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Addressing Variability and Heterogeneity of Induced Pluripotent Stem Cell-Derived Cardiomyocytes

Sherri M. Biendarra-Tiegs, Frank J. Secreto, and Timothy J. Nelson

Abstract

Induced pluripotent stem cells (iPSCs) offer great promise in the areas of disease modeling, basic research, drug development, and regenerative medicine. Much of their value comes from the fact that they can be used to create otherwise inaccessible cell types, such as cardiomyocytes, which are genetically matched to a patient or any other individual of interest. A consistent issue plaguing the iPSC platform, however, involves excessive variability exhibited in the differentiated products. This includes discrepancies in genetic, epigenetic, and transcriptional features, cell signalling, the cell types produced from cardiac differentiation, and cardiomyocyte functionality. These properties can result from both the somatic source cells and environmental conditions related to the derivation and handling of these cells. Understanding the potential sources of variability, along with determining which factors are most relevant to a given application, are essential in advancing iPSC-based technologies.

Keywords

Cardiomyocytes · Cellular microenvironment · Differentiation · Induced pluripotent stem cells · Phenotypic variability · Stem cell heterogeneity

S. M. Biendarra-Tiegs
Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, Rochester, MN, USA

Program for Hypoplastic Left Heart Syndrome-Center for Regenerative Medicine, Mayo Clinic, Rochester, MN, USA

F. J. Secreto
Program for Hypoplastic Left Heart Syndrome-Center for Regenerative Medicine, Mayo Clinic, Rochester, MN, USA

Department of Internal Medicine, Division of General Internal Medicine, Mayo Clinic, Rochester, MN, USA

T. J. Nelson (✉)
Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, Rochester, MN, USA

Program for Hypoplastic Left Heart Syndrome-Center for Regenerative Medicine, Mayo Clinic, Rochester, MN, USA

Department of Cardiovascular Medicine, Mayo Clinic, Rochester, MN, USA

Department of Pediatric and Adolescent Medicine, Division of Pediatric Cardiology, Mayo Clinic, Rochester, MN, USA

Department of Internal Medicine, Division of General Internal Medicine, Mayo Clinic, Rochester, MN, USA
e-mail: nelson.timothy@mayo.edu

Abbreviations

AP	action potential
APD	action potential duration
cGMP	current Good Manufacturing Practice
CNV	copy number variation
DEG	differentially expressed gene
EB	embryoid body
ECM	extracellular matrix
ESA	etoposide sensitivity assay
ESC	embryonic stem cell
ESC-CM	embryonic stem cell-derived cardiomyocyte
FACS	fluorescence-activated cell sorting
iPSC	induced pluripotent stem cell
iPSC-CM	induced pluripotent stem cell-derived cardiomyocyte
mtDNA	mitochondrial DNA
SNV	single nucleotide variation
XCI	X-chromosome inactivation

1 Introduction

Since the original discovery of human induced pluripotent stem cells (iPSCs) in 2007 (Takahashi et al. 2007; Yu et al. 2007), this revolutionary technology has been adopted in a wide variety of settings including disease modeling, drug discovery and toxicity testing, and cell-based therapies (Musunuru et al. 2018; Yoshida and Yamanaka 2017). Much of the power of this platform lies in the ability to produce iPSCs from individuals with unique genetic backgrounds and subsequently differentiate them into a myriad of cell types, including those difficult to obtain by other means, such as cardiomyocytes. This is highly relevant to the pursuit of individualized medicine, including autologous cell-based therapies. Furthermore, the ability to capture variation in the human population and study genotype-phenotype relationships has led to the use of iPSCs in modeling a wide variety of diseases. For example, iPSC-derived cardiomyocytes (iPSC-CMs) have been applied to model diseases including long QT syndrome (Moretti et al. 2010), other channelopathies such as Timothy syndrome (Yazawa et al. 2011), cardiomyopathies (Wyles et al. 2016), ventricular tachycardia (Jung et al. 2012), mitochondrial diseases including Barth syndrome (Wang et al. 2014), and even structural heart disease (Hrstka et al. 2017).

However, while the natural variation captured by iPSCs and their differentiated cardiomyocyte progeny is of great value, heterogeneity and variability of these cellular populations can also be problematic. Ideally, it would be advantageous to produce iPSC-CMs in a reliable and consistent manner. In terms of disease modeling, it is important to be able to understand the relationship between genotype and phenotype without confounding variables distorting the results. Furthermore, for disease modeling it is often desirable to have cells that closely resemble a particular cardiomyocyte subtype, including cells resembling those found in the ventricular or atrial chambers of the heart (Marczenke et al. 2017). This is also vital for reproducibility of the results, an ongoing concern in scientific research (Osterloh and Mulelane 2018). In terms of drug development applications, it is important to be working with iPSC-CMs of a sufficient developmental state to behave in a manner that is predictive of human heart tissue. Cardiomyocyte subtype is a concern in this setting, as well (Denning et al. 2016). In terms of cell-based therapies, it is critical to minimize any safety concerns such as genetically abnormal cells (Merkle et al. 2017). There has also been concern that mixed subtypes of cardiomyocytes could lead to arrhythmias (Liu et al. 2018).

In this review, we discuss sources and types of variability and heterogeneity in pluripotent stem cells and differentiated cardiomyocytes, as well as approaches which have been proposed to aid in retaining pertinent genetic variability while maximizing consistency. While the focus will be on human iPSC-CMs, much of what has been learned from embryonic stem cells (ESCs) or even murine pluripotent stem cells can be applied to the iPSC-CM platform. Ultimately, the goal will be to determine what facets of heterogeneity or variability are or are not permissible to a particular application, and tailor cell production or the study design accordingly.

2 Intrinsic and Acquired Variation in Pluripotent Stem Cell Lines

2.1 Genetic and Epigenetic Abnormalities

One potentially troubling source of variation in iPSC lines is abnormalities at the genetic or epigenetic level. Such variations have been discovered between different iPSC lines, different passages of the same iPSC line, or even different subpopulations within an iPSC culture. These include aneuploidy, chromosomal rearrangement, sub-chromosomal copy number variation (CNV), single nucleotide variation (SNV), variable X-chromosome inactivation, and aberrant DNA methylation. Any of these could potentially result in unexpected cellular properties such as acquisition or disappearance of disease-related phenotypes (Liang and Zhang 2013; Nguyen et al. 2013).

Large-scale studies have provided much insight into the frequency and specific natures of these abnormalities. For example, karyotypic analysis of over 1,700 iPSC and ESC cultures from 97 investigators in 29 labs revealed that for both cell types, approximately 12% of cultures were abnormal. In terms of the types of abnormalities, there were both similarities and differences between iPSCs and ESCs. Trisomy 12 was predominant for both, partial gain of chromosome 12 and trisomy 20q were also seen in both, trisomy 8 was more common for iPSCs than for ESCs, an additional chromosome X was more common in female ESCs, and trisomy 17 was only seen in ESCs (Taapken et al. 2011). In another study, 12 of 38 (~32%) of ESC lines and 13 of 66 (~20%) of iPSC lines had chromosomal aberrations, with 6 iPSC lines having a full trisomy of chromosome 1, 3, 9, or 12 (Mayshar et al. 2010). Rate of aneuploidy has also been shown to increase with higher passage (Mayshar et al. 2010). It has been suggested that karyotypic abnormalities could be derived from culture adaptation, were present in the parental cells, or arose from selective pressure during the reprogramming process (Mayshar et al. 2010). Studies have indeed demonstrated that chromosomal abnormalities are in some cases present in the original somatic cells (Vitale et al. 2012). Reprogramming method and culturing substrate were not found to have a notable role in some studies (Mayshar et al. 2010; Taapken et al. 2011), while some other studies have found that certain passaging methodologies can lead to chromosomal abnormalities (Mitalipova et al. 2005). Karyotypic abnormalities can have functional consequences, as well. For example, spontaneously differentiated normal and abnormal ESC lines demonstrated differences in expression of differentiation-related genes and different propensities for particular lineages (Fazeli et al. 2011).

CNVs have been observed in pluripotent stem cells as well. For example, one study applied single-cell array-based comparative genomic hybridization to reveal notable fractions of both somatic cells and ESCs with diverse megabase-scale chromosomal abnormalities. The authors identified replication break fork collapse and breakage-induced replication as a potential cause, possibly a result of sub-optimal culture conditions (Jacobs et al. 2014). Genomic analysis of 58 iPSC lines from 10 laboratories revealed CNVs that were donor-specific and others that varied between lines from the same donor. There were some genomic loci that were frequently affected, suggesting a basis in the reprogramming process. In some cases, the deletion of tumor suppressors or duplication of cell growth-related genes suggested a survival or proliferative advantage (Salomonis et al. 2016). Another study similarly concluded that CNVs were produced in the reprogramming process and provided a selective advantage, but also found more CNVs in early-passage iPSCs (Hussein et al. 2011). An evaluation of 711 cell lines from 301 healthy individuals reported lower levels of genetic aberrations than had been detected in some previous studies, likely because the authors also had access to donor-matched reference samples and were thus able to identify germline copy number variations. Most of the aberrations they did find were unique to individual iPSC lines, but some alterations were found in

multiple cell lines from the same donor. The number of these alterations was not associated with passage number, donor age, gender, or the results of the quality control assay PluriTest (Kilpinen et al. 2017). The prevalence of SNVs has also been investigated, and one study identified between 1058 and 1808 heterozygous SNVs in each iPSC line examined, with 50% of these being synonymous changes. Since the SNVs were not shared between iPSC lines from the same donor, the abnormalities were deemed likely to have resulted from the reprogramming process (Cheng et al. 2012).

Epigenetic differences between iPSC lines, including variable levels of aberrant DNA methylation, have also been described. For example, a genomic analysis of 58 iPSC lines from 10 laboratories showed that while ESCs and iPSCs were generally indistinguishable at the level of global gene expression, there were notable differences in methylation profiles (Salomonis et al. 2016). Differences in DNA methylation have also been found for iPSCs derived from distinct source cell types (neonatal dermal fibroblasts, adult dermal fibroblasts, and $CD34^+$ cells from peripheral blood mononuclear cells) via different reprogramming technologies, with this heterogeneity reduced after prolonged culture to a more ESC-like DNA methylation state (Tesarova et al. 2016). Conversely, another group found that epigenetic patterns of different iPSC lines were similar to each other and to ESCs, regardless of source cell, although in that case the same reprogramming approach was used for all the iPSC lines. There was some random aberrant hypermethylation observed at early passages, but this was decreased with additional passaging (Nishino and Umezawa 2016). Transcriptional profiling of 317 human iPSC lines from 101 individuals revealed transcriptional variability in Polycomb repressive complex 2 (PRC2) and H3K27me3 targets, which appeared to be independent of genetic background, suggesting that the reprogramming process could be the source. In this same study, some genes showed allelic imbalance while others demonstrated biallelic expression. These patterns were in some cases consistent within individuals, but different across individuals (Carcamo-Orive et al. 2017). This mixture of genes with monoallelic or biallelic expression had previously been seen for both iPSCs (Pick et al. 2009) and ESCs (Kim et al. 2007). One study reported low frequency loss of imprinting in some iPSC lines, which was stable in culture (Hiura et al. 2013). These epigenetic differences can have functional relevance, since it has been shown that epigenetic features can be used to identify iPSC lines with particular differentiation capacities and perhaps even maturation capacity (Nishizawa et al. 2016). For example, histone modifications H3K27me3 and H3K4me3 at lineage-associated and pluripotency genes in ESCs influence developmental potential towards particular lineages (Hong et al. 2011).

The X-chromosome status of female iPSCs and ESCs has also been an area of extensive characterization (Wutz 2012). This was originally described by Silva et al., who showed that ESCs tend to lose XIST RNA expression during culture, leading to three different classes of cells. Class I is pre X-chromosome inactivation (XCI) with a capacity to recapitulate XCI upon differentiation. Class II cells show elevated XIST-positive cells and XCI status. Class III cells have lost XIST expression but still have an inactivated X-chromosome which is not reactivated upon differentiation. Some of these class III lines demonstrate poor spontaneous differentiation in embryoid bodies (EBs) (Silva et al. 2008). Analysis of dozens of iPSC lines has shown notable variation in XIST expression, H3K27me3, and XCI status (Geens et al. 2016; Mayshar et al. 2010; Salomonis et al. 2016). Single cell-derived iPSC clones from the same donor show various states of XCI right after clonal isolation, with both pre- and post-XCI cells within individual colonies (Andoh-Noda et al. 2017). Other studies have similarly noted a mixture of cells with different XCI status in the same passage or even the same colony (Geens et al. 2016; Tanasijevic et al. 2009). In some cases XCI is acquired over time, with no reactivation with repeated passaging (Andoh-Noda et al. 2017). In other cases erosion of XCI in culture has been reported, with this being a stable

condition that cannot be restored by differentiation or reprogramming (Geens et al. 2016; Mekhoubad et al. 2012). One of these studies which reported some reactivation of X-chromosomes additionally noted that clusters of genes in certain chromosomal areas were being reactivated sooner than those in others (DeBoever et al. 2017). Another study, while reporting activation of X-chromosomes in some iPSCs upon reprogramming, did not note any correlation of XCI status to passage number, culture, conditions, or reprogramming method (Bruck and Benvenisty 2011). X-chromosome status can have functional implications for these iPSCs. Loss of XIST in female iPSCs is correlated with upregulation of X-linked oncogenes, downregulated tumor suppressors, accelerated growth rate in vitro, and poorer differentiation in teratomas (Anguera et al. 2012). Developmental genes are also differentially methylated in female iPSC lines with different XIST expression and XCI status (Salomonis et al. 2016).

2.2 Contribution of Source Cells to iPSC Properties

The main sources of genomic, epigenetic, and transcriptional variation between different iPSC lines remains a major question within the field, although a number of studies have helped to provide insight. Transcriptional profiling of 317 human iPSC lines from 101 individuals revealed that ~50% of genome-wide expression variability could be explained by the variation across individuals (Carcamo-Orive et al. 2017). It was even possible to identify expression quantitative trait loci contributing to this variation, which could be conducive to studying variants identified in genome-wide association studies (Carcamo-Orive et al. 2017; DeBoever et al. 2017). Other variables such as donor age, body mass index, sex, ancestry, reprogramming batch and technician, RNA preparation technician, Sendai virus lot, and reprogramming cell source influenced expression variation for only a small number of genes. There were, however, differences in the degree of similarity between iPSC lines derived from the same individual, with Polycomb targets playing a major role in non-genetic variability both within and between individuals (Carcamo-Orive et al. 2017). When genomic analysis was performed on 58 cell lines from 10 laboratories, donor, sex, reprogramming technology, and originating laboratory, but not passage number, were major driving covariates in mRNA, miRNA, and methylation profiling. In regards to methylation profiling, cell of origin played a contributing role but there was no clear connection to differences in somatic methylation profiles (Salomonis et al. 2016). According to an analysis of 711 lines from 301 healthy individuals, between 5 and 46% of variation in iPSC phenotypes including genome-wide assays, protein immunostaining, differentiation capacity, and cellular morphology was due to differences between individuals, and this donor variance was primarily due to genetic differences (Kilpinen et al. 2017).

Other studies have likewise found that genetic differences between individuals are a major contributing factor to variation between cell lines, such as in mRNA levels, splicing, and imprinting (Rouhani et al. 2014). For example, three iPSC clones from the same individual could not be distinguished by transcriptional profiling and functional pathway analysis, and were distinct from ESCs and iPSCs from different donors. These differences between unique donors were retained after differentiation to all three germ layers in embryoid bodies (Schuster et al. 2015). Generated isogenic ESC and iPSC lines have been shown not to have significantly different gene expression in either an undifferentiation or differentiated state, and have little difference in methylations profiles while undifferentiated (Mallon et al. 2014).

On the other hand, the role of epigenetic memory derived from the somatic cell of origin in the variability between cell lines has been more controversial. It is known that different somatic cells have distinct epigenetic profiles, even for the same cell type from different locations. For example, genome-wide DNA methylation and transcriptome data on matched pairs of dural and

scalp fibroblasts showed strong epigenetic memory based on sampling location. More epigenetic variability was observed with age, especially for the scalp-derived cells (Ivanov et al. 2016). However, it has also been found that epigenetic memory is not necessarily a major contributor to transcriptional variation (DeBoever et al. 2017; Rouhani et al. 2014). One study examined matched iPSCs from fibroblasts and blood from multiple donors and observed that lines from the same donor were highly transcriptionally and epigenetically similar, but that different donors had specific transcriptome and methylation patterns that contribute to distinct differentiation capacities (Kyttala et al. 2016). Similarly, in another study very few differences in DNA methylation states or gene expression patterns were detected between iPSCs derived from lymphoblastoid cell lines and from fibroblasts. Again, genetic variation was found to be the largest contributor to differences between different cell lines (Burrows et al. 2016). In fact, if variation between individuals is not corrected for appropriately, transcriptional differences between iPSCs and ESCs and between iPSCs from different somatic tissues of origin seem much larger than in actuality (Rouhani et al. 2014). When considering genetically matched ESC and iPSC lines, genetic background had a larger impact on transcriptional variation than either somatic origin or Sendai virus reprogramming method (Choi et al. 2015). Putting genetic contributions aside, however, iPSCs derived from murine ventricular cardiomyocytes demonstrate a higher propensity to spontaneously differentiate into ventricular-like cardiomyocytes than genetically matched ESCs or iPSCs from tail-tip fibroblasts. This was thought to potentially be due to distinct transcriptomes and DNA methylation, including at promoters of cardiac genes (Xu et al. 2012). Cardiac differentiation efficiency has also been shown to be higher for cardiac progenitor cell-derived iPSCs than for fibroblast-derived iPSCs, possibly due to differential methylation at the NKX2-5 promoter. However, these epigenetic differences decreased with passaging and there were no significant differences in morphology, calcium handling, or electrophysiology of the resulting cardiomyocytes. Moreover, these cells had a similar therapeutic effect in a murine myocardial infarction model (Sanchez-Freire et al. 2014). In order to address this source of variability, our laboratory devised an approach to negate the influence of somatic origin on methylation and transcriptional profiles of the resultant iPSCs, via comparison of murine iPSC clones against a standardized gene expression profile. Expression levels of two pluripotency genes, Oct4 and Zfp42, were identified to indicate increased cardiogenicity regardless of cell source or reprogramming strategy, thus allowing a way to address clonal variability (Hartjes et al. 2014).

The role of somatic cell source in cellular aberrations is another potential concern. Blood-derived iPSCs have been found to be less likely to acquire aberrant DNA methylations than iPSCs from other somatic sources (Nishizawa et al. 2016). In terms of genomic aberrations, it has been determined that an average iPSC line has two CNVs that are not apparent in the originating fibroblasts, although by using more sensitive techniques it can be seen that at least 50% of those CNVs are actually low frequency somatic genomic variants in the parental fibroblasts which are revealed due to the clonal origin of iPSCs. It has been estimated that about 30% of fibroblasts have somatic CNVs (Abyzov et al. 2012). Examinations of SNVs in murine iPSCs have also suggested that most mutations occur prior to reprogramming (albeit at very low allele frequency) and are captured by the clonal nature of iPSCs, although some mutations can occur later on (Li et al. 2015; Young et al. 2012). One study identified 4 somatic mutation classes: clonal, subclonal (which would have arisen during reprogramming or culturing), UV-damage mutations, and copy number alterations. Most point mutations were found to be in areas of repressed chromatin and thus not influence gene expression in iPSCs, although subclonal mutations were associated with altered gene expression to a greater degree. Furthermore, over a third of the genes overlapped by copy number alterations had altered expression. Still, mutations that did not influence gene expression in iPSCs could still potentially have effects in

differentiated tissues, so they should not necessarily be discounted. As for the UV-damage mutations, these were found in ~50% of iPSCs from skin fibroblast. However, the number of mutations in cancer genes was not significantly different than what would be expected by random chance (D'Antonio et al. 2018).

It has become apparent in the past few years that mutations in mitochondrial DNA (mtDNA) can also vary across iPSC lines. Studies have shown that that individual fibroblasts can carry unique mutations, and that mutations in iPSCs can be found in very low levels in parental fibroblasts (and thus may not even be detectable when analyzing whole tissue). These mutations may even be homoplasmic or present in high heteroplasmy. iPSCs from older adults have been reported to exhibit more mtDNA mutations than those derived from younger individuals, and even blood-derived iPSC lines may harbor mitochondrial mutations. These mutations can subsequently lead to defects in metabolic function and respiration (Kang et al. 2016). It was previously shown that while somatic murine cells with high mtDNA mutation load can be reprogrammed to iPSCs, the resultant cells have slower proliferation and differentiation defects (Wahlestedt et al. 2014). Our laboratory reported that low levels of mtDNA mutations in fibroblasts, even from healthy individuals, are detectable following reprogramming into iPSCs. While cardiac differentiation potential was not impacted by mtDNA mutations, this could lead to impaired mitochondrial respiration in iPSC-CMs. Additionally, we observed that a subset of iPSC clones derived from patients diagnosed with mitochondrial disease exhibit low levels of mtDNA heteroplasmy, and thus do provide a representative model system (Perales-Clemente et al. 2016).

3 The Dynamic Transcriptional State of Pluripotent Stem Cells

Of course, the ultimate uses of iPSCs typically involve the differentiation of these cells into a somatic cell type such as cardiomyocytes. The iPSCs must thus be receptive to developmental cues at the time of initiation of differentiation, and progress fully down a desired trajectory. Therefore, in addition to genetic and epigenetic properties, transcriptional heterogeneity of iPSCs at the time of initiation can have a notable impact upon how the cells respond to those cues (Fig. 1).

Recently, it was discovered that iPSC cultures contain two subtypes of cells which differ in morphology, cell-matrix and cell-cell adhesion, pluripotency, and gene expression. Both of these can differentiate into all 3 germ layers, but have different propensities towards these different germ layers when undergoing spontaneous differentiation (Yu et al. 2018a). Along these lines, it had previously been shown that murine ESCs can be described by one of two transcriptional states, and that DNA methylation plays an important role in maintaining these states (Singer et al. 2014). The Nucleosome Remodeling and Deacetylation (NuRD) complex was also found to modulate transcriptional heterogeneity and the expression of pluripotency of genes in murine ESCs, thus controlling response to differentiation signals (Reynolds et al. 2012). These findings suggest that even within a single culture, different cells can have distinct responses to the same differentiation cues. Moreover, they represent a sampling of a much broader body of work describing heterogeneity in pluripotent and development factors, as well as signalling molecules (Singh 2015).

In particular, studies have focused on the concept of heterogenous pluripotency factor gene expression. For example, it has been reported that some ESCs exhibit high Nanog expression levels, while others display levels considerably lower than expected, with the latter group being particularly prone to undergo spontaneous differentiation. Transitions from the high to the low state were modeled to be rare and stochastic, while transitions in the opposite direction were predicted to be frequent (Kalmar et al. 2009). Model simulations have further shown that low-Nanog cells act as an intermediate state to reduce the barrier of transition in the differentiation process (Yu et al. 2018b). However, it has also been suggested that Nanog heterogeneity

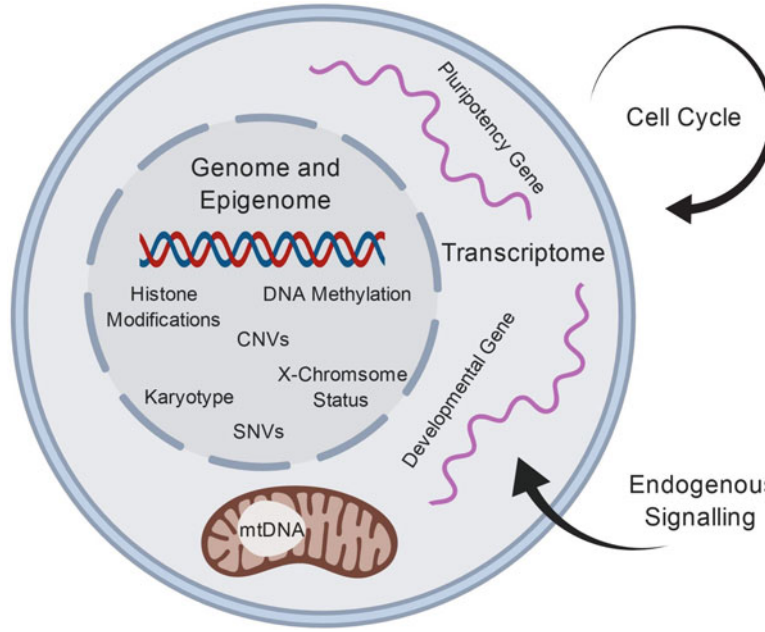

Fig. 1 Sources of variation in iPSCs at the time of initiation of differentiation. Differences in genomes, epigenomes, and transcriptomes between iPSC lines, or even different cells within the same culture, can influence response to differentiation cues. Endogenous signalling and cell cycle position have also been found to exert a noticeable effect upon differentiation trajectories

could be due to specific *in vitro* culture conditions and may not be functionally significant (Smith 2013). The reporters used in such studies can also disturb the cell states they are intended to model. For example, genetic reporters for Nanog can influence behavior of pluripotency-related positive feedback loops and lead to a bifurcation that results in heterogeneous Nanog expression (Smith et al. 2017). Still, some researchers continue to assert that these Nanog fluctuations are real and functionally relevant, based on the fact that similar fluctuations are not seen for some other pluripotency genes, mathematical models have supported bimodal distribution of Nanog, and single-cell RNA seq in murine ESCs suggests bimodal expression (Yu et al. 2018b).

Developmental genes have also been reported to exhibit heterogeneous expression in pluripotent stem cells. For example, Hes1 is a developmental factor that regulates cell proliferation and differentiation in embryogenesis, and along with its downstream gene targets, demonstrates an oscillating expression pattern in murine ESCs. High levels promote mesodermal differentiation whereas low levels promote neural differentiation through modulation of Notch signalling and the cell cycle (Kobayashi and Kageyama 2010; Kobayashi et al. 2009). Modeling approaches have also shown that intrinsic noise in the Hes1 gene regulatory network could explain heterogeneity in murine ESC differentiation (Sturrock et al. 2013). In human ESCs, a Wnt reporter has been used as a read-out of heterogeneity in endogenous Wnt signalling activity, even in cells with similar expression of pluripotency markers. Moreover, the level of Wnt signalling activity in pluripotent stem cells correlates with lineage propensity in differentiation, with high Wnt expression promoting endoderm and cardiac differentiation, and low Wnt enhancing neuroectodermal differentiation (Blauwkamp et al. 2012; Paige et al. 2010).

Interestingly, studies have found that when pluripotent stem cells are exposed to signals which induce differentiation, they activate developmental pathways in an asynchronous manner. Recent evidence has suggested that this is linked to the cell cycle (Dalton 2015). It has been observed that G1 cells are more responsive to differentiation cues, which could help explain heterogeneity in expression of developmental factors. It is possible that developmental genes

are transcriptionally primed in G1 and/or that some pluripotency markers could be diminished in G1, and that a favorable epigenetic and nuclear architecture state in G1 promotes activation of developmental programs (Dalton 2015; Singh et al. 2014). One group used the FUCCI reporter system to show that differentiation capacity of ESCs and iPSCs varies throughout the cell cycle, with early G1 cells having a propensity towards endoderm/mesoderm and late G1 cells tending to differentiate towards neuroectoderm. They found that cells in G2/S/M, on the other hand, responded poorly to differentiation signals. The authors further focused on the differences between early and late G1, and ultimately found that in early G1, level of cyclin D is low, so Smad2/3 can bind and activate endoderm genes, whereas in late G1 cyclin D is high and CDK4/6 is activated and phosphorylates Smad2/3, thus preventing nuclear entry (Pauklin and Vallier 2014). Using the FUCCI system combined with fluorescence-activated cell sorting (FACS) and RNA-seq, other researchers found that heterogeneous expression of developmental regulators in cells which also express pluripotency genes is linked to cell cycle position, with increased expression of these regulators in G1. Major changes in global 5-hydroxymethylcytosine, namely upregulation of 5-hydroxymethylation in G1, were linked to both cell-cycle progression and expression of developmental factors. G1 was seen to be a window of time when the cells could respond to external differentiation signals via gene activation, possibly due to chromatin being in a more permissive state (Singh et al. 2014). Interestingly, it has been reported that when ESCs and iPSCs are cultured with DMSO, this activates the retinoblastoma (Rb) protein, increases proportion of cells in early G1 phase, and improves differentiation efficiency across all germ layers. Such culture manipulation has been used to differentiate cardiomyocytes from an ESC line predicted to be impaired in mesodermal differentiation ability (Chetty et al. 2013). A recent study describing a comparison of various cell cycle inhibitors in human pluripotent stems ultimately identified nocodazole as an efficient and non-toxic means to synchronize these cells in the G2/M phase. This may provide a valuable framework for further investigation into the relationship between cell cycle and differentiation (Yiangou et al. 2019).

Over the past several years, single-cell transcriptional profiling has provided additional insight into heterogeneity of pluripotent stem cells. One such study reported that heterogeneity in expression levels for a number of pluripotency genes was greater in iPSCs than ESCs, and that significant cell-to-cell variability exists even in cells positive for Tra-1-60 and SSEA-4 (Narsinh et al. 2011). From single cell RNA-seq analysis, it has been observed that genes with a higher coefficient of variation in human and murine ESCs form co-expression clusters and partly explain bivalency of gene expression. This data aligns with the idea that pluripotent stem cells alternate between different transient and reversible cell states, although this does not appear to involve lineage priming since genes with a high coefficient of variation were not shown to be enriched for any particular biological process (Mantsoki et al. 2016). Moving forward, it has been suggested that integrative network models—namely gene network models involving epigenetic, transcriptional, and signaling information—from single cell data will be very important for better understanding how self-renewal and differentiation are regulated (Espinosa Angarica and Del Sol 2016).

4 Extrinsic Influences on Pluripotency and Differentiation

Given the extent of reported heterogeneity between pluripotent stem cells, numerous studies have scrutinized the role of extrinsic factors on their properties. One study examining gene expression profiles of 66 iPSC lines found that the lines clustered together according to laboratory and study of origin (Mayshar et al. 2010). Likewise, a reanalysis of microarray gene expression data from seven labs showed strong correlation between gene expression signatures and lab of origin for both ESCs and iPSCs (Newman and

Cooper 2010). Part of this could be due to the culture conditions used. For example, prior to the availability of commercially produced media designed specifically for maintaining pluripotent stem cells, labs employed a variety of in-house developed cell culture media types. Notably, though, a comparison involving eight reported in-house culture methods and two widely available commercial medias (mTeSR1 and STEMPRO) demonstrated that the commercial medias were superior in supporting the maintenance of pluripotent stem cells (International Stem Cell Initiative C 2010). More recently, single-cell RNA-seq of murine ESCs showed enhanced heterogeneity of pluripotency and differentiation marker gene expression for cells cultured in serum as compared to serum free conditions. The most variable of these genes had distinct chromatin state signatures (Guo et al. 2016). A comparison of defined media and media with serum, enzymatic and mechanical passaging, and feeder-free and mouse embryonic fibroblast (MEF) substrates for iPSC and ESC culture demonstrated differences in genomic stability for these different conditions, with more genetic instability in particular for cells subjected to single-cell enzymatic passaging with Accutase (Garitaonandia et al. 2015). Oxygen levels have also been shown to influence properties of pluripotent stem cells, with hypoxic conditions resulting in increased expression of pluripotency markers, reduced chromosomal abnormalities, and reduced transcriptional heterogeneity for ESCs (Forsyth et al. 2008; Lim et al. 2011).

Furthermore, both high and low pH can influence pluripotency of murine and human ESCs, although reports regarding the effects of lactate levels have been more conflicting (Chen et al. 2010; Gupta et al. 2017). One of these reports showed that media acidification due to accumulation of lactic acid from high culture density leads to DNA damage and genomic alterations in ESCs grown on feeders, even over the course of a single passage. This was not seen for a feeder-free system, however (Jacobs et al. 2016). The presence of other metabolites in media can also influence pluripotency. For example, secreted factors from cell culture of ESCs lead to decreased pluripotency marker expression in a system of multiplexed culture chambers (Titmarsh et al. 2013). Build-up of metabolites in media can also influence pluripotency in murine ESCs by priming them for differentiation (Yeo et al. 2013). Some of these effects can be addressed via perfusion culture (Gupta et al. 2017; Yeo et al. 2013). In addition to increasing metabolite levels in media, high density culture results in a higher proportion of cells in G1 (Jacobs et al. 2016; Laco et al. 2018; Wu et al. 2015) and thus could potentially influence differentiation capacity.

Notably, one group found that levels of Wnt fluctuate according to the cell cycle and that higher-density pluripotent stem cells exhibited more cell death and required lower doses of the GSK3β inhibitor CHIR99021 to induce cardiac differentiation. Conversely, cultures consisting of a greater percentage of cells in S/G2/M, along with exhibiting high expression of NANOG and OCT4a, demonstrated an increased propensity for undergoing cardiac differentiation. The authors were therefore able to increase efficiency of more confluent cultures by decreasing concentration of CHIR99021. Ultimately, they discovered that CHIR99021 treatment increased expression of Cyclin D1, promoted cell-cycle progression, and increased genetic instability from acidified media in high-density culture, ultimately leading to cell death. Lower confluence along with increased S/G2/M phase enhanced expression of Wnt inhibitors TCF7L1/2, so those less dense cultures required more CHIR in order to induce higher β-catenin levels via GSK3β inhibition, and ultimately achieve suppression of TCF7L1/2. This then allowed sufficient activation of Wnt target gene expression. Variations in TCF7L1/2 levels and/or cell cycle could thus lead to different differentiation results for the same CHIR concentration. Interestingly, mesoderm development was not found to be as affected by confluency and cell cycle as was full progression to cardiac differentiation (Laco et al. 2018).

In terms of embryoid body (EB) differentiations, outputs can also be influenced by culture conditions, namely colony and EB sizes. Gata6 and Pax6 expression are both impacted by colony size, with

higher input Gata6/Pax6 being connected to more endoderm gene expression. Conversely, there is enhanced mesoderm and cardiac induction at larger EB sizes (Bauwens et al. 2008). Interestingly, the same group later found that efficient cardiac differentiation in EBs is promoted by endogenous extra-embryonic endoderm-like cells which are influenced by aggregate size (Bauwens et al. 2011). A comparison of EB and monolayer cardiac differentiation demonstrated more efficient cardiac differentiation and maturation, as well as homogeneity in cell structure, for the monolayer differentiations (Jeziorowska et al. 2017). Extracellular matrix (ECM) can also potentially influence differentiation ability. For murine ESCs, collagen I and III were individually correlated with decreased cardiac differentiation efficiency, but increased differentiation efficiency when combined. Similar findings were found for the combination of high fibronectin, Wnt2a, and Activin A, suggesting that interactions between growth factors and ECM signalling pathways could modulate stem cell fate (Flaim et al. 2008).

5 The Diverse Nature of Cardiac Differentiations from Pluripotent Stem Cells

5.1 Heterogeneous Cell Populations Resulting from Cardiac Differentiation

Even once iPSCs are successfully differentiated to a cardiac fate, there is still a wide range of variability and heterogeneity in the resultant cell populations. One aspect of this is that cardiac differentiations typically produce a combination of cardiomyocytes and non-cardiomyocytes at varied proportions. There is evidence that these non-cardiomyocytes can impact properties of the cardiomyocytes themselves. For instance, one study reported that when non-cardiomyocytes were removed from a EB-based differentiation of ESCs, development/maturation of electrophysiology and calcium handling were stunted, but these phenotypes were rescued when non-cardiomyocytes were added back (Kim et al. 2010). A second group likewise found that non-cardiomyocytes had an effect upon iPSC-CM electrophysiology and contractility, although they observed optimal properties in several parameters around ~70% cardiomyocytes (Iseoka et al. 2018). However, it is possible that these effects could be cell line-dependent. A study with murine embryonic stem cell-derived cardiomyocytes (ESC-CMs) showed that one line had shortened action potential duration (APD) associated with purification of cardiomyocytes (αMHC+ cells), but another line had a slightly prolonged APD and increased action potential (AP) maximum upstroke velocity when cultured using the same conditions (Hannes et al. 2015). Beyond functional properties, one of these studies also reported an increased proportion of cardiomyocytes expressing ventricular versus atrial myosin light chain for co-cultures with higher cardiomyocyte purity (Iseoka et al. 2018). In an earlier report, it was also found that contaminating non-cardiomyocytes release NRG-1β, which can promote development of working-type (ventricular and atrial) cardiomyocytes (Zhu et al. 2010). Interestingly, one study discovered that BRAF-mutant fibroblast-like cells from cardiac-directed iPSC differentiation promote cardiomyocyte hypertrophy phenotypes via TGFβ paracrine signaling, and that examining purified cardiomyocytes could mask the contributions of non-cardiomyocytes to cardiomyocyte disease processes (Josowitz et al. 2016). This suggests that non-cardiomyocytes may be particularly relevant to some disease modeling applications. There is still much to be learned regarding the interactions between cardiomyocytes and non-cardiomyocytes derived from iPSCs, and this is likely to be an ongoing focus of investigation.

Regarding the cardiomyocytes themselves, there can also be heterogeneity between cultures and within the same culture in when it comes to of properties typically associated with atrial, ventricular, or nodal/pacemaker cardiomyocyte subtypes. Traditionally, it has been asserted that cardiac differentiations produce a heterogeneous population of these subtypes with distinct functional and molecular properties. In order to facilitate

phenotyping of hiPSC-CMs, Kane, et al. have recently proposed a semi-quantitative system for wholistically classifying cardiomyocytes into specific subtypes based on a variety of parameters including AP morphology, gene/protein marker expression, cell morphology, calcium transients, and conduction (Kane and Terracciano 2017).

In terms of electrophysiology, there can be significant variability in APs both between distinct clusters of ESC-CMs and even within the same cluster, with individual clusters frequently having multiple types of APs (Vestergaard et al. 2017; Zhu et al. 2016). Some groups have used signal processing and machine learning to develop platforms to evaluate and classify the electrophysiology of ESC-CMs, and subsequently demonstrated that most cultures exhibit multiple AP phenotypes and even display a continuum of properties between different AP morphologies (Gorospe et al. 2014). However, various studies use different parameters to categorize AP profiles as atrial-like, ventricular-like, or nodal-like, with some researchers questioning whether chamber specificity can be determined via AP morphologies alone (Du et al. 2015; Kane et al. 2016).

One study examined the concordance between electrophysiology and expression of the proposed pacemaker markers HCN4 and Isl1 at Days 40 and 60 of differentiation by acquiring APs of single cells optically, then assessing protein expression via immunofluorescence in the same cell. The researchers saw that HCN4 expression was higher in the cells with pacemaker-like APs initially but that differences decreased with downregulation of HCN4 over time. Conversely, Isl1 expression was initially not different for cells with different AP profiles, but became statistically higher in electrophysiologically pacemaker-like versus ventricular-like cells over time. Therefore, they deemed that neither protein marker was sufficient to identify pacemaker-like cells. Interestingly, they saw that differences in AP properties of the collective groups between Day 40 and 60 seemingly reflected an increase in ventricular- and atrial-like cardiomyocytes, suggesting that subtype may not be determined by Day 40 (Yechikov et al. 2016). Other studies have also found that subtype classification by AP morphology is influenced by time in culture. In one case it was reported that time in culture lead to a transition from nodal-like to ventricular-like APs, with a transient atrial-like phenotype appearing between Days 57–70. That group also performed flow cytometry analysis of cTnT (cardiomyocyte marker), HCN3 (nodal marker), MYL2 (ventricular marker), and MYL7 (atrial marker), which further supported a transition from nodal to atrial/ventricular-like phenotypes from Day 30 to Day 60. Both approaches also revealed some cells with intermediate phenotypes, and ultimately led to the conclusion that AP profiles could not be categorized into three distinct groups (Ben-Ari et al. 2016).

It is possible that culture conditions or microenvironment could further have an impact upon AP properties. For example, AP morphologies of iPSC-CMs seeded at different densities demonstrate distinct distributions, with these differences seemingly not due to gap junction conductance (Du et al. 2015). It has also been observed that similar APs can be found in local regions within clusters of ESC-CMs, with a continuous gradient of AP shapes between regions with distinct AP profiles (Zhu et al. 2016).

5.2 The Quest for Pure Cardiomyocyte Populations

This heterogeneity has led to the development of a variety of approaches to purify cardiomyocytes from the cardiac differentiation process and to enrich for or specifically differentiate cardiomyocytes with the properties associated with a particular cardiomyocyte subtype. These efforts would also aid in addressing variability in cellular distributions between independent differentiations. An early approach to enrich for cardiomyocytes was to use a Percoll density gradient with centrifugation, but this could only enrich to 40–70%(Ban et al. 2017). Mitochondrial staining via the TMRM dye was also proposed fairly early on, but later studies showed that this approach could not robustly discriminate cardiomyocytes early in differentiation from

non-cardiomyocytes and undifferentiated ESCs (Elliott et al. 2011). Other proposed solutions have included expression of a drug resistance gene or fluorescent reporter gene driven by a cardiomyocyte reporter (followed by drug treatment or FACS), but these have the caveat of needing to genetically modify the cells (Ban et al. 2017).

One of the more common, non-invasive approaches is antibody-based enrichment via fluorescent activated cell sorting (FACS) or magnetic-activated cell sorting. Multiple papers from 2011 reported the identification of SIRPA (CD172a) and VCAM1 (CD106) as iPSC and ESC-derived cardiomyocyte cell-surface markers, respectively (Dubois et al. 2011; Elliott et al. 2011; Uosaki et al. 2011). While these markers can be useful, it should be kept in mind that they are not completely specific or selective. One of these papers reported that ~71% of NKX2-5 eGFP^{+} ESC-CMs express VCAM1 and ~85% express SIRPA at Day 14 of differentiation, with only ~37% being dual-positive. Furthermore, only ~67% of VCAM+SIRPA+ cells were also eGFP+, and eGFP+SIRPA+ cells had higher expression of endothelial and smooth muscle markers (Elliott et al. 2011). Later reports have shown that VCAM1 is more highly expressed at earlier stages of differentiation (before Day 25) and that SIRPA expression exists as a continuum, which makes gating based on that alone to be difficult (Veevers et al. 2018).

Another promising non-invasive approach is to take advantage of metabolic differences between cardiomyocytes and non-cardiomyocytes. Differences in glucose and lactate metabolism between non-cardiomyocytes and cardiomyocytes from murine and human pluripotent stem cells allow for cardiomyocyte enrichment in glucose-depleted media with supplementation of lactate (Tohyama et al. 2013). A subsequently-developed protocol involving glucose- and glutamine-depleted media plus lactose was shown to also kill pluripotent stem cells remaining after differentiation (Tohyama et al. 2016). Other methods such as molecular beacons to label cardiomyocyte-specific mRNAs, miRNA-based enrichment, and microfluidic systems are still in relatively early stages of development, but may prove to be useful in the future (Ban et al. 2017).

Likewise, numerous different approaches have been pursued in order to isolate cardiomyocytes with properties of a specific cardiomyocyte subtype. These have included an SLN reporter for atrial-like cardiomyocytes, a cGATA6 reporter for nodal-like cardiomyocytes, and an MLC-2v reporter for ventricular-like cardiomyocytes (Bizy et al. 2013; Josowitz et al. 2014; Zhu et al. 2010). A molecular beacon approach has been investigated in this context as well, namely the use of molecular beacons targeting *Irx4* mRNA in murine ESCs to select for ventricular-like cardiomyocytes. However, a high load of molecular beacons per cell were needed in order to achieve significant signal (Ban et al. 2015). Another group recently used an ESC line for which GFP expression was driven by the MYL2 promoter in order to screen for cell-surface markers of ventricular cardiomyocytes. They found that a CD77+/CD200- population was >97+ cTNI+ with 65% expression MYL2-GFP, allowing for selection of a nearly pure cardiomyocyte population which was enriched for ventricular-like cells. While this approach worked well for the ESC lines they tested, the two iPSC lines they attempted to use interestingly had little-to-no CD77 expression. This enrichment approach was amenable to both EB and monolayer-based differentiations, but with somewhat less efficiency in the monolayer differentiation (Veevers et al. 2018). Other researchers took a unique approach where instead of trying to sort out specific subpopulations, they aimed to identify them *in situ*. To that end, they used subtype-specific promoters (MLC-2v, SLN, and SHOX2) to express a voltage-sensitive fluorescent protein in iPSC-CMs for subtype-specific optical AP recordings (Chen et al. 2017).

Other groups have taken a more developmental biology-informed approach and thereby developed differentiation protocols tailored to the production of particular cardiomyocyte subtypes. Initial work with neonatal rat ventricular myocytes and murine ESCs showed that overexpression of *Tbx18* or *Isl1* transcription

factors was associated with development of the nodal subtype (Dorn et al. 2015; Kapoor et al. 2013). Inhibition of NRG-1β/ErbB signalling can also enhance the proportion of nodal-like cells, as can co-modulation of BMP, RA, and FGF signalling pathways (Protze et al. 2017; Zhu et al. 2010). Interestingly, iPSCs co-cultured with the visceral endoderm-like cell line END-2 produced primarily nodal-like cells, as well (Schweizer et al. 2017).

Protocols have also been proposed for the targeted production of working-type cardiomyocytes. There have been a couple protocols that involved modulation of canonical Wnt signalling by the small molecule IWR-1 in order to produce ventricular-like cardiomyocytes from ESCs and iPSCs (Karakikes et al. 2014; Weng et al. 2014). Gremlin 2 has been reported to upregulate pro-atrial transcription factors and downregulate atrial fate-repressive transcription factors during the differentiation of murine ESCs via stimulation of JNK signaling (Tanwar et al. 2014). More studies, though, have focused on the role of retinoid signaling in atrial verus ventricular development from pluripotent stem cells. Protocols involving retinoic acid treatment can promote atrial-like phenotypes, whereas protocols which include treatment with a retinoic acid receptor antagonist can promote ventricular-like development (Devalla et al. 2015; Lemme et al. 2018; Zhang et al. 2011). A subsequent study showed that atrial and ventricular-like cardiomyocytes develop optimally from specific mesoderm populations (CD235a+/CYP26a1+ for ventricular-like and RALDH2+ for atrial-like), and that these different mesoderms can be specified with different concentrations of BMP4 and Activin A. The RALDH2+ mesoderm responds to retinol to thus make atrial-like cardiomyocytes, since only cells with ALDH expression can synthesize retinoic acid from retinol. Retinoic acid can specify both mesoderms to an atrial fate, but the RALDH2+ mesoderm is more efficient for the production of atrial-like cells. Conversely, without retinoid signalling the RALDH2+ mesoderm can produce ventricular-like cardiomyocytes, but at low efficiency. Importantly, this study also showed that differential cell lines may have variable expression of endogenous Nodal/Activin A and that different cytokine lots can have different activity, and thus optimization of differentiation reagents is necessary (Lee et al. 2017). One group has even used a reporter for the atrial transcription factor COUP-TFII to further enrich atrial-like hESC-CMs from a retinoic acid-directed cardiac differentiation. Interestingly, though, they also found that COUP-TFII was not required for atrial specification of the hESCs, highlighting that the processes associated with development of different cardiomyocyte subtypes have not yet been fully elucidated (Schwach et al. 2017).

5.3 Phenotypic Variability of Cardiomyocytes

Beyond the consideration of different cardiomyocyte subtype-like populations arising from cardiac differentiation, there is also quite a bit of variability and heterogeneity in other aspects of pluripotent stem cell-derived cardiomyocyte properties. A number of studies have focused on evaluating electrophysiological properties in particular, which have been demonstrated to differ between differentiations (with different cell lines or differentiation protocols) for both mouse and human (Hannes et al. 2015; Pekkanen-Mattila et al. 2010). For example, one study reported that there was heterogeneity in electrophysiological phenotypes of ESC-CMs differentiated with two different methods, with approximately one third of cells demonstrating fairly mature electrophysiological properties (maximum diastolic potential < -70 mV and upstroke velocity >140 V/S) but others appearing more embryonic-like (Pekkanen-Mattila et al. 2010). Even cell lines with similar gene expression profiles at the pluripotent cell state can have distinct electrophysiological properties, which was the case for an ESC line and an iPSC line differentiated to cardiomyocytes in one particular study. This comparison revealed great differences in APs and sodium currents at Day 60 of differentiation, with higher sodium currents in the iPSC-CMs and

differential responsiveness to lidocaine and tetrodotoxin. There was also variation in AP frequency and APD, as well as differences in subtype classification between the lines and as a function of time (Sheng et al. 2012). AP profiles can change in numerous ways as the cardiomyocytes undergo maturation with time in culture, due to ongoing development of multiple electrophysiological currents in terms of current density and properties (Sartiani et al. 2007). Such variability can have implications for the application of these cells, where it is often important to elucidate which differences are biologically meaningful. For example, there can be variability in APD and drug responses for iPSC-CMs from LQT3 patients. In response to this, one research group created an *in silico* model to identify plausible mechanisms, and henceforth identified currents with possible differences at baseline or in response to drug treatment (Paci et al. 2017). Culture conditions can have a profound effect upon electrophysiological properties of hiPSC-CMs, which can be a particularly important consideration for drug-screening applications. In one study, it was discovered that more drugs prolonged field potential duration of iPSC-CMs in serum-containing media than in serum-free media, with some drugs also inducing arrhythmias at lower concentrations in the serum-containing media. This was a result of the media formulation impacting both compound availability (dissolved drug concentrations were surprisingly lower in the serum-free media) and baseline electrophysiology (the cells in serum-containing media had longer field potential durations) (Schocken et al. 2018).

Much of the heterogeneity in pluripotent-stem cell-derived cardiomyocytes can be attributed to the maturation status of these cells. With changes in maturation come changes in numerous cardiomyocyte properties including cell morphology (size, shape, nucleation), gene expression, contractility (sarcomere organization, myosin light chain isoforms, troponin T isoforms), electrophysiology (ion channels, APs, cell-cell coupling, conduction velocity), calcium handling, metabolism (including mitochondrial maturity), and proliferation. Numerous different approaches have been taken to modulate and enhance the maturity of these cells and there have been several informative reviews on this topic, including recent reviews by Scuderi et al. and Tu et al. (Scuderi and Butcher 2017; Tu et al. 2018).

One simple approach is to culture the cells for extended periods of time, even months. This can lead to changes in morphology, contractile properties, calcium handling, electrophysiology, and gene expression (Lundy et al. 2013). Culture substrate can also have a notable impact upon cardiomyocyte development, since the use of substrates with physiological stiffness, micro- or nano-patterned surfaces, incorporation of native cardiac extracellular matrix components, and culture in 3D scaffolds can be used to promote advanced maturation (Carson et al. 2016; Fong et al. 2016; Nunes et al. 2013; Ribeiro et al. 2015; Ruan et al. 2015; Tiburcy et al. 2017; Zhang et al. 2013). In an effort to even further mimic physiology, both electrical and mechanical stimulation have been used to promote cardiomyocyte maturation (Mihic et al. 2014; Nunes et al. 2013; Ruan et al. 2015, 2016; Shen et al. 2017). Even increasing the conductivity of the culture system can enhance maturation, for example through the incorporation of trace amounts of electrically conductive silicon nanowires into scaffold-free cardiac spheroids (Tan et al. 2015).

In addition to physical influences upon the cardiomyocytes and their development, chemical influences designed to mimic *in vivo* maturation factors can also be quite impactful. For example, both tri-iodo-L-thyronine and dexamethasone (thyroid and glucocorticoid hormones, respectively) can enhance multiple measures of cardiomyocyte maturation. The combination of the two with a Matrigel mattress protocol is able to promote development of a T-tubule network (Parikh et al. 2017), which has historically been a bottleneck in the maturation of these cells (Scuderi and Butcher 2017). Some miRNAs are also able to impact cardiomyocyte maturation, as was found to be the case for overexpression of Let-7 miRNA family members (Kuppusamy et al. 2015).

A recent examination of cardiac differentiation from human pluripotent stem cells via single-cell RNA-seq was able to provide great insight into

the transcriptional heterogeneity of the cells arising from this differentiation process, in particular revealing the role of HOPX in late stages of cardiac maturation (Friedman et al. 2018). A second recent study applied both single-cell RNA-seq and bulk RNA-seq over the course of a cardiac differentiation of iPSCs, and thereby identified distinct subpopulations of cardiomyocytes which were enriched for specific cardiac transcription factors and represented distinct maturation states. Through a variety of follow-up experiments, the authors furthermore found evidence that two of these transcription factors, *NR2F2* and *HEY2*, can promote atrial and ventricular transcriptional and electrophysiological phenotypes, respectively (Churko et al. 2018). Both of these studies provide a wealth of new information, and the continued use of single-cell RNA-seq will likely provide additional insight into the heterogeneity of iPSC-CMs and allow for generation of new hypotheses regarding how to better control the output of the differentiation process.

6 Looking Ahead: Approaches to Improve Consistency and Reproducibility

6.1 Improving and Validating the Starting Material

Overall, the creation of iPSCs from somatic cells and subsequently differentiation of these cells into cardiomyocytes involves taking mosaic cells from genetically diverse individuals and subjecting them to a wide variety of procedures and environmental conditions over the course of several months. Furthermore, there are no universally defined standards for these processes and culture conditions, which can differ markedly between groups or even individuals (Fig. 2). It should therefore not be surprising that different batches of iPSCs and iPSC-CMs demonstrate considerable variability, and that heterogeneity can even be present within a single population.

Moving forward, there will continue to be a need to reduce undesired variability within the iPSC-derived cardiomyocyte platform, in order to highlight true biological differences that are relevant for the given application. A component of this will be ensuring that the starting iPSCs are of high quality and meet a certain set of desired standards such as pluripotency and differentiation capacity. It has been suggested that one approach to this would be to choose cellular starting material that is less likely to have accumulated mutations or abnormalities (such as multipotent stem cells) (Silva et al. 2015). Quality control assays are also very useful in this regard. The teratoma assay is an established gold-standard for the capacity of pluripotent stem cells to differentiate into all three germ layers. However, it has been shown that it is not necessarily sufficient as a stand-alone means of evaluating pluripotent stem cell quality. For example, one study found that 45/46 evaluated cell lines could form teratomas with all three germ layers, yet 23 of those cell lines had contamination, karyotypic abnormalities, or features suggestive of spontaneous differentiation in culture (Salomonis et al. 2016). It has also been reported that murine iPSCs can demonstrate differences in cardiogenic potential despite a lack of variability in teratoma formation (Hartjes et al. 2014). In response to this, one group created a quantitative scorecard (TeratoScore) based on gene expression data from *in vivo* cell types in order to differentiate pluripotent stem cell-derived teratomas from malignant tumors. This approach could even differentiate between normal and abnormal karyotype (Avior et al. 2015).

A variety of other types of assays are now available to provide additional quality information on pluripotent stem cells. For instance, one group established an unbiased approach to evaluate colony morphology of human pluripotent stem cells using automated live-cell, label-free imaging and analysis algorithms (Kato et al. 2016). The PluriTest was created in order to evaluate pluripotency based on gene expression profiles, using both a "pluripotency score" and a "novelty score", which quantifies how different the gene expression profile of the sample is from the historic data used by the algorithm (Muller et al. 2011). The ScoreCard assay also uses gene

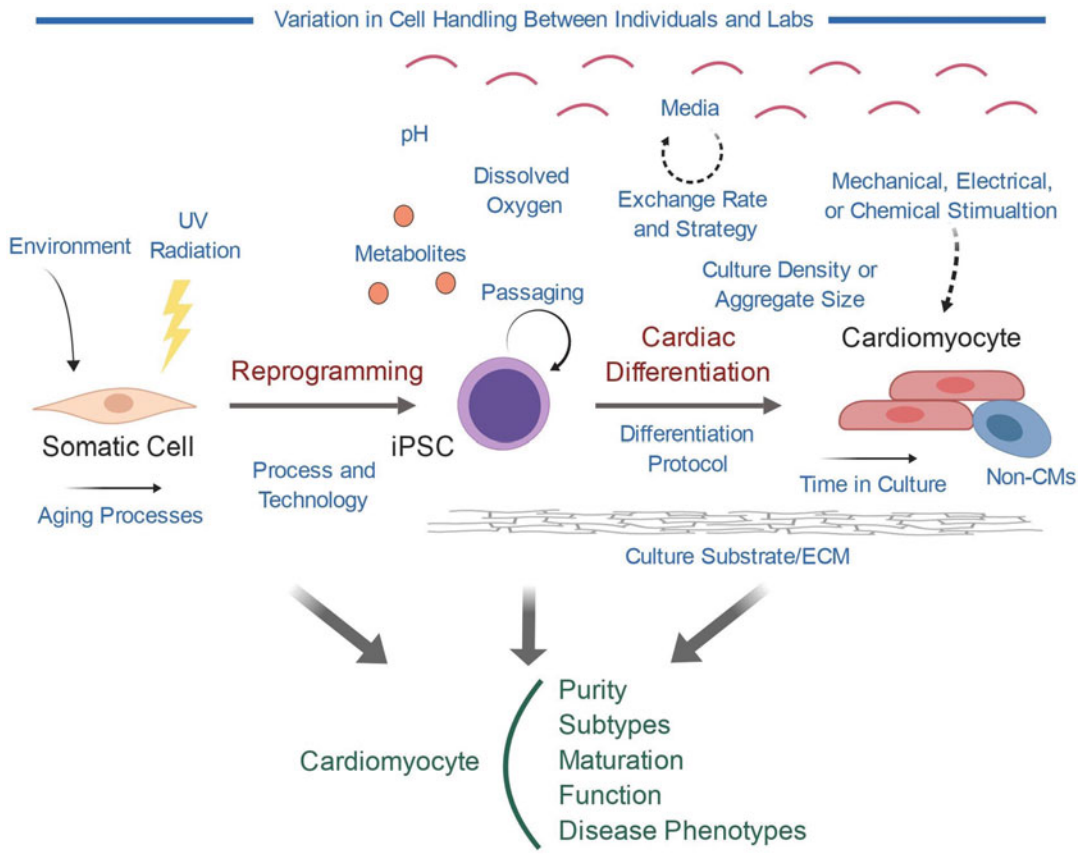

Fig. 2 Role of extrinsic factors in cellular properties. Numerous environmental and technical factors have been shown to influence the molecular and functional properties of somatic cells, iPSCs, and iPSC-derived cardiomyocytes. Altogether, these can ultimately modulate the final cardiomyocyte product and impact its utility for the desired applications

expression signatures, but to determine differentiation capacity (Tsankov et al. 2015). The Etoposide Sensitivity Assay (ESA) developed by our group takes advantage of the fact that pluripotent stem cells are hypersensitive to the topoisomerase inhibitor etoposide, and thus can be used to distinguish good quality iPSC clones from malignant teratocarcinoma clones. This is in contrast to PluriTest, which was shown to be unable to distinguish pluripotent teratocarcinoma cell lines or those with a considerable amount of spontaneous differentiation (Secreto et al. 2017).

Another study, though, highlighted the fact that some of these assays may not be sufficient in isolation. The authors profiled 18 cell lines which had variation in endogenous pluripotency gene expression and other properties, including those which were only partially reprogrammed and had low SSEA4 expression. However, this variability did not have any bearing on other criteria for evaluating pluripotency such as teratoma formation and the PluriTest assay. However, for the lines which did fulfill the most stringent pluripotency criteria, there was low interclonal and inter-individual variability. The authors concluded that thorough analyses of pluripotency are necessary and that proper characterization is vital to be able to distinguish differences between individuals from disease-associated differences (Vitale et al. 2012).

Recently, the International Stem Cell Initiative performed a detailed comparison of several

different quality control assays via blinded analyses by independent experts in iPSCs and ESCs in four laboratories. Of the four methods the evaluated (PluriTest, 'Spin EB' system plus adapted lineage ScoreCard method, histological assessment of teratomas, and TeratoScore assessment of teratomas), all could be used to show pluripotency and each provided some information about differentiation potential. The authors suggested that the particular approach should be chosen based on the final application of the cells. For example, they asserted that a teratoma assay would be vital for cells that are intended for clinical purposes, since only that approach could evaluate both pluripotency and malignant potential (International Stem Cell I 2018).

There has been much focus on figuring out how to maintain pluripotent stem cell quality and consistency through the culture system used, for example by developing fully defined and integration-free conditions for iPSC reprogramming, and implementing pluripotent stem cell culture systems using chemically-defined media, attachment surfaces, and splitting reagents. These approaches have the potential to minimize the batch-to-batch variation that can be seen in culture systems which use serum-containing and Matrigel for cell attachment (Chen et al. 2011). One study reported the creation of current Good Manufacturing Practice (cGMP)-compliant iPSC lines for clinical purposes and tested differentiation capacity into cell types from all 3 germ layers. The authors proposed that the creation of repositories of well-characterized iPSC lines that could be expected to respond predictably to standard differentiation protocols. However, this must be taken with the caveat that they based their assertion that cGMP iPSC lines behave in a predictable manner on only two iPSC lines (Rao et al. 2018). Several chemically-defined differentiation conditions for pluripotent stem cells involving chemically defined medium and small molecules have also been described and subsequently shown to produce reproducible differentiation efficiency across 10+ iPSC lines differentiated repeatedly at multiple passages (Burridge et al. 2014; Lian et al. 2015, 2017). Some researchers even used an albumin-free and chemically-defined medium for ventricular- and atrial-directed differentiations (using either retinoic acid or a retinoic acid inhibitor). They were able to achieve higher efficiency, higher cardiomyocyte yield, and lower inter-experimental variation as compared to differentiations performed using a B27-supplemented medium (Pei et al. 2017).

Significant effort has been devoted in general to the creation of new and improved cardiac differentiation protocols, with the aim of increasing efficiency, yield, and reproducibility. For instance, one early study involved the optimization of >45 different variables for cardiac differentiation of iPSCs and ESCs (Burridge et al. 2011). However, variability between cell lines can hinder the pursuit of universal differentiation protocols, and in some cases modified protocols have been created to enhance cardiac differentiation of specific cell lines which respond poorly to standard protocols (Hrstka et al. 2017; Yassa et al. 2018). Some suggestions have been made as to how to address this challenge in a more systematic way. One group applied a cytokine screening strategy to optimize cardiac output for murine ESC lines with differences in endogenous signaling of Activin/Nodal and BMP (Kattman et al. 2011).

Another group created a high-throughput platform to screen pluripotent stem cells in different microenvironments in order to optimize colony size, cell density, media composition, and substrate, and ultimately quantify endogenous signaling pathways and differentiation bias. They found that endogenous signaling is a major source of variability in how cells respond to exogenous induction conditions, and could therefore use their system to improve differentiation of difficult cell lines, including along a cardiac lineage (Nazareth et al. 2013). A different group focused on addressing the challenges associated with cardiac differentiation which can be posed by high density monolayer culture. They found that using rapamycin (mTOR inhibition) and CHIR99021 together improved efficiency and yield by reducing p53- and DNA damage-depended apoptosis in high density culture through reduction of p53 accumulation and mitochondrial ROS production. A similar effect could potentially be

achieved by hypoxia and control over the nutrients present, instead of rapamycin treatment (Qiu et al. 2017).

6.2 Prioritizing Robust Study Designs and Cellular Manufacturing

Although the variable features of pluripotent stem cell-derived cardiomyocytes can pose a challenge, it should also be kept in mind that these may reflect true biological differences between individuals, an advantage of the iPSC system. For example, not only is beat rate variation present in iPSC-CMs, but heart rate variation can be observed *in vivo* (Binah et al. 2013). Additionally, an examination of ECGs from over 12,000 subjects undergoing routine medical exams for occupation purposes furthermore revealed natural variation in QT interval, down to 335 ms (Gallagher et al. 2006). In the case of diseased populations, electrophysiological recordings from sinus rhythm and chronic atrial fibrillation patients have been shown to exhibit inter-subject variability in AP morphology. Mathematical modeling has been used to determine possible causes of this, and revealing that variability in several different ion currents could modulate variability in APD and triangulation (Sanchez et al. 2014).

Ideally, studies involving iPSC-CMs should be carefully designed in order to highlight relevant differences between healthy and disease states, without being overshadowed by other inter-individual differences. It has been posited that variability among small cohorts of iPSCs could lead to inaccurate conclusions due to inherent differences arising from genetic variability (Kyttala et al. 2016). A number of studies have suggested that it is preferable to utilize cell lines from more individuals, rather than generating and studying multiple iPSC lines from the same individual, in order to differentiate disease mechanisms from the effect of genetic background in disease modeling (Burrows et al. 2016; Rouhani et al. 2014; Schuster et al. 2015).

A recent study concluded that use of more than one clone per individual can actually negatively impact the robustness of findings for transcriptionally-focused studies. Since differences between individuals play large roles in transcriptional variance, comparison of unrelated individuals, as generally done in disease modeling studies, will result in some differentially expressed genes (DEGs) that are not relevant to the disease of interest. They found that using more than one clone per individual actually increased spurious DEGs. While the use of multiple clones per individual can increase sensitivity (although not more than using more individuals), there is a larger loss in specificity. When multiple clones must be used per individual, the authors suggested using analysis methods that take into account the interdependence of the samples, such as an R package that they developed. The choice of controls was found to be another significant issue. An analysis of 77 studies published in 2016 showed that 79% of them used only unrelated controls. This is notable since very few spurious DEGs were found for the comparison of isogenic clones as opposed to a comparison between unrelated individuals. The authors suggested using two clones per individual with a mixed-models approach in order to obtain similar results to the use of isogenic controls, with at least 3 individuals per group in order to reduce false positives. When single clones from unrelated individuals are used, they suggested having at least 4 individuals per group, although having more than 6–7 per group did not improve performance (Germain and Testa 2017).

Other studies have likewise found the choice (or lack-thereof) of controls in iPSC disease modeling studies to be an issue. An analysis of 117 studies revealed that the median and average number of controls in such studies were only 1 and 1.6, respectively, and did not generally account for age, gender, or ethnicity. These authors suggested use of at least 3 controls from 3 separate subjects which are matched for such demographic factors. They proposed that these should be from unaffected family members whenever possible, and when not possible, as many as 12 or more individual donor lines should be used

for controls since it has previously been reported that differences between iPSC and ESCs are negligible when that many lines are evaluated. Furthermore, for differentiated cells it may be essential to compare cells which have been in culture for similar amount of time, in order to reduce effect of maturation-related differences (Johnson et al. 2017).

Some researchers have suggested that when participants in iPSC studies are selected based on the presence or absence of polygenic disease the patients may be genetically heterogeneous and phenotypically variable, thus decreasing statistical power to detect the differences between cases and controls. Instead, they suggested the selection of patients with a known genetic variant with high penetrance and large effect size, or patients with high polygenic risk based on common genetic variants. They proposed an ideal study design with 4 different groups: patients with and without the disease penetrant variant or high polygenic risk, and controls with and without the same. The use of family members as controls could also help to control for genetic heterogeneity (Hoekstra et al. 2017). Finally, regardless of the number of clones per individual used in a study, it may be necessary to produce and screen multiple iPSC lines for chromosomal, nuclear gene, and mtDNA defects, any of which could potentially lead to misleading phenotypes (Kang et al. 2016).

While choice of the number and identity of samples and controls is highly relevant for disease modeling studies involving iPSCs, different considerations exist for the production of iPSCs and iPSC-CMs for therapeutic uses, high-throughput drug screening purposes, or other such applications. In these cases, improving consistency and quality in the cell manufacturing processes is of particular concern. To that end, various approaches to automating aspects of pluripotent stem cell culture have been investigated, including automated approaches to iPSC cell reprogramming, cell seeding, medium changes, passaging, differentiation, imaging, and harvesting (Konagaya et al. 2015; Kowalski et al. 2012; Paull et al. 2015; Serra et al. 2010). Some of these efforts have indeed been shown to reduce well-to-well, plate-to-plate, and line-to-line variability (Crombie et al. 2017; Kowalski et al. 2012; Paull et al. 2015). Automated versus manual cell handling approaches have even been shown to differentially influence expression of pluripotency and differentiation marker expression in iPSCs (Archibald et al. 2016). In addition to adaptation of existing methods to automated approaches, ongoing improvements are being made to the methods themselves. For example, there was a recent report demonstrating that using dextran sulfate during cell seeding was able to control aggregate size and reduce heterogeneity and variability in suspension cultures of pluripotent stem cells. This is due to the fact that greater homogeneity in aggregates allows for more control over nutrient gradients and the prevention of large aggregate formation, in which the cells tend to lose pluripotency and undergo increased apoptosis (Lipsitz et al. 2018). Suspension culture methods will become increasingly valuable as a means to more efficiently produce large batches of pluripotent stem cell-derived cardiomyocytes, and thus improved methods in this area will also be of notable impact.

From a broader standpoint, generating cellular products requires overcoming variability in starting materials, reagents, microenvironment, and stochastic variability. Silva et al. have made some suggestions to help overcome these challenges, such as developing approaches for standardized comparative evaluation of cell product quality during the production process, and the need for robust and scalable standardized platforms for selection, purification, and validation of iPSCs (Silva et al. 2015). French et al. have provided an overview of the types of physical standards (reference materials) which will aid in improving reproducibility and consistency in the creation of differentiated cells from pluripotent stem cells. “Product” reference materials are representative of the product and can aid in evaluating its identity or potency. For example, these could be samples of specific batches, pooled populations from multiple batches, or other cell populations that are biologically equivalent in relevant properties. On the other hand, “method” reference materials, such as fixed cells or RNA

samples, can be used to validate and define criteria for particular assays and perform necessary calibrations (French et al. 2015).

Building upon these ideas, Lipsitz et al. have been proponents of using quality-by-design principles to design cell manufacturing processes, an approach already commonly used by small-molecule pharmaceutical manufacturers. Quality-by-design integrates both scientific knowledge and risk analysis and involves product and process description, characterization, design, and monitoring. It also highlights the need to understand desired characteristics of the end product, attributes that influence safety and efficacy, and what parameters influence those attributes. In the case of pluripotent stem cell-derived cardiomyocytes, potency is now often evaluated via electrophysiological read-outs, but force-of-contraction assays may be of value for applications where they cells are intended to be used as a therapy and ultimately act as new heart tissue. One of the major issues for the use of pluripotent stem cell-derived cardiomyocytes in clinical applications is purity, since nodal cells and non-cardiomyocytes could potentially promote arrhythmias, and undifferentiated pluripotent stem cells can lead to teratomas. Ultimately, these authors highlighted a need to understand the influence of various factors such as dissolved oxygen, pH, metabolic by-products, and media exchange rate and strategy, and then monitor and control them if necessary, with the ability to reduce or at least understand the effect of variability in reagents being of equal importance (Lipsitz et al. 2018).

7 Final Remarks: Strategically Matching Approach to Application

Despite the challenges associated with the derivation and use of iPSC-derived cardiomyocytes, they have proven to be an extremely powerful platform in basic and translational science. Not only have these cells been used to model a wide variety of cardiac diseases, but they show great promise for drug safety testing and have demonstrated efficacy in large animal pre-clinical models (Gao et al. 2018; Ishida et al. 2018; Liu et al. 2018; Musunuru et al. 2018; Yoshida and Yamanaka 2017). Moreover, while there is still much to learn, there has been a progressively detailed understanding of how variability and heterogeneity in the iPSC-CM platform arises, and a number of proposed approaches to further enhance desired characteristics in the final cellular products. However, it should be recognized that it may not be feasible to validate and optimize all possible parameters and cellular properties for every single cell line. Therefore, it will become increasingly necessary to define which criteria are most important for a given study or application, and produce iPSC-CMs with those considerations in mind. This approach of developing purpose-built iPSC-CM products will require identifying which cellular features or functionalities are needed to achieve the ultimate purpose, and choosing the materials, protocols, and quality control measures based on those desired end properties. For instance, disease modeling relies heavily on careful selection of both the patient and control cell lines in order to uncover disease phenotypes and differentiate those from other aspects of inter-individual or inter-clonal variability. Conversely, for drug screening applications, the particular cell lines used may not matter as much as being able to achieve high batch-to-batch consistency.

There must also be an element of being able to balance risk versus benefit, which becomes particularly relevant for more translational or clinical applications. As one reflection of this, it may be appropriate to pursue the first clinical trials of iPSC-CMs in patient populations with severe disease and limited alternative treatment options, since those patients have the potential for achieving the greatest potential benefit despite the risks of a novel therapeutic modality. Then as quality control criteria for achieving maximal safety and efficacy are established, the number of suitable disease indications may grow.

Currently, the systems are in place to successfully create purpose-built iPSC-CMs for many applications. In fact, some of the challenges

associated with these cells can actually be considered assets in expanding their versatility. For example, non-cardiomyocytes can be leveraged to modulate cardiomyocyte properties or reveal disease phenotypes, genetic variability can be used to recapitulate diverse populations *in vitro*, and the ability of these iPSC-CMs to display properties of varied cardiac subtypes and maturation states means that the cells can act as models of distinct regions of the heart across developmental stages. The future will only aid in refining these cells and expanding their utility. Therefore, as the field continues to discover what factors impact cardiomyocyte differentiation, purity, and ultimate phenotypes, as well as develop additional means by which to evaluate and control these factors, the potential of this platform will only grow.

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