State of the heart : the promise of pluripotent stem cell-derived cardiomyocytes in disease modelling, differentiation and development

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CHAPTER 1

Introduction

Embryonic stem cells

Pluripotent stem cells (PSCs) are defined by two distinct properties – their ability to self-replicate indefinitely; and their ability to develop into cells from all three germ layers, such as cardiomyocytes and hematopoietic cells (mesoderm), pancreatic and hepatic cells (endoderm), and neurons (ectoderm). This makes these cells excellent models for studying differentiation in vitro.

For many years the only type of PSCs with these properties was embryonic stem cells (ESCs). These are derived from the inner cell mass (ICM) from blastocyst stage embryos. Whilst ESCs were firstly derived from mice [1,2], subsequently rabbit, canine, rat and non-human primate ESC were also described [3,4]. To demonstrate their pluripotency, mouse ESCs (mESCs) were injected into a mouse blastocyst and the chimeric embryos transplanted into the uterus of mice. The resulting pups were then examined to see whether the injected mESCs have contributed to normal development. Stringent evidence of pluripotency was chimerism in the germ line where the ESCs also formed gametes so that the chimeras' offspring gave rise to pups that were entirely ESC derived [5,6]. It took another 17 years before human ESCs (hESCs) were derived from human blastocysts. While their pluripotency could not be confirmed by (germ line) chimerism, it was demonstrated by the formation of teratomas, a tumour with tissue from all three germ layers, following injection in immune deficient mice [7].

Maintaining ESCs in a pluripotent state requires precise control of the pluripotent regulatory circuit, which is governed by the transcription factors OCT4, SOX2, and NANOG through specific signal-transduction pathways [8,9]. Mouse PSCs exist in two states. The naïve ICM-like state requires leukemia inhibitory factor (LIF) and bone morphogenetic protein (BMP) to maintain self-renewal in vitro, while the "primed" (epiblast-like) state is dependent on activin/nodal and fibroblast growth factor (FGF) signalling. The main difference between mouse PSCs in these two pluripotency states is that "primed" mESCs are unable to give rise to germ line chimeras [10,11]. In vitro hESCs are thought to be more epiblast-like (epiSC) and closer to the primed rather than the naïve state, based on gene expression profiles, epigenetic regulation and required signalling pathways for maintaining pluripotency [12,13]. Therefore hESCs need alternative growth factors to mESCs to remain undifferentiated in culture. Like mEpiSCs, hESCs depend on FGF and activin/nodal signalling for self-renewal, and cannot be maintained in media containing LIF and BMP [14].

Induced pluripotent stem cells

Until almost a decade ago, ESCs were the only type of pluripotent stem cells that had been derived. However a real game changer in the stem cell field was the discovery that it was possible to reprogram normal somatic cells to a pluripotent state by transiently overexpressing genes known to play crucial roles in maintaining pluripotency in ESCs. These cells, known as induced pluripotent stem cells (iPSCs) were first generated from mouse [15,16] and then from human [17] fibroblasts isolated from skin biopsies. These cells were derived using a retroviral system overexpressing the transcription factors, Oct4, Sox2, c-Myc and Klf4. These genes provide instructions that are sufficient to reprogram adult cells into an embryonic-like pluripotent cell state, that can self-renew in culture and differentiate into specialised cell types. Like their mESC counterparts, miPSCs were also demonstrated to be truly pluripotent through their ability to generate (germ line) chimeras and undergo "tetraploid complementation", a process by which diploid 2-cell mouse embryos are fused to form one tetraploid embryo and the addition of mESCs results in embryos that are 100% chimeric [18].

While initially requiring retroviral or lentiviral expression constructs that integrated into the genome to deliver the pluripotency genes (also referred to as the "Yamanaka factors"), non-integrating delivery methods such as Sendai virus [19], episomal vectors [20] and mRNA are now commonly used. Certain small molecules have been shown to increase efficiency of reprogramming. These include the inhibition of histone deacetylation [21] and the transforming growth factor-β (TGF-β) and MAPK/Erk kinase (MEK) signalling pathways [22,23]. Many somatic cell types have now been reprogrammed including cells isolated from urine, blood, hair and even teeth – all samples that can be collected relatively non-invasively [24,25].

The discovery of induced pluripotency by Yamanaka et al., was rewarded with the Nobel Prize in 2012, in part because it opened many immediate opportunities for stem cell research. The ability to derive PSCs from any individual that also capture their genome provided an exciting way to generate disease models for any disease with a genetic origin. Not only would the gene be known, but also the clinical features of the disease in the patient, such as severity, age of onset and which pharmaceutical drugs had been successful or unsuccessful in treating the condition. Indeed, combined with genetic approaches to correct known mutations, an increasing number of disease modelling studies are now performed using isogenically matched controls which differ from the disease cells only in the gene of interest [26]. This reduces the chance of misinterpretation resulting from normal biological variance in cellular phenotypes.

Furthermore, hiPSC models provide opportunities to develop drug-screening assays for mutation- or disease-specific treatments. In studies of cardiac disease, the use of hiPSC-derived cardiomyocytes is expanding, with these cells now frequently employed for modelling, safety pharmacology and drug discovery [27,28]. This is despite the physiological immaturity of

the hiPSC-cardiomyocytes [29]. Human iPSCs are also being used in cell therapies. The first trial of therapeutic transplantation of hiPSC-derived retinal pigment epithelial (RPE) cells in a patient with age-related macular degeneration has taken place. This was preceded by in vitro studies and a pre-clinical study in animals for investigating the safety and suitability of the iPSCs in clinical research [30,31]. Macular degeneration can lead to blindness and it is hoped that transplantation of these RPE cells can at least delay the rate at which this occurs. However transplantation of other cell types derived from hPSC, such as cardiomyocytes, remains challenging.

Differentiation to cardiomyocytes and their applications

Removing the signalling cues that maintain both hESCs and hiPSCs in a pluripotent state combined with factors that direct lineage specification, leads to differentiation into various more committed cell types. The most successful differentiation procedures are those that mimic the key developmental events that occur in the early embryo [32]. The heart is one of the first organs to form from mesodermal cells after gastrulation. Key signalling pathways that regulate mesoderm and cardiomyocyte formation are TGF-β, wingless/INT proteins (WNTs) and the FGFs that are essential in the activation of *NKX2-5* and *GATA4* (Figure 1). The earliest mesoderm progenitors express *BRACHYURY* and homeodomain protein *MIXL1*, with mesoderm posterior 1 (*MESP1*) expressed in the cardiac mesoderm stage [33]. The expression of *MESP1* is one of the first detectable markers for cardiogenesis and the earliest *MESP1*-expressing cells form the first heart field (FHF), while later $MESP1^{+}$ cells contribute to the second heart field (SHF). Subsequent inhibition of Wnt/β-catenin signalling leads these cells to further specify into cardiomyocytes. The cells of the FHF primarily contribute to the left ventricle and express transcription factors such as *TBX5* and *NKX2-5*. The SHF contributes to the right ventricle, both atria, as well as the outflow and inflow myocardium and expresses amongst others, the transcription factor *ISL1* [34]. Other transcription factors that are important in chamber and tract formation are *TBX20*, *NKX2-5* and *GATA4*. Transcription factors of the myocyte enhancer factor 2 (*MEF2*) family also regulate cardiomyocyte differentiation by regulating cardiac structural genes.

These complex signalling networks can be mimicked to differentiate PSCs towards cardiomyocytes in vitro. Differentiation protocols continue to improve and refine over time. For example, it is now becoming possible to replace many of the growth factors that initiate signalling pathways with small chemically-synthesised molecules [35-37].

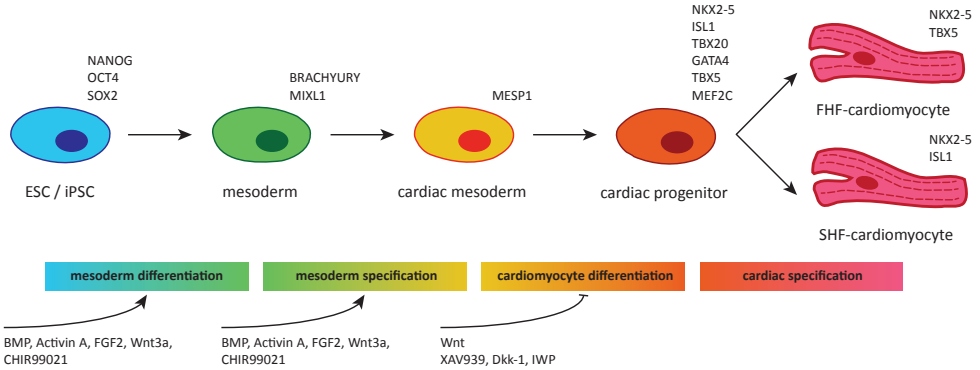

Figure 1. Sequential steps that occur when differentiating pluripotent stem cells to cardiomyocytes
Differentiation from embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSC) resembles cardiac development in the embryo. They are induced from the pluripotent state to mesoderm by BMP, Activin A, FGF2 and canonical Wnt signalling pathways. Specification of mesoderm to cardiac progenitors is regulated by Wnt inhibition and cardiac progenitors further develop into first heart field (FHF) and second heart field (SHF) cardiomyocytes. Key transcription factors for each stage are indicated.

Our ability to efficiently generate cardiomyocytes from hPSCs has also improved dramatically over the last five years thanks to the better understanding of key signals involved [38]. This has been aided by the generation of targeted fluorescent reporter hESC lines that mark the expression of transcription factors crucial for cardiac development, such as *MESP1*, *ISL1* and *NKX2-5* [39-41]. For example, the generation of two hESC lines in which the sequence encoding eGFP was targeted to the *NKX2-5* locus has greatly assisted the development of new differentiation procedures, either as 3-dimensional cell aggregates or 2-dimensional monolayer cultures, due to the ease in monitoring the efficiency of the protocols in real time [39]. As these cells express GFP when differentiated to cardiomyocytes, this also makes it possible to isolate enriched populations of cardiomyocytes in large quantities. Much of the work in this thesis has been based on the use of these genetically marked cells. This has enabled the study of early cardiac development and offered the possibility to unveil cues that are important in lineage specification.

Aside from using hESCs as a model to understand cardiac differentiation in human development, it has long been hoped that these cells could be used in cell therapies aimed to replace cardiomyocytes lost after myocardial infarction [42] (Figure 2). Aside from ethical and safety concerns, there has also turned out to be important technical challenges. For example, whilst it has been possible to generate cardiomyocytes from hESCs for almost 15 years, it has proven complex to transfer these cells to the adult heart and have them survive and integrate. Transplanted cardiomyocytes not only must survive the hostile environment of possibly damaged heart tissue, but must also align properly and contract synchronously with the heart muscle by connecting with the resident heart cells, incorporate a vasculature

network, and not be rejected by the immune system or form teratomas. Each of these issues are major hurdles to overcome and it is likely that studies in non-human primates will be necessary as an intermediate step. These are ongoing in several groups, most notably that of Murry et al. in the US [43].

However, the ability to generate cardiomyocytes from somatic cells does provide the opportunity to use them in disease modelling (Figure 2). The first iPSCs related to cardiac disease were generated from a patient with LEOPARD syndrome, a genetic disorder causing hypertrophic cardiomyopathy [44]. Since then, many patient specific hiPSC models of cardiovascular diseases have been generated, and these have provided insights into the mechanisms underlying the disease [45]. Part of the work in this thesis focuses on one specific cardiac disease in which we derived hiPSC from a patient affected by a "channelopathy" caused by a mutation in an ion channel gene important for heart cells.

Finally, hiPSC-derived cardiomyocytes provide opportunities to investigate drugs and develop new therapeutic interventions [46] (Figure 2). They are of growing interest to the pharmaceutical industry and can also contribute to drug screening, safety tests and eventually more personalized medicine [47].

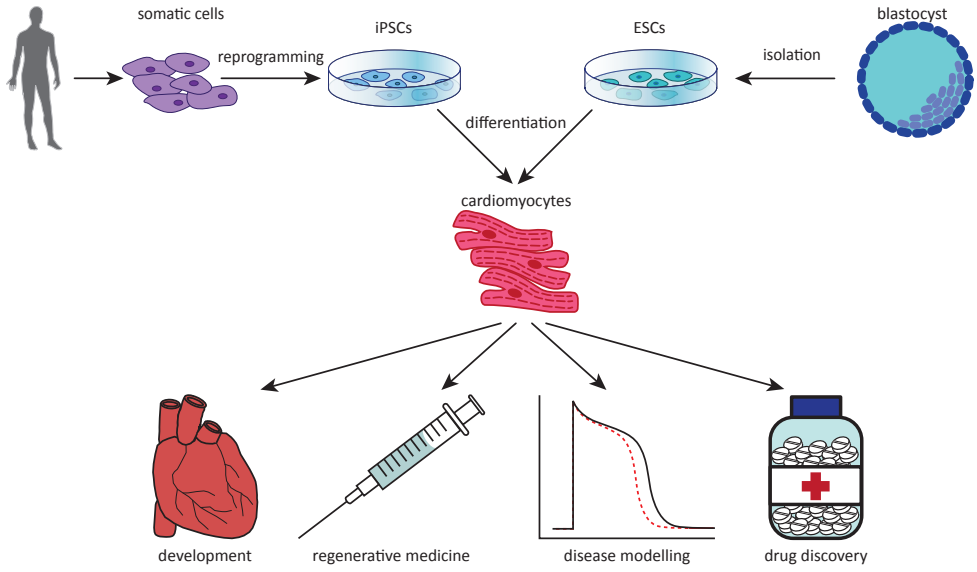

Figure 2. Derivation and differentiation of pluripotent stem cells and their applications
Adult somatic cells from patients or healthy individuals can be reprogrammed into induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs) can be derived from blastocyst stage embryos. Both types of pluripotent stem cell can be differentiated into cardiomyocytes that can be applied in 1) studying development in a dish; 2) regenerative medicine in transplantation; 3) modelling disease pathogenesis; and 4) therapeutic drug development or toxicity screening.

Aim and scope of the thesis

While there have been many comparisons of hESC and hiPSCs reported in the literature, few studies have compared their differentiated derivatives obtained using the same protocol. The overall aim of this thesis was to investigate development and cardiac disease models using both mouse and human ESC- and iPSC-derived cardiomyocytes. The chapters in this thesis describe these studies, with one specifically directly comparing hiPSC and hESC-derived cardiomyocytes. Furthermore gene expression in the developing human heart was examined with the view to establish the developmental stage of hPSC-derived cardiomyocytes in culture.

Chapter 2 reviews the opportunities that have arisen because of the ability to generate PSC-derived cardiomyocytes from both patients and healthy individuals in culture, in particular with regards to the study of cardiac diseases, cardiac drug discovery and development.

In **chapter 3** the opportunity to use PSCs in cardiac disease modelling for an ion channel disease is explored. Our results demonstrated that cardiomyocytes from both a mouse ESC and iPSC line, as well as a human iPSC line carrying a mutation in the cardiac sodium channel gene *SCN5A*, captured essential features of the complex phenotype in patients. PSC-derived cardiomyocytes with just a single mutation showed both prolonged QT interval and symptoms of Brugada syndrome and are thus suitable in vitro models for studying complex sodium channel mutations, despite their electrophysiological maturity.

Chapter 4 contains a detailed description of a directed cardiac monolayer differentiation protocol for human pluripotent stem cells that generates cultures comprising of ~50 % cardiomyocytes.

Chapter 5 focuses on an aspect of pluripotent stem cell biology not often examined, namely by investigating how the differentiation of hiPSC and hESC compare directly and assessing the suitability of these cell lines to model early human cardiac development in vitro. NKX2.5-eGFP fluorescent reporter hESC and hiPSC lines offered the opportunity to isolate different cardiac populations and assess the true degree of similarity between lineages. Both cell lines were maintained and differentiated under defined conditions using the protocol described in chapter 4, so that any differences between hiPSC and hESC-cardiomyocytes were not due to the differentiation protocol used for their generation.

Finally, in **chapter 6**, the gene expression profiles of human fetal heart from the first and second trimester of development are examined and both hESC- and hiPSC-derived cardiomyocytes were compared with this data to assign specific stages of fetal development.

In **chapter 7** the results of the studies are summarized and discussed in the light of implications for future research.

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