make iPSC, cardiac progenitors or hESC a better source for cell therapy, since these cells can form cardiomyocytes (hESC-CM) or cardiac progenitor cells (hESC-CPC) for repair. Studies have shown that hESC-CM introduced into mouse or rat hearts will survive and mature [114-116]. However the cells are often separated from the animal heart by fibrotic tissue, and these hearts have only shown short term functional improvements [116]. While most studies have only been performed in small animals, a recent study in porcine showed improved cardiac function after intramyocardial injection of hESC-CM compared to the controls [117] although long term follow-up was not described.

Despite these transient improvements in cardiac function following hESC-CM transplantation in animals, there are a number of other obstacles which need to be overcome before transfer to humans may even be considered. *In vivo*, hESC-CM trigger an immune response. Therefore all studies thus far have involved immune suppression or used immunocompromised animals. The discovery of iPSC may help overcome this by creating immune-matched cells for transplantation but a large cell bank would still be necessary to match most of the population since hiPSC lines for individual patients, at present, would not be economically viable. Not only would cheaper reagents be necessary for scaling up cell production, but the whole procedure would also have to be done under conditions of Good Manufacturing Practice (GMP). These are by themselves costly and labor intensive. A second obstacle is the removal of all undifferentiated hESC (or hiPSC) before introduction into the body as described above, since undifferentiated pluripotent stem cells can give rise to teratomas when injected into a living organism, animal or human. Most important though in human patients is the potential risk of arrhythmias, which can be caused by the introduction of immature cardiomyocytes with intrinsic pacemaker-like activity into the human heart, as well as anisotropy and other scar-implanted cell interactions.

Conclusions and future perspectives

Even after more than a decade of research, the potential contribution of human embryonic stem cells to understanding cardiac disease and as a cell therapy is far from fully realized. Restricted in some countries by ethical issues, research into these cells was slow to start and often lacked financial support. The discovery of iPSC technology has helped jump start the field in recent years and has added much to our understanding of pluripotency and manipulation of embryonic stem cells. Even though many questions still remain unanswered there is already much learned from hESC and this will likely accelerate implementation of pluripotent cells in general and in the cardiac arena. Most important and immediate is likely to be the contribution of these cells to drug discovery since this is a significant unmet need for new drugs as the incidence of myocardial infarct and heart failure increase in the aging population. The number of new drugs entering the market for heart disease is decreasing, whilst investment by the pharmaceutical industry in this area continues to increase. This is perhaps the strongest evidence that present systems on which drug discovery is based are inadequate for future needs. In the context of safety pharmacology, big pharma is generally conservative and will not be keen to have additional safety tests imposed if they do not replace those in present use. In addition, replacing present tests may increase their risk of litigation should the new predictors prove wrong.

Whilst many scientists began research with hESC with view to their potential in therapy, experiments over the last decade have indicated that this will be very challenging for many types of disease. Exceptions likely include macular degeneration (age-related blindness), since it is fairly easy to induce differentiation of hESC to retinal pigment epithelial cells and the eye is hardly sensitive to immune attack, certain types of spinal cord lesion, for which the first transplantation has recently taken place in the US, and diabetes, for which the transplantation technique is straightforward and some groups are now able to generate β-islet-like cells with

some reproducibility. The heart is certainly among the more challenging organs to repair because integrating cardiomyocytes with host tissue may require more than simple injection. Thus drugs derived from research on stem cell-derived cardiomyocytes may then have the greatest clinical impact.

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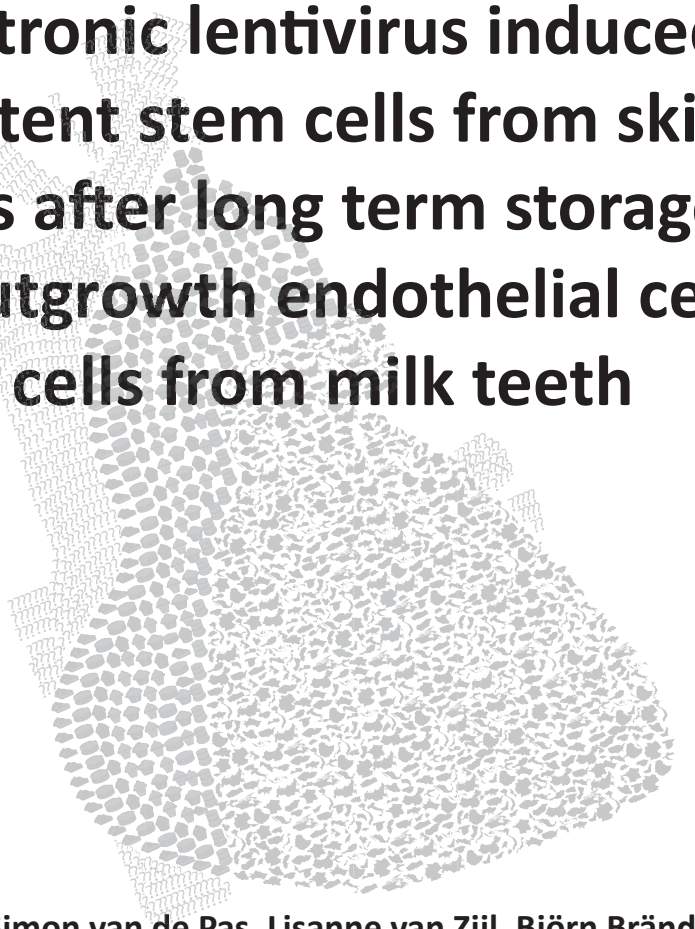
Chapter 3:
Polycistronic lentivirus induced pluripotent stem cells from skin biopsies after long term storage, blood outgrowth endothelial cells and cells from milk teeth



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Abstract

The generation of human induced pluripotent stem cells (hiPSCs) requires the collection of donor tissue but clinical circumstances in which the interests of patients have highest priority may compromise the quality and availability of cells that are eventually used for reprogramming. Here we compared (i) skin biopsies stored in standard physiological salt solution for up to 2 weeks (ii) blood outgrowth endothelial cells (BOECs) isolated from fresh peripheral blood and (iii) children's milk teeth lost during normal replacement for their ability to form somatic cell cultures suitable for reprogramming to hiPSCs. We derived all hiPSC lines using the same reprogramming method (a conditional (FLPe) polycistronic lentivirus) and under similar conditions (same batch of virus, fetal calf serum and feeder cells). Skin fibroblasts could be reprogrammed robustly even after long-term biopsy storage. Generation of hiPSCs from juvenile dental pulp cells gave similar high efficiencies but that of BOECs was lower. In terms of invasiveness of biopsy sampling, biopsy storage and reprogramming efficiencies skin fibroblasts appeared best for the generation of hiPSCs but where non-invasive procedures are required (e.g. for children and minors) dental pulp cells from milk teeth represent a valuable alternative.

Introduction

Human induced pluripotent stem cells (hiPSCs) generated from patients with genetic diseases hold great promise for disease modeling, safety pharmacology and drug discovery [1-3]. This is particularly relevant for cells of the internal organs, for which biopsies are not routinely available and therefore analysis of the disease phenotype is hampered. hiPSCs are similar to human embryonic stem cells (hESCs) [4] in that they self-renew and can differentiate into all somatic cell types of the human body.

Since the first derivation of hiPSCs in 2007 using fibroblasts cultured from skin biopsies and the retroviral expression of four pluripotency genes *Oct3/4, Sox2, Klf4* and *c-Myc* [5], considerable research has been devoted to reprogramming other somatic cell types, also using other methods of gene delivery to the host cell. These include integrating methods (e.g. using lentiviruses or transposons) and a variety of non-integrating approaches (adenovirus, plasmid, protein, episomal vectors and RNA; reviewed in [6]). The obvious advantages of non-integrating methods are still limited by their relatively low efficiencies, high cost and labor intensity. In addition, general transfection methods require relatively large numbers of somatic cells. Integrating methods by contrast are reasonably efficient but the quality of the resultant iPSC lines may be compromised by random integration of transgenes, altering endogenous gene expression, or resulting in incomplete silencing of transgenes after reprogramming [7-9]. Excisable systems for removing transgenes represent an important improvement in this respect.

In addition to skin fibroblasts, reprogramming of keratinocytes, hepatocytes, T-cells from peripheral blood, adipose tissue-derived stem cells, dental pulp from adult teeth [10] and other cell types has been reported (reviewed in [1]. Criteria for selecting which somatic cell type to reprogram include (i) tissue accessibility and invasiveness of the biopsy procedure, (ii) whether the tissue sample can be stored for prolonged periods during transport from clinic to laboratory, (iii) whether cell culture conditions are adequate for supporting proliferation of the somatic cells and (iv) the reprogramming efficiency of the particular somatic cell type.

Direct comparison of different methods is hampered by variability in reagents and experimental protocols. Here we studied the isolation and reprogramming efficiencies of three easily accessible somatic cell types with view to examining how normal constraints in the clinic impact the experimental outcome. For reprogramming we used an excisable, polycistronic lentiviral vector, which can be easily produced in large quantities at low cost and only requires a small number of somatic cells. An additional dTomato reporter gene in the vector also enabled real-time monitoring of transduction efficiencies and silencing of transgenes. Skin fibroblasts and blood outgrowth endothelial cells (BOECs) were obtained by minimally invasive procedures (4 mm punch biopsy and collection of peripheral blood, respectively) and milk teeth were obtained after natural loss. Isolation of cells from all tissues was straightforward. More importantly, skin fibroblasts could be readily isolated at any time within two weeks after collection of the biopsy and reprogrammed with robust efficiencies even after storage of the biopsy for the entire period simply in cold physiological buffered saline. Similarly, cells from milk teeth could be easily isolated and reprogrammed at efficiencies comparable to skin fibroblasts. Isolation of BOECs showed patient-to-patient variability and required a longer period in culture than skin fibroblasts or dental pulp cells to obtain sufficient cells for reprogramming. Reprogramming of BOECs was also less efficient than that of skin fibroblasts or dental pulp cells. However, hiPSC lines from all three tissues displayed expression of typical markers of embryonic stem cells and differentiated readily into derivatives of ectoderm, endoderm and mesoderm in vitro. Although much of the methodology used is routine in many hiPSC laboratories, we have validated protocols here that (i) facilitate tissue collection and transport from distant sites and (ii) provide an additional non-invasive approach for use in minors.

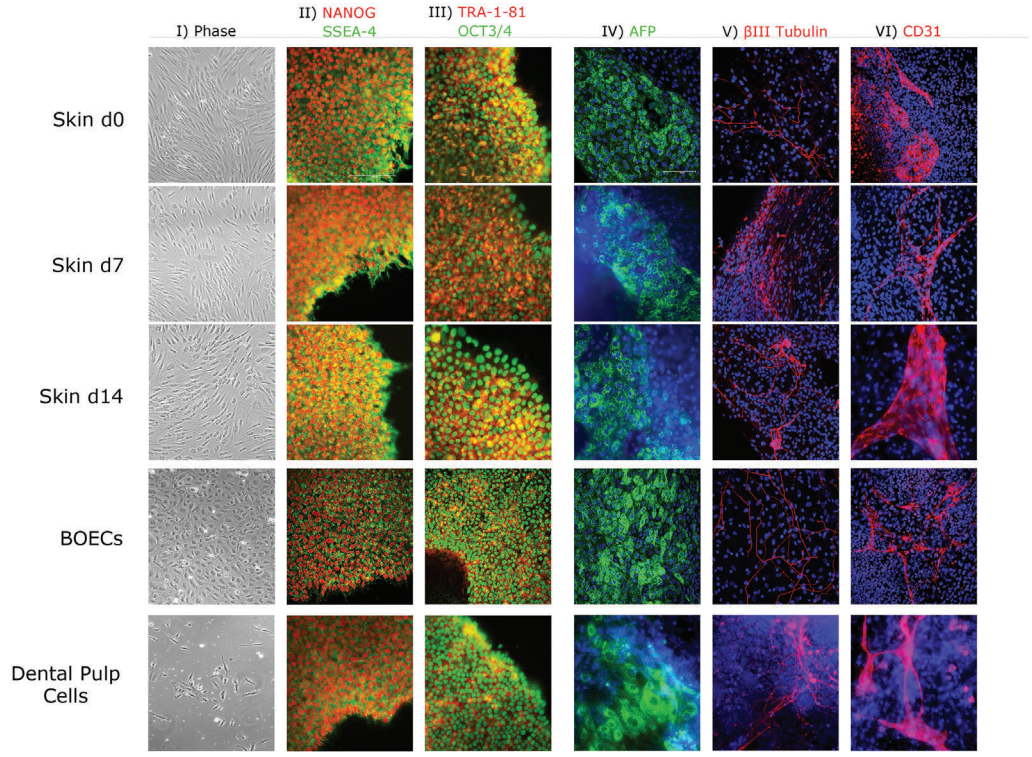

Figure 1: Characterization of hiPSCs derived from skin fibroblasts, dental pulp cells or BOECs. Bright field images of the somatic cells before transduction (column I). Undifferentiated hiPSCs express typical markers of hESCs (red: NANOG, TRA-1-81; green: OCT3/4, SSEA-4) as shown by immunofluorescent staining (columns II and III). Immunofluorescent stainings of spontaneous differentiation of hiPSCs into derivatives of the three germ layers (columns IV-VI: AFP (green): endoderm; βIII-TUBULIN (red): ectoderm; CD31 (red): mesoderm; nuclei (DAPI, blue)). BOECs: Blood outgrowth endothelial cells; AFP: alpha feto-protein. Scale bar: 100 µm.

Material and Methods:

Isolation of skin fibroblasts

Nine 4 mm punch biopsies were obtained from anonymously donated skin (from the Department of Dermatology, LUMC) and processed immediately (d0) as described below or stored in phosphate buffered saline (PBS) at 4 °C for 7 and 14 days (d7, d14, respectively), before being processed (three biopsies per time point).

For fibroblast isolation skin pieces were incubated overnight at 4 °C in 25 U/ml of dispase (Gibco) dissolved in Dulbecco's Modified Eagle Media/F12 (DMEM/F12, Gibco). The next morning the biopsy was rinsed with PBS and the epidermis removed and discarded. Using a scalpel the dermis was minced into small pieces of approximately 0.5 mm by 0.5 mm, then incubated in 0.75% collagenase A (Roche) and 2 U/ml of dispase (Gibco) in PBS for one hour in a shaking water bath at 37 °C. The samples were mixed by gently vortexing every ten minutes during incubation. During this period, the pieces disintegrated. At the end of incubation fibroblast growth medium (Dulbecco's Modified Eagle Media (DMEM) supplemented with 2 mM L-glutamine, 10 mM non-essential amino acids (NEAA), 75 U/ml penicillin, 75 µg/ml streptomycin, 50 µg/ml gentamicin, 1 mM sodium pyruvate (all Invitrogen), 10 µg/ml ascorbic acid and 10 % fetal calf serum (FCS) (both Sigma)) was added. Cells were centrifuged for 5 min at 200 x g, resuspended in fibroblast growth media and plated in a T25 flask. Cells attached within one or two days. Upon reaching confluence, cells were passaged according to standard procedures using trypsin/EDTA (Gibco) at a split ratio of 1:3. Fibroblasts at passage 2 were used for reprogramming.

Isolation of dental pulp cells from milk teeth
Milk teeth from a 9 year old boy and a 10 year old girl were collected anonymously and stored dry at 4 °C overnight. Each tooth was washed with PBS, wrapped in Parafilm and plastic bags and mechanically crushed using a hammer. The pieces were incubated in a mixture of 4 mg/ml dispase (Gibco) and 3 mg/ml collagenase A (Roche) for 1 hour in a shaking water bath at 37 °C, with gentle vortexing every 10 minutes. At the end of incubation, fibroblast growth media was added; the cells were centrifuged at 200 x g for 10 minutes and resuspended in fibroblast growth media containing 2.5 mg/L amphotericin B (Sigma). Cells were plated in a 6 cm dish until reaching confluence and subsequently split 1:3 using trypsin/EDTA according to standard procedures. Amphotericin B was removed after the first passaging. For reprogramming we used dental pulp cells at passage 3.

Isolation of BOECs
BOECs were isolated from anonymously donated peripheral blood as described previously [11] with the exception that FCS (Sigma) was used instead of pooled human platelet lysate. In brief, 20 ml of blood was collected in a BD Vacutainer NH 170 iU and then mixed with complete EGM-2 media (Lonza) supplemented with 10 % FCS and additional heparin (100U/ml, Biochrom AG). After 24 hours erythrocytes were removed by gentle washes with PBS and the remaining cells cultured in EGM-2 with 10 %FCS until colonies of cells with cobblestone morphology appeared 14-21 days later. Once colonies had reached diameters of approximately 1 cm, cells were passaged using trypsin/EDTA. For reprogramming we used BOECs at passage 1 to 5. Ficoll-based isolation of BOECs was performed as described (Wang et al., submitted).

Flow cytometry analysis of BOECs
BOECs were trypsinized and incubated with an antibody against CD31 (CD31-APC, eBioscience) or CD34 (CD34 PerCPC/Cy5.5, BD Pharmingen) for 30 min at 4 °C and subsequently analyzed with a LSRII FACS (BD Pharmingen).

Reprogramming vector and lentivirus production
Lentiviruses containing a self-inactivating polycistronic cassette encoding *Oct3/4*, *Sox2*, *Klf4*, and *c-Myc* [12] were produced using polyethyleneimine to cotransfect HEK/293 T cells with the expression vector pRRL.PPT.SF.hOKSM.idTomato.preFRT and the helper vectors pCMV-VSVG, pMDLg-RRE, pRSV-REV. The virus was harvested after 48 hours. The amount of virus particles was measured by ELISA detecting HIV p24 using a kit (Zeptometrix) according to the manufacturer's protocol and the virus titer was estimated by multiplying the p24 concentration (ng/ml) with a factor of 2500 [13].

Reprogramming skin fibroblasts
For lentiviral infection, 2 x 10^4 fibroblasts were seeded into one well of a 12-well plate and transduced 4 to 6 hours later with the lentivirus at 0.4 multiplicity of infection (MOI) in the presence of 4 µg/ml polybrene (Sigma-Aldrich) in fibroblast media. The virus was removed after 24 hours. Six days after transduction, the cells were harvested and subjected to flow cytometry analysis (LSRII FACS, BD) for quantification of dTomato expression. After determining efficiencies 10,000 fibroblasts were seeded on mouse embryonic fibroblasts (MEFs; 2 x 10^6 MEFs/10 cm dish) and cultured in hESC KOSR Medium (Dulbecco's Modified Eagle Media (DMEM)/F12 supplemented with Glutamax, 10 mM NEAA, 25 U/ml penicillin, 25 µg/ml streptomycin, 100 µM β-mercaptoethanol, 20 % knockout serum replacement (KOSR; Invitrogen) and 10 ng/ml basic FGF (PreproTech)). Medium was changed every other day until the appearance of hESC-like colonies.

hiPSC lines were named according to the nomenclature suggested by Luong et al. [14]. The data shown in Figures 1 and 2 was generated from hiPSC line C (LUMC0016iCTRL).

Reprogramming of dental pulp cells from milk teeth
Dental pulp cells from milk teeth were reprogrammed as described above for skin fibroblasts with MOIs of 0.5, 1 and 5. The hiPSC line G (LUMC0012iCTRL) was used to generate data shown in Figures 1 and 2.

Reprogramming of BOECs
BOECS were reprogrammed as described for skin fibroblasts except that MEFs were added after attachment of transduced BOECs. Four days after transduction, BOECs were harvested and seeded on gelatinized 10 cm dishes in EGM-2/10 % FCS medium. The following day, MEFs were added at a density of 1.7 x 10^6 cells/10 cm in MEF medium (DMEM supplemented with 2 mM L-glutamine, 10 mM non essential amino acids, 25 U/ml penicillin, 25 µg/ml streptomycin, 10 % FCS (Sigma)) After 24 hours cells were cultured in hESC KOSR medium until the appearance of hiPSC colonies. The hiPSC line F (LUMC0018iCTRL) was used to generate data shown in Figures 1 and 2.

Picking of hiPSC colonies and expansion
hESC-like colonies were picked manually, and cultured and expanded in mTeSR1 according to the manufacturer's protocol (Stem Cell Technologies).

RT-PCR
Total RNA was isolated from undifferentiated hiPSCs using the NucleoSpin RNAII kit (Macherey-Nagel) according to the manufacturer's instructions. cDNA was synthesized from 2 µg of RNA using the iScript cDNA synthesis kit (Invitrogen). Primers and PCR conditions were as previously described [15]. PCRs were performed with SilverStar Taq polymerase (Eurogentec).
We used the Nkx 2-5$^{eGFP/w}$ hESC line [16;16] as a standard for the expression of typical markers of undifferentiated pluripotent stem cells.

Microarray and PluriTest
For whole genome microarray total RNA was isolated from 1 x 10^6 cells with a RNA isolation kit (Qiagen) and hybridized on Illumina HT12v4 microarrays following the manufacturer's protocol and as previously described [17] The raw microarray data was analyzed with the PluriTest algorithm as described [17].

Alkaline phosphatase (AP) staining and calculation of reprogramming efficiencies
Citrate-acetone-formaldehyde fixed colonies were exposed to an alkaline-dye mixture according to the manufacture's instructions (Sigma-Aldrich, 86R-1KT). The reprogramming efficiencies were calculated as the number of AP positive colonies normalized to the number of cells seeded on MEFs after transduction.

Southern blot
DNA from hiPSC colonies was collected using phenol chloroform extraction. DNA was digested by SphI and run on a 0.7 % TAE gel. The DNA was then transferred onto a nylon membrane and incubated with a ^{32}P-labeled probed against RRE.

Spontaneous differentiation of hiPSCs into derivatives of the three germ layers
hiPSC colonies were passaged as described and pieces cultured in mTESR1 on Matrigel coated chamber slides (BD Falcon) for two days. Subsequently cells were cultured in DMEM/F12 (Gibco) supplemented with 20 % FCS to induce mesoderm or in DMEM/F12 supplemented with 1 % FCS and 50 ng/ml Activin A (R&D systems) to induce both ectoderm and endoderm.

The medium was changed every other day. After 3 weeks the cells were fixed with 2 % PFA for 30 min at room temperature.

Table 1: Effect of skin biopsy storage on reprogramming efficiencies.			
	Number of fibroblasts 7 days after isolation	Transduction efficiency (%)	Reprogramming efficiency (%)
Day 0 (A)	1,090,000	3.2	0.02
Day 0 (B)	266,250	5.9	0.58
Day 0 (C)	236,250	11	0.84
Day 7 (A)	100,00	3.6	0.016
Day 7 (B)	109,375	4.7	0.33
Day 7 (C)	90,625	6.4	0.58
Day 14 (A)	100,000*	2.4	0.012
Day 14 (B)	37,500	2.6	0.18
Day 14 (C)	17,500	7.8	0.55
*number of cells after 14 days in culture			

Skin fibroblasts were isolated immediately after biopsy sampling (d0) or after 7 or 14 days of storage in PBS (d7 and d14) from three individuals (A, B, and C; left column). The total number of skin fibroblasts from each time point was counted one week after isolation (second column from left). Transduction efficiencies (% of cells expressing dTomato) were determined by flow cytometry analysis six days after transduction with 0.4 MOI (third column from left). Reprogramming efficiencies based on the number AP expressing colonies normalized to the number of cells seeded on MEFs (right column). AP: alkaline phosphatase; MOI: multiplicity of infection; MEFs: mouse embryonic fibroblasts.

Immunofluorescent staining

Immunofluorescent staining was performed according to standard procedures. Briefly PFA fixed cells were permeabilized with Triton X-100, blocked with 4 % normal goat serum for 1 hour before overnight incubation with the primary antibody (NANOG (1:500, Peprotech), SSEA-4 (1:30, Biolegend), OCT3/4 (1:100, Santa Cruz), TRA-1-81 (1:25, Biolegend), βIII-TUBULIN (1:2000, Covance), AFP (1:25, Quarttet), CD31 (1:100, DAKO), von Willebrand factor (VWF, 1:1000 DAKO), VE-cadherin (1:200 Santa Cruz)) at 4 °C. The next day, secondary antibodies labeled with Cy3 (1:200, Jackson Immuno Research), Alexa 568 (1:200, Invitrogen) or Alexa 488 (1:500, Invitrogen) were added for 1 hour at room temperature. Nuclei were stained with DAPI before mounting slides with Mowiol (Calbiochem).

Results:

Reprogramming cells from skin biopsies after long-term storage

Skin fibroblasts were isolated from three 4 mm punch biopsies under local anesthesia, which are regarded as minimally invasive and do not require stitching. Since immediate processing of biopsies is not always possible, for example if patients are in clinics distant from the iPSC laboratory or even in different countries, we tested the effect of prolonged biopsy storage on fibroblast isolation and reprogramming efficiencies. In order to assess variability between skin samples of different origins, we took biopsies from three healthy individuals (samples A-C, Table 1). Fibroblasts were isolated from samples from each individual either immediately (d0) or after seven (d7) or 14 day (d14) storage in PBS at 4 °C. At each time point fibroblasts were isolated enzymatically, plated and counted after one week. As expected, fibroblasts grew readily from all d0 biopsies with samples yielding between ~ 2.5 x 10^5cells and 1 x 10^6cells (Table 1, samples A-C).

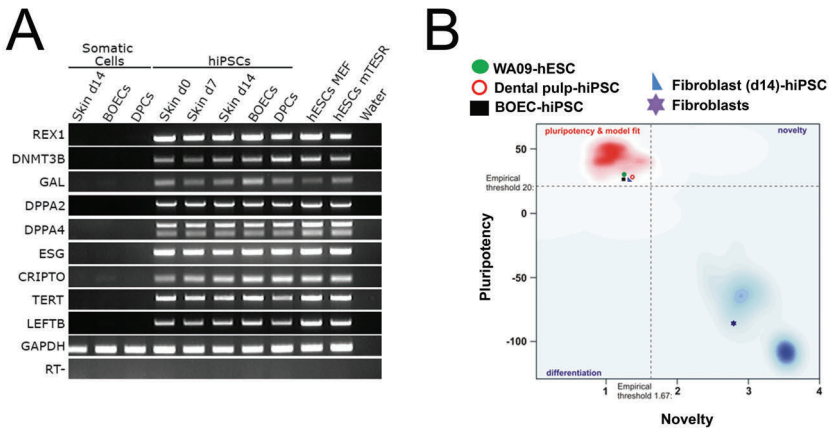

Figure 2: Undifferentiated hiPSCs express markers of pluripotent stem cells. A) RT-PCR analysis of the expression of pluripotent stem cell markers in somatic cells, hiPSCs and hESCs. GAPDH was used as a loading control. B) PluriTest analysis of hiPSCs derived from skin fibroblasts (d14), BOECs and dental pulp cells. All hiPSC lines have a high Pluripotency Score and a low Novelty Score indicating that they resemble normal pluripotent stem cells. The scores of a hESC line and fibroblasts, both from the reference pool are shown for comparison. BOECs: Blood outgrowth endothelial cells; DPCs: Dental Pulp Cells; hESCs: human embryonic stem cells; hiPSCs: human induced pluripotent stem cells; MEF: mouse embryonic fibroblasts.

Surprisingly, sufficient fibroblasts for reprogramming could also be isolated from biopsies after long-term storage, although the efficiency was clearly reduced. At d7 of processing we obtained ~ 1 x 10^5 cells from each biopsy corresponding to 60 % (Table 1, samples B, C) and 90 % reduction compared to d0 processing (sample A). Fibroblast numbers were further reduced when the biopsy was processed after 14 days of storage (Table 1). Nevertheless, fibroblast numbers obtained after 2 weeks storage were sufficient for reprogramming since we only used 1-2 x 10^4 cells for lentiviral transduction. The lifespan of fibroblasts in culture is limited by the Hayflick's factor (the maximum number of population doublings that a normal cell can undergo) and cells typically go into senescence after prolonged periods of culture [18;19]. Yet a prerequisite for cellular reprogramming is that somatic cells are actively dividing [20;21]. Since increased cell death during long-term storage of biopsies is likely to reduce the starting number of fibroblasts in the samples even before isolation, it can be inferred that a higher number of population doublings before reprogramming could take place. We therefore checked whether fibroblasts isolated from biopsies after long-term storage displayed any visible signs of senescence. As shown in Figure 1, cells from d7 and d14 had typical spindle-like fibroblast morphologies similar to the d0 cells and did not show any obvious signs of senescence. We were thus able to obtain sufficient numbers of proliferating fibroblasts from small skin biopsies even after prolonged storage. Next we tested whether cells isolated at different time points could be transduced with a polycistronic lentivirus carrying the four Yamanaka factors and a dTomato reporter [12]. Equal numbers of cells from d0, d7 or d14 were plated and transduced overnight at a MOI of 0.4. In an initial experiment we found that MOIs between 0.2 and 0.4 generate hiPSCs with one or two proviral integrations at favorable efficiencies (supplementary Fig. 1). Fibroblasts were harvested six days post-transduction and subjected to flow cytometry for quantification of dTomato-expressing cells. In general the number of dTomato-positive cells did not exceed 11 % (Table 1). For two out of three samples, transduction efficiencies in fibroblasts from d7 or d14 were lower compared to d0 fibroblasts with a maximal reduction of roughly 50 % (Table 1, samples B, C). The transduction efficiency of sample A fibroblasts was low (d0 and d7) and was further reduced only at d14 (Table 1). Next, we tested whether fibroblasts isolated after long-term storage (d7 and d14) could still be reprogrammed to hiPSCs and whether the efficiencies differed from that

of freshly isolated ones (d0). After flow cytometry analysis, transduced fibroblasts were seeded on MEFs and cultured in hESC media. When hESC-like colonies emerged, we used AP staining to quantify the number of hiPSC colonies. We found that AP staining closely overlaps with silencing of the transgenes (as determined by the absence of dTomato expression) indicating that AP staining can indeed serve as a preliminary marker to estimate the efficiency of reprogramming (supplementary Fig. 2). Silencing of the transgenes is required for successful reprogramming [21]. In a first experiment we reprogrammed fibroblasts from sample A. For freshly isolated cells (d0) we obtained a reprogramming efficiency of 0.02 % (Table 1). Reprogramming efficiencies were similar for fibroblasts isolated from biopsies, which had been stored for 7 or 14 days (Table 1). Since the reprogramming efficiency for this particular sample was low compared to previous experiments (data not shown) we generated hiPSC lines from samples B and C. For both, reprogramming efficiencies were high (~0.6-0.8 %, d0, Table 1). Reprogramming efficiencies were only slightly decreased when hiPSCs were generated from d7 or d14 fibroblasts (Table 1). Still, even at the lowest reprogramming efficiency (0.18 %, 14d, sample B) we obtained eighteen hiPSC colonies from 10,000 cells seeded on MEFs. This is far more than the average amount of three clones generally used to assess clonal variation. Colonies with hESC-like morphology were picked from d0, d7 and d14 samples between 28 and 42 days after transduction and could be readily expanded on Matrigel and mTESR (Fig. 3B). Undifferentiated hiPSCs derived from d0, d7 or d14 fibroblasts expressed markers typical of hESCs as assessed by immunofluorescent staining (Fig. 1) and RT-PCR (Fig. 2A). In addition all lines were able to differentiate effectively into derivatives of the three germ layers in vitro (Fig. 1). In order to verify the pluripotent state, we used PluriTest to analyze undifferentiated hiPSCs derived from d14 fibroblasts. The most stringent assay of pluripotency in human pluripotent stem cells (PSCs) is generally regarded as teratoma formation after injection of undifferentiated cells into immunocompromised mice. Teratomas of hPSCs should contain derivatives of all three germ layers. However, aside from requiring animal use and being time consuming, the results are difficult to quantify. By contrast PluriTest is a bioinformatic assay which predicts pluripotency based on the comparison of the microarray data of a query sample with the expression profiles from more than 250 pluripotent stem cell lines as well as from non-pluripotent cells [17]. The resulting “Pluripotency Score” indicates the extent to which the query sample contains a pluripotent signature whereas the “Novelty Score” reports whether the tested cells resemble normal PSCs [17]. As shown in Figure 2B, hiPSCs derived from 14d fibroblasts had a high Pluripotency Score whereas their Novelty Score was low, indicating that they were indistinguishable from normal hESCs. In summary, we have developed an efficient protocol to generate hiPSCs from fibroblasts isolated from biopsies even after prolonged storage in very simple and readily available conditions. To our knowledge this is the first report showing that skin fibroblasts can be efficiently reprogrammed even when their isolation from biopsies is delayed for up to 14 days.

Reprogramming of blood outgrowth endothelial cells (BOECs)

Although punch biopsies are minimally invasive, the procedure can be unpleasant and not all patients agree to donate tissue in this way. Taking blood is even less invasive and often part of routine patient examinations. We therefore investigated whether peripheral BOECs could be reprogrammed into hiPSCs and how the efficiency compared with skin fibroblasts. To isolate BOECs, we used a method similar to that described by Reinisch et al. [11]. Briefly 10-20 ml of blood was mixed at a ratio of 1:4 with EGM-2 endothelial growth media containing FCS and then cultured in endothelial growth media until colonies of cells with cobblestone-like morphologies emerged about three weeks later. Cells expressed von Willebrand factor (VWF) and CD31 and were able to form typical tubular networks on Matrigel (not shown). Flow cytometry analysis revealed that the majority of the cells expressed CD31 (~80 %) whereas a smaller proportion was double positive for CD31 and CD34 (14 %, data not shown). In a first experiment, we transduced BOECs at MOIs of 1, 10 or 25 and obtained a single hiPSC colony four weeks post transduction at 10 MOI, corresponding to a reprogramming efficiency of 0.001 % (Table 2, sample D). The colony

was picked and expanded on Matrigel in mTESR1 media. hiPSCs expressed typical markers of pluripotency, as above, and were able to differentiate into the three germ layers in vitro (not shown). The low reprogramming efficiency could have been caused by virus overload, thus we aimed at optimizing the amount of virus in the subsequent experiment: BOECs from a different individual were transduced with 1, 2 or 4 MOI. At 1 MOI 40 % of the cells expressed dTomato and the percentage further increased up to 70 % at 4 MOI (table 2). hiPSC colonies appeared late (4-7 weeks post transduction, Fig. 3B) and were again scarce. Due to the low numbers, we only quantified hiPSC colonies based on morphology and silencing of transgenes (absence of dTomato). Reprogramming efficiencies were generally low, with ~0.002 % at 1 MOI and a maximum of 0.008 % at 2 MOI (Table 2, sample E). hiPSC colonies were picked and expanded on Matrigel in mTESR1 media. Cells expressed the typical set of pluripotency markers and were able to differentiate into derivatives of all three germ layers in vitro (supplementary Fig. 3).

Table 2 :Transduction and reprogramming efficiencies for dental pulp cells and BOECs.

	Transduction efficiency (%)			Reprogramming efficiency (%)		
	MOI			**MOI**		
	1	**10**	**25**	**1**	**10**	**25**
BOECs (D)	N/A	N/A	N/A	0	0.0011	0
	1	**2**	**4**	**1**	**2**	**4**
BOECs (E)	42	61	70	0.0019	0.0083	0.0058
BOECs (F)	2.5	4.9	16.2	0.012	0.064	0
	0.5	**1**	**5**	**0.5**	**1**	**5**
Tooth (G)	2.5	4.9	16.2	0.012	0.064	0
Tooth (H)	3.2	4.8	16.7	0.11	0.30	0.36

BOECs or dental cells were transduced with increasing MOIs as indicated and transduction efficiencies determined by flow cytomtery analysis of dTomato expressing cells (second column from left). Reprogramming efficiencies are based on the number of hESC-like colonies (BOECs) or AP expressing cells (dental pulp cells) normalized to the number of cells seeded on MEFs (right column). AP: alkaline phosphatase; MOI: multiplicity of infection; MEFs: mouse embryonic fibroblasts.

Using the method for isolation of BOECs described above, we were unable to obtain BOECs from about half of the blood samples. In addition the number of BOEC-colonies clearly varied between patients and approximately $1x10^5$ cells could be obtained in the best case. We therefore tried to isolate BOECs from 80 ml of blood with a standard Ficoll-based method. We were then able to obtain BOECs in four out of five blood samples. Lower amounts of blood dramatically decreased the success rate for the isolation of BOECs (data not shown). For reprogramming, Ficoll-isolated BOECs were transduced with the lentivirus at 1, 2 or 4 MOI (table 2, sample F). Although transduction efficiencies were low, we obtained two colonies at both 2 and 4 MOI corresponding to a reprogramming efficiency of 0.018 and 0.022 %, respectively. Again colonies were pluripotent as assessed by immunofluorescent staining (Fig. 1) and RT-PCR (Fig. 2A) and readily differentiated into derivatives of the three germ layers (Fig. 1). In addition, BOEC-derived hiPSCs were pluripotent as assessed by PluriTest (Fig. 2B).

hiPSCs can thus be generated from BOECs isolated using different methods, although reprogramming efficiencies in general tend to be lower compared to skin fibroblasts under the same conditions.

Reprogramming cells from milk teeth

In the Netherlands and possibly elsewhere, medical ethics committees will generally not permit punch biopsies from children and minors unless part of a surgical procedure. In addition a volume of 40 ml is regarded as the maximum acceptable for pediatric blood collection. Previously reprogramming of cells obtained from dental pulp from adult individuals has been reported [10] Here we investigated whether milk teeth obtained from children after natural loss could also be used for this purpose. Fibroblast-like cells were isolated from teeth stored dry overnight at 4 °C and these expanded readily in culture reaching numbers between $4.5x10^5$ and $7.5x10^5$ cells after 16 or 19 days, respectively, with the capacity for further expansion as necessary for storage or tissue banking. Next we added increasing amounts of a lentivirus carrying the four Yamanaka factors and a dTomato reporter (0.5, 1 or 5 MOI). The transduction efficiency analyzed by flow cytometry at day 6 post transduction was directly correlated with the amount of virus although not linearly. Cells from both samples showed similar transduction efficiencies with a maximum of ~ 16 % at 5 MOI (Table 2). Transduced cells were plated on feeders and the number of hiPSC colonies was quantified with AP staining. Despite similar transduction rates, we observed a patient-specific variation in reprogramming efficiencies (Table 2): Whereas one sample was reprogrammed at an efficiency similar to that of skin fibroblasts (sample (H), Table 2) the reprogramming efficiency of the second sample was about 5 times lower (sample G). hiPSC colonies from sample H (from all MOIs) were picked 32-42 days after transduction indicating that reprogramming kinetics of juvenile dental pulp cells and skin fibroblasts are approximately similar (Fig. 3B). Colonies expressed the typical set of pluripotency markers as assessed by immunofluorescent staining (Fig. 1) and RT-PCR (Fig. 2A) and differentiated readily into derivatives of the three germ layers in vitro (Fig. 1). Again PluriTest identified these hiPSCs as closely resembling normal hESCs (Fig. 2B). Thus hiPSCs can be generated from teeth obtained in a completely non-invasive manner from children at efficiencies similar to reprogramming of skin fibroblasts.

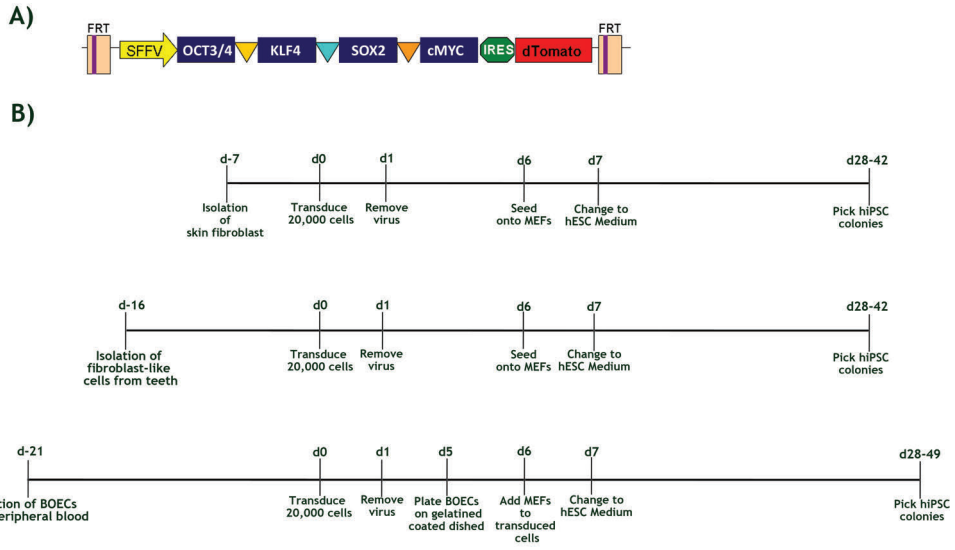

Figure 3: Lentiviral vector and kinetics of somatic cell isolation and reprogramming. A) Schematic of the polycistronic lentiviral vector used to generate iPSCs. B) Isolation and reprogramming timelines for skin fibroblast, dental pulp cells, and BOECs, respectively. hiPSCs: human induced pluripotent stem cells; BOECs: Blood outgrowth endothelial cells.

Discussion

In this study, we isolated cells from three easily accessible somatic tissue sources and compared reprogramming efficiencies using a polycistronic lentivirus. Skin fibroblasts and cells from milk teeth could be readily isolated and reprogrammed at similar efficiencies even if the skin samples had been stored for up to 14 days in a simple salt solution. By contrast the isolation efficiency of BOECs was variable from individual to individual in our hands and reprogramming efficiencies in general tended to be lower compared to the other two cell types.

Numerous reprogramming methods with different somatic cell types have been published [22-24]. Nevertheless comparisons of efficiencies between laboratories remain difficult, even if the same reprogramming methods are used. The main reasons for this are differences in experimental procedures and the use of undefined cell culture components, which display high batch-to-batch variability. Although we did not reprogram skin fibroblasts, BOECs and dental pulp cells simultaneously, all of the hiPSC lines from the three tissue sources were generated with the same reprogramming method and under similar conditions that is with the same batches of FCS, lentivirus and MEFs. Our results show that skin fibroblasts and dental pulp cells can be isolated rapidly and are reprogrammable with similar kinetics and at similar efficiencies. As we had observed earlier for skin fibroblasts, low amounts of virus were sufficient to generate hiPSC with a maximum of two proviral integrations. For most of the clones however one transgene copy was sufficient for reprogramming. Since we used an excisable lentivirus, this offers the possibility of generating transgene-free hiPSCs by addition of the FLPe enzyme. This has been done routinely in independent experiments generating disease hiPSC lines (Dambrot et al. and Mikkers et al., unpublished).

Between the fibroblasts isolated from different individuals we observed some variability in reprogramming efficiencies although numbers of independent experiments were limited. For fibroblasts from individual A the efficiency of hiPSC generation was more than tenfold lower compared to samples B and C. Nonetheless transduction efficiencies were within the same range. This variability may therefore have been individual specific.

Compared to skin fibroblasts, dental pulp cells were derived in a completely non-invasive manner and thus represent an excellent source of somatic tissue for children. The reprogramming of dental pulp cells from adult teeth has been reported previously [10] but the results here show that this is also efficient from juvenile teeth at the end of their lifespan even after dry storage at 4 °C. This is important since the time point of natural loss of teeth is often not precisely predictable and experimental planning may be more difficult for this type of tissue. We demonstrated that immediate processing is not required. In addition dental pulp cells can also be isolated from teeth, for which extraction for pathological reasons is scheduled. One disadvantage of this type of tissue however, is a higher risk of bacterial and fungal contamination since teeth are directly exposed to pathogens. Treatment with additional antibiotics and fungicides immediately after isolation can help overcome this problem.

Compared to the skin fibroblasts and dental pulp cells, isolation of BOECs from small amounts of blood was only successful in some of the samples and outgrowth of the cells was more time-consuming. The reason for this is possibly the low abundance of BOECs in peripheral blood. Indeed BOECs represent only a small fraction of mononuclear cells and their numbers vary considerably between donors [25]. This may be due to pathological conditions which influence the abundance of the cells in the blood [26]. It is likely that higher blood volumes would increase the efficiency for isolation of BOECs. Unfortunately this would require high amounts of EGM-2 media, which is expensive. As an alternative method we therefore tried to isolate BOECs from 80 ml of blood with a standard Ficoll-based method. Indeed this increased the success rate for isolation of BOECs. Nevertheless not every adult individual may agree to donate such a relatively large volume of blood. For pediatric samples a volume of 40 ml is regarded as the maximum. Thus for children, blood as used here is not a likely tissue source

for reprogramming. Another disadvantage of blood samples maybe their limited storage. For the isolation of BOECs, processing of the blood is initiated within two hours after blood collection [11]. In addition, culture of BOECs requires specific growth medium, which is relatively expensive. Importantly, compared to fibroblasts and dental pulp cells, reprogramming of BOECs was slower and tended to be less efficient. Our experiments therefore indicated that BOECs may be less suitable for reprogramming than skin fibroblasts and dental pulp cells, at least with this lentiviral vector and experimental conditions used here. On the other hand, these cells could be ideal for reprogramming in other circumstances, for example, if differentiation of hiPSCs into the endothelial lineage is required. Effects of epigenetic memory, which predispose iPSCs to favor differentiation towards their cell of origin, have been reported for various cell types [27]. In addition, BOEC-iPSCs lack irreversible rearrangements in genes involved in T cell and B lymphocyte function, contrary to iPSCs generated from these blood cell types [28]. Finally BOECs are not exposed to UV irradiation of sunlight as in the case for skin fibroblasts. These cells may therefore have a lower risk of acquiring additional non-systemic mutations.

Since this work was completed, Geti et al. reported the generation of hiPSCs with retroviral vectors from late outgrowth endothelial progenitor cells (L-EPCs, also called BOECs) from peripheral blood [29]. Reprogramming efficiencies for L-EPCs were higher compared to skin fibroblasts. In our hands the polycistronic lentivirus was able to reprogram skin fibroblasts and dental pulp cells at high efficiencies whereas hiPSC generation from BOECs tended to be low. The efficiency of reprogramming in individual cell types is likely to depend on the reprogramming method. Here we used a polycistronic lentivirus, which offers the possibility of transgene excision after reprogramming. In addition all reprogramming factors are on the same virus reducing the number of necessary integrations and eliminating potential variability between clones due to differences in factor stoichiometry.

Beside simple, inexpensive and fast isolation of cells and high reprogramming efficiencies for most samples, we show here that skin samples also offer the possibility of prolonged storage before processing. Although cell numbers were reduced, fibroblasts isolated after long-term storage in a simple salt solution, often the most readily available option in a standard hospital clinic, did not show any visible signs of senescence and were reprogrammed at similar or only slightly reduced efficiencies as fresh cells. Thus we have developed a robust protocol for obtaining actively dividing cells from small skin pieces which are equally suitable for reprogramming as freshly isolated samples. In some cases, immediate processing is impossible because of long distances between the patient center and the reprogramming facility. The ability to store skin samples overcomes this problem. Isolation of cell types from other somatic tissues, e.g. BOECs or T cells from blood, often requires almost immediate processing, excluding these tissues in such a case.

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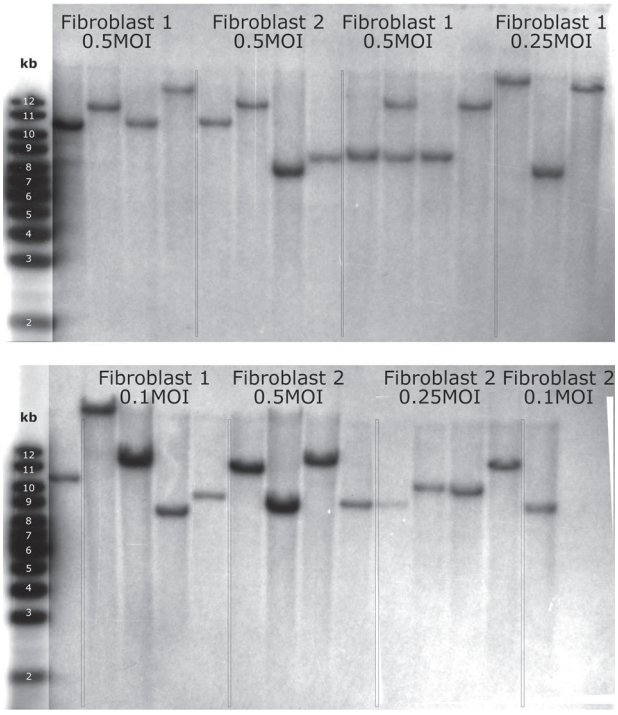
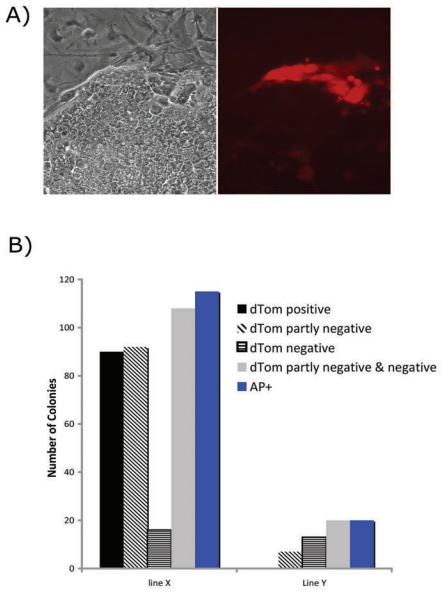
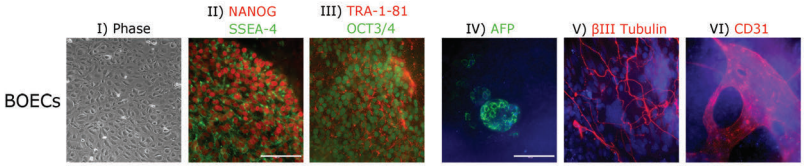
Supplementary Figures



Supplementary Figure 1: Transduction of skin fibroblasts with low MOI leads to the generation of hiPSC clones with a maximum of one or two proviral integrations. Southern blot of 29 hiPSC clones from 2 different fibroblast samples after transduction with MOI ranging from 0.1 to 0.5 confirming low integration number. MOI: multiplicity of infection.



Supplementary Figure 2: AP staining closely overlaps with silencing of the transgenes (absence of dTomato) A) An example of a partially dTomato negative colony is shown. These colonies are nevertheless likely to be fully reprogrammed because of their overall morphological similarity to hESCs. B) Quantification of colonies per 10 cm dish. Twenty four days after seeding, 10,000 transduced fibroblasts on a 10 cm dish with MEFs, the number of colonies which (i) expressed dTomato ("positive"), (ii) were partially negative or (iii) were completely negative for dTomato were counted. Subsequently AP-expressing colonies on the same plates were quantified. The sum of partially negative and negative colonies was similar (line X) or equal (line Y) to the number of AP expressing colonies. We therefore used AP staining as a preliminary marker to estimate reprogramming efficiencies in subsequent experiments. AP: alkaline phosphatase; dTom: dtomato.



Supplementary Figure 3: Characerization of hiPSC derived from BOECs (line E). Bright field images of the somatic cells before transduction (column I). Undifferentiated hiPSCs express typical markers of hESCs (red: NANOG, TRA-1-81; green: OCT3/4, SSEA-4) as shown by immunofluorescent staining (columns II and III). Immunofluorescent staining of hiPSCs following "spontaneous" differentiation into derivatives of the three germ layers (columns IV-VI: AFP (green): endoderm; βIII-TUBULIN (red): ectoderm; CD31 (red): mesoderm; nuclei (DAPI, blue)). BOECs: Blood outgrowth endothelial cells; AFP: alpha feto-protein. Scale bar: 100 µm.

Chapter 4:
Strategies for rapidly mapping proviral integration sites and assessing cardiogenic potential of nascent human induced pluripotent stem cell clones

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Abstract

Recent methodological advances have improved the ease and efficiency of generating human induced pluripotent stem cells (hiPSCs), but this now typically results in a greater number of hiPSC clones being derived than can be wholly characterized. Additionally, individual clones display biological variability including their capacity to differentiate, with genetic heterogeneity thought to be a contributing factor. It is therefore imperative that methods are developed which facilitate rapid selection of the hiPSC clones that are most suited to the downstream research aims. Here we describe a useful combination of procedures that enables the simultaneous screening of multiple iPSC clones to determine their genomic integrity as well as their cardiac differentiation potential within two weeks of the putative reprogrammed colonies initially appearing in culture. By coupling a splinkerette-PCR method with Ion Torrent sequencing, we could ascertain the number of and map the proviral integration sites in lentiviral-reprogrammed hiPSCs, enabling identification of clones containing single copy integrations in genomic regions devoid of known genes or transcriptional control elements. In parallel, we developed an effective cardiac differentiation protocol that generated functional cardiomyocytes within 10 days without requiring line-specific optimization for any of 6 independent human pluripotent stem cell lines tested. Finally, to demonstrate the scalable potential of these procedures, we picked 20 nascent iPSC clones and performed these independent assays concurrently. Before the clones required passaging, we were able to identify clones with a single integrated copy of the reprogramming vector and robust cardiac differentiation potential for further analysis.

Introduction

Since the initial reports that human fibroblasts could be reprogrammed to a self-renewing, pluripotent state [1,2], interest in induced pluripotent stem cells (iPSC) has grown exponentially as their many applications in biomedical research and regenerative medicine have become evident. However, a remaining challenge for the field is to address the technical issues that contribute to individual cell line differences and thereby confound our ability to distinguish disease-associated variability from background noise. These include methods for rapidly assessing the quality and genomic integrity of the reprogrammed cell lines, and robust protocols for differentiation that are completely defined and readily transferable across multiple iPSC lines.

Most reprogramming methods generate a large number of putative iPSC clones that appear morphologically similar to embryonic stem cells (ESCs); however only a subset of these have comparable molecular and functional features [3]. To distinguish bona fide iPSCs from those that are only partially reprogrammed, detailed characterization of the clones is required, which is often lengthy and expensive. This typically limits the number of clones that are examined to fewer than six, with the selection of clones for further analysis predominantly based on subjective criteria like morphology and culture characteristics [4,5]. While this is generally sufficient to identify at least one genuine iPSC clone that can indefinitely self-renew and is pluripotent, it does not assess the clonal variability in differentiation efficiency that is often detected when pluripotent stem cells (PSCs) undergo further guided differentiation to functional cell subtypes [6-9]. One potential source of this clonal variation if integrative reprogramming methods have been used might be insertional mutagenesis caused by the reprogramming factors [10,11]. However, the genomic integrity of iPSCs is frequently not assessed, despite studies demonstrating that the integration of viral vectors can result in the dysregulation of adjacent genes [12,13]. This altered gene expression could lead to perturbed signalling pathways that may alter the pluripotency or differentiation potential of the iPSCs, similar to that observed in some virally-transduced hematopoietic stem cell lines [14].

To address these issues, we developed a novel combination of methods to aid in selecting hiPSC clones for studying cardiogenesis. Furthermore, both procedures are scalable, allowing many potential iPSC clones to be screened simultaneously. To demonstrate this, the number and location of the proviral integrations, as well as the cardiac differentiation potential of 20 putative iPSC clones was determined within 2 weeks following their emergence during reprogramming. These methods require minimal amounts of starting material and can assist in identifying the optimal iPSC clones for further characterization. Furthermore, these procedures potentially could be applied to aid in the selection of transgenic human PSCs in which, for example, overexpression constructs, therapeutic genes or ectopic reporters have been randomly integrated.

Materials and Methods

Ethics Statement

Human skin biopsies were obtained from patients after individual written permission using standard informed consent procedures, and following approval for use in this study by the Leiden University Medical Center's medical ethics committee. Control skin samples and milk teeth were obtained as waste tissue from donors in accordance with the Dutch Federation of Biomedical Scientific Societies "Use of human tissue for scientific research" and "Code of good use" directives. All samples were collected and anonymized by the treating physician.

Human iPSC derivation

Human dermal fibroblasts or dental pulp cells were reprogrammed to iPSCs using the pRRL.PPT. SF.hOKSMidTomato-preFRT polycistronic lentiviral vector as previously published [15,16]. Based on morphology, human (h)ESC-like colonies were manually picked 30 days after transduction. All human samples were obtained after informed consent.

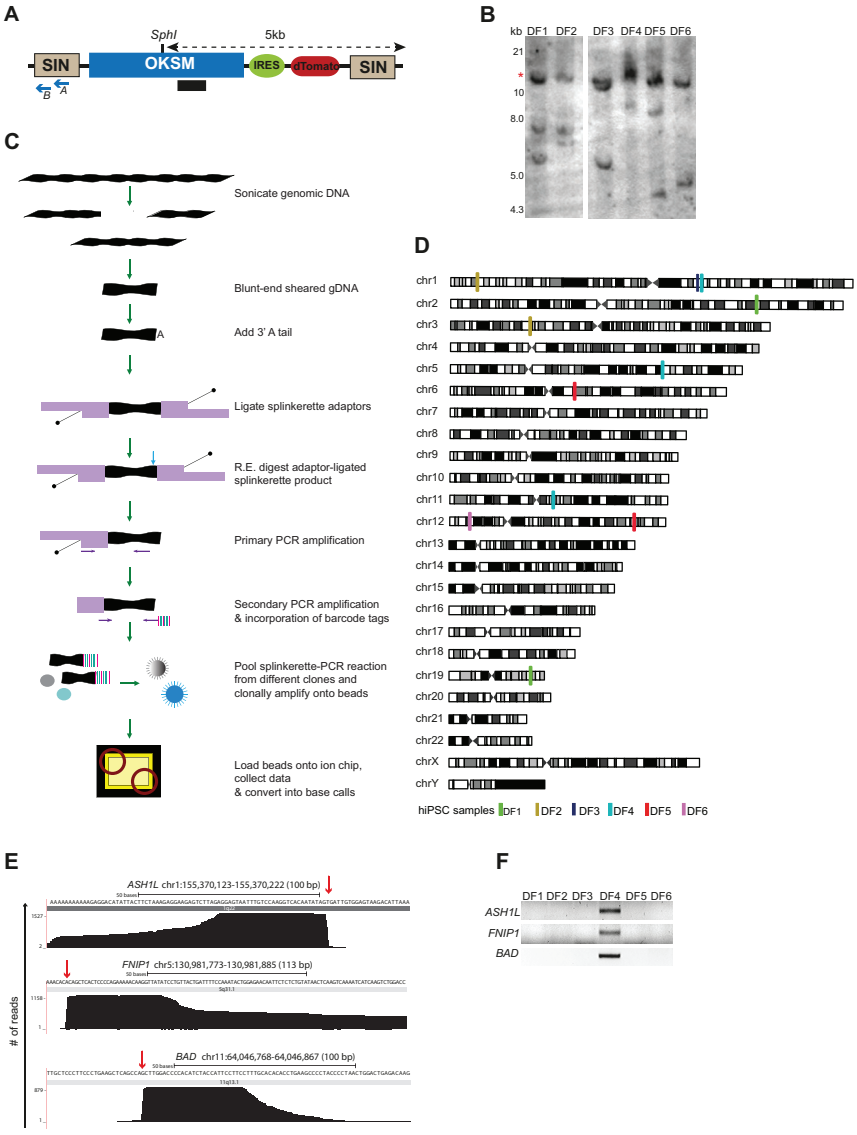

Figure 1. Genomic mapping of proviral integration sites in lentiviral-generated hiPSCs. A) Schematic representation of the polycistronic lentiviral vector used to generate the hiPSCs following integration into the host genome. The four reprogramming factors (OKSM) are linked by 2A peptide sequences, while a red fluorescent protein (dTomato) is downstream of an IRES sequence. The position and distance of the SphI site from the 3' end of the integrated provirus, as well as the probe (thick black line) used for Southern blot analysis are shown. Also depicted are the primers (A, B) within the viral LTR used in the amplification of the adaptor-ligated splinkerette products (SIN, self-inactivating element; OKSM, OCT4/KLF4/SOX2/c-MYC; IRES, internal ribosome entry site). B) Southern blot analysis of 6 control hiPSC lines to assess the number of viral integrations. Genomic DNAs were digested with SphI and hybridized with a probe to c-myc. The * indicates the location of the band corresponding to the endogenous c-MYC locus. C) Schematic representation of the shear-splinkerette procedure coupled to the fragment preparation for multiplexed PCR amplification and Ion Torrent sequencing. D) Idiogram depicting the genomic integration sites of the lentivirus for the 6 control hiPSC lines as determined by shear-splink PCR combined with Ion Torrent sequencing. E) Visualization of the mapped reads obtained for hiPSC line, DF4 following shear-splink PCR and Ion Torrent sequencing. Reads mapped to three genomic loci. The red arrows indicate the position in the genome where the provirus integrated. F) Validation of the lentivirus integration sites identified in E by genomic PCR analysis using primer pairs specific for each integration locus. Insertion of the lentivirus at these three genomic loci was only detected in the hiPSC line, DF4.

PSC culture and differentiation

PSCs were cultured on Matrigel (BD Biosciences)-coated tissue culture dishes in mTeSR1 according to the manufacturer's protocol (Stem Cell Technologies). The control hESC and dermal fibroblast (DF)-derived hiPSC lines used in this study are listed in Table S1. To initiate differentiation to cardiomyocytes, the PSCs were dissociated into small clusters of cells and seeded onto a Matrigel-coated cell culture dish in mTeSR1. Three days later (differentiation day(d) 0), the medium was replaced with low-insulin (1 mg/L), (LI)-BPEL medium [17] and supplemented with BMP4, Activin A, CHIR99021 and XAV939 as indicated. If the cultures were to be maintained for more than 16 days, the cells were overlaid with additional Matrigel (1:100) on differentiation d3 to prevent detachment from the dishes. From differentiation d6, the LI-BPEL medium was refreshed twice a week.

Southern blot

Genomic DNA was digested with SphI (Promega) overnight, resolved by gel electrophoresis and transferred to Hybond-N+ Nylon membrane (GE Healthcare). Hybridization and detection of the probe, encompassing the coding sequences of c-Myc, was performed as previously described [18].

Amplification and sequencing of vector integration sites

To determine the lentiviral integration sites in either the DF hiPSC lines or the putative iPSC clones, genomic DNA was isolated with the DNAeasy Blood and Tissue Kit (Qiagen) and splinkerette PCR performed [19-21]. Oligonucleotides and PCR amplification conditions used are listed in Tables S2 and S3. To fragment the DNA, either 2 µg was sheared for a target peak of 200bp using a Covaris S2 sonicator according to the manufacturer's protocol, or 5µg was digested with the restriction enzymes CviQI, AseI and BclI (all from New England Biolabs (NEB)). The resulting DNA fragments were concentrated, blunt-ended using the NEBNext End Repair module (NEB) and modified at the 3' ends with the addition of an adenosine (NEBNext dA-Tailing Module; NEB). Splinkerette adaptors were ligated to the DNA fragments using Quick T4 DNA Ligase (NEB). To prevent amplification of internal lentiviral sequences, samples were digested overnight with BglII. Primary amplification of the adaptor-ligated splinkerette products was performed using Platinum Taq HiFi (Invitrogen) with the primer pairs, PCR_A1 and PCR_A2, followed by secondary amplification with the primer pair, PCR_B1 and PCR_B2.

To de-multiplex the splinkerette-PCR products, the amplified products were either cloned into the vector pCR®2.1-TOPO® (Invitrogen), transformed into bacteria and sequenced using Sanger sequencing, or barcoded and sequenced using ion semi-conductor sequencing [22]. For Ion Torrent sequencing (Life Technologies) of pooled splinkerette-PCR products from multiple iPSC lines, "barcode" and adaptor sequences were incorporated into the primers PCR_B1 and PCR_B2. Adaptor and barcode sequences are listed in Table S4.

Mapping and Processing of Sequence Reads

The Ion Torrent sequence data was processed in several steps to identify the provirus integration sites in the hiPSC genome. First, the custom 3' splinkerette adaptor sequence was identified and removed from the sequence reads by the Ion Torrent Suite software (v3.4.2). The dataset was demultiplexed into the individual sample sets and the provirus sequences removed from the reads. Reads with a minimal length of 30 bases were aligned to the human genome (Hg19) using the TMAP sequence aligner with standard settings. The Bed-tools package [23] was used to retrieve regions with a peak height of at least 10% reads relative to the total number of aligned reads and a peak base width of at least 40 nucleotides. The selected candidate integration sites were annotated to the nearest gene or transcriptional control element. Lentiviral insertion loci in the DF-iPSCs were confirmed by genomic PCR using lentiviral-specific and genomic-specific primers (Table S5).

Flow cytometry
Differentiation d13 cells were dissociated using TrypLE Select (Invitrogen) and filtered through a 35 µm cell strainer (BD Bioscience). Intracellular flow cytometry with mouse anti-bovine cardiac troponin T (cTnT; Hybridomabank) was performed using the FIX & PERM kit (Invitrogen) according to manufacturer's protocol, with the primary antibody detected with phycoerythrin-conjugated goat anti-mouse IgG2a (Jackson ImmunoResearch). Cells were gated based on forward and side scatter, and flow cytometric gates were set using control cells not expressing GFP, or differentiated cells labeled with the isotype control. Samples were measured using a LSRII flow cytometer (BD Bioscience), and results analyzed using FlowJo 7.6.5 (TreeStar).

Immunofluorescence analysis
Differentiation d20 NKX2-5eGFP/w hESCs were dissociated with TrypLE Select, seeded on Matrigel-coated coverslips and cultured for a minimum of 72 h before fixing with 2% PFA. Coverslips were stained with antibodies against α-Actinin (mouse-α-rabbit), sarcomeric Tropomyosin (mouse-α-chick; both from Sigma Aldrich), Troponin I (rabbit-α-human), Nkx2.5 (rabbit-α-human; both from Santa Cruz), β-MHC (mouse-α-human; Millipore), MLC2a (rabbit-α-mouse; provided by S.W. Kubalak), as previously published [24]. Primary antibodies were detected with IgG-conjugated Cy3 (donkey-α-rabbit, Jackson Immunoresearch) or Alexa 488 (donkey-α-mouse, Invitrogen) antibodies. Images were captured on a Leica SP5 confocal laser scanning microscope (Leica Microsystems).

Table 1: The number of confirmed proviral insertions in the lentiviral-generated DF-hiPSC lines as determined using different mapping approaches.

Clone	Number of inserts as determined by:		
	Southern blot	RE-splinkerette; TOPO Subcloning	Shear-splink; Ion Torrent sequencing
DF1	2	2	2
DF2	2	2	2
DF3	1	0	1
DF4	1	2	3
DF5	2	1	2
DF6	1	1	1

RE: restriction enzyme

Electrophysiology and Ca2+ imaging
Action potential measurements were performed on single cardiomyocytes 7-15 days after cell dissociation and analyzed as described previously [25]. For Ca2+ imaging, differentiation d20 NKX2-5eGFP/w hESCs were dissociated with TrypLE Select, seeded (40,000 cells/well) into a Matrigel-coated 96 well imaging plate (BD Bioscience), and cultured for a further 7 days. Cells were loaded with 5µM Fluo-4 AM (Invitrogen) and imaged in Tyrode's solution using a confocal microscope (Leica AF6000) as previously published [26]. Spontaneous Ca2+ transients were recorded at 37° C for 20 s.

Results

High throughput mapping of lentiviral integration sites in iPSCs
To validate and establish a procedure for high-throughput sequencing and mapping of the lentiviral insertion sites, we initially quantified and mapped the genomic locations of the reprogramming cassettes in six control hiPSC lines (DF1-6) by Southern blot analysis, and by

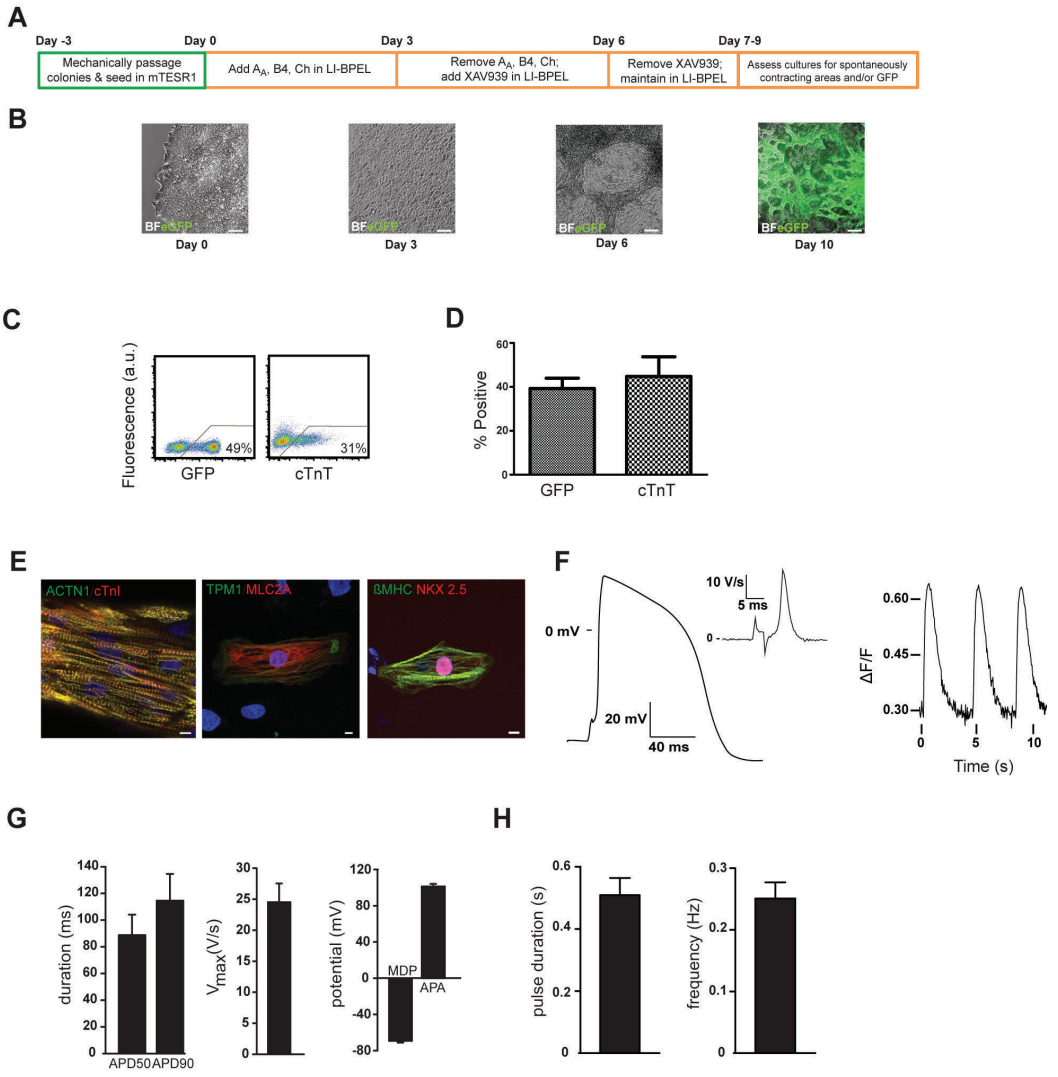

Figure 2. Efficient cardiogenesis of NKX2-5eGFP/w hESCs maintained as colonies in mTeSR1. A) Schematic representation of the optimized differentiation protocol for generating cardiomyocytes from human PSCs. B) Overlaid bright field (BF) and green fluorescence (eGFP) images showing the typical morphology and eGFP expression of NKX2-5eGFP/w hESCs at various time points during the differentiation. Scale bars, 100 µm. C) Representative flow cytometry profiles showing the correlation in the percentage of cells expressing eGFP (NKX2-5) and cardiac troponin T (cTnT) at differentiation d14. D) Histogram showing the mean percentage of differentiation d14 cells expressing eGFP (NKX2-5) or cTnT. Data represents the mean ± SEM from 5 independent experiments. E) Immunofluorescence images of the cardiac markers α-actinin (ACTN1), troponin I (cTnI), sarcomeric tropomyosin (TPM1), myosin regulatory light chain 2, atrial isoform (MLC2A), beta-myosin heavy chain (βMHC), and NKX2-5 in cardiomyocytes differentiated using the protocol depicted in A. All cells were co-stained with the nuclear dye DAPI (blue). Scale bars represent 5 µm. F) Representative electrophysiological recordings from cardiomyocytes derived using the protocol depicted in A. The left panel shows a typical example of an action potential and an upstroke velocity (inset) from a cardiomyocyte triggered at 1Hz. The right panel shows the calcium transient of a spontaneously contracting cardiomyocyte. G) Average data at 1Hz for action potential duration at 50% and 90% repolarization (APD50, APD90), maximal upstroke velocity (Vmax), maximal diastolic potential (MDP), and action potential amplitude (APA) in cardiomyocytes derived from NKX2-5eGFP/w hESCs (n=7, mean ±SEM). H) Summary of spontaneous whole cell Ca2+ pulse duration and frequency in differentiated NKX2-5eGFP/w hESC cardiomyocytes (n=34, mean ±SEM).

combining splinkerette-PCR with TOPO-shotgun cloning and amplicon sequencing. These hiPSC lines had been generated following transfection of fibroblasts with a polycistronic lentivirus at MOIs ranging from 0.25 to 5 (Figure 1A). We had previously ascertained that MOIs above 0.4 would frequently generate hiPSCs containing multiple proviral integrations [16], enabling us to assess the sensitivity of the above procedures.

Southern blot analysis confirmed that each of the lines contained at least 1-3 proviral integrations (Figure 1B). However, a limitation of this technique is that it only provides information on the number of integrations and not their genomic location. Such data is critical to fully assess the risk of insertional mutagenesis in virally-transduced cell lines. Additionally, if two or more integrations result in a similar sized DNA fragment when digested, this can lead to an inaccurate estimation of the total number of integrations in that clone. To resolve these issues, splinkerette PCR was performed on genomic DNA that was fragmented using restriction endonucleases (REs), either individually or in combination [19,20] (Figure S1). Splinkerette PCR is a variant of ligation-mediated PCR and is commonly used in forward genetic screens to map the genomic sites in which insertional mutagens such as viruses or transposons have integrated [19,21]. More recently the technique has been employed to identify the integration sites in iPSCs reprogrammed using transposons, enabling the excision of the reprogramming cassette following re-expression of the transposase to be monitored [18,27].However neither of these splinkerette procedures identified the minimum number of insertion sites as determined by Southern blot analysis for all six clones (Table 1), most likely due to amplification biases resulting from the uneven genomic distribution of RE recognition sites [21].

To limit these amplification and sequencing biases, we applied a modified splinkerette method, termed “shear-splink”, that employed random fragmentation of the genomic DNA resulting in controllable size distribution of DNA fragments for all insertions [21]. By incorporating ‘barcode’ sequences into the primers, the resulting splinkerette PCR products from the different clones could be pooled together and sequenced using ion sequencing technology (Figure 1C). All insertion sites previously identified using RE-splinkerette procedures were also detected using this high-throughput method. Furthermore, the shear-splink method mapped additional insertions not detected either by RE-splinkerette or Southern blot (Figure 1D & E, Table 1, and Table S6). For example, with DF4, Southern blot analysis identified one insert while shear-splink analysis identified three. All insertion events were confirmed by locus-specific genomic PCR (Figure 1F and Figure S2). These results indicated that combining shear-splink with ion semi-conductor sequencing can efficiently uncover proviral insertion sites and is amenable to high-throughput adaptation.

Robust and Scalable Protocol to assess the Cardiac Differentiation Efficiency of human PSCs

To test the cardiac differentiation potential of hiPSC clones as they emerge from the reprogramming process, a rapid and reproducible method requiring no optimization between the clones is needed. Building on previous findings [28-30], we developed a monolayer differentiation protocol that could be carried out directly on mechanically passaged PSC colonies grown in mTeSR1 culture medium (Figure 2A). The method was initially optimized using a hESC line in which GFP reports the expression of the cardiac associated transcription factor NKX2-5 (NKX2-5eGFP/w hESCs) [28]. We then confirmed the procedure also effectively induced cardiac differentiation in a further 5 human PSC lines (Figure S3).

Three days after mechanical passaging, the medium on the colonies was replaced with BPEL medium supplemented with 20 ng/ml Activin A, 20 ng/ml BMP4 and 1.5 μM Chir99021 for 72 h. Although this growth factor combination was sufficient to generate cardiomyocytes in some PSC lines, the yield was very low and variable between individual differentiation experiments. Because it has previously been demonstrated the tankyrase inhibitor XAV939 could promote cardiogenesis when added after mesoderm formation [30,31], we supplemented the medium

with 5μM XAV at day 3 for 72 h. Phase contrast photomicrographs of cultures at various time points during the protocol demonstrate the typical appearance of the cells during the differentiation process (Figure 2B). By differentiation d6, GFP- web-like colonies were visible that subsequently expanded, becoming GFP+ and forming a sheet of contracting cells by differentiation d10. Using this optimized differentiation protocol, on average ~40% of the cells expressed NKX2-5(eGFP) and cTnT by differentiation d14 (Figure 2C & D). Immunofluorescence analysis confirmed these cells expressed sarcomeric markers (Figure 2E). Typical examples of action potential, upstroke velocity and Ca2+ transients in the resulting cardiomyocytes are shown in Figure 2F. The action potential properties of these cells, as well as the spontaneous whole cell Ca2+ pulse duration and frequency (Fig 2G & H), were comparable to those previously reported for human PSC-derived cardiomyocytes [32-34].

Mapping proviral integration sites and assessing the cardiac differentiation potential of 20 newly reprogrammed hiPSC clones

To demonstrate the applicability of these methods to identify the lentiviral insertion sites and assess the cardiac differentiation potential of prospective iPSC clones rapidly, 20 putative iPSC clones were selected from reprogrammed dental pulp cells based on morphology. Colony pieces from these clones were either plated for the cardiac differentiation assay or used to isolate genomic DNA (Figure 3A). The shear-splink procedure in combination with ion semi-conductor sequencing identified that 1 of the 18 clones with mapped reads had 2 proviral insertions, while the remainder had only 1 insertion (Figure 3B). The predominance of single copy integrations was due to a MOI of 0.25 being used for reprogramming. Based on the genomic locations to which the lentivirus was mapped, 11 of these clones were unique with the remaining 6 being subclones. Of these 11 clones, 2 (clones 5 & 17) had the provirus integrated outside any known genes or non-coding RNA sequences. A complete overview of insert locations is summarized in Table S7.

As early as day 10 after initial plating, contracting clusters were observed in the differentiation assay with some of the clones. Analysis of cTnT expression at day 13 by flow cytometry indicated the cardiac differentiation efficiencies of the putative iPSC clones ranged from ~0-22% (Figure 3C & D). Based on these results, several iPSC clones (9, 11, 12, 17, and 19) were identified as being the most suitable for further characterization, while several other clones (1, 4, 6, 8, 13, 14 and 20) were designated as least suitable based on their poor cardiac differentiation efficiency. Combining these findings with the Ion Torrent sequencing data, the lentivirus had inserted within intragenic regions for clones 9 and 19 (NPLOC4 and AP1G1 respectively), while for clone 11 the lentivirus was in exon 2 of ZDBF2. Clone 12 had two proviral integrations within introns of PRPSAP1 and PEX14. Only clone 17 contained a viral insert outside any annotated genes or transcriptional control elements, and also had relatively high cardiac differentiation efficiency.

Discussion

Current methods to identify fully reprogrammed iPSCs following viral infection of somatic cells rely on expensive and often lengthy protocols, highlighting the need for methods that will streamline the selection process. During reprogramming, hundreds of clonal iPSCs are typically generated, often with variable differentiation capacities possibly due to genetic and epigenetic differences [6,9,35]. However representative iPSC clones are generally selected based on morphology, at a time when little or no information is available on the number and location of the proviral integrations or on the differentiation efficiency to particular lineages.

Using shear-splink PCR coupled with high-throughput ion semi-conductor sequencing [21,22], we were able to quickly and reliably determine proviral insert number and genomic location for multiple hiPSC clones simultaneously. Indeed this combined procedure identified integrations that were undetected when the clones were analyzed by Southern blot or RE-based splinkerette procedures.

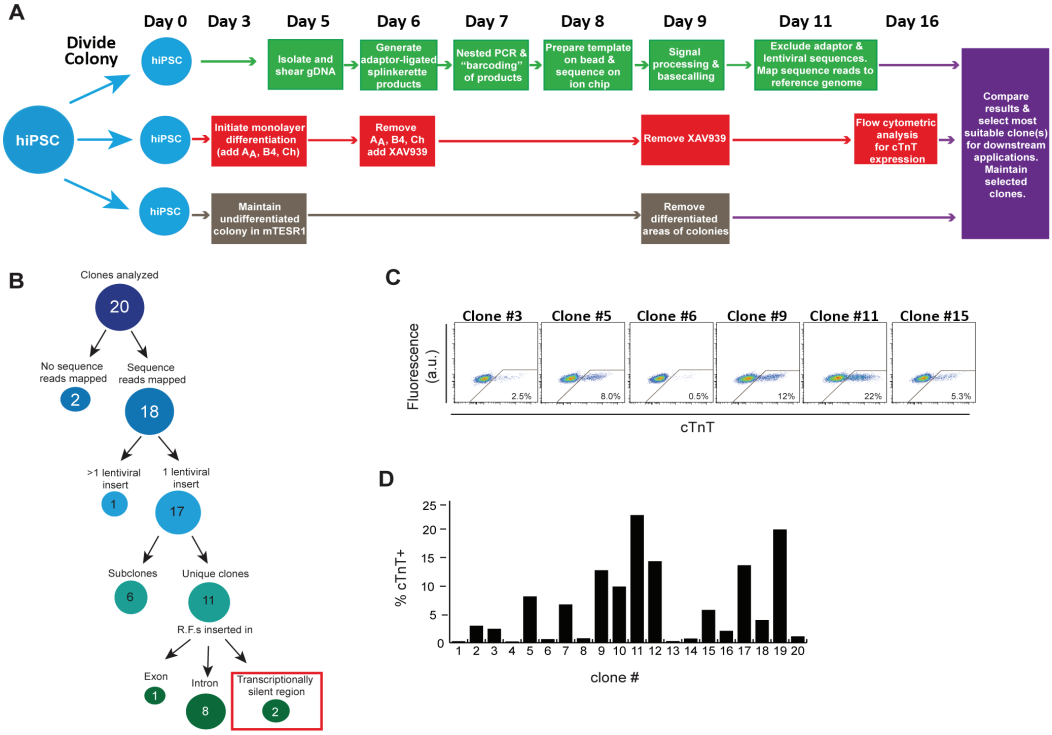

Figure 3. Simultaneous mapping of proviral integration sites and assessment of cardiac differentiation potential for multiple hiPSC clones. A) Schematic representation of the pipeline to assess newly derived hiPSCs. B) Flow chart summarizing the proviral insert number, genomic location and locality of 20 hiPSC as determined by shear-splink and Ion Torrent sequencing. The red box indicates the clones most suitable for further evaluation (RFs, reprogramming factors) C) Representative flow cytometry plots showing the percentage of cardiac troponin T-positive (cTnT+) cells after 13 days of differentiation for a selection of the 20 hiPSC clones analyzed. D) Histogram showing the mean percentage of cells expressing cTnT at day 16 for the 20 hiPSC clones assessed for cardiac differentiation potential.

Significant inter-clonal variability in the differentiation efficiency of hiPSCs, including in the generation of cardiomyocytes has been well documented [6,9,36]. Inadvertently selecting representative patient-specific iPSC clones with poor cardiac differentiation efficiency could adversely influence the ability to distinguish disease-associated phenotypes. Here we developed a cardiac differentiation protocol that was fast and required no optimization between human PSC lines. We favored a monolayer differentiation-based technique due to its simplicity and scalability [28,37,38]. Previously published protocols often required prior adaptation of the cells to enzymatic passaging to enable the seeding of single or small clusters of cells, necessitating an additional 1-4 weeks to establish [39,40]This makes these protocols unsuitable for rapidly determining the cardiac differentiation potential of emerging iPSC colonies, since at this stage the colonies are still manually passaged. Aside from the time required, maintaining >20 colonies while assessing the cardiac potential is not practical and prohibitively expensive for high throughput development.

By using LI-BPEL, a serum-free differentiation medium, we limited the variability due to undefined components present in serum, as well as enhanced the effectiveness of the recombinant growth factors and small molecules [17,41]. While the combination of BMP4, Activin A and either bFGF or WNT3A can induce cardiogenesis in monolayer cultures of PSCs [28,29,37], optimization of the concentrations of BMP4 and activin A for individual cell lines as well as the time of addition

and removal of the growth factors has proved to be important [42]. Although small molecule modulation of the regulatory elements of the Wnt/β-catenin signaling pathway has been shown also to result in more robust cardiac differentiation in multiple human PSC lines [38,43] than the method described here, these protocols as mentioned earlier, have all required prior adaptation of the cells to enzymatic passaging. We found combining both approaches (staged exposure to BMP4, activin A and the GSK3β inhibitor, CHIR99021, followed by the tankyrase inhibitor, XAV939) resulted in robust cardiac differentiation from 6 human PSC lines without the need to individually optimize timing or concentrations for each line. This procedure could successfully induce cardiomyocyte differentiation from hiPSC colonies maintained either with MEFs in KOSR-containing medium or feeder-free with mTeSR1 medium, as well as from PSC lines passaged enzymatically, without any adaptations to the protocol. It is likely that the efficiency of cardiac development could be further optimized for each cell line by determining the optimal concentration and time of application of the growth factors and small molecule [42]. However such an approach is not feasible when screening multiple clones and is more suitable once an iPSC line is established. Similarly, reducing the level of insulin in the differentiation media further might lead to additional improvements in cardiac differentiation efficiency.

To demonstrate that both procedures could be incorporated into the current workflow for identifying fully reprogrammed iPSCs, we analyzed 20 nascent iPSC colonies that were selected for further characterization based on morphology. Within 16 days and prior to the clones requiring passaging, the cardiac potential along with mapping the proviral integration sites had been ascertained. It should be noted that the results from performing both of these procedures does not conclusively demonstrate that the poor cardiac differentiation capabilities of a clone is due to the integration site of the provirus. Impaired differentiation could also be due to the clone not being fully reprogrammed, or other genetic or epigenetic abnormalities [9]. The purpose of performing such a screen is to enable the researcher to make a more informed decision on which iPSC clones to select for further characterization based on the downstream applications of the cell line. From the iPSC colonies that were screened, clone 11 was the most efficient at generating cardiomyocytes. However with this clone, the lentivirus had inserted into exon 2 of the zinc finger gene, ZDBF2. Little is known about the function of ZDBF2, although there is evidence that it is an imprinted gene predominantly expressed in the brain and muscle [44]. It has also been identified as a transcript upregulated in embryonic cardiac fibroblasts from microarray studies [45]. Therefore while disruption of ZDBF2 does not appear to be detrimental to cardiomyocyte differentiation, it could influence the phenotype if the cell line was to be used in developing cardiac models for example in “organ-on-a-chip” formats. Alternatively, clone 17 also had relatively high cardiac differentiation efficiency along with a proviral integration site outside any known coding regions, potentially making this clone more suitable for such applications.

Furthermore, both of these techniques are not limited to evaluating the genomic integrity and differentiation potential of reprogrammed iPSCs. With little modification, these procedures could also be readily incorporated into a screening strategy of human PSCs that have been genetically modified by either viral or transposon-based transfection. Here there is also a recognized need to generate and analyze several transgenic lines, preferably in a short timespan, since positional effects resulting from genomic integration could impact on their differentiation potential.

Conclusion

It is widely acknowledged that hiPSCs hold great promise for disease modeling, safety pharmacology and drug discovery, particularly with respect to the cardiovascular system [46-48]. However, methods to identify the most suitable iPSC clones for further study are often lengthy and laborious. Here, we have described a rapid and scalable pipeline consisting of two independent assays that can simultaneously be applied directly to hiPSC clones as they appear following reprogramming, enabling clones to be selected based on the number and genomic

location of the integrated reprogramming cassette, as well as the cardiac differentiation potential. Solutions to a number of the remaining technical issues, such as those covered here, could contribute to more rapid realization of the potential of iPSCs by reducing the time required between obtaining primary cell cultures for reprogramming and selecting the best hiPSC lines for the question to be addressed.

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Supplementary Figures:

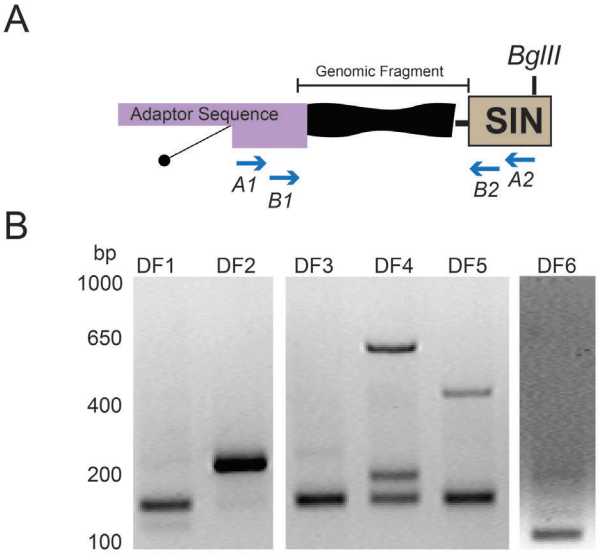

Figure S1. Genomic mapping of proviral integration sites in the lentiviral-generated DF-hiPSCs using restriction enzyme (RE)-splinkerette PCR. A) Schematic representation of the adaptor-ligated splinkerette products generated following RE digestion of the genomic DNA. The position of the BglII within the SIN sequence, as well as the splinkerette adaptor (purple box with angled black line) are shown. Also depicted are the PCR primers (A1, A2, B1, B2) used to amplify the junction sequences between the endogenous genome and the proviral insertion site (SIN, Self inactivating element). B) Agarose gel electrophoresis depicting the fragments generated following primary and secondary PCR amplification of the adaptor-ligated splinkerette products for DF-iPSC clones 1-6. Note, following cloning of these products into a TOPO vector, transformation into bacteria and subsequent sequencing, some fragments corresponded to non-specific amplification of genomic DNA sequences. Additionally, some bands corresponded to multiple proviral insertions. The column "RE-splinkerette; TOPO subcloning" in Table 1 lists the number of unique proviral inserts identified per clone using this technique.

F

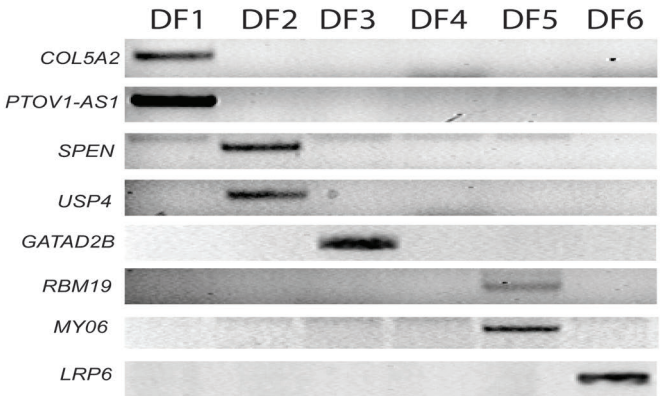

Figure S2. Validation of the lentivirus integration sites identified by Ion Torrent sequencing for the DF-hiPSC clones and listed in **Table S6**, using locus-specific PCR analysis. Results for clone DF4 are shown in **Figure 1F.**

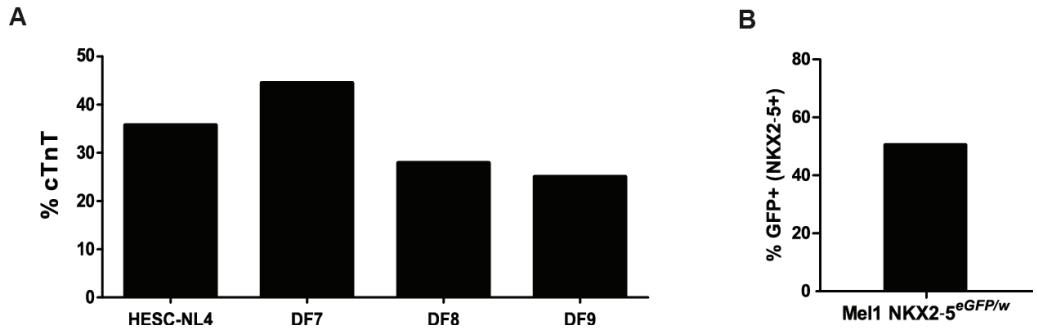

Figure S3. The monolayer differentiation protocol efficiently induces cardiogenesis for multiple hESC and hiPSC lines without requiring line-specific optimisation. A) The histogram shows the mean percentage of cells expressing cTnT at differentiation d14 for an additional 4 hPSC lines assessed for cardiac differentiation potential. B) The histogram shows the mean percentage of cells expressing eGFP (NKX2-5) at differentiation d14 from the hESC line, Mel1 NKX2-5eGFP/w. Data represents the mean ± SEM from 2 independent experiments.

Table S1: Pluripotent stem cell lines used in this study.

PSC type	Identification within article	Full name of clone*
hESC	*NKX2-5$^{eGFP/w}$* hESCs	HES3 *NKX2-5$^{eGFP/w}$* [1]
hESC	HESC-NL4	HESC-NL4 [2]
hESC	Mel1 *NKX2-5$^{eGFP/w}$*	Mel1 *NKX2-5$^{eGFP/w}$* [1]
hiPSC	DF1	LUMC0035iMyBPC01
hiPSC	DF2	LUMC0034iMyBPC02
hiPSC	DF3	LUMC0033iMyBPC09
hiPSC	DF4	LUMC0034iMyBPC12
hiPSC	DF5	LUMC0033iMyBPC13
hiPSC	DF6	LUMC0004ictrl03
hiPSC	DF7	LUMC0006ictrl01
hiPSC	DF8	LUMC0004ictrl10
hiPSC	DF9	FLB243#1 [3]

* Nomenclature of iPSC lines based on the standardized naming system proposed by Luong et al, Cell Stem Cell, 2011. Where applicable, the reference describing the initial characterization of the cell line is included in brackets.

[1] Elliott et al, Nat Methods, 2011; [2] Freund et al, Stem Cells, 2008; [3] Freund et al Neth Heart J, 2010

Table S2: Oligonucleotides used for splinkerette-PCR

Name	Sequence
Splink adaptor (Long)	*5'*-CCTCCACTACGACTCACTGAAGGGCAAGCAGTCCTAACAACCATGT-*3'*
Splink adaptor (Short)	*5*'-Phosphate-CATGGTTGTTAGGACTG-Amino-*3'*
PCR_A1	*5'*-CCTCCACTACGACTCACTGAAGGGC-*3*'
PCR_A2	*5*'-GCAGATCTTGTCTTCGTTGGGAGTT-*3'*
PCR_B1	*5*'-GGGCAAGCAGTCCTAACAACCATG-*3'*
PCR_B2	*5*'-CTTTCTAGAGAATAGGAACTTCGG-*3*'

Table S3: Amplification conditions for primary and secondary splinkerette-PCRs

Primary Amplification

Step	Temperature	Time	# of cycles
Initial denaturation	96°C	2'00"	1
Denaturation	92°C	0'40''	
Annealing	60°C (-1°C per cycle)	0'40''	10
Elongation	68°C	2'00"	
Denaturation	92°C	0'40''	
Annealing	63°C (-0.5°C per cycle)	0'40''	10
Elongation	68°C	1'00"	
Denaturation	92°C	0'40''	
Annealing	50°C	0'40''	25
Elongation	68°C	1'00"	
Elongation	68°C	10'00"	1

Secondary Amplification

Step	Temperature	Time	# of cycles
Initial denaturation	96°C	2'00"	1
Denaturation	92°C	0'40''	
Annealing	66°C (-1°C per cycle)	0'40''	6
Elongation	68°C	1'00"	
Denaturation	92°C	0'40''	
Annealing	59°C	0'40''	14
Elongation	68°C	1'00"	
Elongation	68°C	10'00"	1

Table S4: Adaptor and barcode sequences incorporated into primers PCR_B1 and PCR_B2 for sequencing splinkerette products using the Ion Torrent.

Adaptor	Sequence	Comment
Y	*5'*-CCTCTCTATGGGCAGTCGGTGAT -3'	Adaptor sequence attached to the 5'end of primer PCR_B1
Z	*5'*- CCATCTCATCCCTGCGTGTCTCCGACTCAGXXX-3'	Adaptor and barcode sequence attached to the 5'end of primer PCR_B2, where XXX refers to the barcodes listed below.

Barcode	Sequence	Barcode	Sequence
1	*5'*-ACGAGTGCGT-*3'*	14	*5'*-TCACGTACTA-*3'*
2	*5'*-ACGCTCGACA-*3'*	15	*5'*-CGTCTAGTAC-*3'*
3	*5'*-AGACGCACTC-*3'*	16	*5'*-TCTACGTAGC-*3'*
4	*5'*-AGCACTGTAG-*3'*	17	*5'*-TGTACTACTC-*3'*
5	*5'*-ATCAGACACG-*3'*	18	*5'*-ACGACTACAG-*3'*
6	*5'*-ATATCGCGAG-*3'*	19	*5'*-CGTAGACTAG-*3'*
7	*5'*-CGTGTCTCTA-*3'*	20	*5'*-TACGAGTATG-*3'*
8	*5'*-CTCGCGTGTC-*3'*	21	*5'*-TACTCTCGTG-*3'*
9	*5'*-TCTCTATGCG-*3'*	22	*5'*-TAGAGACGAG-*3'*
10	*5'*-TGATACGTCT-*3'*	23	*5'*-TCGTCGCTCG-*3'*
11	*5'*-CATAGTAGTG-*3'*	24	*5'*-ACATACGCGT-*3'*
12	*5'*-CGAGAGATAC-*3'*	25	*5'*-ACGCGAGTAT-*3'*
13	*5'*-ATACGACGTA-*3'*		

Table S5: Oligonucleotides used to confirm proviral genomic insert locations.

Gene	Forward Primer	Reverse Primer
RBM19	*5'*-TCTGGTTTCCCTTTCGCTTTCAGGT-*3'*	*5'*- GAGTTTGCATGCTCATCCTGTGG-*3'*
FINP1	*5'*-TCTGGTTTCCCTTTCGCTTTCAGGT-*3'*	*5'*-CTGTGAATTTTGGTTACATTTATG-*3'*
SPEN	*5'*-TCTGGTTTCCCTTTCGCTTTCAGGT-*3'*	*5'*-GCTGATCATCCAGCAAAAGCAG-*3'*
ASH1L	5'-AGCTCGGTACCTTTAAGACCAATGACTT-3'	*5'*-TCTGGTTTCCCTTTCGCTTTCAGGT-*3'*
USP4	*5'*-TCTGGTTTCCCTTTCGCTTTCAGGT-*3'*	*5'*-CAGCCTTGTGGTTTTTCACA-*3'*
COL5A2	*5'*-TCTGGTTTCCCTTTCGCTTTCAGGT-*3'*	*5'*- CATTTGGTTCATATATTTGACTTA-*3'*
PTOV1-AS1	*5'*- CGATGCTCTCGACACTTCC-*3'*	*5'*-TCTGGTTTCCCTTTCGCTTTCAGGT-*3'*
LRP6	*5'*-TCTGGTTTCCCTTTCGCTTTCAGGT-*3'*	*5'*-AAAAATAGCCATCTCCATGAGC-*3'*
MY06	*5'*-CTGTTCGGGCGCCACTGCTAG-*3'*	*5'*-GGAAAAAGTAACCGGGCACAGTGGC-*3'*
BAD	*5'*-CTGTTCGGGCGCCACTGCTAG-*3'*	*5'*-GCTTGTCTCAGTCCAGTTAGGGGTAG-*3'*
GATAD2B	*5'*-CTCCCGGCTGCCTTCCCTTTTT-*3'*	*5'*-CTGTTCGGGCGCCACTGCTAG-*3'*

Table S6: The proviral insert locations identified for the 6 DF-iPSC clones investigated in Figure 1.

Clone	Genome Location	Nearest Gene	Present in
DF1	chr19:503428454	*PTOV1-AS1*	Exon
	chr2:190023157	*COL5A2*	Intron
DF2	chr1:16232602	*SPEN*	Intron
	chr3:49349283	*USP4*	Exon
DF3	chr1: 153863681	*GATAD2B*	Intron
DF4	chr1:155370199	*ASH1L*	Intron
	chr11:64046798	*BAD*	Intron
	chr5:130981785	*FNIP1*	Intron
DF5	chr12:114276140	*RBM19*	Intron
	chr6:76532084	*MYO6*	Intron
DF6	chr12:12290145	*LRP6*	Intron

Table S7: Overview of the proviral insert locations determined by shear-splink and Ion Torrent sequencing for the 20 putative hiPSC clones.

Clone	Genome Location	Nearest Gene	Present in
1	N/A		
2	chr2: 207144375	*ZDBF2*	Exon
3	chr2: 207144375	*ZDBF2*	Exon
4	chr6: 44392477	*CDC5L*	Intron
5	chr12: 28151923	upstream of *PTHLH*	Non-coding region
6	chr1: 150653834	*GOLPH3L*	Intron
7	chr16: 71769591	*AP1G1*	Intron
8	chr1: 1330539	*CCNL2*	Intron
9	chr17: 79562823	*NPLOC4*	Intron
10	chr2: 207144375	*ZDBF2*	Exon
11	chr2: 207144375	*ZDBF2*	Exon
12	chr17: 74311940	*PRPSAP1*	Intron
	chr1: 10660290	*PEX14*	Intron
13	chr2: 207144375	*ZDBF2*	Exon
14	chr2: 42438527	*EML4*	Intron
15	chr19: 1121879	*SBNO2*	Intron
16	N/A		
17	chr5: 116648220	upstream of *CTC-504A5.1*	Non-coding region
18	chr1: 1330539	*CCNL2*	Intron
19	chr16: 71769591	*AP1G1*	Intron
20	chr17: 36589101	*ARHGAP23*	Intron

Chapter 5: Cardiomyocyte differentiation of human pluripotent stem cells

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OVERVIEW

Human cardiomyocytes differ significantly from those of rodents, most obviously in their electrophysiological properties. For this reason, many potential biomedical applications of cardiomyocytes would benefit from using human rather than rodent cells. This is of growing interest as new opportunities arise for using induced pluripotent stem cells (iPSCs) carrying cardiac disease-associated mutations to understand the pathophysiology of heart disease and to develop new treatment modalities.

There is an obvious need for robust and efficient cardiac differentiation protocols for human pluripotent stem cells, preferably under defined, serum free conditions. Although protocols that are effective on multiple pluripotent cell lines are beginning to emerge, none has yet been shown to be universally applicable without individual optimization, and most still require a cell aggregation step to initiate differentiation. More than 10 years of research on human embryonic stem cells (hESCs) has, however, provided important clues as to which parameters are candidates for further improvement. Many of the recent methodological advances made using hESCs are proving to be transferable to human iPSCs with minor modifications. Thus, it is now possible to produce cardiomyocytes from most hESC and hiPSC lines, although the efficiencies with which individual cell lines differentiate can still be highly variable.

Most protocols mimic in some way the signals that take place during fetal heart development. Among the first was one that recapitulated signals from endoderm adjacent to the developing heart: mechanically passaged hESC colonies were co-cultured with a mouse visceral endoderm-like cell line (END2) in serum-free culture medium. This method is effective in most hESC and hiPSC lines and will yield 5–15% cardiomyocytes depending on the individual line. This technique provides a simple way to check for cardiomyocyte differentiation capacity of multiple hiPSC clones while they are still at very early (mechanical passage) stages of development. For producing larger numbers of cardiomyocytes from pluripotent cells already adapted to enzymatic passage on feeder cells, however, a more effective method is based on centrifuging the cells to force them to make aggregates, which are called “Spin embryoid bodies”. Human pluripotent cells have less inclination to form aggregates spontaneously in suspension than mouse pluripotent cells and forced aggregation makes cells attach more firmly to one another. In the cardiomyocyte differentiation protocol, the aggregates do not become true embryoid bodies, since they are not cultured long enough to induce all three germ layers.

In this chapter we outline a basic protocol for the differentiation of hESCs into cardiomyocytes. The method is based on the formation of aggregates of a standard size using a fixed number of undifferentiated cells in each aggregate. The aggregates are formed by centrifugation of the cells into embryoid body (EB)-like structures and are referred to as “Spin EBs”. Key to the procedure, and most difficult, is the prior adaption of the undifferentiated cells to a standardized single cell passaging bulk culture system in which cells are passaged enzymatically and grown on feeder layers. The method we recommend for making spin EBs after bulk culture adaption is straightforward and has worked well in our hands for multiple hESC lines and several hiPSC lines. The method requires titration of growth factor concentrations for each line to optimize efficiencies. We also discuss alternative methods and reagents that work but are not routinely used in our laboratories. In addition, we discuss aspects of the protocol that could be further optimized. The key variables that we outline in this chapter are:

- Basal culture conditions
- Adaption to bulk passage
- Generating spin “embryoid bodies” (Spin EBs)
- Growth factor titration curves
- Dissociation of EBs and replating cardiomyocytes.

While optimizing and standardizing conditions, it is important to keep in mind that changing one reagent or parameter in the system may unexpectedly impact the outcome of the entire protocol.

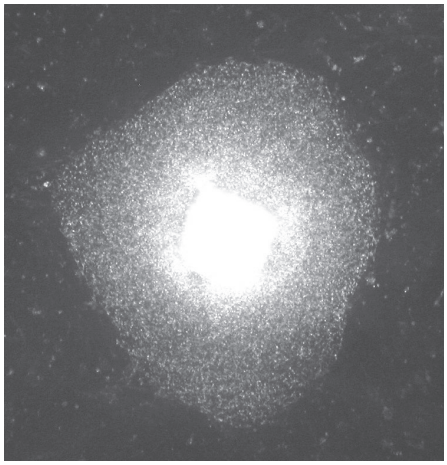

Figure: 1
Example of a healthy looking hESC colony with a small amount of differentiation in the center of the colony.

Procedures

Basal Culture Conditions

In our laboratory, many of our stock hESC lines (e.g. HES2, HES3, HES4, NL-HES1) are grown and maintained as mechanically passaged "cut and paste" colonies in KnockOut Serum Replacement-based medium on irradiated or mitomycin C-treated mouse embryonic feeder cells (MEFs). The colonies are cut into small pieces using glass needles and, using a pipette, are transferred to new feeder layers once weekly. See Chapters 1 and 2. For details see Mummery et al., 2007.

From Mechanical Colonies to Bulk Culture: Tips for Successful Adaption Prior to Generating Spin EBs

Several of the hESC lines we use are routinely passaged enzymatically, twice weekly on MEFs using TrypLE Select. These cells can be used immediately in the spin EB protocol for cardiomyocyte differentiation. For cell lines that are not enzymatically passaged, the spin EB protocol requires that the undifferentiated colonies be adapted to single cell enzymatic passage on feeder layers (referred to as bulk culture).

It is important to select hPSC lines for adaption that have been well-maintained with timely medium refreshment and regular mechanical passaging. The colonies should look healthy with little debris and few differentiated cells around the colony edges (Figure 1).

For best results use 80–100 mechanically-passaged colonies per line to start bulk adaption of a line.

MEFs are inactivated by prior irradiation at 3000 rads in suspension culture.
Plate irradiated MEFs the day before bulk culture adaption to allow attachment and spreading.

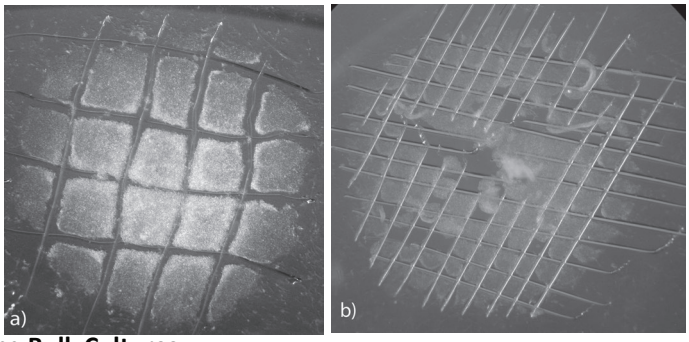

Figure: 2
(A) Example of the size of pieces for mechanical passaging (pieces approximately 400–500 µm in length). (B) Example of grid made for bulk culture initiation (pieces approximately 100–150 µm in length).

Starting Bulk Cultures

1. Cut away any residual differentiated parts of the colonies. These are usually the flat cells around the edge and the cluster at the center.
2. Cut the colonies into extremely small square pieces in a grid (cut closely spaced lines first in one direction over the whole colony then similarly spaced lines in the perpendicular direction) with standard tungsten or glass needles. Note that the squares should be much smaller than those generated during routine mechanical passaging (Figure 2).
3. Lift the cut pieces from the dish using a p1000 pipette and place in a 15 mL tube.
4. Collect all of the pieces from a total of 80–100 colonies.
5. Centrifuge for 3 minutes at 250 *g*.
6. Aspirate medium and resuspend in HESC-medium using a p1000 to pipette colony pieces up and down gently several times.
7. Plate all of the pieces in a T25 flask seeded the day before with 2-3 x 10^4 irradiated MEFs (density of MEFs depends on mouse strain used to produce MEFs and should be optimized empirically) per cm2 with the appropriate amount of HESC-medium.
8. Gently passage the cells in the flask 4 days later in a 1:1 ratio by incubation in TrypLE Select and resuspend in hESC-medium to spread the small colonies, which tend to pile up, over the surface of the flask (Figure 3).
9. The cultures should be observed and the medium should be changed daily. The cells should be passaged at a low ratio (1:2 or 1:3) on new feeder layers in HESC-medium when the islands of undifferentiated cells start to fill the flask (Figure 4).

- **Note**

This low split ratio should be continued until the cells adapt (possibly up to five or six passages). This depends somewhat on the individual cell line; some lines accept a higher split ratio after only two passages. The cells are then cultured according to the procedure below (see Maintaining Bulk Cultures).

Maintaining Bulk Cultures

Bulk cultures should be passaged approximately every 3–4 days as follows:

1. Wash once with phosphate buffered saline (PBS).
2. Add 1–1.5 mL of TrypLE Select to the T25 flask.
3. Place at 37°C for 5 minutes.
4. Tap the flask with fingers to dislodge cells.
5. Resuspend cells in 5 mL of culture medium containing 10% serum and place in a 15 mL tube.
6. Rinse the flask with an additional 3–5 mL of the same serum-containing medium and add to the same tube.
7. Centrifuge for 3 minutes at 250 *g*.

8. Gently resuspend the pellet in the appropriate amount of HESC-medium.
9. Plate down in an appropriate sized flask, plate, or dish pre-plated with
10. 2-3 x 10^4 irradiated MEFs per cm^2.

- **Note**

If the bulk cultures were started using T25 flasks, the flask size can easily be increased for cryopreservation or scaled down to 6-well plates for general maintenance. One well of a 6-well plate contains a sufficient number of cells for the next step of the procedure and reduces the number of MEF feeder cells required at each passage.

Generating Spin EBs

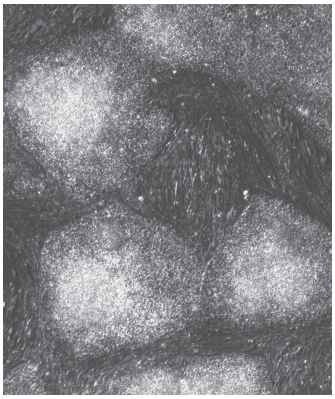

Figure 3:
Example of a bulkculture after 4 days, just before the first passaging.

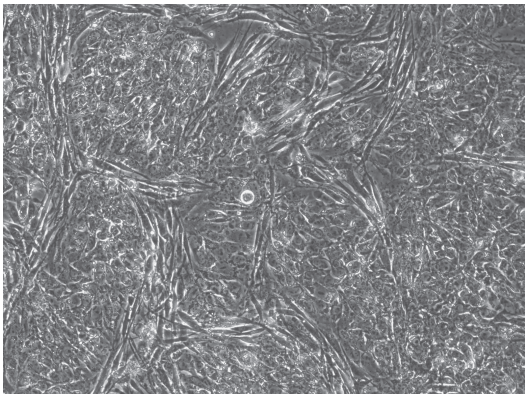

Figure 4:
Example of bulk culture that has been adapted and is ready to be passaged.

One Day Before Starting the Spin EB Differentiation Protocol

Deplete cultures of MEF feeder cells by passaging the bulk cultures and plating on to Matrigel-coated dishes or flasks (ratio about 1:2/1:3 or about 8 x 10^5 cells per well of a 6-well plate) (Figure 5). The following protocol uses one well of a 6-well plate.

Day 0

1. Early morning on the day of differentiation: refresh the medium on the bulk culture in the well of the 6-well plate to maintain culture quality and wash away dead cells.
2. Prepare the growth factor solutions prior to harvesting the cells.
3. Midday on the day of differentiation, start harvesting the cells by washing the culture once with PBS.
4. Add 1 mL of TrypLE™ Select (this is for one well of a 6-well plate; adjust as necessary to the size of culture flask, dish, or well) and incubate for 5 minutes at 37°C.
5. Add 3 mL (adjusting again as necessary to the size of culture flask, dish, or well) of serum-containing medium to collect cells.
6. Wash the flask again with 3 mL of serum-containing medium to collect remaining cells.
7. Centrifuge for 3 minutes at 250 *g*.
8. Resuspend cells in 2 mL of BPEL medium. BPEL medium is basal medium with BSA, Polyvinylalcohol (PVA), and Essential Lipids (see Recipes).
9. Centrifuge for 3 minutes at 250 *g*.
10. Resuspend cells in BPEL medium (1 mL per well of 6-well plate).
11. Count cells.

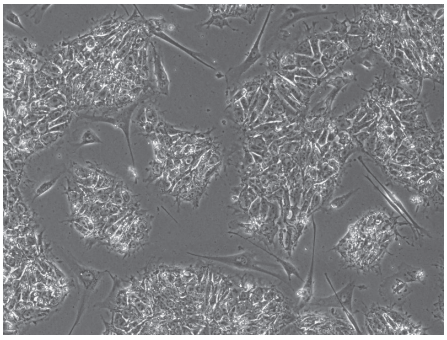

Figure 5:
Bulk cultures one day after plating on Matrigel™ for MEF feeder cell depletion.

12. Plate cells at a final density of 3000 cells in 50 µL of BPEL medium plus growth factors in each V-shaped well of a 96-well plate from a stock solution containing the required number of cells in the correct volume for all wells to be seeded.

- **Note**

For a complete plate only use the 60 inner wells and add 100 µL of PBS to the outer wells to compensate for any evaporation of culture medium.

13. Centrifuge plate for 3 minutes at 450 *g* to form aggregates (Spin EBs) (Figure 6A).
14. Place in the incubator at 37°C for 3 days.

Day 3

15. Remove the BPEL medium containing growth factors and add 100 µL of growth factor-free BPEL medium.

Day 7

16. Plate the Spin EBs in BEL medium (BPEL without PVA) on 0.1% gelatincoated 96-well plates with flat-bottomed wells (Figure 6B).

- **Note**

For practical reasons BPEL can also be used but PVA is not needed after 7 days for the continuous differentiation of the EBs.

Day 8

17. Begin checking for beating areas in the cell aggregates under a phase contrast microscope (203 objective) (Figure 6C).

- **Note**

Beating may begin up to 20 days later, depending on the cell line. HES3 Spin EBs usually start to beat around day 8 and reach a maximum at day 12 (that is, all EBs containing cardiomyocytes are beating). Note that EBs that do not beat visibly do not usually contain cardiomyocytes if stained retrospectively with an antibody against sarcomeres.

18. After 14 days, refresh wells with BEL or BPEL medium once a week.

Growth Factor Titration Curves

Titration of Activin A and BMP4 is usually needed to determine the optimal concentrations for the induction of beating cells. Titration will be required for each new batch of growth factors (which are supplied on the basis of protein concentration and not specific activity) and each new cell line or clone:

	Final (ng/mL)	Stock (ng/µL)
BMP4	Determine using titration	30
VEGF	30	50
SCF	40	40
Activin A	Determine using titration	25

One way in which a titration can be done is to test new growth factor batches or cell lines in the middle 60 wells (6 wells by 10 wells) of a 96-well plate. Vary the concentration of Activin A (0–30 ng/mL) in one direction and BMP4 (0–30 ng/mL) in the other direction. Duplicate rows and columns so that there are multiple samples of each combination.

Constant concentrations of VEGF (30 ng/mL) and SCF (40 ng/mL) are present in the Spin-EB medium. PVA is an essential component and should not be omitted (Figure 7).

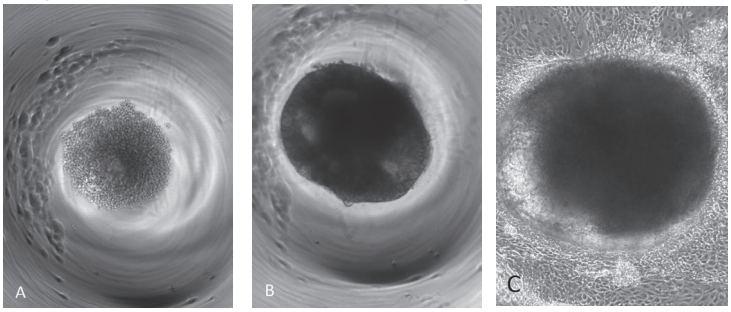

Figure 6:
(A) Example of Spin EB in 96 well V-wells at day 0 after centrifuging. (B) Example of spin EB in V-wells on day 7 (C) Example of Spin EB after plating onto gelatin on day 8.

Dissociation of Beating Spin EBs for Experimentation on Single Cardiomyocytes

1. Wash Spin EBs with PBS.
2. Add TrypLE Select for 20 min at 37°C (this depends on the size of the EBs, days of differentiation, etc.). Break up the EBs by gentle pipetting.

- **Note**
  The time needed for dissociation increases with the age of the EBs and thus the time mentioned above should be monitored.

3. Neutralize the enzyme by resuspending cells in serum-containing medium and place in a 15 mL tube.

- **Note**
  Pipette up and down vigorously (maximum three times) if clumps of EBs are still visible

4. Centrifuge for 3 minutes at 250 *g*.

- **Note**
  For use in FACS, begin standard FACS staining protocol after this step.

5. Resuspend in BEL medium containing 5 µM Rock Inhibitor.
6. Plate dissociated cells on the appropriate 0.1% gelatin-coated cell culture treated dish, plate, or flask.

- **Note**
  5000 cells per cm2 gives a nice spread of single cells although the ideal cell density depends on the subsequent experiments that will be carried out. Coating dishes, plates, or flasks with other ECM materials (e.g. Matrigel or fibronectin) may improve attachment in some cases.

7. Refresh medium, removing the Rock Inhibitor 24 hours later.

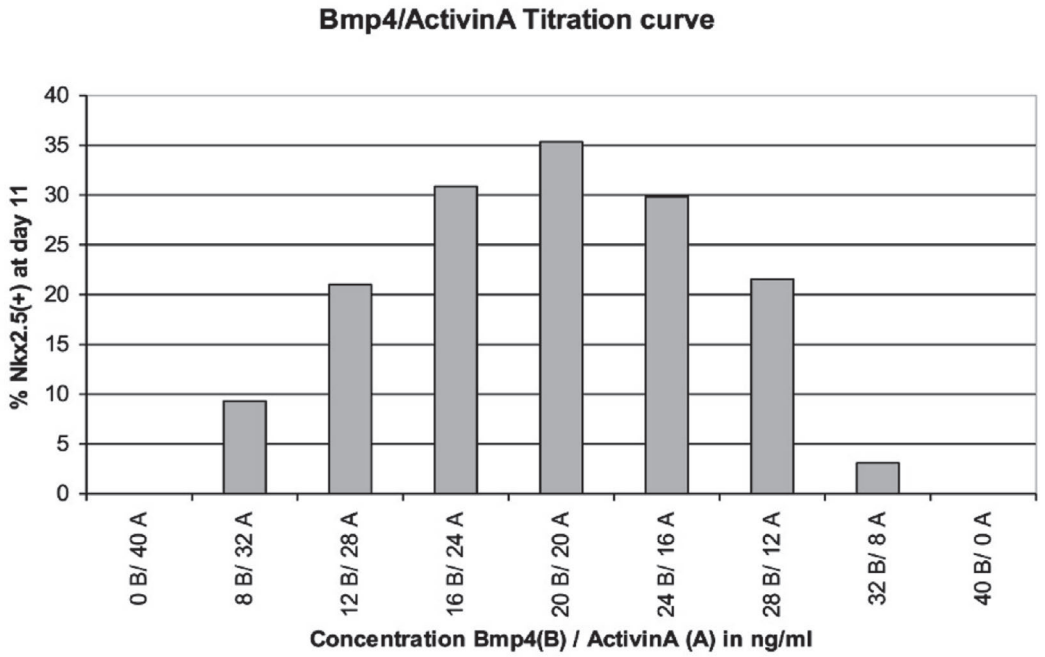

FIGURE 7
The outcome of a typical titration curve of BMP4 and Activin A, using HES3 cells, showing optimal BMP4/ Activin A concentrations of 20 ng/mL for each. Higher Activin A concentrations in HES3 lead to endoderm formation. Lower Activin A and higher BMP4 concentrations lead to other mesoderm derivatives like blood. Here, the number of Nkx2.5 1 cells present was determined by FACS analysis of GFP (NKX2.5-GFP-Hes3 line [Elliot et al., 2011], but it may also be done using an Nkx2.5 antibody) in a single cell suspension (dissociated beating Spin EBs, see protocol below). For other cell lines, similar titration experiments showed concentrations as low as 5 ng/mL for these factors to be optimal for inducing cardiomyocyte differentiation.

ALTERNATIVE PROCEDURES

Use of APEL Instead of BPEL

The use of Bovine Serum Albumin (BSA) in the BPEL medium has some drawbacks, including batch-to-batch variation and the fact that it is an animal product. Replacing BSA with human recombinant albumin may decrease the batch-to-batch variation observed with BSA. Human recombinant albumin may also produce higher efficiencies of differentiation. However, human recombinant albumin is much more expensive than BSA. Stem Cell Technologies™ also supplies pretested APEL medium on a commercial basis.

PITFALLS AND ADVICE

Defined Media for hESC Cultures

The use of defined media for culture of hESCs is ideal for reducing the dependence on MEFs, eliminating the unknown components they produce and reducing variability in cultures over time. There are two commercial media, mTeSR and Nutristem, which we have used to grow hESC cultures under defined, feeder-free conditions for up to 20 or so passages. Long-term passage in either media may be more likely to cause earlier karyotypic abnormalities compared to MEF-based mechanically passaged cultures because it is thought that the total number of cell divisions in a given time period may be higher in defined media. In addition, the potential for subsequent differentiation using other protocols can vary depending on the media used, possibly due to the differences in media components. For example, higher bFGF levels in mTeSR have been found by some to impact the efficiency of differentiation to cardiomyocytes and endoderm derivatives.

Other Factors Influencing Differentiation Efficiency

While we have optimized the Spin EB method using specific growth factors, there are a number of factors which could still be added to help improve efficiency further. These include the addition of ascorbic acid or small molecular inhibitors of transforming growth factor β receptors. The presence of Wnts or Wnt inhibitors has also been reported to enhance cardiomyocyte differentiation if present during the earliest phases of mesoderm formation. Efficient nascent mesoderm formation is a prerequisite for subsequent patterning to cardiogenic mesoderm.

EQUIPMENT

- Tissue culture hood: Class II A/B3
- Tissue culture incubator, 37°C, 5% CO2, in humidified air
- Inverted phase/contrast microscope with 43, 103 and 203 objectives
- Centrifuge, 250 to 450 *g*
- Water bath, 37°C
- Pipettes, such as Eppendorf p2, p20, p200, p1000, multichannel
- Pipette Aid, automatic pipettor for use in measuring and dispensing media
- Aspirator in the hood, with flask
- Eight-channel plastic aspirator
- Roller bank
- Refrigerator, 4°C
- Freezers:-20°C, -80°C

REAGENTS AND SUPPLIES

Supplies

- 5 mL, 10 mL, 25 mL sterile disposable pipettes
- 6-well culture dishes
- T25 culture flask
- 96-well Greiner bioone V-shaped bottom low attachment tissue culture plates
- 96-well flat-bottomed tissue culture plates
- 15 mL sterile conical tubes
- 50 mL sterile conical tubes
- Sterile 9” Pasteur pipettes (autoclaved)
- Pipette tips for Eppendorf or similar pipette (use blue and yellow low attachment polypropylene pipette tips from Corning)
- Hemocytometer
- 0.22 µm Stericup filtration unit
- Other filters and syringes
- Reagent reservoir, optional

Recommended Reagents

Item	Supplier	Catalog #
Basal medium		
DMEM/high glucose (Dulbecco's Modified Eagle's Medium)	Life Technologies	11960-044
DMEM/F12 1:1 (GlutaMAX, no HEPES)	Life Technologies	31331-028
IMDM, L-glutamine, 25 mM HEPES, no phenol red (Iscove's Modified Dulbecco's Medium)	Life Technologies	21056-023
F12 Nutrient Mixture (Ham), GlutaMAX	Life Technologies	31765-027
Serum components		
Knockout Serum Replacement (contains bovine products)	Life Technologies	10828-028
Fetal Bovine Serum (FBS) (batch selected)	Sigma-Aldrich	F7524
L-Glutamine 200 mM	Life Technologies	25030-024
GlutaMAX™-I Supplement 200 mM	Life Technologies	35050-038
MEM-Non-essential amino acids 100x	Life Technologies	11140-035
2-Mercaptoethanol 50 mM (1000x)	Life Technologies	31350-010
D-PBS (Dulbecco's Phosphate- Buffered Saline without Calcium and Magnesium)	Life Technologies	15230-089
Distilled H_2O	Invitrogen	15230-089
Basic FGF (FGF-2) Recombinant human	PeproTech	100-18B
Albumin from bovine serum, powder	Sigma	A3311
Resin Beads	Bio-Rad	142-6425
Poly(vinyl alcohol) (PVA)	Sigma-Aldrich	P8136
Recombinant Human VEGF 165	R&D systems	293-VE
Recombinant Human SCF	PeproTech	300-07
Recombinant Human/Mouse/Rat Activin A	R&D systems	338-AC
Recombinant Human BMP-4, CF	R&D systems	314-BP
Fasudil, Monohydrochloride Salt, >99%	LC Laboratories	F-4660
BD Matrigel Matrix Growth Factor Reduced (GFR)	BD Biosciences	354230
Chemically Defined Lipid Concentrate	Invitrogen	11905-031
TrypLE Select (1x)	Invitrogen	12563-029

1-Thioglycerol	Sigma-Aldrich	M6145
Insulin-Transferrin-Selenium-X Supplement (100x)	Invitrogen	51500-056
Gelatin from porcine skin	Sigma-Aldrich	G1890
PFHM II: Protein free Hybridoma medium 1x	Invitrogen	12040-051
L-Ascorbic acid 2-phosphate	Sigma-Aldrich	A8960
Pen/Strep (100x)	Life Technologies	15070-063

RECIPES

- **Note**

Glassware should be dedicated to tissue culture use only. If glassware is to be used instead of pre-sterilized plasticware, do not expose bottles to detergent.

Stock Solution Human Basic FGF (bFGF) 100 µL

Component	Amount	Stock Concentration
Human bFGF	10 µg	100 ng/µL

1. Dissolve 10 µg of human basic FGF in 10 µL of 5 mM Tris (according the product sheet).
2. Add 90 µL of PBS +0.1% BSA.
3. Aliquot in 5, 10 and 25 µL samples.
4. Store thawed aliquots at 4°C for up to 2 weeks.
5. Store frozen aliquots at -80°C (stable for up to 12 months).

Stock Solution 10% Deionized BSA 45 mL

Component	Amount	Stock Concentration
10% Deionized BSA	4.5 g	10% w/v in IMDM

1. Dissolve 4.5 *g* in 45 mL of IMDM at 37°C in a 50 ml tube.
2. Vortex to help mix.
3. After completely dissolved, add Bio-Rad Resin Beads in excess (.2 grams).
4. Set rolling at 4°C on a roller bank until beads turn yellow (beads aresaturated).
5. Remove the supernatant and place in a new Falcon tube.
6. Add new beads and set rolling on a roller bank at 4°C.
7. Repeat until beads remain blue.

Stock Solution 5% PVA 50 mL

Component	Amount	Stock Concentration
PVA	2.5 g	5% PVA in dH2O

1. Dissolve 2.5 *g* in 25 mL of dH2O (tissue culture qualified).

- **Note**

Add the powder to the dH2O and move the tube while adding the PVA powder).

2. Mix slightly.
3. Set rolling at 4°C on a roller bank until completely dissolved and adddH2O up to 50 ml (can take up to 2 days).

Stock Solution 1-Thioglycerol 10 mL

Component	Amount	Stock Concentration
1-Thioglycerol	130 µL	0.15 M

1. Add 130 µL of 1-Thioglycerol to 10 mL IMDM (making these larger volumes of 130 µL is more accurate than smaller volumes because the Thioglycerol is highly viscous).

Stock Solution BD Matrigel Matrix Growth Factor Reduced (GFR) 10 mL

Component	Amount	Stock Concentration
BD Matrigel Matrix Growth Factor Reduced (GFR)	10 mL	Undiluted

1. Thaw 10 mL stock overnight on ice and place in icebox at 4°C. Place Eppendorf tubes and pipette tips at -20°C.
2. Aliquot stock into 100, 200, and 500 µL samples on ice in ice-cold Eppendorf tubes and store at -20°C for up to 3 months.

For use in coating plates:

3. Thaw 100 µL aliquot on ice.
4. Using cold pipettes and medium add 100 µL of Matrigel to 10 mL of DMEM/F12 basal medium.
5. Using a cold pipette, add 2 mL of medium to each well of a 6-well plate.
6. Leave at room temperature for at least 45 minutes.
7. Plates can be used immediately or stored for 2 weeks at 4°C.

- **Note**

Stored plates should be left out at room temperature for 45 minutes before use to allow them to warm up.

Stock Solution Recombinant Human VEGF 165 200 µL

Component	Amount	Stock Concentration
Recombinant Human VEGF 165	10 µg	50 ng/µL

1. Centrifuge prior to opening.
2. Dissolve in 200 µL of dH2O (according to the product sheet).
3. Aliquot in 5 and 10 µL samples.
4. Store thawed aliquots at 4°C for up to 1 week.
5. Store frozen aliquots at 280°C (stable for up to 12 months).

Stock Solution Recombinant Human SCF 250 µL

Component	Amount	Stock Concentration
Recombinant Human SCF	10 µg	40 ng/µL

1. Centrifuge prior to opening.
2. Dissolve in 250 µL of dH2O (according to the product sheet).
3. Aliquot in 5, 10, and 20 µL samples.
4. Store thawed aliquots at 4°C for up to 1 week.
5. Store frozen aliquots at -80°C (stable for up to 12 months).

Stock Solution Recombinant Human/Mouse/Rat Activin A 400 µL

Component	Amount	Stock Concentration
Recombinant Human/Mouse/Rat Activin A	10 µg	25 ng/µL

1. Centrifuge prior to opening.
2. Dissolve in 400 µL of PBS +0.1% BSA (according to the productsheet).
3. Aliquot in 5, 10, and 20 µL samples.
4. Store thawed aliquots at 4°C for up to 1 week.
5. Store frozen aliquots at -80°C (stable for up to 12 months).

Stock Solution Recombinant Human BMP4 330 µL

Component	Amount	Stock Concentration
Recombinant Human BMP-4, CF	10 µg	30 ng/µL

1. Centrifuge prior to opening.
2. Dissolve in 330 µL of PBS10.1% BSA (according to the productsheet).
3. Aliquot in 5 and 10 µL samples.
4. Store thawed aliquots at 4°C for up to 1 week.
5. Store frozen aliquots at -80°C (stable for up to 12 months).

Stock Solution Fasudil, Monohydrochloride Salt, >99% 5 mL, ROCK Inhibitor

Component	Amount	Stock Concentration
Fasudil, Monohydrochloride Salt, >99%	8.15 mg	5 mM

1. Add 8.15 mg to 5 mL of dH2O.
2. Mix until dissolved.
3. Filter using 0.22 µm.
4. Aliquot in 5 and 10 µL samples.
5. Store at -20°C (stable up to 3 months).

Stock Solution "Knockout Serum Replacement" (Invitrogen 10828-028)

This product can be kept (after thawing) at 4°C for 1 week.

1. After 1 week freeze aliquots into appropriate volumes in 50 mL tubes andstore at 220°C.
2. Thaw at 37°C just prior to use.

Stock Solution L-Glutamine (200 mM) (Invitrogen 25030-081)

L-glutamine is unstable and must be stored frozen.

1. Thaw the bottle completely and aliquot in 10 mL tubes. Store at -20°C.
2. Thaw a tube just prior to use.
3. Do not refreeze tube, store at 4°C and discard unused glutamine after 2 weeks.

HESC Medium 100 mL

Component	Amount	Stock Concentration
DMEM/F12 1:1 (GlutaMAX, no HEPES)	78 mL	
KnockOut Serum Replacement	20 mL	20%
100x MEM-Non-Essential amino acids	1 mL	10 mM
50 mM 2-Mercaptoethanol	200 µL	0.1 mM
Human bFGF (100 ng/µL)	10 µL	10 ng/mL
Pen/Strep (100x)	0.5 mL	1:200

1. Prepare all media in the tissue culture hood using aseptic techniques.
2. Combine the basal medium and all the components except bFGF in the filter top. Filter sterilize using a 0.22 µm Stericup filtration unit.
   Add 10 ng/mL bFGF after filtering.
3. Store at 4°C for up to 2 weeks.

BPEL Medium: Bovine Serum Albumin (BSA) Polyvinylalchohol Essential Lipids 100 mL

Component	Amount	Stock Concentration
IMDM, L-glutamine, 25 mM HEPES, no phenol red (Iscove's Modified Dulbecco's Medium)	42.6 mL	
F12 Nutrient Mixture (Ham), GlutaMAX	42.6 mL	
PFHM II: Protein free Hybridoma medium 1x	5 mL	1:20
10% Deionized BSA in IMDM	2.5 mL	0.25% w/v
5% PVA in dH2O	2.5 mL	0.125% w/v
Chemically Defined Lipid Concentrate	1 mL	1:100
Insulin-Transferrin-Selenium-X Supplement (100x)	100 µL	1:1000
0.15 M 1-Thioglycerol	300 µL	450 µM
5 mg/mL L-Ascorbic acid 2-phosphate	1 mL	0.05 mg/mL
200 mM GlutaMAX-I Supplement	1 mL	2 mM
Pen/Strep (100x)	0.5 mL	1:200

1. Prepare all media in the tissue culture hood using aseptic techniques.
2. Combine all components and filter sterilize using 0.22 µm filter.
3. Store at 4°C for up to 2 weeks.

BEL medium: Bovine Serum Albumin (BSA), Essential Lipids 100 mL

Component	Amount	Stock Concentration
IMDM, L-glutamine, 25 mM HEPES, no phenol red (Iscove's Modified Dulbecco's Medium)	42.6 mL	
F12 Nutrient Mixture (Ham), GlutaMAX	42.6 mL	
PFHM II: Protein free Hybridoma medium 1x	5 mL	5%
10% Deionized BSA in IMDM	5 mL	0.5% w/v
Chemically Defined Lipid Concentrate	1 mL	1:100
Insulin-Transferrin-Selenium-X Supplement (100x)	1 mL	1:100
0.15 M 1-Thioglycerol	300 µL	450 µM
5 mg/mL L-Ascorbic acid 2-phosphate	1 mL	0.05 mg/mL
200 mM GlutaMAX-I Supplement	1 mL	2 mM
Pen/Strep (100x)	0.5 mL	1:200

1. Prepare all media in the tissue culture hood using aseptic techniques.
2. Combine all components and filter sterilize using 0.22 µm filter.
3. Store at 4°C up to 2 weeks.

Serum-Containing Medium 100 mL

Component	Amount	Stock Concentration
DMEM/high glucose (Dulbecco's Modified Eagle's Medium)	87.5 mL	
FBS	10 mL	10%
100 x MEM-Non-essential amino acids	1 mL	10 mM
200 mM L-Glutamine	1 mL	2 mM
Pen/Strep (100 x)	0.5 mL	1:200

1. Prepare all media in the tissue culture hood using aseptic techniques.
2. Store at 4°C up to 4 weeks

QUALITY CONTROL METHODS

Lot-to-Lot Variability of Reagents

Growth factors are sold on the basis of weight/volume, not specific activity. Effective concentrations of growth factors required for growth and differentiation will then depend on how and how long the reagent has been stored. Thus, be careful to test each lot and record lot numbers.

READING LIST

Co-Culture

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Activin/BMP/Wnt signaling

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Ren, Y., Lee, M.Y., Schliffke, S., Paavola, J., Amos, P.J., Ge, X., et al., 2011. Small molecule Wnt inhibitors enhance the efficiency of BMP-4-directed cardiac differentiation of human pluripotent stem cells. J. Mol. Cell. Cardiol. 51 (3), 280–287.

Chapter 6:
Human induced pluripotent stem cells from patients with familial hypertrophic cardiomyopathy

Cheryl Dambrot, Stefan R. Braam, Richard P. Davis, Eszter Varga, Leon G.J. Tertoolen, Daniela Salvatori, Christian Freund, Saskia Maas, Simone van de Pas, Dorien Ward-van Oostwaard, Douwe E. Atsma, Christine L. Mummery

Abstract

Hypertrophic cardiomyopathy (HCM) is a genetic cardiac disease characterized by increased heart size and wall thickening in the absence of other cardiac or systemic diseases. Many cases of familial HCM are caused by mutations in the myosin binding protein C (MYBPC) gene. The paucity of appropriate *in vitro* or animal models and difficulties in obtaining cardiac biopsies from patients have limited the opportunities to study underlying molecular mechanisms that may determine severity and onset of symptoms. Here, we derived human induced pluripotent stem cells (iPSC) from three HCM patients by reprogramming fibroblasts from skin biopsies; two of the patients were related, the other was not but had the same mutation. MYBPC type 3 protein levels were significantly reduced by Western blotting in HCM-iPSC cardiomyocytes compared to the control, consistent with reduced MYBPC3 levels previously found in heart biopsies from patients. Dissociated and replated single HCM-iPSC derived cardiomyocytes had a cell surface area more than 2-fold greater than control cardiomyocytes. In addition, calcium signaling was altered in the disease cardiomyocytes compared with controls, consistent with previously published data. Cardiomyocytes from HCM-iPSC thus show essential features of the disease and provide a basis for further studies on the nature of HCM caused by mutations in MYBPC3.

Introduction

Hypertrophic cardiomyopathy (HCM) is a genetic cardiac disease characterized by increased heart size and cardiac wall thickening [1]. It is the most common cardiomyopathy, affecting 1 in 500 people [2]. HCM is associated with mutations in one of the 11 sarcomeric genes, the most prevalent mutations being found in the β-myosin heavy chain (βMHC) and myosin binding protein C3 (MYBPC3) genes [3]. In the Netherlands, three founder mutations have been found in the MYBPC3 gene, a cardiac sarcomeric protein that is believed to modulate contractile force through phosphorylation-regulated intracellular calcium although the exact mechanism and function of MYBPC3 in contraction is still unclear [2,4,5]. The most common of these founder mutations is 2373insG. It accounts for 25% of familial HCM cases in the Netherlands [6]. The mutation in exon 25 is predicted to cause protein truncation although truncated protein has not been detected in heart biopsies. The clinical phenotype is currently regarded as being due to haploinsufficiency (Figure 1) [6,7]. This mutation is mostly associated with late onset of symptoms with most individuals only displaying clinical complaints when thirty or older [6]. The exact mechanism of the disease remains unclear and it is not known what causes the onset of clinical symptoms.

There are a number of MYBPC3 mutant mouse models [8-10], although none for this Dutch founder mutation. Since access to human heart biopsies from patients is limited, we sought to establish a model based on human induced pluripotent stem cells (hiPSC) [11,12]. hiPSC, like human embryonic stem cells (hESC), can self-renew and differentiate to derivatives of the three germ layers, including cardiomyocytes. They can also be generated from any individual of any genetic background including those carrying mutant genomes. hiPSC lines have already been generated from patients with a variety of cardiac diseases including LQT [13-15], Brugada syndrome [16], Catecholaminergic Polymorphic Ventricular Tachycardia (CPTV) [17,18] and one form of HCM [19]. In addition hiPSC lines have been derived from patients with multiorgan mutations that cause syndromes with heart failure or other heart defects as one of their clinical features [20].

In this study we generated hiPSC from three patients with the same MYBPC3 mutation (2373insG) but each with a different clinical phenotype: they included a father and son with different ages of disease onset and one unrelated patient. After successful generation of the hiPSC lines, the disease phenotype was assessed in clones in which the reprogramming genes were still present or had been removed. MYBPC3 protein levels, cardiomyocyte cell surface area and alterations in calcium signaling/handling responses were determined. MYBPC3 protein levels were reduced in the diseased hiPSC derived cardiomyocytes as expected, and this was accompanied an increase in cell surface area on a culture substrate as well as altered calcium handling. This recapitulated the essential features of the disease and contributed to validating hiPSC from HCM patients as an *in vitro* model for investigating the mechanism underlying the disease pathology.

Material and Methods:

Ethics Statement

Heart biopsies and human skin biopsies were obtained from patients after individual written permission using standard informed consent procedures and approval of the Leiden University Medical Center's medical ethics committee. All samples were collected and anonymized by the treating physician. The control skin sample was obtained as waste tissue from an anonymized male donor.

Histology

Paraffin-embedded blocks from the left ventricle of a 72 year-old male (cause of death pneumonia) and the interventricular septum of patient 2 collected during Morrow surgery were provided by the LUMC Pathology Department. 5µm sections were cut and stained using hematoxylin and eosin (HE) staining solution and for Sirius red as previously published [21].

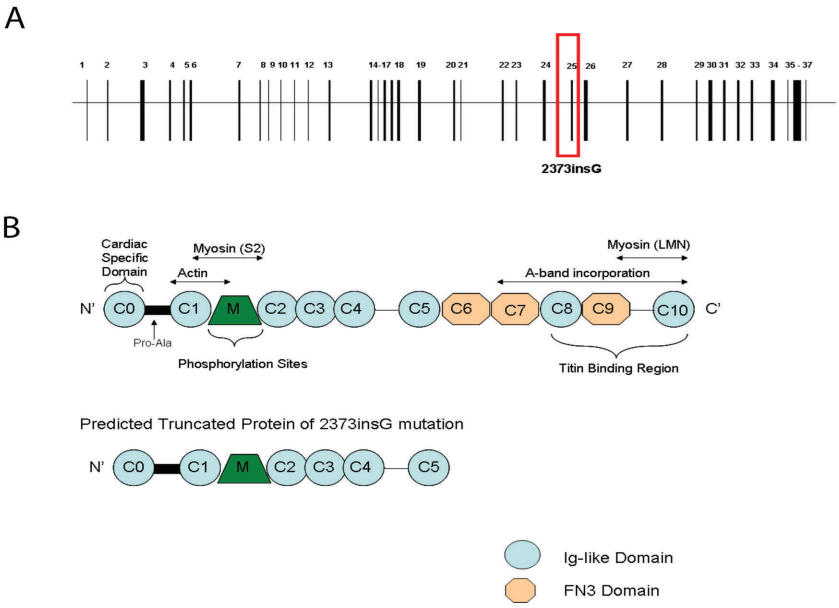

Figure 1: Schematic representations of MYBPC3. **A**) Schematic representation of the 37 exons on the MYBPC3 gene and the location of the Dutch Founder mutation 2373insG. **B)** The MYBPC3 protein and the predicted truncated protein created by the 2373insG mutation.

Isolation of dermal fibroblasts

Dermal fibroblast were isolated as previously described [22]. Fibroblasts at passage 2 were used for reprogramming.

Lentiviral production

Lentiviruses containing a self-inactivating multicistronic cassette encoding OCT3/4, SOX2, KLF4, and c-MYC were produced using polyethyleneimine to cotransfect HEK/293 T cells with the expression vector pRRL.PPT.SF.hOKSM.idTomato.preFRT and the helper vectors pCMV-VSVG, pMDLg-RRE, pRSV-REV. The virus was harvested after 48 h. The quantity of virus particles was measured by ELISA detecting HIV p24 using a kit (Zeptometrix) according to the manufacturer's protocol. The MOI was estimated by multiplying the p24 concentration (ng/ml) by a factor of 2500 [23].

hiPSC generation and maintenance

For lentiviral infection, 1.25 x 10^5 fibroblasts were seeded into one well of a 6-well plate and transduced 24 h later with the lentivirus at 5 MOI in the presence of 4 µg/ml polybrene (Sigma-Aldrich) in fibroblast medium. The virus was removed after 24 h. Six days after transduction, 20,000 fibroblasts were seeded on mouse embryonic fibroblasts (MEFs; 2 x 10^6 MEFs/10 cm dish) and cultured in hESC knock out serum replacement (KOSR) medium (DMEM/F12 supplemented with Glutamax, 10 mM NEAA, 25 U/ml penicillin, 25 µg/ml streptomycin, 100 µM β-mercaptoethanol, 20% KOSR (Invitrogen) and 10 ng/ml basic FGF (PreproTech)). Medium was changed every other day until the appearance of hESC-like colonies. Individual colonies were mechanically picked and placed on Matrigel in mTeSR (Stem Cell Technologies) and subcultured according to the manufacturer's protocol. hiPSC lines used in this study were denoted LUMC0004iCtrl (ctrl), LUMC0033iMyBPC (HCM1), LUMC0034iMyBPC (HCM2), LUMC0035iMyBPC (HCM3).

Determining insert number

Insert number was determined using splinkerette PCR. DNA was collected from each clone using Purecore Kit B from Qiagen according to the manufacturer's protocol (Qiagen). 5 µg of genomic

DNA was digested overnight in 10 U/µl CViQI (NEB) at 25°C or 10 U/µl DPNII (NEB) at 37°C. 300 ng of digest was then used for overnight T4 DNA ligase reaction in 40 µl as previously published [24]. Following a second digestion to prevent amplification of internal lentiviral sequences, 5 µl of the ligated product was amplified using touchdown PCR. After successful amplification, the PCR product was run in a second touchdown PCR (Dambrot et al, in preparation). This product was then run on a 2.5% tris-acetate-EDTA (TAE) gel with 0.5 µg/ml of ethidium bromide to determine insert number.

Transgene removal

hiPSC clones with either 1 or 2 inserts were first adapted to enzymatic passage on MEFs [25]. hiPSC were transfected with pLV.hCMV-IE.FLPe.IRES.PurR.hHBVPRE [26] using FuGENE 6 (Roche) as previously described [27]. In brief, 20,000 hiPSC/cm^2 were seeded on MEFs (density 30,000 cells/cm^2. Following overnight attachment, the cells were transfected using FuGENE 6 according to the manufacturer's protocol. The following day, the cells were exposed for 24 h to puromycin (final concentration 1 µg/ml). Colonies reappeared 1 to 2 weeks after puromycin treatment and were screened for successful transgene removal using specific PCR primers (OCT3/4-KLF4: forward: 5'-TCGTGAGAGTGTGGTTCTGC-3', reverse: 5'-CTCCCGCCATCTGTTGTTAG-3'; FRT (triple primer set): 5'-CGAGTCGGATCTCCCTTTGGGC-3', 5'-TGGAAGGGCTACGTAGCTAGC-3', 5'-GGTTCCCTAGTTAGCCAGAGAGC-3'). Transgene-free colonies were mechanically picked and placed on Matrigel with mTeSR for further expansion. To test for possible integration of the vector, hiPSC colonies were exposed to puromycin (final concentration of 1 µg/ml) for 24 h of selection.

Karyotype analysis

Karyotype analysis was performed using COBRA-FISH as previously described [28]. 20 metaphase spreads for each sample were analyzed.

Mutation Confirmation

DNA from hiPSC was collected using a phenol chloroform-based method. MYBPC3 exon 25 was amplified by PCR (forward primer: 5'-CCTGTGGCGGTTAGTTGG-3', reverse primer: 5' CACCGGTAGCTCTTCTTCTTCTTG-3') and used for Sanger sequencing with the forward primer. The mutation was also confirmed using mutation-specific PCR primers [6] (Forward primer: 5'-AGGACTCCTGCACAGTACAGGT- 3', reverse primer: 5'- CACCGGTAGCTCTTCTTCTTCTTG-3').

Spontaneous Differentiation of hiPSC into derivatives of the three germ layers

Pieces of hiPSC colonies were passaged onto Matrigel coated chamber slides (BD Falcon) in mTeSR for two days, then cultured in DMEM/F12 with 20% FCS for 3 weeks, changing medium every other day. Cells were then fixed for 30 minutes in 2% paraformaldehyde (PFA).

Table 1: Clinical Data of HCM patients with 2373insG mutation in MYBPC3

Patient	AGE	SEX	Interventricular Septum in Diastole, mm	LVEDD, mm	LVESD, mm	% FS	LVEF%
1	44	m	13	54	32.9	39	56
2	14	m	35	41	24	41	58
3	42	m	24	55	42	24	48
Healthy Range			<13	36-56	20-40	25-43	55-70

% Fractional shortening (FS) = (LVEDD-LVESD)/LVEDD*100%

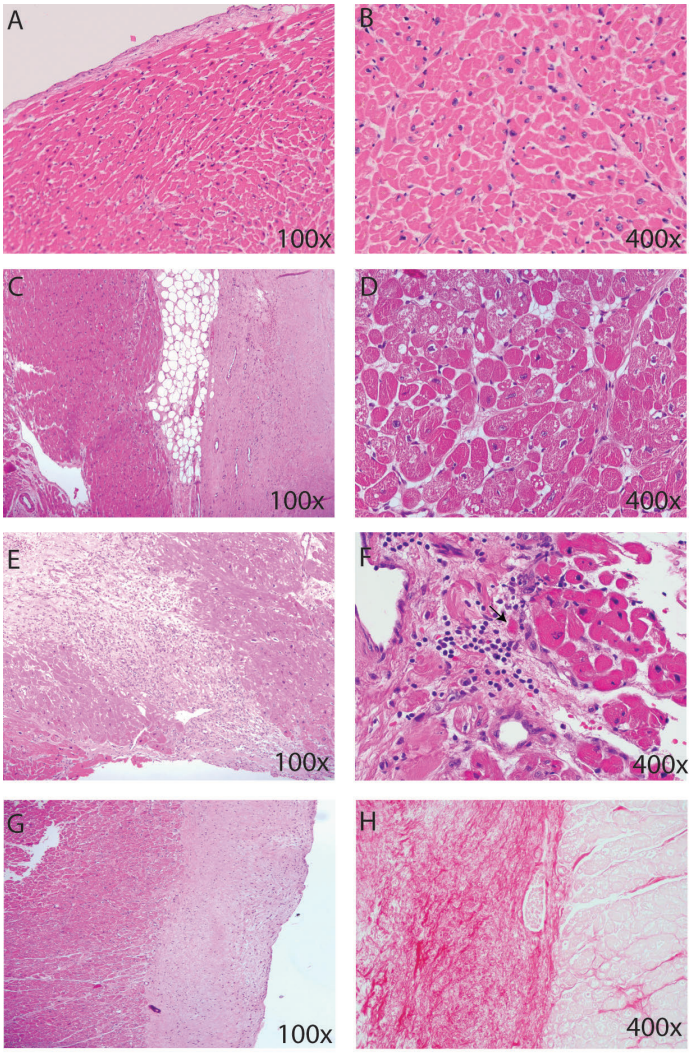

Figure 2: Histological sections of heart tissue from normal heart tissue and heart tissue from HCM patient 2 collected during a Morrow operation. **A&B)** Hematoxylin and eosin (HE) staining of healthy heart tissue of the left ventricle, 100x and 400x magnifications, respectively, demonstrating uniformly shaped myocardial fibers and a moderate amount of collagen deposition, **C)** HE staining of a section (100x magnification) from HCM patient 2 showing a severely thick band of collagen adjust to the myocardial fibers**. D)** Sirius red staining of a subsequent section to the HE staining of image C, confirming the presence of collagen extending from the endocardium into the myocardial fibers. **E&F)** HE staining of a second piece of heart tissue from patient 2 (100x and 400x magnification, respectively) indicating large areas of fibrosis with infiltrating inflammatory cells including lymphocytes, plasma cells and macrophages (as indicated by the black arrow). **G)** HE staining of a third piece of heart tissue displaying adipose tissue between the collagen band and myocardial fibers (100x magnification). **H)** HE staining of fourth piece of hearth tissue (400x magnification) demonstrating irregularly shaped myocardial fibers with fragmented cytoplasm.

Monolayer Cardiac Differentiation

hiPSC were plated in mTeSR on a Matrigel-coated plate. After 3 days, the medium was removed and BEL medium (Iscove's Modified Dulbecco's Medium (IMDM) supplemented with L-glutamine and 25mM HEPES (Invitrogen,), F12 Nutrient Mixture (HAM) supplemented with Glutamax (Invitrogen), 5% protein free hybridoma medium (Invitrogen), 0.25% deionized albumin from BSA (Sigma,) in IMDM, 1% Chemically Defined Lipid Concentrate (Invitrogen), 0.1%

Insulin-Transferrin-Selenium-X supplement (Invitrogen), 450 µM 1-Thioglycerol (Sigma), 5mg/ml L-ascorbic acid 2-phosphate (Sigma), 1% Glutamax (Invitrogen), 25 U/ml penicillin, 25 µg/ml streptomycin (both Invitrogen) plus 20ng/ml Activin A (R&D systems), 20ng/ml BMP4 (R&D systems) and 1.5 µM CHIR99021 (Axon). The growth factors were removed 72 h later and BEL plus 5 µM XAV939 and Matrigel (1:100) was added for 72 h to prevent cells detaching from the dish. The cells were then refreshed twice weekly with BEL.

Immunostaining

Immunofluorescent staining was performed as previously [16]. In brief, PFA-fixed cells were permeabilized with Triton X-100, blocked with 4% normal goat serum for 1 h before overnight incubation with the primary antibody (NANOG (1:500, Peprotech), SSEA-4 (1:30, Biolegend), OCT3/4 (1:100, Santa Cruz), TRA-1-81 (1:25, Biolegend), βIII-TUBULIN (1:2000, Covance), AFP (1:25, Quarttet), CD31 (1:100, DAKO) α-Actinin (Sigma), Troponin I (Santa Cruz), β-MHC (Millipore), Nkx2.5 (Santa Cruz), MYBPC3 (provided by J. van de Velde), sarcomeric Tropomyosin (Sigma Aldrich)) at 4 °C. The next day, secondary antibodies labeled with Cy3 (1:200, Jackson Immuno Research), Alexa 568 (1:200, Invitrogen) or Alexa 488 (1:500, Invitrogen) were added for 1 h at room temperature. For cell surface area measurements, α-Actinin stained cells were co-stained with pre-conjugated Phalloidin-Alexa 488 (Invitrogen) following the secondary antibody (30 m room temperature). Nuclei were stained with DAPI before mounting slides with Mowiol (Calbiochem).

Protein Collection and Western Blot

Protein samples were collected 30 days after the start of differentiation. Protein samples were collected in Baeuerle Buffer (20mM HEPES, pH 7.9, 350 mM NaCl, 20% Glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1% NP-40) plus proteinase inhibitors (Proteinase inhibitor (Invitrogen, 1:100), Orthovanadate (Sigma, 1mM), and Dithiothreitol (DTT, Roche, 0.1mM), using flash freezing in liquid nitrogen. Protein concentration was determined using Protein Assay buffer (Bio-rad) according to the manufacturer's protocol. 100 µg of protein was run on 10% acrylamide gel and transferred to a Polyvinylidene fluoride (PVDF) membrane overnight at 55 mAmps. Blots were blocked with 5% Milk for 1 h followed by overnight staining with primary antibody (MYBPC3, α-actinin, (as above), and actin (Millipore)) in 5% Milk. The next day, the appropriate horseradish peroxidase-linked secondary (Cell Signaling) in 5% Milk was added at room temperature for 1h. The bands were detected using SuperSignal West Pico Chemiluminescent Substrate (according to the manufacturer's protocol, Thermo Scientific) and analyzed using ImageQuant software.

Cell Surface Area Measurement

Cardiomyocytes from beating monolayers were dissociated into single cells 20 days after the start of differentiation using TrypLE Select (Invitrogen) for 30 minutes at 37 °C and plated onto Matrigel-coated coverslips. After 1 week, 100 µM PE was added for 72 h. The PFA-fixed cells were fluorescently stained with α-actinin-cy3 (Sigma and Jackson ImmunoResearch, respectively) and pre-conjugated Phalloidin-Alexa 488 (Invitrogen) as described above. Cells were visualized using Nikon Eclipse T*i*-S microscope. Single plane of 1200x1600 pixels images at 313nm/pixel resolution were recorded using a Plan fluor 20x/0.50 lens and analyzed using ImageJ software. Cell surface area was determined by outlining the cell membrane of α-actinin positively stained cells and using ImageJ software to subsequently measure the area.

Responses to changes in external calcium

Beating areas were dissected from cultures at day 20 and plated onto Matrigel-coated coverslips. 7 to 10 days later, the beating clumps were perfused with Tyrode's solution (140mM NaCl, 10mM glucose, 5mM HEPES, 5.4mM KCl, 1.2mM $MgCl_2$, 1.8mM $CaCl_2$, pH 7.4) with decreasing, then increasing amounts of external calcium (1.8mM, 1.0mM, 0.5mM, 0.3mM, 0.1mM, 0.5mM, and 1.8mM) for 30 m intervals. After 30 m of perfusion, two-minute videos were captured and the beat rate determined from these as beats per minute. Results were normalized to beats per minute of the first 1.8mM recording.

Table 2: Number of inserts identify by Splinkerette PCR

HCM1		***HCM2***		***HCM3***	
Clone	Number of Inserts	Clone	Number of Inserts	Clone	Number of Inserts
1	N/A	**1**	N/A	**1*‡**	2
2	2	**2*‡**	2	**2**	1
3	N/A	**3**	N/A	**3**	3
4	N/A	**4**	N/A	**4***	2
5	N/A	**5**	N/A	**5**	2
6	N/A	**6**	N/A	**6**	N/A
7	N/A	**7**	3	**7**	N/A
8	N/A	**8**	3	**8**	N/A
9*	1	**9**	N/A	**9**	3
10	N/A	**10**	N/A	**10**	4
11	N/A	**11**	N/A	**11**	2
12	N/A	**12***	2	**12**	5
13*‡	1	**13**	N/A	**13**	1
14	2	**14**	N/A	**14**	4
15	3	**15**	2	**15**	2

* Clones selected for transgene removal.
‡ Clones used for phenotype assessment after transgene removal.

Internal Calcium Measurements
Cardiomyocytes from beating monolayers were dissociated into single cells 20 days after the start of differentiation using TrypLE Select for 30 minutes at 37°C and plated onto Matrigel coated 96-well plates (40,000 cells/cm^2). 10 days after plating internal calcium was measured using Fluo-4am (Invitrogen) according to the manufacturer's protocol and previously published [19]. In brief, 5 µM Fluo 4am plus 0.02% w/v Pluronic (Invitrogen) was added to each well and the cells were incubated for 15 minutes at 37°C, washed with Tyrode's solution (same as above) and recorded using a Leica AF6000 microscope equipped with a Hamamatsu EM-CCD camera. Caffeine (10mM) was added after 20 s of video capture and the capture continued for an additional minute. Raw calcium transients were analyzed using ImageJ software and Z-profile_ImJ software (tailored for use here) to convert the raw data to ΔF/F measurements for comparative analysis.

Statistical Analysis
Results are expressed as mean ±SEM. Comparisons were made using one-way ANOVA or two-way repeated measures ANOVA as indicated. Values of $p \leq 0.01$ were considered significant. Statistical analyses were performed using GraphPad Prism.

Results

Clinical manifestation of the disease
The three patients included were previously identified by the LUMC Cardiology Department as carrying the 2373insG mutation in the MYBPC3 gene. Each patient displayed a different clinical phenotype. Patient 1 (HCM1) was at the time of collection a 44 year old male with an interventricular septum (IVS) of 13mm. Patient 2 (HCM2) was a 14 year old male, the son of patient 3, with an incident of out of hospital cardiac arrest and a 44mm IVS. Patient 3 (HCM3) was a 41 year old male (HCM2's father) with a 29mm IVS. With the exception of increased IVS, other clinical cardiac characteristics (such as fractional shortening) were within the normal range (Table 1).

Due to the large size of patient 2's IVS, he underwent a Morrow surgery, which removes pieces of the thickened heart wall. Some of these pieces were embedded in paraffin for histological studies. The histological sections from patient 2 were compared with healthy left ventricular heart tissue (Figure 2)). The HE staining of the healthy heart tissue displayed uniformly shaped myocardial fibers and a moderate amount of collagen deposition among the muscle fibers (Figure 2A and B). Stained histological sections from Patient 2's heart showed many abnormalities, including a thick band of collagen that extended from the endocardium into the myocardial fibers (Figure 2C and D) as well as large areas of fibrosis with infiltrating inflammatory cells (lymphocytes, plasma cells and macrophages) (Figure 2E and F). Furthermore adipose tissue was seen between the collagen band and the myocardial fibers (Figure 2G) and many of the myocardial fibers were angularly arranged, irregular in diameter and shape and in some, fragmented cytoplasm was visible (Figure F and H).

Table 3: The proviral insert locations identified for the HCM clones

Clone	Insert location
HCM1 #9	*GATAD2B* chr1: 153863681-153863723
HCM1 #13	*RBM19:* chr12:114276140-114276269
HCM2 #2	*SPEN* chr1:16232602-16232707 *USP4* chr3:49349283-49349394
HCM2 #12	*ASH1L* chr1:155370084-155370192 *FNIP1* chr5:130981785-130981902
HCM3 #1	*PTOV1-AS1* chr19:50342702-50342854 *COL5A2* chr2:190023157-190023269
HCM3 #4	NUMA1 Chr11: 71776625-71776694

Induced pluripotent stem cell generation, proviral insert identification and removal

30 days after transduction of the reprogramming genes [29] 15 embryonic stem cell-like clones were selected from each patient and the proviral insert number was determined. The HCM-iPSC clones contained between 1 and 5 inserts (Table 2) with the majority of clones containing 2 or more inserts. Based on these results, 2 clones containing 1 or 2 inserts were selected for TOPO TA cloning and sequencing to determine the insert location as shown in Table 3. These clones were then used in the subsequent experiments.

The transgenes were successfully removed from 5 out of 6 clones attempted, using FLPe, with efficiencies ranging from 20-98% (see Table 4 for individual efficiencies). Specific PCR and sequencing of exon 25 of the MYBPC3 genes showed the 2373insG mutation was still present in the HCM-iPSC lines (figure 3A & B). All HCM-iPSC lines expressed pluripotency markers (NANOG, SSEA4, OCT3/4 and TRA1-81, figure 3C), and could undergo spontaneous differentiation resulting in expression of markers of the three germ layers (AFP, βIII Tubulin, CD31, figure 3A) comparable to that of a hiPSC derived from a gender and age-matched (to patient 1 and 3) control line. Additionally, these lines were karyotypically normal (figure 3D) after transgene removal.

Cardiac Differentiation and assessment of phenotype

HCM-iPSC and ctrl-iPSC lines were induced to differentiate into cardiomyocytes that showed spontaneous beating and positive immunostaining for the cardiac markers: α-actinin, NKX 2.5, Troponin T, βMHC, Tropomyosin, and MYBPC3 (Figure 4).

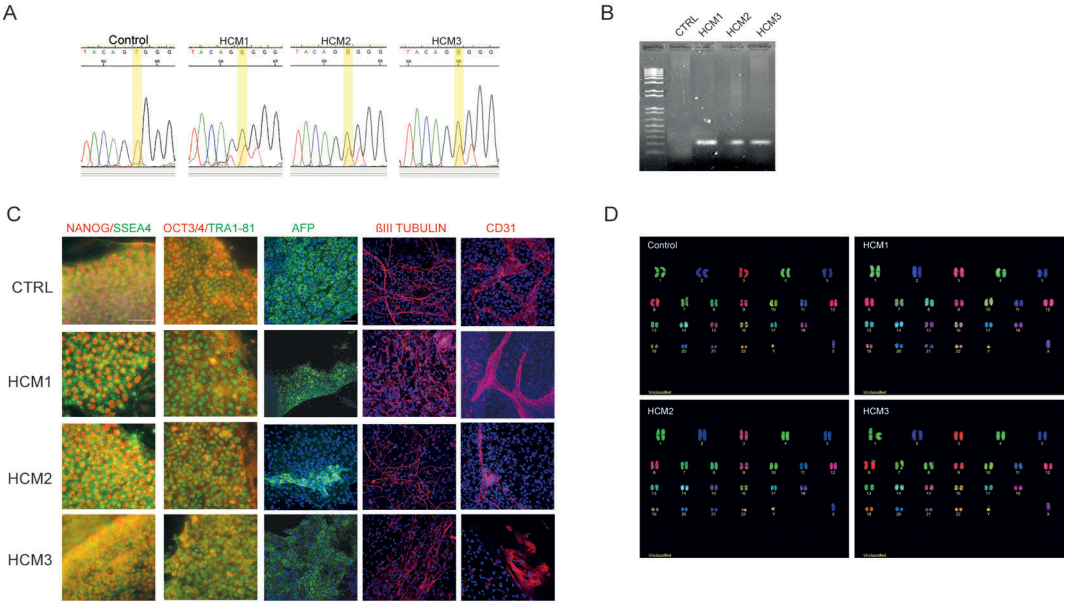

Figure 3: Characterization of hiPSC. **A)** Sanger sequencing results of exon 25 of MYBPC3 of control and HCM-iPSC. **B)** PCR analysis of 2373insG mutation using mutation specific PCR primer pair. **C)** Immunofluorescence images of undifferentiated hiPSC expressing typical pluripotency markers (red: NANOG, TRA1-81; green: OCT3/4, SSEA-4) and spontaneously differentiated hiPSC stained for derivatives of the three germ layers (alpha feto-protein (AFP, green); βIII-Tubulin (red); CD31 (red); nuclei (DAPI, blue). Scale bar: 50µm). **D)** Karyogram generated by combined binary ratio labeling fluorescence *in situ* hybridization (COBRA-FISH) of HCM and ctrl hiPSC showing a normal 46XY karyotype.

The HCM phenotype of patients with this MYBPC3 mutation is thought to be due to haploinsufficiency in MYBPC3 protein levels. To examine this in cardiomyocytes from HCM-iPSC lines, protein samples were collected 30 days after the start of differentiation. MYBPC3 protein levels in HCM-iPSC cardiomyocytes (based on normalization to α-actinin protein levels to correct for possible differences in cardiomyocyte number and differentiation efficiencies) were compared to the healthy hiPSC-CM. MYBPC3 protein levels were 48-58% (n=4) lower in all HCM-iPSC-CM compared to the ctrl-iPSC-CM (Figure 5A). There was no significant change in this outcome after the removal of the transgenes although patient 3 then showed an 80% drop in protein level (n=3) (Figure 5B).

Table 4: The efficiency of transgene removal

Clone	Efficiency (%)
HCM1 #9	0
HCM1 #13	40
HCM2 #2	24
HCM2 #12	43
HCM3 #1	98
HCM3 #4	20

An increase in heart size or wall thickening is a characteristic of HCM in vivo in mouse disease models and in patients [30-32]. However, the demonstration of hypertrophy in cardiomyocytes *in vitro* is more difficult as most measurements are surrogates and a combination of evidence

will generally be required. Others have previously described cell surface area in culture as evidence for hypertrophic phenotypes, with cell size in two-dimensions as the surrogate measure [19,20,33,34]. Dissociated, single HCM-iPSC-CM from all three patients showed a significant 2- to 3-fold increase in cell surface area compared to the control cardiomyocytes in the absence of hypertrophic stimuli (figure 5C). Upon addition of 100 µM phenylephrine (PE) for 72 h, the ctrl-hiPSC-CM demonstrated a 2-fold increase in cell surface area compared to the untreated cells. There was no significant change in cell surface area for the HCM-iPSC-CM (figure 5C). Nevertheless, the untreated HCM-iPSC-CM remained significantly larger than the PE treated healthy ctrl-hiPSC-CM. Upon removal of the reprogramming transgene, the cell surface areas of single untreated HCM-iPSC-CM was still significantly greater than those of the healthy individual, though in this case only between 1.5 and 2-fold (one-way ANOVA $p \leq 0.05$). PE had similar effects in the absence of the transgene as in its presence, demonstrating that its removal had no effect on the overall outcome of the experiment although the HCM-iPSC-CM were no longer larger than the PE treated ctrl-hiPSC-CM (figure 5D).

The lack of PE-response was also evident in their chronotropic responses. Ctrl-hiPSC-CM and HCM-iPSC-CM from patients 2 and 3 were treated with 100 µM PE or 100nM ISO and their automatic responses immediately measured. While the ctrl-hiPSC-CM responded as expected with significant increase in beating frequency upon treatment with adrenergic stimuli, HCM-iPSC-CM showed a diminished response and failed to reach statistical significance (Figure 5E). Even strong stimuli such as serum (5 or 20%) failed to produce a response in the HCM-iPSC-CM although serum significantly increased the beating frequency of the ctrl-hiPSC-CM (Figure 5E).

HCM derived cardiomyocytes display an altered calcium response

MYBPC3 modulates heart contraction through calcium; although the mechanism is still unclear there have been a number of studies which demonstrated an alteration in the calcium signaling in primary animal and human cardiomyocytes [32,35]. In order to assess the calcium handling,

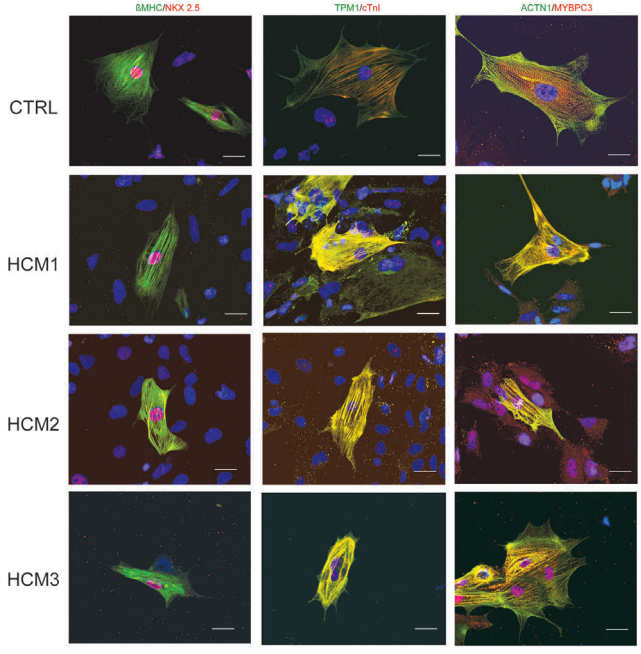

Figure 4: Immunofluorescence images of the cardiac markers α-actinin (ACTN1), troponin I (cTnI), sarcomeric tropomyosin (TPM1), myosin binding protein C3 (MYBPC3), beta-myosin heavy chain (β-MHC), and NKX2.5 in cardiomyocytes. Scale bar: 25µm.

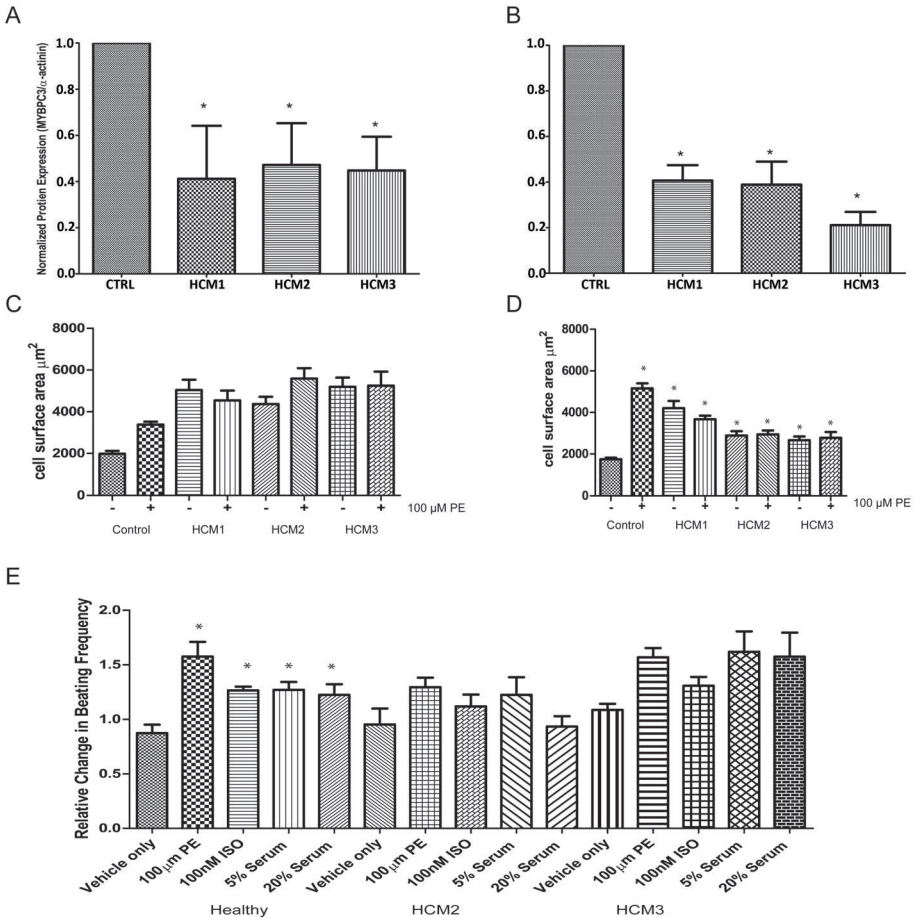

Figure 5: Assessment of disease phenotype of HCM-hiPSC-CM. **A)** Relative expression of MYBPC3 (n=4) of transgene containing hiPSC *: p<0.05. **B)** Relative expression of MYBPC3 (n=4) of hiPSC after transgene removal *: p<0.05). **C)** Cell surface area of transgene containing hiPSC ± 100 μM PE *: p<0.05. **D)** Cell surface area of transgene-free hiPSC ± 100 μM PE *: p<0.05. **E)** Relative change in beating frequency of hESC-CM immediately after addition of 100 μM PE, 100nM ISO, 5% serum, 20% serum or vehicle only control. Results normalized to initial beating frequency. *=p<0.05 vehicle only vs. treated n≥7 measurements.

beating clumps of hiPSC-CM were exposed to decreasing and then increasing concentrations of external calcium (stepwise 1.8, 1, 0.5, 0.3, 0.1 mM) and their beating frequency determined. As external calcium decreased, cardiomyocytes from control and all disease lines showed an expected reduction in beat rate, but the disease lines displayed a significantly altered response (two way ANOVA p=0.01). All of the HCM-iPSC-CM decreased their beating frequency more rapidly compared to the ctrl-hiPSC-CM but displayed a faster recovery as the external calcium was returned to normal levels (figure 6A).

Since changes in external calcium could affect other ion channel responses involved in beating frequency, such as sodium or potassium channels, we investigated the HCM-iPSC-CM response to calcium more directly by measuring the caffeine induced calcium release using a Fluo-4am dye (Invitrogen). As expected, the addition of caffeine to the cells released the internal calcium stores resulting a detectable increase in fluorescence. This increase was significantly larger in the HCM-iPSC-CM (between 38-79%) than those derived from the healthy ctrl-hiPSC-CM ((between 16-46%, Figure 6B-D), one-way ANOVA p≤0.05). Furthermore, there was no difference between lines in the absolute beating frequency or the recovery time after the addition of caffeine (Figure 6E-F).

Discussion

Quantitative and predictive assessment of cardiac hypertrophic disease phenotypes in patient derived hiPSC is still a challenge despite the increasing availability of a variety of cell lines from patients with genetic forms of this disease. One important issue is to ensure that the way the hiPSC lines have been generated, in particular whether the reprogramming transgenes are present or not, has not impacted the surrogate phenotype measurements. Whilst this is increasingly being overcome by using non-integrating methods for reprogramming, in some cases the lines already exist and returning to the original somatic cells for reprogramming is not an option. Here we generated hiPSC lines from three patients with a Dutch founder mutation in the MYBPC3 gene that is associated with HCM and compared cardiomyocytes from these lines with a healthy control individual. We showed that the reprogramming transgene could be removed from 5 out 6 lines using a FLPe plasmid and that this did not affect karyotype, the ability to differentiate efficiently into cardiomyocytes, or the basic hypertrophic phenotype measured as a function of protein levels and cell surface area.

The splinkerette PCR method was shown to be useful for determining the number of inserts during reprogramming, although the method contains some amplification bias and may have missed some of the inserts. We identified clones with up to 5 proviral inserts, which could make complete transgene removal difficult. Since it was unclear at the start of our experiments whether the presence of the transgenes would affect directed differentiation or the identification of a disease phenotype, we considered effective transgene removal as critical. Using transient transfections with FLPe-puromycin plasmid we successfully removed the transgenes from 5 out of 6 lines (with 1 to 3 inserts) with at least 20% efficiency with the exception of one line. The efficiency of removal appeared largely independent of insert number in the 1-3 range. For example, several transfections with various FLPe-puromycin constructs were made but were unable to remove the transgenes from one clone even though only one insert was present (data not shown). However the transgene could be removed from another clone generated in the same reprogramming experiment and from the same fibroblast. This implies that the problem was most likely with this particular clone and its insertion site (in the GATA2B gene).

While the histological sections from patient 2 showed an increase in interstitial fibrosis which is characteristic of HCM [36], there was a lack of myocyte disarray and no obvious hypertrophic cardiomyocytes. Additionally a number of the other characteristics present in patient 2's histological sections, such as the presence of adipose tissue between the collagen band and myocardial fibers and the fragment cytoplasm, are uncharacteristic of HCM. Furthermore the thickened collagen band seen is more characteristic of endocardial fibroelastosis and not HCM [37]. These non-characteristic features could explain the unusual early onset of the disease symptoms in patient 2 and imply that other factors contributed to onset and severity.

In cardiomyocytes from all disease lines, we observed a decrease in the MYBPC3 protein levels, in line with the haploinsufficiency believed to be the cause of the disease phenotype [6]. Additionally these results concur with those of van Dijk et al (2009) where heart biopsies collected from several patients with the 2373insG MYBPC3 mutation showed significantly lower MYBPC3 protein levels compared with that in heart biopsies from control individuals without the mutation [7]. The results therefore demonstrated the ability of hiPSC-CM to recapitulate the observations in patients.

It is still unclear whether the hypertrophic phenotype in patients with the 2373insG mutation in MYBPC3 is due directly to the mutation or is a secondary effect caused by additional stress on the heart due to generalized disruption of cardiomyocyte function. Since our results showed an increase in cell surface area of HCM-iPSC-CM at a single cell level without the addition of hypertrophic stimulus, it would seem likely that the cells do have an intrinsic hypertrophic phenotype that is not only a secondary effect of overload on the heart. Additionally our results

showed no difference in the disease phenotype (in Western blot or cell surface area) before or after transgene removal. This implies that for our clones transgene removal may not have been necessary to assess this phenotype using these criteria.

Previous studies in primary cardiomyocytes from patients with mutations in the MYBPC3 gene [5,8] as well as patient with this particular Dutch Founder mutation [7] reported higher calcium sensitivity with respect to force of contraction: as external calcium increased the force of contraction increased more rapidly in cardiomyocytes from patients than controls [32,38]. Our results show similarly higher calcium sensitivity with respect to autonomic beating frequency

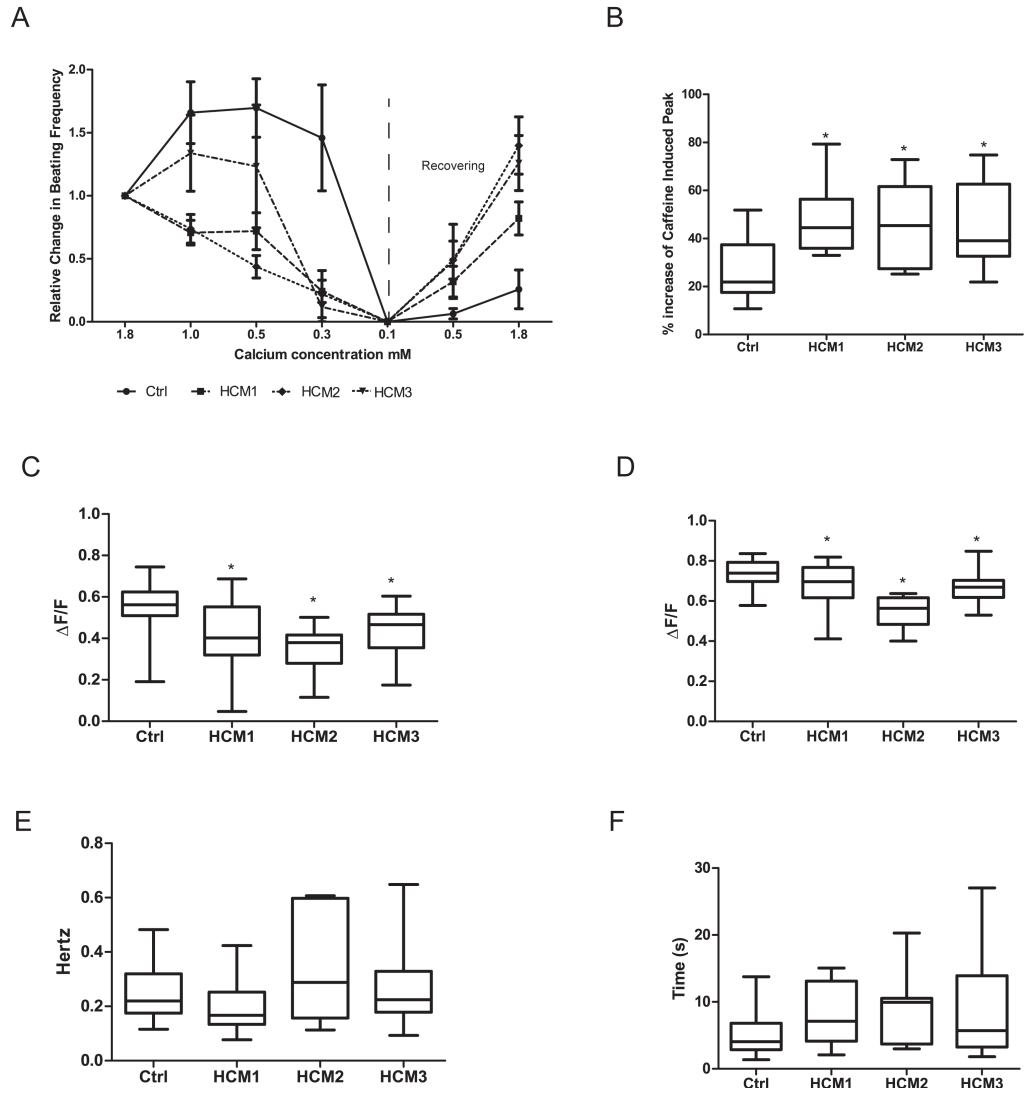

Figure 6: Assessment of calcium signaling/handling of HCM-hiPSC-CM compared to ctrl-hiPSC-CM. **A)** Changes in beating frequency in response to decreasing, then increasing external calcium. n=3-7, Two-Way ANOVA p=0.0065. **B)** The relative increase in calcium transient size on caffeine-stimulation of transgene free hiPSC-CM. n=7 *p<0.05, ctrl vs. HCM. **C)** Average initial amplitude of hiPSC-CM before treatment with caffeine n≥9 *p<0.05 ctrl vs. HCM. **D)** Average amplitude immediately after the addition of caffeine n≥9 *p<0.05 ctrl vs. HCM. **E)** Average beating frequency of hiPSC-CM. **F)** Recovery time (in seconds) of beating after the addition of caffeine.

only when calcium was reintroduced into a calcium-deprived system. The initial decrease in external calcium starting from physiological calcium concentration (1.8mM) displays a different effect, implying that the HCM cells are more profoundly affected by calcium than the control line (contrary to published results on isolated primary cardiomyocytes, as mentioned above). One possible reason for this apparent discrepancy is that the isolation procedure of primary cardiomyocytes involves using a low calcium buffer; the published experiments began at low external calcium and then the concentrations were increased [38]. No experiments on the force contraction of primary human cardiomyocytes to date have started with external calcium at physiological level and then decreased it. This may be due to fragility of the primary adult cardiomyocytes in culture after dissociation and plating compared with the more immature hiPSC-derived cardiomyocytes. Thus by using hiPSC-CM we may have revealed a new aspect of the calcium handling/signaling in these diseased cells.

Further investigation into the altered calcium response involved the examination of caffeine-induced calcium release. Upon the addition of caffeine to cardiomyocytes there is a release of the calcium stores. This has been shown previously in both hESC- and hiPSC-derived cardiomyocytes [39,40]. Interestingly when caffeine was added to HCM-hiPSC-CM, there was a greater relative increase in amplitude compared to ctrl-hiPSC-CM. A larger increase in response to caffeine has been linked to greater maturity in hESC-CM [41]. Yet still other studies have demonstrated maturation of the immature stem cell derived cardiomyocytes can be induced by hypertrophic stimuli [39,42]. Thus this difference in response to caffeine may show a greater intrinsic hypertrophic response of the disease cardiomyocytes. Additional caffeine-induced calcium experiments comparing different time points may help to explain these finding further.

We have presented here aspects of how hiPSC could be used for modeling HCM in humans, a first step in exploring the use of these cells to model this disease. We performed basic characterization of the HCM disease phenotype. We could show that HCM-hiPSC-CM displayed distinct characteristics that are different from the control cardiomyocytes under the same conditions. However, we were unable to show a difference in phenotype between the individual patients based on the clinical severity of their symptoms. Other reports of disease models have shown cardiomyocytes obtained from hiPSC lines derived from asymptomatic patients (with LQT syndromes and HCM) to be symptomatic in culture [15,19]. Whether our assays, as well as those used in the previous reports, are not sensitive enough to see any differences between individuals or culture conditions promote full revelation of the phenotype independent of its clinical severity in patients, is not clear. Furthermore, there are likely extrinsic (lifestyle) factors that may precipitate disease onset and severity in patients, which we are unable to capture in these relatively simple in vitro assays. It is likely we may have to make the disease cardiomyocytes undergo prolonged mechanical work or perhaps some kind of stress before any differences are revealed.

The exact mechanism and onset of HCM is still unknown. Correcting this mutation either genetically or pharmacologically could make it possible to determine whether the mutation is the only cause of the varying severity of the disease phenotype or whether other mutations, particularly in the case of patient 2, have a significant impact. No other mutations were found in the hotspot region of the sarcomeric gene that are known to be associated with HCM in patient 2 (data not shown) and it is currently unclear why patient 2 had early onset of what is normally a late onset disease.

Conclusions

hiPSC from HCM patients displayed features of hypertrophy in culture without the experimental addition of known hypertrophic stimuli independent of the presence or absence of the reprogramming transgenes. Cardiomyocytes from the disease lines all exhibited alterations in

calcium responsiveness. These results illustrate the ability of hiPSC to recapitulate the HCM cardiac disease phenotype in much the same way as primary adult human heart cardiomyocytes from patients with this disease. hiPSC cardiomyocytes are however, very immature with very low forces of contraction [43] so that alteration of contraction force to external calcium, using the current methods for adult cardiomyocytes, is presently difficult to determine. It is expected that various biophysical and engineering approaches may address this in the future [44]. These future advance will hopefully make it possible to better understand the clinical manifestation of cardiac diseases, an important component for using hiPSC in disease modeling.

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Chapter 7:
Serum supplemented culture medium masks hypertrophic phenotypes in human pluripotent stem cell derived cardiomyocytes

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Abstract

It has been known for over 20 years that fetal calf serum can induce hypertrophy in cultured cardiomyocytes but this is rarely considered when examining cardiomyocytes derived from pluripotent stem cells (PSC). Here, we determined how serum affected cardiomyocytes from human embryonic- (hESC) and induced pluripotent stem cells (hiPSC) and hiPSC from patients with hypertrophic cardiomyopathy linked to a mutation in the MYBPC3 gene. We first confirmed previously published hypertrophic effects of serum on cultured neonatal rat cardiomyocytes evidenced as increased cell surface area and beating frequency. We then found that serum increased the cell surface area of hESC- and hiPSC-derived cardiomyocytes and their spontaneous contraction rate. Phenylephrine, which normally induces cardiac hypertrophy, had no additional effects under serum conditions. Likewise, hiPSC-derived cardiomyocytes from 3 MYBPC3 patients which had a greater surface area than controls in the absence of serum as predicted by their genotype, did not show this difference in the presence of serum. Serum can thus alter the phenotype of human PSC derived cardiomyocytes under otherwise defined conditions such that the effects of hypertrophic drugs and gene mutations are underestimated. It is therefore pertinent to examine cardiac phenotypes in culture media without or in low concentrations of serum.

Introduction

Human pluripotent stem cells (hPSC), particularly those derived as induced pluripotent stem cells (hiPSC) from patients with genetic diseases, are increasingly regarded as useful for disease modeling and drug target discovery. hiPSC are considered particularly valuable because they capture the genome of the individual from whom they are derived. They have the ability to self-renew over long periods and differentiate into all cell types of the body and thus represent a permanent resource for modeling human disease. Although directed differentiation is still challenging for many lineages, cardiomyocytes were among the first functional cells to be derived and characterized from human embryonic stem cells (hESC) [1-3] and they can now be generated efficiently from hiPSC using similar protocols. Cardiomyocytes derived from human (h)PSC have been successful in mimicking reported drug responses in patients [4-7] including the positive chronotropic response to phenylephrine (PE), an α-adrenergic agonist, and isoproterenol (ISO), a β-adrenergic agonist [3]. These adrenergic stimuli have also been shown to induce pathological hypertrophy in cultured cardiomyocytes [8-10]. Pathological hypertrophy is an abnormal increase in cell size accompanied by the re-expression of fetal cardiac genes [11]. Aside from drug-induced cardiac hypertrophy, cardiomyocytes from hiPSC of patients with mutations associated with hypertrophic cardiomyopathy (HCM) also show features of hypertrophy and are thus potentially useful disease models [12]. Cardiomyocytes from patient hiPSC have already increased our knowledge of cardiac diseases [12-14], complementing and extending previous work based on clinical data and primary human cardiomyocyte cultures.

Whilst the uses of hPSC-derived cardiomyocytes are becoming well established, there is still no single method to derive these cells in culture. Although differentiation protocols are increasingly based on defined media and timed growth factor addition [15-17; reviwed in 18], fetal calf serum (FCS) is still often present, either during differentiation or during long-term maintenance to support survival as the cardiomyocytes attain some degree of maturity. Of 119 articles published using hPSC-CM in 2012 and 2013, 54 used 5% or higher concentrations of serum in the differentiation and/or maintenance medium. In others serum was present but at concentrations ≤2%. Since the exact composition of serum is unknown and varies considerably from batch to batch [19], its presence can confound interpretation of experiments investigating disease phenotype or drug responses. For example, it has long been known that serum or serum deprivation can profoundly affect primary neonatal rat cardiomyocytes (Rat-CM) in culture [20-24]. Cell size and protein-to-cell ratio can increase and additional stress-fiber-like structures may be induced in response to serum, which is indicative of cardiac hypertrophy [21, 24]. Other studies have used serum as a hypertrophic stimulus for cardiomyocytes[20, 25, 26], in place of the more commonly used hypertrophic stimuli PE or ISO [10]. The impact of serum on the cardiomyocyte phenotype and, more specifically, on the manifestation of the disease in the case of cardiomyocytes derived from patient hiPSC, remains unreported.

Here we examined the effects of serum and PE in the widely used Rat-CM model and confirmed findings reported previously. We then investigated the effects of serum on healthy- and HCM patient-derived hPSC-CM obtained under serum-free, defined conditions. Parameters examined included cell surface area, sarcomeric structure and beating frequency. These are common descriptors of phenotype in the literature. The effects of serum were compared with those induced by the hypertrophic drugs PE and ISO. We found that serum induced increased cell surface area and beating frequency and disrupted sarcomeric structure, indicating a hypertrophic response in both hESC-CM and hiPSC-CM. This masked the disease-associated hypertrophy of HCM-hiPSC-CM that was evident in the absence of serum. These results have important implications for conditions under which disease phenotypes are examined.

Material and Methods

Ethics statement

The isolation of neonatal rat cardiomyocytes was approved by the Animal Experiment Committee of Leiden University Medical Center and complies with the Guide for the Care and Use of Laboratory Animals as stated by the US National Institutes of Health. Human skin biopsies were obtained from patients after individual written permission using standard informed consent procedures following approval for use in this study by Leiden University Medical Center's medical ethics committee; this conforms to the Declaration of Helsinki. Control skin samples were obtained as waste tissue from donors in accordance with the Dutch federation of Biomedical Scientific Societies "Use of human tissue for scientific research" and "Code of good use" directives. All samples were collected by the treating physician and then anonymized.

Neonatal rat cardiomyocytes

Neonatal rat cardiomyocytes were isolated as previously described [27]. Briefly, rats were anaesthetized with inhaled isoflurane (4-5%) and adequate anesthesia was confirmed by the absence of pain reflexes before excising the hearts. Ventricles from the dissected hearts were minced and dissociated using collagenase and DNase and then suspended in Ham's F10 medium (ICN Biomedicals) with 10% horse serum (Invitrogen) and 10% FCS. To allow for preferential

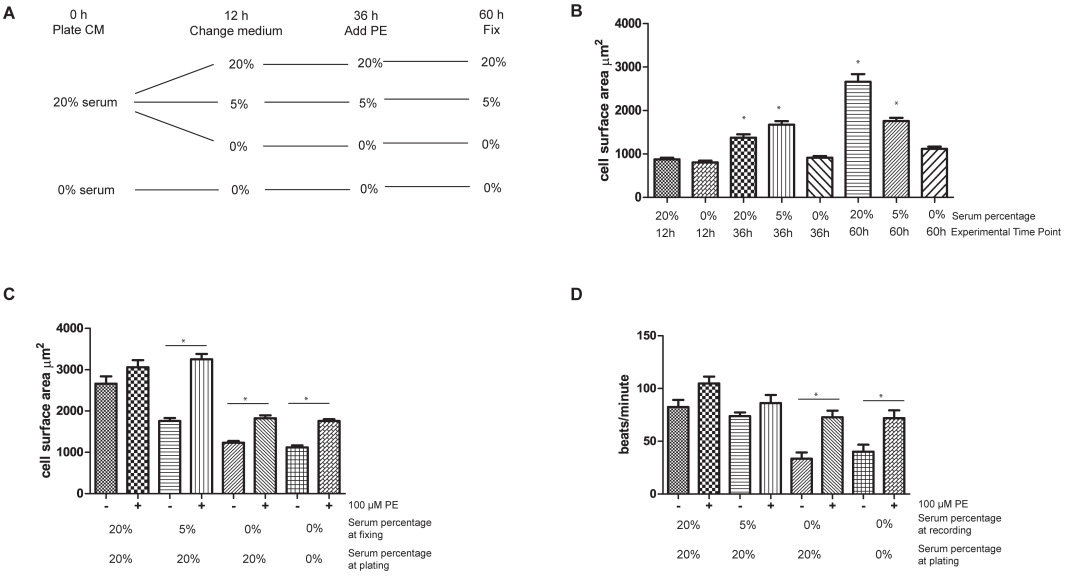

Figure 1) Responses of neonatal Rat-CM to serum. A) Schematic timeline of treatment procedure. Percentages indicate the serum concentrations. In parallel controls, PE was omitted. B) Cell surface area of Rat-CM in 20%, or 0% serum at 12h, 36h and 60h after plating and 5% serum 36h and 60h after plating. *=p<0.05 20% serum (12h) vs. other conditions (n≥67). C) Cell surface area of neonatal Rat-CM (60h) maintained in isolation medium (20% serum) throughout or subsequently reduced to 5% or 0% serum, or initially plated in 0% serum ±100 µM PE for 24 h. *=p<0.05 untreated vs. PE-treated (n≥59 cells). D) Beating frequency of Rat-CM (60h) ± 100 µM PE. *=p <0.05 untreated vs. PE-treated (n≥7 measurements).

attachment and negative selection of the non-cardiomyocytes, the cell suspension was pre-plated for 1h in a tissue culture dish. The non-adherent cardiomyocytes were then collected and plated on Matrigel coated coverslips (plating density of 20,000 cells/cm2) in isolation medium or serum-free BEL medium (Iscove's Modified Dulbecco's Medium (IMDM) supplemented with L-glutamine and 25mM HEPES (Invitrogen,), F12 Nutrient Mixture (HAM) supplemented with Glutamax (Invitrogen), 5% protein free hybridoma medium (Invitrogen), 0.25% deionized albumin from BSA (Sigma,) in IMDM, 1% Chemically Defined Lipid Concentrate (Invitrogen),

0.1% Insulin-Transferrin-Selenium-X supplement (Invitrogen), 450 µM 1-Thioglycerol (Sigma), 5mg/ml L-ascorbic acid 2-phosphate (Sigma), 1% Glutamax (Invitrogen), 25 U/ml penicillin, 25 µg/ml streptomycin (both Invitrogen)). 12 h later the medium was replaced and the cells were maintained in the original isolation medium (20% serum), 5% FCS in BEL or serum-free medium BEL for 24 h before treatment with 100 µM PE for an additional 24 h.

hESC culture

The Nkx 2-5eGFP/w hESC line [28] was maintained as a single cell culture in Dulbecco's Modified Eagle Medium (DMEM)/F12 (Invitrogen) containing 20% Knockout Serum replacement (Invitrogen), 1x MEM-Non-Essential amino acids (Invitrgoen) 0.1 mM 2-Mercaptoethanol (Invitrogen), 10 ng/ml Human basic fibroblast growth factor (bFGF) (Peprotech) , 25 U/ml penicillin and 25 µg/ml streptomycin (both Invitrogen) and passaged every 3-4 days, as previously described [29].

Human iPSC Derivation

Human dermal fibroblasts were isolated from biopsies from a control subject and patients carrying a MYBPC3 gene mutation and displaying an HCM phenotype. Fibroblasts were reprogrammed to hiPSC as previously described [30]. hiPSC were routinely cultured on Matrigel (BD Biosciences)-coated tissue culture dishes in mTESR according to the manufacturer's protocol (Stem Cell Technologies). The cells were mechanically passaged weekly using 1 mg/ml Dispase (Gibco).

Transgene removal and confirmation

Based on morphology, hESC-like colonies were manually picked 30 days after transduction. Cells were adapted to enzymatic culture on mouse embryonic feeder cells [29] and then transfected with pLV.hCMV-IE.FLPe.IRES.PurR.hHBVPRE [31] to remove the reprogramming cassette. Removal was confirmed by PCR of the FRT region (triple primer set): 5'-CGAGTCGGATCTCCCTTTGGGC-3', 5'-TGGAAGGGCTACGTAGCTAGC-3', 5'-GGTTCCCTAGTTAGCCAGAGAGC-3'). Transgene-free clones were readapted to mechanical passage on Matrigel with mTESR. Frozen stocks were made at passage 5 and cells were used for experimentation between passage 10 and 30. LUMC0004iCtrl (Healthy), LUMC0033iMyBPC (HCM1), LUMC0034iMyBPC (HCM2), LUMC0035iMyBPC (HCM3) hiPSC were used in this study.

Generation of Cardiomyocytes

Cardiomyocytes were generated from the Nkx 2-5eGFP/w hESC line [28] and hiPSC using a monolayer method in defined medium with timed addition of growth factors and small molecules. hESC were plated at a density of 10,000 cells/cm2 and hiPSC were dissociated into small clumps three days prior to differentiation and allowed to attach on Matrigel coated dishes in mTESR (~1.5 clumps/cm2) before starting the differentiation procedure. Differentiation was induced in low-insulin (1mg/L), "BEL medium" [16] plus 20 ng/ml Activin A (R&D systems), 20 ng/ml BMP4 (R&D systems) and 1.5 µM CHIR99021 (Axon). These factors were removed after 3 days and replaced with BEL plus 5 µM XAV939 (R& D systems) and Matrigel (BD bioscience) (1:100) to prevent cell detachment. The cells were refreshed with BEL three days later and subsequently maintained in BEL (changing medium twice a week). On average the differentiation efficiency of this method is 28-44% over several hPSC lines as determined by flow cytometry for cardiac troponin T expression (cTnT) (Fig S1).

Cell Stimulation and Cell Surface Area

Cardiomyocytes were dissociated into single cells 20 days after the start of differentiation using TrypLE Select (Invitrogen) for 30 minutes at 37°C and plated onto Matrigel-coated coverslips (25,000 cell/cm2) in serum-free BEL medium. After 10 days, the culture medium was placed with BEL containing 100 µM PE (Sigma),100 nM ISO (Sigma), 5% serum, 20% serum (Greiner (Serum A) or Gibco (Serum B)), or left untreated for 72 h. After 72 h, all coverslips were fixed in 2% paraformaldehyde for 30 minutes. For long-term experiments, cells on coverslips were exposed to serum for 7 days followed by 72 h treatment ±PE prior to fixation. For reversibility

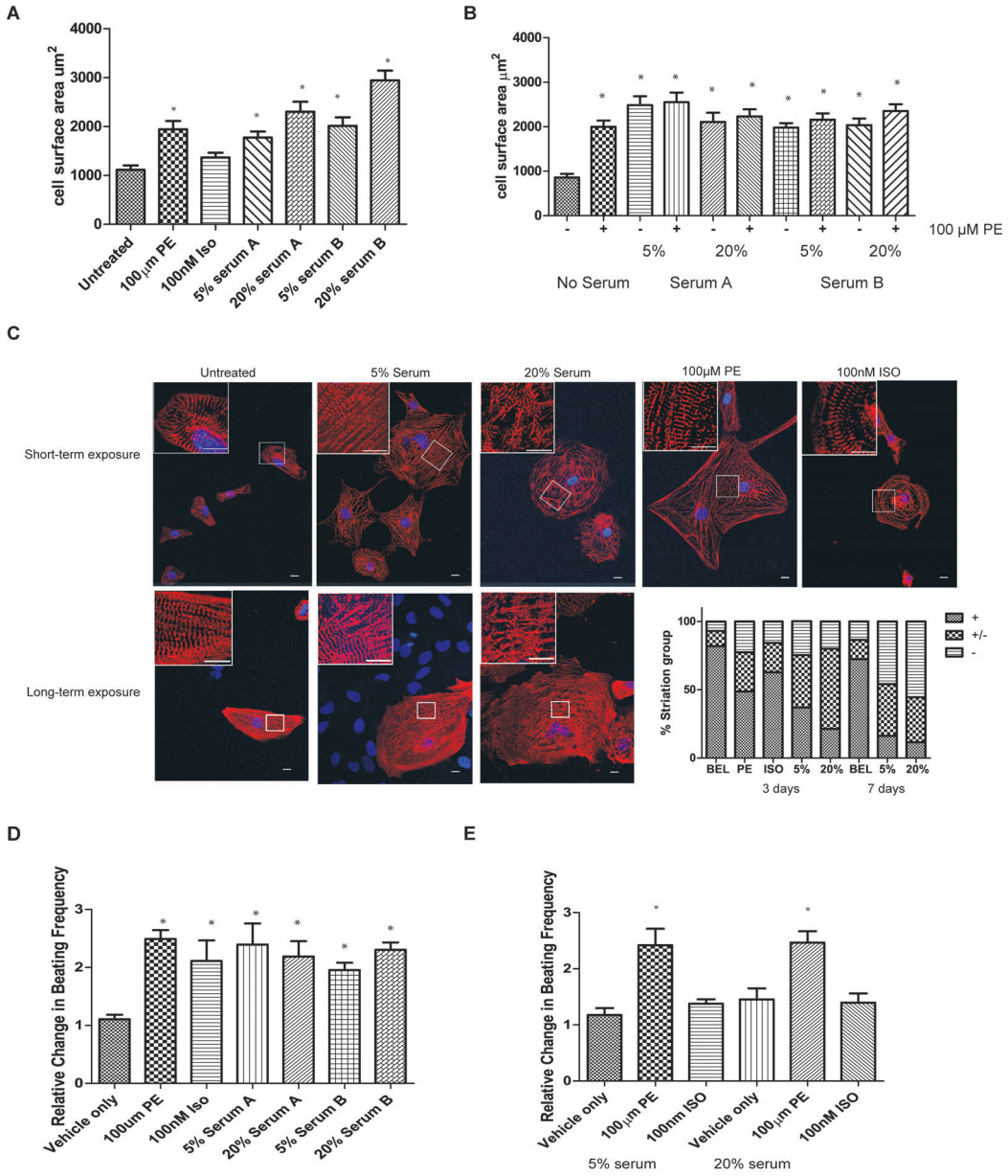

Figure 2) Response of hESC-CM to serum. A) Cell surface area of hESC-CM treated with 100 µM PE, 100nM ISO, 5% serum or 20% serum for 72 h compared to untreated n≥72 cells. * = p<0.05 untreated vs. treated. B) Cell surface area of hESC-CM treated with 5% or 20% serum for 7 days 100µM PE for 72h.*=p<0.05 no PE added vs. PE-treated, n≥40. C) Representative immunofluorescent images of hESC-CM treated with 100 µM PE, 100 nM ISO, 5% serum or 20% serum for 72 h (short-term exposure) or 5% serum and 20% serum for 7 days (long-term exposure). Stacked bar graph of the percentage of cells with sarcomeric striation (visible well-organized striations (+), some (disorganized) striations (+/-), and poor/non-existent striations (-)); n≥72 cells. Red: α-actinin, Blue: DAPI, scale bar: 10 µm. D) Relative change in beating frequency of hESC-CM after immediate addition of 100 µM PE, 100 nM ISO, 5% serum, 20% serum or vehicle only control. Results normalized to initial beating frequency. *=p<0.05 vehicle only vs. treated, n≥7 measurements. E) Relative change in beating frequency of long-term serum treated hESC-CM after immediate addition of 100 µM PE, 100 nM ISO or vehicle only control. Results normalized to initial beating frequency. *=p<0.05 vehicle only vs. treated, n≥5 measurements.

experiments, cells were treated as above and subsequently refreshed with serum-free BEL medium for 3, 5, or 10 days.

For determination of cardiomyocyte cell surface area, fixed cells were labeled with anti-α actinin (1:800; Sigma) followed by Cy3 (ImmunoResearch) secondary as previously described [32]. Nuclei were labeled with DAPI. For cell surface area measurements, 9 to 15 areas were randomly selected from each 10 mm coverslip and visualized using a Nikon Eclipse Ti-S microscope. Single plane of 1200x1600 pixels images at 313nm/pixel resolution were recorded using a Plan fluor 20x/0.50 lens and analyzed using ImageJ software. Representative sarcomere images were visualized using a Leica TCS SP5 confocal microscope; single planes of 1024x1024 pixels images at 44nm/pixel resolution were recorded using a Plan-Apochromat 40x/1.25 oil and 100x/1.4 oil lens. Sarcomeric structures of cardiomyocytes were divided into three categories, visible well-organized striations (+; category 1), some striations mostly disorganized (+/-; category 2) and poor or non-existent striations (-; category 3).

Calcium imaging

Cell cultures undergoing cardiac differentiation were dissociated after 20 days using TrypLE Select and replated on 96-well Matrigel-coated imaging plates (BD Bioscience, 40,000 cells/cm2). In order to determine calcium transient/contraction frequency 13 days after plating, cells were loaded with 5 µM fluo-4am (Invitrogen) for 15 minutes in the presence of 0.2% Pluronic in Tyrode's solution (140 mM NaCl, 10 mM glucose, 5 mM HEPES, 5.4m M KCl, 1.2 mM MgCl2, 1.8 mM CaCl2, pH 7.4). The cells were then washed 3 times with Tyrode's solution and maintained in 100µl of Tyrode's solution. With the plate maintained at 37ºC, time-lapse of Fluo-4am fluorescence was recorded using a Leica AF6000 microscope equipped with a Hamamatsu EM-CCD camera. Contracting areas were identified and imaged consecutively for 1.4 minutes (1500 images at 51ms per cycle). After recording a baseline for 20 seconds drug or serum additions were made to achieve final concentrations of 100 nM ISO, 100 µM PE, 5% serum, 20% serum, or vehicle only. Calcium transient frequency before and after treatment was determined using ImageJ and Z-profile_ImJ software, tailored for use here to convert raw data to dF/F results [33].

Statistical Analysis

Results are expressed as mean±SEM. Comparisons were made using one-way ANOVA with Bonferroni's multiple comparison post test. Values of $p \leq 0.05$ were considered significant. Statistical analyses were performed using GraphPad Prism.

Results

A hypertrophic response to serum in neonatal rat cardiomyocytes

We first confirmed well established hypertrophic effects of serum and PE on Rat-CM using cell surface area and beating frequency as standard surrogate measures. Rat-CM were plated in the original isolation medium containing 20% serum or in the absence of serum (Fig 1A, 0h). After 12 h, the cells were transferred to 20, 5 or 0% serum and maintained at this serum concentration until the end of the experiment (60h). Although the initial plating of these cells with or without serum (12 h exposure) did not affect cell surface area (Fig 1B), by 36h, cells in serum (5% or 20%) were significantly larger than those in absence of serum (Fig 1B). By 60h, a concentration-dependent effect was evident with cells maintained in 20% serum now significantly the largest (Fig 1C) there was no observed difference in the sarcomeric structures or organization between the groups (Fig S2).

Exposure of cardiomyocytes to PE has been previously shown to increase their cell surface area [34]. We observed a similar hypertrophic effect with exposure to PE for cells maintained in 0% or 5% but not 20% serum (Fig 1C). It was further noted that Rat-CM beat significantly more slowly in the absence than in the presence of serum (Fig 1D, $n \geq 7$, $p < 0.05$). PE exerted an expected positive chronotropic action under serum-free conditions (Fig 1D, $n \geq 7$, $p < 0.05$), effects which were similar to serum. Cells maintained in serum were refractory to the positive chronotropic effect of PE.

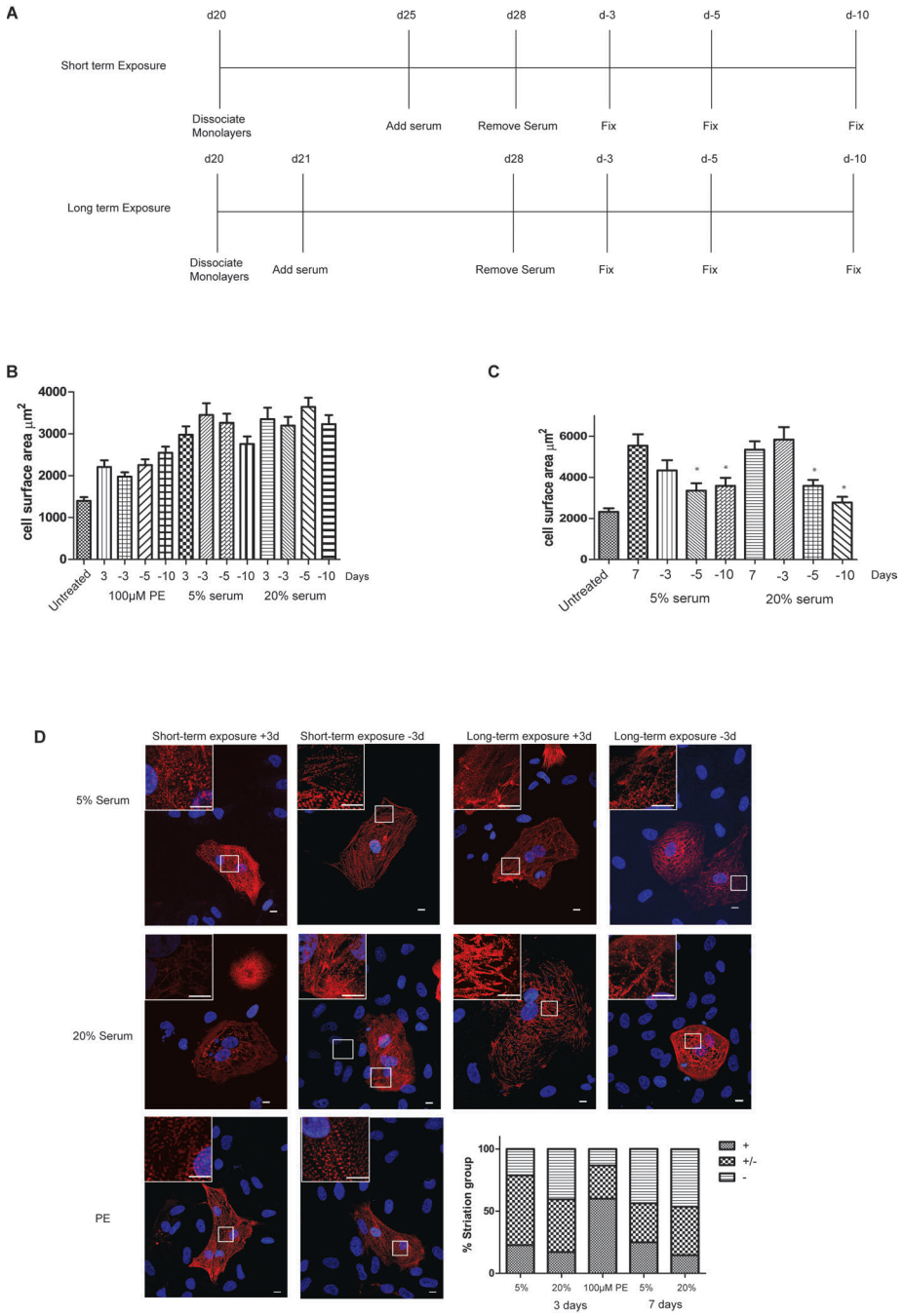

Figure 3) The stability of serum-induced alterations in hESC-CM. A) Schematic of treatment protocol. Cell surface area of hESC-CM treated with 5% or 20% serum for 3 days (n≥81) (B) or 7 days (n≥42) (C) and then removed for 3, 5, or 10 days. *=p<0.05 serum vs. -removal. D) Representative immunofluorescent images of hESC-CM treated with 100 0M PE, 5%, and 20% serum for 72 h +3 days (short-term exposure +3 days) or 5 % or 20% serum for 7 days +3 days (long-term exposure +3days) and then removed for 3 days (short-term exposure-3d and long-term exposure-3d, respectively). Stacked bar graph of the percentage of cells of different sarcomeric structural class (visible well-organized striations (+; category 1), some (disorganized) striations (+/-; category 2), and poor/non-existent striations (-; category 3)); n≥72. Red: α-actinin, Blue: DAPI, scale bar: 10µm.

A hypertrophic and positive chronotropic response of hESC-CM to serum containing medium
We assessed whether hESC-CM responded similarly to Rat-CM on exposure to serum or known adrenergic stimuli. When measured after 72h, cells maintained in 5% or 20% serum (2 batches, Sigma (Serum A) and Gibco (Serum B)) or PE all showed increased cell surface area (Fig 2A). The β-adrenergic agonist ISO failed to induce a significant increase (Fig 2A). Combined exposure of cells to both serum (>7days) and PE (72h) did not induce any additional increase in cell surface area (Fig 2B).

As results for serum brands A and B were similar, only serum B was used in subsequent experiments. Interestingly, besides the increased cell surface area, hESC-CM exposed to 5% or 20% serum developed abnormal sarcomeric structures. These effects were quantified and cells were divided into three categories: 1) visible well-organized striations, 2) some (disorganized) striations, and 3) poor/non-existent striations. The percentage of cells in category 1 was reduced and in category 3 was increased (Fig 2C) as early as 72 h after serum addition and was further exacerbated by long term serum exposure (Fig 2C).

Stimulation of the α-adrenergic system with PE or the β-adrenergic system with ISO has been shown previously to induce a positive chronotropic response in hESC-derived cardiomyocytes [3]. We confirmed these responses in this model. 100 μM PE induced a 2.25 fold relative increase in beating frequency (n=11 beating clusters) and 100 nM ISO induced a 1.9 fold relative increase (n=11 beating clusters) (Fig 2D). Furthermore, we found that serum addition to a serum-free culture caused a similar increase in beating frequency. Following chronic exposure to serum (>7 days) the positive chronotropic effect of PE was still evident but the effect of ISO was completely lost (Fig 2E). Besides the changed in beating frequency all other measurement parameters of the calcium transient (e.g. upstroke, slope decay and amplitude) showed no significant difference between treated and untreated groups (data not shown).

The hypertrophic effect of serum on hESC-CM may be irreversible
To assess the stability of the serum- or PE-induced hypertrophy we measured cardiomyocyte surface area upon withdrawal of these factors (Fig. 3A). For cells exposed to serum or PE for 72 h, there was no significant decrease in cell surface area at any time point and the cells remained significantly larger than the untreated cells (Fig 3C). However cells treated with serum for 7 days and then deprived of serum showed a significant decrease in cell surface after 5 days in serum-free medium (Fig 3B). By this time point these cells were no longer significantly larger than the untreated cells.

The sarcomeric structures which had already remodeled in the presence of serum appeared even more disrupted, with many more cells showing disorganized or missing striations (Fig 3D). This loss of sarcomeric structure was increased the longer the cells were deprived of serum (data not shown). The removal of PE from the cells had little or no effect on the sarcomeric structure (Fig 3D).

Serum effects on cardiac hypertrophic disease modeling.
Since hiPSC-CM from patients with gene mutations affecting the heart are now being increasingly used to model cardiac disease, it is important to determine whether serum, still widely used in the culture of cardiomyocytes, would affect the measurement of the disease phenotype in culture. To address this, we used a series of control and disease hiPSC lines recently derived in our laboratory (Chapter 6), differentiated them to cardiomyocytes and assessed the impact of exposure to serum or adrenergic agonists on cell surface area and automaticity. hiPSC-CM derived from a healthy individual and three patients with a mutation in the MYBPC3 gene linked to HCM (Chapter 6) were kept in serum-free medium and were treated with 5% or 20% serum or left untreated for 3 or 7 days. After 3 days, HCM-iPSC-CM kept serum-free were significantly larger than the ctrl-hiPSC-CM with a ≥1.5 fold increase in cell surface area (Fig4A). The healthy control hiPSC-CM displayed a 3-fold increase in surface area on exposure to either 5% or 20% serum (Fig 4B and C). The hiPSC-CM from the HCM disease patients were significantly larger than

the control in serum-free conditions, suggesting that they had become hypertrophic without additional stimuli, but these cells failed to show a further increase in surface area in response to serum (Fig 4A-C). Similarly to the hESC-CM under serum-free conditions, the surface area of the control hiPSC-CM increased in response to a 72h exposure to 100μM PE (Fig 4D and 100nM of ISO showed no effect (Fig S3)), whereas the hiPSC-CM from the three disease patient lines showed no response (Fig 4D and Fig S3).

Longer-term (>7 days) exposure of hiPSC-CM to serum produced similar results as short-term exposure (72 h): the healthy control hiPSC-CM showed an increase in cell surface area while the HCM-hiPSC-CM showed no change upon serum exposure (Fig 4E). Reflecting the hESC-CM results in Fig 2B, hiPSC-CM also failed to show an additional hypertrophic response to PE when maintained in serum (Fig 4E).

Upon examination of the sarcomeric structures of the hiPSC-CM the percentage of cells in category 3 was increased after 72 h treatment with serum only in the healthy control hiPSC-CM while the percentage of category 3 cells remained relatively the same in the HCM-hiPSC-CM (Fig 4F-H).

Discussion

There are numerous reports documenting the adverse effects of serum on the efficiency of cardiac differentiation of PSCs [35;36]. Nevertheless serum is often used as a standard medium additive to improve cardiomyocyte viability both during and after differentiation by providing growth factors, nutrients and hormones [19]. However, the effect of serum on cardiomyocytes during their long-term maintenance in culture has not been investigated in much depth even though several studies have described hypertrophic effects of serum on primary rodent cardiomyocytes in culture [37;38]. For this reason, serum concentrations are usually reduced or absent in experiments carried out on these cells. In this study we first confirmed the published effects of serum and PE on primary neonatal Rat-CM and then investigated the effects of serum on cardiomyocytes derived from hESC and hiPSC, both from a healthy individual and patients with a mutation in the MYBPC3 gene causing HCM. We found that the hypertrophic and physiological effects on the Rat-CM were as reported but the effects on the hPSC derived cardiomyocytes varied depending on how long they were exposed to serum (72h or >7 days) and which concentration had been present. Hypertrophic and physiological effects of serum were only seen in healthy hPSC and not in cardiomyocytes from the hiPSC lines derived from patients carrying the HCM associated mutation.

hESC-CM and hiPSC-CM derived from the healthy individual showed similar responses to serum, with an increased cell surface area and altered beat rate. They were also similar in their responses to PE and ISO. However the hiPSC-CM derived from patients with a mutation causing HCM showed no response to the addition of serum; they were larger than controls at the outset in serum free conditions and this did not increase further. These cells were also unresponsive to PE and ISO. The underlying mechanism for this is not clear but is being investigated in an independent study.

In the hESC-CM and healthy hiPSC-CM, short-term serum (72 h) exposure resulted in similar changes in cell surface area and beating frequency as PE, a known adrenergic agonist and inducer of hypertrophy in cardiomyocytes in vitro. This effect was not reversed by the removal of serum, even after 10 days. In addition, ISO had no effect on the beating frequency of hPSC-CM exposed to serum while in the absence of serum cells doubled their beating frequency as expected (Fig 2D and 2E). Our results suggest serum may control cardiac hypertrophy through the adrenergic pathway while additional unknown components in serum, and not present in the PE or ISO conditions, could cause the adverse effects on the sarcomeric structure. In concordance, previous studies demonstrated that isolating and maintaining Rat-CM in serum-free medium resulted in a decrease in stress-like fibers which are more commonly seen in cardiac non-muscle cells [21]. Moreover, studies in isolated human and chick cardiomyocytes have demonstrated the presence of stress-fibers both in unhealthy [39] and immature [40] cardiomyocytes. On the

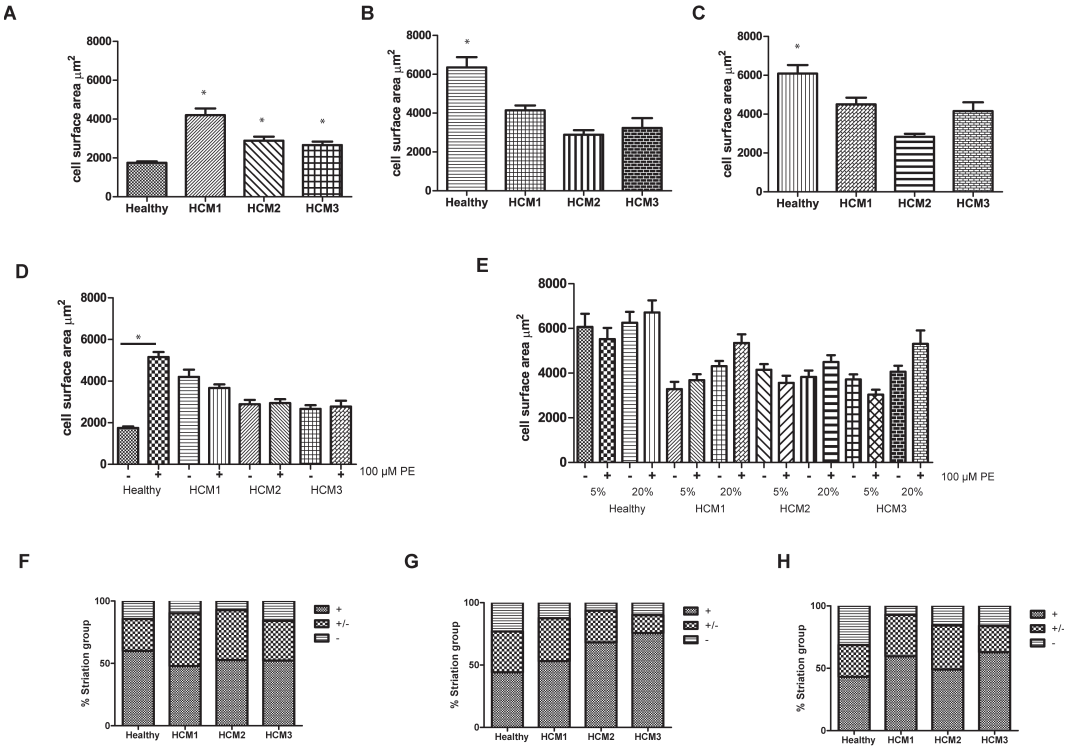

Figure 4) Response of hiPSC-CM to serum. Cell surface area of hiPSC-CM derived from a healthy individual (healthy) and three patients with hypertrophic cardiomyopathy (HCM1, HCM2, HCM3) in (A) serum-free medium *p=0.05 healthy vs. HCM n≥60 cells. (B) treated with 5% for 72 h. *p=0.05 healthy vs. HCM, n≥66 cells. (C) treated with 20% serum for 72 h. *p=0.05 healthy vs. HCM, n≥64 cells. D) Cell surface area of hiPSC-CM ± 100 µM PE for 72 h. *p=0.05 no PE added vs. PE-treated, n≥50 cells. E) Cell surface area of hiPSC-CM treated with 5% or 20% serum for 7 days ± 100µM PE for 72h, n≥39 cells. F-H) Stacked bar graph of the percentage of cells of different sarcomeric structural class (visible well-organized striations (+; category 1), some (disorganized) striations (+/-; category 2), and poor/non-existent striations (-; category 3)); F: no serum; G: treated with 5% serum; H: treated with 20% serum; n≥47.

other hand, it is possible that the breakdown of sarcomere structure may only be remodeling of cell structure due to chronotropic or inotropic changes caused by unknown components in the serum. However the complexity of the adrenergic pathway and the undefined factors in serum require additional investigation. Previous studies on Rat-CM have revealed various parts of the hypertrophy pathway with differing responses to serum [37;38;41]. In one report, the endothelin 1 hypertrophic pathway was suppressed by retinoic acid but serum-induced hypertrophy was not suppressed [38]. In a later study PE and serum-induced hypertrophy were both found to be impaired by the overexpression of CHAMP [41]. Taken together with our results and the lack of an effect of ISO on the beating frequency of hESC-CM after long-term exposure to serum, it might be inferred that serum affects the same pathway as β-adrenergic stimuli rather than α-adrenergic stimuli.

When hESC-CM or healthy hiPSC-CM were treated with serum for longer periods of time (> 7 days) they also showed an increase in cell surface area but lost their augmented response to PE. While other drugs were not tested, these results implied that serum in cell cultures could mask, or otherwise alter, drug induced effects. Ren et al (2012) for example showed glucocorticoids induced hypertrophy in rat embryonic cardiomyocytes (H9C2 cells) cultured in serum but in the absence of serum, glucocorticoids protected the cardiomyocytes from apoptosis. Furthermore, this increased cell surface area reverted when the serum was removed for at least 5 days [23. However the exact cause of this phenomenon is unclear. It may be that the sudden withdrawal

of growth factors from the serum leads to problems in basic cell metabolism and the synthesis and trafficking of sarcomeric proteins has become comprised. Thus the cardiomyocytes with longer exposure to serum may be on their way to losing viability. Others have previously reported similar results in Rat-CM in which serum-deprivation lead to increased apoptosis [42].

In addition to PSC-CM from healthy individuals, we investigated the effects of serum on cells derived from three patients with HCM caused by a mutation in MYBPC3 gene. Clinically these patients had an increase interventricular septum thickness but other heart functions, such as fractional shortening, left ventricular systolic-end and diastolic-end diameter, remained within the normal range [43]. Their derivative hiPSC-CM were resistant to PE and ISO induced hypertrophy and chronotropic effects and they also lacked the response to serum seen in the controls and primary cardiomyocytes. Thus their enlarged cell surface areas and altered beat rates relative to controls in the absence of serum were no longer evident in the presence of serum. This represents a cautionary note on the culture conditions used to compare diseased and control cardiomyocytes derived from pluripotent stem cells. In addition, some experiments described in the literature use primary rodent cells ±24 h after transfer to serum-free medium but this may not always be the case. Improved consistency in the results may be achieved by carrying out the experiments under serum-free or low serum conditions.

In conclusion, we have demonstrated that the addition of serum to PSC-CM derived under defined and conventional serum-free conditions can significantly alter the phenotype of cardiomyocytes: their surface area, sarcomeric structure and beating frequency are all parameters that show relevant alterations. These are among the parameters widely used to report phenotypes in cardiomyocytes derived from patient hiPSC. Therefore it is highly desirable to control experimental conditions and to culture hPSC derived cardiomyocytes in fully defined culture media.

Acknowledgements

Rat-CM were kindly provided by the experimental cardiology laboratory of the LUMC. We thank C. Baum and A. Schambach (Department of Experimental Hematology, Hannover Medical School, Hannover, Germany) for the lentiviral vector used for reprogramming, C. Freund for advice and S. van de Pas from the LUMC iPSC core facility for generation of control hiPSC and Jolanda van der Velden (Department of Physiology, VUmc) for antibodies used in study. This work was supported by the Netherlands Institute for Regenerative Medicine (NIRM) to CLM and SRB, and the Netherlands Organization for Health Research and Development (ZonMW grant no. 114000111 to LGJT, and grant 11.600.1016 to DEA).

Disclosures

SRB and CLM are cofounders of Pluriomics BV.

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Supplementary Figures:

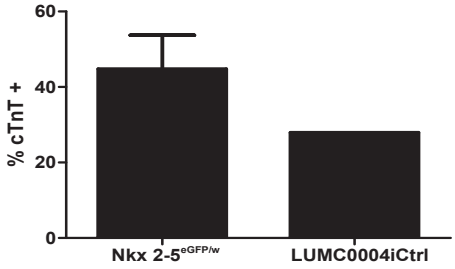

Fig S1) Cardiac differentiation efficiency of the Nkx 2-5eGFP/w hESC line and LUMC0004ictrl determined by flow cytometry of cardiac troponin T (cTnT) expression (n=11 for Nkx 2-5eGFP/w, two independent experiments for LUMC0004ictrl).

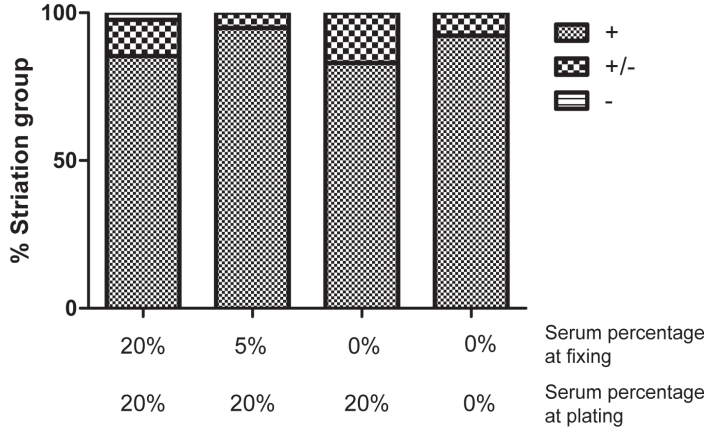

Fig S2) Stacked bar graph of the percentage of RAT-CM with sarcomeric structural class (visible well-organized striations (+; category 1), some (disorganized) striations (+/-;category 2), and poor/non-existent striations (-;category 3)); n≥41 cells.

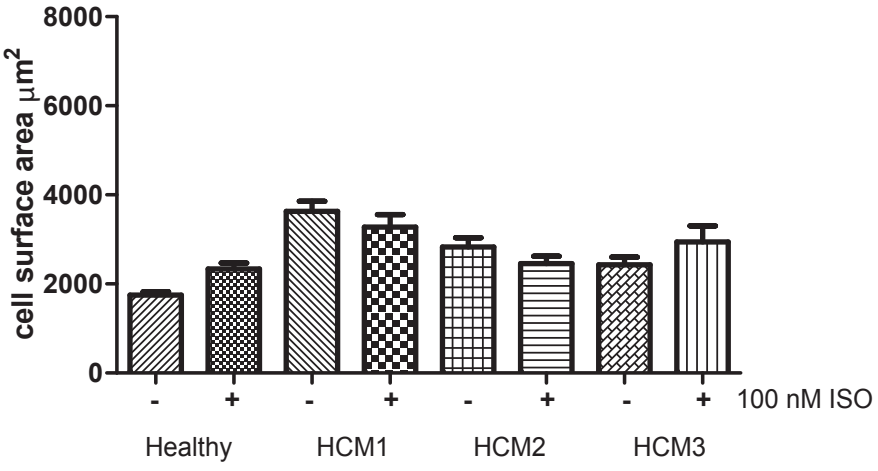

Fig S3: Response of hiPSC-CM to isoproterenol (ISO). Cell surface area of hiPSC-CM ± 100 nM ISO for 72 h (n≥25 cells).

Chapter 8:
Selection of pluripotent stem cell-derived cardiomyocytes using an adeno-associated virus vector containing a puromycin resistance cassette driven by a myocyte-specific promoter

Cheryl Dambrot, Antoine A.F. de Vries, Jim Swildens, Stefan R. Braam, Douwe E. Atsma, and Christine L. Mummery

Abstract:
Simple methods for selection of cardiomyocytes from mixed populations of differentiated human pluripotent stem cells (PSC-CMs) are still limited. Although several surface marker combinations with some specificity for cardiomyocytes have been identified (such as SIRPA and VCAM1), selection by flow cytometry has been proven to be difficult due to poor survival and reattachment of the sorted cells. Alternatively, transgenic approaches with selectable marker genes are used but the generation of transgenic cell lines is labor-intensive. Here, we developed a universal selection system for PSC-CMs with high cell survival and low toxicity based on adeno-associated virus (AAV) vectors. These vectors can efficiently transduce dividing as well as post-mitotic cells without the need to integrate their DNA into the target cell genome. We generated an AAV serotype 2 (AAV2) vector with reduced susceptibility to proteasomal degradation carrying a striated muscle-specific promoter (*i.e.* MHCK7)-driven puromycin resistance gene for selecting PSC-CMs. We showed that this vector transduced PSC-CMs better than fibroblasts. In initial experiments, puromycin selection resulted in ≥97% pure cardiomyocyte populations but in later experiments it proved difficult to reproducibly replate the cardiomyocytes following puromycin treatment. Suggestions are given for further improvement of the vector and selection procedure, which could ultimately result in an efficient AAV vector-based PSC-CM selection method.

Introduction:

Human pluripotent stem cells (hPSCs) hold great promise as model systems for studying human development and genetic disorders, as source of autologous cells to replace damaged, diseased or aged tissue and to evaluate the effectiveness and safety of newly developed drugs. Embryonic stem cells were the first pluripotent stem cells to be studied (Thomson et al, 1998) but ethical concerns limited research on hPSCs until 2006 with the discovery of induced pluripotent stem cells (iPSCs) [1-3]. hPSCs have the unique ability to self-renew in culture over long periods and to differentiate into derivatives of all three germ layers. This has made them a good model system for studying normal and abnormal differentiation in human development. However, a major problem facing researchers studying PSC derivatives is the heterogeneity of the differentiated cell populations they produce [4]. For some cells types such as endothelial cells, there are specific cell surface markers which allow sorting of these cells from the rest of the population but for many other cell types, cell surface marker proteins for which there are specific antibodies to select only one population by immunological means are limited. Such is the case for cardiomyocytes, which until recently before reports of partial enrichment with SIRPA- and VCAM1-specific antibodies [5,6] could not be selected out using surface markers.

Recent advances in hPSC technology (*e.g.* single cell culturing, zing finger nuclease-, TALEN- and CRISPR/Cas9-based genome editing strategies) have made it easier to genetically modify PSC lines [7]. This has allowed lineage-specific expression of selectable markers (*e.g.* fluorescent proteins and proteins conferring antibiotic resistance) in the differentiated progeny of hPSCs. One example is the Nkx2.5 $^{GFP/w}$ human embryonic stem cell line published by Elliot and co-workers [6] of which only the cardiomyogenic progeny display green fluorescence. Similarly, fluorescence and/or antibiotic resistance constructs have been combined with the myosin heavy chain [8,9] and myosin light chain 2v [10] promoters to identify cardiomyocytes. These cells can be separated from other hPSC derivatives by fluorescence-activated cell sorting (FACS) or antibiotic addition.

A disadvantage of the methods described above is that they depend on the permanent genetic modification of hPSCs with genes encoding exogenous proteins. Alternative (physical and biochemical) methods to enrich the progeny of hPSCs include the use of Percoll density gradients [11], mitochondrial dyes [12], cell surface markers [5,6] and special medium (low glucose, high lactate)[13] that supports selective cardiomyocyte survival. However, low purities, poor cell survival after cell sorting and disruption of cell integrity make these methods far from ideal.

Another possibility for selecting cardiomyocytes is by using viral vector technology. Viral vectors can be engineered to contain cell-type specific promoters driving the expression of selectable marker genes. Adeno-associated virus (AAV) vectors are especially well suited for selection purposes because (i) they can efficiently transduce a wide variety of mammalian cell types, (ii) their genomes remain essentially episomal minimizing the risk of insertional mutagenesis, (iii) the absence in their genomes of viral genes encoding potentially cytotoxic proteins and (iv) their excellent *in vivo* safety [14]. In this study, we have developed a capsid-modified AAV serotype 2 (AAV2) vector in which a hybrid striated (*i.e.* cardiac and skeletal) muscle-specific promoter (MHCK7) (Salva et al., 2007) is linked to the coding sequence of *Streptomyces alboniger* puromycin N-acetyltransferase (PAC) in order to purify hPSC-derived cardiomyocytes (hPSC-CMs) from the rest of the differentiated hPSC progeny; non-cardiomyocytes should die in the presence of puromycin. Although this enrichment strategy was shown to be effective in principle, *i.e.* treatment with puromycin resulted in cell cultures with >95% cardiomyocytes, poor survival upon replating of the cardiomyocytes negatively affected reproducibility so that further optimization would clearly be required to make the method of practical use.

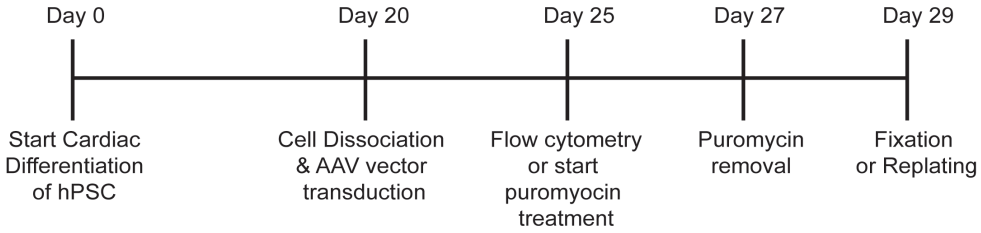

Figure 1: Schematic timeline of experimental setup.
hPSCs: human pluripotent stem cells; AAV: adeno-associated virus.

Materials and Methods:

Human PSCs

The human iPSC (hiPSC) line used in the experiments was derived previously from human dermal fibroblasts using the pRRL.PPT.SF.hOKSMidTomato-preFRT polycistronic lentiviral vector also known as LUMC004ictrl [15,16]. The generation of the Nkx2-5$^{GFP/w}$ human embryonic stem cell line has been described elsewhere [6]. This cell line has the coding sequence of the *Aequorea victoria* enhanced green fluorescent protein (GFP) inserted in exon 1 of the NKX2-5 gene, which codes for a transcription factor that is almost exclusively expressed in cardiac progenitor cells and cardiomyocytes.

PSC culture and differentiation

PSCs were cultured on growth factor reduced-Matrigel (BD Biosciences)-coated tissue culture dishes in mTeSR1 according to the manufacturer's protocol (Stem Cell Technologies). To initiate differentiation to cardiomyocytes, the PSCs were dissociated into small clusters of cells and seeded onto a Matrigel-coated cell culture dish in mTeSR1. Three days later, the medium was replaced with low-insulin (1 mg/L) BEL medium (LI-BEL)[17] supplemented with 20 ng/ml Activin A (R&D systems), 20 ng/ml BMP4 (R&D systems) and 1.5 µM CHIR99021 (Axon). Since the cultures were to be maintained for more than 16 days, the cells were overlaid with additional Matrigel (1:100) on differentiation day 3 to prevent their detachment from the bottom of the culture dishes. From differentiation day 6, the LI-BPEL medium was refreshed twice a week.

Construction of AAV vector shuttle plasmids

As scaffold for the construction of plasmids pDD.hEEF1A1.eGFP.WHVPRE.synpA and pDD.MHCK7.PurR.WHVPRE.synpA, we used the AAV vector shuttle plasmid pDD345 [18]. In pDD.hEEF1A1.eGFP.WHVPRE.synpA the GFP-coding sequence is preceded by the ubiquitous promoter of the human eukaryotic translation elongation factor 1α1 gene (hEEF1A1) and followed by the woodchuck hepatitis virus post-transcriptional regulatory element and a synthetic polyadenylation signal derived from the rabbit hemoglobin β gene [19]. pDD.MHCK7.PurR.WHVPRE.synpA has the same overall genetic makeup as pDD.hEEF1A1.eGFP.WHVPRE.synpA except for the replacement of the hEEF1A1 gene promoter and GFP open reading frame by the striated muscle-specific MHCK7 promoter (Salva et al., 2007) and the *Streptomyces alboniger* PAC-coding sequence, respectively. The nucleotide sequences of pDD.hEEF1A1.eGFP.WHVPRE.synpA and pDD.MHCK7.PurR.WHVPRE.synpA are available upon request.

AAV vector production

The capsid-modified AAV2 vectors AAV2/2mt. hEEF1A1.eGFP.WHVPRE.synpA (Fig 2A) and AAV2/2mt.MHCK7.PurR.WHVPRE.synpA (Fig 3A) were produced by co-transfecting 293T cells with pAAV2/2Y444500730F(+)[20], pAdΔF6 (Penn Vector Core; http://www.med.upenn.edu/gtp/vectorcore/Catalogue.shtml#AAVplasmids) and either pDD.hEEF1A1.eGFP.WHVPRE.synpA or pDD.MHCK7.PurR.WHVPRE.synpA using polyethyleneimine as transfection agent. Three days after transfection, the supernatant and cells were harvested and subjected to three freeze-thaw cycles to release the AAV vector particles from the cells. After pelleting of the cell debris by

low-speed centrifugation, the resulting supernatant was mixed with one-fourth of volume of 2.5 M NaCl, 40% polyethyleneglycol 8000 and incubated overnight at 4°C with gentle stirring and concentrated overnight in a 40% Polyethylene glycol solution. The AAV vector particles were then pelleted by centrifugation at 5000×*g* for 30 minutes. Subsequently, the concentrated AAV preparation was treated for 30 min with 100 U of OmniCleave Endonuclease (EPICENTRE Biotechnologies) per mL of sample at 37 °C before purifying the AAV particles using an iodixanol block gradient. Following purification, the AAV vector particles were washed with PBS + 5% sucrose and concentrated in three steps to approximately 200 µl per AAV vector in an Amicon Ultra-15 filter unit with a nominal molecular weight limit of 100 kDa (Millipore) according to the manufacture's proctol. The physical titer of the concentrated vector preparations (*i.e.* genome copies [GCs]/ml) was determined using a dot-blot assay. Two µL of AAV stock was treated with 10 U OmniCleave in 30 µl of 50 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$ for 30 minutes at 37°C to digest any unpacked DNA. The AAV vector genomes were then released by a 1 hour treatment at 37°C with 1 µg/µl proteinase K. Following denaturation of the vector DNA, the sample was diluted 1:1 with 20× saline-sodium citrate buffer (3M NaCL, 300mM triosodium citrate (pH 7.8)) and blotted onto a Hybond-N+ nylon membrane (GE Healthcare) using a Bio-Dot microfiltration apparatus (Bio-Rad Laboratories). The membrane was incubated for 2 h at 80°C to fix the vector DNA to the membrane. The DNA was quantified using AlkPhos direct labelling and detection system (GE healthcare). A 772-bp fragment specific for MHCK7 (primers: *5'*-acccttcagattaaaaataactga-*3'* and

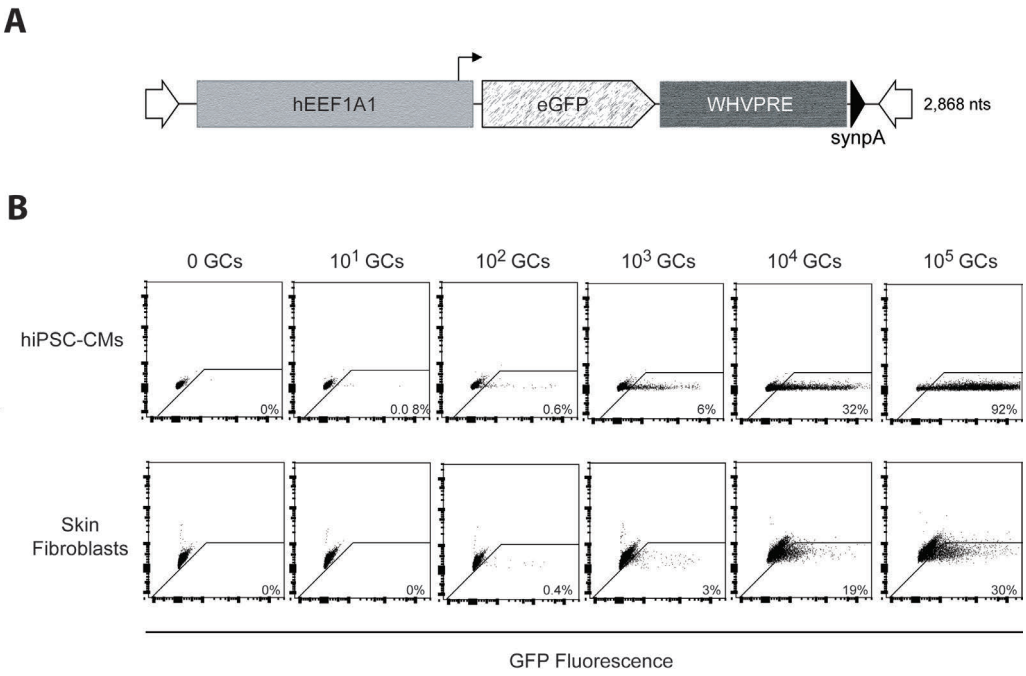

Figure 2: A) Vector map of AAV2/2mt.hEEF1A1.eGFP.WHVPRE.synpA. B) Flow cytometry plots showing the percentage of GFP-positive cells detected 5 days after transduction of hiPSC-CMs and skin fibroblasts with different doses of AAV2/2mt. hEEF1A1.eGFP.WHVPRE.synpA.

5'-tcaggagccagccagc-*3'*) obtained by PCR and isolated from agarose gel was used as probe. The membrane was prehybridized for 30 min at 55°C followed by an overnight hybridize at 55°C of 150ng of the thermostable alkaline phosphatase-labelled probe. Stringency washes and signal detection was performed according to the manufacturer's recommended protocol. ImageQuant software was used to quantify the DNA.

Transduction of hPSC-CMs with AAV vectors

Twenty days after the start of differentiation the monolayer was treated with TrypLE Select (Invitrogen-Life Technologies) for 30 minutes to produce single cells. The single cell suspension was exposed to different doses of AAV vector and immediately afterwards cells were plated on

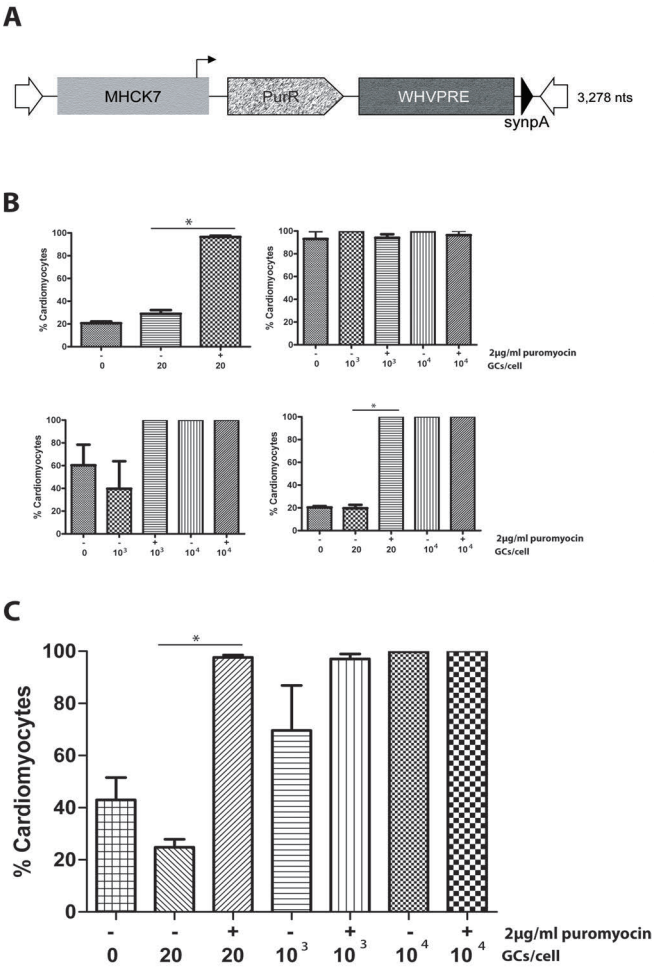

Figure 3: Purification of hESC-CMs AAV2/2mt.MHCK7.PurR.WHVPRE.synpA. A) Vector map of AAV2/2mt.MHCK7.PurR.WHVPRE.synpA. B) Percentage of cardiomyocytes after treatment with different doses (GCs/cell) of AAV2/2mt.MHCK7.PurR.WHVPRE.synpA and exposure to 2 µg/ml puromycin for 48 hours for four individual experiments. *p<0.05 puromycin added vs no puromycin added, n=3. C) The average percentage of cardiomyocytes from the experiment in B. *p<0.05 puromycin added vs no puromycin added, n≥2.

Matrigel-coated plates in LI-BEL medium. Forthy-eight hours later the medium was replaced by fresh LI-BEL without viral vector particles. Five days after transduction, the cells were treated with 2 µg/ml puromycin for 2 days. Following puromycin treatment, cells were left to recover for 2 days before either fixing them by a 30-minute treatment with 2% paraformaldehyde (PFA) for immunostaining or dissociation of the cells with TrypLE Select to replate them for additional experiments (Fig. 1).

Fixed cells were stained for α-actinin and filamentous actin to determine cardiomyocyte number and total cell number, respectively. Six areas (190,000 µm^2 per area) per condition were analyzed.

Immunostaining

Immunofluorescent staining was performed according to standard procedures. Briefly, PFA-fixed cells were permeabilized with Triton X-100, blocked with 4% normal goat serum (NGS) for 1 hour before overnight incubation at 4°C with α-actinin-specific mouse monoclonal antibody (Sigma-Aldrich) diluted 800-fold in PBS plus 4%NGS. After washing, the cells were incubated

for 1 hour at room temperature with Cy3-conjugated secondary antibody (1:200, Jackson ImmunoResearch). The cells were washed again and subsequently incubated with Alexa Fluor 488 Phalloidin (Invitrogen-Life Technologies) for 30 minutes at room temperature. Nuclei were stained with DAPI before mounting slides with Mowiol (Calbiochem-Merck4Biosciences).

Results:

AAV2mt transduction of cardiomyocytes versus fibroblasts

hiPSC-CMs were dissociated into single cells at differentiation day 20, transduced with different amounts of AAV2/2mt.hEEF1A1.eGFP.WHVPRE.synpA (*i.e.* 0, 10^1, 10^2, 10^3, 10^4, 10^5 GCs/cell, for vector map, see Fig 2A) and analyzed by flow cytometry 5 days later. In parallel, skin fibroblasts from the cell batch that serves as source of the hiPSC line were transduced with the same AAV vector at various doses. Only at vector doses ≥10^3 GCs/cell, the hiPSC-CMs and dermal fibroblasts showed evidence of successful transduction. There appeared to be a direct relationship between the vector dose and the percentage of cells transduced for both the cardiomyocytes and the fibroblasts with the highest vector dose (*i.e.* 10^5 GCs/cell) resulting in 92 and 30% GFP-positive cells, respectively. Thus, the cardiomyocytes were more easily transduced than the fibroblasts (Fig. 2B). It is important to note the some degree of toxicity was observed in both the cardiomyocyte and fibroblast cultures at vector doses ≥ 10,000 GCs/cell based on the decrease in the number of cells available for flow cytometry analysis at day 25 compared to the other samples (data not shown).

Cardiomyocyte selection using AAV2/2mt.MHCK7.PurR.WHVPRE.synpA

Based on the titration experiment described above, hESC-CMs were transduced with 20, 10^3 or 10^4 GCs/cell of AAV2/2mt.MHCK7.PurR.WHVPRE.synpA (for vector map, see Fig. 3A) and treated after 5 days with 2 µg/ml of puromycin for 48 h.. Following a recovery period of 2 days, the cells were stained for α-actinin and filamentous actin. As is shown in Fig. 3B and C, after treatment with puromycin, 97-100% of hESC-CM population consisted of cardiomyocytes irrespective of the vector dose used while the percentage of cardiomyocytes in the untransduced cell cultures ranged from 21-100% (Fig. 3B).

Parallel cultures of cardiomyocytes that were not fixed were dissociated and replated for functional assays. However, after successful replating of cardiomyocytes in the pilot experiment (20 GCs/cell, Fig.4), the AAV vector-transduced and puromycin-treated hESC-CMs did not reproducibly attach to the dishes or form confluent monolayers anymore, precluding their use in most functional assays.

Discussion

One of the major problems facing PSC-CMs is the purity of the differentiated cell population [4]. In this study, we generated a capsid-modified AAV2 vector for the selective expression of puromycin N-acetyltransferase in cardiomyocytes. When applied to differentiated hPSCs containing a subpopulation of PSC-CMs, selection of cardiomyocytes to greater than 97% purity was achieved. However, we were unable to confirm the cardiomyocyte identity of these cells in functional assays because of unexplained technical issues associated with low replating efficiency of the PSC progeny in culture.

Comparison of the transduction efficiency of PSC-CMs and fibroblasts (as surrogate for the non-myocytes present in the mixed populations of differentiated cells) with AAV2/2mt. hEEF1A1.eGFP. WHVPRE.synpA showed that transduction of the cardiomyocytes was somewhat more efficient. This could be due to differences in the various steps of the AAV vector transduction process (*e.g.* cell attachment, endocytosis, intracellular trafficking, capsid processing, release of the vector genome in the cell nucleus and second-strand DNA synthesis [21]), there may be a higher activity of the hEEF1A1 gene promoter in cardiomyocytes than in fibroblasts, or differences in division rate between the two cell types. Fibroblasts divide more rapidly than cardiomyocytes causing

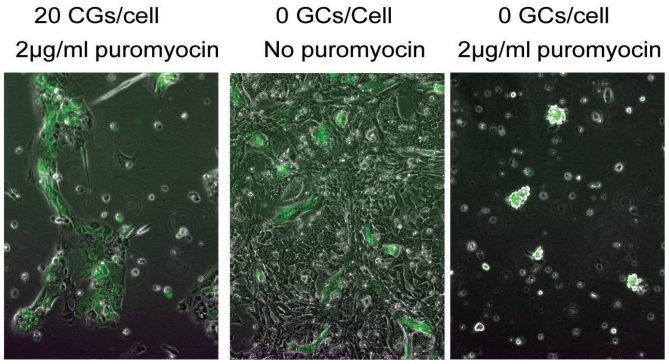

Figure 4: Replated Nkx 2-5$^{eGFP/w}$-derived cardiomyocytes after mock-transduction or transduction with 20 GCs/cell of AAV2/2mt.MHCK7.PurR.WHVPRE.synpA and exposure or no exposure to 2 µg/ml puromycin for 2 days.

more rapid dilution/loss of the episomal vector genomes. Of note, in the present study, genetic variation could not play a part in the observed differences in transduction efficiency between the dermal fibroblasts and the hiPSC-CMCs as both were derived from the same individual.

Although MHCK7 has been shown to be highly active in both cardiomyocytes and skeletal muscle cells [22], this does not pose a problem in selecting cardiomyocytes from the mixed derivatives of hPSCs since it is well-established that the currently used methods to differentiate hPSCs into cardiomyocytes yield only a very small percentage of skeletal myocytes [6,17,23].

While the use of AAV2/2mt.MHCK7.PurR.WHVPRE.synpA allowed selection of the cardiomyocytes from a mixed population of differentiated hPSC derivatives, the application of high vector doses resulted in extensive cell death. Moreover, the large variation in cardiomyogenic differentiation efficiency (ranging from 20% to nearly 100%; Fig. 3B, leftmost bars) between individual experiments makes it hard to draw definitive conclusions and may have contributed to some of the discrepancies between the titration results (Fig. 2) and the cardiomyocytes selection results (Fig. 3). Additionally, after treatment with puromycin the cardiomyocytes could not be reproducibly replated, making additional experiments on the cells difficult. To be of practical use, the AAV vector-based selection method described herein clearly required further technical optimization. It may, for instance, be possible that cardiomyocytes have a higher tolerance to puromycin than the other cells derived during hPSC differentiation [24-26], possibly due to their slower propagation rate compared to the non-myocytes in culture. It has been shown that different cell types response differently to stimuli which could also explain the higher tolerance [27]. Either of these points might explain why the selection procedure appeared to work at a vector dose of only 20 GCs/cell while the titration experiment indicated that at such a low vector dose the transduction rate was close to zero. This may also explain why the cardiomyocytes did not survive the replating procedure. Furthermore figure 3 seems to indicate that while at low vector doses puromycin selection leads to cardiomyocyte enrichment (accompanied by massive cell loss), high vector doses themselves provide a selective advantage to the hPSC-derived cardiomyocytes even in the absence of puromycin selection. This may indicate that viral transduction and/or puromycin-N-acetyltransferase activity per se provide a selective advantage to the hPSC-derived cardiomyocytes or a selective disadvantage to the hPSC-derived non-cardiomyocytes, similarly to effects which have been seen with other aminoglycoside resistance selection markers [28].

The seemingly unexpected effectiveness of the AAV2/2mt.MHCK7.PurR.WHVPRE.synpA at 20GCs/cell may have also been due additional proteins specifically expressed by cardiomyocytes and not the non-myocytes. Not unlike the results of Wang et al. in which showed that the transduction of cells with a “gutless” viral vector is not a “neutral” event but may have a large impact on the target cell’s biological properties [29]. It may thus be possible that the transduction with AAV2/2mt vector particles enhances the cardiomyogenic differentiation of hPSC-derived cells.

In spite of the fact that we were unable to consistently replate the AAV2/2mt.MHCK7.PurR.WHVPRE.synpA-transduced cardiomyocytes after puromycin selection, these pilot results indicated that AAV vectors do have potential for the transient genetic modification of hPSC-CMs, if the technical problems described above can be resolved. Moreover, since the AAV2/2mt.MHCK7.PurR.WHVPRE.synpA genome was too large to be packaged in the vector particle as transcriptionally competent dimers [30] the cells could not be treated with puromycin until 5 days after transduction. This additional 5-day incubation time may have contributed to the low replating survival/attachment rate of the hPSC-CMs. The generation of a self-complementary version of the AAV2/2mt.MHCK7.PurR.WHVPRE.synpA would decrease this 5-day incubation period and may allow for better replating attachment and survival. Since AAV vectors are non-integrating and will not replicate within the infected cells, any cell division will decrease the number of AAV particles present in a cell. If the long incubation time and the replating problem were resolved, this technology could prove very useful in selecting cardiomyocytes from differentiated hPSCs.

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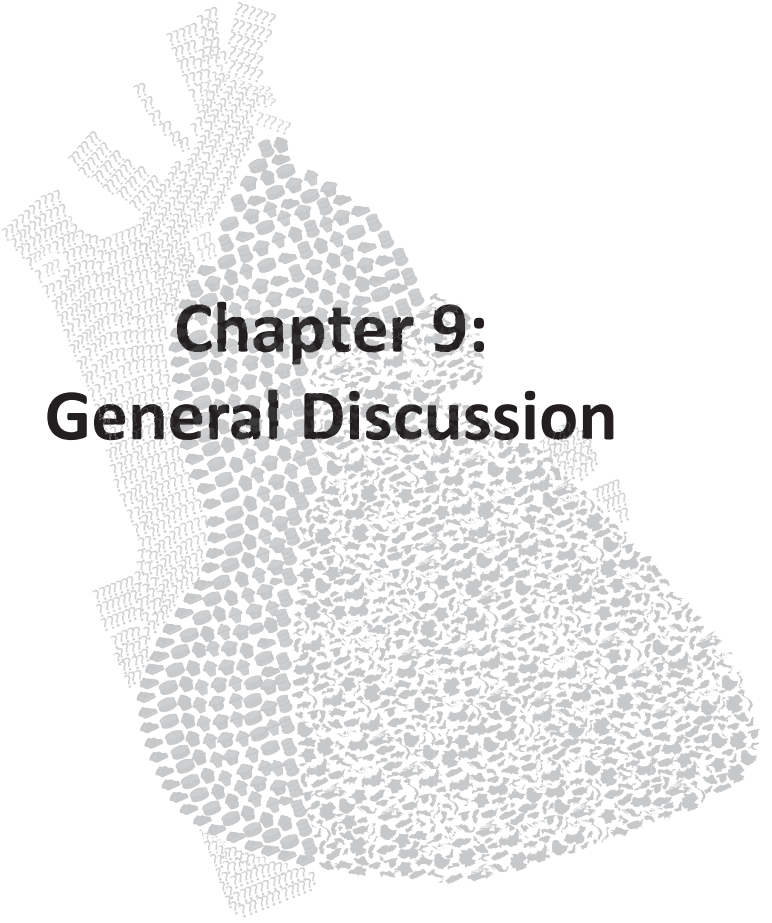

Chapter 9:
General Discussion

Induced Pluripotent Stem Cells

Over the last several years, research on human pluripotent stem cells (both embryonic and induced) has begun to fall into two distinct areas (i) basic stem cell biology, such as understanding the control of differentiation in development and the process of reprogramming, understanding pluripotency and finding ways to improve the reprogramming process itself to obtain a more "perfect" iPSC closer to hESC [1] and (ii) iPSC and genetically modified hESC as a tool to study/model disease [2]. While some aspects of the approaches and methodologies may be similar, their goals are very different so that their requirements of the iPSC and hESC they use may also be different. The work described in this thesis is in the second group: using human (h)iPSC as a model for human (cardiac) diseases so that the primary feature and requirement of the hiPSC used here is that the cell lines form cardiomyocytes efficiently and reproducibly, independent of the way in which the hiPSC were derived.

While it is important to have a fully reprogrammed hiPSC to decrease the variation between the different patients and the control lines, the need for the hiPSC to remain in a ground state of absolute pluripotency is not nearly as important as the ability to differentiate into the desired cell type. In this thesis, cardiomyocyte differentiation is the principle requirement of the hPSC lines. By contrast, those researchers studying the reprogramming process itself [3] or are trying to push hiPSC into a more naïve state rather more like mouse (m)iPSC or ESC [4] may require more stringent reprogramming and pluripotency tests as well as improved reprogramming methods which do not leave any footprint behind in the host DNA [5;6].

For many purposes, the tissue source of somatic cells for reprogramming can be of major importance. Dermal fibroblasts for example are easily derived and grow well in culture so that they are easy to reprogram to pluripotency but in the case of minors or young children, it may not be possible to obtain permission to collect skin biopsies. Some patients may be willing to take part in basic research by donating tissues samples but may be nervous of skin biopsy collection or injections for local anesthetic. In these cases, a non- or minimally invasive source of somatic cells would be preferable. Part of the research described in this thesis concerned selecting the cell sources that could be collected easily and reliably reprogrammed; this work is described in Chapter 3. We found that skin biopsies could still successfully provide cells able to be reprogrammed at about the same efficiency as freshly isolated fibroblasts, even after 14 days of storage in buffered saline solution (a basic salt solution). We also investigated the use of milk teeth and blood as a possible sources of somatic cells for reprogramming and while cells isolated from milk teeth were successfully reprogrammed at similar efficiencies to skin fibroblast, blood proved in our hands to be a more challenging cell source for reprogramming and we needed large starting volumes for success. This was an empirical finding and we did not find a reason for it. Easily accessible and minimally invasive tissue sources, such as hair follicle keratinocytes or small quantities of blood would be allowed for use in both children and adults. Although these tissue sources had been described in the literature as being successfully reprogrammed [7;8], we found it difficult to grow cells from these tissues and to cryopreserve and store them for later use in reprogramming. Banking the isolated somatic cells is a more feasible option when trying to collect a large number of samples either from patients or control samples. Fibroblasts could be dependably isolated from skin biopsies as small as 4mm, were easily stored and recovered well from frozen storage. So while we have successfully generated hiPSC from milk teeth as well as blood (Chapter 3) and others have generated hiPSC from other more easily accessible cell sources like hair keratinocytes [7], urine [9] and T-cells [10], fibroblasts were readily available and most reliable for the studies described in this thesis.

Aside from the tissue cell source, the method used for reprogramming and the technique used to induce differentiation could impact the use of iPSC technology for generating disease models. There has been much development since the first published articles claiming somatic cells could be reprogrammed to embryonic-like stem cells (ESC-like)[11-13]. There has been a variety

of protocols and methods of reprogramming factor delivery reported, including viral vectors (retro-, lenti-,adeno-, Sendai) mRNA, and episomes, with a range of reported efficiencies [14], which could be related their ability to recruit the Mbd3/NuRD repressor complex [15]. However most studies compare experiments with those described in the literature previously, and are not direct comparisons of methods in single reprogramming experiments [16]. This paucity of data that can be directly compared as well as a lack of a consistent method for calculating and presenting efficiencies make statements on efficiencies open to misinterpretation. For example, the total number of cells at the start of the reprogramming is sometimes compared to the number of morphologically identified ESC-like colonies and this is sometimes reported as "efficiency" but the number of cells identified as taking up the reprogramming factors vs number of morphologically identified ESC-like colonies has also been reported as a measure of efficiency. This makes comparing methods difficult and decisions on which methods are 'best' depends highly on the experience of the laboratory performing the experiments and whether it even matters, depends on the purpose for which the reprogrammed cells will be used. At the time at which the work in this thesis was initiated the best method for reprogramming which left only a minimal scar in the genome was using an excisable lentiviral vector. Later it became clear that non-integrating methods such as episomal [17] and Sendai virus-based protocols [6] which leave no trace in the genome at all could be used at a high efficiency and are now being routinely used to produce hiPSC in our lab as well.

With the development of transgene free methods which have been found to be robust and reproducible (e.g. Sendai virus, [6], the question arises if there is a need to develop techniques to identify insert number and locations such as in Chapter 4. In chapter 4 we found that by using a combination of splinkerette-PCR and ion torrent sequencing we could rapidly and accurately identify not only the number of lentiviral inserts in our hiPSC but also the location. While most groups are now implementing transgene free methods, there is a large number of lines which have already been generated and that for various reasons cannot be reprogrammed again (e.g. the loss or lack of source material). To the end of 2013, >90% of the published iPSC lines were generated using viral vectors.

There are several studies that have compared hiPSC lines to hESC to address the question on the extent to which they are similar or differ. There is at present no consensus but it seems the more lines that are compared, the smaller the differences between them become [16]. Some discrepancies have also been attributed to incomplete reprogramming, the insert number or the site in the genome that the reprogramming genes were inserted. Most of these studies have compared undifferentiated hESC and hiPSC lines but reports of characterization of their differentiated derivatives, such as cardiomyocytes, are now becoming more prevalent in literature, allowing comparisons of different lines at a different level. If differences are found, knowing and understanding the insert number and location could help explain some of these controversies.

Cardiac Disease Modeling

Most information on cardiac diseases is from clinical data and transgenic mouse models. While both can provide useful information they both have their disadvantages. Clinical assessment of disease is limited to symptoms (which may be explained by a number of diseases) and genetic testing; information regarding mechanism or cellular functionality is limited or unavailable. Genetically modified mouse models provide a wealth of knowledge by providing a continuous supply of cells as well as providing a full body model in which to study disease development and progression; however the differences between animals and humans, especially with respect to the heart is great [18;19].

There are a number of physiological, metabolic and protein marker expression differences between the human and rodent heart. The most obvious difference between the mouse (or any

small rodents) and human heart is heart rate. The mouse heart beats at 5-600 beats per minute (b/m) while the human heart beats at 60-70 b/m. The high heart rate of the mouse makes studying human arrhythmias caused by specific gene mutations difficult even when the mutated gene is successfully introduced. Furthermore, the differences in ion channels composition and their contribution to the action potential puts channelopathy studies solely carried out in the mouse in question. Another difference is the expression of myosin heavy chain (MHC) isoforms. βMHC is the isoform predominantly expressed in fetal mouse hearts whilst αMHC is predominant in the adult; the reverse is seen in humans [20].

In the past two decades our knowledge of cardiac development and disease has been greatly aided by pluripotent stem cell derived cardiomyocytes (PSC-CM). However there are still many issues which must be addressed before PSC-CM can contribute fully to cardiac research. In this thesis we have attempted to address a few of these points (Chapter 4-8). One of the first factors to consider is which differentiation method to use [21]. In Chapter 4 and 5, we described two different differentiation methods, both using defined medium and specific growth factors, however with striking differences. In chapter 4 we describe a new rapid method for cardiac differentiation of hPSC directly from mechanically passaged colonies on mTESR without the need for adaption to enzymatic passage. We showed this method to produce cardiomyocytes with at least 30% efficiency from 5 established hPSC lines as well as from a newly reprogrammed (less than passage 2) hiPSC line. The cardiomyocytes produced were positive for specific cardiac markers (e.g. α-actinin, troponin T and Nkx2.5) and basic electrophysiological properties (e.g. action potential and calcium transients) could be measured.

The method described in Chapter 5 used an embryoid body (EB) based system. It required optimizing the concentrations and timing of the growth factors, a necessity for many differentiation protocols due to the variation in endogenously produced factors as well as varying kinetics of the differentiation process itself [22]. This method yielded a large number of uniform, beating EBs. It is very suitable when a large number of cardiomyocytes is required from one PSC line, such as for drug discovery or toxicity testing and in combination with bioreactors,to be scaled to produce millions of cells. It is proving especially useful in this context when a cardiomyocyte-expressed promoter driving an antibiotic resistance gene is included that allows selective survival of cardiomyocytes, as described in chapter 8. This allows the large-scale derivation of pure populations of cardiomyocytes. The other differentiation method described in Chapter 4, does not require optimization or adaptation and can result in very rapid production of cardiomyocytes. This method is well suited to/for cases when a large number of different cell lines or clones (for screening after genetic modification) are being studied, for example in disease modeling when it is considered that minimally three clones from three different individuals are required for proper statistical comparison [23;24].

Another important point in phenotypic comparison and functional characterization of cardiomyocytes is the use of proper maintenance media (Chapter 7). Much effort has gone into optimizing the differentiation protocols for hPSC into cardiomyocytes [25-27]; however we are only beginning to analyze in detail the resulting cardiomyocytes that are being generated. In Chapter 7, we examined the effects of fetal calf serum (FCS), commonly used for cardiomyocyte maintenance to support survival and/or attain some degree of maturity, on PSC-CM. We found that treatment of PSC-CM derived using defined medium with FCS not only had hypertrophic effects similar to the addition of the known hypertrophic stimulus phenylephrine which was irreversible but also produced a positive chronotropic response. FCS is a common medium supplement with incompletely known and batch-to-batch variable composition [28]. It has been shown to cause hypertrophy in cultured cardiomyocytes [29;30] and yet is still often used to maintain PSC-CM for extended periods of time [31-33]. By comparing the effects of FCS on PSC-CM derived and maintained in defined medium, we highlighted the importance of the need to understand the effects of FCS components, which we often overlook, can have on results. In

chapter 7, we clearly showed that the disease phenotype that was described in chapter 6 was masked when FCS was present in the culture medium. This brings into question the results and conclusions of other groups (e.g. K. Laugwitz, SE Harding, and C. Denning) that have routinely used FCS or other sera in the cardiomyocyte culture. Lastly,we attempted to develop a generic method for purifying the population of cardiomyocytes among differentiated cells by selection using transiently expressed adeno-associated viruses (Chapter 8). Preliminary data using this construct was promising but the technology needs to be developed further and additional testing is needed before wider use of this virus for selecting cardiomyocytes. Researchers have often used other ways to get around having impure populations by using single cell assays or normalizing population data (for example in Western blots or RT-PCR) for the number of cardiomyocytes using cardiomyocyte-specific gene or protein expression; however this does not negate the need for a ubiquitous selection method which has low toxicity and can be easily applied to multiple PSC lines.

PSC-CM use in drug discovery is increasingly well documented [34;35] and their use in disease modelling has been further developed with the increased use of hiPSC from patients [2;24]. In this thesis we have also shown how hiPSC can be used in modeling hypertrophic cardiomyopathy (HCM). In chapter 6 we describe the reprogramming and basic disease characterization of hiPSC derived from patients with HCM. Fibroblasts from three patients (2 related and one unrelated male) carrying the Dutch founder mutation 2373inG in myosin binding protein C3 (MYBPC3) were collected and reprogrammed into hiPSC. Subsequently cardiomyocytes were derived. We found the cardiomyocytes from the HCM patients contained less MYBPC3 protein and were larger in cell surface area compared to cardiomyocytes from a healthy hiPSC line, comparable to the clinical situation. Also, the HCM-derived cardiomyocytes had an altered response to changes in external calcium as well as a larger caffeine-induced calcium release. Our results were comparable to those found in cardiac samples retrieved from patients [36;37]. However the mechanism of disease is still unknown and is currently being investigated along with more in depth disease characterization by other researchers in the lab.

The research described in this thesis is just the first step in modeling the HCM disease. We showed basic phenotypic characteristics but the exact disease mechanism and the reason for differences in clinical manifestation of the disease in patients are still unknown. However, these hiPSC-CM contain/provide the potential to answer these questions which have eluded research and clinician thus far.

Future Perspective

With the first successful cardiac differentiation of PSC [38], their use for regenerative medicine, drug testing and disease modeling became just a matter of time. The use of PSC-derived cardiomyocytes in cell therapy has unfortunately been fraught with difficulties and has had limited success so far [39]. However, their use in drug testing and disease modeling is a real possibility. Nevertheless there are still many questions that need to be answered to improve their use in drug testing and disease modeling.

In this thesis we have addressed some of these issues but there are still many which need to be investigated. One of the major obstacles to full use of PSC-CM is their immaturity [40;41]. While there have been many developments in improving differentiation procedures, most cardiomyocytes produced, like those generated in this thesis (Chapters 4-8), are still in an immature or fetal-like state based on electrophysiological - [42], physical and gene expression characteristics. While the immature cells have been able to mimic the phenotype of some late onset diseases (Chapter 6 of this thesis and [24] it is still unclear if the current state of maturity is enough to assay all phenotypes. If the field of PSC-CM research is to move forward, understanding of the immature state of PSC-CM and how to mature them in culture is key.

Additionally several reports to date have demonstrated a cardiac disease phenotype in culture [23;24;43] while disease phenotypes have not always been evident in patients[24;33]. This can be intrinsic to the technology or could indicate that patients will develop symptoms only in later life, or that there are lifestyle factors which trigger symptoms (extreme exercise for example). Future development in engineering technology that could modify stress/stretch, pacing or tissue environment combined with increased sensitivity of current assays, may resolve these issues. On the other hand it could be the lack of cellular maturity, mentioned earlier in this section, which has not allowed disease phenotypes to be fully revealed. Lastly it could be the absence of environmental factors, which cannot be replicated in culture that will remain a shortcoming of PSC technology. However which of these possibilities is correct will remain unanswered until the field progresses further and we have the tools that truly mimic all aspects of cardiomyocyte biology in culture.

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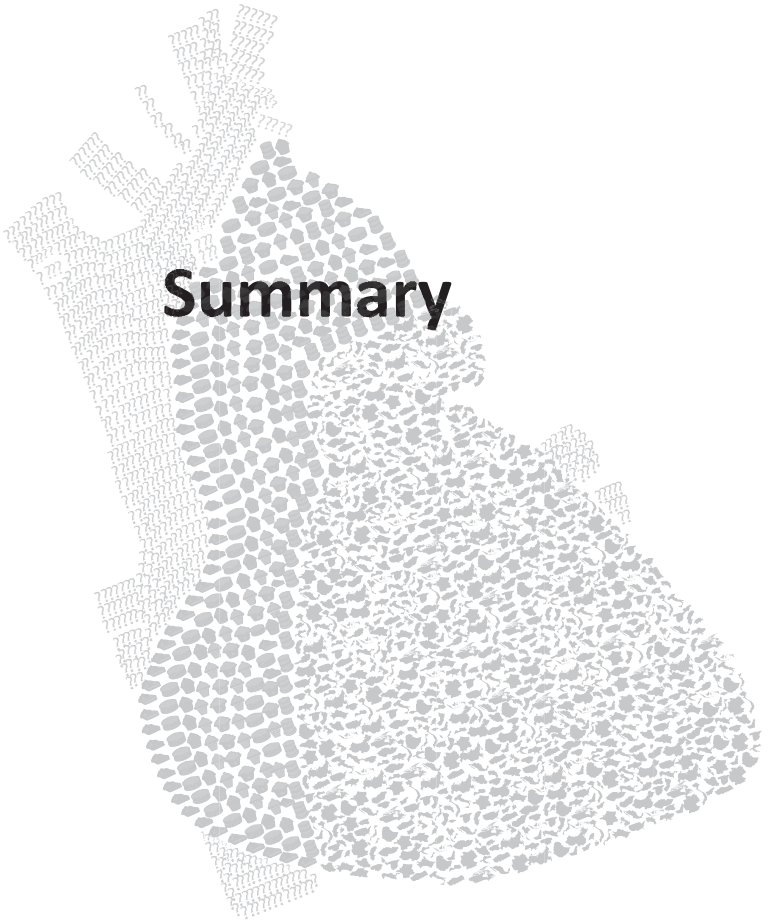


Summary

Since the first reports of human induced pluripotent stem cells (hiPSC), the field of pluripotent stem cell (PSC) research has grown in leap and bounds, particularly in the area of (cardiac) disease modeling. This is in part because it is fairly easy to produce cardiomyocytes from hPSC and also there are now more assays available which can determine phenotypic behavior. This thesis describes and discusses improvements to reprogramming technology as well as its use in cardiac development and disease modeling. **Chapter 1 and 2** give a general background on PSCs, covering embryonic stem cells (ESC) and the discovery and development of induced pluripotent stem cells, with a particular focus on their use in studying cardiac development and disease modeling.

In **chapter 3** we examined some practical aspects relevant to the collection of tissues from patients in the clinic. We compared (i) skin biopsies stored in standard physiological salt solution for up to 2 weeks (ii) blood outgrowth endothelial cells (BOECs) isolated from fresh peripheral blood and (iii) children's milk teeth lost during normal replacement, for their ability to form somatic cell cultures suitable for reprogramming to hiPSCs. All hiPSC lines were derived using the same reprogramming method (a conditional (FLPe) polycistronic lentivirus) and under similar conditions (same batch of virus, fetal calf serum and feeder cells). Skin fibroblasts could be reprogrammed robustly even after long-term biopsy storage. Generation of hiPSCs from juvenile dental pulp cells gave similar high efficiencies but that of BOECs was lower. In terms of invasiveness of biopsy sampling, biopsy storage and reprogramming efficiencies, skin fibroblasts appeared best for the generation of hiPSCs but where non-invasive procedures are required (e.g. for children) dental pulp cells from milk teeth represent a valuable alternative.

In **chapter 4** we described a useful combination of procedures that enables the simultaneous screening of multiple iPSC clones to determine their genomic integrity as well as their cardiac differentiation potential within two weeks of the putative reprogrammed colonies initially appearing in culture. By coupling a splinkerette-PCR method with Ion Torrent sequencing, we could ascertain the number of and map the proviral integration sites in lentiviral-reprogrammed hiPSCs, enabling identification of clones containing single copy integrations in genomic regions devoid of known genes or transcriptional control elements. In parallel, we developed an effective cardiac differentiation protocol that generated functional cardiomyocytes within 10 days without requiring line-specific optimization for any of 6 independent human pluripotent stem cell lines tested. Finally, to demonstrate the scalability of these procedures, we picked 20 nascent iPSC clones and performed these independent assays concurrently. Before the clones required passaging, we were able to identify clones with a single integrated copy of the reprogramming vector and robust cardiac differentiation potential for further analysis.

Chapter 5 describes another cardiac differentiation method. Starting from mechanically passaging PSC grown on mouse embryonic fibroblasts, this chapter describes adapting PSC to enzymatic passaged bulk cultures in order to generation spin EBs for directed defined cardiac differentiation. This method is useful for the generation of large numbers of uniformly produced cardiomyocytes from a single cell line, as each line would most likely require optimization of the growth factors used (also described in this chapter).

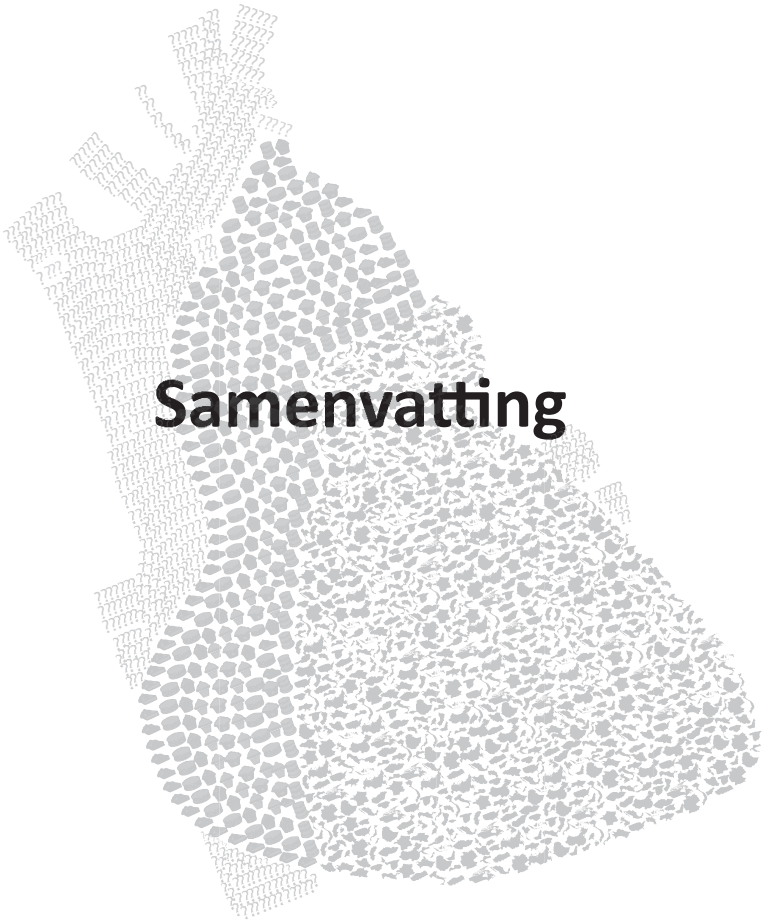
In **chapter 6** we derived hiPSC from three hypertrophic cardiomyopathy (HCM) patients by reprogramming fibroblasts from skin biopsies; two related patients and an unrelated individual who also had the same Dutch founder mutation (2373insG) in the myosin binding protein C type 3 (MYBPC type 3) gene. MYBPC type 3 protein levels were significantly reduced by Western blotting in HCM-iPSC cardiomyocytes compared to the control, consistent with reduced MYBPC3 levels previously found in heart biopsies from patients. Dissociated and replated single HCM-iPSC derived cardiomyocytes had a cell surface area more than 2-fold greater than control cardiomyocytes. In addition, calcium signaling was altered in the disease cardiomyocytes compared with controls, consistent with previously published data. Cardiomyocytes from HCM-

iPSC thus show essential features of the disease and provide a basis for further studies on the nature of HCM caused by mutations in MYBPC3.

In **chapter 7** we examined the direct effects of serum on cardiomyocytes from hESC, hiPSC and hiPSC from patients with HCM linked to a mutation in the MYBPC type 3 gene to understand how they might affect interpretation of phenotypes in culture. We first confirmed previously published hypertrophic effects of serum on cultured neonatal rat cardiomyocytes evidenced by increased cell surface area and beating frequency. We then found that serum induced similar and poorly reversible increases in the cell surface area of hESC- and hiPSC-derived cardiomyocytes and significantly increased their rate of contraction. Phenylephrine, which normally induces hypertrophy in cultured cardiomyocytes, had no additional effects under these serum conditions. We further showed that hiPSC-derived cardiomyocytes from 3 MYBPC3 patients had a greater surface area than control cardiomyocytes in the absence of serum, indicating hypertrophy as predicted by their genotype, but this difference was not evident in the presence of serum. Serum can significantly alter the phenotype of human PSC derived cardiomyocytes under otherwise defined conditions such that the effects of drugs and gene mutations affecting hypertrophy may be underestimated. It is pertinent therefore to examine cardiac phenotypes, whenever possible, in fully defined culture media without or in low concentrations of serum.

In **chapter 8** we developed a universal selection system for PSC-CMs with high cell survival and low toxicity based on adeno-associated virus (AAV) vectors. The generated AAV serotype 2 (AAV2) vector had a reduced susceptibility to proteasomal degradation and carried a striated muscle-specific promoter (i.e. MHCK7)-driven puromycin resistance gene for selecting PSC-CMs. We showed that this vector transduced PSC-CMs better than fibroblasts. In initial experiments, puromycin selection resulted in ≥97%, pure cardiomyocyte populations but in later experiments it proved difficult to reproducibly replate the cardiomyocytes following puromycin treatment. Suggestions are given for further improvement of the vector and selection procedure, which could ultimately result in an efficient AAV vector-based PSC-CM selection method.

Finally **chapter 9** is a general discussion linking findings in the different chapters in this thesis as well as discussing future perspectives and obstacles which still limit some uses of PSC technology principally in regards to obtaining information on diseases from disease bearing hPSC lines e.g. immaturity of PSC-CM and disease phenotype in clinically asymptomatic patients.



Samenvatting

Sinds de eerste studies naar humane geïnduceerde pluripotente stamcellen (hiPSC) is het onderzoeksgebied van pluripotente stamcellen (PSC) met grote stappen vooruitgegaan, met name op het gebied van (hart)ziektemodellering. Dit komt deels doordat het vrij eenvoudig is om hartspiercellen te produceren uit humane (h)PSC en deels doordat er een aantal testen beschikbaar zijn die fenotypisch gedrag kunnen analyseren. In dit proefschrift worden verbeteringen in herprogrammeringstechnologie beschreven en besproken, evenals het gebruik van deze technologieën in hartontwikkeling en ziektemodellering. In **hoofdstuk 1 en 2** wordt de algemene achtergrond van PSC besproken. Hierbij wordt aandacht besteed aan embryonale stamcellen (ESC) en de ontdekking en ontwikkeling van geïnduceerde pluripotente stamcellen (iPSC) voornamelijk gericht op hun gebruik in het bestuderen van hartontwikkeling en ziektemodellering.

In **hoofdstuk 3** wordt verder ingegaan op enkele praktische aspecten met betrekking tot het verzamelen van weefsels van patiënten in de kliniek. Er wordt een vergelijking gemaakt tussen (i) huidbiopten die maximaal twee weken zijn opgeslagen in reguliere fysiologische zoutoplossing (ii) endotheelcellen geïsoleerd uit vers perifeer bloed (BOEC) en (iii) melktanden verkregen uit normaal verlopende tandwisseling om het vermogen te bepalen somatische celculturen te vormen die geschikt zijn voor het herprogrammeren naar hiPSC. Alle hiPSC-lijnen zijn verkregen volgens dezelfde herprogrammeringsmethode (een conditioneel (FLPe) polycistronisch lentivirus) en onder soortgelijke omstandigheden (dezelfde batch virus, foetaal kalfsserum en feeder-cellen). Huidfibroblasten konden, zelfs na langdurige opslag van het biopt, krachtig worden geherprogrammeerd. Het ontwikkelen van hiPSC uit kindertandmerg cellen leverde een vergelijkbaar hoog rendement op, maar dat van BOEC lag lager. Ten aanzien van de invasiviteit van de biopsies, de opslag van biopten en de efficiëntie van herprogrammering, blijken huidfibroblasten het meest geschikt voor het ontwikkelen van hiPSC. Waar niet-invasieve procedures vereist zijn, bijvoorbeeld bij kinderen, vormen tandmergcellen van melktanden een waardevol alternatief.

In **hoofdstuk 4** wordt een nuttige combinatie van procedures beschreven die de gelijktijdige screening van meerdere iPSC-klonen mogelijk maakt om hun genomische integriteit en hun cardiaal differentiatiepotentieel binnen twee weken na het verschijnen van de vermoedelijk geherprogrammeerde kolonies in de kweek te bepalen. Door het combineren van een splinkerette-PCR-methode met Ion Torrent sequencing, konden we het aantal vaststellen van en de provirale integratiegebieden van lentivirale geherprogrammeerde hiPSC in kaart brengen. Hierdoor werd de identificatie van klonen mogelijk gemaakt die single copy-integraties bevatten in genomische regio's zonder bekende genen of transcriptionele controle-elementen. Tegelijkertijd ontwikkelden we een effectief hartcel differentiatieprotocol dat binnen 10 dagen functionele hartspiercellen genereert waarbij geen lijnspecifieke optimalisatie nodig was voor de 6 geteste, onafhankelijke humane pluripotente stamcellijnen. Om de mogelijkheden voor het opschalen van deze procedures aan te tonen, hebben we tot slot 20 beginnende iPSC-klonen geselecteerd en daarop deze onafhankelijke testen gelijktijdig uitgevoerd. Voordat het doorzetten van de klonen nodig was, waren we in staat om klonen met een single intergrated copy van de herprogrammeringsvector en een breed cardiaal differentiatiepotentieel te identificeren voor verdere analyse.

In **hoofdstuk 5** wordt een andere differentiatiemethode voor hartcellen beschreven. Als eerste wordt ingegaan op de mechanische passage van PSC gekweekt op embryonale muis-fibroblasten. Vervolgens wordt beschreven hoe PSC worden omgezet in enzymatische bulk-kweken om EBs te genereren voor gerichte gedefinieerde hartcel differentiatie. Deze methode is nuttig voor het genereren van grote aantallen uniform geproduceerde hartspiercellen uit dezelfde cellijn, aangezien voor elke lijn hoogstwaarschijnlijk optimalisatie van de gebruikte groeifactoren nodig is (tevens beschreven in dit hoofdstuk).

In **hoofdstuk 6** wordt gekeken naar het verkrijgen van hiPSC van drie patiënten met hypertrofische cardiomyopathie (HCM) door het herprogrammeren van fibroblasten uit de

huidbiopten. Twee van de patiënten behoorden tot dezelfde familie, de derde patiënt niet, maar deze had wel dezelfde Nederlandse foundermutatie (2373insG) in het myosinebindend eiwit C Type 3-gen (MYBPC3). Met behulp van western blotting konden we detecteren dat in vergelijking met de controlegroep de MYBPC3-eiwitniveaus significant verlaagd waren in HCM-iPSC hartspiercellen (HCM-iPSC-CM). Dit komt overeen met eerder gevonden verminderde MYBPC3-niveaus in hartbiopten van patiënten. De gedissocieerde en uitgeplate enkele hartspiercellen van HCM-iPSC hadden een celoppervlak dat tweemaal groter was dan dat van de controlehartspiercellen. Bovendien was in zieke hartspiercellen de calciumsignalering veranderd ten opzichte van de controlecellen, wat aansluit bij eerder gepubliceerde gegevens. Hartspiercellen van HCM-iPSC vertonen dus essentiële kenmerken van de ziekte en bieden een basis voor verder onderzoek naar de aard van HCM veroorzaakt door mutaties in MYBPC3.

In **hoofdstuk 7** worden de directe gevolgen van serum op hartspiercellen van menselijk ESC, gezonde hiPSC en hiPSC van patiënten met HCM door een mutatie in het gen MYBPC3 besproken om te begrijpen hoe ze van invloed kunnen zijn op de interpretatie van het fenotype gedurende de kweek. Als eerste hebben we de eerder gepubliceerde hypertrofische effecten van serum, die blijken uit een vergroot celoppervlak en hogere slagfrequentie, bevestigd bij gekweekte hartspiercellen van neonatale ratten. Vervolgens hebben we aangetoond dat serum soortgelijke en slecht omkeerbare vergrotingen van het celoppervlak van hartspiercellen uit hESC- en hiPSC veroorzaakte, evenals een significante verhoging van het tempo van samentrekking. Fenylefrine, dat normaliter hypertrofie veroorzaakt in gekweekte hartspiercellen, had geen additioneel effect onder deze serumomstandigheden. Daarnaast hebben we aangetoond dat hartspiercellen van iPSC van drie MYBPC3-patiënten een groter celoppervlak hadden dan de controlehartspiercellen bij afwezigheid van serum. Dit wijst op hypertrofie zoals voorspeld door hun genotype, maar dit verschil was niet aanwezig bij gebruik van serum. Serum kan het fenotype van hartspiercellen van hPSC zodanig significant veranderen onder anderszins omstandigheden dat de effecten van geneesmiddelen- en genmutaties die van invloed zijn op hypertrofie mogelijk onderschat worden. Het is daarom belangrijk om cardiale fenotypes, waar mogelijk, te onderzoeken in volledig gedefinieerde cultuurmedia zonder of met lage concentraties serum.

Hoofdstuk 8 richt zich op de ontwikkeling van een universeel selectiesysteem voor hartcellen van PSC (PSC-CM) met hoge celoverleving en lage toxiciteit gebaseerd op adeno-associated virusvectoren (AAV). De gegenereerde AAV serotype 2 vector (AAV2) was minder gevoelig voor proteasomale degradatie en had een dwarsgestreept spierweefsel promotorgedreven (d.w.z. MHCK7) puromycineresistentiegen voor het selecteren van PSC-CM. We hebben aangetoond dat deze vector beter PSC-CM transduceerde dan fibroblasten. Tijdens de eerste experimenten resulteerde puromycineselectie in een ≥ 97%-zuivere hartspiercelpopulatie, maar in vervolgexperimenten bleek het moeilijk om de hartspiercellen reproduceerbaar uit te platen na de puromycinebehandeling. In dit hoofdstuk worden eveneens suggesties gedaan voor verdere verbetering van de vector en selectieprocedure, wat uiteindelijk kan leiden tot een effectieve op AAV-vector gebaseerde PSC-CM selectiemethode.

Ten slotte worden in **hoofdstuk 9** in een algemene discussie de bevindingen van de verschillende hoofdstukken van dit proefschrift samengebracht en worden eveneens de toekomstperspectieven en belemmeringen besproken. Het gebruik van PSC-technologie maakt het bestuderen van ziekte van de ziektedragende hPSC-lijnen lastig, vooral door moeilijkheden met immaturiteit van PSC-CM en met ziektefenotype in klinisch asymptomatische patiënten.

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Curriculum Vitae

Cheryl S. Dambrot was born in Plainview, New York, United States on April 19, 1984. After receiving her high school diploma in 2002, she started her Bachelor's studies in Biomolecular Sciences with a minor in Science, Technology and Society at Clarkson University (Potsdam, NY, USA). During her Bachelor's studies she had the opportunity to take part in research projects in Analytical Chemistry and Cell and Molecular Biology as well as a semester abroad at the City University of Hong Kong and an internship in Organic Chemistry at the University of Ljubljana, Slovenia (resulting in a co-authorship). After receiving her Bachelor's diploma (with great distinction) in 2006, Cheryl moved to the Netherlands to continue her studies at Leiden University.

During her Master's in Biomedical Sciences, she was involved in numerous research projects at Leiden University Medical Center, resulting in several co-authorships. Cheryl researched BRCAx with the Human Genetic Department, maggot excretions/secretions and their effects on bacterial biofilms with the Department of Infectious Disease and myoblast-mesenchymal stem cell fusion (the subject of her Master's thesis) at the Molecular Cell Biology Department. She obtained her Master's Degree in 2009. In the same year Cheryl started her PhD training at Leiden University Medical Center as part of a collaborative project between the Department of Cardiology and the Department of Anatomy and Embryology under the supervision of Prof. Dr. Christine Mummery and Prof. Dr. Douwe Atsma. The results of this research are presented in this thesis.

Since August 2014, Cheryl is a postdoctoral associate in Natalia Ivanova's group at Yale University in New Haven, CT, USA.