Generation and Application of Human Pluripotent Stem Cell-Derived Cardiomyocytes

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Abstract

Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) are a powerful technology with established applications in regenerative medicine, disease modeling, drug testing and discovery, and developmental biology. The in vitro generation of human cardiomyocytes has progressed rapidly over the last 15 years, and cardiac differentiation of human pluripotent stem cells (hPSC) is now a simple and routine process for many laboratories, generating contracting fetal-like cardiomyocytes that recapitulate a variety of known cardiovascular disease phenotypes. Here we discuss the history and existing knowledge in the field on the generation of cardiomyocytes from human pluripotent stem cells. We cover the potential clinical and investigational applications of hPSC-CMs, as well as the role of the pluripotent state in differentiation, and current methodologies for differentiation, purification, directed cardiomyocyte subtype specification and maturation, and large-scale production. We conclude with a discussion on the future of the field.

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4.1 Introduction

The last two decades have witnessed a sea change in cardiology, in which the heart has gone from being perceived as incapable of regenerating new cardiomyocytes to an organ which contains potential for cardiomyocyte replacement by proliferation of existing cardiomyocytes, differentiation of resident or circulating stem and progenitor cells, the transdifferentiation of endogenous cardiac fibroblasts, or the engraftment of exogenous human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs). Heart disease remains the leading cause of death in both men and women in the USA (Mozaffarian et al. 2016). It has been estimated that 1–1.7 billion cardiomyocytes have been lost in a failing heart (Murry et al. 2006; Beltrami et al. 1994). Yet, in the face of such a large contractile cell deficit, the level of in situ cardiomyocyte renewal in humans remains low. While medical therapies for coronary heart disease and heart failure have improved mortality related to heart disease since 1980 (Mozaffarian et al. 2016), these therapies usually do not represent cures. Furthermore, a number of genetic cardiac diseases have limited treatment options available. Progress in treating these diseases is hindered by the limitations inherent to studying animal models that are often inadequate mimics of human disease and may involve different pathophysiology despite a similar phenotype.

Several cell types have been proposed as candidates for use in cardiac regenerative therapies, and most of these have demonstrated limited success in generating new cardiomyocytes in vivo, particularly in large animal studies (Bolli et al. 2013; Johnston et al. 2009; Leiker et al. 2008; Hatzistergos et al. 2010), with observed improvements in cardiac parameters being largely attributed to paracrine effects (Gallina et al. 2015; Broughton and Sussman 2016; Khanabdali et al. 2016; Tang et al. 2010). Among these cells, hPSCs have proven to have the greatest potential for cardiomyocyte differentiation and myocardial repair. Initially, this option was limited to embryonic stem cells (ESC); however, their use for therapeutic purposes entails an allogenic therapy with concerns over immune rejection and the need for immunosuppression. In 2006, Takahashi and Yamanaka opened the field of induced pluripotent stem cells (iPSC) with their creation of cells that behaved like mouse embryonic stem cells (mESC) from mouse fibroblasts that had been retrovirally transduced with four genes: *Pou5f1 (Oct4)*, *Sox2*, *Klf4*, and *Myc* (Takahashi and Yamanaka 2006). These cells showed gene expression profiles similar to mESCs, potential to form teratomas and differentiate into cells from all three germ layers, and had clonogenic potential. Subsequently, germline competence was established by selecting for *Nanog*- or native *Oct4*-expressing miPSCs (Okita et al. 2007; Wernig et al. 2007; Maherali et al. 2007). In 2007 and 2008, reports of iPSCs derived from human cells (hiPSC) were published using the same *OCT4*, *SOX2*, *KLF4*, and *MYC* combination of factors (Takahashi et al. 2007; Park et al. 2008). Despite the identification of numerous other reprogramming gene combinations, the use of the four "OSKM" Yamanaka factors with non-integrating Sendai viruses for transduction (Fusaki et al. 2009) remains the most common approach for generation of hiPSCs today.

The advances in hiPSCs make autologous regenerative cardiac therapies more plausible and also introduce a platform upon which to model genetic cardiac

diseases by way of creating disease-specific and even patient-specific models for investigation. Despite this progress, there remains a great deal of work to be done to improve our understanding of the genetics and epigenetics of human heart development, the generation of de novo cardiomyocytes both in vitro and in vivo, and factors involved in cardiac repair before safe and effective approaches for human cardiac regenerative medicine can be developed for large-scale use.

4.2 Applications

The creation of hiPSC-CMs has opened the floodgates to possibilities for therapeutic applications of these cells as well as never-before-available models for investigation of development, disease processes, and drug and toxicity screening. The ability to easily and reproducibly generate large numbers of human cardiomyocytes from hiPSCs has placed a powerful tool in the hands of investigators to accelerate progress in a number of areas, several of which are outlined here.

4.2.1 Regenerative Medicine

While regenerating over a billion cardiomyocytes in a failing heart is a lofty goal, current evidence would support its pursuit. Multiple stem and progenitor cell populations have been proposed as candidates to meet this challenge, including bone marrow-derived stem cells (e.g., mesenchymal stem cells, c-KIT$^+$ cells, CD34$^+$ cells, Hoechst exclusion or "side population cells"), resident cardiac progenitor cells (e.g., c-KIT$^+$, CD34$^+$, ISL1$^+$, Hoechst exclusion, and cardiosphere-derived cells), cord blood cells, and fat-derived stem cells. Indeed, several of these cell types have shown modest improvements in cardiac function in post-injury models, as measured by parameters such as left ventricular ejection fraction (LVEF), regional systolic function, or scar reduction following myocardial infarction (MI). However, scant evidence supports clinically meaningful levels of cardiomyocyte differentiation of any of these cell types in vivo (Bolli et al. 2013; Johnston et al. 2009; Leiker et al. 2008; Hatzistergos et al. 2010; Gallina et al. 2015; Broughton and Sussman 2016; Khanabdali et al. 2016; Tang et al. 2010).

In contrast, ESCs and iPSCs have demonstrated far greater capacity to generate de novo cardiomyocytes in vitro (Kattman et al. 2011; Lian et al. 2012, 2013; Hudson et al. 2012; Burridge et al. 2014), as well as in vivo (Chong et al. 2014; Carpenter et al. 2012). Despite the successes with in vitro cardiomyocyte differentiation, in vivo data demonstrating significant regeneration of cardiac tissue is less plentiful. Nevertheless, there is a reason for optimism. Since a defining characteristic of PSCs is the ability to form teratomas, most in vivo studies have focused on delivery of PSC-CMs, rather than PSCs themselves. Delivery of 2×10^6 hiPSC-CMs to a rat heart post-MI demonstrated the presence of cardiomyocyte markers in engrafted cells as late as 10 weeks after injection, although there was no significant improvement in cardiac function by MRI (Carpenter et al. 2012). However,

Ong and colleagues delivered the same dose of hiPSC-CMs to an immunodeficient mouse heart post-MI and observed poor engraftment at 35 days using bioluminescent imaging despite improvements in cardiac function by MRI and invasive hemodynamic monitoring (Ong et al. 2015). Further analyses suggested that release of proangiogenic and antiapoptotic factors by the transplanted cells might underlie the observed improvement in cardiac function after injury (Ong et al. 2015). In the first study in nonhuman primates, delivery of 1 billion hESC-CMs to post-MI macaque hearts resulted in extensive remuscularization with human cardiomyocytes with evidence of electrical integration with host myocardium (Chong et al. 2014). This study demonstrated the feasibility of generation of large numbers of hPSC-CMs in addition to providing strong evidence of myocardial regeneration.

A growing body of evidence supports the notion that combination cell therapy may improve transplanted cell retention and cardiac repair. Ye et al. (2014) delivered a combination of hiPSC-derived cardiomyocytes, endothelial cells, and smooth muscle cells to a porcine heart following MI. In addition, they placed a fibrin patch containing IGF1-releasing gelatin microspheres over the site of injury prior to cell injection. The use of the fibrin patch resulted in improved cell retention (~9% of injected cells vs. ~4% without the patch, as assessed by quantitative PCR for human Y chromosome in tissue digests). Histological analyses confirmed hiPSC-CMs within muscle fibers in the heart. This treatment resulted in significant improvements in LVEF, wall stress, apoptosis, myocardial energetics, and arteriole density.

4.2.2 Biological Pacemaker Development

In addition to treating disorders of cardiac mechanical pump function, there has also been interest in using cell-based therapies to treat electrical heart problems. Patients who suffer from sinoatrial node dysfunction and bradycardia currently rely on electronic pacemakers to take over pacing of the heart. While these devices are highly effective, they do have shortcomings which include limited battery life necessitating generator changes, the potential for devastating infections, lack of autonomic responsiveness, pacing-induced cardiomyopathy, and lead fracture or dislodgement, which is particularly problematic in the pediatric population as they grow and attempt to carry out a normal active life (Rosen et al. 2011). The ability to biologically replace a failing sinoatrial node might alleviate several of these problems. In the case of genetic causes of sinus node dysfunction, nodal cells could be created from autologous hiPSCs that have been genetically modified to correct the defect and restore normal pacing function. For example, overexpression of HCN4 (the ion channel responsible for the pacemaker current, I_f, which has been implicated in sick sinus syndrome) in mESC-CMs increased their spontaneous beating rate and allowed them to pace hiPSC-CMs in co-culture (Saito et al. 2015). These cells also demonstrated β-adrenergic responsiveness.

To date, all efforts at cardiomyocyte generation from hiPSCs have produced a mixed population of cardiac myocytes which resemble ventricular, atrial, and nodal cells, based on their action potential characteristics. On the one hand, this heterogeneity raises concerns about potential arrhythmia generation in clinical applications (see "Cardiomyocyte Subtype Specification" below). However, this also highlights the fact that the creation of nodal cardiomyocytes from hPSCs appears to be possible in vitro, and ongoing efforts to control the subtype of cardiomyocytes produced will likely allow for the generation of pure ventricular as well as nodal cells for their respective applications. Kehat and colleagues were the first to demonstrate the principle that hPSC-CMs could provide cardiac pacing by delivering hESC-CMs to porcine hearts with complete atrioventricular block (Kehat et al. 2004). The delivered cells integrated into the cardiac syncytium and paced the hearts, as demonstrated by 3D electrical mapping. Similar results might be expected from hiPSC-derived cells, although Lee et al. (2011) provide reason for caution. They investigated the calcium-handling characteristics of hiPSC- and hESC-derived cells and found that the calcium handling of hiPSC-CMs was relatively immature compared to hESC-CMs (Lee et al. 2011). This could limit their potential as biological pacemakers (Barbuti and Robinson 2015), although efforts to improve maturation of hiPSC-CMs cells are currently a major focus of research by several groups.

4.2.3 Understanding of Human Cardiac Development

While the use of hiPSCs for regenerative therapies may be their most obvious application in the cardiovascular space, there are multiple other areas in which these cells are already proving valuable. Improving the efficiency of hiPSC differentiation into cardiomyocytes has relied upon a thorough understanding of normal cardiac development. Human heart development is uniquely difficult to study, as embryonic and fetal heart tissue is difficult to obtain and raises obvious ethical concerns. Even adult primary cardiomyocytes are difficult to isolate and can only be maintained for days in active cell culture. These facts have forced the research community to utilize animal models to understand cardiac developmental biology, focusing largely on murine, avian, and frog cardiogenesis. The discovery and successful culture of ESCs opened new possibilities to study cardiac development in a human cell model. At present, much of what we know about human cardiac development has been discovered or verified by investigating hESC signaling and gene expression profiles. This knowledge has guided approaches to cardiomyocyte differentiation of hPSCs, while hPSC-CMs in turn provide ever improving models of cardiogenesis. Furthermore, hiPSCs provide a valuable model for studying cardiac development in both normal and diseased states. Human iPSCs can be created from patients with congenital or developmental heart disease and used to study abnormal development or the effects of various environmental toxins or pharmacologic agents on development in the setting of various patient-specific genetic backgrounds that are known to produce a phenotype, thus greatly enhancing our understanding of these diseases.

4.2.4 Disease Modeling

Human iPSCs provide a platform of unprecedented breadth for the study of virtually any disease process with a genetic association. The creation of hiPSC-CMs derived from patients allows for the study of their specific pathophysiology, whether it involves a genetic or acquired cardiomyopathy, arrhythmia, defect in development, or metabolic disorder. Several cardiac disease phenotypes have already been modeled in hiPSC-CMs from affected patients.

Familial cardiomyopathies have been associated with several known mutations, often involving genes that encode for proteins that comprise the contractile apparatus of the cell. In one of the first examples of the use of patient-derived hiPSCs to study cardiac disease, Sun et al. created hiPSC-CMs from seven members of a family affected by familial dilated cardiomyopathy (DCM), including controls and individuals carrying a point mutation (R173W) in the cardiac troponin T (*TNNT2*) gene (Sun et al. 2012). The hiPSC-CMs from patients with the mutation showed impaired myofilament regulation, contractility, and calcium handling compared to controls, although the cardiomyocytes were notably immature. Subsequent work from this group demonstrated abnormal β-adrenergic signaling and contractile function in these DCM hiPSC-CMs and a possible role for nuclear localization of the mutated TNNT2 in epigenetic modifications resulting in upregulation of phosphodiesterase 2A and 3A, which related to compromised β-adrenergic signaling and contractility (Wu et al. 2015).

Other mutations involved in DCMs have since been modeled in hiPSC-CMs as well, including a mutation in the gene encoding lamin A/C (*LMNA*), demonstrating increased electrical stimulation-induced nuclear senescence and apoptosis in hiPSC-CMs, which could be reduced or eliminated by pharmacologic blockade of the MEK1/ERK1/2 pathway (Siu et al. 2012). A mutation in the gene for desmin (*DES*) was identified in another DCM patient, and hiPSC-CMs derived from this patient demonstrated structural and functional abnormalities due to protein misfolding that recapitulated findings from pathologic specimens (Tse et al. 2013). Sarcomere insufficiency was identified as a cause of DCM in patients who carry truncating mutations in the gene for titin (*TTN*), a major structural protein in myofibrils (Hinson et al. 2015). In another example, hiPSC-CMs from a DCM patient carrying a phospholamban (*PLN*) mutation demonstrated abnormal Ca^{2+} handling, electrical instability, and abnormal cytoplasmic distribution of phospholamban protein, recapitulating findings in primary cardiomyocytes of patients with *PLN* mutations and DCM (Karakikes et al. 2015).

These efforts highlight the fact that there are now over 50 genes which have been implicated in familial DCMs (Skrzynia et al. 2015), and these various mutated genes confer nearly indistinguishable clinical phenotypes via very different mechanisms. The use of hiPSCs has been instrumental in defining these causative factors and may aid in identifying unique treatments for individual patients carrying specific mutations. As such, the ability to create cardiomyocytes from hiPSCs represents one of the greatest steps toward the realization of personalized medicine, in which

treatments for diseases can be tailored to an individual patient based on their genetics or the specifics of their pathophysiology.

Several other cardiac diseases have now been modeled in hiPSCs, including hypertrophic cardiomyopathy (Lan et al. 2013; Han et al. 2014; Birket et al. 2015a; Tanaka et al. 2014; Ojala et al. 2016); arrhythmogenic right ventricular cardiomyopathy (Kim et al. 2013; Ma et al. 2013a; Wen et al. 2015); diabetic cardiomyopathy (Drawnel et al. 2014); familial long QT syndrome type 1 (Moretti et al. 2010; Egashira et al. 2012; Ma et al. 2015), type 2 (Itzhaki et al. 2011; Matsa et al. 2011; Lahti et al. 2012; Matsa et al. 2014; Jouni et al. 2015; Mehta et al. 2014), type 3 (Ma et al. 2013b; Fatima et al. 2013; Terrenoire et al. 2013; Malan et al. 2016), and type 8 (Timothy syndrome) (Yazawa et al. 2011); catecholaminergic polymorphic ventricular tachycardia types 1 (CPVT1) (Fatima et al. 2011; Jung et al. 2012; Itzhaki et al. 2012; Kujala et al. 2012; Zhang et al. 2013; Di Pasquale et al. 2013; Penttinen et al. 2015; Novak et al. 2015) and 2 (CPVT2) (Novak et al. 2012, 2015); hypoplastic left heart syndrome (Jiang et al. 2014; Kobayashi et al. 2014; Theis et al. 2015); and the cardiac phenotypes of Pompe disease (Huang et al. 2011; Raval et al. 2015; Sato et al. 2015) and Duchenne muscular dystrophy (Dick et al. 2013; Guan et al. 2014; Lin et al. 2015; Hashimoto et al. 2016).

Human iPSC-CMs also allow investigators to go beyond simply recreating disease physiology in human cells and tissues in vitro to demonstrating causality of a given mutation or signaling pathway by providing a platform for human gene knockdown and overexpression techniques or the introduction of mutations into control human cardiomyocytes to demonstrate reproducibility of a given phenotype. This is particularly important when one considers that observed differences between diseased hiPSC-CMs and control cells could be due to off-target genetic differences. For example, selective expression of a mutated titin gene in control hiPSC-CMs resulted in reduced contractile function, recapitulating the DCM phenotype seen in diseased cells (Hinson et al. 2015), and knockdown of LMNA in control hiPSC-CMs using shRNA recreated the phenotypic changes and susceptibility to electrical stress seen in *LMNA* mutant hiPSC-CMs (Siu et al. 2012). Similarly, normal genes can be introduced into diseased hiPSC-CMs to demonstrate rescue of a disease phenotype. Karakikes et al. used both a TALEN-mediated gene correction and a combination strategy of knockdown of the mutant *PLN* and addition of the normal gene into hiPSCs prior to cardiomyocyte differentiation to confirm the role of the *PLN* mutation in the DCM phenotype (Karakikes et al. 2015). This approach rescued Ca^{2+} handling and PLN distribution and fully restored normal cardiac phenotype in hiPSC-CMs.

4.2.5 Drug Discovery, Pharmacogenomics, and Cardiotoxicity Screening

Human iPSC-CMs provide an unprecedented ability to study cellular signaling, metabolism, and ion channel function in human cardiomyocytes. Therefore, it is not surprising that several of the hiPSC-CM disease models outlined above have resulted

in the identification of novel drugs or potential therapeutic targets which may correct or improve the cardiac defects in question. Studying type 1 long QT syndrome (LQT1), Ma et al. observed decreased slowly activating delayed rectifier potassium channel current (I_{Ks}) in hiPSC-CMs from an LQT1 patient with a novel loss of function mutation in *KCNQ1* (Ma et al. 2015). This mutation resulted in prolonged action potential duration, which confers an increased risk of fatal arrhythmias in these patients. Application of the novel drug ML277 increased I_{Ks} in the LQT1 hiPSC-CMs and shortened action potential duration, identifying this as a potential therapy for LQT1. In a hiPSC-CM model of CPVT1, dantrolene was shown to suppress arrhythmogenic spontaneous Ca^{2+} release events and abolish delayed afterdepolarizations and spontaneous action potentials in CPVT1 hiPSC-CMs carrying an N-terminal mutation in the cardiac ryanodine receptor (Jung et al. 2012). And while recombinant enzyme replacement therapy effectively corrects lysosomal storage in Pompe disease, its effect is incomplete. The use of hiPSC-CMs from a patient with Pompe disease allowed investigators to screen for additional therapies, identifying L-carnitine as having potential benefit due to its ability to rescue mitochondrial function in diseased cardiomyocytes (Huang et al. 2011).

Just as hiPSCs can aid in the discovery of new drugs and drug targets, they also have great potential to improve the efficiency of bringing new therapies to market. Recent estimates suggest that the average cost to bring one new drug to market in the USA, including the cost of failures, is $1.24 billion (Kaitin 2010). Considering that only one in six drugs that made it into clinical trials in the USA actually made it to market (DiMasi et al. 2010), while the costs of clinical trials have been rising in recent years (Kaitin 2010), the wasted R&D expenditures involved are alarming. The high drug attrition rate is at least in part due to unforeseen toxicities encountered during clinical trials that were not predicted by suboptimal screening assays which rely on the use of nonhuman cells such as Chinese hamster ovary (CHO) cells or immortalized transgenically modified human cell lines, such as human embryonic kidney (HEK) cells, during drug development. Of particular concern is the potential for pharmacologically induced cardiac arrhythmias. Human iPSCs provide a model for testing drug toxicity in virtually any human tissue or cell type without having to rely on animal models which may produce very different results. As an example of such use, a recent initiative called the Comprehensive In Vitro Proarrhythmia Assay (CiPA) was begun in an effort to more accurately predict the risk of the potentially fatal cardiac arrhythmia, torsades de pointes (TdP). TdP has been associated with a long list of medications based on surrogate markers for TdP risk, which include inhibition of the potassium channel hERG and prolongation of the QT interval on an electrocardiogram (Fermini et al. 2016). Common practice has been to test an investigational drug on non-cardiomyocyte cells such as hERG transgenic CHO cells (Danker and Moller 2014) and monitor QT intervals in clinical trials. While this method is sensitive for TdP risk, it is not very specific and ignores potential for effects on other ion channels (Fermini et al. 2016). As a result, several drugs that may not truly increase risk of TdP have been forced to go through additional phase IIb and III trials to assess risk of QT prolongation and TdP, at a cost of approximately $1 billion (Fermini et al. 2016). The CiPA initiative was designed to assess drug effects

on seven ion channels (not just hERG), generate in silico models of cardiac repolarization from these data, and utilize standardized hiPSC-CM protocols to assess effects on repolarization in an effort to screen out drugs with unacceptable risks of toxicity prior to reaching clinical trials. Combining such an approach with current state-of-the-art technology such as commercially available high-throughput robotic patch-clamp systems and microelectrode arrays could aid in rapid screening of multiple drugs at multiple doses using a panel of hiPSC-CMs from subjects with varying genetic backgrounds (Mordwinkin et al. 2013).

Importantly, patients with underlying cardiac disease or rare genetic mutations may not be included in investigational drug trials, making identification of cardiotoxicity risk virtually impossible until post-market analyses are performed. Furthermore, Liang et al. demonstrated increased susceptibility to cardiotoxicity in diseased cardiomyocytes by creating hiPSC-CMs from healthy subjects and patients with hereditary long QT syndrome, familial HCM, and familial DCM and performing assessments of the effects of known cardiotoxic drugs on ion channel currents in these cells (Liang et al. 2013). Diseased hiPSC-CMs demonstrated increased propensity for arrhythmias, which mimicked that of the patients from whom they were derived, and were superior to hERG assays alone. This work illustrates how high-throughput technologies could be valuable in identifying cardiotoxicities in patients with varying cardiac conditions *before* they are given a new drug.

As an example, hiPSC-CMs have demonstrated benefit in modeling, and even predicting, chemotherapy-induced cardiotoxicity. Anthracyclines are effective in treating a wide range of malignancies, but a subset of patients go on to develop cardiomyopathy and heart failure. Burridge et al. showed that hiPSC-CMs from breast cancer patients who had developed anthracycline-induced cardiomyopathy actually recapitulated the increased predilection toward cardiotoxicity relative to patients who received similar treatment but did not develop cardiotoxicity, suggesting that hiPSCs from cancer patients could be used to screen for risks of serious treatment side effects (Burridge et al. 2016). Such an in vitro model of chemotherapeutic-induced toxicity could be useful in screening cells from large numbers of individuals to identify particular gene variants or single nucleotide polymorphisms that might identify patients at risk for toxicity, moving healthcare closer to the goal of personalized medicine.

4.3 Cardiomyogenesis during Mammalian Heart Development

The heart is the first functioning organ to develop in an embryo, bearing the responsibility for pumping oxygen and nutrients to the other developing tissues. The heart forms through multiple stages of morphogenesis which begin with development of cardiac progenitor cells, formation of the linear heart tube, cardiac looping, chamber formation, septation, and finally maturation. Cardiac development begins following the formation of the blastocyst, when the inner cell mass (from which ESCs are obtained) undergoes gastrulation to form the three primitive germ layers:

endoderm, mesoderm, and ectoderm. Initiation of gastrulation and induction of the mesoderm layer is heavily dependent on NODAL, bone morphogenetic protein (BMP), Wnt, and fibroblast growth factors (FGF) signaling (Kimelman 2006; Noseda et al. 2011; Brade et al. 2013). Mesodermal induction is marked by expression of the T-box transcription factor Brachyury (T), which is itself a target of Wnt/β-catenin signaling. The mesoderm contains precursors of the first and second heart fields (FHF and SHF), which will ultimately give rise to the majority of the atria, ventricles, and the outflow tract myocardium (Brade et al. 2013).

The commitment and differentiation of mesodermal cells to a cardiac lineage is dependent on a complex interaction of signaling pathways. The T^+ cells require NOTCH-mediated inhibition of canonical Wnt/β-catenin signaling and activation of noncanonical Wnt pathways for induction of cardiac transcription factors (Fig. 4.1, upper panel), leading to expression of KDR (FLK1) (Brade et al. 2013; Kwon et al. 2009; Gessert and Kuhl 2010). These T^+/KDR^+ cells give rise to the $MESP1^+$ cardiogenic mesoderm, and finally the FHF and SHF, which form the cardiac crescent. The more anterolaterally located FHF cells are exposed to BMP and FGF signals as well as canonical Wnt pathway inhibitors which further drives their differentiation, and they begin to express NKX2-5, GATA4, and TBX5, as well as contractile proteins myosin light chain-2a (MYL7) and sarcomeric myosin heavy chain (Brade et al. 2013). The FHF cells of the cardiac crescent give rise to the linear heart tube, while the SHF cells migrate into the heart tube later, where FGF signaling maintains progenitor cell proliferation, while Sonic hedgehog-mediated signals from the endoderm and canonical Wnt signaling from the midline neural tube inhibit differentiation and stimulate proliferation (Brade et al. 2013; Kelly 2012). As

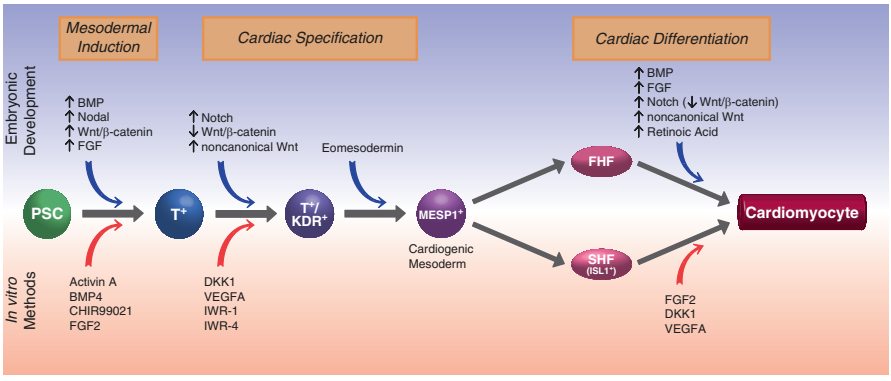

Fig. 4.1 Signaling pathways involved in embryonic cardiomyocyte development (*top*) and factors shown to influence differentiation in vitro (*bottom*). Increases or decreases in the pathways indicated have been shown to promote advancement of cell differentiation during embryonic development. In many cases, successful in vitro cardiomyocyte differentiation strategies have recapitulated these embryonic events. Abbreviations: *BMP* bone morphogenetic protein, *DKK1* Dickkopf-related protein-1, *FGF* fibroblast growth factor, *FHF* first heart field, *IWR* inhibitor of Wnt response, *PSC* pluripotent stem cell, *SHF* second heart field, *VEGFA* vascular endothelial growth factor

with the FHF, BMPs, NOTCH, and noncanonical Wnt signals promote cardiac differentiation of the SHF cells. The later stages of cardiac development include cardiac looping and chamber formation, as well as formation/septation of the outflow tract, which are reviewed elsewhere (Schleich et al. 2013).

4.4 Control of the Pluripotent State

Though all pluripotent cells harbor the ability to differentiate into somatic cell lineages, it is now increasingly evident that pluripotency can exist in a small number of diverse stable states. Of these, two pluripotent phases corresponding to successive stages in development have been described: the naïve state, which corresponds to the pre-implantation blastocyst in mice, and the primed state, which remains pluripotent but has undergone epigenetic modifications.

Historically, mESCs have been acquired from embryonic day 3.5 (E3.5) pre-implantation embryos at the blastocyst stage by explanting cells from the inner cell mass (ICM) and allowing for subsequent outgrowth (Evans and Kaufman 1981). These mESCs, still in the naïve state, form dome-shaped colonies and were classically cultured in media containing fetal bovine serum (FBS) and leukemia inhibitory factor (LIF) (Smith et al. 1988) or bone morphogenetic protein 4 (BMP4) and LIF (Ying et al. 2003). These cells are now commonly maintained by a combination of MAPK/ERK kinase (MEK) and glycogen synthase kinase 3 beta (GSK3B) inhibition plus LIF, commonly referred to as "2i/LIF" (Nichols and Smith 2009). In contrast, mESCs acquired from E5.5–7.5 post-implantation epiblasts (EpiSC) (Tesar et al. 2007; Brons et al. 2007) represent the primed state. They grow in monolayer colonies, proliferate in response to Activin/NODAL/TGFβ and FGF2 signaling (Brons et al. 2007), and have diverse propensities for differentiation (Bernemann et al. 2011). The discernable differences in morphology and varying dependence on exogenous factors between these two populations have been ascribed to the developmental stages from which these cells were derived.

In contrast, human PSCs, whether hESCs derived from blastocysts or hiPSCs reprogrammed from adult somatic cells, show morphological, epigenetic, and growth factor requirements reminiscent of the primed EpiSC-like state in mice. Early attempts at capturing a naïve state of human pluripotency similar to that of mESCs relied on the expression of transgenes, such as sustained doxycycline-dependent expression of *OCT4, SOX2, KLF4,* and *MYC* (De Los et al. 2012). However, recent transgene-free methods of deriving human naïve pluripotent cells have been described, and methods for transferring human pluripotent cells between the naïve and primed states are being developed (Guo et al. 2016; Gafni et al. 2013; Theunissen et al. 2014).

In a recent study, Hanna and colleagues (Gafni et al. 2013) used an *OCT4*-GFP reporter with a doxycycline-inducible system to express reprogramming factors previously shown to convert cells to a naïve-like state. A subsequent screen for conditions that allowed for sustained GFP expression in the absence of doxycycline found that a combination of 2i/LIF, TGFB1, FGF2, along with JNK and p38 inhibitors was

sufficient to maintain reporter construct expression and sustain a naïve-like state in primed hiPSCs. Human ESCs and hiPSCs grown under these conditions exhibited molecular and epigenetic features similar to those of naïve mESCs, including utilization of the *OCT4* distal enhancer and a lack of X chromosome inactivation. Furthermore, these cells generated cross-species chimeric mouse embryos and displayed a distinct gene expression pattern when compared to primed hESCs and hiPSCs, underscoring their pluripotency potential and suggesting a distinct pluripotent state.

In another study, a screen for factors that maintain the expression of a reporter of *OCT4* distal enhancer activity identified five kinase inhibitors (of MEK, GSK3B, ROCK, BRAF, and SRC) that, in combination with Activin A and LIF, allow for the maintenance of hESCs in a naïve state as well as for the interconversion between primed and naïve states (Theunissen et al. 2014). Human ESCs cultured under these conditions exhibited an upregulation of transcription factors associated with naïve pluripotency in mice, including *STELLA*, *DPPA2*, *DPPA5*, *REX1*, *KLF4*, *KLF5*, *TFCP2L1*, and *NANOG*. However, they expressed a distinct gene expression pattern when compared to hiPSCs and previously described naïve hESC states. Contrary to findings in mESCs, these cells showed an upregulation of the X-chromosome inactivator *XIST* and inactivation of X-linked gene expression. While this may indicate that the relationship between X inactivation and the naïve pluripotent state may not be evolutionarily conserved, the authors point out that it may also be attributable to X chromosome erosion in their late passage cells and thus warrants further investigation. These reports illustrate the challenges in capturing a naïve state in hPSCs and the lack of consensus regarding what this state might look like.

Refining the relationship between the naïve and primed state provides the opportunity to explore the epigenetic and transcription factor networks governing the stabilization of pluripotency (Guo et al. 2016; Gafni et al. 2013; Theunissen et al. 2014). Notably, a number of pathways appear to play contradictory roles in maintaining the naïve versus primed state. FGF2 signaling, classically used to maintain the pluripotent state of primed hESCs and hiPSCs, plays an antagonistic role in maintaining the naïve state cells (Guo et al. 2016; Gafni et al. 2013). BMP4, which can be used to support naïve cells (Ying et al. 2003), results in the mesoderm or trophectoderm differentiation of primed cells (Xu et al. 2002; Yu et al. 2011), while BMP inhibitors promote self-renewal of hPSCs (Frank et al. 2012). Likewise, JNK and p38 inhibition results in the stabilization of the naïve state but is not required for maintenance of primed cells (Gafni et al. 2013) (Fig. 4.2).

Further investigation of the distinct mechanisms by which these two pluripotent states are sustained may help explain subtle differences in their susceptibility to differentiation and could allow for the derivation of pluripotent states with increased stability and wider differentiation potential. For example, in mice, BMP signaling through SMAD has been shown to be dispensable for pluripotency and instead has been implicated in lineage priming predisposition via control of DNA methylation (Gomes Fernandes et al. 2016). Because the elucidation of signaling networks governing pluripotency is complicated by the use of different media formulations and cell attachment matrices across studies, systematic design of experiments (DOE) approaches using chemically defined media will help better characterize the naïve state. If successfully captured and described, the naïve state of human pluripotency

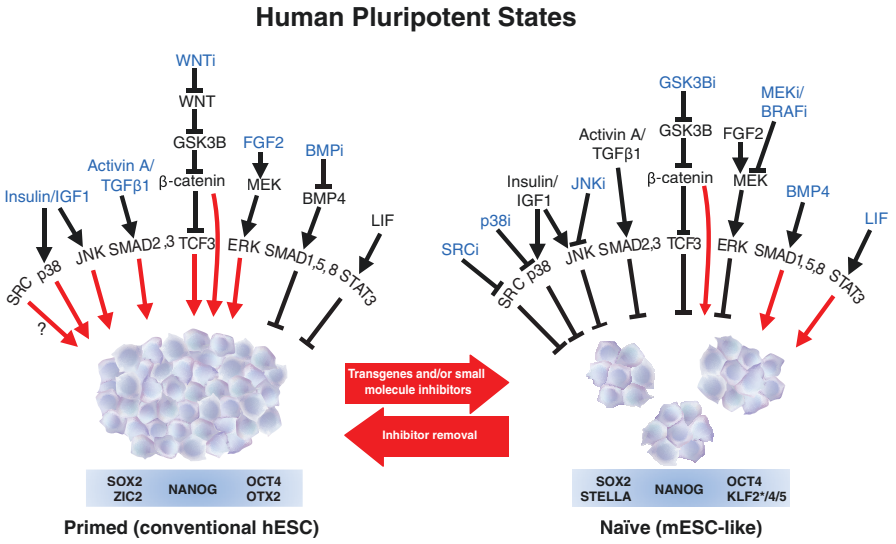

Fig. 4.2 Signaling pathways and reprogramming factors involved in deriving and sustaining the primed and naïve pluripotent states in humans. Multiple signaling pathways influence the primed and naïve states of hPSC. Notably, most of these pathways have opposite effects on stabilization of the primed vs. naïve state. *Blue* text indicates factors that have been utilized in vitro to induce or maintain the respective pluripotent state. *Red arrows* indicate pathways that promote the indicated pluripotent state. Genes in *blue boxes* have been shown to be expressed in the respective state. *KLF2, while not expressed in human pre-implantation epiblast cells (Blakeley et al. 2015), can promote the naïve pluripotent state when overexpressed

may supply a source of unobstructed cells in a stable pluripotent state with the capacity for unrestricted and unbiased differentiation.

4.5 Human Pluripotent Growth Media

The use of patient-derived hiPSCs in regenerative medicine will require the homogeneous production of large quantities of cells cultured in defined and xeno-free environments (Fig. 4.3). Initial conditions for culturing hESCs relied heavily on factors known to support pluripotency in mESCs and involved the use of DMEM supplemented with fetal bovine serum (FBS), glutamine, β-mercaptoethanol (BME), and nonessential amino acids. The use of a mouse embryonic fibroblast (MEF) feeder layer as a source for cell adhesion and of soluble factors was deemed necessary for maintaining pluripotency in both the presence and absence of leukemia inhibitory factor (LIF) (Thomson et al. 1998). Although these culture conditions provided a source of cells suitable for studying human development, the use of xenogenic factors rendered the cultures inadequate for clinical applications.

The elimination of FBS by KnockOut serum replacement (KSR), as well as the addition of human recombinant FGF2, improved the cloning efficiency of cells cultured in KnockOut DMEM (KO-DMEM) (Amit et al. 2000). Likewise, the use of a Matrigel matrix eliminated the need for an MEF feeder layer and established an important role

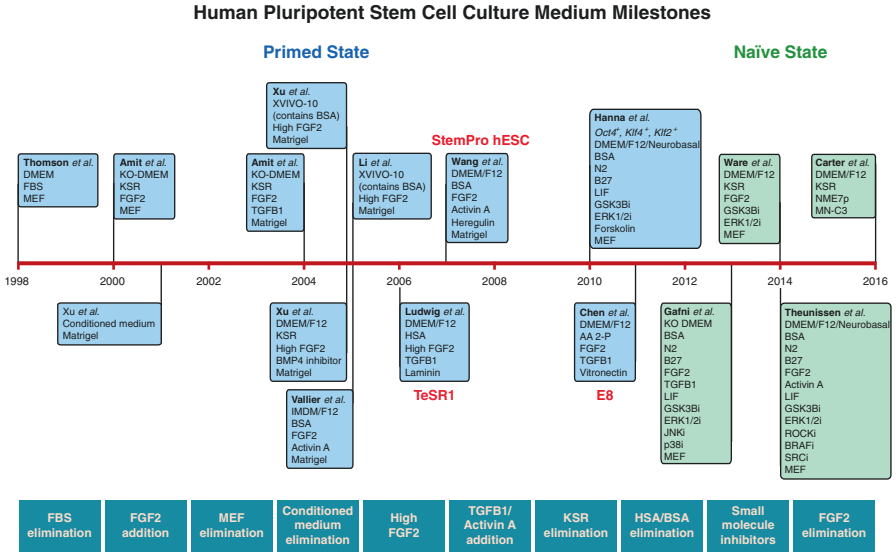

Fig. 4.3 Milestones in human pluripotent stem cell culture. Important publications in the evolution of pluripotent stem cell media. Included are key media components, growth factors, and small molecules employed to sustain pluripotency. *Red text* indicates the name of the media formulation when commercialized. *Blue boxes* indicate media derived for primed hPSCs, with naïve hPSC media in *green boxes*. Boxes below the timeline depict important milestones. Abbreviations: *AA 2-P* L-ascorbic acid 2-phosphate, *BMP4* bone morphogenetic protein 4, *BSA* bovine serum albumin, *FBS* fetal bovine serum, *FGF2* basic fibroblast growth factor, *HSA* human serum albumin, *KO-DMEM*, KnockOut DMEM, *KSR* KnockOut serum replacement (Xu et al. 2001, 2005a, b; Amit et al. 2004; Hanna et al. 2010; Ware et al. 2014; Carter et al. 2016)

for laminin in mediating cell-matrix interactions but required the use of conditioned media from heterologous sources (Xu et al. 2001). This obstacle was overcome by the addition of transforming growth factor β1 (TGFB1) (Amit et al. 2004) as well as the inclusion of higher levels of FGF2 (Xu et al. 2005a) and suppression of BMP signaling using NOGGIN (Xu et al. 2005b). While these factors allowed for the feeder-free maintenance of hESCs in basal media, the use of KSR and Matrigel remained problematic, as both contain undefined components of animal origin. Nonetheless, the shift away from conditioned media spurred the identification of key factors necessary to maintain pluripotency, such as the cooperation of the Activin/Nodal and FGF2 pathways. This allowed for the replacement of KSR and BMP inhibition with bovine serum albumin (BSA) and insulin (Vallier et al. 2005), as well as for the culture of hESCs in serum-free medium (X-VIVO 10) supplemented with high levels of human recombinant FGF2 (Li et al. 2005). The optimized development of defined medium containing DMEM/F12, human serum albumin (HSA), high FGF2, and TGFB1 (TeSR1) by Ludwig et al. marked the first time hESCs had been both derived and maintained in defined conditions free of nonhuman products (Ludwig et al. 2006), and its commercialization laid the groundwork for establishing standardized methods of culturing hESCs. A focus on highly expressed receptor tyrosine kinases in hESCs led to the development of pluripotency media containing FGF2 and Activin A as well as the ERBB2/ERBB3 ligand heregulin1β (commercialized as StemPro hESC) and established an important role for insulin signaling in maintaining pluripotency in the absence of KSR (Wang et al. 2007).

However, the use of human or bovine serum albumin in these media limited their large-scale use for therapeutic applications. The careful examination of basal (DMEM/F12) and TeSR components allowed for the formulation of albumin-free media by revealing BME-mediated toxicity in the absence of BSA and allowed for the identification of components necessary to maintain pluripotency (Chen et al. 2011). The resulting formulation (E8) consists of chemically defined components including FGF2, TGFB1, insulin, L-ascorbic acid 2-phosphate, selenium, and transferrin and is free of xenogenic factors, making it suitable for use in clinical applications (Chen et al. 2011). Although hESCs and hiPSCs can now be maintained in chemically defined conditions, the further optimization of components using DOE approaches may result in formulations that allow for improved culture conditions (Marinho et al. 2015).

4.6 Priming for Cardiac Differentiation

Just as optimization of culture conditions can improve maintenance of the pluripotent state, the right conditions can also bias pluripotency toward cardiac cells. This approach requires the stabilization of a pluripotent cell in a state that is poised to become mesoderm. The derivation of such a cell state must consider determinants of fate, including the relative expression of pluripotency factors, as these can play both inductive and inhibitive roles in lineage determination.

Pluripotency has proven to be quite diverse. Various studies have noted differences in directed differentiation efficiency among hiPSC lines as well as in the distribution of cell types within teratomas formed by these cells. Furthermore, lineage-associated genes and pluripotency factors can be co-expressed and can fluctuate within a single culture (Montserrat et al. 2013; Cahan and Daley 2013). While it is unclear whether the diversity in gene expression and differentiation potential arises due to donor- or clone-specific gene expression, time in culture, variability in culture conditions, or is simply a reflection of pluripotency in vivo, these observations lend support for the idea that cells can take on various stable states that are primed, yet undifferentiated. Therefore, the challenge in deriving a cell line primed specifically for cardiac differentiation lies in minimizing the heterogeneity observed and driving pluripotency toward a specific state that is exclusively poised for differentiation into the cell of interest.

Establishing a primed mesodermal state will require a delicate balance of expressed pluripotency factors and mesodermal genes. Pluripotency factors, including *OCT4*, *SOX2*, and *NANOG*, were identified based on their expression in the pluripotent state. However, it is now clear that these factors also play a role in specifying lineage commitment. OCT4 and SOX2 have been associated with the induction of mesendoderm and neuroectoderm fates, respectively (Thomson et al. 2011); therefore, their relative expression will need to be regulated in a way that allows for both pluripotency and facilitated entry into mesodermal lineages.

Recent reports show that pluripotency can also be achieved by overexpression of genes associated with embryonic lineages, such as the mesendodermal lineage specifier *GATA3* (Montserrat et al. 2013; Shu et al. 2013). This suggests a model in which transcription factors can act as lineage specifiers and repressors rather than as direct inducers of pluripotency (Montserrat et al. 2013; Thomson et al. 2011; Shu et al. 2013). The successful reprogramming of human fibroblasts using lineage determinants to replace

core pluripotency factors *OCT4* and *SOX2* (Montserrat et al. 2013) implies that a mesodermally primed state may be achieved by sustaining pluripotency via controlled expression of optimal relative levels of mesodermal and endodermal lineage specifiers. Although cells reprogrammed via lineage specifiers appear indistinguishable from traditionally derived hiPSCs by genome-wide transcription analysis (Montserrat et al. 2013), it is possible that differences in gene expression may become evident only upon differentiation, as is observed in different hESC lines with varied differentiation propensities (Osafune et al. 2008). Comparisons of directed differentiation success in hiPSC lines stabilized via different lineage determinants could address this issue and aid in the elucidation of conditions best suited for cardiac differentiation. Likewise, the development of computational methods for predicting transcription regulatory networks and lineage specifiers for subpopulations within heterogeneous cultures (Okawa and del Sol 2015) has the potential to increase our understanding of how the pluripotent state can be stabilized and expedite the discovery of favorable conditions for cardiac priming.

4.7 Maintenance of hiPSC-Derived Mesodermal Intermediates and Cardiac Progenitor Cells

A remaining barrier in the application of hiPSC-CMs is the variability in differentiation success when using large numbers of hiPSC lines at varying passages. The development of an expandable intermediate mesodermal cell line to provide a renewable source of cells fated to become mesodermally derived tissues could overcome this obstacle. An hiPSC-derived cardiac progenitor cell (CPC) line could also potentially be used directly in regenerative therapies. Although the stabilization of such cell lines has been challenging, several groups have recently reported diverse methods for effectively capturing and expanding the mesoderm and cardiac progenitor states.

Cao and colleagues described the stabilization of self-renewing MESP1/MESP2$^+$, SSEA1$^+$, GATA4$^+$, MEF2C$^+$, ISL1$^+$ CPCs through BMP4, Wnt, and MEK-ERK activation in defined conditions (Cao et al. 2013). In this approach, hESCs were cultured in medium containing BMP4, CHIR99021, and ascorbic acid and showed a loss in expression of pluripotency markers followed by an upregulation of early mesodermal genes within 3 days of differentiation. Cells were subsequently stabilized and propagated for >15 passages with Activin/NODAL and BMP inhibition along with Wnt activation (CHIR99021). The resulting CPCs lacked in vivo tumorigenicity and readily differentiated into smooth muscle cells, endothelial cells, and beating, cross-striated NKX2-5$^+$/TNNT2$^+$ cardiomyocytes. However, while promising, the success of this protocol has still yet to be replicated.

More recently, a high-throughput screening method was used to identify a combination of small molecules and growth factors that allow for the sustained expression of T in mesoderm cells (Kumar et al. 2015). Cells cultured in RPMI/B27 with a combination of FGF2 and the GSK3B inhibitor CHIR98014 on an extracellular matrix composed of fibronectin, vitronectin, and collagens I, III, IV, and V retained the ability to expand and stably express the mesodermal markers MESP1, MIXL1, and LHX1. Although the mesodermal progenitor cells successfully differentiated into renal cells, they failed to differentiate into other mesodermally derived lineages, including cardiac. However, these conditions could help increase our understanding

of development along mesodermal lineages and possibly improve differentiation into cardiac cells. Using a similar approach, Birket and colleagues demonstrated the sustained culture of PDGFRA$^+$, pre–NKX2-5$^+$ CPCs using controlled *MYC* expression in the presence of IGF1 and hedgehog signaling (Birket et al. 2015b). In this study, MYC expression at differentiation day 4.75 blocked CPCs from transitioning to an NKX2-5$^+$ state for over 40 population doublings (Birket et al. 2015b). While this discovery will aid in an increased understanding of cardiac development, the use of transgenic elements currently precludes its use in clinical applications.

In contrast, two very recent studies focused on reprogramming mouse fibroblasts directly to an expandable CPC state in defined conditions. A candidate gene approach to identify transcription factors and chromatin remodelers that allow for reprogramming of mouse adult cardiac fibroblasts into CPCs revealed that five cardiac factors (*Mesp1*, *Tbx5*, *Gata4*, *Nkx2-5*, and *Smarcd3* (*Baf60c*)) along with JAK/STAT and Wnt signaling (LIF and BIO) gave rise to *Nkx2-5*$^+$ CPCs (Lalit et al. 2016). Although these CPCs could be passaged and successfully differentiated into cardiomyocytes, smooth muscle cells, and endothelial cells, they did not spontaneously contract, suggesting that further optimization is necessary. Similarly, a second group has reported that the use of BMP4, Activin A, and Wnt activation (CHIR99021), along with FGF, VEGFA, and PDGF inhibition, allows for the sustained culture of KDR$^+$, PDGFRA$^+$ CPCs derived from murine fibroblasts (Zhang et al. 2016).

The approaches outlined above all result in the stabilization of a mesodermal precardiac state; however, each employs varied combinations of growth factors and small molecules, resulting in populations defined by different markers. This suggests that the resulting CPCs represent different stages of mesodermal differentiation, and continued analysis is necessary to define optimal conditions for deriving CPCs restricted to differentiation into intended cardiac subtypes. While transgene-free and chemically defined methods of capturing the mesodermal or cardiac precursor state in humans remain to be elucidated, the studies above are an auspicious beginning.

4.8 Directed Cardiac Differentiation of Human Pluripotent Stem Cells

The current efficiency of hiPSC-CM generation owes in large part to previous efforts to understand the pathways involved in cardiac development, including modulation of the TGFβ/Activin/NODAL, BMP, Wnt, NOTCH, FGF, vascular endothelial growth factor (VEGF), and Dickkopf-related protein-1 (DKK1) pathways (Fig. 4.1). Many of the methods used in differentiating hiPSCs along various lineages stems from earlier work using hESCs. Early on, growth of hESCs in suspension was found to produce spherical aggregates of cells termed embryoid bodies (EB) (Itskovitz-Eldor et al. 2000) and was shown by Kehat et al. in 2001 to result in spontaneous contractions in 8–10% of the EBs (Kehat et al. 2001). Cardiac-specific gene expression and calcium transients were observed in cells from the contracting EBs. Efforts to improve on this technique soon followed (Table 4.1). EBs of differing sizes resulted in inconsistent differentiation efficiencies, so attempts were made to control the size of EBs using forced aggregation techniques, in which a known number of hESCs were plated in U- or V-bottomed wells (Ng et al. 2005; Burridge et al. 2007). This method significantly improved the

Table 4.1 Methods for static cardiac differentiation of human pluripotent stem cells

Pink cells indicate positive inducers (growth factors, FBS, and Wnt-inducing small molecules). Yellow cells indicate inhibitors. Green cells indicate media used. Abbreviations: *BME* β-mercaptoethanol, *BMP4* bone morphogenetic protein 4, *DKK1* Dickkopf-related protein-1, *EB* embryoid body, *END-2* endoderm-like cell line, *FBS* fetal bovine serum, *FGF2* basic fibroblast growth factor, *HF* human fibroblasts, *IWR-1* inhibitor of Wnt response-1, *KO-DMEM,* KnockOut DMEM, *KSR* KnockOut Serum Replacement, *MEF* mouse embryonic fibroblasts, *MEF-CM* MEF conditioned medium, *MTG* α-monothioglycerol, *NEAA* non-essential amino acids, *N/S* not stated, *PFHM* Protein-Free Hybridoma Medium, *PVA* polyvinyl alcohol, *SCF* stem cell factor, *VEGFA* vascular endothelial growth factor (Kattman et al. 2011; Lian et al. 2012; Hudson et al. 2012; Burridge et al. 2014; Kehat et al. 2001; Burridge et al. 2007; Mummery et al. 2003; Yang et al. 2008; Laflamme et al. 2007; Zhang et al. 2011; Zhang et al. 2012; Elliott et al. 2011; Uosaki et al. 2011; Zhang et al. 2015; Graichen et al. 2008; Xu et al. 2008; Takei et al. 2009; Tran et al. 2009; Burridge et al. 2011; Ren et al. 2011; Willems et al. 2011; Cao et al. 2012; Gonzalez et al. 2011; Willems et al. 2012; Minami et al. 2012; Fonoudi et al. 2013; Lian et al. 2015; Aguilar et al. 2015; van den Berg et al. 2016; Lin et al. 2016)

Milestone	Pluripotency Media	Differentiation protocol, growth factors, small molecules and media by day of protocol (d0 d1 d2 d3 d4 d5 d6 d7 d8 d9 d10 d11 d12)	Efficiency	Reference
Spontaneous differentiation	KO-DMEM, 20% FBS	20% FBS KO-DMEM	8% OF EBs	Kehat et al., 2001 [135]
END-2 co-culture	DMEM, 20% FBS	20% FBS, END-2 cells DMEM	N/S	Mummery et al., 2003 [138]
Growth factor-directed mesoderm induction, forced aggregation	MEF-CM	Activin A, FGF2 | 20% FBS CDM-PVA, ascorbic acid | DMEM	24% of EBs	Burridge et al., 2007 [137]
Growth factor monolayer	MEF-CM	Activin A | BMP4 RPMI+B27	30% MYH7+	Laflamme et al., 2007 [141]
First application of a small molecule (p38 MAPKi)	KO-DMEM, 20% KSR, FGF2	SB203580 END-2 conditioned: DMEM, NEAA, insulin, transferrin, sodium selenite, BME	22% MYH6+	Graichen et al., 2008 [192]
First chemically-defined medium, insulin has negative effect on differentiation	KO-DMEM, 20% KSR, FGF2	PGI2, SB203580 DMEM, NEAA, transferrin, sodium selenite, BME	11% MYH6+	Xu et al., 2008 [193]
High efficiency, growth factor optimization, Wnt inhibition	DMEM/F12, 20% KSR, FGF2	BMP4 | BMP4, Activin A, FGF2 | DKK1, VEGFA | DKK1, VEGFA, FGF2 StemPro-34, ascorbic acid	50% TNNT2+	Yang et al., 2008 [140]
Use of BMP4	DMEM, 20% KSR, FGF2	BMP4, 20% FBS | 20% FBS DMEM, NEAA, BME	98% of EBs	Takei et al., 2009 [194]
Serum and insulin inhibit differentiation	DMEM/F12, 20% KSR, FGF2	WNT3A, 15% FBS | 15% FBS | 5% FBS | 0.2% BSA KO-DMEM, NEAA, BME	N/S	Tran et al., 2009 [195]
Developmental stage-specific signaling requirements	DMEM/F12, 20% KSR, FGF2	BMP4 | BMP4, Activin A, FGF2 | DKK1, VEGFA | VEGFA, FGF2 StemPro-34, ascorbic acid	50-70% TNNT2+	Kattman et al., 2011 [19]
High efficiency, thorough optimization, chemically defined media	MEF-CM	BMP4, FGF2 | 20% FBS or HSA RPMI+PVA | RPMI, ascorbic acid | RPMI, insulin, lipids	94% of EBs	Burridge et al., 2011 [196]
Small molecule Wnt inhibition	DMEM/F12, 20% KSR, FGF2	20% FBS, BMP4 | 20% FBS | 20% FBS, IWR-1 | 20% FBS | 2.5% FBS KO-DMEM, NEAA, BME	16% TNNT2+	Ren et al., 2011 [197]

Small molecule Wnt inhibition, serum-free	KO-DMEM, 20% KSR, FGF2	BMP4 / BMP4, Activin A, FGF2 / IWR-1 / IWR-1, FGF2, VEGFA / FGF2, VEGFA / 2% FBS StemPro-34 / DMEM	30% MYH6+	Willems et al., 2011 [198]
First successful monolayer for multiple lines	MEF-CM	Activin A / BMP4 / DKK1 RPMI+B27 without insulin	54% TNNT2+	Uosaki et al., 2011 [164]
EB and monolayer protocol	DMEM/F12, 20% KSR, FGF2	BMP4, WNT3A, Activin A, VEGFA, SCF LI-APEL (IMDM/F12, PFHM-II, PVA, BSA, lipids, ITS, MTG) ascorbic acid / LI-AEL (IMDM/F12, PFHM-II, BSA, lipids, ITS, MTG), ascorbic acid	~25% GFP+	Elliott et al., 2011 [163]
Cardiomyocyte subtype specification	MEF-CM	BMP4, / Activin A / Noggin / DKK1, BMS-189453 or retinoic acid / DKK1 RPMI+B27	83% V or 94% A	Zhang et al., 2011 [142]
Defined pluripotency media, monolayer small molecule Wnt inhibition	mTeSR1	BMP4, Activin A / IWP-4 RPMI+B27	60% MYH6+	Hudson et al., 2012 [22]
Mechanism of ascorbic acid	DMEM/F12, 20% KSR, FGF2	20% FBS / 5% FBS DMEM/F12, KSR / DMEM, ascorbic acid	90-100% of EBs	Cao et al., 2012 [199]
Small molecule only, monolayer	MEF-CM	CHIR99021 / IWR-1, SB431542, purmorphamine DMEM/F12+N2+B27 / RPMI+B27	70-80% NKX2-5+	Gonzalez et al., 2011 [200]
Small molecule only, monolayer	mTeSR1	CHIR99021 / IWP-4 RPMI+B27 without insulin / RPMI+B27	85% TNNT2+	Lian et al., 2012 [20]
Matrigel sandwich	mTeSR1	Activin A / BMP4 RPMI+B27 without insulin / RPMI+B27	98% TNNT2+	Zhang et al., 2012 [150]
TGFB1 inhibition	KO-DMEM, 20% KSR, FGF2	BMP4 / Activin A, BMP4, FGF2, ITD-1 / VEGFA, DKK1, FGF2 StemPro34, NEAA, BME, ascorbic acid	60% TNNT2+	Willems et al., 2012 [201]
Novel Wnt inhibitor	DMEM/F12, 20% KSR	CHIR99021, BIO / KY02111, XAV939 IMDM, NEAA, BME, HSA or BSA	98% TNNT2+	Minami et al., 2012 [202]
Transcription factor transduction	MEF-CM	Activin A / ISL1, BMP4 / ISL1 RPMI+B27	75% TNNT2+	Fonoudi et al., 2013 [203]
Small molecule, chemically defined	E8	CHIR99012 / Wnt-C59 RPMI, recombinant HSA, ascorbic acid 2-phosphate	95% TNNT2+	Burridge et al., 2014 [23]
Without albumin, chemically defined	FTDA	BMP FGF CHIR / IWP-2 or Wnt-C59 KO-DMEM, ascorbic acid, transferrin, sodium selenite (insulin and Y27632 for first day)	80-95% TNNT2+	Zhang et al., 2015 [172]
Small molecule, without albumin, chemically defined	E8 or mTeSR1	CHIR99021 / IWP-2 RPMI, putrescine, progesterone, sodium selenite, / RPMI, insulin, ascorbic acid 2-phosphate	88-98% TNNT2+	Lian et al., 2015 [204]
Use of BMP inhibitor	mTeSR1	CHIR99021 / DMH1 DMEM/F12+B27	75% TNNT2+	Aguilar et al., 2015 [205]
Multiple pluripotent culture methods	Any	BMP4, Activin A, CHIR99021 / XAV939 IMDM/F12, PFHM-II, BSA, lipids, low insulin, transferrin, sodium selenite, MTG	84% TNNT2+	van den Berg et al., 2016 [206]
E8-based media	E8	CHIR99021 / IWP-2 DMEM/F12, ascorbic acid, lipids, transferrin, sodium selenite, sodium bicarbonate (heparin from d1-d7)	94% TNNT2+	Lin et al., 2016 [207]

reproducibility of EB formation within a given hESC line but highlighted the variability in CM differentiation between hESC lines (Burridge et al. 2007).

Turning to developmental biology, modulators of key signaling pathways involved in mesodermal induction and cardiomyocyte differentiation were added to the culture conditions. Considering the importance of signals from neighboring endodermal cells during cardiac development, Mummery et al. co-cultured hESCs with a visceral endoderm-like cell line (END-2 cells), which resulted in cardiomyocyte differentiation, although the efficiency remained low (Mummery et al. 2003). The combination of Activin A and BMP improved cardiomyocyte differentiation of hEB-derived cells (Burridge et al. 2007; Yao et al. 2006). As protocols incorporated additional signaling pathways and improvements in the timing of their modulation, the efficiency of differentiation greatly improved. Using a staged approach, differentiation could be boosted by mesodermal induction with Activin A and BMP4, followed by canonical Wnt inhibition with DKK1, with subsequent FGF2 addition to promote expansion of the developing cardiomyocytes (Yang et al. 2008). This approach improved the frequency of contracting cardiomyocytes to over 50%.

Although successful, the method of EB formation followed by selection of beating EBs and separation of cardiomyocytes was very labor intensive. Therefore, attempts to differentiate hESCs in monolayer cultures using defined media were undertaken. Human ESCs grown on Matrigel and treated with Activin A for 24 h, followed by BMP4 for 4 days, yielded >30% CMs (Laflamme et al. 2007). As with the EB approach, additional cytokines including FGF2 and DKK1 improved monolayer yields as well (Zhang et al. 2011). The small molecules CHIR99021, an inhibitor of the downstream canonical Wnt pathway inhibitor glycogen synthase kinase 3 (GSK3) (Lian et al. 2012; Lian et al. 2013), and inhibitor of Wnt Production-4 (IWP4) and -2 (IWP2) (Lian et al. 2012, 2013; Hudson et al. 2012) were also shown to be effective and in sequential combination could drive differentiation efficiency as high as 98%.

The efforts with hiPSCs began in 2007, when Takahashi et al. first demonstrated evidence of spontaneous beating and cardiac gene expression in hiPSCs (Takahashi et al. 2007). Zhang et al. followed with a more thorough characterization of hiPSC-CMs in vitro (Zhang et al. 2009). They observed spontaneous beating in up to ~10% of hiPSC-EBs, comparable to hESC-EBs (Zhang et al. 2009). From there, investigations of hiPSCs largely followed the work being done on hESCs. The role of Activin/Nodal and BMP signaling in hiPSCs was confirmed (Kattman et al. 2011), and small molecule Wnt and GSK3B inhibitors proved effective in hiPSCs as well (Lian et al. 2012).

In keeping with the move away from serum-containing media, effective cardiac differentiation protocols were established using chemically defined media. Burridge et al. developed a protocol for highly efficient and reproducible differentiation using a low-cost fully chemically defined medium (Burridge et al. 2014). Supplementing the basal medium RPMI 1640 with recombinant human albumin and L-ascorbic acid 2-phosphate and use of sequential CHIR99021 and the Wnt inhibitor Wnt-C59, cardiomyocyte yields as high as 80–95% could be produced. Metabolic selection techniques could further enrich the cells to >95% pure cardiomyocytes (Burridge et al. 2014).

Rao et al. provided further insight into the molecular basis of cardiomyocyte differentiation when they demonstrated that early BMP and Wnt activation in hESCs drives mesodermal induction via cooperative rapid silencing of *SOX2* (Rao et al.

2016). Further, they demonstrated that Wnt inhibition is necessary at the mesodermal stage to restrict MSX1 and CDX2/CDX1 upregulation and allow for cardiac lineage induction. These findings suggest additional targets for modulation to more precisely control hPSC fate in vitro.

Another approach to inducing cardiac differentiation involves the use of cardiac transcription factors to "forward program" the cells. In 2011, Dixon et al. demonstrated that lentivirus-mediated expression of the four transcription factors *GATA4*, *TBX5*, *NKX2–5*, and *BAF60C* (collectively known as "GTNB") in hESCs and hiPSCs could generate 6–12% cardiomyocytes in culture conditions that typically maintain pluripotency (Dixon et al. 2011). Subsequently, similar results were achieved with only the transcription factors *BAF60C*, *GATA4*, and *MESP1* using an electroporation method or even with further reduction to just *GATA4* and *MESP1*, although with lower efficiency (Hartung et al. 2013). Demonstrating potential for cardiomyocyte subtype specification, forward programming using the human *TBX3* gene in mouse ESCs combined with *Myh6*-based antibiotic selection resulted in generation of >80% pure nodal-like cells (Jung et al. 2014), although a similar result has yet to be achieved with hPSCs. These efforts demonstrate a completely separate, and perhaps complementary, avenue for controlling cardiomyocyte differentiation, and it is conceivable that optimal control of cardiomyocyte differentiation and even subtype specification may involve some combination of cytokines, small molecules, and transient transcription factor expression.

4.9 Gene Expression during Differentiation

Human iPSC-CMs exhibit properties of isolated primary cardiomyocytes including expression of contractile proteins such as cardiac troponin T (Takahashi et al. 2007; Kattman et al. 2011; Lian et al. 2012; Burridge et al. 2014; Ye et al. 2014; Zhang et al. 2009), sarcomeric α-actinin (Lian et al. 2012; Burridge et al. 2014; Ye et al. 2014; Zhang et al. 2009), and myosin heavy and light chains (Lian et al. 2012; Burridge et al. 2014; Ye et al. 2014; Zhang et al. 2009); sarcomeric organization (Lian et al. 2012; Burridge et al. 2014; Ye et al. 2014; Zhang et al. 2009); spontaneous action potential generation (Lian et al. 2012; Burridge et al. 2014; Zhang et al. 2009); contraction/electromechanical coupling (Takahashi et al. 2007; Lian et al. 2012; Ye et al. 2014; Zhang et al. 2009); calcium transients; β-adrenergic signaling/responsiveness (Zhang et al. 2009); and intracellular connections via connexin 43 (Ye et al. 2014). Differentiation of cardiomyocytes from hiPSCs progresses in a stepwise fashion consistent with patterns seen in embryonic development and differentiation of hESCs. On induction of differentiation, downregulation of the pluripotency genes *OCT4* (Lian et al. 2012; Burridge et al. 2014; Zhang et al. 2009), *NANOG* (Lian et al. 2012; Zhang et al. 2009), and *SOX2* (Lian et al. 2012; Rao et al. 2016) occurs along with upregulation of mesodermal genes, including *T* and *MIXL1* (Lian et al. 2012; Burridge et al. 2014). Expression of the cardiac mesoderm marker *MESP1* soon follows (Burridge et al. 2014). At the mesodermal stage, *MSX1* and *CDX2/CDX1* are also restricted to allow further progression (Rao et al. 2016). Differentiating hiPSCs have been shown to progress through a cardiac progenitor state (Lian et al. 2012), as indicated by expression of *ISL1* (Lian et al. 2012; Burridge et al. 2014) and *WT1* (Lian et al. 2012). Other early cardiac genes also become

activated around this time, including *KDR* (Burridge et al. 2014) and *GATA4* (Lian et al. 2012; Burridge et al. 2014). The later cardiac markers *NKX2-5* (Takahashi et al. 2007; Zhang et al. 2009; Lian et al. 2012; Burridge et al. 2014), *TBX2* (Lian et al. 2012), *TBX5* (Lian et al. 2012; Burridge et al. 2014), *MEF2C* (Takahashi et al. 2007; Lian et al. 2012; Burridge et al. 2014), and *PLN* (Lian et al. 2012; Zhang et al. 2009) appear between days 3 and 8. Finally, cardiac myofilament mRNA levels increase after day 8. Transcripts for *TNNT2* (Burridge et al. 2014; Zhang et al. 2009), *TNNI2* and *TNNI3* (Lian et al. 2012), *MYH6* (Burridge et al. 2014), *MLC2A* (*MYL7*) (Takahashi et al. 2007; Lian et al. 2012; Zhang et al. 2009), *MLC2V* (*MYL2*) (Lian et al. 2012; Burridge et al. 2014; Zhang et al. 2009), and *ACTN2* (Zhang et al. 2009) have all been demonstrated. Human iPSC-CMs also express the cardiac ion channel genes *HCN1*, *HCN4*, *KCNQ1*, and *KCNH2* (Burridge et al. 2014). In general agreement with these patterns, a recent study using gene ontology on hESCs evaluated 2917 gene transcripts with significant expression changes at four specified time points: hESCs, primitive mesoderm, cardiac mesoderm, and differentiated CMs (Tompkins et al. 2016). The data demonstrated an upregulation of genes for cardiac development, cardiac structural proteins, glycolysis, and mitochondrial oxidative phosphorylation, while mitotic and organelle fission genes were suppressed with differentiation. This finding is consistent with known characteristics of cardiomyocytes. Corresponding with mRNA transcripts, cardiac protein translation has also been confirmed in differentiated hiPSCs, as expected (Takahashi et al. 2007; Kattman et al. 2011; Lian et al. 2012; Burridge et al. 2014; Ye et al. 2014; Zhang et al. 2009).

As myofilament protein expression stabilizes and the cells mature, *ISL1* expression extinguishes by day 30 post-induction (Lian et al. 2012). Likewise, it has been noted that both myosin light chain mRNA and protein expression shifts from predominantly MLC2A early after differentiation to MLC2V by 45–60 days after induction, consistent with progression of the cells from an unspecified or atrial cardiac phenotype to a more mature ventricular phenotype (Lian et al. 2012; Burridge et al. 2014). Markers of the various cardiomyocyte subtypes have been identified in populations of hiPSC-CMs as well, with expression of the atrial-specific genes *NPPA*, *CX40*, and *SLN*, ventricle-specific genes *MLC2V* and *IRX4*, and the nodal gene *TBX18* (Burridge et al. 2014) all observed. This indicates that hiPSCs have the potential to form all of these cardiomyocyte subtypes, while efforts to more precisely control subtype specification are ongoing (see below).

4.10 Large-Scale Production of hiPSC-Derived Cardiomyocytes

Beginning with the earliest hESC cardiac differentiation protocols, there was a consideration for scale-up, with the knowledge that EBs are a suitable format for large-scale bioreactors commonly used in commercial cell production (Burridge et al. 2007). Despite this, monolayer protocols have proved popular as they vastly simplify media changing regimes, do not have the complex requirements of EB formation, and allow for simple assessment of differentiation success (Laflamme et al. 2007). It is now well accepted in the field that monolayer differentiation, commonly performed in six-well plates, does not scale up well past 100 cm^2 surface area. It is theorized that this is due to the small molecule methodologies currently in use being dependent on

paracrine effects, with the CHIR99021-induced GSK3B inhibition allowing Wnt signaling to occur and forming a positive feedback loop with Activin/NODAL/TGFβ, BMP, and FGF production and signaling (Burridge et al. 2012, 2014). In addition, a major barrier to scale up has been the widespread use of prohibitively expensive media, such as mTeSR1 for pluripotent growth (Lian et al. 2012; Zhang et al. 2012) and StemPro-34 (Yang et al. 2008) or RPMI + B27 for differentiation (Lian et al. 2012; Zhang et al. 2012). There are now multiple efforts to replace these media with cost-effective alternatives such as chemically defined E8 for pluripotent growth, which can be made in-house for less than $50 per liter, and various chemically defined media for cardiac differentiation, such as CDM3 (Burridge et al. 2014) costing as little as $11 per liter. Another cost consideration has been the use of growth factors, which become prohibitively expensive at large volumes, although at least for cardiac differentiation, these have largely now been replaced with small molecules. In each case, there has been an obligation to prove that novel media have the minimal number of components necessary to simplify the formulation and optimize results.

There has been significant progress with cardiac differentiation in stirred suspension vessels (Kempf et al. 2016), as hiPSCs easily adapt to suspension-based culture using either cell aggregates (hEBs) or attachment to matrix-coated microcarriers (Lam et al. 2016; Lecina et al. 2010). Both techniques have resulted in efficient differentiation, although microcarriers substantially increase the complexity of handling (Lecina et al. 2010; Lam et al. 2014; Leahy et al. 1999). Early techniques began with FBS-based spontaneous differentiation (Niebruegge et al. 2009), progressed through growth factor-based systems (Chen et al. 2012), and most recently, protocols have been demonstrated adopting the small molecule-based approach using CHIR99021 for 24 h followed by a Wnt inhibitor such as IWP-2 or IWP-4 and media volumes of ~100 ml (Chen et al. 2015; Kempf et al. 2014; Kempf et al. 2015). These techniques primarily involve simple Corning spinner flasks (Chen et al. 2015) or full stirred bioreactors such as the DASGIP platform (Kempf et al. 2014). These methods commonly have a pluripotent culture step of 3–4 days for hEB formation, with seeding at ~0.3–0.5 × 10^6 cells/ml, and result in cardiomyocyte production with purities of ~50–90% TNNT2$^+$ in the 1.0 × 10^6 cells/ml range (Chen et al. 2015; Kempf et al. 2014, 2015). In particular, bioreactors allow constant perfusion of media, with online monitoring of key process parameters such as pH, dissolved oxygen, glucose consumption, and lactate accumulation potentially allowing a reduction in differentiation variability.

4.11 Purification of hiPSC-Derived Cardiomyocytes

Current cardiac differentiation methodologies can achieve purities of ~80–90% TNNT2$^+$ cardiomyocytes, yet there is still considerable variation in differentiation reproducibility and variability between hiPSC lines. Numerous techniques have been adopted to purify cardiomyocytes from contaminating cell types such as fibroblasts, endothelial cells, or smooth muscle cells. One of the first techniques was the use of the mitochondrial membrane potential sensor TMRM, based on the hypothesis that cardiomyocytes have a higher mitochondrial number per cell than the contaminating cell types (Hattori et al. 2010) although this was later demonstrated to be less effective in hESC-CM than

animal-derived cardiomyocytes (Dubois et al. 2011). Also used have been genetically encoded sensors such as *MYH6-GFP* or *NKX2-5-GFP* (Elliott et al. 2011). Considerable effort has been expended to discover unique hiPSC-CM cell surface markers, which has led to the discovery of SIRPA (Dubois et al. 2011) and VCAM1 (Uosaki et al. 2011) as suitable markers, which can be very effective when used in combination (Elliott et al. 2011). Metabolic selection has proven particularly successful, as replacing D-glucose in common media (DMEM) with 4 mM L-lactic acid from around d20 to ~d27 of differentiation can achieve purities of up to 98.9% ACTN2^{+} cells (Tohyama et al. 2013). This simple process is also well suited to suspension cultures (Hemmi et al. 2014). One common approach in commercial hiPSC-CM cell production has been the use of antibiotic selection (Anderson et al. 2007; Ma et al. 2011), using a cardiac-specific promoter such *MYH6* or *TNNT2* linked to a puromycin resistance gene or similar strategy (Kita-Matsuo et al. 2009). With these commercial cells becoming more ubiquitous, this genetic modification has now become acceptable within the industry.

4.12 Cardiomyocyte Subtype Specification

After cardiomyocyte generation, the next major step is determining how to produce the specific cardiomyocyte subtypes found in the heart, such as ventricular, atrial, and nodal. Subtype isolation could prove to be useful for transplantation purposes, as hPSC-derived ventricular cardiomyocytes could be used to repair ischemic damage to the ventricular myocardium following MI. The production of pure isolates of specific subtypes may be critical for the prevention of arrhythmias in therapeutic applications, as injection of a mixed population of hESC-CMs resulted in ventricular arrhythmias in a primate MI model (Chong et al. 2014). Likewise, hPSC-derived nodal cells could be used to restore proper cardiac rhythm and electrical conduction, and hPSC-derived atrial cells could be used to assess drugs which target the atria (Devalla et al. 2015). Common cardiac differentiation protocols produce predominantly ventricular cells with ~15–20% atrial cells and ~5% nodal cells when assessed by patch-clamp electrophysiology (Burridge et al. 2014). The divergence of atrial and ventricular markers between mouse and human models has slowed progress toward the production of specific subtypes. For example, in mice, MYL7 (MLC2A) is expressed in the atria during development, and MYL2 (MLC2V) is expressed in the ventricles, whereas in humans, MYL7 is expressed throughout the atria, ventricles, and outflow tract, and only MYL2 is specific for the ventricles (Chuva de Sousa Lopes et al. 2006). In hiPSC-CMs, the expression of MYL7 decreases from ~d15 to d60 of differentiation, while the expression of MYL2 increases (Burridge et al. 2014; Zhang et al. 2012; Zhang et al. 2015) with little change in the subtype ratios as detected by patch clamp. Reporter gene constructs such as *MLC2V*-GFP have been successfully employed to isolate ventricular cells from hPSCs (Bizy et al. 2013). Similarly, sarcolipin (*SLN*)-tdTomato has been used to isolate atrial cells (Josowitz et al. 2014). Other genes that are subtype specific include *IRX4* (ventricular), *HCN4* and *TBX18* (nodal), and *NPPA (ANF)* and *NPPB (BNF)* (ventricular and atrial). Modification of existing differentiation protocols to skew cardiomyocyte sub-populations has also been successful. Using factors such as the small molecule AG1478 to inhibit neuregulin signaling

can increase the nodal population to ~50% (Zhu et al. 2010). Similarly, inhibiting retinoic acid signaling using the small molecule BMS-189453 increased the ventricular population to 83%, while additional retinoic acid increased the atrial population to 94% (Zhang et al. 2011). In mESCs, *Tbx3* overexpression has been used to increase the population of nodal cells to ~80% (Jung et al. 2014), and *Shox2* has been similarly employed (Ionta et al. 2015).

Measuring cardiomyocyte subtype by action potential morphology derived from patch clamping has been a mainstay of the hiPSC-CM field (Moretti et al. 2010). Recently the reliability of this technique in the cells has been called into question (Du et al. 2015), suggesting that the atrial, ventricular, and nodal designations are in fact based on the arbitrary clustering of continuous variables (Kane et al. 2016).

4.13 Cardiomyocyte Maturation

It is now well established that cardiomyocytes generated from hPSCs by any of the current differentiation protocols are of an immature, fetal type in terms of automaticity, proliferation, metabolism, gene expression, sarcomere organization, shape/aspect ratio, calcium handling, lack of T-tubules, and electrophysiological properties (Yang et al. 2014a) and that these hPSC-CMs can mature with extended time in culture (>80 days) (Lundy et al. 2013). Numerous media-based methodologies have been developed for improving cardiomyocyte maturation. The addition of triiodothyronine (T_3) (Yang et al. 2014b; Ivashchenko et al. 2013), which binds the THRA and THRB receptors, results in the downregulation of MYH7 and upregulation of MYH6 and SERCA2A (ATP2A2), along with numerous ion channels. In addition to T3, the application of dexamethasone has also been shown to increase maturation metrics (Kosmidis et al. 2015). The combination of insulin and dexamethasone (Kim et al. 2013; Wen et al. 2015) or IGF1, dexamethasone, and T3 (Birket et al. 2015a) have been shown to enhance the electrophysiologic properties, bioenergetics, and contractile force of hiPSC-CM.

The lack of Kir2.1 (I_{K1}), an inward-rectifier potassium ion channel, is thought to be a main contributor to the observed immature electrophysiological properties of hPSC-CMs, and forced expression *KCNJ2* that encodes Kir2.1 has been demonstrated to be effective in inducing maturation (Lieu et al. 2013). Other techniques such as adrenergic stimulation, electrical stimulation, stretch, and micropatterning have also been employed to successfully improve maturation (Zhu et al. 2014). In particular, soft surfaces have been demonstrated to hold potential as a simple method for maturation including thick layers of matrix such as the "Matrigel mattress" (Feaster et al. 2015) or a layer of PDMS below Matrigel (Herron et al. 2016).

Adult cardiomyocytes primarily derive their ATP from oxidative phosphorylation, breaking down fatty acids such as palmitate and linoleic acid to acetyl-CoA via beta oxidation. In contrast, hiPSC-CM and fetal cardiomyocytes derive their ATP from glycolysis. Numerous attempts have been made to switch hiPSC-CMs to non-glucose metabolism. One method that has been demonstrated is to replace the D-glucose in the medium with D-galactose, resulting in ATP net-neutral glycolysis and the production of pyruvate. Pyruvate can then be decarboxylated to acetyl-CoA, which can enter the citric acid cycle and cause a shift to mitochondrial metabolism (Rana et al. 2012; Wang et al. 2014). Another approach under active investigation is

to force hPSC-CMs into a mature metabolic profile by combining the D-galactose usage with the presence of oleic and palmitic fatty acids (Rana et al. 2012), allowing the use of beta oxidation.

4.14 Future Directions and Challenges

Although the progress in generating cardiomyocytes from hPSCs in the past decade has been tremendous, a great deal of work remains. To achieve their full clinical and translational potential, the production of hPSC-CMs must be scaled up considerably while simultaneously improving the interline reproducibility of cardiac differentiation. This will no doubt necessitate the discovery of new techniques for growth and differentiation as well as identification of additional culture factors and fine-tuning of doses to control the pluripotent state and steer differentiation more precisely. Along these lines, greater control over cardiomyocyte subtype specification must also be achieved to maximize safety in cell-based therapeutic applications, as well as the reliability of developmental, disease modeling, and drug and toxicity screening data. The identification of ventricular arrhythmias in macaques that received non-subtype selected hESC-CMs post-MI may illustrate the importance of this issue (Chong et al. 2014). While Ye et al. saw no arrhythmias in a porcine MI model with delivery of a 100-fold lower dose of hiPSC-CMs (Ye et al. 2014), the use of smaller numbers of cells undermines the problem of regenerating large amounts of myocardium. Going one step beyond subtype specification, it will also be necessary to demonstrate that the electrophysiologic characteristics of a given hPSC-CM subtype closely resemble those of the intended delivery site (e.g., ventricle), as electrical heterogeneity could also predispose to arrhythmia generation. This leads us to the need to improve our ability to rapidly mature hPSC-CMs to an adult phenotype for most clinical applications. However, maturation is also important for in vitro studies to accurately model certain disease phenotypes, in particular those that present during adulthood such as ARVC and HCM, as well as for generating relevant results from drug and toxicity screening applications. While time in culture leads to hPSC-CM maturation, waiting over 80 days after hiPSC generation and subsequent differentiation is suboptimal for most applications. For all of these challenges, high-throughput screening assays will prove indispensable.

Finally, another area of great effort not touched upon in this chapter involves determination of the best method for delivery of hPSC-CMs to the diseased heart, as well as elucidation of ways to improve long-term cell retention. A constant theme in all cardiac cell therapy attempts to date is the poor long-term engraftment rates. Precise analyses of cell engraftment are difficult in vivo due to the difficulties in tracking all of the delivered cells. However, the few available estimates of PSC retention suggest that rates are as low as <1–2% as few as 2–4 weeks after basic intramyocardial injection (Chong et al. 2014; Ye et al. 2014; Xiong et al. 2013). As noted above, co-injection of hiPSC-derived cardiomyocytes, smooth muscle cells, and endothelial cells through an IGF1-releasing fibrin patch improved retention estimates to about 9% (Ye et al. 2014). While the efficacy of this method needs to be validated using more direct cell tracking techniques, it represents a step in the right direction. Certainly, improvement of cell engraftment warrants ongoing efforts. Considering how remarkably far this field has come in the last 10 years, the

prospects of overcoming these challenges for cardiac regeneration and therapeutic discovery in the near future are very encouraging.

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Compliance with Ethical Standards

Conflict of Interest P.W.B. is on the scientific advisory board of Pluriomics B.V. and a shareholder in Stem Cell Theranostics.

Ethical Approval This article does not contain any studies with human participants performed by any of the authors.

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