



Human pluripotent stem cells: Prospects and challenges as a source of cardiomyocytes for in vitro modeling and cell-based cardiac repair[☆]



Matthew E. Hartman^{a,b,c,d}, Dao-Fu Dai^{c,d,e}, Michael A. Laflamme^{c,d,e,*}

^a Division of Cardiology, Department of Medicine, USA

^b Department of Genome Sciences, USA

^c Institute for Stem Cell and Regenerative Medicine, USA

^d Center for Cardiovascular Biology, USA

^e Department of Pathology, University of Washington, Seattle, WA, USA

ARTICLE INFO

Article history:

Received 5 March 2015

Accepted 7 May 2015

Available online 14 May 2015

Keywords:

Embryonic stem cells

Induced pluripotent stem cells

Cardiac regeneration

Cardiac repair

Heart failure

Cardiovascular disease

Drug discovery

Disease modeling

ABSTRACT

Human pluripotent stem cells (PSCs) represent an attractive source of cardiomyocytes with potential applications including disease modeling, drug discovery and safety screening, and novel cell-based cardiac therapies. Insights from embryology have contributed to the development of efficient, reliable methods capable of generating large quantities of human PSC-cardiomyocytes with cardiac purities ranging up to 90%. However, for human PSCs to meet their full potential, the field must identify methods to generate cardiomyocyte populations that are uniform in subtype (e.g. homogeneous ventricular cardiomyocytes) and have more mature structural and functional properties. For in vivo applications, cardiomyocyte production must be highly scalable and clinical grade, and we will need to overcome challenges including graft cell death, immune rejection, arrhythmogenesis, and tumorigenic potential. Here we discuss the types of human PSCs, commonly used methods to guide their differentiation into cardiomyocytes, the phenotype of the resultant cardiomyocytes, and the remaining obstacles to their successful translation.

© 2015 Elsevier B.V. All rights reserved.

Contents

1.	Introduction	4
2.	Pluripotent stem cells: Definitions, origins, and early evidence for cardiac potential	4
3.	Approaches to obtaining pluripotent stem cell-derived cardiomyocytes	6
3.1.	Lessons from embryology: Epiblast to cardiac mesoderm.	6
3.2.	“Spontaneous” differentiation via embryoid bodies	6
3.3.	Directed differentiation methods	6
3.3.1.	Endoderm-directed differentiation.	6
3.3.2.	Manipulation of nodal/activin and/or BMP4 signaling	6
3.3.3.	“Matrix sandwich” protocol.	6
3.3.4.	Manipulation of Wnt signaling	6
3.3.5.	Hybrid approaches and chemically defined protocols	7
3.3.6.	Embryoid body vs. monolayer differentiation	7
4.	Phenotype of human pluripotent stem cell-derived cardiomyocytes	8
4.1.	Size and structure	8
4.2.	Mechanical properties and force generation	8

[☆] This review is part of the *Advanced Drug Delivery Reviews* theme issue on “Tissue engineering of the heart: from in vitro models to regenerative solutions”.

* Corresponding author at: Center for Cardiovascular Biology, UW Medicine at South Lake Union, Box 358050, Brotman Building, Rm 442, 815 Mercer St. Seattle, WA 98109, USA. Tel.: +1 206 897 1518; fax: +1 206 897 1540.

E-mail address: laflamme@u.washington.edu (M.A. Laflamme).

4.3.	Electrophysiology	8
4.4.	Calcium handling	9
4.5.	Mitochondrial function and metabolism	9
5.	Remaining obstacles to the successful translation of pluripotent stem cell-derived cardiomyocytes as a practical source of heart cells	9
5.1.	Immature phenotype	9
5.2.	Cardiomyocyte subtype diversity	10
5.3.	Arrhythmogenesis	11
5.4.	Teratomas and karyotypic abnormalities	11
5.5.	Immune rejection	11
5.6.	Epigenetic modifications	12
5.7.	Scalability and clinical grade production	12
6.	Conclusions	13
	Acknowledgments	13
	References	13

1. Introduction

Human pluripotent stem cells (PSCs) have garnered a high degree of interest because of their potential use in applications ranging from basic research to novel cell-based therapies and tissue engineering. While the field has developed well-defined protocols for the expansion of human PSCs in the undifferentiated state as well as their subsequent differentiation into cardiomyocytes, we still need improved methods to derive specific cardiac subtypes (i.e. nodal versus ventricular cardiomyocytes) and to promote their maturation into a more adult-like phenotype. These improvements would most likely be applied first to in vitro applications for human PSC-derived cardiomyocytes, for example, drug discovery and cardiotoxicity screening as currently envisioned by the pharmaceutical industry and regulatory agencies [1–3]. Investigators are already using human PSC-derived cardiomyocytes as in vitro models of incompletely understood monogenic diseases, such as channelopathies and cardiomyopathies [4–9]. Moreover, if these cells are to advance to eventual clinical applications, a number of major hurdles must be overcome including graft cell death, immune rejection of the graft cells, and the risk of tumorigenesis. The heart is a particularly challenging target for human PSC-based therapies, at least if bona fide tissue regeneration (“remuscularization”) is the goal, because the graft cardiomyocytes must undergo appropriate electromechanical integration and couple with host myocardium to contribute new force generating units without arrhythmias.

In this review, we will describe early progress in the field that led to the availability of human PSCs and human PSC-derived cardiomyocytes, the various approaches that have been pursued to generate populations of human PSC-derived cardiomyocytes at ever-improving purities and scales, our current understanding of the phenotype of these cardiomyocytes, and the challenges that must be overcome if human PSCs are to become a practical source of heart cells for in vitro modeling and in vivo cardiac regeneration.

2. Pluripotent stem cells: Definitions, origins, and early evidence for cardiac potential

In 1981, Martin reported the successful isolation and expansion of a PSC population from the inner cell mass of mouse blastocysts [10]. These cells were termed mouse embryonic stem cells (ESCs) to distinguish them from the previously described embryonal carcinoma cells, which also exhibit pluripotency (i.e. capacity to differentiate into cell types from all three embryonic germ layers). While mouse ESCs maintain their pluripotent phenotype when grown on a feeder layer of mouse embryonic fibroblasts (MEFs), Doetschman and colleagues found that, when removed from MEFs and placed into suspension culture with a high fraction of fetal bovine serum, mouse ESCs begin to differentiate and spontaneously form three-dimensional aggregates, termed embryoid bodies (EBs) [11]. These EBs contained differentiated cell types from all three embryonic germ layers, including a small fraction of spontaneously beating cardiomyocytes. When transplanted into syngeneic mice, these mouse ESCs formed teratomas that included cardiomyocytes among other cell types. In 1998, Thomson and colleagues reported the successful isolation of human ESCs from the inner cell mass of blastocysts that were left over from in vitro fertilization efforts [12]. As with their mouse ESC counterparts, human ESCs formed EBs when placed in suspension cultures in a high fraction of fetal bovine serum [13]. A subset of these EBs contracted rhythmically and expressed the cardiomyocyte marker α -cardiac actin. Kehat and colleagues more comprehensively phenotyped human ESC-derived cardiomyocytes obtained via the EB approach and showed that they expressed a variety of expected cardiac markers, including α - and β -myosin heavy chain, cardiac troponin I and T, myosin light chain (MLC)-2a and 2v, atrial natriuretic factor, α -actinin, and the transcription factors Nkx2.5 and GATA4 [14]. Human ESC-derived cardiomyocytes also exhibited spontaneous electrical activity and intracellular calcium transients [14].

The National Institutes of Health Human Embryonic Stem Cell Registry currently lists 303 human ESC lines that are eligible for U.S. federal

Table 1

Comparison between two main pluripotent stem cell types as potential cardiomyocyte sources.

	ESCs	iPSCs
Derivation	Isolation from inner cell mass of blastocyst	Somatic cell reprogramming
Cardiac differentiation methods	Similar	Similar
Cardiac differentiation efficiency	Similar	Similar
Phenotype after cardiac differentiation	Similar	Similar
Relative advantages	Reprogramming not required (e.g. no use of viral vectors) Lack somatic epigenetic modifications Potentially more direct regulatory path for clinical use	No ethical issues with derivation Tissue specific derivation Particularly useful for modeling monogenic diseases Potentially useful for autologous cell therapies Possibly less immunogenic

ESCs: embryonic stem cells, iPSCs: induced pluripotent stem cells.

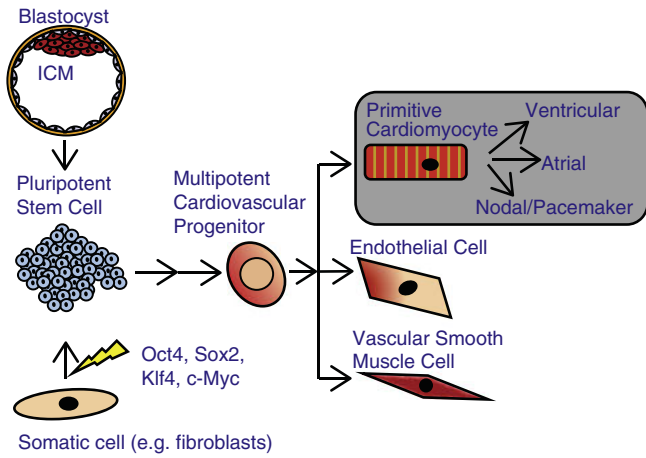


Fig. 1. Human PSCs to cardiomyocytes. Human PSCs can be isolated from the inner cell mass (ICM) of human blastocysts or generated by the reprogramming of somatic cell types via the forced expression of transcription factors (e.g. Oct4, Sox2, Klf4 and c-Myc). Using available protocols, undifferentiated PSCs can be efficiently guided into mesodermal progenitors, more restricted multipotent cardiovascular progenitor cells, and ultimately the various parenchymal cell types that populate the heart, including cardiomyocytes. Work is also ongoing to further control their differentiation into specialized cardiac subtypes including ventricular, atrial and nodal cardiomyocytes.

research support [15]. All but one of these lines (the Elf1 human ESC line [16]) are considered to be “primed” stage PSCs. Primed-stage PSCs are thought to be analogous to cells in the post-implantation epiblast and are exemplified by mouse epiblast stem cells [17]. Cells representing the preimplantation inner cell mass are termed “naïve”-stage stem cells and are typified by mouse ESCs. Naïve stem cells can contribute

to all lineages when injected into a blastocyst [17], have two active X chromosomes in female cells [17], express Oct4 regulated by the distal rather than the proximal enhancer [18], and exhibit a number of other important differences from their primed-stage counterparts [16, 19, 20]. More recently, three groups of investigators have independently reported the de novo derivation of naïve-stage human ESCs as well as culture conditions for their maintenance [16, 18, 19]. Gafni and colleagues demonstrated that these naïve human PSCs could contribute to cross-species chimeras by microinjection into mouse morulas with subsequent organogenesis and embryo viability to E10.5 [19]. Different primed-stage human ESC lines have different cardiomyocyte differentiation potential [21, 22] and naïve human ESCs may have a more broad pluripotent potential comparatively [16]. However, to date, naïve human ESCs have only been differentiated in teratoma assays and we do not know how they will respond to the cardiomyocyte differentiation protocols that have been developed for primed-stage human ESCs.

The reprogramming of adult somatic cells into an ESC-like state was first described by Takahashi and Yamanaka in 2006, when they created so-called induced pluripotent stem cells (iPSCs) by the forced expression of four transcription factors (Oct3/4, Sox2, c-Myc, and Klf4) in mouse fibroblasts [23]. In 2007, both the Yamanaka [24] and Thomson groups [25] reported the successful generation of human iPSCs, albeit with slightly different sets of transcription factors. These early iPSCs were made with integrating viral vectors, which raised concerns about potential genetic alterations and neoplastic transformation. More recently, many groups have reported alternative approaches to produce iPSCs including the use of non-integrating viral and non-viral vectors or reprogramming via protein-, mRNA-, and small molecule-based methods [26]. Importantly, equivalent methods can be used to induce the cardiac differentiation of both human ESCs and iPSCs, and the resultant cardiomyocytes have comparable phenotypes, at least to first

	Time (days): 0 1 2 3 4 5 6 7 8 9									
A	Culture on MEFs		Suspension culture, aggregates					Adherent EBs		
	Knockout DMEM +20% FBS									
B	Monolayer	AA	BMP4			No factors				
	MEF-CM +bFGF	RPMI +B27 -Ins			RPMI +B27 +Ins					
C	Monolayer	Matrigel	AA*	BMP4 bFGF			No factors			
	mTeSR1™ +Y-27632		RPMI +B27 -Ins			RPMI +B27 +Ins				
D	Feeder-depletion	BMP4	AA	BMP4	bFGF	Dkk1 VEGF		Dkk1 VEGF bFGF		
	DMEM/F12 +20% KO		EBs in StemPro™-34, 5% O ₂							
E	Monolayer + Synthemax™		CHIR	No factors		IWP	No factors			
	mTeSR1™		RPMI +B27 -Ins			RPMI +B27 +Ins				
F	Monolayer	CHIR		Wnt-C59		No factors				
	E8 media		RPMI +rhAlbumin +Ascorbic acid							
G	Monolayer	CHIR	AA	BMP4	CHIR	XAV939		No factors		
	MEF-CM +bFGF		RPMI +B27 -Ins			RPMI +B27 +Ins				
	<i>Pre-differentiation</i>				<i>Differentiating conditions</i>					

Fig. 2. Human PSC-cardiomyocyte differentiation protocols. Diagram depicting the most commonly used methods for obtaining cardiomyocytes from human ESCs and iPSCs, including the A) embryoid body [14], B) activin A- and BMP4-based monolayer [46], C) “matrix sandwich” [50], D) Wnt inhibition [35], E) small-molecule Wnt modulation [56], F) chemically-defined, small-molecule [58], and G) “hybrid” [59] cardiac differentiation protocols. The top shaded bar of each protocol indicates the specific factors and culture conditions employed, while the bottom bar indicates the culture media and additional culture specifications used. These various interventions are depicted relative to the time-line above (with day 0 indicating the initial induction of cardiac differentiation). Abbreviations: AA, activin A; AA*, activin A with Matrigel; BMP4, bone morphogenetic protein 4; bFGF, basic fibroblast growth factor; CHIR, CHIR99021, glycogen synthase kinase 3β inhibitor; Dkk1, dickkopf homolog 1; DMEM, Dulbecco’s modified eagle’s medium; EBs, embryoid bodies; FBS, fetal bovine serum; IWP, inhibitor of Wnt protein; KO, knockout serum; MEFs, mouse embryonic fibroblasts; MEF-CM, MEF conditioned medium; RPMI, Roswell Park Memorial Institute 1640 medium; VEGF, vascular endothelial growth factor; Wnt-C59, Wnt-3a inhibitor; XAV939, tankyrase inhibitor; Y-27632, Rho kinase inhibitor.

approximation [27]. Notwithstanding these similarities, there are important practical advantages and disadvantages to consider when selecting between ESC- and iPSC-derived cardiomyocytes for any potential application (summarized in Table 1).

In sum, there are at least two practical sources of human PSCs. As detailed in the next section, multiple ever-improving protocols have been developed to efficiently guide these PSCs into cardiomyocytes as well as the other major parenchymal cell types present in myocardium (Fig. 1).

3. Approaches to obtaining pluripotent stem cell-derived cardiomyocytes

3.1. Lessons from embryology: Epiblast to cardiac mesoderm

At the earliest stages of development, the inner cell mass of the blastocyst expands to form the epiblast that will eventually give rise to all fetal tissue structures. After this expansion, the embryo begins the process of gastrulation, which is initiated by the formation of the primitive streak at the epiblast–extraembryonic border. The primitive streak, marked by expression of the transcription factor Brachyury T [28], expands in the developing embryo in part due to the contribution of neighboring epiblast cells that undergo an epithelial–mesenchymal transition (EMT) and migrate into the primitive streak [29]. Cells exiting the primitive streak initially contribute to extraembryonic structures, but they later give rise to embryonic structures including the heart [30]. The heart forms from the cardiac mesoderm which arises from the anterior primitive streak. The formation and patterning of this cardiac mesoderm is largely controlled by gradients of a number of critical signaling molecules including Nodal, Wnt, and bone morphogenetic protein 4 (BMP4) (for more details about these signaling events, the reader is referred to an excellent review by Gadue and colleagues [31]). Perhaps not surprisingly, investigators in the stem cell field have found that these same signaling molecules are required for the cardiac differentiation of human PSCs, and this has led to the development of multiple guided cardiac differentiation protocols based on the manipulation of Nodal/activin, Wnt, and/or BMP4 signaling [31–35].

3.2. “Spontaneous” differentiation via embryoid bodies

As noted above in Section 2, Doetschman and colleagues first demonstrated that cardiomyocytes could be obtained spontaneously by placing mouse ESCs into suspension cultures and forming EBs [11]. This aggregation leads to non-directed cardiomyocyte differentiation in a process that somewhat recapitulates normal embryonic development. Kehat and colleagues later used a similar approach to obtain human ESC-derived cardiomyocytes (Fig. 2A) [14]. Briefly, human ESCs are removed from the MEF feeder layer used to maintain pluripotency and placed into suspension cultures for 7–10 days to form EBs. These EBs are then collected and replated onto gelatin-coated tissue culture plates. While this approach does produce cardiomyocytes, it is quite inefficient with only approximately 10% of the replated EBs showing foci of spontaneous contractile activity [14]. Because the resultant cardiomyocytes represent at best 1% of the total cell population, these spontaneous beating areas must be manually microdissected to obtain sufficient cardiomyocytes for even small-scale *in vitro* experiments. Xu and colleagues later refined these EB-based methods and added a Percoll gradient centrifugation step to obtain enriched populations of up to 70% human ESC-derived cardiomyocytes [36].

3.3. Directed differentiation methods

3.3.1. Endoderm-directed differentiation

Endoderm-derived signals are known to play a critical role in cardiogenesis [37–39]. Mummery and colleagues exploited this information by exposing human ESCs to factors from mitotically inactivated END-2 cells, visceral endoderm-like cells derived from an embryonal

carcinoma cell line [40]. Human ESCs, when co-cultured with END-2 cells or exposed to serum-free medium conditioned by END-2 cells, differentiate into cardiomyocytes with an efficiency of approximately 25% [41]. To work effectively, this protocol required the elimination of insulin and insulin-like growth factor signaling, which otherwise favors neuroectodermal differentiation [42].

3.3.2. Manipulation of nodal/activin and/or BMP4 signaling

While the preceding cardiac differentiation protocols involving fetal bovine serum or endoderm-derived factors did yield human ESC-derived cardiomyocytes, they were inefficient, poorly scalable and not suitable for clinical grade cell production. As such, these early methods have since been largely supplanted in the field by alternative approaches that employ defined signaling factors and serum-free culture conditions. As noted above in Section 2, these newer protocols build on insights from developmental biology, including prior work showing the critical roles of Nodal and BMP4 signaling in cardiogenesis [31, 43–45]. The first of these directed differentiation methods was reported by our group and involved the serial application of activin A (as a substitute for Nodal) and BMP4 [46]. In brief, in our protocol (Fig. 2B), human ESCs are cultured in MEF-conditioned medium on Matrigel-coated substrates [47] until they form a compact monolayer (Matrigel is an extract of basement membrane proteins secreted by Engelbreth–Holm–Swarm mouse sarcoma cells [48]). The human ESC cultures are then switched to differentiation conditions by replacing the MEF-conditioned medium with serum-free, defined medium (RPMI 1640 medium supplemented with B27 serum substitute) containing activin A. 24-hours later, the activin A-supplemented medium is removed and replaced with medium instead supplemented with BMP-4. The latter is left on for 4 days, and then the cultures are re-fed every other day with fresh media with no exogenous growth factors. By this protocol, spontaneous beating is typically observed approximately 8–12 days after the initial induction of differentiation with activin A. This protocol reliably results in populations of >30% cardiomyocytes (range: 30–60%) that can be further enriched to >80% cardiomyocyte by Percoll gradient centrifugation. We estimated that each starting undifferentiated human ESC yields three cardiomyocytes, an efficiency that greatly exceeds that obtained with historical, non-directed (“spontaneous”) EB-based methods [49].

3.3.3. “Matrix sandwich” protocol

Zhang and colleagues [50] reasoned that the extracellular matrix is important for cardiomyocyte differentiation since it: 1) interacts with surrounding cells to support cell migration, 2) enables cross-talk between receptors for extracellular matrix and growth factors, 3) is able to sequester cytokines and growth factors, 4) transduces mechanical forces to associated cells, and 5) provides a dynamic environment over the course of differentiation providing additional cues to local cells. With this in mind, they modified the monolayer-based approach described in the preceding section with the addition of a Matrigel overlay step at two critical points in the differentiation process: when the initial human ESC monolayer approaches confluence and at the time of activin A treatment (Fig. 2C). This addition of Matrigel was shown to promote an EMT akin to that in gastrulation and boosted the efficiency of cardiogenesis across multiple human ESC and human iPSC lines. Cardiomyocyte purities ranged from approximately 40 to 90%, and the authors estimated yields of up to 11 cardiomyocytes per starting undifferentiated cell. However, the application of Matrigel specifically has two potential drawbacks: its non-human origin raises potential concerns about xenogeneic pathogens, plus it exhibits significant lot-to-lot variation. Hence, for *in vivo* applications, it would be helpful to identify a better defined or recombinant human matrix alternative with comparable efficacy in this protocol.

3.3.4. Manipulation of Wnt signaling

Wnt/ β -catenin signaling plays a biphasic role in heart development with early canonical Wnt ligands stimulating mesoderm induction,

while Wnt activation at later timepoints inhibits cardiogenesis [32, 35, 51, 52]. Indeed, the preceding Nodal/activin- and BMP4-based cardiac differentiation protocols are likely dependent on endogenous Wnt signaling, as evidenced by the fact that human ESC cultures showing poor cardiac induction by these methods are distinguished by minimal to no upregulation of *Wnt1*, *Wnt3a*, and *Wnt8a* [32]. Keller and colleagues exploited this information in the development of differentiation protocols for mouse [53] and human [35] PSCs that combine manipulation of Nodal/activin, BMP4 and Wnt signaling in a sequential manner, i.e. as the cultures go through specific stages of cardiac differentiation including primitive streak and mesoderm formation, cardiac mesoderm specification, and cardiac lineage specification. In brief, in their methods (Fig. 2D), human EBs are formed by feeder depletion followed by low-attachment culture in serum-free medium supplemented with BMP4 in 5% oxygen [35]. After 24 h, the BMP4 is replaced with activin A and bFGF for 3 days to induce primitive-streak and mesoderm formation. The cells are then treated with the Wnt inhibitor Dkk1, VEGF and later bFGF to support cardiovascular lineage expansion. With this approach, they reliably obtained human ESC- and iPSC-derived populations of >40% cardiomyocyte purity [35]. An advantage of this protocol is that they identified the specific stage at which a multipotent cardiovascular progenitor cell could be isolated. Keller and colleagues showed that these progenitors give rise not just to cardiomyocytes, but also endothelial and vascular smooth muscle cells [35], making them an attractive source of cells for study in cardiac tissue engineering and cell-based cardiac repair.

3.3.5. Hybrid approaches and chemically defined protocols

Although the preceding directed differentiation protocols involving growth factors and defined media represented a major advance over earlier non-directed (“spontaneous”) EB-based methods (as in Section 3.3.2), they nonetheless have significant limitations. The efficacy of these protocols varies somewhat from line to line even in experienced hands, plus the high cost of recombinant growth factors imposes a challenge to cost-effective scalability [32, 54]. In an effort to reduce

variability and cell production costs, a number of investigators have sought to identify small molecule alternatives to recapitulate the signaling described above. For example, the small molecule CHIR99021 is a glycogen synthase kinase 3 β inhibitor that mimics Wnt signaling and stimulates mesoderm induction in human PSCs [55]. Lian and colleagues explored directed cardiac differentiation based on temporal Wnt signaling modulation with CHIR99021, followed sequentially by the small-molecule Wnt inhibitors IWP-2 and IWP-4 [56, 57]. By this approach, they were able to guide human ESCs into populations of 82–98% pure cardiomyocytes (Fig. 2E) [56, 57]. Importantly, these authors were able to obtain similar cardiac differentiation efficiencies using dishes coated with Matrigel and Synthemax, a synthetic surface. In another step toward a protocol with high translational potential, BurrIDGE and colleagues have reported cardiac differentiation methods involving entirely chemically-defined reagents (Fig. 2F) [58]. They also found no difference between Matrigel and Synthemax across multiple human PSC lines. They reported cardiomyocyte purities of routinely >90% using this approach with an average yield of 44 cardiomyocytes for every starting undifferentiated human PSC. Finally, a “hybrid” protocol has been described that combines sequential activin A and BMP4 treatment, supplemented by early Wnt activation using CHIR99021, followed later by Wnt inhibition with XAV939, a small-molecule tankyrase inhibitor (Fig. 2G) [59]. In our experience, the latter methods significantly reduce both the cost of cardiomyocyte production and lot-to-lot variation in terms of yield and purity.

3.3.6. Embryoid body vs. monolayer differentiation

Some of the above directed differentiation protocols use an EB-based method while others use a monolayer-based method. Each of these methods has potential pros and cons that should be considered when choosing a differentiation method. On the one hand, monolayer-based methods may have practical advantages in that they are technically easier to perform, avoid aggregation and potential replating steps, and may allow more uniform exposure to exogenous soluble factors. On the other hand, EB-based methods provide a three-dimensional context

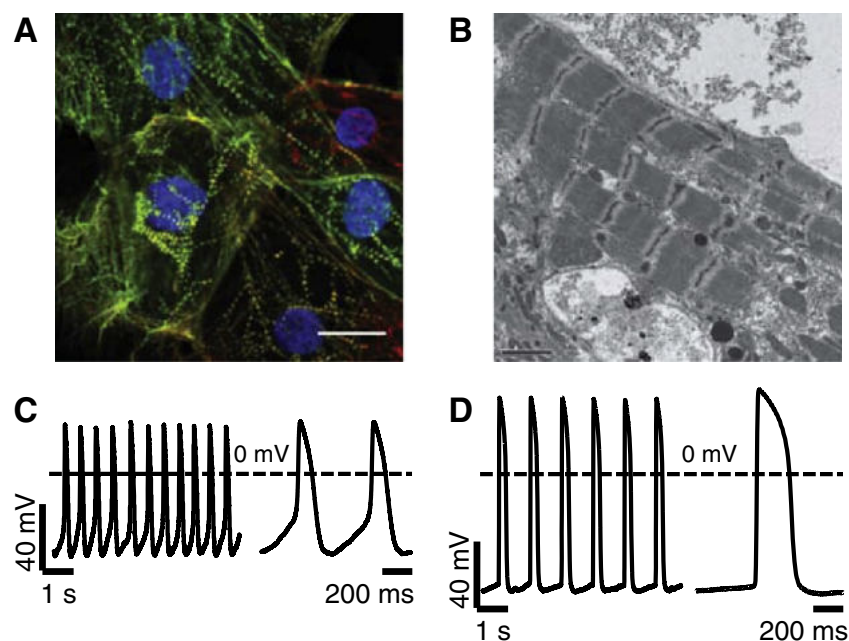


Fig. 3. Phenotype of human PSC-derived cardiomyocytes. A) Confocal photomicrograph of a representative early-stage human ESC-derived cardiomyocytes expressing α -actinin (immunostain, green), F-actin (phalloidin, red), and nuclei (Hoechst, blue). Scale bar: 100 μ m. B) Ultrastructure of a late-stage human ESC-derived cardiomyocyte. Scale bar: 2 μ m. Representative action potential recording from an early-stage human ESC-derived C) nodal cardiomyocyte (adapted from Zhu and colleagues [119]) and D) working cardiomyocyte. Early-stage cardiomyocytes had undergone 3–5 weeks of in vitro maturation, while late-stage cardiomyocytes had undergone 12–15 weeks of in vitro maturation.

that may more faithfully recapitulate the three-dimensional environment of the developing embryo. Finally, EB-based methods or at least protocols that involve the formation and culture of small aggregates of cells may be more amenable to scaled production via bioreactors (as described below in [Section 5.7](#)).

4. Phenotype of human pluripotent stem cell-derived cardiomyocytes

The vast majority of published studies have examined human PSC-derived cardiomyocytes at relatively short timepoints, typically after only 2 to 3 weeks of culture under differentiating conditions. At this stage, these cells have an unambiguously immature phenotype, roughly akin to that of cardiomyocytes in the very early fetal heart. That said, the phenotype of human PSC-derived cardiomyocytes is obviously somewhat of a moving target, as the cells show progressive maturation with duration in culture, even in the absence of exogenous pro-maturation stimuli [60–63]. Indeed, as the field has recognized the need to improve the maturation of these cells, a small but ever-increasing number of reports have examined their phenotype at later timepoints [27, 60–64] and/or after chemical or physical interventions intended to promote their maturation (discussed further in [Section 5.1](#) below). In our own work [60], we have found it convenient to distinguish between early- and late-stage human PSC-derived cardiomyocytes, roughly corresponding to cultures at 3–5 weeks versus 12–15 weeks following cardiac induction, respectively. Because cardiomyocytes at these two stages show such dramatically different structural and functional properties [60–63, 65] and because others in the field have embraced similar terminology [66], we will use it in the following sections.

4.1. Size and structure

Ventricular cardiomyocytes in the adult human heart are large (~130 μm in length), brick-shaped cells with sarcomeres that are readily apparent by phase-contrast microscopy [67]. By contrast, early-stage human ESC-derived cardiomyocytes look quite distinct with much smaller dimensions (~15 \times 10 μm) and typically a round or triangular shape [40, 60, 68, 69]. With time in culture, they become larger, longer, and more rectangular [60, 63, 69]. Desmosomes and gap junctions can be seen by electron microscopy in early-stage human ESC-derived cardiomyocytes [14]. The myofibrillary architecture of early-stage human PSC-derived cardiomyocytes is significantly different from that in adult ventricular cardiomyocytes with the former showing disorganized and relatively short (~1.68 μm) sarcomeres where present ([Fig. 3A](#)) [14, 40, 60, 69]. Morphological maturation has been seen over time with late-stage human PSC-derived cardiomyocytes showing hypertrophy, elongation, greater myofibril density, increased sarcomeric content, better organized sarcomeres ([Fig. 3B](#)) [60] and improved mitochondrial alignment [69]. In long-term culture (up to 180 days), human iPSC-derived cardiomyocytes from EB-based differentiation exhibit impressive ultrastructural maturation characterized by the appearance of mature Z-, A-, H- and I-bands, as well as more tightly packed, parallel-oriented myofibril arrays [70]. Concomitant with structural and functional maturation, proliferative capacity decreases, and nuclei positively labeled by [3H] thymidine or Ki-67 decreased from 50 to 60% in early-stage to <1% in late-stage human PSC-derived cardiomyocytes [69].

4.2. Mechanical properties and force generation

Particularly for in vivo applications, human ESC-derived cardiomyocytes would ideally closely recapitulate the force generation capacity of adult human cardiomyocytes. It is often challenging to compare measures of force generation in the literature because of differences in preparation and experimental techniques [71–73]. The most common and straightforward comparisons that have been made between human adult ventricular and human PSC-derived cardiomyocytes have

employed single-cell measurements, so we will focus on that data here. Most estimates have placed the maximal force generated by individual primary human ventricular cardiomyocytes in the range of 12 to 26 μN per cell (adapted from force measures reported in kN per cross-sectional area) [71, 73]. By comparison, Rodriguez and colleagues used a microarray post-system to obtain force measurements of ~15 nN per cell for human iPSC-derived cardiomyocytes, a value that is approximately three orders of magnitude smaller than their adult counterparts [59]. Other investigators have used independent techniques to arrive at similar estimates of force generation in human PSC-derived cardiomyocytes [74] or at least values within an order of magnitude of that reported by Rodriguez and colleagues [75, 76]. Some recent studies have suggested that the mechanical properties of these cells can be somewhat improved by maturation within a more biomimetic tissue-engineered patch [77], but even these values are dwarfed by those reported for adult ventricular myocytes. In summary, even the most optimistic assessments suggest that the force generating capacity of human PSC-derived cardiomyocytes is approximately two-orders of magnitude smaller than in adult myocytes. The need to improve this parameter remains a major hurdle in the field.

4.3. Electrophysiology

There have been surprisingly few experimental measurements of the action potential (AP) properties of normal adult human ventricular myocytes, but the best available data suggest these cells have maximum diastolic potentials (MDPs) of ~-80 mV, AP amplitudes ranging from 100 mV to 135 mV, AP upstroke velocities (dV/dt_{max}) from 230 to 400 V/s, and AP durations to 90% repolarization (APD_{90}) from 280 to 400 ms [78–81] (the precise values for these parameters obviously vary with rate and location in the ventricles). By comparison, human PSC-derived cardiomyocytes show far greater heterogeneity and immaturity in their electrophysiological properties, especially in early-stage cultures. The cardiomyocytes that result from all currently available cardiac differentiation protocols are diverse in that they include cells with distinct ventricular-, atrial- and nodal-like action potentials and patterns of gene expression [5, 27, 40, 50, 64]. However, even if one corrects for this diversity and considers only the subset of human PSC-derived cardiomyocytes that would be classified as ventricular-like, these cells still exhibit more variable and immature action potential properties than their adult counterparts ([Fig. 3C](#)). For example, Kamp and colleagues have studied ventricular-like cells from human ESCs [64] and iPSCs [27] and, while there was substantial variability, these cardiomyocytes typically had relatively depolarized MDPs (~-60 mV), narrow AP amplitudes (<100 mV), slow dV/dt_{max} values (~15 to 50 V/s), and APD_{90} of ~250 to 300 ms. As discussed further below in [Section 5.3](#), this significant electrical mismatch with adult ventricular myocytes raises concerns about arrhythmogenic potential of human PSC-based cardiac therapies. Human PSC-derived cardiomyocytes are also distinguished from adult cardiomyocytes by their automaticity (spontaneous electrical activity). This is another indicator of their relative immaturity, as automaticity is a property of early fetal cardiomyocytes that later becomes restricted to specialized pacemaking centers such as the sinoatrial node. In our hands, early-stage human PSC-derived cardiomyocytes show automaticity at rates of ~100 beats per minute (bpm), while their late-stage counterparts fire at rates of less than 10 bpm or even lose automaticity altogether [60].

Underlying these differences in the rate and AP properties of human PSC-derived versus adult cardiomyocytes are corresponding differences in ion channel gene expression and function. In general, human PSC-derived cardiomyocytes are distinguished by their relatively low levels of the inward rectifier potassium current (I_{K1}) [62, 82] and high levels of the pacemaker current (I_f) [62, 83]. The functional consequence is a relatively depolarized MDP and greater electrical instability. On the other hand, current densities reported for a number of other major ionic currents including the fast sodium (I_{Na}) [84–86], the L-type

calcium (I_{CaL}) [85, 87], transient outward potassium (I_{to}) [82] and delayed rectified potassium (I_{Kr} and I_{Ks}) currents [85] are not greatly dissimilar between human PSC-derived and adult cardiomyocytes; although in some instances there are more subtle differences in isoform expression and/or kinetics. Indeed, modeling experiments suggest that the single-cell electrophysiological properties of human PSC-derived cardiomyocytes could be largely “normalized” if they can be induced to have more adult-like expression levels of I_{K1} and pacemaking currents [88, 89].

In addition to these important differences at the single-cell level, multicellular preparations of human PSC-derived cardiomyocytes also show significantly slower electrical propagation than their adult counterparts. Conduction velocity in two-dimensional cultures of human PSC-derived cardiomyocytes has been measured at anywhere from ~2 to 20 cm/s [90–92]. This is an obviously wide range (likely due in part to differences in cardiomyocyte purity), but all reported values are significantly less than the >50 cm/s reported for comparably formed monolayers of postnatal cardiomyocytes [93]. The slower conduction velocities exhibited by human PSC-derived cardiomyocyte cultures is likely a consequence of their smaller cell size, lower expression levels of the gap junction protein connexin-43, and isotropic cell orientation.

4.4. Calcium handling

Excitation–contraction coupling in adult mammalian ventricular cardiomyocytes is initiated by membrane depolarization and the opening of L-type calcium channels. The resultant calcium influx is amplified via calcium-induced calcium release (CICR) from sarcoplasmic reticulum (SR) stores that are gated by caffeine- and ryanodine-sensitive release channels (i.e. ryanodine receptors) [94]. Importantly, this CICR operates via a tight “local control” mechanism in which a local rise in cytosolic calcium following trans-sarcolemmal influx activates only nearby ryanodine receptors, rather than a regenerative, all-or-none calcium response throughout the cell. During systole, these localized calcium release events (calcium “sparks”) summate to produce the “whole-cell” calcium transient and contraction. Moreover, because the involved L-type calcium channels are clustered in the transverse tubules (or t-tubules) with ryanodine receptors immediately adjacent, these calcium sparks occur predominantly near the t-tubules and in register with the Z-line [95]. These mechanisms of excitation–contraction coupling distinguish adult mammalian ventricular cardiomyocytes from ventricular cardiomyocytes in fish and amphibians, in which cytosolic calcium transients result almost entirely from trans-sarcolemmal calcium influx (i.e. without significant amplification by CICR) [96–98]. Calcium handling is also different in mammalian cardiomyocytes that lack t-tubules (e.g. Purkinje fibers, atrial cardiomyocytes, and neonatal ventricular cardiomyocytes). In cardiomyocytes without t-tubules, there is an initial rise in cytosolic calcium near the plasma membrane at the periphery of the cell that then propagates more slowly throughout the cytoplasm by either diffusion or sequential CICR events [95].

Very early reports suggested that human PSC-derived cardiomyocytes lack significant SR calcium cycling [99, 100], implying mechanisms of excitation–contraction coupling similar to those operational in fish and amphibian hearts. However, our group and other investigators subsequently demonstrated that human PSC-derived cardiomyocytes do in fact have robust caffeine- and ryanodine-sensitive SR calcium stores [87, 101, 102] and that CICR in these cells operates via the aforementioned tight “local control” mechanism of adult ventricular cardiomyocytes [87, 102]. That said, there are important differences in the calcium handling properties of human PSC-derived versus adult cardiomyocytes. First, while reports have varied as to whether L-type calcium channels and ryanodine receptors are co-localized in human PSC-derived cardiomyocytes [87, 103], they definitely lack a significant t-tubule network, resulting in spatially heterogeneous and incompletely synchronized calcium transients [103, 104]. Second, relative to their adult counterparts, human PSC-derived cardiomyocytes show greatly reduced expression and function of the SR calcium ATPase pump

(SERCA) that mediates SR calcium reuptake, so they show correspondingly slower calcium transient kinetics during diastole [60, 100, 104]. Interestingly, SERCA expression and calcium transient kinetics in these cells can be somewhat improved by maturation over time in culture [60, 101] and/or electromechanical conditioning [105]. Human PSC-derived cardiomyocytes also express very low levels of calsequestrin, the most abundant calcium-binding protein in the SR of adult cardiomyocytes and an important component of a luminal SR calcium load sensor that regulates ryanodine receptor calcium release [106]. Of note, overexpression of calsequestrin in these cells has been shown to enhance their calcium handling properties, as evidenced by increases in calcium transient amplitude and kinetics [107]. Finally, while one recent report suggested that there may be important differences in calcium handling between human ESC- and iPSC-derived cardiomyocytes [104], this observation has not been supported in subsequent work [6, 108] and so warrants further study.

4.5. Mitochondrial function and metabolism

A major metabolic switch occurs in the developing heart with glycolysis predominating in the fetal heart and fatty acid metabolism in the adult [109, 110]. This transition is accompanied by a tremendous increase in mitochondrial content, termed mitochondrial biogenesis, that results in mitochondria eventually occupying as much as 23% of total cell volume in the adult ventricular cardiomyocyte [111]. The corresponding changes in metabolism and mitochondrial biology during the differentiation and maturation of human PSC-derived cardiomyocytes are just beginning to be investigated. Interestingly, chemical enhancers of mitochondrial biogenesis have been shown to help promote mesendodermal differentiation in early human ESCs [112]. Although the relationship of mitochondrial biogenesis to cardiomyocyte-specific human PSC differentiation has not been specifically investigated, a recent study demonstrated that the number and surface area of mitochondria increased when human iPSCs differentiate into cardiomyocytes [113]. However, mitochondria still comprise only 7% of the cell volume in early-stage human iPSC-derived cardiomyocytes (after ~20 days of in vitro differentiation) [74]. The mitochondria in these cells appear immature with a long, slender morphology and a disorganized arrangement around the nucleus [114]. By contrast, late-stage human PSC-derived cardiomyocytes show more of the expected localization of mitochondria between the myofibrils [69]. This placement of mitochondria between the myofibrils is thought to promote more efficient diffusion of the high-energy phosphate to the myofibrils during muscle contraction. Interestingly, with reprogramming of somatic cells into human iPSCs, mitochondria revert to immature, embryonic-like structures [115].

Mitochondrial metabolic function has only been studied in the mouse ESC-system, in which it shows significant changes during cardiogenesis. During cardiomyocyte differentiation, the energy source shifts from anaerobic glycolysis to mitochondrial respiration, along with an increase in the number of mitochondria [116]. This metabolic switch is required to meet the increasing energy demands of cardiomyocytes. In addition, amino acid and carbohydrate metabolism are most prominent in undifferentiated ESCs, whereas fatty acid metabolism predominates in mouse ESC-derived cardiomyocytes [116], similar to the metabolism observed in adult mouse cardiomyocytes. Clearly, this is an area that needs further study with specific attention to the metabolic function of human PSC-derived cardiomyocytes.

5. Remaining obstacles to the successful translation of pluripotent stem cell-derived cardiomyocytes as a practical source of heart cells

5.1. Immature phenotype

In Section 4.1, we discussed one of the major limitations of currently available human PSC-derived cardiomyocytes, their immature phenotype. Although we noted how their maturation does improve

with prolonged duration in culture, this is a very slow process and even late-stage cardiomyocytes do not fully recapitulate the adult cardiomyocyte phenotype. For example, as noted above, late-stage human PSC-derived cardiomyocytes still have somewhat depolarized MDPs [27, 64], lack t-tubules [103, 104], and exhibit reduced mitochondrial mass and function [74, 114]. To address this limitation of phenotypic immaturity, the field is actively exploring new approaches to accelerate and/or enhance their maturation in vitro. These pro-maturation strategies can be broadly divided into three categories: treatment with chemical agents such as growth factors, electromechanical conditioning, and culture within more biomimetic environments (e.g. three-dimensional tissue engineering) [61, 68, 74, 77, 117, 118]. These approaches are obviously not mutually exclusive, and we speculate that the most efficacious maturation scheme will likely incorporate elements of all three.

One example of a chemical factor that has been shown to promote the maturation of human PSC-derived cardiomyocytes is the thyroid hormone triiodothyronine (T3). The treatment of these cells with T3 promotes increases in cardiomyocyte size, anisotropy, and sarcomere length, as well as robust increases in the kinetics of calcium release and reuptake, maximal contractile force generation, and the kinetics of contraction. T3 also significantly increases maximal mitochondrial respiratory capacity, without changing mitochondrial DNA copy numbers [74]. Of note, T3 is present in the serum substitute, B-27 (Life Technologies), which is used as a media constituent in multiple directed cardiomyocyte differentiation protocols. Other factors have been reported to promote the maturation of at least some parameters in cardiomyocytes from mouse or human PSCs include neuregulin [119, 120], angiotensin II [63], and the alpha-adrenergic agonist phenylephrine [63]. Finally, while it has been suggested that non-myocytes secrete as yet undefined factors that favor human PSC-derived cardiomyocyte maturation [61], our own data demonstrate that these factors are not absolutely required for maturation [60].

It has been known for many years that electromechanical stimulation promotes the maturation of primary neonatal cardiomyocyte cultures [121–123], so this intervention represents a logical strategy for enhancing the maturation of human PSC-derived cardiomyocytes as well. Indeed, when exposed to chronic electrical pacing, human iPSC-derived cardiomyocytes respond by elongating, assuming a higher cytoplasm-to-nucleus ratio, and forming better organized sarcomeres with visible M-bands by electron microscopy [117]. The latter structural changes are accompanied by evidence of functional maturation including an ~50% increase in force generation, reduced automaticity, a rightward shift of the Ca^{2+} -response curve, and an increased inotropic response to β -adrenergic stimulation [117]. Nunes and colleagues have recently described similar structural and functional maturation following the electrical stimulation of human iPSC-derived cardiomyocytes cultured in collagen hydrogel “biowires” [124].

Human ESC-derived cardiomyocytes grown in three-dimensional patches even without electrical stimulation exhibit improved maturation compared with those in two-dimensional arrangements, as evidenced by their former's higher conduction velocities, longer sarcomeres, higher maximal contraction amplitudes, and enhanced expression of genes involved in cardiac contractile function, including cardiac troponin T, α -myosin heavy chain, calsequestrin, and sarcoendoplasmic reticulum ATPase [77]. Engineered human cardiac tissues made of human ESC-derived cardiomyocytes mixed with collagen and cultured on three dimensional elastomeric devices exhibit microstructural features, mRNA expression, and electromechanical properties comparable to neonatal human myocardium [118]. For more details about physical cues and maturation, the reader is referred to a recent review by Zhu and colleagues [125].

5.2. Cardiomyocyte subtype diversity

All currently available cardiac differentiation protocols for human PSCs produce a mixture of different cardiac subtypes, e.g., ventricular,

atrial, nodal and Purkinje cardiomyocytes [22, 50, 64, 119, 126]. Although ventricular cardiomyocytes usually predominate and increase in abundance over time [70, 127], this subtype diversity nonetheless represents a significant challenge for most in vitro and in vivo applications, in which a highly purified population of ventricular cardiomyocytes would better recapitulate the ventricular myocardium being modeled or replaced. The delivery of a high fraction of nodal cells to a recently infarcted heart might increase the risk of graft-related arrhythmias. Conversely, a number of investigators have envisioned employing human PSC-derived cardiomyocytes in the creation of a biological pacemaker, and, for this application, cells with a stable nodal phenotype would obviously be desirable [128, 129].

With this in mind, our group and others in the field have worked to identify methods to reliably guide the differentiation of human PSCs not just into cardiomyocytes but into specific cardiac subtypes. These efforts build on decades of prior work in developmental biology aimed at understanding the signaling pathways that regulate this specialization (for a comprehensive recent review, please see Rana and colleagues [130]). A large number of transcription factors have been implicated in this process including Nkx2.5, Isl1, Hand1/2, Irx4, Cited1, COUP transcription factor-2, CHF1/Hey2, and the T-box family among many others [131–142], and a subset of these have been manipulated in the PSC system. For example, in differentiating mouse ESC-derived cardiomyocytes, Nkx2-5 represses Isl1 resulting in ventricular cardiomyocyte formation [143]. When Nkx2-5 is not present, the Isl1+ cells have a greater propensity to become nodal cells [143]. Overexpression of the transcription factor SHOX2 has also been shown to promote nodal differentiation in the mouse ESC system [144].

A number of growth factors have also been shown to regulate cardiac subtype specialization in the PSC system including neuregulin [119, 145], endothelin [146], retinoic acid (RA) [147, 148] and canonical Wnts [126, 149]. Our group has shown that endogenous neuregulin normally drives ventricular maturation in differentiating human ESC-derived cardiomyocyte cultures, but that inhibition of neuregulin (with either a small molecule antagonist or a neutralizing anti-neuregulin antibody) greatly increases the proportion of cardiomyocytes with a nodal phenotype [119]. Interestingly, neuregulin stimulation has been shown to drive the conversion of committed ventricular cardiomyocytes into Purkinje fibers in mouse embryo cultures [145], inviting speculation that this signal might be able to mediate similar effects in human PSC-derived ventricular cardiomyocytes if appropriately timed. Interestingly, Maass and colleagues recently described the successful isolation and tracking of mouse ESC-derived cardiac Purkinje cells based on the marker contactin-2 [150], and this might be a useful tool for testing this hypothesis.

Retinoic acid (RA) and Wnt signaling appear to play a critical role in determining atrial versus ventricular cardiomyocyte fate in differentiating PSCs [147, 148]. Ma and colleagues reported that the treatment of human ESC-derived cardiomyocytes with a pan-RA receptor antagonist produce an increase in the fraction of ventricular-type cells based on AP profiling and expression of the ventricular markers Mlc-2v and Irx4 [148]. Conversely, treatment with RA seems to favor atrial differentiation in human and mouse ESCs [147, 148]. Hudson and colleagues found that treating differentiating human ESC cultures with two different small-molecule Wnt inhibitors, IWP-4 and IWR-1, results in cardiomyocyte populations with differing expression levels of the atrial marker Mlc-2a and the ventricular marker Mlc-2v [149]. Another group found that treatment with IWR-1, 4.5 days after induction of differentiation with activin A, resulted in 100% ventricular cardiomyocytes based on action potential phenotyping [126]. More work is required to dissect the pharmacological basis of these intriguing differential responses to Wnt inhibitors, and more rigorous phenotyping should be applied (including comprehensive electrophysiological endpoints).

5.3. Arrhythmogenesis

One major rationale for obtaining highly purified ventricular cardiomyocytes from human PSCs is that the transplantation of these cells might prove less likely to promote dangerous tachyarrhythmias than with the transplantation of cell preparations including significant quantities of nodal or other non-ventricular cardiomyocytes. There is good reason for this concern: the introduction of a population of cells with sustained pacemaking activity and the unique neurohormonal responsiveness of nodal tissue could indeed induce ventricular ectopy. However, there are actually multiple mechanisms by which PSC-derived cardiac grafts (even if highly ventricular cardiomyocyte-enriched) could promote electrical instability, and the risk of graft-related arrhythmias has emerged as arguably the greatest barrier to the successful development of PSC-based cardiac therapies.

The principal advantage of PSC-derived cardiomyocytes over other candidate cell types that have been considered for use in cell-based cardiac repair is that these cells are capable of “remuscularizing” the infarct scar with functionally integrated new force-generating units [151]. Our group provided the first direct evidence that human ESC-derived cardiomyocytes can couple electrically and contract synchronously with host myocardium following transplantation in both intact and injured hearts [152–155]. However, this capacity of human PSC-derived cardiomyocytes for electromechanical integration is a double-edged sword. The graft myocardium obviously needs to integrate and activate synchronously with the host to restore the lost systolic function. On the other hand, electrically-integrated graft tissue could promote arrhythmias, particularly when that graft tissue has the relatively immature electrophysiological properties discussed in Section 4.3. As noted above, all human PSC-derived cardiomyocytes exhibit some degree of automaticity, at least at early timepoints, so even nodal-depleted grafts represent a potential source of ventricular ectopy. Moreover, *in vitro* and *in silico* models suggest that ectopic foci firing at rates slower than the host sinus rhythm may be capable of driving tachyarrhythmias under conditions likely to arise in graft tissue including poor coupling, source-sink electrical mismatch and/or network heterogeneity [156, 157]. PSC-derived cardiomyocytes could also be prone to afterdepolarizations and triggered arrhythmias [158], particularly if stressed during the initial period post-transplantation (e.g. by ischemia).

Reentry is another plausible mechanism for graft-related arrhythmias, since human PSC-derived cardiomyocyte grafts exhibit several properties expected to favor its initiation. Our group has used optical methods to examine propagation in graft tissue and have obtained estimates of graft conduction velocity that are substantially slower than that in host myocardium [152, 153]. This is perhaps not surprising given that human PSC-derived cardiomyocytes are relatively small, immature cells with low levels of isotropically distributed connexins [46, 152, 153, 159, 160]. They also form irregular strands of graft muscle that represent abnormal conduction pathways, and their intrinsic electrophysiological heterogeneity seems likely to result in non-uniform repolarization properties within the graft, further exacerbating the risk of electrical instability.

These concerns about graft-related arrhythmias based on first principles were recently confirmed by our group following the intra-cardiac transplantation of human ESC-derived cardiomyocytes in a large animal model [154]. While our early work in a small animal model had suggested that these cells might exert an arrhythmia-suppressive effect [152], we found that their transplantation in a non-human primate infarct model resulted in transient bouts of ventricular tachycardia [154]. While these events were non-lethal and seemed to greatly dissipate by 2 to 3 weeks post-transplantation, their occurrence nonetheless underscores the need to obtain human PSC-derived cardiomyocytes with more mature electrophysiological properties, better matched to that of the recipient heart.

5.4. Teratomas and karyotypic abnormalities

A major concern that must always be considered with any potential clinical therapies based on PSCs is the risk of tumorigenesis. Indeed, undifferentiated PSCs are operationally defined by their ability to give rise to teratomas (i.e. benign tumors including elements of all three embryonic germ layers) following transplantation into immunodeficient mice. Although some early studies suggested that the heart may be a cardio-instructive environment capable of efficiently guiding undifferentiated ESCs into cardiomyocytes [161, 162], it is now accepted that this is not the case and the intra-cardiac injection of PSCs instead gives rise to the expected teratomas [163, 164]. On the other hand, there is now a large number of studies in which investigators have transplanted enriched populations of human PSC-derived cardiomyocytes [152–154, 159, 165–169]. In all of these instances, cardiomyocytes successfully engrafted, but no teratomas were detected. Importantly, this includes transplantation studies from our group involving the delivery of $\sim 10^8$ and $\sim 10^9$ human ESC-derived cardiomyocytes to injured guinea pig and primate hearts, respectively [152–154]. This suggests that there may be minimal risk of teratoma formation even when injecting large quantities of cells, as long as they are of sufficient cardiomyocyte purity. In sum, while more study is required (including experiments involving large doses of cells and a longer duration of follow-up), early experience in the field has been reassuring with regard to the risk of teratomas. Moreover, if it is determined that guided differentiation protocols still leave an unacceptable level of non-myocytes, the field has identified a number of cardiomyocyte-specific cell surface markers suitable for magnetic- and/or fluorescence-activated cell sorting [170, 171].

That said, even if safety concerns about teratoma formation can be addressed, vigilance is still needed. PSCs also show significant karyotypic instability with prolonged duration in culture [172], raising separate concerns about potential neoplastic transformation and/or dysregulation of gene expression [173, 174]. This may be less problematic for *in vitro* applications such as disease modeling or drug screening, but it would be potentially devastating in the context of clinical PSC-based therapies. The frequency of such chromosomal alterations is highly variable and appears to be a function of both cell line and culture conditions. Taapken and colleagues found karyotypic abnormalities in about 13% of 1715 cultures analyzed from 259 different human PSC lines [172]. Of note, they found human ESC cultures with abnormal karyotypes as early as after three passages post-derivation and normal karyotypes as late as after 240 passages. While they did not find a set of karyotypic abnormalities common to both human ESCs and iPSCs, the abnormalities in cultured human PSCs differed from those observed in human embryos [175].

As discussed in Section 2, naïve human ESCs may be less prone to developing karyotypic abnormalities than primed-stage human ESCs and human iPSCs. Indeed, Ware and colleagues reported that the Elf1 naïve human ESC line was still karyotypically normal after more than 90 passages [16]. Gafni and colleagues demonstrated karyotypic stability in their naïve human ESCs for at least 46 passages and in human iPSC cultures grown in naïve human ESC culture conditions for over 50 passages [19]. However, enthusiasm is somewhat tempered by the experience of Theunissen and colleagues, who reported finding karyotypic abnormalities in both human ESCs converted to the naïve state as well as in embryo-derived naïve human ESCs [18]. The three naïve human ESC lines are very new and were each derived under somewhat different conditions, so more investigation will be necessary to determine if these cells represent a solution to the challenge of human PSC karyotypic stability.

5.5. Immune rejection

Although human PSC-derived cardiomyocytes have shown considerable promise in preclinical models of myocardial infarction, most of these studies have employed immunodeficient or heavily immunosuppressed

animals [46, 152, 154, 160]. While it is possible that PSC-based therapies will evoke a less robust immune reaction than whole-organ transplantation (particularly if the injectate is devoid of antigen-presenting cells such as endothelial cells) [176, 177], the successful clinical translation of these cells will undoubtedly require dealing with the immune system. Conventional pharmacological immunosuppression could be employed, but this approach would bring along with it increased risks of infection and malignancy [178]. It would very likely limit the target patient population for PSC-based cardiac therapies to the end-stage heart failure patient that is currently considered appropriate for heart transplantation. This strategy would likely also require the banking of diverse human leukocyte antigen (HLA) typed PSCs, and many dozens of human PSC lines would be needed to ensure a reasonable chance of a suitable match even in patient populations with relatively low ethnic diversity [179, 180] (for example, it has been estimated that a bank of ~150 lines would suffice to match >90% of the population in the United Kingdom with minimal requirement for immunosuppression). The size of such a bank could be possibly reduced by the use of HLA-homozygous ESCs derived from human parthenogenetic blastocysts [181].

It is theoretically possible to create autologous cell-based therapies for every person with iPSC technology, but the cost to create, test, and store these human iPSCs would be prohibitive in reality. There are also practical regulatory hurdles to individualized iPSC-based therapies [182]. Moreover, there have been conflicting reports regarding the ability of syngeneic iPSCs to avoid immune rejection in the first place. In a provocative study, Zhao and colleagues transplanted mouse iPSCs (generated via non-integrating reprogramming methods) into syngeneic recipients and reported that the resultant teratomas evoked a brisk immune response, including T-cell infiltration [183]. Liu and colleagues described similar immune rejection following the transplantation of iPSCs and iPSC-derived cardiomyocytes in syngeneic mice [184]. On the other hand, more recent work in the field has called into question the veracity of the preceding reports. For example, Guha and colleagues performed similar transplantation experiments into syngeneic recipients and did not find any evidence of immune rejection using cells derived from three mouse iPSC lines [185], including one line previously employed by Zhao and colleagues. Similarly, Araki and colleagues generated ten mouse iPSC and seven mouse ESC lines and found no difference in the immunogenicity of iPSCs versus ESCs in syngeneic recipients [186].

A number of other strategies to overcome immune rejection of human PSC-based therapies have been envisioned. For example, combined blockade of T-cell activation using cytotoxic T lymphocyte antigen 4-immunoglobulin (CTLA4-Ig) fusion protein and anti-LFA-1 (co-stimulatory ligand) has been shown to improve human ESC-derived cardiomyocyte engraftment in mouse models [187]. Cardiomyocytes derived from human ESCs expressing CTLA4-Ig and programmed death ligand-1, a T-cell inhibitory protein, evoke a significantly reduced immune response when transplanted into mice with humanized immune systems [177]. The induction of immune tolerance via the transplantation of human ESC-derived dendritic cells has also been suggested as a possibility [188]. Another strategy to evade T-cell activation is through the knock-down of HLA-class I proteins as shown with human ESC xenotransplants into mice [189]. Lu and colleagues disrupted β -2 microglobulin in human ESCs and generated a cell line that has impaired surface expression of HLA-class I proteins [190]. They showed that cells derived from these human ESCs were less immunogenic when assayed with human peripheral blood mononuclear cells. This last approach may provide a “universal donor” human ESC line.

While the wide range of options for overcoming graft cell rejection gives reason for optimism, the precise nature and strength of the immune response to allogeneic PSCs or PSC-derived cardiomyocytes in humans remains unknown. Given this, while much has been learned from xenotransplantation experiments, the field needs to undertake more studies involving PSC-derivatives in allogeneic recipients (for

example, allotransplantation of primate PSC-derived cardiovascular progenitors into immunosuppressed non-human primate recipients [191]).

5.6. Epigenetic modifications

Epigenetic modifications may limit the ability of human PSCs to differentiate into cardiomyocytes or specific cardiomyocyte subtypes, which would diminish their utility as a source of heart cells. Early-passage mouse iPSCs retain some of the epigenetic modifications of the cell from which they were derived, and these iPSCs differ in their overall differentiation potential [192]. Human iPSCs generated from cardiac progenitor cells have been reported to have a greater cardiomyocyte differentiation efficiency than iPSCs from dermal fibroblasts, although these differences appear to lessen with prolonged duration in culture in the undifferentiated state [193]. Leschik and colleagues demonstrated that the activating histone 3 lysine 4 trimethylation marks and the repressive histone 3 lysine 27 trimethylation marks on cardiac-related transcription factors differed between two human ESC lines with different cardiac potential [21]. An improved understanding of how epigenetic modifications regulate cell state might provide new opportunities to manipulate the cardiac differentiation of PSCs, their fate decisions with regard to cardiac subtype specialization, and subsequent maturation. Consistent with this, the treatment of differentiating human ESC-derived ventricular cardiomyocytes with a histone deacetylase inhibitor, which increases DNA accessibility for transcription factors, resulted in a larger cell size and increased expression of calcium-handling and ion channel genes [194]. Given the dynamic nature of epigenetic modifications on human PSCs and their association with differences in differentiation potential, further study will be required if we are to use the epigenetic regulation of DNA to our advantage.

5.7. Scalability and clinical grade production

The average human heart contains approximately 4 billion cardiomyocytes [195], and the typical infarct involves the loss of approximately one-quarter of these cells, a situation that all too often initiates progressive heart failure [196]. Human PSC-derived cardiomyocytes do proliferate in vivo [46, 159, 197], but any such expansion is at least counterbalanced by cell death shortly after transplantation [198]. Therefore, the delivery of ~1 billion cardiomyocytes per patient does not seem unreasonable if remuscularization is the therapeutic goal. Our group recently demonstrated the feasibility of cardiomyocyte production and transplantation at this scale in a preclinical study in the non-human primate model [154]. While this study provided useful proof-of-concept, our approach to cell production was highly labor-intensive, expensive and not clinical grade. To obtain the required numbers of cells, we employed the monolayer-based cardiac differentiation protocol described above in Section 3.3.2 and harvested approximately one-dozen 150 cm² flasks of human ESC-derived cardiomyocytes to obtain the cell dose for each primate. Cardiomyocytes were prepared ahead of time and cryopreserved until shortly before injection, using methods previously shown to result in reasonable viability [199].

While the preceding methods suffice for early preclinical work, they are obviously not suitable for the large-scale and clinical-grade production that would be required for eventual human patients. To meet this challenge, a number of groups have turned to the use of suspension culture systems that have become the standard for industrial-grade production of other cell types. Indeed, suspension bioreactor-based systems have been used to grow large scale numbers of human PSCs (with yields of ~1–10 × 10⁷ cells per flask) [200] and to produce significant quantities of human PSC-derived cardiomyocytes (~0.4–1 × 10⁶ cells/ml) at cardiac purities of up to 85% using cyclic perfusion feeding [201]. However, the efficiency of differentiation of human PSCs in suspension systems has been variable [201–204]. Correia and colleagues differentiated human iPSCs to

cardiomyocytes using the Cellbag™-WAVE bioreactor (GE Healthcare) in 4% oxygen, rather than continuous or intermittent agitation in a stirred-tank bioreactor [205]. They reported obtaining a purity of ~75% and an impressive yield of 60 cardiomyocytes per input undifferentiated cell. In short, while much more work remains to be done, the field can clearly generate quantities of human PSC-derived cardiomyocytes that would have seemed impossible only a decade ago.

6. Conclusions

Human pluripotent stem cells present an exciting opportunity for basic research into various diseases, the discovery of new therapies and mechanisms, and possibly providing a cure to conditions that today are considered chronic. These opportunities exist because of the hard work of multiple investigators leading to the isolation of PSCs, first from the mouse and then later from humans, followed by the development of robust protocols for cardiomyocyte generation. However, as discussed in Section 5, a number of major hurdles must be overcome before the full potential of these cells can be realized. On the other hand, the field has moved remarkably fast, and there are exciting technologies on the horizon that promise to accelerate scientific discovery and unlock new possibilities. For example, the emergence of novel biomaterials and tissue bioengineering technologies seem likely to greatly improve the efficiency of cardiac differentiation and maturation. By employing insights from genome sciences and bioinformatics, the field hopes to decode the “program” of epigenetic modifications and deconstruct cellular regulatory networks that will bring us one step closer to being able to create very specific cellular states. Recent reports describing the direct reprogramming of unrelated somatic cell types (e.g. fibroblasts) into cardiomyocytes both in vitro and in vivo support the feasibility of this approach [206–208]. Finally, emerging high-precision genome-engineering technologies open up the possibility of creating new cardiovascular disease models as well as transformative PSC-based cell populations such as HLA-deficient “universal donor” cells.

Acknowledgments

The authors would like to thank Scott Lundy, José David Otero-Cruz, Sung G. Park, Benjamin Van Biber and Wei-Zhong Zhu for providing valuable comments and/or assistance with figure preparation. This work was supported by funding from the National Institutes of Health (grants R01-HL064387, P01-HL094374, U01-HL100405, R01-HL117991, and U54-HG007010).

References

- [1] P. Liang, F. Lan, A.S. Lee, T. Gong, V. Sanchez-Freire, Y. Wang, S. Diecke, K. Sallam, J.W. Knowles, P.J. Wang, P.K. Nguyen, D.M. Bers, R.C. Robbins, J.C. Wu, Drug screening using a library of human induced pluripotent stem cell-derived cardiomyocytes reveals disease-specific patterns of cardiotoxicity, *Circulation* 127 (2013) 1677–1691.
- [2] I. Caverio, H. Holzgreffe, Comprehensive in vitro proarrhythmia assay, a novel in vitro/in silico paradigm to detect ventricular proarrhythmic liability: a visionary 21st century initiative, *Expert Opin. Drug Saf.* 13 (2014) 745–758.
- [3] H. Landgren, P. Sartipy, Can stem-cell-derived models revolutionize drug discovery? *Expert Opin. Drug Discovery* 9 (2014) 9–13.
- [4] X. Carvajal-Vergara, A. Sevilla, S.L. D'Souza, Y.S. Ang, C. Schaniel, D.F. Lee, L. Yang, A.D. Kaplan, E.D. Adler, R. Rozov, Y. Ge, N. Cohen, L.J. Edelmann, B. Chang, A. Waghray, J. Su, S. Pardo, K.D. Lichtenbelt, M. Tartaglia, B.D. Gelb, I.R. Lemischka, Patient-specific induced pluripotent stem-cell-derived models of LEOPARD syndrome, *Nature* 465 (2010) 808–812.
- [5] A. Moretti, M. Bellin, A. Welling, C.B. Jung, J.T. Lam, L. Bott-Flugel, T. Dorn, A. Goedel, C. Hohnke, F. Hofmann, M. Seyfarth, D. Sinnecker, A. Schomig, K.L. Laugwitz, Patient-specific induced pluripotent stem-cell models for long-QT syndrome, *N. Engl. J. Med.* 363 (2010) 1397–1409.
- [6] A. Fatima, G. Xu, K. Shao, S. Papadopoulos, M. Lehmann, J.J. Arnaiz-Cot, A.O. Rosa, F. Nguemo, M. Matzkies, S. Dittmann, S.L. Stone, M. Linke, U. Zechner, V. Beyer, H.C. Hennies, S. Rosenkranz, B. Klauke, A.S. Parwani, W. Haverkamp, G. Pfister, M. Farr, L. Cleemann, M. Morad, H. Milting, J. Hescheler, T. Saric, In vitro modeling of ryanodine receptor 2 dysfunction using human induced pluripotent stem cells, *Cell. Physiol. Biochem.* 28 (2011) 579–592.
- [7] I. Itzhaki, L. Maizels, I. Huber, L. Zwi-Dantsis, O. Caspi, A. Winterstern, O. Feldman, A. Gepstein, G. Arbel, H. Hammerman, M. Boulos, L. Gepstein, Modelling the long QT syndrome with induced pluripotent stem cells, *Nature* 471 (2011) 225–229.
- [8] N. Sun, M. Yazawa, J. Liu, L. Han, V. Sanchez-Freire, O.J. Abilez, E.G. Navarrete, S. Hu, L. Wang, A. Lee, A. Pavlovic, S. Lin, R. Chen, R.J. Hajjar, M.P. Snyder, R.E. Dolmetsch, M.J. Butte, E.A. Ashley, M.T. Longaker, R.C. Robbins, J.C. Wu, Patient-specific induced pluripotent stem cells as a model for familial dilated cardiomyopathy, *Sci. Transl. Med.* 4 (2012) (130ra47).
- [9] F. Lan, A.S. Lee, P. Liang, V. Sanchez-Freire, P.K. Nguyen, L. Wang, L. Han, M. Yen, Y. Wang, N. Sun, O.J. Abilez, S. Hu, A.D. Ebert, E.G. Navarrete, C.S. Simmons, M. Wheeler, B. Pruitt, R. Lewis, Y. Yamaguchi, E.A. Ashley, D.M. Bers, R.C. Robbins, M.T. Longaker, J.C. Wu, Abnormal calcium handling properties underlie familial hypertrophic cardiomyopathy pathology in patient-specific induced pluripotent stem cells, *Cell Stem Cell* 12 (2013) 101–113.
- [10] G.R. Martin, Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells, *Proc. Natl. Acad. Sci. U. S. A.* 78 (1981) 7634–7638.
- [11] T.C. Doetschman, H. Eistetter, M. Katz, W. Schmidt, R. Kemler, 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 (1985) 27–45.
- [12] J.A. Thomson, J. Itskovitz-Eldor, S.S. Shapiro, M.A. Waknitz, J.J. Swiergiel, V.S. Marshall, J.M. Jones, Embryonic stem cell lines derived from human blastocysts, *Science* 282 (1998) 1145–1147.
- [13] J. Itskovitz-Eldor, M. Schuldiner, D. Karsenti, A. Eden, O. Yanuka, M. Amit, H. Soreq, N. Benvenisty, Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers, *Mol. Med.* 6 (2000) 88–95.
- [14] I. Kehat, D. Kenyagin-Karsenti, M. Snir, H. Segev, M. Amit, A. Gepstein, E. Livne, O. Binah, J. Itskovitz-Eldor, L. Gepstein, Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes, *J. Clin. Invest.* 108 (2001) 407–414.
- [15] NIH human embryonic stem cell registry, http://grants.nih.gov/stem_cells/registry/current.htm (Accessed: February 24, 2015).
- [16] C.B. Ware, A.M. Nelson, B. Mechem, J. Hesson, W. Zhou, E.C. Jonlin, A.J. Jimenez-Caliani, X. Deng, C. Cavanaugh, S. Cook, P.J. Tesar, J. Okada, L. Margaretha, H. Sperber, M. Choi, C.A. Blau, P.M. Treuting, R.D. Hawkins, V. Cirulli, H. Ruohola-Baker, Derivation of naive human embryonic stem cells, *Proc. Natl. Acad. Sci. U. S. A.* 111 (2014) 4484–4489.
- [17] J. Nichols, A. Smith, Naive and primed pluripotent states, *Cell Stem Cell* 4 (2009) 487–492.
- [18] T.W. Theunissen, B.E. Powell, H. Wang, M. Mitalipova, D.A. Faddah, J. Reddy, Z.P. Fan, D. Maetzke, K. Ganz, L. Shi, T. Lungjangwa, S. Imsoonthornruksa, Y. Stelzer, S. Rangarajan, A. D'Alessio, J. Zhang, Q. Gao, M.M. Dawlaty, R.A. Young, N.S. Gray, R. Jaenisch, Systematic identification of culture conditions for induction and maintenance of naive human pluripotency, *Cell Stem Cell* 15 (2014) 471–487.
- [19] O. Gafni, L. Weinberger, A.A. Mansour, Y.S. Manor, E. Chomsky, D. Ben-Yosef, Y. Kalma, S. Viukov, I. Maza, A. Zviran, Y. Rais, Z. Shipony, Z. Mukamel, V. Krupalnik, M. Zerbib, S. Geula, I. Caspi, D. Schneir, T. Shwartz, S. Gilad, D. Amann-Zalcenstein, S. Benjamin, I. Amit, A. Tanay, R. Massarwa, N. Novershtern, J.H. Hanna, Derivation of novel human ground state naive pluripotent stem cells, *Nature* 504 (2013) 282–286.
- [20] J. Hanna, A.W. Cheng, K. Saha, J. Kim, C.J. Lengner, F. Soldner, J.P. Cassidy, J. Muffat, B.W. Carey, R. Jaenisch, Human embryonic stem cells with biological and epigenetic characteristics similar to those of mouse ESCs, *Proc. Natl. Acad. Sci. U. S. A.* 107 (2010) 9222–9227.
- [21] J. Leschik, L. Caron, H. Yang, C. Cowan, M. Puceat, A view of bivalent epigenetic marks in two human embryonic stem cell lines reveals a different cardiogenic potential, *Stem Cells Dev.* 24 (2015) 384–392.
- [22] J.C. Moore, J. Fu, Y.C. Chan, D. Lin, H. Tran, H.F. Tse, R.A. Li, Distinct cardiogenic preferences of two human embryonic stem cell (hESC) lines are imprinted in their proteomes in the pluripotent state, *Biochem. Biophys. Res. Commun.* 372 (2008) 553–558.
- [23] K. Takahashi, S. Yamanaka, Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors, *Cell* 126 (2006) 663–676.
- [24] K. Takahashi, K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda, S. Yamanaka, Induction of pluripotent stem cells from adult human fibroblasts by defined factors, *Cell* 131 (2007) 861–872.
- [25] J. Yu, M.A. Vodyanik, K. Smuga-Otto, J. Antosiewicz-Bourget, J.L. Frane, S. Tian, J. Nie, G.A. Jonsdottir, V. Ruotti, R. Stewart, I.I. Slukvin, J.A. Thomson, Induced pluripotent stem cell lines derived from human somatic cells, *Science* 318 (2007) 1917–1920.
- [26] Y.Y. Zhou, F. Zeng, Integration-free methods for generating induced pluripotent stem cells, *Genomics Proteomics Bioinformatics* 11 (2013) 284–287.
- [27] J. Zhang, G.F. Wilson, A.G. Soerens, C.H. Koonce, J. Yu, S.P. Palecek, J.A. Thomson, T.J. Kamp, Functional cardiomyocytes derived from human induced pluripotent stem cells, *Circ. Res.* 104 (2009) e30–e41.
- [28] D.G. Wilkinson, S. Bhatt, B.G. Herrmann, Expression pattern of the mouse T gene and its role in mesoderm formation, *Nature* 343 (1990) 657–659.
- [29] S.M. Evans, D. Yelon, F.L. Conlon, M.L. Kirby, Myocardial lineage development, *Circ. Res.* 107 (2010) 1428–1444.
- [30] S.J. Kinder, T.E. Tsang, G.A. Quinlan, A.K. Hadjantonakis, A. Nagy, P.P. Tam, The orderly allocation of mesodermal cells to the extraembryonic structures and the anteroposterior axis during gastrulation of the mouse embryo, *Development* 126 (1999) 4691–4701.
- [31] P. Gadue, T.L. Huber, M.C. Nostro, S. Kattman, G.M. Keller, Germ layer induction from embryonic stem cells, *Exp. Hematol.* 33 (2005) 955–964.
- [32] S.L. Paige, T. Osugi, O.K. Afanasiev, L. Pabon, H. Reinecke, C.E. Murry, Endogenous Wnt/beta-catenin signaling is required for cardiac differentiation in human embryonic stem cells, *PLoS One* 5 (2010) e11134.

- [33] T. Sumi, N. Tsuneyoshi, N. Nakatsuji, H. Suemori, Defining early lineage specification of human embryonic stem cells by the orchestrated balance of canonical Wnt/ β -catenin, Activin/Nodal and BMP signaling, *Development* 135 (2008) 2969–2979.
- [34] M. Nakanishi, A. Kurisaki, Y. Hayashi, M. Warashina, S. Ishiura, M. Kusuda-Furue, M. Asashima, Directed induction of anterior and posterior primitive streak by Wnt from embryonic stem cells cultured in a chemically defined serum-free medium, *FASEB J.* 23 (2009) 114–122.
- [35] L. Yang, M.H. Soonpaa, E.D. Adler, T.K. Roepke, S.J. Kattman, M. Kennedy, E. Henckaerts, K. Bonham, G.W. Abbott, R.M. Linden, L.J. Field, G.M. Keller, Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population, *Nature* 453 (2008) 524–528.
- [36] C. Xu, S. Police, N. Rao, M.K. Carpenter, Characterization and enrichment of cardiomyocytes derived from human embryonic stem cells, *Circ. Res.* 91 (2002) 501–508.
- [37] C.L. Mummery, T.A. van Achterberg, A.J. van den Eijnden-van Raaij, L. van Haaster, A. Willemse, S.W. de Laat, A.H. Piersma, Visceral-endoderm-like cell lines induce differentiation of murine P19 embryonal carcinoma cells, *Differentiation* 46 (1991) 51–60.
- [38] Y. Sugi, J. Lough, Anterior endoderm is a specific effector of terminal cardiac myocyte differentiation of cells from the embryonic heart forming region, *Dev. Dyn.* 200 (1994) 155–162.
- [39] A. Arai, K. Yamamoto, J. Toyama, Murine cardiac progenitor cells require visceral embryonic endoderm and primitive streak for terminal differentiation, *Dev. Dyn.* 210 (1997) 344–353.
- [40] C. Mummery, D. Ward-van Oostwaard, P. Doevendans, R. Spijker, S. van den Brink, R. Hassink, M. van der Heyden, T. Opthof, M. Pera, A.B. de la Riviere, R. Passier, L. Tertoolen, Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells, *Circulation* 107 (2003) 2733–2740.
- [41] C.L. Mummery, D. Ward, R. Passier, Differentiation of human embryonic stem cells to cardiomyocytes by coculture with endoderm in serum-free medium, *Curr. Protoc. Stem Cell Biol.* (2007) (Chapter 1, Unit 1F.2).
- [42] C. Freund, D. Ward-van Oostwaard, J. Monshouwer-Kloots, S. van den Brink, M. van Rooijen, X. Xu, R. Zweigerdt, C. Mummery, R. Passier, Insulin redirects differentiation from cardiogenic mesoderm and endoderm to neuroectoderm in differentiating human embryonic stem cells, *Stem Cells* 26 (2008) 724–733.
- [43] G. Winnier, M. Blessing, P.A. Labosky, B.L. Hogan, Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse, *Genes Dev.* 9 (1995) 2105–2116.
- [44] F.L. Conlon, K.M. Lyons, N. Takaesu, K.S. Barth, A. Kispert, B. Herrmann, E.J. Robertson, A primary requirement for nodal in the formation and maintenance of the primitive streak in the mouse, *Development* 120 (1994) 1919–1928.
- [45] C.C. Lu, E.J. Robertson, Multiple roles for Nodal in the epiblast of the mouse embryo in the establishment of anterior–posterior patterning, *Dev. Biol.* 273 (2004) 149–159.
- [46] M.A. Laflamme, K.Y. Chen, A.V. Naumova, V. Muskheli, J.A. Fugate, S.K. Dupras, H. Reinecke, C. Xu, M. Hassanipour, S. Police, C. O'Sullivan, L. Collins, Y. Chen, E. Minami, E.A. Gill, S. Ueno, C. Yuan, J. Gold, C.E. Murry, Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts, *Nat. Biotechnol.* 25 (2007) 1015–1024.
- [47] C. Xu, M.S. Inokuma, J. Denham, K. Golds, P. Kundu, J.D. Gold, M.K. Carpenter, Feeder-free growth of undifferentiated human embryonic stem cells, *Nat. Biotechnol.* 19 (2001) 971–974.
- [48] H.K. Kleinman, G.R. Martin, Matrigel: basement membrane matrix with biological activity, *Semin. Cancer Biol.* 15 (2005) 378–386.
- [49] W.Z. Zhu, B. Van Biber, M.A. Laflamme, Methods for the derivation and use of cardiomyocytes from human pluripotent stem cells, *Methods Mol. Biol.* 767 (2011) 419–431.
- [50] J. Zhang, M. Klos, G.F. Wilson, A.M. Herman, X. Lian, K.K. Raval, M.R. Barron, L. Hou, A.G. Soerens, J. Yu, S.P. Palecek, G.E. Lyons, J.A. Thomson, T.J. Herron, J. Jalife, T.J. Kamp, Extracellular matrix promotes highly efficient cardiac differentiation of human pluripotent stem cells: the matrix sandwich method, *Circ. Res.* 111 (2012) 1125–1136.
- [51] S. Ueno, G. Weidinger, T. Osugi, A.D. Kohn, J.L. Golob, L. Pabon, H. Reinecke, R.T. Moon, C.E. Murry, Biphasic role for Wnt/ β -catenin signaling in cardiac specification in zebrafish and embryonic stem cells, *Proc. Natl. Acad. Sci. U. S. A.* 104 (2007) 9685–9690.
- [52] A.T. Naito, I. Shiojima, H. Akazawa, K. Hidaka, T. Morisaki, A. Kikuchi, I. Komuro, Developmental stage-specific biphasic roles of Wnt/ β -catenin signaling in cardiomyogenesis and hematopoiesis, *Proc. Natl. Acad. Sci. U. S. A.* 103 (2006) 19812–19817.
- [53] S.J. Kattman, T.L. Huber, G.M. Keller, Multipotent flk-1+ cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages, *Dev. Cell* 11 (2006) 723–732.
- [54] S.J. Kattman, A.D. Witty, M. Gagliardi, N.C. Dubois, M. Niapour, A. Hotta, J. Ellis, G. Keller, Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines, *Cell Stem Cell* 8 (2011) 228–240.
- [55] A.Q. Lam, B.S. Freedman, R. Morizane, P.H. Lerou, M.T. Valerius, J.V. Bonventre, Rapid and efficient differentiation of human pluripotent stem cells into intermediate mesoderm that forms tubules expressing kidney proximal tubular markers, *J. Am. Soc. Nephrol.* 25 (2014) 1211–1225.
- [56] X. Lian, C. Hsiao, G. Wilson, K. Zhu, L.B. Hazeltine, S.M. Azarin, K.K. Raval, J. Zhang, T.J. Kamp, S.P. Palecek, Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical Wnt signaling, *Proc. Natl. Acad. Sci. U. S. A.* 109 (2012) E1848–E1857.
- [57] X. Lian, J. Zhang, S.M. Azarin, K. Zhu, L.B. Hazeltine, X. Bao, C. Hsiao, T.J. Kamp, S.P. Palecek, Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/ β -catenin signaling under fully defined conditions, *Nat. Protoc.* 8 (2013) 162–175.
- [58] P.W. Burridge, E. Matsa, P. Shukla, Z.C. Lin, J.M. Churko, A.D. Ebert, F. Lan, S. Diecke, B. Huber, N.M. Mordwinkin, J.R. Plews, O.J. Abilez, B. Cui, J.D. Gold, J.C. Wu, Chemically defined generation of human cardiomyocytes, *Nat. Methods* 11 (2014) 855–860.
- [59] M.L. Rodriguez, B.T. Graham, L.M. Pabon, S.J. Han, C.E. Murry, N.J. Sniadecki, Measuring the contractile forces of human induced pluripotent stem cell-derived cardiomyocytes with arrays of microposts, *J. Biomech. Eng.* 136 (2014) 051005.
- [60] S.D. Lundy, W.Z. Zhu, M. Regnier, M.A. Laflamme, Structural and functional maturation of cardiomyocytes derived from human pluripotent stem cells, *Stem Cells Dev.* 22 (2013) 1991–2002.
- [61] C. Kim, M. Majidi, P. Xia, K.A. Wei, M. Talantova, S. Spiering, B. Nelson, M. Mercola, H.S. Chen, Non-cardiomyocytes influence the electrophysiological maturation of human embryonic stem cell-derived cardiomyocytes during differentiation, *Stem Cells Dev.* 19 (2010) 783–795.
- [62] L. Sartiani, E. Bettioli, F. Stillitano, A. Mugelli, E. Cerbai, M.E. Jaconi, Developmental changes in cardiomyocytes differentiated from human embryonic stem cells: a molecular and electrophysiological approach, *Stem Cells* 25 (2007) 1136–1144.
- [63] G. Foides, M. Mioulane, J.S. Wright, A.Q. Liu, P. Novak, B. Merkely, J. Gorelik, M.D. Schneider, N.N. Ali, S.E. Harding, Modulation of human embryonic stem cell-derived cardiomyocyte growth: a testbed for studying human cardiac hypertrophy? *J. Mol. Cell. Cardiol.* 50 (2011) 367–376.
- [64] J.Q. He, Y. Ma, Y. Lee, J.A. Thomson, T.J. Kamp, Human embryonic stem cells develop into multiple types of cardiac myocytes: action potential characterization, *Circ. Res.* 93 (2003) 32–39.
- [65] X. Yang, L. Pabon, C.E. Murry, Engineering adolescence: maturation of human pluripotent stem cell-derived cardiomyocytes, *Circ. Res.* 114 (2014) 511–523.
- [66] C. Robertson, D.D. Tran, S.C. George, Concise review: maturation phases of human pluripotent stem cell-derived cardiomyocytes, *Stem Cells* 31 (2013) 829–837.
- [67] A.M. Gerdes, T. Onodera, T. Tamura, S. Said, T.J. Bohlmeyer, W.T. Abraham, M.R. Bristow, New method to evaluate myocyte remodeling from formalin-fixed biopsy and autopsy material, *J. Card. Fail.* 4 (1998) 343–348.
- [68] T.G. Otsuji, I. Minami, Y. Kurose, K. Yamauchi, M. Tada, N. Nakatsuji, Progressive maturation in contracting cardiomyocytes derived from human embryonic stem cells: qualitative effects on electrophysiological responses to drugs, *Stem Cell Res.* 4 (2010) 201–213.
- [69] M. Snir, I. Kehat, A. Gepstein, R. Coleman, J. Itskovitz-Eldor, E. Livne, L. Gepstein, Assessment of the ultrastructural and proliferative properties of human embryonic stem cell-derived cardiomyocytes, *Am. J. Physiol. Heart Circ. Physiol.* 285 (2003) H2355–H2363.
- [70] T. Kamakura, T. Makiyama, K. Sasaki, Y. Yoshida, Y. Wuriyanghai, J. Chen, T. Hattori, S. Ohno, T. Kita, M. Horie, S. Yamanaka, T. Kimura, Ultrastructural maturation of human-induced pluripotent stem cell-derived cardiomyocytes in a long-term culture, *Circ. J.* 77 (2013) 1307–1314.
- [71] J. van der Velden, L.J. Klein, M. van der Bijl, M.A. Huybregts, W. Stooker, J. Witkop, L. Eijmsman, C.A. Visser, F.C. Visser, G.J. Stienen, Isometric tension development and its calcium sensitivity in skinned myocyte-sized preparations from different regions of the human heart, *Cardiovasc. Res.* 42 (1999) 706–719.
- [72] N. Piroddi, A. Belus, B. Scellini, C. Tesi, G. Giunti, E. Cerbai, A. Mugelli, C. Poggesi, Tension generation and relaxation in single myofibrils from human atrial and ventricular myocardium, *Pflügers Arch.* 454 (2007) 63–73.
- [73] E.R. Witjas-Paalberends, N. Piroddi, K. Stam, S.J. van Dijk, V.S. Oliviera, C. Ferrara, B. Scellini, M. Hazebroek, F.J. ten Cate, M. van Slegtenhorst, C. dos Remedios, H.W. Niessen, C. Tesi, G.J. Stienen, S. Heymans, M. Michels, C. Poggesi, J. van der Velden, Mutations in MYH7 reduce the force generating capacity of sarcomeres in human familial hypertrophic cardiomyopathy, *Cardiovasc. Res.* 99 (2013) 432–441.
- [74] X. Yang, M. Rodriguez, L. Pabon, K.A. Fischer, H. Reinecke, M. Regnier, N.J. Sniadecki, H. Ruohola-Baker, C.E. Murry, Tri-iodo-L-thyronine promotes the maturation of human cardiomyocyte-derived from induced pluripotent stem cells, *J. Mol. Cell. Cardiol.* 72 (2014) 296–304.
- [75] J. Liu, N. Sun, M.A. Bruce, J.C. Wu, M.J. Butte, Atomic force mechanobiology of pluripotent stem cell-derived cardiomyocytes, *PLoS One* 7 (2012) e37559.
- [76] R.E. Taylor, K. Kim, N. Sun, S.J. Park, J.Y. Sim, G. Fajardo, D. Bernstein, J.C. Wu, B.L. Pruitt, Sacrificial layer technique for axial force post-assay of immature cardiomyocytes, *Biomed. Microdevices* 15 (2013) 171–181.
- [77] D. Zhang, I.Y. Shadrin, J. Lam, H.Q. Xian, H.R. Snodgrass, N. Bursac, Tissue-engineered cardiac patch for advanced functional maturation of human ESC-derived cardiomyocytes, *Biomaterials* 34 (2013) 5813–5820.
- [78] M. Nabauer, D.J. Beuckelmann, P. Uberfuhr, G. Steinbeck, Regional differences in current density and rate-dependent properties of the transient outward current in subepicardial and subendocardial myocytes of human left ventricle, *Circulation* 93 (1996) 168–177.
- [79] E. Drouin, G. Lande, F. Charpentier, Amiodarone reduces transmural heterogeneity of repolarization in the human heart, *J. Am. Coll. Cardiol.* 32 (1998) 1063–1067.
- [80] G.R. Li, J. Feng, L. Yue, M. Carrier, Transmural heterogeneity of action potentials and Ito1 in myocytes isolated from the human right ventricle, *Am. J. Physiol.* 275 (1998) H369–H377.
- [81] V. Piacentino III, C.R. Weber, X. Chen, J. Weisser-Thomas, K.B. Margulies, D.M. Bers, S.R. Houser, Cellular basis of abnormal calcium transients of failing human ventricular myocytes, *Circ. Res.* 92 (2003) 651–658.
- [82] J.M. Cordeiro, V.V. Nesterenko, S. Sicouri, R.J. Goodrow Jr., J.A. Treat, M. Desai, Y. Wu, M.X. Doss, C. Antzelevitch, J.M. Di Diego, Identification and characterization of a transient outward K⁺ current in human induced pluripotent stem cell-derived cardiomyocytes, *J. Mol. Cell. Cardiol.* 60 (2013) 36–46.

- [83] D. Weisbrod, A. Peretz, A. Ziskind, N. Menaker, S. Oz, L. Barad, S. Eliyahu, J. Itskovitz-Eldor, N. Dascal, D. Khananshvil, O. Binah, B. Attali, SK4 Ca^{2+} activated K^{+} channel is a critical player in cardiac pacemaker derived from human embryonic stem cells, *Proc. Natl. Acad. Sci. U. S. A.* 110 (2013) E1685–E1694.
- [84] J. Satin, I. Kehat, O. Caspi, I. Huber, G. Arbel, I. Itzhaki, J. Magyar, E.A. Schroder, I. Perlman, L. Gepstein, Mechanism of spontaneous excitability in human embryonic stem cell derived cardiomyocytes, *J. Physiol.* 559 (2004) 479–496.
- [85] M.K. Jonsson, M.A. Vos, G.R. Mirams, G. Duker, P. Sartipy, T.P. de Boer, T.A. van Veen, Application of human stem cell-derived cardiomyocytes in safety pharmacology requires caution beyond hERG, *J. Mol. Cell. Cardiol.* 52 (2012) 998–1008.
- [86] X. Sheng, M. Reppel, F. Nguemo, F.I. Mohammad, A. Kuzmenkin, J. Hescheler, K. Pfannkuche, Human pluripotent stem cell-derived cardiomyocytes: response to TTX and lidocaine reveals strong cell to cell variability, *PLoS One* 7 (2012) e45963.
- [87] W.Z. Zhu, L.F. Santana, M.A. Laflamme, Local control of excitation–contraction coupling in human embryonic stem cell-derived cardiomyocytes, *PLoS One* 4 (2009) e5407.
- [88] G.C. Bett, A.D. Kaplan, A. Lis, T.R. Cimato, E.S. Tzanakakis, Q. Zhou, M.J. Morales, R.L. Rasmuson, Electronic “expression” of the inward rectifier in cardiocytes derived from human-induced pluripotent stem cells, *Heart Rhythm*. 10 (2013) 1903–1910.
- [89] R.M. Meijer van Putten, I. Mengarelli, K. Guan, J.G. Zegers, A.C. van Ginneken, A.O. Verkerk, R. Wilders, Ion channelopathies in human induced pluripotent stem cell derived cardiomyocytes: a dynamic clamp study with virtual IK1, *Front. Physiol.* 6 (2015) 7.
- [90] O. Caspi, I. Itzhaki, I. Kehat, A. Gepstein, G. Arbel, I. Huber, J. Satin, L. Gepstein, In vitro electrophysiological drug testing using human embryonic stem cell derived cardiomyocytes, *Stem Cells Dev.* 18 (2009) 161–172.
- [91] A. Mehta, Y.Y. Chung, A. Ng, F. Iskandar, S. Atan, H. Wei, G. Dusing, W. Sun, P. Wong, W. Shim, Pharmacological response of human cardiomyocytes derived from virus-free induced pluripotent stem cells, *Cardiovasc. Res.* 91 (2011) 577–586.
- [92] P. Lee, M. Klos, C. Bollensdorff, L. Hou, P. Ewart, T.J. Kamp, J. Zhang, A. Bizy, G. Guerrero-Serna, P. Kohl, J. Jalife, T.J. Herron, Simultaneous voltage and calcium mapping of genetically purified human induced pluripotent stem cell-derived cardiac myocyte monolayers, *Circ. Res.* 110 (2012) 1556–1563.
- [93] M. Valderrábano, Influence of anisotropic conduction properties in the propagation of the cardiac action potential, *Prog. Biophys. Mol. Biol.* 94 (2007) 144–168.
- [94] D.M. Bers, Cardiac excitation–contraction coupling, *Nature* 415 (2002) 198–205.
- [95] F. Brette, C. Orchard, T-tubule function in mammalian cardiac myocytes, *Circ. Res.* 92 (2003) 1182–1192.
- [96] M. Morad, Y.E. Goldman, D.R. Trentham, Rapid photochemical inactivation of Ca^{2+} -antagonists shows that Ca^{2+} entry directly activates contraction in frog heart, *Nature* 304 (1983) 635–638.
- [97] M. Nabauer, M. Morad, Modulation of contraction by intracellular Na^{+} via Na^{+} – Ca^{2+} exchange in single shark (*Squalus acanthias*) ventricular myocytes, *J. Physiol.* 457 (1992) 627–637.
- [98] G.L. Galli, E.W. Taylor, H.A. Shiels, Calcium flux in turtle ventricular myocytes, *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 291 (2006) R1781–R1789.
- [99] K. Dolnikov, M. Shilkrut, N. Zeevi-Levin, S. Gerech-Nir, M. Amit, A. Danon, J. Itskovitz-Eldor, O. Binah, Functional properties of human embryonic stem cell-derived cardiomyocytes: intracellular Ca^{2+} handling and the role of sarcoplasmic reticulum in the contraction, *Stem Cells* 24 (2006) 236–245.
- [100] J. Liu, J.D. Fu, C.W. Siu, R.A. Li, Functional sarcoplasmic reticulum for calcium handling of human embryonic stem cell-derived cardiomyocytes: insights for driven maturation, *Stem Cells* 25 (2007) 3038–3044.
- [101] J. Satin, I. Itzhaki, S. Rapoport, E.A. Schroder, L. Izu, G. Arbel, R. Beyar, C.W. Balke, J. Schiller, L. Gepstein, Calcium handling in human embryonic stem cell-derived cardiomyocytes, *Stem Cells* 26 (2008) 1961–1972.
- [102] X.H. Zhang, S. Haviland, H. Wei, T. Saric, A. Fatima, J. Hescheler, L. Cleemann, M. Morad, Ca^{2+} signaling in human induced pluripotent stem cell-derived cardiomyocytes (iPS-CM) from normal and catecholaminergic polymorphic ventricular tachycardia (CPVT)-afflicted subjects, *Cell Calcium* 54 (2013) 57–70.
- [103] D.K. Lieu, J. Liu, C.W. Siu, G.P. McNeerney, H.F. Tse, A. Abu-Khalil, T. Huser, R.A. Li, Absence of transverse tubules contributes to non-uniform Ca^{2+} wavefronts in mouse and human embryonic stem cell-derived cardiomyocytes, *Stem Cells Dev.* 18 (2009) 1493–1500.
- [104] Y.K. Lee, K.M. Ng, W.H. Lai, Y.C. Chan, Y.M. Lau, Q. Lian, H.F. Tse, C.W. Siu, Calcium homeostasis in human induced pluripotent stem cell-derived cardiomyocytes, *Stem Cell Rev.* 7 (2011) 976–986.
- [105] Y.C. Chan, S. Ting, Y.K. Lee, K.M. Ng, J. Zhang, Z. Chen, C.W. Siu, S.K. Oh, H.F. Tse, Electrical stimulation promotes maturation of cardiomyocytes derived from human embryonic stem cells, *J. Cardiovasc. Transl. Res.* 6 (2013) 989–999.
- [106] I. Gyorke, N. Hester, L.R. Jones, S. Gyorke, The role of calsequestrin, triadin, and junctin in conferring cardiac ryanodine receptor responsiveness to luminal calcium, *Biophys. J.* 86 (2004) 2121–2128.
- [107] J. Liu, D.K. Lieu, C.W. Siu, J.D. Fu, H.F. Tse, R.A. Li, Facilitated maturation of Ca^{2+} handling properties of human embryonic stem cell-derived cardiomyocytes by calsequestrin expression, *Am. J. Physiol. Cell Physiol.* 297 (2009) C152–C159.
- [108] M.C. Ribeiro, L.G. Tertoolen, J.A. Guadix, M. Bellin, G. Kosmidis, C. D’Aniello, J. Monshouwer-Kloots, M. Goumans, Y. Wang, A.W. Feinberg, C.L. Mummery, R. Passier, Functional maturation of human pluripotent stem cell derived cardiomyocytes in vitro – correlation between contraction force and electrophysiology, *Biomaterials* 51 (2015) 138–150.
- [109] D.J. Fisher, Oxygenation and metabolism in the developing heart, *Semin. Perinatol.* 8 (1984) 217–225.
- [110] G.D. Lopaschuk, M.A. Spafford, D.R. Marsh, Glycolysis is predominant source of myocardial ATP production immediately after birth, *Am. J. Physiol.* 261 (1991) H1698–H1705.
- [111] J. Schaper, E. Meiser, G. Stammeler, Ultrastructural morphometric analysis of myocardium from dogs, rats, hamsters, mice, and from human hearts, *Circ. Res.* 56 (1985) 377–391.
- [112] A.B. Prowse, F. Chong, D.A. Elliott, A.G. Elefanty, E.G. Stanley, P.P. Gray, T.P. Munro, G.W. Osborne, Analysis of mitochondrial function and localisation during human embryonic stem cell differentiation in vitro, *PLoS One* 7 (2012) (e52214).
- [113] P. Kerscher, B.S. Bussie, K.M. DeSimone, D.A. Dunn, E.A. Lipke, Characterization of mitochondrial populations during stem cell differentiation, *Methods Mol. Biol.* 1264 (2015) 453–463.
- [114] M. Gherghiceanu, L. Barad, A. Novak, I. Reiter, J. Itskovitz-Eldor, O. Binah, L.M. Popescu, Cardiomyocytes derived from human embryonic and induced pluripotent stem cells: comparative ultrastructure, *J. Cell. Mol. Med.* 15 (2011) 2539–2551.
- [115] C.D. Folmes, A. Martinez-Fernandez, R.S. Faustino, S. Yamada, C. Perez-Terzic, T.J. Nelson, A. Terzic, Nuclear reprogramming with c-Myc potentiates glycolytic capacity of derived induced pluripotent stem cells, *J. Cardiovasc. Transl. Res.* 6 (2013) 10–21.
- [116] J.A. Gaspar, M.X. Doss, J.G. Hengstler, C. Cadenas, J. Hescheler, A. Sachinidis, Unique metabolic features of stem cells, cardiomyocytes, and their progenitors, *Circ. Res.* 114 (2014) 1346–1360.
- [117] M.N. Hirt, J. Boeddinghaus, A. Mitchell, S. Schaaf, C. Bornchen, C. Muller, H. Schulz, N. Hubner, J. Stenzig, A. Stoehr, C. Neuber, A. Eder, P.K. Luther, A. Hansen, T. Eschenhagen, Functional improvement and maturation of rat and human engineered heart tissue by chronic electrical stimulation, *J. Mol. Cell. Cardiol.* 74 (2014) 151–161.
- [118] I.C. Turnbull, I. Karakikes, G.W. Serrao, P. Backeris, J.J. Lee, C. Xie, G. Senyei, R.E. Gordon, R.A. Li, F.G. Akar, R.J. Hajjar, J.S. Hulot, K.D. Costa, Advancing functional engineered cardiac tissues toward a preclinical model of human myocardium, *FASEB J.* 28 (2014) 644–654.
- [119] W.Z. Zhu, Y. Xie, K.W. Moyes, J.D. Gold, B. Askari, M.A. Laflamme, Neuregulin/ErbB signaling regulates cardiac subtype specification in differentiating human embryonic stem cells, *Circ. Res.* 107 (2010) 776–786.
- [120] O. Iglesias-Garcia, S. Baumgartner, L. Macri-Pellizzeri, J.R. Rodriguez-Madoz, G. Abizanda, E. Guruceaga, E. Albiasu, D. Corbacho, C. Benavides-Valive, M. Soriano-Navarro, S. Gonzalez-Granero, J.J. Gavira, B. Krausgrill, M. Rodriguez-Manero, J.M. Garcia-Verdugo, C. Ortiz-de-Solorzano, M. Halbach, J. Hescheler, B. Pelacho, F. Prosper, Neuregulin-1beta induces mature ventricular cardiac differentiation from induced pluripotent stem cells contributing to cardiac tissue repair, *Stem Cells Dev.* 24 (2015) 484–496.
- [121] T.B. Johnson, R.L. Kent, B.A. Bubolz, P.J. McDermott, Electrical stimulation of contractile activity accelerates growth of cultured neonatal cardiocytes, *Circ. Res.* 74 (1994) 448–459.
- [122] S. Kato, C.T. Ivester, G. Cooper IV, M.R. Zile, P.J. McDermott, Growth effects of electrically stimulated contraction on adult feline cardiocytes in primary culture, *Am. J. Physiol.* 268 (1995) H2495–H2504.
- [123] M. Radisic, H. Park, H. Shing, T. Consi, F.J. Schoen, R. Langer, L.E. Freed, G. Vunjak-Novakovic, Functional assembly of engineered myocardium by electrical stimulation of cardiac myocytes cultured on scaffolds, *Proc. Natl. Acad. Sci. U. S. A.* 101 (2004) 18129–18134.
- [124] S.S. Nunes, J.W. Miklas, J. Liu, R. Aschar-Sobbi, Y. Xiao, B. Zhang, J. Jiang, S. Masse, M. Gagliardi, A. Hsieh, N. Thavandiran, M.A. Laflamme, K. Nanthakumar, G.J. Gross, P.H. Backx, G. Keller, M. Radisic, Biowire: a platform for maturation of human pluripotent stem cell-derived cardiomyocytes, *Nat. Methods* 10 (2013) 781–787.
- [125] R. Zhu, A. Blazeski, E. Poon, K.D. Costa, L. Tung, K.R. Boheler, Physical developmental cues for the maturation of human pluripotent stem cell-derived cardiomyocytes, *Stem Cell Res. Ther.* 5 (2014) 117.
- [126] I. Karakikes, G.D. Senyei, J. Hansen, C.W. Kong, E.U. Azeloglu, F. Stillitano, D.K. Lieu, J. Wang, L. Ren, J.S. Hulot, R. Iyengar, R.A. Li, R.J. Hajjar, Small molecule-mediated directed differentiation of human embryonic stem cells toward ventricular cardiomyocytes, *Stem Cells Transl. Med.* 3 (2014) 18–31.
- [127] Z. Weng, C.W. Kong, L. Ren, I. Karakikes, L. Geng, J. He, M.Z. Chow, C.F. Mok, W. Keung, H. Chow, A.Y. Leung, R.J. Hajjar, R.A. Li, C.W. Chan, A simple, cost-effective but highly efficient system for deriving ventricular cardiomyocytes from human pluripotent stem cells, *Stem Cells Dev.* 23 (2014) 1704–1716.
- [128] M.R. Rosen, R.B. Robinson, P.R. Brink, I.S. Cohen, The road to biological pacing, *Nat. Rev. Cardiol.* 8 (2011) 656–666.
- [129] T. Xue, H.C. Cho, F.G. Akar, S.Y. Tsang, S.P. Jones, E. Marban, G.F. Tomaselli, R.A. Li, 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 (2005) 11–20.
- [130] M.S. Rana, V.M. Christoffels, A.F. Moorman, A molecular and genetic outline of cardiac morphogenesis, *Acta Physiol (Oxf.)* 207 (2013) 588–615.
- [131] A. Sizarov, H.D. Devalla, R.H. Anderson, R. Passier, V.M. Christoffels, A.F. Moorman, Molecular analysis of patterning of conduction tissues in the developing human heart, *Circ. Arrhythm. Electrophysiol.* 4 (2011) 532–542.
- [132] M.L. Bakker, G.J. Boink, B.J. Boukens, A.O. Verkerk, M. van den Boogaard, A.D. den Haan, W.M. Hoogaars, H.P. Buermans, J.M. de Bakker, J. Seppen, H.L. Tan, A.F. Moorman, P.A. ‘t Hoen, V.M. Christoffels, T-box transcription factor TBX3 reprogrammes mature cardiac myocytes into pacemaker-like cells, *Cardiovasc. Res.* 94 (2012) 439–449.
- [133] N. Kapoor, W. Liang, E. Marban, H.C. Cho, Direct conversion of quiescent cardiomyocytes to pacemaker cells by expression of Tbx18, *Nat. Biotechnol.* 31 (2013) 54–62.
- [134] V. Vedantham, G. Galang, M. Evangelista, R.C. Deo, D. Srivastava, RNA sequencing of mouse sinoatrial node reveals an upstream regulatory role for Islet-1 in cardiac pacemaker cells, *Circ. Res.* 116 (2015) 797–803.

- [135] D.O. Nelson, D.X. Jin, K.M. Downs, T.J. Kamp, G.E. Lyons, *Irx4* identifies a chamber-specific cell population that contributes to ventricular myocardium development, *Dev. Dyn.* 243 (2014) 381–392.
- [136] D. Lai, X. Liu, A. Forrai, O. Wolstein, J. Michalick, I. Ahmed, A.N. Garratt, C. Birchmeier, M. Zhou, L. Hartley, L. Robb, M.P. Feneley, D. Fatkin, R.P. Harvey, Neuregulin 1 sustains the gene regulatory network in both trabecular and nontrabecular myocardium, *Circ. Res.* 107 (2010) 715–727.
- [137] C.L. Cai, X. Liang, Y. Shi, P.H. Chu, S.L. Pfaff, J. Chen, S. Evans, *Isl1* identifies a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart, *Dev. Cell* 5 (2003) 877–889.
- [138] B.G. Bruneau, M. Logan, N. Davis, T. Levi, C.J. Tabin, J.G. Seidman, C.E. Seidman, Chamber-specific cardiac expression of *Tbx5* and heart defects in Holt–Oram syndrome, *Dev. Biol.* 211 (1999) 100–108.
- [139] B.G. Bruneau, Z.Z. Bao, D. Fatkin, J. Xavier-Neto, D. Georgakopoulos, C.T. Maguire, C.I. Berul, D.A. Kass, M.L. Kuroski-de Bold, A.J. de Bold, D.A. Conner, N. Rosenthal, C.L. Cepko, C.E. Seidman, J.G. Seidman, Cardiomyopathy in *Irx4*-deficient mice is preceded by abnormal ventricular gene expression, *Mol. Cell. Biol.* 21 (2001) 1730–1736.
- [140] B.G. Bruneau, Z.Z. Bao, M. Tanaka, J.J. Schott, S. Izumo, C.L. Cepko, J.G. Seidman, C.E. Seidman, Cardiac expression of the ventricle-specific homeobox gene *Irx4* is modulated by *Nkx2-5* and *dHand*, *Dev. Biol.* 217 (2000) 266–277.
- [141] S.P. Wu, C.M. Cheng, R.B. Lanz, T. Wang, J.L. Respress, S. Ather, W. Chen, S.J. Tsai, X.H. Wehrens, M.J. Tsai, S.Y. Tsai, Atrial identity is determined by a COUP-TFII regulatory network, *Dev. Cell* 25 (2013) 417–426.
- [142] N. Koibuchi, M.T. Chin, *CHFI1/Hey2* plays a pivotal role in left ventricular maturation through suppression of ectopic atrial gene expression, *Circ. Res.* 100 (2007) 850–855.
- [143] T. Dorn, A. Goedel, J.T. Lam, J. Haas, Q. Tian, F. Herrmann, K. Bundschu, G. Dobreva, M. Schiemann, R. Dirschinger, Y. Guo, S.J. Kuhl, D. Sinnecker, P. Lipp, K. Laugwitz, M. Kuhl, A. Moretti, Direct *Nkx2-5* transcriptional repression of *Isl1* controls cardiomyocyte subtype identity, *Stem Cells* 33 (2015) 1113–1129.
- [144] V. Ionta, W. Liang, E.H. Kim, R. Rafie, A. Giacomello, E. Marban, H.C. Cho, *SHOX2* overexpression favors differentiation of embryonic stem cells into cardiac pacemaker cells, improving biological pacing ability, *Stem Cell Rep.* 4 (2015) 129–142.
- [145] S. Rentschler, J. Zander, K. Meyers, D. France, R. Levine, G. Porter, S.A. Rivkees, G.E. Morley, G.I. Fishman, Neuregulin-1 promotes formation of the murine cardiac conduction system, *Proc. Natl. Acad. Sci. U. S. A.* 99 (2002) 10464–10469.
- [146] N. Gassanov, F. Er, N. Zagidullin, U.C. Hoppe, Endothelin induces differentiation of ANP–EGFP expressing embryonic stem cells towards a pacemaker phenotype, *FASEB J.* 18 (2004) 1710–1712.
- [147] N. Gassanov, F. Er, N. Zagidullin, M. Jankowski, J. Gutkowska, U.C. Hoppe, Retinoid acid-induced effects on atrial and pacemaker cell differentiation and expression of cardiac ion channels, *Differentiation* 76 (2008) 971–980.
- [148] Q. Zhang, J. Jiang, P. Han, Q. Yuan, J. Zhang, X. Zhang, Y. Xu, H. Cao, Q. Meng, L. Chen, T. Tian, X. Wang, P. Li, J. Hescheler, G. Ji, Y. Ma, Direct differentiation of atrial and ventricular myocytes from human embryonic stem cells by alternating retinoid signals, *Cell Res.* 21 (2011) 579–587.
- [149] J. Hudson, D. Titmarsh, A. Hidalgo, E. Wolvetang, J. Cooper-White, Primitive cardiac cells from human embryonic stem cells, *Stem Cells Dev.* 21 (2012) 1513–1523.
- [150] K. Maass, A. Shekhar, J. Lu, G. Kang, F. See, E. Kim, C. Delgado, S. Shen, L. Cohen, G.I. Fishman, Isolation and characterization of ESC-derived cardiac Purkinje cells, *Stem Cells* 33 (2015) 1102–1112.
- [151] M.A. Laflamme, C.E. Murry, Heart regeneration, *Nature* 473 (2011) 326–335.
- [152] Y. Shiba, S. Fernandes, W.Z. Zhu, D. Filice, V. Muskheli, J. Kim, N.J. Palpant, J. Gantz, K.W. Moyes, H. Reinecke, B. Van Biber, T. Dardas, J.L. Mignone, A. Izawa, R. Hanna, M. Viswanathan, J.D. Gold, M.I. Kotlikoff, N. Sarvazyan, M.W. Kay, C.E. Murry, M.A. Laflamme, Human ES-cell-derived cardiomyocytes electrically couple and suppress arrhythmias in injured hearts, *Nature* 489 (2012) 322–325.
- [153] Y. Shiba, D. Filice, S. Fernandes, E. Minami, S.K. Dupras, B.V. Biber, P. Trinh, Y. Hirota, J.D. Gold, M. Viswanathan, M.A. Laflamme, Electrical integration of human embryonic stem cell-derived cardiomyocytes in a guinea pig chronic infarct model, *J. Cardiovasc. Pharmacol. Ther.* 19 (2014) 368–381.
- [154] J.J. Chong, X. Yang, C.W. Don, E. Minami, Y.W. Liu, J.J. Weyers, W.M. Mahoney, B. Van Biber, N.J. Palpant, A.J. Gantz, J.A. Fugate, V. Muskheli, G.M. Gough, K.W. Vogel, C.A. Astley, C.E. Hotchkiss, A. Baldessari, L. Pabon, H. Reinecke, E.A. Gill, V. Nelson, H.P. Kiem, M.A. Laflamme, C.E. Murry, Human embryonic-stem-cell-derived cardiomyocytes regenerate non-human primate hearts, *Nature* 510 (2014) 273–277.
- [155] W.Z. Zhu, D. Filice, N.J. Palpant, M.A. Laflamme, Methods for assessing the electromechanical integration of human pluripotent stem cell-derived cardiomyocyte grafts, *Methods Mol. Biol.* 1181 (2014) 229–247.
- [156] A. Pumir, A. Arutunyan, V. Krinsky, N. Sarvazyan, Genesis of ectopic waves: role of coupling, automaticity, and heterogeneity, *Biophys. J.* 89 (2005) 2332–2349.
- [157] V.N. Biktashev, A. Arutunyan, N.A. Sarvazyan, Generation and escape of local waves from the boundary of uncoupled cardiac tissue, *Biophys. J.* 94 (2008) 3726–3738.
- [158] Y.M. Zhang, C. Hartzell, M. Narlow, S.C. Dudley Jr., Stem cell-derived cardiomyocytes demonstrate arrhythmic potential, *Circulation* 106 (2002) 1294–1299.
- [159] O. Caspi, I. Huber, I. Kehat, M. Habib, G. Arbel, A. Gepstein, L. Yankelson, D. Aronson, R. Beyar, L. Gepstein, Transplantation of human embryonic stem cell-derived cardiomyocytes improves myocardial performance in infarcted rat hearts, *J. Am. Coll. Cardiol.* 50 (2007) 1884–1893.
- [160] L.W. van Laake, R. Passier, J. Monshouwer-Kloots, A.J. Verkleij, D.J. Lips, C. Freund, K. den Ouden, D. Ward-van Oostwaard, J. Korving, L.G. Tertoolen, C.J. van Echteld, P.A. Doevendans, C.L. Mummery, Human embryonic stem cell-derived cardiomyocytes survive and mature in the mouse heart and transiently improve function after myocardial infarction, *Stem Cell Res.* 1 (2007) 9–24.
- [161] J.Y. Min, Y. Yang, K.L. Converso, L. Liu, Q. Huang, J.P. Morgan, Y.F. Xiao, Transplantation of embryonic stem cells improves cardiac function in postinfarcted rats, *J. Appl. Physiol.* 92 (2002) (1985) 288–296.
- [162] A. Behfar, L.V. Zingman, D.M. Hodgson, J.M. Rauzier, G.C. Kane, A. Terzic, M. Puceat, Stem cell differentiation requires a paracrine pathway in the heart, *FASEB J.* 16 (2002) 1558–1566.
- [163] J. Nussbaum, E. Minami, M.A. Laflamme, J.A. Virag, C.B. Ware, A. Masino, V. Muskheli, L. Pabon, H. Reinecke, C.E. Murry, Transplantation of undifferentiated murine embryonic stem cells in the heart: teratoma formation and immune response, *FASEB J.* 21 (2007) 1345–1357.
- [164] R.J. Swijnenburg, M. Tanaka, H. Vogel, J. Baker, T. Kofidis, F. Gunawan, D.R. Lebl, A.D. Caffarelli, J.L. de Bruin, E.V. Fedoseyeva, R.C. Robbins, Embryonic stem cell immunogenicity increases upon differentiation after transplantation into ischemic myocardium, *Circulation* 112 (2005) 1166–1172.
- [165] L.W. van Laake, R. Passier, P.A. Doevendans, C.L. Mummery, Human embryonic stem cell-derived cardiomyocytes and cardiac repair in rodents, *Circ. Res.* 102 (2008) 1008–1010.
- [166] J. Luo, M.S. Weaver, B. Cao, J.E. Dennis, B. Van Biber, M.A. Laflamme, M.D. Allen, Cobalt protoporphyrin pretreatment protects human embryonic stem cell-derived cardiomyocytes from hypoxia/reoxygenation injury in vitro and increases graft size and vascularization in vivo, *Stem Cells Transl. Med.* 3 (2014) 734–744.
- [167] S.H. Moon, S.W. Kang, S.J. Park, D. Bae, S.J. Kim, H.A. Lee, K.S. Kim, K.S. Hong, J.S. Kim, J.T. Do, K.H. Byun, H.M. Chung, The use of aggregates of purified cardiomyocytes derived from human ESCs for functional engraftment after myocardial infarction, *Biomaterials* 34 (2013) 4013–4026.
- [168] L. Ye, Y. Chang, Q. Xiong, P. Zhang, L. Zhang, P. Somasundaram, M. Lepley, C. Swingen, L. Su, J. Wendel, J. Guo, A. Jang, D. Rosenberg, L. Greder, J. Dutton, J. Zhang, T. Kamp, D. Kaufman, Y. Ge, J. Zhang, Cardiac repair in a porcine model of acute myocardial infarction with human induced pluripotent stem cell-derived cardiovascular cells, *Cell Stem Cell* 15 (2014) 750–761.
- [169] L. Citro, S. Naidu, F. Hassan, M.L. Kuppasamy, P. Kuppasamy, M.G. Angelos, M. Khan, Comparison of human induced pluripotent stem-cell derived cardiomyocytes with human mesenchymal stem cells following acute myocardial infarction, *PLoS One* 9 (2014) e116281.
- [170] N.C. Dubois, A.M. Craft, P. Sharma, D.A. Elliott, E.G. Stanley, A.G. Elefanty, A. Gramolini, G. Keller, SIRPA is a specific cell-surface marker for isolating cardiomyocytes derived from human pluripotent stem cells, *Nat. Biotechnol.* 29 (2011) 1011–1018.
- [171] H. Uosaki, H. Fukushima, A. Takeuchi, S. Matsuoka, N. Nakatsuji, S. Yamanaka, J.K. Yamashita, Efficient and scalable purification of cardiomyocytes from human embryonic and induced pluripotent stem cells by VCAM1 surface expression, *PLoS One* 6 (2011) e23657.
- [172] S.M. Taapken, B.S. Nisler, M.A. Newton, T.L. Sampsel-Barron, K.A. Leonhard, E.M. McIntire, K.D. Montgomery, Karyotypic abnormalities in human induced pluripotent stem cells and embryonic stem cells, *Nat. Biotechnol.* 29 (2011) 313–314.
- [173] M.B. Upender, J.K. Habermann, L.M. McShane, E.L. Korn, J.C. Barrett, M.J. Difilippantonio, T. Ried, Chromosome transfer induced aneuploidy results in complex dysregulation of the cellular transcriptome in immortalized and cancer cells, *Cancer Res.* 64 (2004) 6941–6949.
- [174] A. Letourneau, F.A. Santoni, X. Bonilla, M.R. Sailani, D. Gonzalez, J. Kind, C. Chevalier, R. Thurman, R.S. Sandstrom, Y. Hibaoui, M. Garier, K. Popadin, E. Falconnet, M. Gagnebin, C. Gehrig, A. Vannier, M. Guipponi, L. Farinelli, D. Robyr, E. Migliavacca, C. Borel, S. Deutsch, A. Feki, J.A. Stamatoyannopoulos, Y. Herauld, B. van Steensel, R. Guigo, S.E. Antonarakis, Domains of genome-wide gene expression dysregulation in Down's syndrome, *Nature* 508 (2014) 345–350.
- [175] E. Vanneste, T. Voet, C. Le Caignec, M. Ampe, P. Konings, C. Melotte, S. Debrock, M. Amyere, M. Vikkula, F. Schuit, J.P. Fryns, G. Verbeke, T. D'Hooghe, Y. Moreau, J.R. Vermeesch, Chromosome instability is common in human cleavage-stage embryos, *Nat. Med.* 15 (2009) 577–583.
- [176] M. Drukker, H. Katchman, G. Katz, S. Even-Tov Friedman, E. Shezen, E. Hornstein, O. Mandelboim, Y. Reisner, N. Benvenisty, Human embryonic stem cells and their differentiated derivatives are less susceptible to immune rejection than adult cells, *Stem Cells* 24 (2006) 221–229.
- [177] Z. Rong, M. Wang, Z. Hu, M. Stradner, S. Zhu, H. Kong, H. Yi, A. Goldrath, Y.G. Yang, Y. Xu, X. Fu, An effective approach to prevent immune rejection of human ESC-derived allografts, *Cell Stem Cell* 14 (2014) 121–130.
- [178] J. Chinen, R.H. Buckley, Transplantation immunology: solid organ and bone marrow, *J. Allergy Clin. Immunol.* 125 (2010) S324–S335.
- [179] C.J. Taylor, E.M. Bolton, S. Pocock, L.D. Sharples, R.A. Pedersen, J.A. Bradley, Banking on human embryonic stem cells: estimating the number of donor cell lines needed for HLA matching, *Lancet* 366 (2005) 2019–2025.
- [180] F. Nakajima, K. Tokunaga, N. Nakatsuji, Human leukocyte antigen matching estimations in a hypothetical bank of human embryonic stem cell lines in the Japanese population for use in cell transplantation therapy, *Stem Cells* 25 (2007) 983–985.
- [181] B. Daughtry, S. Mitalipov, Concise review: parthenote stem cells for regenerative medicine: genetic, epigenetic, and developmental features, *Stem Cells Transl. Med.* 3 (2014) 290–298.
- [182] M.L. Condic, M. Rao, Regulatory issues for personalized pluripotent cells, *Stem Cells* 26 (2008) 2753–2758.
- [183] T. Zhao, Z.N. Zhang, Z. Rong, Y. Xu, Immunogenicity of induced pluripotent stem cells, *Nature* 474 (2011) 212–215.
- [184] Z. Liu, X. Wen, H. Wang, J. Zhou, M. Zhao, Q. Lin, Y. Wang, J. Li, D. Li, Z. Du, A. Yao, F. Cao, C. Wang, Molecular imaging of induced pluripotent stem cell immunogenicity with in vivo development in ischemic myocardium, *PLoS One* 8 (2013) e66369.

- [185] P. Guha, J.W. Morgan, G. Mostoslavsky, N.P. Rodrigues, A.S. Boyd, Lack of immune response to differentiated cells derived from syngeneic induced pluripotent stem cells, *Cell Stem Cell* 12 (2013) 407–412.
- [186] R. Araki, M. Uda, Y. Hoki, M. Sunayama, M. Nakamura, S. Ando, M. Sugiura, H. Ideno, A. Shimada, A. Nifuji, M. Abe, Negligible immunogenicity of terminally differentiated cells derived from induced pluripotent or embryonic stem cells, *Nature* 494 (2013) 100–104.
- [187] B.C. Huber, J.D. Ransohoff, K.J. Ransohoff, J. Riegler, A. Ebert, K. Kodo, Y. Gong, V. Sanchez-Freire, D. Dey, N.G. Kooreman, S. Diecke, W.Y. Zhang, J. Odegaard, S. Hu, J.D. Gold, R.C. Robbins, J.C. Wu, Costimulation–adhesion blockade is superior to cyclosporine A and prednisone immunosuppressive therapy for preventing rejection of differentiated human embryonic stem cells following transplantation, *Stem Cells* 31 (2013) 2354–2363.
- [188] J.I. Pearl, L.S. Kean, M.M. Davis, J.C. Wu, Pluripotent stem cells: immune to the immune system? *Sci. Transl. Med.* 4 (2012) (164ps25).
- [189] T. Deuse, M. Seifert, D. Tyan, P.S. Tsao, X. Hua, J. Velden, T. Eiermann, H.D. Volk, H. Reichenspurner, R.C. Robbins, S. Schrepfer, Immunobiology of naive and genetically modified HLA-class-I-knockdown human embryonic stem cells, *J. Cell Sci.* 124 (2011) 3029–3037.
- [190] P. Lu, J. Chen, L. He, J. Ren, H. Chen, L. Rao, Q. Zhuang, H. Li, L. Li, L. Bao, J. He, W. Zhang, F. Zhu, C. Cui, L. Xiao, Generating hypoimmunogenic human embryonic stem cells by the disruption of beta 2-microglobulin, *Stem Cell Rev.* 9 (2013) 806–813.
- [191] G. Blin, D. Nury, S. Stefanovic, T. Neri, O. Guillevic, B. Brinon, V. Bellamy, C. Rucker-Martin, P. Barbry, A. Bel, P. Bruneval, C. Cowan, J. Pouly, S. Mitalipov, E. Gouadon, P. Binder, A. Hagege, M. Desnos, J.F. Renaud, P. Menasche, M. Puceat, A purified population of multipotent cardiovascular progenitors derived from primate pluripotent stem cells engrafts in postmyocardial infarcted nonhuman primates, *J. Clin. Invest.* 120 (2010) 1125–1139.
- [192] J.M. Polo, S. Liu, M.E. Figueroa, W. Kulalert, S. Eminli, K.Y. Tan, E. Apostolou, M. Stadtfeld, Y. Li, T. Shioda, S. Natesan, A.J. Wagers, A. Melnick, T. Evans, K. Hochedlinger, Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells, *Nat. Biotechnol.* 28 (2010) 848–855.
- [193] V. Sanchez-Freire, A.S. Lee, S. Hu, O.J. Abilez, P. Liang, F. Lan, B.C. Huber, S.G. Ong, W.X. Hong, M. Huang, J.C. Wu, Effect of human donor cell source on differentiation and function of cardiac induced pluripotent stem cells, *J. Am. Coll. Cardiol.* 64 (2014) 436–448.
- [194] M.Z. Chow, L. Geng, C.W. Kong, W. Keung, J.C. Fung, K.R. Boheler, R.A. Li, Epigenetic regulation of the electrophysiological phenotype of human embryonic stem cell-derived ventricular cardiomyocytes: insights for driven maturation and hypertrophic growth, *Stem Cells Dev.* 22 (2013) 2678–2690.
- [195] C.E. Murry, H. Reinecke, L.M. Pabon, Regeneration gaps: observations on stem cells and cardiac repair, *J. Am. Coll. Cardiol.* 47 (2006) 1777–1785.
- [196] J.B. Caulfield, R. Leinbach, H. Gold, The relationship of myocardial infarct size and prognosis, *Circulation* 53 (1976) 1141–1144.
- [197] M.A. Laflamme, J. Gold, C. Xu, M. Hassanipour, E. Rosler, S. Police, V. Muskheili, C.E. Murry, Formation of human myocardium in the rat heart from human embryonic stem cells, *Am. J. Pathol.* 167 (2005) 663–671.
- [198] T.E. Robey, M.K. Saiget, H. Reinecke, C.E. Murry, Systems approaches to preventing transplanted cell death in cardiac repair, *J. Mol. Cell. Cardiol.* 45 (2008) 567–581.
- [199] C. Xu, S. Police, M. Hassanipour, Y. Li, Y. Chen, C. Priest, C. O'Sullivan, M.A. Laflamme, W.Z. Zhu, B. Van Biber, L. Hegerova, J. Yang, K. Delavan-Boorsma, A. Davies, J. Lebkowski, J.D. Gold, Efficient generation and cryopreservation of cardiomyocytes derived from human embryonic stem cells, *Regen. Med.* 6 (2011) 53–66.
- [200] V.C. Chen, L.A. Couture, The suspension culture of undifferentiated human pluripotent stem cells using spinner flasks, *Methods Mol. Biol.* 1283 (2015) 13–21.
- [201] H. Kempf, R. Olmer, C. Kropp, M. Ruckert, M. Jara-Avaca, D. Robles-Diaz, A. Franke, D.A. Elliott, D. Wojciechowski, M. Fischer, A. Roa Lara, G. Kensah, I. Gruh, A. Haverich, U. Martin, R. Zweigerdt, Controlling expansion and cardiomyogenic differentiation of human pluripotent stem cells in scalable suspension culture, *Stem Cell Rep.* 3 (2014) 1132–1146.
- [202] F.B. Myers, J.S. Silver, Y. Zhuge, R.E. Beygui, C.K. Zarins, L.P. Lee, O.J. Abilez, Robust pluripotent stem cell expansion and cardiomyocyte differentiation via geometric patterning, *Integr. Biol. (Camb.)* 5 (2013) 1495–1506.
- [203] R. Pal, M.K. Mamidi, A.K. Das, R. Bhonde, Comparative analysis of cardiomyocyte differentiation from human embryonic stem cells under 3-D and 2-D culture conditions, *J. Biosci. Bioeng.* 115 (2013) 200–206.
- [204] M. Lecina, S. Ting, A. Choo, S. Reuveny, S. Oh, Scalable platform for human embryonic stem cell differentiation to cardiomyocytes in suspended microcarrier cultures, *Tissue Eng. Part C Methods* 16 (2010) 1609–1619.
- [205] C. Correia, M. Serra, N. Espinha, M. Sousa, C. Brito, K. Burkert, Y. Zheng, J. Hescheler, M.J. Carrondo, T. Saric, P.M. Alves, Combining hypoxia and bioreactor hydrodynamics boosts induced pluripotent stem cell differentiation towards cardiomyocytes, *Stem Cell Rev.* 10 (2014) 786–801.
- [206] M. Ieda, J.D. Fu, P. Delgado-Olguin, V. Vedantham, Y. Hayashi, B.G. Bruneau, D. Srivastava, Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors, *Cell* 142 (2010) 375–386.
- [207] L. Qian, Y. Huang, C.I. Spencer, A. Foley, V. Vedantham, L. Liu, S.J. Conway, J.D. Fu, D. Srivastava, In vivo reprogramming of murine cardiac fibroblasts into induced cardiomyocytes, *Nature* 485 (2012) 593–598.
- [208] K. Song, Y.J. Nam, X. Luo, X. Qi, W. Tan, G.N. Huang, A. Acharya, C.L. Smith, M.D. Tallquist, E.G. Neilson, J.A. Hill, R. Bassel-Duby, E.N. Olson, Heart repair by reprogramming non-myocytes with cardiac transcription factors, *Nature* 485 (2012) 599–604.