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Induced Pluripotent Stem Cell-derived Cardiomyocytes: Cardiac Applications, Opportunities and Challenges

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Abstract

Chronic diseases are the primary cause of mortality worldwide, accounting for 67% of deaths. One of the major challenges in developing new treatments is the lack of understanding of the exact underlying biological and molecular mechanisms. Chronic cardiovascular diseases are the single most common cause of death worldwide, and sudden deaths due to cardiac arrhythmias account for approximately 50% of all such cases. Traditional genetic screening for genes involved in cardiac disorders is laborious and frequently fails to detect the mutation that explains or causes the disorder. However, when mutations are identified, human induced pluripotent stem cells (hiPSCs) derived from affected patients make it possible to address fundamental research questions directly relevant to human health. As such, hiPSC technology has recently been used to model human diseases and patient-specific hiPSC-derived cardiomyocytes (hiPSC-CMs) thus offer a unique opportunity to investigate potential disease-causing genetic variants in their natural environment. The purpose of this review is to present the current state of knowledge regarding hiPSC-CMs, including their potential, limitations, and challenges and to discuss future prospects.

Keywords: hiPSCs , Cardiac tissue , Electrophysiology , Cardiac arrhythmias , Cardiomyocytes, Sodium channels, Na_v

Introduction

Industrialized societies are increasingly confronted with major challenges posed by chronic health-care problems such as cancer, cardiovascular and neurodegenerative diseases and metabolic, pain, and developmental disorders. Accounting for 50% of all sudden deaths, cardiac arrhythmias constitute a major cause of mortality (Estes 2011). One of the major challenges facing the scientific community in their quest to develop novel treatment modalities is the lack of understanding of the exact biological and molecular mechanisms relevant to human diseases that underlie these pathologies whose primary outcome is death or disability. The pivotal finding by Takahashi and Yamanaka (Takahashi and Yamanaka 2006) that human somatic cells (fibroblasts) can be reprogrammed to generate human induced pluripotent stem cells (hiPSCs) was a crucial first step toward exploring mechanisms relevant to human (**Fig.1**). For the first time, hiPSCs became a unique tool for studying chronic diseases in depth. Research on human cell replacement therapy has given rise to two major stem cell-based technologies: human embryonic stem cells (hESCs) and hiPSCs (Bongso et al. 1994; Takahashi and Yamanaka 2006; Thomson et al. 1998), which are invaluable adjuncts for dissecting the complex cellular and molecular events occurring during early human organogenesis. The relative ease with which hiPSCs can be generated from a wide variety of human somatic cells and coaxed to differentiate into various cell types such as neuronal cells and cardiomyocytes has changed the way we study chronic diseases. hiPSCs provide an unlimited supply of healthy and patient-specific derived cells from the same family with the same genetic background. The generation of patient-specific hiPSC-CMs has the advantage to investigate potential disease causing variants in their natural environment. However, hiPSC technology also comes with its own challenges and limitations. The limited availability of human cardiac tissues, the difficulty in maintaining fully differentiated cardiomyocytes in culture, and the circumstantial biopsy availability are barriers to understanding cardiac pathophysiology. However, reprogrammed fibroblasts obtained directly from individuals with a potential disease-causing mutation can now be used to model the disease (Takahashi et al. 2007). Indeed, recent proof-of-principle studies have shown that patient-specific hiPSCs can be successfully differentiated into hiPSC-CMs that reproduce the molecular mechanisms of disorders such as long QT syndrome (Itzhaki et al. 2011; Keller et al. 2003) and Brugada syndrome (Davis et al. 2012), as well as catecholaminergic polymorphic ventricular tachycardia (Itzhaki et al. 2012).

Since hiPSCs capture and retain the genetic background of a patient, they are well suited for developing:

1) Disease models: hiPSCs can be coaxed to differentiate into various cell types, including cardiomyocytes, which as mentioned above are usually available in limited quantities. Post-mortem heart tissues are not always available and often embody the end stage of cardiac disease, combining the consequences of the mutation and the consequences of pathological secondary remodeling. On the other hand, murine and rodent models do not fully recapitulate the human phenotype. In addition, molecular pathways in human and rodent tissues can be different (Zimmer et al. 2014). In the other hand, murine models do not fully recapitulate the human phenotype. In addition, molecular pathways in human and rodent tissues can be different. For example, when focused only on the example of Na_v channels, important differences of Na_v channels expression have been demonstrated in mouse when compared to human (Zimmer et al. 2014). Since Na_v isoforms possess their own biophysical characteristics, changing their balance in the heart could be expected to affect studied parameters (pharmacology, electrical signals, and protein partners). Dissociated mammalian cardiac myocytes however constitute cells coming from an extremely rich and complex environment. Such model potentially represents the closer model to physiological conditions, well suited notably for short term studies on morphological aspects or contractile parameters. Similarly, studies focused on neonatal rodent cardiomyocytes benefit from close physiological conditions. However, when combined to the species specificities, their immaturity (with morphological and functional differences) is a limitation. Immortalized animal cardiac cells such as HL-1 or H9C2 cells, constitute other models used to study physiological or pathological cardiac mechanisms (Claycomb et al. 1998; Kimes and Brandt 1976). HL-1 cells were initially presented as spontaneously beating atrial derived from AT-1 cells (cells from an atrial tumor) (Claycomb et al. 1998). H9C2 quiescent cells derives from rat embryonic heart tissue but are considered as myoblasts since these cells fuse to form multinucleated myotubes as shown in the original publication (Kimes and Brandt 1976). Even if HL-1 and H9C2 cells can be easily replicated, maintained for very long times, and display electrical properties as same as more or less organized contractile proteins, they necessarily present important limitations due to their animal origin. Furthermore, extensive morphological and functional alterations rapidly occur after cell isolation (Bugaisky and Zak 1989; Viero et al. 2008). Such distance with native physiological conditions is thus expected for these

immortalized cell. This is why disease models using patient-derived hiPSCs are needed to study human diseases pathophysiology. Moretti et al. generated patient-specific hiPSCs from members of a family affected by long-QT syndrome type 1 (LQT1) and induced them to differentiate into hiPSC-CMs. They showed that patient-derived cells recapitulated the electrophysiological characteristics of the disorder (Moretti et al. 2010), which led to a better understanding of the molecular mechanisms underlying the pathology and a proof-of-concept that validated the relevance of this cellular model.

2) Drug screening: hiPSCs are a superior alternative to conventional toxicology and drug tests. They are also better suited for safety assessments since they provide a genetic background that recapitulates human physiological conditions, thereby ideal for drug screening. For instance, hiPSCs differentiated into hiPSC-CMs *in vitro* can be used for cardiac toxicity tests (Rajamohan et al. 2013). hiPSC-CMs have also been used to study drug-induced QT prolongation (Braam et al. 2010; Raschi and De Ponti 2012). In addition, screening drugs for toxicity against transformed hiPSC derivatives harboring the same genetic background as the source patients will accelerate the investigational new drug (IND) filing process for clinical trials (Fermini et al. 2016). Furthermore, translational applications of hiPSC technology show potential for developing short therapeutic decoy biologics such as small molecule therapeutics, antisense oligonucleotides, and specific antibodies. In addition, hiPSC-CM have recently been made commercially available, which constitutes an easy way to access to hiPSC-CMs, while some issues can still exist. Indeed, commercial hiPSC-CMs are in some cases genetically modified for a purpose of purification or could also be derived from a clone which demonstrated a more efficient differentiation capabilities (Ma et al. 2011).

3) Disease treatments: hiPSCs offer better hope for the field of regenerative medicine, (tissue replacement, grafts, and cell therapies). A recent study provided evidence that iPSC-CMs transplanted into cynomolgus monkeys (*Macaca fascicularis*) show no signs of immune rejection and become electrically coupled with host cardiomyocytes (Shiba et al. 2016). However, the authors observed an increase in ventricular arrhythmias in hearts harboring the transplanted iPSC-CMs, suggesting that more studies are required to prevent the unintended outcome, arrhythmias. Transplanted nodal cells derived from hiPSCs may function as biological pacemakers. In this respect, two pilot studies have reported promising results using embryonic stem cell-derived cardiomyocytes (Kehat et al. 2004; Xue et al. 2005). Lastly, hiPSC cultures

coupled with genetic editing of mutations using Clustered regularly-interspaced short palindromic repeats (CRISPR)/Cas9 or transcription activator-like effector nucleases (TALENs) technology are opening the way to the development of innovative cell therapies and regenerative medicine (Te Riele et al. 2017).

4) Personalized medicine: hiPSC-CMs are well suited for identifying the responses of several drugs to specific known markers, and may help improve the personalization of treatments. This is of particular importance for cardiac diseases. Prospective evaluations of the most efficient treatment using a patient's own cells are just emerging. Getting deeper in the personalized medicine will however require further studies aimed to demonstrate that genetic modifications and maintenance conditions do not alter the phenotype and usefulness of hiPSCs.

Previous differentiation techniques: Obtaining cardiomyocytes from stem cells is a daunting challenge. Protocols were initially developed to study stem cells and the physiological processes that occur during the commitment and differentiation of cardiomyocytes (Doetschman et al. 1985; Maltsev et al. 1993). The very first technique relied on the formation of stem cell aggregates called embryoid bodies (EBs) (Doetschman et al. 1985; Maltsev et al. 1993). Many procedures to generate EBs were subsequently developed, including hanging drops, cell spinning, and specifically designed microwells (**Fig. 2, Panel A**) (Mohr et al. 2010; Mummery et al. 2012). hiPSC-CM differentiation using EBs has been used for many years despite several limitations. Since EBs are usually used to initiate the formation of the three germ layers (endoderm, ectoderm, and mesoderm), these protocols provide low levels of hiPSC-CM purity. This may be due to the presence of diffusional barriers in EBs, which prevent the formation of homogenous concentrations of molecules and compounds near the cells. hiPSC-CM differentiation protocols using EBs have since been refined, and high differentiation efficiencies have been reported (Burridge et al. 2011), although these protocols still produce tightly packed cells in culture dishes. As such, after several weeks of culture, hiPSC-CMs are often hard to work with because it is difficult to dissociate and isolate the cells. hiPSC-CMs have also been differentiated by co-culturing mouse stem cells with visceral-endoderm-like cells such as END-2 cells (**Fig.2, Panel B**) (Mummery et al. 1991). This technique has since been adapted for the differentiation of human stem cells but, like the EB method, differentiation efficiency is poor and cell clusters often need to be micro-dissected and re-plated before use (Mummery et al. 2003).

The co-culture technique may be suitable for early differentiation tests since it only requires a few cells and can be performed using feeder-cultured stem cells (Mummery et al. 2012). However, this technique is rarely used due to major drawbacks.

Currently used differentiation technique: Although the differentiation techniques described above are still in use, they have been largely replaced by the more recent monolayer differentiation method (**Fig. 2, Panel C**) (Burridge et al. 2014; Denning et al. 2016; Lian et al. 2013; Moreau et al. 2017), which relies on the differentiation of single cell passaged hiPSCs that eventually form a more or less confluent 2D cells layer. This method seems to be a refined and more user-friendly hiPSC culture technique. Although hiPSCs were initially cultured on feeder cells, with daily changes of homemade medium or MEF conditioned medium, major R&D investments resulted in the commercialization of more convenient culture products. hiPSCs are now usually cultured in feeder-free conditions using commercial media and are often single cell passaged, meaning that the daily cleaning of hiPSC cultures is no longer required. 2D cultures also ensure homogenous exposure to the small molecules and compounds required for the differentiation process, ensuring a more uniform and reproducible differentiation of hiPSC-CMs. After a pre-differentiation step (usually ranging from 3 to 5 days) consisting of single cell passaging and plating of hiPSCs, differentiation is initiated. This differentiation process is based on the modulation of the Wnt pathway to drive hiPSCs toward mesodermal specification and then to cardiomyocyte differentiation (Burridge et al. 2014; Denning et al. 2016; Kadari et al. 2015; Lian et al. 2012; Lian et al. 2013). Although several factors have been reported to modulate differentiation into cardiomyocytes, the Wnt pathway appears to be an important player throughout the process (Burridge et al. 2012). Early differentiation steps require Wnt activation to obtain mesodermal cells. However, Wnt must be inhibited later in the process to allow the generation of cardiac progenitors. The culture media used in these protocols are insulin-depleted since insulin inhibits early mesodermal specification. There are two main approaches for modulating the Wnt pathway: (1) the addition of factors such as BMP4, FGF2, activating A, or ascorbic acid to the medium; and (2) the addition of small molecules such as CHIR99021, Wnt-C59, IPW2, or IPW4 (Denning et al. 2016). Although both approaches modulate the Wnt pathway and lead to identical hiPSC-CM differentiation, small molecules are less expensive, slightly more efficient, and generate more reproducible results (Denning et al.

2016; Lian et al. 2012). This protocol generates a contractile monolayer that initiates beating after 7 days in culture. Since the cells form a 2D structure, the hiPSC-CMs are easier to work with compared to the 3D structure formed using EB techniques. A simple enrichment process based on a glucose-depleted medium supplemented with lactate has also been developed (Kadari et al. 2015; Tohyama et al. 2013). The differentiation efficiency using this protocol (with or without enrichment) ranges from 70 to 90% (Burridge et al. 2014; Denning et al. 2016; Hinson et al. 2015; Lian et al. 2012; Lian et al. 2013). The 2D structure also avoids the need to micro-dissect beating areas and provides more cells that can be maintained in culture for several months (Bedada et al. 2014). Producing such high differentiation efficiencies using a relatively simple procedure has completely changed the way hiPSCs are handled and has opened the way for more extensive use of hiPSC-CMs. However, it is important to remember that each hiPSC line or clone must be fine-tuned prior to differentiation. Even if these protocols become more accessible, the maintenance, differentiation, and handling of hiPSCs remain complicated and require a great deal of experience.

hiPSC-CM populations: As mentioned earlier, several different differentiation protocols are commonly used to differentiate hiPSC-CMs (Burridge et al. 2014). Except for the monolayer process, hiPSC-CM differentiation is usually not very efficient and does not generate large numbers of cardiomyocytes (Mummery et al. 2012). Since only 20 to 40% of cells are hiPSC-CMs, the remaining 60 to 80% are considered as "contaminant" cells. These differentiation protocols are not designed to specifically enhance mesoderm specification and cardiomyocyte production. They thus result in the generation of many different cell types from the endoderm and the ectoderm (Doetschman et al. 1985). In addition, beating hiPSC-CM clusters need to be micro-dissected, making the experimental procedure more difficult and time-consuming (Moretti et al. 2010). On the other hand, the monolayer-based differentiation technique often results in high differentiation efficiencies ranging from 60 to more than 90% (Burridge et al. 2014; Denning et al. 2016; Hinson et al. 2015; Lian et al. 2012; Lian et al. 2013). This technique relies on mesoderm specification, limiting the sources of "contaminant" cells and resulting in a more homogenous cell population.

Given the diversity of cardiac cells types, studies on hiPSC-CM differentiation have shown that nodal-, atrial-, and ventricular-like cells can all be generated (**Fig. 3**) (Bett et al. 2013; Burridge

et al. 2014; Lian et al. 2012; Ma et al. 2011; N. Sun et al. 2012; Yechikov et al. 2016), although ventricular-like hiPSC-CMs usually predominate (Burridge et al. 2014; Lian et al. 2012; Yechikov et al. 2016). Specific cardiac cells subtype diversity has also been reported to evolve throughout the differentiation process. The ratio of nodal-, atrial-, and ventricular-like cells appears to be balanced during early differentiation, while ventricular-like cells predominate in older cell cultures (Burridge et al. 2014; Lian et al. 2012; Yechikov et al. 2016). It is important to note that there is no consensus regarding the classification of hiPSC-CMs into subtypes. Indeed, Du et al. recently proposed that differences in action potential characteristics do not depend on cardiac chamber specificity but rather on the density of the plated hiPSC-CMs (Du, Hellen, Kane, and Terracciano, 2015). They also published a comment to the editor questioning the existence of different chamber-specific hiPSC-CMs (Kane, Du, Hellen, and Terracciano, 2016). However, a very elegant recent study that combined electrophysiology, single cell RT-PCR, and optical action potential recordings showed that action potential characteristics are related to the expression of ventricular-, atrial-, and nodal-specific markers (Chen et al. 2016). Chen et al. notably used a fluorescent voltage sensor domain driven by chamber-specific promoters such as the myosin-light chain 2v (mlc2v, for ventricular cells), sarcolipin (SLN, for atrial cells), and short stature homeobox 2 (SHOX2, for nodal cells) to verify whether the voltage sensor domain is expressed in the appropriate cell subtypes. They then compared action potential characteristics and specific cardiac chamber markers using single cell PCR. While this does not constitute definitive proof, this study clearly showed that cardiac chamber-specific hiPSC-CM subtypes do exist (Chen et al. 2016). However, given the varied action potential morphologies and characteristics, appropriate and robust methods to compare chamber-specific hiPSC-CM subtypes are required. Indeed, when plated at the same density, different hiPSC-CMs (controls and patient-specific, for example) may still display differences in their action potential characteristics. As highlighted by Kane et al. hiPSC-CM classifications are often based on subjective arbitrary cut-offs (presence of a plateau phase or the shape of the depolarizing phase). It is, however, possible to use a mathematical approach such as the Calinski-Harabasz criterion, to objectively analyze hiPSC-CM clusters (**Fig. 4**). Other similar objectives and automated frameworks have been developed to separate hiPSC-CM populations into clusters (Gorospe et al. 2014). These approaches make it possible to analyze several clusters of cells at the same time based on the input parameters. Cluster numbers and types can then be attributed to cardiac-

specific chambers. Although such extrapolations rely on subjective interpretations, this does not invalidate the comparison of action potentials.

Lastly, inducing cardiac chamber-specific differentiation or efficiently sorting specific hiPSC-CM subtypes are two other ways to avoid heterogeneous hiPSC-CM populations and eliminate "contaminant" cells that can bias results. Cell sorting is used in immunofluorescence studies where, for example, *mlc2v*, *mlc2a*, and *HCN4* are often employed as ventricular-, atrial-, and nodal-specific markers, respectively (Chen et al. 2016; Moreau et al. 2017). However, this approach is terminal in that the cells are fixed and permeabilized, precluding further functional work. A second approach involves genetic modifications to drive the expression of a fluorescent protein under the control of a chamber-specific marker (Maass et al. 2015). Although this approach is efficient and the cells can be subsequently sorted, it requires major genetic modifications that prevent its use on a regular basis. A third and simpler approach involves the surface expression of chamber-specific markers such as $K_v1.5$ and $K_{ir3.1/3.4}$ (atrial markers), *HCN4* (ventricular marker), or *CD166* (nodal marker), which are used to identify and sort a particular cell subtype. However, specific surface markers are not available for all chamber-specific subtypes. A fourth approach involves directed differentiation for generating specific cardiac-chamber cell subtypes. Several studies based on the use of hESCs rather than hiPSCs have shown that it is possible to specifically differentiate atrial-like cardiomyocytes using retinoic acid (Birket et al. 2015; Devalla et al. 2015; Zhang et al. 2011). Other studies have generated mostly ventricular-like cardiomyocytes while others have shown that the modulation of the neuregulin-1 β /endothelin (NRB1 β /ErbB) pathway in hESCs promotes the formation of nodal-like cardiomyocytes (Zhu et al. 2010). These experimental approaches have also made it possible to work with cardiac Purkinje cells. Usually, only ~2% of cardiomyocytes derived from hiPSCs are considered to be Purkinje cells based on *contactin2* expression (Maass et al. 2015). However, modulating cAMP levels raises the proportion of Purkinje cells to ~20% (Tsai et al. 2015).

hiPSC-CM maturation: The hiPSC-CM maturation process is an important step following the initiation of hiPSC-CM differentiation and before carrying out experiments. Once differentiated, hiPSC-CMs can be kept in culture for several months (Bedada et al. 2014; Kamakura et al. 2013). During differentiation, hiPSC-CMs rapidly express contractile proteins (**Fig. 5**), but the

cells undergo important morphological and functional changes. Action potential morphology is one of the markers attesting to these changes. Several studies have reported that action potential diversity is modified by the time spent in culture and that shorter action potentials tend to make way for longer action potentials with longer culture time (Burrige et al. 2014). The β -adrenergic stimulation pathway also undergoes major modifications during the differentiation process (Jung et al. 2016). Longer culture times are known to greatly improve the morphology and function of hiPSC-CMs (Kamakura et al. 2013; Lundy et al. 2013). Lundy et al. reported that hiPSC-CM circularity decreases with aging and is accompanied by an increase in myofibril density and organization (Lundy et al. 2013). While the myofibrillar M-band is usually not observed in hiPSC-CMs, Kamakura et al. reported that it can take up to one year to observe these structures in some hiPSC-CMs (Kamakura et al. 2013). With aging, action potentials display a faster depolarization slope, a hyperpolarized maximum diastolic potential, and an increased amplitude while the spontaneous beating rate of hiPSC-CMs decreases. Aging also increases the contractile performance of hiPSC-CMs (Lundy et al. 2013). These characteristics are usually indicative of a good maturation process, with cells evolving toward an adult cardiomyocyte phenotype. It is however, important to point out that even though hiPSC-CMs are often characterized as immature cells with fetal or neonatal cardiomyocyte properties, their real maturation stage remains unknown. Since these cells are generated *in vitro*, it is difficult to attribute a specific maturation stage to them. It is also possible that their properties are unique, and that their exact maturation stage cannot be determined in physiological conditions. However, it is important to study and describe them as precisely as possible in order to assess their relevance as a cellular model. For example, while action potentials can be recorded from hiPSC-CMs, some ion conductances differ from those of native cardiomyocytes. For example, K_{ir} channel expression is lower in hiPSC-CMs than in native cardiac cells (Bett et al. 2013; Denning et al. 2016). This could explain the more depolarized resting membrane potential since K_{ir} channels mediate the I_{K1} currents that are responsible for the late repolarization phase and the setting of the resting membrane potential (Denning et al. 2016; Fermini et al. 2016; Sun and Nunes 2016). Increased expression of K_{ir} channels also affects beating automaticity, promoting a quiescent state (Lieu et al. 2013; Vaidyanathan et al. 2016). Furthermore, hiPSC-CM action potentials display a slower depolarization phase than those of native cardiomyocytes while hiPSC-CMs robustly express sodium channels, with densities similar to native cardiomyocytes (Denning et al. 2016; Ma et al.

2011; Moreau et al. 2017). The lower dV/dT can also be explained by a partial lack of an I_{K1} current and the associated depolarized resting membrane potential, which inactivates a fraction of Na_v channels. There are also potential differences between the Na_v channels of hiPSC-CMs and native cardiomyocytes. In addition to a strong (dominant) expression of cardiac-specific $Na_v1.5$ channels, hiPSC-CMs also express neuronal $Na_v1.7$ channels, which likely account for the fraction of tetrodotoxin-sensitive Na^+ currents in hiPSC-CMs (Moreau et al. 2017).

hiPSC-CMs clearly possess characteristics that differ from native human cardiac myocytes. To minimize these differences, many maturation procedures have been proposed (Denning et al. 2016; Ribeiro et al. 2015; Sun and Nunes 2016; Veerman et al. 2015). These procedures include aging, electrical stimulations, applied stretching, the use of chemical compounds, co-culturing with non-cardiac cells, overexpressing cardiac proteins, and modulating the cell support/matrix (3D support, micro-patterning, stiffness) (Veerman et al. 2015). Improvements to the maturation status of hiPSC-CMs depend on the method used. Studies rarely describe potential benefits for all major characteristics (morphology, electrical signals, and contractility). This might be due to the fact that the human heart is a rich and complex environment difficult to reproduce *in vitro*. The maturation status can be further improved when several techniques such as biological wires and electrical stimulations are combined (Sun and Nunes, 2016). Unfortunately, there is no consensus on an optimal maturation process. The methods are often costly and difficult to apply routinely to large numbers of hiPSC-CMs. In addition, hiPSC-CMs are mainly used to model diseases and to better understand the molecular mechanisms involved. A balance between the maturation status and the ability to work with the cells should be forthcoming in the near future. Several maturation procedures lead to tightly packed hiPSC-CMs, resulting in cells that are difficult to dissociate. Culturing hiPSC-CMs with non-cardiac cells to mimic a physiological environment would be a more natural way of promoting or inducing maturation. In addition to efforts to fine-tune the maturation process to obtain the required proportions of different cells, major efforts are being made to enhance the purity of hiPSC-CMs. It should also be remembered that the addition of non-cardiac "contaminant" cells, even if controlled, might lead to unanticipated problems.

Conclusions:

Although the differentiation efficiency of hiPSC-CMs has increased considerably with succeeding generations of differentiation protocols, the issue of cardiac cell heterogeneity remains. Indeed, while 80 to 90% of cardiac cells are expected when using these protocols, it is still not possible to robustly guide cells toward a specific ventricular, atrial, or nodal phenotype. Furthermore, so far, no reliable specific surface markers have been identified that can help in efficiently purifying a given cardiac cell subtype.

The second main challenge is the maturation stage of hiPSC-CMs. Several cellular characteristics indicate that cardiomyocyte-like cells do not reach an "adult" maturation stage but more likely display a fetal-like stage. These characteristics notably include cellular morphology, depolarized RMP and spontaneous contractile activity, low expression of several K_v channels, and expression of cardiac troponin isoforms. However, hiPSC-CM technology is constantly evolving and improving, and a large number of techniques to improve the maturational status of hiPSC-CMs have been proposed, including modulating the form and stiffness of the culture substrate and investigating various chemical compounds. Collectively, there are indications that, in the near future, reliable consensus techniques will provide an efficient way to provide hiPSC-CMs with an "adult" maturation stage.

Current research projects should, however, focus on the strengths of hiPSC-CMs while acknowledging the associated pitfalls in order to leverage the full benefit offered by hiPSC-CMs. Lastly, despite the immature features described above, hiPSC-CMs remain the most relevant model for studying human cardiomyocytes and related pathologies.

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References:

- Bedada, F. B., Chan, S. S., Metzger, S. K., Zhang, L., Zhang, J., Garry, D. J., et al. 2014. Acquisition of a quantitative, stoichiometrically conserved ratiometric marker of maturation status in stem cell-derived cardiac myocytes. *Stem Cell Reports*, 3(4): 594-605. doi: 10.1016/j.stemcr.2014.07.012.
- Bett, G. C., Kaplan, A. D., Lis, A., Cimato, T. R., Tzanakakis, E. S., Zhou, Q., et al. 2013. Electronic "expression" of the inward rectifier in cardiocytes derived from human-induced pluripotent stem cells. *Heart Rhythm*, 10(12): 1903-1910. doi: 10.1016/j.hrthm.2013.09.061.
- Birket, M. J., Ribeiro, M. C., Verkerk, A. O., Ward, D., Leitoguinho, A. R., den Hartogh, S. C., et al. 2015.. Expansion and patterning of cardiovascular progenitors derived from human pluripotent stem cells. *Nat. Biotechnol.* 33(9): 970-979. doi: 10.1038/nbt.3271.
- Bongso, A., Fong, C. Y., Ng, S. C., and Ratnam, S. 1994. Isolation and culture of inner cell mass cells from human blastocysts. *Hum. Reprod.* 9(11): 2110-2117.
- Braam, S. R., Tertoolen, L., van de Stolpe, A., Meyer, T., Passier, R., and Mummery, C. L. 2010. Prediction of drug-induced cardiotoxicity using human embryonic stem cell-derived cardiomyocytes. *Stem Cell Res.* 4(2): 107-116. doi: 10.1016/j.scr.2009.11.004.
- Bugaisky, L. B., and Zak, R. 1989. Differentiation of adult rat cardiac myocytes in cell culture. *Circ. Res.* 64(3): 493-500.
- Burridge, P. W., Keller, G., Gold, J. D., and Wu, J. C. 2012. Production of de novo cardiomyocytes: human pluripotent stem cell differentiation and direct reprogramming. *Cell Stem Cell*, 10(1): 16-28. doi: 10.1016/j.stem.2011.12.013.
- Burridge, P. W., Matsa, E., Shukla, P., Lin, Z. C., Churko, J. M., Ebert, A. D., et al. 2014. Chemically defined generation of human cardiomyocytes. *Nat. Methods*, 11(8): 855-860. doi: 10.1038/nmeth.2999.
- Burridge, P. W., Thompson, S., Millrod, M. A., Weinberg, S., Yuan, X., Peters, A., et al. 2011. A universal system for highly efficient cardiac differentiation of human induced pluripotent stem cells that eliminates interline variability. *PLoS One*, 6(4): e18293. doi: 10.1371/journal.pone.0018293.
- Chen, Z., Xian, W., Bellin, M., Dorn, T., Tian, Q., Goedel, A., et al. 2016. Subtype-specific promoter-driven action potential imaging for precise disease modelling and drug testing in hiPSC-derived cardiomyocytes. *Eur. Heart J.* doi: 10.1093/eurheartj/ehw189.

Claycomb, W. C., Lanson, N. A., Jr., Stallworth, B. S., Egeland, D. B., Delcarpio, J. B., Bahinski, A., et al. 1998. HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. *Proc. Natl. Acad. Sci. U. S. A.*, 95(6): 2979-2984.

Davis, R. P., Casini, S., van den Berg, C. W., Hoekstra, M., Remme, C. A., Dambrot, C., et al. 2012. Cardiomyocytes derived from pluripotent stem cells recapitulate electrophysiological characteristics of an overlap syndrome of cardiac sodium channel disease. *Circulation*, 125(25): 3079-3091. doi: 10.1161/circulationaha.111.066092.

Denning, C., Borgdorff, V., Crutchley, J., Firth, K. S., George, V., Kalra, S., et al. 2016. Cardiomyocytes from human pluripotent stem cells: From laboratory curiosity to industrial biomedical platform. *Biochim. Biophys. Acta*, 1863(7 Pt B): 1728-1748. doi: 10.1016/j.bbamcr.2015.10.014.

Devalla, H. D., Schwach, V., Ford, J. W., Milnes, J. T., El-Haou, S., Jackson, C., et al. 2015.. Atrial-like cardiomyocytes from human pluripotent stem cells are a robust preclinical model for assessing atrial-selective pharmacology. *EMBO Mol. Med.* 7(4): 394-410. doi: 10.15252/emmm.201404757.

Doetschman, T. C., Eistetter, H., Katz, M., Schmidt, W., and Kemler, R. 1985. The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *J. Embryol. Exp. Morphol.* 87: 27-45.

Du, D. T., Hellen, N., Kane, C., and Terracciano, C. M. 2015.. Action potential morphology of human induced pluripotent stem cell-derived cardiomyocytes does not predict cardiac chamber specificity and is dependent on cell density. *Biophys. J.* 108(1): 1-4. doi: 10.1016/j.bpj.2014.11.008.

Estes, N. A., 3rd. 2011. Predicting and preventing sudden cardiac death. *Circulation*, 124(5) : 651-656. doi: 10.1161/circulationaha.110.974170.

Fermini, B., Hancox, J. C., Abi-Gerges, N., Bridgland-Taylor, M., Chaudhary, K. W., Colatsky, T., et al. 2016. A New Perspective in the Field of Cardiac Safety Testing through the Comprehensive In Vitro Proarrhythmia Assay Paradigm. *J. Biomol. Screening*, 21(1): 1-11. doi: 10.1177/1087057115594589.

Gorospe, G., Zhu, R., Millrod, M. A., Zambidis, E. T., Tung, L., and Vidal, R. 2014. Automated grouping of action potentials of human embryonic stem cell-derived cardiomyocytes. *IEEE Trans. Biomed. Eng.*, 61(9): 2389-2395. doi: 10.1109/TBME.2014.2311387.

Hinson, J. T., Chopra, A., Nafissi, N., Polacheck, W. J., Benson, C. C., Swist, S., et al. 2015. HEART DISEASE. Titin mutations in iPS cells define sarcomere insufficiency as a cause of dilated cardiomyopathy. *Science*, 349(6251): 982-986. doi: 10.1126/science.aaa5458.

Itzhaki, I., Maizels, L., Huber, I., Gepstein, A., Arbel, G., Caspi, O., et al. 2012. Modeling of catecholaminergic polymorphic ventricular tachycardia with patient-specific human-induced pluripotent stem cells. *J. Am. Coll. Cardiol.* 60(11): 990-1000. doi: 10.1016/j.jacc.2012.02.066.

Itzhaki, I., Maizels, L., Huber, I., Zwi-Dantsis, L., Caspi, O., Winterstern, A., et al. 2011. Modelling the long QT syndrome with induced pluripotent stem cells. *Nature*, 471(7337): 225-229. doi: 10.1038/nature09747.

Jung, G., Fajardo, G., Ribeiro, A. J., Kooiker, K. B., Coronado, M., Zhao, M., et al. 2016. Time-dependent evolution of functional vs. remodeling signaling in induced pluripotent stem cell-derived cardiomyocytes and induced maturation with biomechanical stimulation. *FASEB J.* 30(4): 1464-1479. doi: 10.1096/fj.15-280982.

Kadari, A., Mekala, S., Wagner, N., Malan, D., Koth, J., Doll, K., et al. 2015. Robust Generation of Cardiomyocytes from Human iPS Cells Requires Precise Modulation of BMP and WNT Signaling. *Stem Cell Rev. Rep.* 11(4): 560-569. doi: 10.1007/s12015-014-9564-6.

Kamakura, T., Makiyama, T., Sasaki, K., Yoshida, Y., Wuriyanghai, Y., Chen, J., et al. 2013. Ultrastructural maturation of human-induced pluripotent stem cell-derived cardiomyocytes in a long-term culture. *Circ. J.* 77(5): 1307-1314.

Kane, C., Du, D. T., Hellen, N., and Terracciano, C. M. 2016. The Fallacy of Assigning Chamber Specificity to iPSC Cardiac Myocytes from Action Potential Morphology. *Biophys. J.* 110(1): 281-283. doi: 10.1016/j.bpj.2015.08.052.

Kehat, I., Khimovich, L., Caspi, O., Gepstein, A., Shofti, R., Arbel, G., et al. 2004. Electromechanical integration of cardiomyocytes derived from human embryonic stem cells. *Nat. Biotechnol.* 22(10): 1282-1289. doi: 10.1038/nbt1014.

Keller, D. I., Acharfi, S., Delacrétaiz, E., Benammar, N., Rotter, M., Pfammatter, J. P., et al. 2003. A novel mutation in *SCN5A*, delQKP 1507-1509, causing long QT syndrome: Role of Q1507 residue in sodium channel inactivation. *J.Mol.Cell.Cardiol.* 35(12): 1513-1521.

Kimes, B. W., and Brandt, B. L. 1976. Properties of a clonal muscle cell line from rat heart. *Exp. Cell Res.* 98(2): 367-381.

Lian, X., Hsiao, C., Wilson, G., Zhu, K., Hazeltine, L. B., Azarin, S. M., et al. 2012. Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical Wnt signaling. *Proc. Natl. Acad. Sci. U. S. A.*, 109(27): E1848-1857. doi: 10.1073/pnas.1200250109.

Lian, X., Zhang, J., Azarin, S. M., Zhu, K., Hazeltine, L. B., Bao, X., et al. 2013. Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/beta-

catenin signaling under fully defined conditions. *Nat. Protoc.* 8(1): 162-175. doi: 10.1038/nprot.2012.150.

Lieu, D. K., Fu, J. D., Chiamvimonvat, N., Tung, K. C., McNerney, G. P., Huser, T., et al. 2013. Mechanism-based facilitated maturation of human pluripotent stem cell-derived cardiomyocytes. *Circ. Arrhythm. Electrophysiol.* 6(1): 191-201. doi: 10.1161/CIRCEP.111.973420.

Lundy, S. D., Zhu, W. Z., Regnier, M., and Laflamme, M. A. 2013. Structural and functional maturation of cardiomyocytes derived from human pluripotent stem cells. *Stem Cells Dev.* 22(14): 1991-2002. doi: 10.1089/scd.2012.0490.

Ma, J., Guo, L., Fiene, S. J., Anson, B. D., Thomson, J. A., Kamp, T. J., et al. 2011. High purity human-induced pluripotent stem cell-derived cardiomyocytes: electrophysiological properties of action potentials and ionic currents. *Am. J. Physiol. Heart Circ. Physiol.* 301(5): H2006-2017. doi: 10.1152/ajpheart.00694.2011.

Maass, K., Shekhar, A., Lu, J., Kang, G., See, F., Kim, E. E., et al. 2015. Isolation and characterization of embryonic stem cell-derived cardiac Purkinje cells. *Stem Cells*, 33(4), 1102-1112. doi: 10.1002/stem.1921.

Maltsev, V. A., Rohwedel, J., Hescheler, J., and Wobus, A. M. 1993. Embryonic stem cells differentiate in vitro into cardiomyocytes representing sinusnodal, atrial and ventricular cell types. *Mech. Dev.* 44(1): 41-50.

Mohr, J. C., Zhang, J., Azarin, S. M., Soerens, A. G., de Pablo, J. J., Thomson, J. A., et al. 2010. The microwell control of embryoid body size in order to regulate cardiac differentiation of human embryonic stem cells. *Biomaterials*, 31(7): 1885-1893. doi: 10.1016/j.biomaterials.2009.11.033.

Moreau, A., Mercier, A., Thériault, O., Boutjdir, M., Burger, B., Keller, D. I., et al. 2017. Biophysical, molecular, and pharmacological characterization of voltage-dependent sodium channels from induced pluripotent stem cell-derived cardiomyocytes. *Can. J. Cardiol.* 33(2): 269-278. doi: 10.1016/j.cjca.2016.10.001.

Moretti, A., Bellin, M., Welling, A., Jung, C. B., Lam, J. T., Bott-Flugel, L., et al. 2010. Patient-specific induced pluripotent stem-cell models for long-QT syndrome. *N. Engl. J. Med.* 363(15): 1397-1409. doi: 10.1056/NEJMoa0908679.

Mummery, C. L., van Achterberg, T. A., van den Eijnden-van Raaij, A. J., van Haaster, L., Willemse, A., de Laat, S. W., et al. 1991. Visceral-endoderm-like cell lines induce differentiation of murine P19 embryonal carcinoma cells. *Differentiation*, 46(1): 51-60.

Mummery, C. L., Ward-van Oostwaard, D., Doevendans, P., Spijker, R., van den Brink, S., Hassink, R., et al. 2003. Differentiation of human embryonic stem cells to cardiomyocytes: role

of coculture with visceral endoderm-like cells. *Circulation*, 107(21): 2733-2740. doi: 10.1161/01.cir.0000068356.38592.68.

Mummery, C. L., Zhang, J., Ng, E. S., Elliott, D. A., Elefanty, A. G., and Kamp, T. J. 2012. Differentiation of human embryonic stem cells and induced pluripotent stem cells to cardiomyocytes: a methods overview. *Circ. Res.* 111(3): 344-358. doi: 10.1161/circresaha.110.227512.

Rajamohan, D., Matsa, E., Kalra, S., Crutchley, J., Patel, A., George, V., et al. 2013. Current status of drug screening and disease modelling in human pluripotent stem cells. *Bioessays*, 35(3): 281-298. doi: 10.1002/bies.201200053.

Raschi, E., and De Ponti, F. 2012. Cardiovascular toxicity of anticancer-targeted therapy: emerging issues in the era of cardio-oncology. *Intern. Emerg. Med.* 7(2): 113-131. doi: 10.1007/s11739-011-0744-y.

Ribeiro, M. C., Tertoolen, L. G., Guadix, J. A., Bellin, M., Kosmidis, G., D'Aniello, C., et al. 2015. Functional maturation of human pluripotent stem cell derived cardiomyocytes in vitro--correlation between contraction force and electrophysiology. *Biomaterials*, 51: 138-150. doi: 10.1016/j.biomaterials.2015.01.067.

Shiba, Y., Gomibuchi, T., Seto, T., Wada, Y., Ichimura, H., Tanaka, Y., et al. 2016. Allogeneic transplantation of iPS cell-derived cardiomyocytes regenerates primate hearts. *Nature*, 538(7625): 388-391. doi: 10.1038/nature19815.

Sun, N., Yazawa, M., Liu, J., Han, L., Sanchez-Freire, V., Abilez, O. J., et al. 2012. Patient-specific induced pluripotent stem cells as a model for familial dilated cardiomyopathy. *Sci. Transl. Med.* 4(130): 130ra147. doi: 10.1126/scitranslmed.3003552.

Sun, X., and Nunes, S. S. 2016. Biowire platform for maturation of human pluripotent stem cell-derived cardiomyocytes. *Methods*, 101: 21-26. doi: 10.1016/j.ymeth.2015.11.005.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., et al. 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*, 131(5): 861-872. doi: 10.1016/j.cell.2007.11.019.

Takahashi, K., and Yamanaka, S. 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*, 126(4): 663-676. doi: 10.1016/j.cell.2006.07.024.

Te Riele, A. S., Agullo-Pascual, E., James, C. A., Leo-Macias, A., Cerrone, M., Zhang, M., et al. 2017. Multilevel analyses of SCN5A mutations in arrhythmogenic right ventricular dysplasia/cardiomyopathy suggest non-canonical mechanisms for disease pathogenesis. *Cardiovasc. Res.* 113(1): 102-111. doi: 10.1093/cvr/cvw234.

Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., et al. 1998. Embryonic stem cell lines derived from human blastocysts. *Science*, 282(5391): 1145-1147.

Tohyama, S., Hattori, F., Sano, M., Hishiki, T., Nagahata, Y., Matsuura, T., et al. 2013. Distinct metabolic flow enables large-scale purification of mouse and human pluripotent stem cell-derived cardiomyocytes. *Cell Stem Cell*, 12(1): 127-137. doi: 10.1016/j.stem.2012.09.013.

Tsai, S. Y., Maass, K., Lu, J., Fishman, G. I., Chen, S., and Evans, T. 2015. Efficient Generation of Cardiac Purkinje Cells from ESCs by Activating cAMP Signaling. *Stem Cell Rep.* 4(6): 1089-1102. doi: 10.1016/j.stemcr.2015.04.015.

Vaidyanathan, R., Markandeya, Y. S., Kamp, T. J., Makielski, J. C., January, C. T., and Eckhardt, L. L. 2016. IK1-enhanced human-induced pluripotent stem cell-derived cardiomyocytes: an improved cardiomyocyte model to investigate inherited arrhythmia syndromes. *Am. J. Physiol. Heart Circ. Physiol.* 310(11): H1611-1621. doi: 10.1152/ajpheart.00481.2015.

Veerman, C. C., Kosmidis, G., Mummery, C. L., Casini, S., Verkerk, A. O., and Bellin, M. 2015. Immaturity of human stem-cell-derived cardiomyocytes in culture: fatal flaw or soluble problem? *Stem Cells Dev.* 24(9): 1035-1052. doi: 10.1089/scd.2014.0533.

Viero, C., Kraushaar, U., Ruppenthal, S., Kaestner, L., and Lipp, P. 2008. A primary culture system for sustained expression of a calcium sensor in preserved adult rat ventricular myocytes. *Cell Calcium*, 43(1): 59-71. doi: 10.1016/j.ceca.2007.04.001.

Xue, T., Cho, H. C., Akar, F. G., Tsang, S. Y., Jones, S. P., Marban, E., et al. 2005. Functional integration of electrically active cardiac derivatives from genetically engineered human embryonic stem cells with quiescent recipient ventricular cardiomyocytes: insights into the development of cell-based pacemakers. *Circulation*, 111(1): 11-20. doi: 10.1161/01.cir.0000151313.18547.a2.

Yechikov, S., Copaciu, R., Gluck, J. M., Deng, W., Chiamvimonvat, N., Chan, J. W., et al. 2016. Same-Single-Cell Analysis of Pacemaker-Specific Markers in Human Induced Pluripotent Stem Cell-Derived Cardiomyocyte Subtypes Classified by Electrophysiology. *Stem Cells*, 34(11): 2670-2680. doi: 10.1002/stem.2466.

Zhang, Q., Jiang, J., Han, P., Yuan, Q., Zhang, J., Zhang, X., et al. 2011. Direct differentiation of atrial and ventricular myocytes from human embryonic stem cells by alternating retinoid signals. *Cell Res.* 21(4): 579-587. doi: 10.1038/cr.2010.163.

Zhu, W. Z., Xie, Y., Moyes, K. W., Gold, J. D., Askari, B., and Laflamme, M. A. 2010. Neuregulin/ErbB signaling regulates cardiac subtype specification in differentiating human embryonic stem cells. *Circ. Res.* 107(6): 776-786. doi: 10.1161/CIRCRESAHA.110.223917.

Zimmer, T., Haufe, V., and Blechschmidt, S. 2014. Voltage-gated sodium channels in the mammalian heart. *Glob. Cardiol. Sci. Pract.* 2014(4): 449-463. doi: 10.5339/gcsp.2014.58.

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Figure legends:

Figure 1: hiPSC morphology. Images showing the typical morphology of hiPSCs. **(A)** hiPSCs naturally form dense colonies (hiPSCs grown on mouse embryonic feeders). **(B)** hiPSCs are tiny compacted cells with a large nucleus.

Figure 2: Differentiation protocols. The three main differentiation protocols used to obtain hiPSC-CM from hiPSC are as follows: **(A)** The hanging drop technique was initially used to form embryoid bodies (EBs) on the lids of petri dishes. EBs were then plated and allowed to differentiate into many cell types, including hiPSC-CMs. **(B)** Co-culturing hiPSCs with non-cardiac cells such as END-2 cells can also be used to produce hiPSC-CMs. **(C)** The most recent protocol involves the differentiation of hiPSC-CMs from monolayers of hiPSCs. The Wnt pathway is modulated to promote the formation of a mesoderm and then to produce cardiac progenitors.

Figure 3: Action potentials. The spontaneous electrical activity of hiPSC-CMs can be recorded using the patch-clamp technique. Action potentials display different characteristics that can be attributed to cardiac chamber-specific morphologies, i.e. ventricular, atrial, and nodal.

Figure 4: Action potential cluster analysis. The cluster analysis was based on action potential duration (APD) and the presence or absence of a plateau phase. APD_{20} is shown as a function of APD_{90} . hiPSC-CM populations were separated into clusters using a set of data obtained in our laboratory. The K-means++ method (Calinski-Harabasz criterion), gave rise to 5 clusters. The first and second clusters corresponded to nodal- and auricular-like cardiomyocytes, respectively, while the last three clusters corresponded to ventricular-like cardiomyocytes.

Figure 5: hiPSC-CM morphology. The morphology of hiPSC-CMs was examined by immunofluorescence confocal imaging. **(A)** Myosin light-chain 2v (mlc2v, green staining), cardiac troponin T (cTnT, red staining), and $Na_v1.5$ channels are shown. **(B)** As maturation progresses, some hiPSC-CMs display a striated pattern following $Na_v1.5$ staining.

A **B**

Ventricular like**Auricular like****Nodal like**

A**mlc2v****cTnT****merge****Na_v1.5** **B****mlc2v** **Na_v1.5**