Minireview

"The state of the heart": Recent advances in engineering human cardiac tissue from pluripotent stem cells

Dario Sirabella, Elisa Cimetta and Gordana Vunjak-Novakovic

Department of Biomedical Engineering, Columbia University, New York 10032, USA
Corresponding author: Gordana Vunjak-Novakovic. Email: gv2131@columbia.edu

Abstract

The pressing need for effective cell therapy for the heart has led to the investigation of suitable cell sources for tissue replacement. In recent years, human pluripotent stem cell research expanded tremendously, in particular since the derivation of human-induced pluripotent stem cells. In parallel, bioengineering technologies have led to novel approaches for *in vitro* cell culture. The combination of these two fields holds potential for *in vitro* generation of high-fidelity heart tissue, both for basic research and for therapeutic applications. However, this new multidisciplinary science is still at an early stage. Many questions need to be answered and improvements need to be made before clinical applications become a reality. Here we discuss the current status of human stem cell differentiation into cardiomyocytes and the combined use of bioengineering approaches for cardiac tissue formation and maturation in developmental studies, disease modeling, drug testing, and regenerative medicine.

Keywords: Human stem cells, cardiac differentiation, molecular induction, extracellular matrix, paracrine factors, microscale platforms

***Experimental Biology and Medicine* 2015; 240: 1008–1018. DOI: 10.1177/1535370215589910**

Introduction

Heart disease is a major life-threatening condition worldwide. A primary reason for the high incidence of cardiac disease is that the regenerative capacity of adult myocardium is very limited. Instead of regeneration, myocardial infarction is followed by the formation of fibrous scar tissue in the damaged area.[1,2] The need for a therapy has triggered deep investigations to identify the proper cell source and strategy for functional regeneration of the heart. Cardiac stem cells, skeletal myoblasts, bone marrow derived cells, and peripheral mononuclear cells have been studied in animal models and in clinical trials, with limited tissue regeneration.[3,4]

Because of their unlimited capacity of self-renewal while virtually retaining differentiation potency, human pluripotent stem cells (hPSCs) may represent the most promising cell type for cardiac cell replacement therapy. Over the last decade, efforts have been made to develop methods for directed cardiac differentiation of hPSCs. Early studies[5] demonstrated feasibility of cardiomyocyte (CM) differentiation *in vitro*, while subsequent studies have led to the optimization of staged differentiation protocols. Although *in vitro* cardiac differentiation of PSCs has been improved in many respects, the strategies currently available still have limitations that need to be overcome before clinical trials. These limitations can be summarized as: (1) high variability of cardiac differentiation efficiency and reproducibility among different cell lines, (2) heterogeneity of the differentiated cardiac cell population, (3) difficulties in the scale-up to generate clinically relevant numbers of CMs, and (4) immaturity of the cells.

Recent advances in the development of engineered culture devices offer advantages over standard cell culture systems.[6] Combination of canonical stem cell biology with bioengineering tools might help overcome, or at least minimize, the aforementioned problems in cell derivation for potential clinical applicability. The current tissue engineering systems are based on *biomaterial scaffolds*, designed to serve as a cell culture niche, and *bioreactors*, designed to provide precise control of the cell culture environment, supply of nutrients and oxygen, and provision of physiologic regulatory factors. We discuss the impact of hPSC research on *in vitro* cardiac differentiation, and the use of new bioengineering approaches to improve cardiac cell culture and generate a faithful human heart tissue in a dish.

Cardiac development

Understanding heart development is fundamental for directing cardiac specification and differentiation starting from cells at a pluripotent state. Most of our knowledge of cardiomyogenesis comes from studies in the mouse model.

The developmental process of gastrulation first gives rise to the mesoderm.[7,8] At embryonic day 5, mesoderm arises with NODAL signaling in the proximal epiblast, maintaining BMP4 (Bone Morphogenic Protein 4) expression in the extra-embryonic ectoderm. Other signaling, including *Dkk1*, *Cer1,* and *Lefty1*, is responsible for NODAL and WNT (homologue of Drosophila Wingless gene) signaling restriction to the posterior epiblast. Then, before the epithelial–mesenchymal transition of the anterior primitive ectoderm, WNT induces the expression of mesendodermal markers such as *T (Brachyury)*, which together with *Eomes* is then involved in the expression of MESP1,[9,10] described as the principal regulator of cardiac specification.[11] In the presence of DKK1, which inhibits the canonical WNT signaling, MESP can activate the differentiation of the cardiac progenitor cells.[11] Terminal CM differentiation is coordinated by complex interactions of transcription factors including *Nkx2.5*, *Mef2C*, *Gata-4,* and *Tbx5*.[12,13] It has been shown that WNT canonical signaling maintains the cardiac progenitor cell pool while the NOTCH signaling controls their differentiation.[12,13]

The cardiac mesoderm progresses through major morphological changes forming the different heart compartments,[8] and further development toward the complete formation of the myocardium involves the BMP, WNT, and FGF (Fibroblast Growth Factor) pathways.[14] Interestingly, recent molecular analysis identified the BMP, FGF, activin/NODAL, and WNT signaling as the four key signaling mechanisms involved in the cardiac differentiation of induced pluripotent stem cells (iPSCs) and need to be regulated in a timely fashion,[15] indicating a meaningful biomolecular correlation between the natural heart development and the *in vitro* cardiac differentiation of hPSCs.

Deriving CMs from PSCs

Stem cell biology has had a great impact on envisioning new therapeutic modalities for the heart ever since the mouse ESCs were first derived[16] and even more after ESCs were also derived from humans.[17] The significance of these studies is in the isolation of a defined stem cell type that can indefinitely proliferate *in vitro* and at the same time is virtually able to give rise to any tissue, hence representing an ideal source for cell replacement therapy. Today, we are still facing two major obstacles to the use of hESCs for clinical therapy. First, human embryos are required for their derivation, raising ethical concerns. Because the clinical use of ESCs is only possible in heterologous fashion, lifelong immunosuppression of the patient would be necessary. Almost 10 years after the derivation of hESCs, these limitations have been largely overcome through another major advance in stem cell science—generation of the iPSCs that soon after being obtained from mouse[18] were also derived from human.[19,20] The iPSC discovery has profoundly changed our understanding of cell plasticity, has resolved most issues associated with the use of hESCs, and opened the possibility for personalized regenerative medicine by autologous transplantation of patient-derived iPSCs.

CM differentiation of hPSCs can be achieved in different cell culture settings, by controlling key steps of the differentiation progression (Figure 1). Many different methods

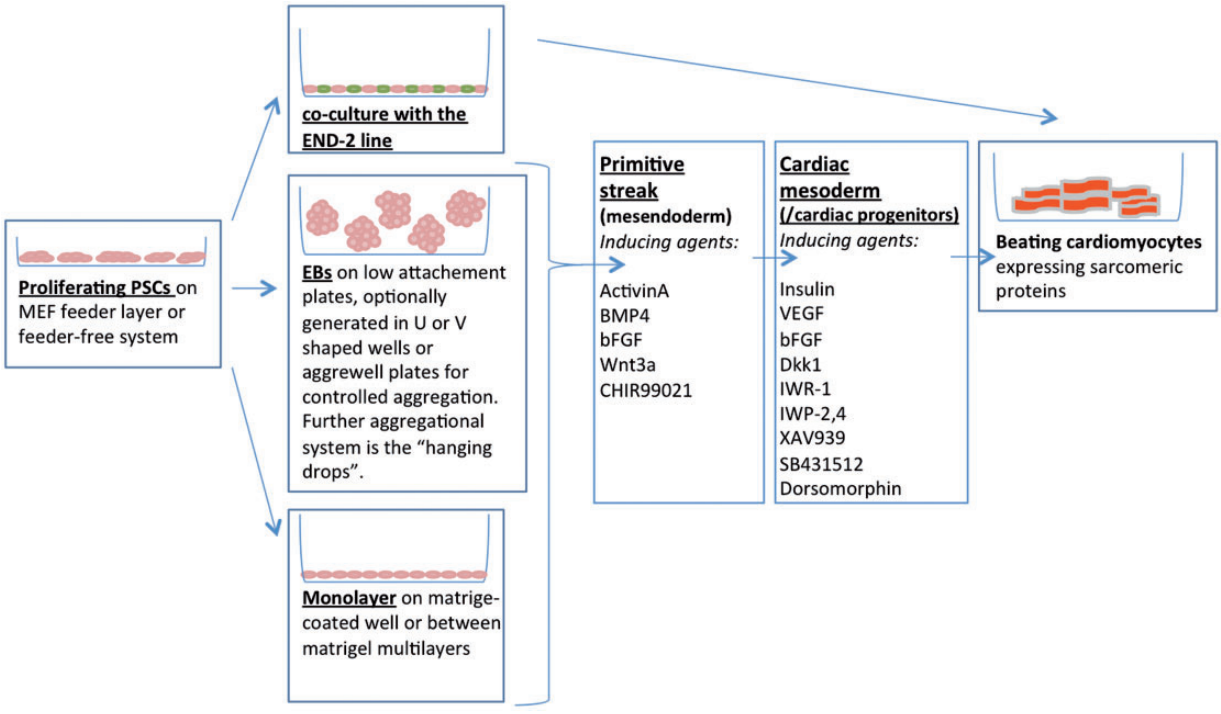

Figure 1 Available cell culture systems and molecular components of the key steps in hPSC cardiomyocyte differentiation. One of the oldest systems used is the co-culture of the PSCs with the inducing endodermal cell line END-2. The EB format is based on a 3D aggregation system. The monolayer is widely used and many different protocols have been established with this format. The reagents here reported are used in different combination (see also Table 1), dosage, and timing depending on the specific protocol. (A color version of this figure is available in the online journal)

Table 1 Established methods of hPSC differentiation into CMs

Differentiation format	Mesoderm (primitive streak/ mesendoderm) inducing factors	Cardiac mesoderm/cardiac precursor specification	References
EB	Spontaneous EB differentiation	Continuation in same culture conditions	Kehat *et al*.[5]
Monolayer co-culture with END-2 cells for several weeks	Spontaneous differentiation	Continuation in same culture conditions	Mummery *et al*.[21]
Monolayer co-culture with END-2 cells for several weeks	FBS removal, insulin removal, ascorbic acid	Continuation in same culture conditions	Passier *et al*.[22]
EB	END-2 conditioned medium, SB203580	Continuation in same culture conditions	Graichen *et al*.[23]
EB	END-2 conditioned medium, SB203580, prostaglandin I2	Continuation in same culture conditions?	Xu *et al*.[24]
EB	EB suspension with Wnt3a, serum	EB attachment, serum free	Tran *et al*.[25]
EB	Activin A, BMP4, bFGF	VEGF, DKK1	Yang *et al*.[26]
EB	Activin A, BMP4, bFGF	VEGF, DKK1, SB431542, dorsomorphin	Kattman *et al*.[27]
EB	Activin A, BMP4, bFGF	IWR-1, IWP-3, XAV939/ triiodotyronine	Willems *et al*.[28]
Forced aggregation	Activin A, bFGF	FBS	Burridge *et al*.[29]
Forced aggregation	BMP4, bFGF	RPMI+FBS or RPMI minus insulin/RPMI +insulin	Burridge *et al*.[30]
Monolayer, matrigel overlay at day –1	Matrigel overlay, Activin A, BMP4, bFGF	Basal medium/insulin	Zhang *et al*.[31]
Monolayer, matrigel overlay at day –1	Activin A/ BMP4, bFGF	DKK1	Uosaki *et al*.[32]
Monolayer	Activin A, BMP4	Basal medium	Laflamme *et al*.[33]
Monolayer	CHIR99021	IWP-2 or 4,	Lian *et al*.[34]
EB	Activin A, BMP4	IWR-1	Karakikes *et al*.[35]
Monolayer	CHIR99021 in CDM3	Wnt-C59 in CDM3	Burridge *et al*.[36]

for PSC differentiation into CMs have been investigated (see Table 1 for an overview of some of the relevant methods). The first protocols adopted were developed using hESCs and were based on the sole spontaneous differentiation.[5] Cells were induced to differentiate in three-dimensional (3D) structures—the embryoid bodies (EBs), or by using an inducing component—the END-2 endodermal cell line.[21,22] In both cases, the processes were relying on poorly controllable paracrine effects, had relatively low differentiation efficiencies, and a well-defined molecular environment was lacking.

Recently established methods are based on the recapitulation of key mechanisms of the heart development in the embryo and involve temporal administration of specific molecules regulating specific signaling cascades. In this regard, the regulation of canonical WNT signaling is the most widely accepted approach to controlled differentiation into CMs. Following this concept, WNT3A, a WNT signaling activator, was applied during the first two days of differentiation of stem cells cultured in the EB format,[25] in order to mimic the early primitive streak/mesendoderm induction. The same pathway was then inhibited at a later stage with the small molecule IWR-1,[37] to achieve the cardiac mesoderm specification.

The laboratory of Dr Keller has developed a serum-free protocol for cardiac differentiation of EBs derived from hESCs and iPSCs at high efficiency and with deeper lineage characterization along the differentiation progression from the pluripotent state to beating differentiated cells.[26,27] They described an early KDR positive cardiovascular progenitor cell population that can give rise to CMs as well as smooth muscle and endothelial cell both *in vitro* and *in vivo* after transplantation.[26] They also identified the fine regulation of the activin/NODAL and BMP signaling as crucial factors for optimal cardiac mesoderm specification and efficient CM differentiation.[27] The process of cardiac differentiation can be monitored by the flow cytometry analysis of a KDR/ PDGFRα positive population. The fine-tuning in downregulation of the aforementioned mechanisms at the cardiac mesoderm specification stage can be further improved with the employment of small molecules such as SB431542 and dorsomorphin (timing of administration between day 3 and 5). The concomitant use of the WNT inhibitor IWP-1 (between day 4 and 10) was also shown to improve the overall efficiency of differentiation. The efficiency of CM differentiation can be evaluated by the detection of the percentage of single cells positive for cardiac specific markers such as cardiac Troponin T (CTNT). The optimized protocol allowed achieving efficiency higher than 60% in CTNT+ cells.

Another system, based on forced cell aggregation in the EB format and the employment of bFGF and BMP4 without

activin A, further improved the differentiation efficiency up to almost 90% of CMs.[30] Another group, again using the EB format with forced cell aggregation, proposed a protocol involving activin A, BMP4, and also WNT3a, KITLG, and VEGF in the first stage and resulting in similar efficiency.[38]

Besides the END-2 co-culture protocol, which gives rise to CMs with a mostly ventricular phenotype, all hPSC-CM differentiation methods result in a mixed population of CM subtypes including atrial, ventricular, and nodal. Remarkably, a recent study proposing a new method for EB-derived hPSC-CM demonstrates how the use of the small molecule IWR-1, a canonical WNT signaling inhibitor, can result in fully ventricular-like CMs while the employment of DKK1 during the exact same temporal window results in almost 50% of ventricular-like, almost 50% of atrial-like, and a very small portion of nodal-like CMs.[38]

Although the 3D EB format reproduces some aspects of the *in vivo* tissue architecture, it also poses several problems related to cell culture preparation such as the control of cell aggregation (both in terms of yield and size uniformity) and the maintenance of the suspension culture. The monolayer format is generally considered more reproducible and, in principle, a more suitable approach for the scale-up for clinical purposes. In addition, a 2D system guarantees a more homogeneous exposure of the cultured cells to the soluble environment and might thus contribute to reducing the differences in quality and quantity of CM differentiation between cell batches and between different cell lines.

In the last few years, many methods adopting the monolayer format have been developed. The first monolayer method proposed was based on high-density PSCs adherent on a Matrigel-coated surface in the Rosewell Park Memorial Institute (RPMI) 1640 medium.[33] The protocol included administration of a high dose of activin A, followed by BMP4 for four days. This protocol resulted in CM differentiation efficiencies of approximately 30%. Improvements were obtained with a Matrigel overlay (the so-called Matrigel sandwich) that facilitated epithelial–mesenchymal transition before the addition of induction media, removal of insulin, addition of bFGF with activin A in the first stage, and addition of DKK1 in the second stage.[32] A similar approach was employed using a Matrigel overlay one day before the addition of differentiation medium and maintaining it also during induction with activin A, followed by a treatment with bFGF and BMP4. This method resulted in CM differentiation efficiencies of up to 98% of CTNT positive cells.[31]

All cardiac differentiation methods, both in 3D and monolayer formats, are based on the use of cytokines for the activation or inhibition of the specific signaling pathways. The cytokines, beside their high cost that affects scale-up of cell differentiation protocols for clinical application, can also cause batch-to-batch variability in the quality of cell preparation. This in turn can hinder reproducibility of cell differentiation protocols even for the same cell line. An alternative approach, recently developed in the Palecek's laboratory, is based on a cytokine-free protocol, where the activation and downregulation of the WNT signaling are achieved by the sole use of small molecules.[34,39]

All protocols for cardiac differentiation of human stem cells in the monolayer format that have been developed thus far require supplementation of B27 to differentiation media, a compound originally designed for neuron cell cultures.[40] It is not fully understood to what extent this component is suitable for CM differentiation. Recent work in Wu's laboratory shows that removal of the B27 supplement and the use of only three basal components (l-ascorbic acid, rice-derived recombinant human albumin, and the classic RPMI 1640 medium) in combination with the small molecules result in CM differentiation efficiencies of over 85%. The cell preparation can be further enriched up to 95% of CMs by metabolic selection.[36] Moreover, this protocol was proven to be effective in 11 different iPSC lines, a remarkable number never reported before for any CM differentiation method. This work represents a major step toward eliminating line-to-line variability of cardiac differentiation in human stem cells.

CM maturation

To date, several research groups have established and validated reliable and efficient protocols for the differentiation of hPSC into CMs. However, the maturation of hPSC-CMs remains a major issue yet to be fully resolved. Postdifferentiation hPSC-CMs are inevitably immature, and the cell phenotype resembles that of a fetal rather than a postnatal cardiac cell. This becomes evident when multiple characteristic traits of the immature and mature CMs are compared. First, the morphological structure of *in vitro* hPSC-CMs is quite different from that of adult CMs. hPSC-CMs tend to have irregular shapes while the adult CMs maintain their cylindrical structure.[41] This difference is also reflected in the poor sarcomeric organization of hPSC-CMs when compared to that of adult hCMs.[42,43] Another important indicator of CM maturation is the presence of T-tubules. Studies in rat CMs show that fetal cells lack T-tubule structures, which are present and fully developed in the postnatal cells. Different reports show that T-tubules are similarly rare or totally absent in hPSC-CMs,[43,45] and that calcium transients are unsynchronized.[46] It has also been described how the metabolism of CMs goes through major changes during development. In the early phases, the metabolism of cardiac cells is mostly glycolytic, while during the later development and terminal differentiation, the mitochondrial oxidative capacity becomes higher and the metabolism relies mostly on fatty acid β-oxidation.[46] A recent paper demonstrated how hiPSC-CMs metabolic activity is mostly glycolytic, thus further proving the low level of maturity of the differentiated iPSC-CMs.[47] Another structural difference is in the localization of gap junction proteins. It is well known that in mature cells they accumulate at the intercalated disks, while in hPSC-CMs they tend to be evenly distributed at the circumference, thus again more closely resembling the structure of fetal cells.[48,49] Finally, mature cells have higher expression levels of cardiac genes than hPSC-CMs. This is particularly evident for sarcomeric proteins, ion transport proteins, and calcium-related genes.[50,51]

The simplest, although not very practical, approach to CM maturation *in vitro* is the long-term culture of the differentiated cells. It has been observed that after 35 days in culture hPSC-CMs display ultrastructural maturation and exit the cell cycle.[43] After 90 days in culture CMs become more electrophysiologically mature in terms of action potential upstroke velocity, diastolic potential, and outward rectifier potassium current.[52] A recent study showed that after 120 days in culture CMs strongly increase their cell size, sarcomere length, percentage of multinucleated CMs, and anisotropy.[53] The simple prolongation of the time in culture can remarkably increase the maturation level of hPSC-CMs but has the undeniable downside of being time consuming and expensive.

Cardiac development is a complex process, evolving from an elaborated system of signaling molecules that have to be tightly coordinated in a spatial and temporal fashion (recently reviewed in the literature[15,54]). In addition, it must be considered that the heart is characterized by interactions of different cell types the function of which is orchestration by multiple signals such as molecular, electrical, and mechanical.[54] The design of an *in vitro* CM differentiation system for pharmacological studies and tissue replacement thus needs to take into account this complex landscape. The engineering approaches reviewed in the following section were designed to study the regulatory cascades and support the development of an effective system for generating hPSC-CMs.

Engineering PSC-derived CMs

Advanced engineering technologies can be exploited in order to increase the degree of control over the cell culture environment and thus obtain functionally mature CMs in an overall system that remains as faithful as possible to the natural heart physiology.[54–56] Several studies conducted in recent years (Figure 2, Table 2) include: the use of biomaterials for proper seeding,[70] orientation and cell–cell and cell–extracellular environment interaction,[57,71,72] bioreactors for constant molecular and fluidic control of the cell culture environment,[59,73,74] and the engineered platforms for providing combinations of mechanical and electrical stimuli.[72,75–77]

A simple example of the use of biomaterials for hPSC culture comes from the attempt to maintain pluripotency of hPSC and improve their growth in culture, while minimizing the amounts of culture media and the exposure to animal-derived components. Matrigel, a gelatinous protein mixture derived from a mouse cell line sarcoma, is the most commonly used substrate for cell adhesion and, in combination with the defined culture medium mTeSR, was identified as the most effective substrate.[60] Considerable efforts have been devoted to the development of hPSC culture systems free of animal-derived components, such as mouse embryonic fibroblasts, typically used as feeder cells, fetal bovine serum, and extracellular matrix proteins of animal source.[78] Alternative approaches use human cell feeder layers and medium supplemented with serum replacements.[79] Purified extracellular matrix molecules such as laminin, vitronectin, and collagen type I are also used as a structural substrate, as well as hyaluronic acid (HA) and calcium alginate hydrogels. Laminin and vitronectin supported the long-term expansion of hESCs in a defined culture medium both in the conventional monolayer culture and on 3D polystyrene microspheres. The expanded cells maintained high expression and karyotypic normality for over 20 passages.[80] A thorough study in which Matrigel was systematically replaced with defined medium supplements and ECM proteins showed that recombinant vitronectin was the optimal functional alternative for supporting sustained self-renewal and pluripotency of three different hESC lines.[81] hESCs were also successfully cultured on a type I collagen substrate without the need of feeders cells and in a defined serum-free medium. The same study also proved that hESCs could proliferate without bFGF supplements if heparin was added.[82] Vunjak-Novakovic's laboratory proposed the use of a completely synthetic hydrogel matrix, HA, to encapsulate and culture hESCs.[83] When encapsulated in 3D HA hydrogels cells preserved their normal karyotype and maintained their full differentiation capacity, which could be induced within the same hydrogel by simply altering soluble factors in culture environment. hESCs were also encapsulated in calcium alginate hydrogels and cultured for up to 260 days.[84] The authors proved the absence of unwanted cell differentiation along with the maintenance of cell pluripotency when exposed to a conditioned environment. ECM-derived hydrogels, a novel class of biomaterials, hold great promise for deriving hPSC-CM. Vunjak-Novakovic's laboratory, for example, used hydrogels composed of native heart ECM obtained from decellularized heart and blended with collagen without the addition of soluble cytokines. Such hydrogels were able to induce hESC differentiation into CMs. Higher contents of heart extracellular matrix resulted in further CM maturation as evidenced by the presence of striation, increased connexin 43 expression, and improved contractile properties.[62] This study brings us to the next key necessity for generating functional *in vitro* heart tissue: the achievement of a physiological 3D architecture of the aggregated cells. The most common approach is based on the suspension of cells in hydrogels in dedicated molds, favoring the spontaneous aggregation of cells in a 3D fashion, and the stabilization of the newly formed structure.[62] When adding two or more anchoring points to the molds, the forming tissue is poised to develop mechanical tension between them, to organize and align and thus push the maturation process further. Bursac's laboratory demonstrated that hESC-CMs seeded around elliptical posts in a 3D fibrin hydrogel system were aligned uniformly and highly organized. They also showed higher conduction velocity, sarcomere length, and contractile force, correlating with their increased expression of genes involved in cardiac contractile function and proving their advanced level of maturation.[63,58] Recent work demonstrated the maturation of hESC-CMs encapsulated in resorbable gelatin hydrogels that were stimulated with cyclic uniaxial stretch.[67] When compared to unstretched controls, the stimulated constructs had an increased percentage of CTNT-positive cells, greater elongation, increased gap junction expression, better contractility, improved calcium handling, and higher

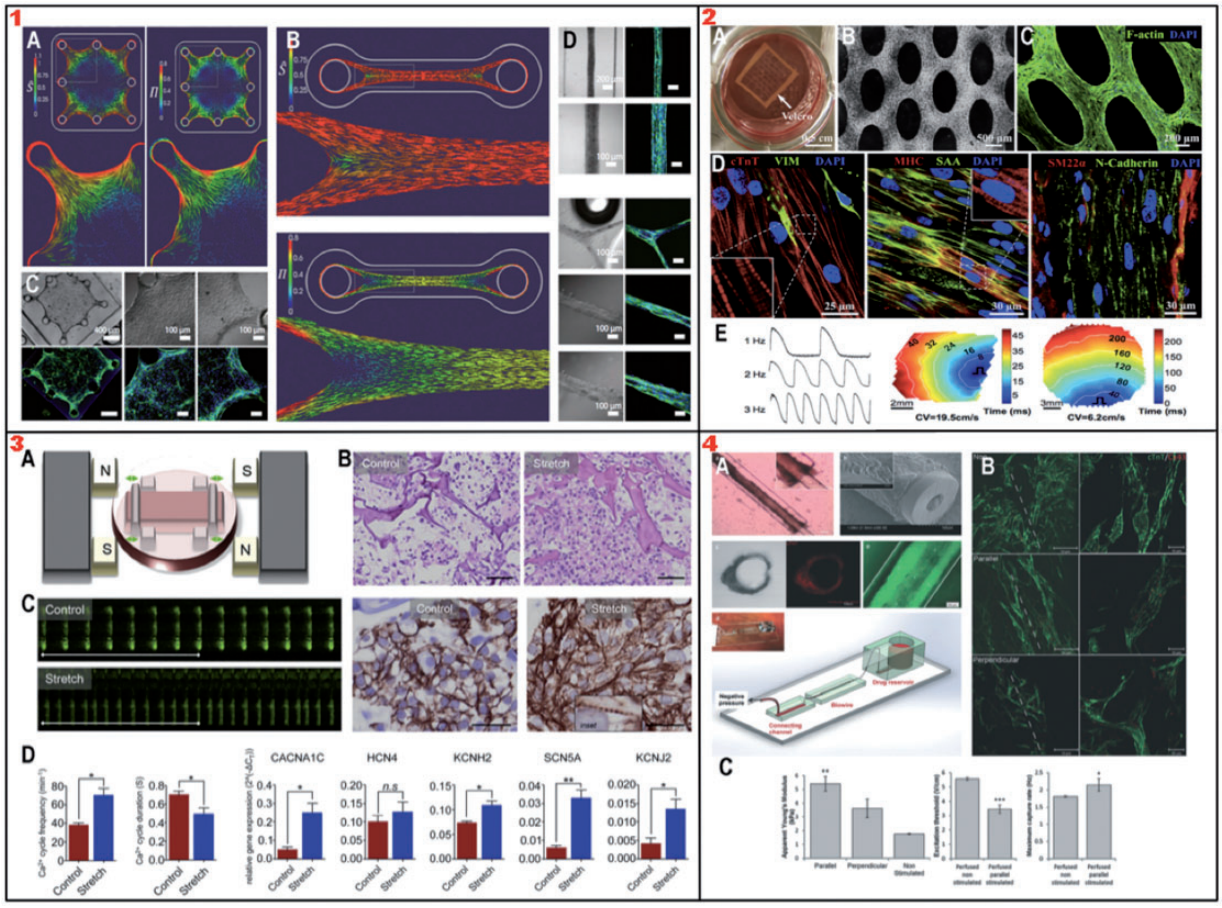

Figure 2 Bioengineered cell culture systems applied to PSC-derived cardiomyocytes. 1. Microtissues composed of CMs in ECM-derived hydrogels exhibit stress-mediated alignment and patterned expression of sarcomeric filaments. (a) Mathematical modeling predicts stress (left) and sarcomeric expression (right) co-localization in border regions, and (b) stress (top) and sarcomeric α-actinin expression (bottom) along the longitudinal axis. (c) Immunostaining for sarcomeric α-actinin (green) confirms localization in border regions. (d) Immunostaining for sarcomeric α-actinin (green; upper) and cardiac troponin T (green; lower) confirms sarcomere protein expression along the longitudinal axis. Nuclei are shown in blue.[57] Reproduced with permission. 2. Microstructured human cardiac tissue patches. (a) Low magnification of a cardiac tissue patch. (b, c) Staggered elliptical pores surrounded by densely packed and aligned cells. (d) hESC-CMs are aligned and show high expression of cardiac Troponin T (cTnT), myosin heavy chain (MHC), and sarcomeric α-actinin (SAA). Fibroblasts positive for vimentin (Vim) and smooth muscle cells (SM22a+) are interspersed among hESC-CMs. N-cadherin shows evidence of mechanical coupling. (e) Representative optically mapped action potential traces in a two-week-old cardiac patch at various pacing rates. Representative isochrone maps during 1 Hz point stimulation of a two-week-old cardiac patch (left) and monolayer (right).[58] Reproduced with permission. 3. Mechanical stretching. (a) The stretching apparatus exerted non-contact electromagnetic forces, simultaneously displacing the construct at both ends. (b) Cross-sectional hematoxylin and eosin staining (top) showed better dispersion of cells in stretched constructs, which also had greater expression of cTnT and well-defined striations with Z-banding running longitudinally along the stretched hESC-CMs (bottom). (c) Line scanning of calcium imaging across multiple cells revealed faster spontaneous contractions in stretched constructs. (d) Stretched constructs cycled calcium faster than controls. mRNA levels of cardiac-specific ion channels (by quantitative real-time PCR) in flow-sorted CMs isolated from the constructs.[67] Reproduced with permission. 4. Perfusion. (a) Generation of perfusable cardiac biowires from neonatal rat cardiomyocytes in collagen type I-based gel compacting around a tubing template. SEM images demonstrate that the cardiac tissue attached to the tubing surface, remodeled and formed an uniformly thick layer. Also shown is the circular morphology of the cross-section of the perfusable cardiac biowire with expression of cardiac Troponin-T (cTnT). (b) Set-up of the perfusion device. The biowire was perfused with FITC-labeled polystyrene beads. Solid lines illustrate the wall of the cell culture channel. FITC-labeled beads are indicated by arrows. Asterisks indicate the auto fluorescence from the cardiomyocytes. (c) Effects of different regimes of electrical stimulation. Young's modulus of cardiac biowires characterized by AFM reveals that the parallel-stimulated biowires had improved mechanical properties compared to controls. Electrically stimulated perfused hESC-CMs biowires had lower excitation threshold and higher maximum capture rate compared to the non-stimulated controls.[59] Reproduced with permission. (A color version of this figure is available in the online journal)

expression level of relevant ion channel-associated genes. Furthermore, implantation of these constructs resulted in increased survival and engraftment of the transplanted cells in the stretched samples.[67]

Similar to hESC-CM, iPSC-CMs also were successfully cultured in 3D hydrogel structures.[64] Cells were assembled in a 3D collagen hydrogel and exposed to uniaxial mechanical stimulation that markedly improved CM alignment, myofibrillogenesis, sarcomere formation, and cell hypertrophy. Interestingly, co-culture with endothelial cells resulted in increased CM proliferation, while the further addition of stromal cells favored the formation of vessel-like structures and simultaneously incremented active force upon increased resting length.

An interesting alternative to the scaffold-based aggregation for the construction of a 3D cardiac tissue is represented by the technology that uses temperature-responsive polymer Poly N-isopropylacrylamide as substrate for cell culture.[85] With this technique, entire sheets of monolayered iPSC-CMs can be detached from the culture surface, fully preserving cell surface and extracellular components, and raising the possibility of fabricating

Table 2 Tissue engineering approaches applied on PSC-derived CMs

PSC type	Tissue engineering system	Effect	Reference
hESCs, iPSCs	Matrigel	PSC growth on feeder-free adherent plate, suitable for monolayer differentiation	Hakala *et al*.[60]
hESCs	Collagen-coated glass perfused with medium	Increased smooth muscle actin and cell density	Figallo *et al*.[61]
hESCs	Native heart ECM-collagen	Sarcomere striation, Cx43 increased expression	Duan *et al*.[62]
hESCs	Fibrin hydrogels in elliptical posts	Increased conduction velocity and contractile force	Liau *et al*.[63]
hESCs	Collagen hydrogel-uniaxial mechanical stimulation, + endothelial cells, +stromal cells	Improved CM alignment and hypertrophy	Tulloch *et al*.[64]
hESCs	Collagen-coated polyacrylamide gel	Gene expression profile similar to that of adult CMs	Jacot *et al*.[65]
hESCs, iPSCs	Polyacrylamide gel	Increased mechanical force	Hazeltine *et al*.[66]
hESCs	Absorbable gelatine sponges stimulated with cyclic uniaxial stretch	Increased elongation, contractility and ion channel expression	Mihic *et al*.[67]
iPSCs	Biowire-electrical stimulation	Improved CM alignment and striation	Nunes *et al*.[68]
hESCs	Thermosensitive PSC-CM cell sheet technology	Potential system aiming at the fabrication of 3D cardiovascular tissue	Matsuura *et al*.[69]

cardiovascular tissues by overlaying multiple sheets.[69,86] Another interesting approach was developed in Murry's laboratory[87] that involves spontaneous aggregation of hESC-CM during suspension culture on a rotating orbital shaker. Cells aggregated forming macroscopic disc-shaped patches of beating tissue with a 300–600 μm thickness. Prevascularization of cell sheets was achieved by co-culturing hESC-CM with human endothelial cells, vascular mural cells, and/or fibroblasts.[88,89] In animal models, microvessels anastomosed with the host coronary circulation and delivered blood to the grafts.[89,90] Cell sheet technology has been used to fabricate viable cardiac constructs from both hESCs and hiPSCs for drug testing[91] and implantation.

It has also been hypothesized that the stiffness of extracellular matrix is related to CM maturation. A major indicator of this concept is the progressive accumulation of collagenous extracellular matrix, and thus a proportionate increase in stiffness, by CMs from the embryonic to the postnatal stage.[46] In mouse studies, the elastic modulus, an indicator of the passive stiffness, has a threefold increase from embryo to the newborn animal.[92] Other studies with polyacrylamide gels with varying elastic moduli showed that hESC-CM morphology was affected in an analogous manner to that of neonatal rat CMs.[65] Following a similar approach, it was recently shown that both hESC- and iPSC-CMs generate more mechanical force when cultured on hydrogels with higher stiffness.[66]

The laboratory of Levchenko[93] developed a nanofabricated PEG hydrogel substratum able to reproduce the nanostructure of the heart extracellular matrix with high fidelity and showed that rat CMs seeded in this substrate align and exhibit cell geometry, conduction velocity, and connexin 43 much more similar to those in the native heart when compared to the non-aligned CMs. It would be interesting to check if this result can be reproduced with iPSC-CMs.

Radisic's laboratory developed a novel engineering platform named Biowire, in which iPSC-CMs are placed in a polydimethylsiloxane channel surrounding a surgical suture in a collagen matrix. This resulted in cardiac cells aligned in 3D and were presenting well-developed striations. Electrical stimulation also improves cardiac tissue parameters, such as myofibril organization, conduction velocity, electrophysiological properties, and calcium handling.[68]

Another important aspect of CM maturation is cellular cross-talk, a process in which non-myocyte cell types in the myocardium play a pivotal role. For example, it has been found that co-culture of non-CM cell populations is necessary during hPSC differentiation for the activation of important calcium handling proteins and ion channel proteins such as HCN4.[94] Interestingly, direct reprogramming of fibroblasts by genetic activation of three key transcription factors, *Gata4*, *Mef2c,* and *Tbx5*, although effective in achieving full differentiation, gives rise to immature hPSC-CMs.[95] The same reprogramming performed *in vivo* generates CMs with morphological and electrophysiological properties similar to those in mature adult cells.[96] This in turn indicates the great importance of establishing a native-like environment for cultured CMs and suggests that further in-depth studies of the key paracrine signals will be necessary to achieve complete *in vitro* maturation of hPSC-CMs.

Summary and future perspectives

Important advances have been made in both stem cell biology and cardiac tissue engineering over the last decade. However, despite the promising results and the great potential, many open questions remain.

In terms of clinical application, the safety of hPSC needs to be addressed. Culture systems must ensure the complete removal of undifferentiated cells in the cell pool prepared for transplantation, in order to avoid potential tumorigenic effect.[97,98] As discussed in this paper, another important issue is the presence of animal-derived components in the culture substrates and differentiation cocktails, which might carry undetected pathogens. Although some of the proposed alternatives to common cultures help approach the golden standard of animal-free conditions, they cannot be considered fully defined. An evident limitation would be the batch-to-batch variability of the components used. An ''ideal'' method would combine animal product-free culture, chemically defined matrix, and serum free medium.[78]

The hiPSCs carry possible immune response potentials because of the genetic or epigenetic modifications required for their generation. The actual immunogenicity of iPSCs has not been fully defined even in the autologous settings. Syngeneic transplantation of undifferentiated iPSCs for teratoma formation resulted in T cell infiltration, even when the cells were generated with the episomal vector.[99] A different study showed no rejection after syngeneic transplantation of differentiated iPSCs and only minor immunogenic response after transplantation of undifferentiated cells.[100] Further studies will be necessary to make the future clinical application of iPSCs safe and effective.

Up until now, hESCs and hiPSCs were the only two hPSC types experimentally available. The laboratory of Mitalipov recently found that somatic cell nuclei can be reprogrammed by nuclear transfer into oocytes in humans, thus generating the so-called nuclear transfer pluripotent stem cells (NT-PSCs).[101] This finding was then confirmed by Egli's laboratory that reprogrammed nuclei of cells from type 1-diabetes patients using the same strategy.[102] NT-PSCs will probably be soon commonly used in research laboratories and in the near future might represent an alternate powerful tool for cell therapy of many tissues, including heart.

Many protocols showed that high efficiency of CM derivation from hPSCs is now possible. Although several published methods claim the reduction or elimination of inter-line variability, high reproducibility and standardization of efficient CM differentiation from different cell lines is still a challenge. It is undeniable that even the most established protocols can lose their effectiveness when adopted by other research laboratories and applied to different lines or the same lines at different passage numbers. Moreover, the scaling up of CM production to billions of cells, as required for cell replacement therapies, together with their integration with the most suitable bioengineering platforms, will need to be achieved. Some efforts have been already made in the CM differentiation approaches[103] and, more in general, the removal of expensive reagents and progressive minimization of the size of culture systems are contributing to lowering the costs and therefore increasing the feasibility of the clinical therapy.

In the last year, a remarkable preclinical study showed that hESC-CMs can extensively remuscularize infracted heart in monkey[104] and electromechanically couple with the host tissue. In addition, the study shows that there was neither tumor formation nor fatal ventricular arrhythmias and that clinical scale production of hPSC-CM is possible,[104] making a major advance toward their applicability in patients. However, the low replica number used due to the high cost of this animal model makes it difficult to achieve statistical significance.[104] Moreover, the size of the injury in this model of ischemia was smaller than the infarcts of patients that would benefit from this type of therapy and that therefore might possibly suffer more severe arrhythmias.[104] Larger studies will be necessary to assess the actual efficacy and safety of this promising therapeutic system.

Approaches at multiple levels are still needed to ensure control of different synergic cues for an efficient reproduction of the natural developmental maturation to achieve full functionality of the cardiac tissue for basic and applicative research purposes. Further interdisciplinary efforts of cell biologists and bioengineers are critically important for further improvements that will make clinical trials of stem cell-based heart regeneration a realistic possibility.

Authors' contribution: DS, EC, and GV-N have jointly planned and written the manuscript. GV-N approved the final version of the manuscript.

ACKNOWLEDGEMENTS

The work described in this paper has been supported by the NIH (grants HL076485, EB002520, and EB17103), NYSTEM (contract C028119), and the Leona M and Harry B Helmsley Charitable Trust.

REFERENCES

1. Murry CE, Soonpaa MH, Reinecke H, Nakajima H, Nakajima HO, Rubart M, Pasumarthi KBS, Virag JI, Bartelmez SH, Poppa V, Bradford G, Dowell JD, Williams DA, Field LJ. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. *Nature* 2004;**428**:664–8
2. Xin M, Olson EN, Bassel-Duby R. Mending broken hearts: cardiac development as a basis for adult heart regeneration and repair. *Nat Rev Mol Cell Biol* 2013;**14**:529–41
3. Malliaras K, Marbán E. Moving beyond surrogate endpoints in cell therapy trials for heart disease. *Stem Cells Transl Med* 2014;**3**:2–6
4. Taghavi S, Sharp TE, Duran JM, Makarewich CA, Berretta RM, Starosta T, Kubo H, Barbe M, Houser SR. Autologous c-kit+ mesenchymal stem cell injections provide superior therapeutic benefit as compared to c-kit+ cardiac-derived stem cells in a feline model of isoproterenol-induced cardiomyopathy. *Clin Transl Sci*, Epub ahead of print 2015. doi: 10.1111/cts. 12251
5. Kehat I, Kenyagin-Karsenti D, Snir M, Segev H, Amit M, Gepstein A, Livne E, Binah O, Itskovitz-Eldor J, Gepstein L. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *J Clin Invest* 2001;**108**:407–14
6. Freed LE, Guilak F, Guo XE, Gray ML, Tranquillo R, Holmes JW, Radisic M, Sefton MV, Kaplan D, Vunjak-Novakovic G. Advanced tools for tissue engineering: Scaffolds, bioreactors, and signaling. *Tissue Eng* 2006;**12**:3285–305
7. Tam PP, Loebel DA. Gene function in mouse embryogenesis: get set for gastrulation. *Nat Rev Genet* 2007;**8**:368–81
8. Buckingham M, Meilhac S, Zaffran S. Building the mammalian heart from two sources of myocardial cells. *Nat Rev Genet* 2005;**6**:826–35

9. Costello I, Pimeisl IM, Dräger S, Bikoff EK, Robertson EJ, Arnold SJ. The t-box transcription factor eomesodermin acts upstream of mesp1 to specify cardiac mesoderm during mouse gastrulation. *Nat Cell Biol* 2011;**13**:1084–91
10. David R, Jarsch VB, Schwarz F, Nathan P, Gegg M, Lickert H, Franz WM. Induction of mesp1 by brachyury(t) generates the common multipotent cardiovascular stem cell. *Cardiovasc Res* 2011;**92**:115–22
11. Bondue A, Lapouge G, Paulissen C, Semeraro C, Iacovino M, Kyba M, Blanpain C. Mesp1 acts as a master regulator of multipotent cardiovascular progenitor specification. *Cell Stem Cell* 2008;**3**:69–84
12. Qyang Y, Martin-Puig S, Chiravuri M, Chen S, Xu H, Bu L, Jiang X, Lin L, Granger A, Moretti A, Caron L, Wu X, Clarke J, Taketo MM, Laugwitz KL, Moon RT, Gruber P, Evans SM, Ding S, Chien KR. The renewal and differentiation of isl1+ cardiovascular progenitors are controlled by a wnt/beta-catenin pathway. *Cell Stem Cell* 2007;**1**:165–79
13. Kwon C, Qian L, Cheng P, Nigam V, Arnold J, Srivastava D. A regulatory pathway involving notch1/beta-catenin/isl1 determines cardiac progenitor cell fate. *Nat Cell Biol* 2009;**11**:951–7
14. Solloway MJ, Harvey RP. Molecular pathways in myocardial development: a stem cell perspective. *Cardiovasc Res* 2003;**58**:264–77
15. Burridge PW, Keller G, Gold JD, Wu JC. Production of de novo cardiomyocytes: human pluripotent stem cell differentiation and direct reprogramming. *Cell Stem Cell* 2012;**10**:16–28
16. Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. *Nature* 1981;**292**:154–6
17. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. *Science* 1998;**282**:1145–7
18. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006;**126**:663–76
19. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007;**131**:861–72
20. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Tomson JA. Induced pluripotent stem cell lines derived from human somatic cells. *Science* 2007;**318**:1917–20
21. Mummery C, Ward-van Oostwaard D, Doevendans P, Spijker R, van den Brink S, Hassink R, van der Heyden M, Opthof T, Pera M, de la Riviere A, Passier R, Tertoolen L. Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells. *Circulation* 2003;**107**:2733–40
22. Passier R, Oostwaard DW, Snapper J, Kloots J, Hassink RJ, Kuijk E, Roelen B, de la Riviere AB, Mummery C. Increased cardiomyocyte differentiation from human embryonic stem cells in serum-free cultures. *Stem Cells* 2005;**23**:772–80
23. Graichen R, Xu X, Braam SR, Balakrishnan T, Norfiza S, Sieh S, Soo SY, Tham SC, Mummery C, Colman A, Zweigerdt R, Davidson BP. Enhanced cardiomyogenesis of human embryonic stem cells by a small molecular inhibitor of p38 MAPK. *Differentiation* 2008;**76**:357–70
24. Xu XQ, Graichen R, Soo SY, Balakrishnan T, Rahmat SN, Sieh S, Tham SC, Freund C, Moore J, Mummery C, Colman A, Zweigerdt R, Davidson BP. Chemically defined medium supporting cardiomyocyte differentiation of human embryonic stem cells. *Differentiation* 2008;**76**:958–70
25. Tran TH, Wang X, Browne C, Zhang Y, Schinke M, Izumo S, Burcin M. Wnt3a-induced mesoderm formation and cardiomyogenesis in human embryonic stem cells. *Stem Cells* 2009;**27**:1869–78
26. Yang L, Soonpaa MH, Adler ED, Roepke TK, Kattman SJ, Kennedy M, Henckaerts E, Bonham K, Abbott GW, Linden RM, Field LJ, Keller G. Human cardiovascular progenitor cells develop from a kdr+ embryonic-stem-cell-derived population. *Nature* 2008;**453**:524–8
27. Kattman SJ, Witty AD, Gagliardi M, Dubois NC, Niapour M, Hotta A, Ellis J, Keller G. Stage-specific optimization of activin/nodal and bmp signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. *Cell Stem Cell* 2011;**8**:228–40
28. Willems E, Spiering S, Davidovics H, Lanier M, Xia Z, Dawson M, Cashman J, Mercola M. Small-molecule inhibitors of the Wnt pathway potently promote cardiomyocytes from human embryonic stem cell-derived mesoderm. *Circ Res* 2011;**109**:360–4
29. Burridge PW, Anderson D, Priddle H, Barbadillo Muñoz MD, Chamberlain S, Allegrucci C, Young LE, Denning C. Improved human embryonic stem cell embryoid body homogeneity and cardiomyocyte differentiation from a novel V-96 plate aggregation system highlights interline variability. *Stem Cells* 2007;**25**:929–38
30. Burridge PW, Thompson S, Millrod MA, Weinberg S, Yuan X, Peters A, Mahairaki V, Koliatsos VE, Tung L, Zambidis ET. A universal system for highly efficient cardiac differentiation of human induced pluripotent stem cells that eliminates interline variability. *PLoS One* 2011;**6**:e18293
31. Zhang J, Klos M, Wilson GF, Herman AM, Lian X, Raval KK, Barron MR, Hou L, Soerens AG, Yu J, Palecek SP, Lyons GE, Thomson JA, Herron TJ, Jalife J, Kamp TJ. Extracellular matrix promotes highly efficient cardiac differentiation of human pluripotent stem cells: the matrix sandwich method. *Circ Res* 2012;**111**:1125–36
32. Uosaki H, Fukushima H, Takeuchi A, Matsuoka S, Nakatsuji N, Yamanaka S, Yamashita JK. Efficient and scalable purification of cardiomyocytes from human embryonic and induced pluripotent stem cells by vcam1 surface expression. *PLoS One* 2011;**6**:e23657
33. Laflamme MA, Chen KY, Naumova AV, Muskheli V, Fugate JA, Dupras SK, Reinecke H, Xu C, Hassanipour M, Police S, O'Sullivan C, Collins L, Chen Y, Minami E, Gill EA, Ueno S, Yuan C, Gold J, Murry CE. Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. *Nat Biotechnol* 2007;**25**:1015–24
34. Lian X, Hsiao C, Wilson G, Zhu K, Hazeltine LB, Azarin SM, Raval KK, Zhang J, Kamp TJ, Palecek SP. Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical wnt signaling. *Proc Natl Acad Sci USA* 2012;**109**:E1848–57
35. Karakikes I, Senyei GD, Hansen J, Kong CW, Azeloglu EU, Stillitano F, Lieu DK, Wang J, Ren L, Hulot JS, Iyengar R, Li RA, Hajjar RJ. Small molecule-mediated directed differentiation of human embryonic stem cells toward ventricular cardiomyocytes. *Stem Cells Transl Med* 2014;**3**:18–31
36. Burridge PW, Matsa E, Shukla P, Lin ZC, Churko JM, Ebert AD, Lan F, Diecke S, Huber B, Mordwinkin NM, Plews JR, Abilez OJ, Cui B, Gold JD, Wu JC. Chemically defined generation of human cardiomyocytes. *Nat Methods* 2014;**11**:855–60
37. Ren Y, Lee M, Schliffke S, Paavola J, Amos PJ, Ge X, Ye M, Zhu S, Senyei G, Lum L, Ehrlich BE, Qyang Y. Small molecule wnt inhibitors enhance the efficiency of bmp-4-directed cardiac differentiation of human pluripotent stem cells. *J Mol Cell Cardiol* 2011;**51**:280–7
38. Elliott DA, Braam SR, Koutsis K, Ng ES, Jenny R, Lagerqvist EL, Biben C, Hatzistavrou T, Hirst CE, Yu QC, Skelton RJ, Ward-van Oostwaard D, Lim SM, Khammy O, Li X, Hawes SM, Davis RP, Goulburn AL, Passier R, Prall OW, Haynes JM, Pouton CW, Kaye DM, Mummery CL, Elefanty AG, Stanley EG. Nkx2-5(egfp/w) hescs for isolation of human cardiac progenitors and cardiomyocytes. *Nat Methods* 2011;**8**:1037–40
39. Lian X, Zhang J, Azarin SM, Zhu K, Hazeltine LB, Bao X, Hsiao C, Kamp TJ, Palecek SP. Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating wnt/β-catenin signaling under fully defined conditions. *Nat Protoc* 2013;**8**:162–75
40. Brewer GJ, Torricelli JR, Evege EK, Price PJ. Optimized survival of hippocampal neurons in b27-supplemented neurobasal, a new serum-free medium combination. *J Neurosci Res* 1993;**35**:567–76
41. Louch WE, Sheehan KA, Wolska BM. Methods in cardiomyocyte isolation, culture, and gene transfer. *J Mol Cell Cardiol* 2011;**51**:288–98
42. Gherghiceanu M, Barad L, Novak A, Reiter I, Itskovitz-Eldor J, Binah O, Popescu LM. Cardiomyocytes derived from human embryonic and induced pluripotent stem cells: comparative ultrastructure. *J Cell Mol Med* 2011;**15**:2539–51
43. Snir M, Kehat I, Gepstein A, Coleman R, Itskovitz-Eldor J, Livne E, Gepstein L. Assessment of the ultrastructural and proliferative properties of human embryonic stem cell-derived cardiomyocytes. *Am J Physiol Heart Circ Physiol* 2003;**285**:H2355–63
44. Lieu DK, Liu J, Siu CW, McNerney GP, Tse HF, Abu-Khalil A, Huser T, Li RA. Absence of transverse tubules contributes to non-uniform ca(2+)

wavefronts in mouse and human embryonic stem cell-derived cardiomyocytes. *Stem Cells Dev* 2009;**18**:1493–500
45. Satin J, Itzhaki I, Rapoport S, Schroder EA, Izu L, Arbel G, Beyar R, Balke CW, Schiller J, Gepstein L. Calcium handling in human embryonic stem cell-derived cardiomyocytes. *Stem Cells* 2008;**26**:1961–72
46. Yang X, Pabon L, Murry CE. Engineering adolescence: maturation of human pluripotent stem cell-derived cardiomyocytes. *Circ Res* 2014;**114**:511–23
47. Kim C, Wong J, Wen J, Wang S, Wang C, Spiering S, Kan NG, Forcales S, Puri PL, Leone TC, Marine JE, Calkins H, Kelly DP, Judge DP, Chen HS. Studying arrhythmogenic right ventricular dysplasia with patient-specific ipscs. *Nature* 2013;**494**:105–10
48. Chen HS, Kim C, Mercola M. Electrophysiological challenges of cell-based myocardial repair. *Circulation* 2009;**120**:2496–508
49. Zwi L, Caspi O, Arbel G, Huber I, Gepstein A, Park IH, Gepstein L. Cardiomyocyte differentiation of human induced pluripotent stem cells. *Circulation* 2009;**120**:1513–23
50. Reiser PJ, Westfall MV, Schiaffino S, Solaro RJ. Tension production and thin-filament protein isoforms in developing rat myocardium. *Am J Physiol* 1994;**267**:H1589–96
51. Angst BD, Khan LU, Severs NJ, Whitely K, Rothery S, Thompson RP, Magee AI, Gourdie RG. Dissociated spatial patterning of gap junctions and cell adhesion junctions during postnatal differentiation of ventricular myocardium. *Circ Res* 1997;**80**:88–94
52. Sartiani L, Bettiol E, Stillitano F, Mugelli A, Cerbai E, Jaconi ME. Developmental changes in cardiomyocytes differentiated from human embryonic stem cells: a molecular and electrophysiological approach. *Stem Cells* 2007;**25**:1136–44
53. Lundy SD, Zhu WZ, Regnier M, Laflamme MA. Structural and functional maturation of cardiomyocytes derived from human pluripotent stem cells. *Stem Cells Dev* 2013;**22**:1991–2002
54. Cimetta E, Godier-Furnémont A, Vunjak-Novakovic G. Bioengineering heart tissue for in vitro testing. *Curr Opin Biotechnol* 2013;**24**:926–32
55. Hirt MN, Hansen A, Eschenhagen T. Cardiac tissue engineering: State of the art. *Circ Res* 2014;**114**:354–67
56. Khademhosseini A, Langer R, Borenstein J, Vacanti JP. Microscale technologies for tissue engineering and biology. *Proc Natl Acad Sci USA* 2006;**103**:2480–87
57. Thavandiran N, Dubois N, Mikryukov A, Massé S, Beca B, Simmons CA, Deshpande VS, McGarry JP, Chen CS, Nanthakumar K, Keller GM, Radisic M, Zandstra PW. Design and formulation of functional pluripotent stem cell-derived cardiac microtissues. *Proc Natl Acad Sci USA* 2013;**110**:E4698–707
58. Zhang D, Shadrin IY, Lam J, Xian HQ, Snodgrass HR, Bursac N. Tissue-engineered cardiac patch for advanced functional maturation of human esc-derived cardiomyocytes. *Biomaterials* 2013;**34**:5813–20
59. Xiao Y, Zhang B, Liu H, Miklas JW, Gagliardi M, Pahnke A, Thavandiran N, Sun Y, Simmons C, Keller G, Radisic M. Microfabricated perfusable cardiac biowire: a platform that mimics native cardiac bundle. *Lab Chip* 2014;**14**:869–82
60. Hakala H, Rajala K, Ojala M, Panula S, Areva S, Kellomäki M, Suuronen R, Skottman H. Comparison of biomaterials and extracellular matrices as a culture platform for multiple, independently derived human embryonic stem cell lines. *Tissue Eng Part A* 2009;**15**:1775–85
61. Figallo E, Cannizzaro C, Gerecht S, Burdick JA, Langer R, Elvassore N, Vunjak-Novakovic G. Micro-bioreactor array for controlling cellular microenvironments. *Lab Chip* 2007;**7**:710–9
62. Duan Y, Liu Z, O'Neill J, Wan LQ, Freytes DO, Vunjak-Novakovic G. Hybrid gel composed of native heart matrix and collagen induces cardiac differentiation of human embryonic stem cells without supplemental growth factors. *J Cardiovasc Transl Res* 2011;**4**:605–15
63. Liau B, Christoforou N, Leong KW, Bursac N. Pluripotent stem cell-derived cardiac tissue patch with advanced structure and function. *Biomaterials* 2011;**32**:9180–7
64. Tulloch NL, Muskheli V, Razumova MV, Korte FS, Regnier M, Hauch KD, Pabon L, Reinecke H, Murry CE. Growth of engineered human myocardium with mechanical loading and vascular coculture. *Circ Res* 2011;**109**:47–59
65. Jacot JG, Kita-Matsuo H, Wei KA, Chen HS, Omens JH, Mercola M, McCulloch AD. Cardiac myocyte force development during differentiation and maturation. *Ann N Y Acad Sci* 2010;**1188**:121–7
66. Hazeltine LB, Simmons CS, Salick MR, Lian X, Badur MG, Han W, Delgado SM, Wakatsuki T, Crone WC, Pruitt BL, Palecek SP. Effects of substrate mechanics on contractility of cardiomyocytes generated from human pluripotent stem cells. *Int J Cell Biol* 2012;**2012**:508294
67. Mihic A, Li J, Miyagi Y, Gagliardi M, Li SH, Zu J, Weisel RD, Keller G, Li RK. The effect of cyclic stretch on maturation and 3d tissue formation of human embryonic stem cell-derived cardiomyocytes. *Biomaterials* 2014;**35**:2798–808
68. Nunes SS, Miklas JW, Liu J, Aschar-Sobbi R, Xiao Y, Zhang B, Jiang J, Massé S, Gagliardi M, Hsieh A, Thavandiran N, Laflamme MA, Nanthakumar K, Gross GJ, Backx PH, Keller G, Radisic M. Biowire: a platform for maturation of human pluripotent stem cell-derived cardiomyocytes. *Nat Methods* 2013;**10**:781–7
69. Matsuura K, Wada M, Shimizu T, Haraguchi Y, Sato F, Sugiyama K, Konishi K, Shiba Y, Ichikawa H, Tachibana A, Ikeda U, Yamato M, Hagiwara N, Okano T. Creation of human cardiac cell sheets using pluripotent stem cells. *Biochem Biophys Res Commun* 2012;**425**:321–7
70. Castells-Sala C, Semino CE. Biomaterials for stem cell culture and seeding for the generation and delivery of cardiac myocytes. *Curr Opin Organ Transplant* 2012;**17**:681–7
71. Turner WS, Wang X, Johnson S, Medberry C, Mendez J, Badylak SF, McCord MG, McCloskey KE. Cardiac tissue development for delivery of embryonic stem cell-derived endothelial and cardiac cells in natural matrices. *J Biomed Mater Res B Appl Biomater* 2012;**100**:2060–72
72. Serena E, Cimetta E, Zatti S, Zaglia T, Zagallo M, Keller G, Elvassore N. Micro-arrayed human embryonic stem cells-derived cardiomyocytes for in vitro functional assay. *PLoS One* 2012;**7**:e48483
73. Cimetta E, Sirabella D, Yeager K, Davidson K, Simon J, Moon RT, Vunjak-Novakovic G. Microfluidic bioreactor for dynamic regulation of early mesodermal commitment in human pluripotent stem cells. *Lab Chip* 2013;**13**:355–64
74. Radisic M, Marsano A, Maidhof R, Wang Y, Vunjak-Novakovic G. Cardiac tissue engineering using perfusion bioreactor systems. *Nat Protoc* 2008;**3**:719–38
75. Tandon N, Cannizzaro C, Chao PH, Maidhof R, Marsano A, Au HT, Radisic M, Vunjak-Novakovic G. Electrical stimulation systems for cardiac tissue engineering. *Nat Protoc* 2009;**4**:155–73
76. Wang B, Wang G, To F, Butler JR, Claude A, McLaughlin RM, Williams LN, de Jongh Curry AL, Liao J. Myocardial scaffold-based cardiac tissue engineering: application of coordinated mechanical and electrical stimulations. *Langmuir* 2013;**29**:11109–17
77. Lu L, Mende M, Yang X, Körber HF, Schnittler HJ, Weinert S, Heubach J, Werner C, Ravens U. Design and validation of a bioreactor for simulating the cardiac niche: a system incorporating cyclic stretch, electrical stimulation, and constant perfusion. *Tissue Eng Part A* 2013;**19**:403–14
78. Amit M, Itskovitz-Eldor J. Embryonic stem cells: isolation, characterization and culture. *Adv Biochem Eng Biotechnol* 2009;**114**:173–84
79. Villa-Diaz LG, Pacut C, Slawny NA, Ding J, O'Shea KS, Smith GD. Analysis of the factors that limit the ability of feeder cells to maintain the undifferentiated state of human embryonic stem cells. *Stem Cells Dev* 2009;**18**:641–51
80. Heng BC, Li J, Chen AK, Reuveny S, Cool SM, Birch WR, Oh SK. Translating human embryonic stem cells from 2-dimensional to 3-dimensional cultures in a defined medium on laminin- and vitronectin-coated surfaces. *Stem Cells Dev* 2012;**21**:1701–15
81. Braam SR, Zeinstra L, Litjens S, Ward-van Oostwaard D, van den Brink S, van Laake L, Lebrin F, Kats P, Hochstenbach R, Passier R, Sonnenberg A, Mummery CL. Recombinant vitronectin is a functionally defined substrate that supports human embryonic stem cell self-renewal via alphavbeta5 integrin. *Stem Cells* 2008;**26**:2257–65
82. Furue MK, Na J, Jackson JP, Okamoto T, Jones M, Baker D, Hata R, Moore HD, Sato JD, Andrews PW. Heparin promotes the growth of human embryonic stem cells in a defined serum-free medium. *Proc Natl Acad Sci USA* 2009;**105**:13409–14
83. Gerecht S, Burdick JA, Ferreira LS, Townsend SA, Langer R, Vunjak-Novakovic G. Hyaluronic acid hydrogel for controlled self-renewal and

differentiation of human embryonic stem cells. *Proc Natl Acad Sci USA* 2007;**104**:11298–303
84. Siti-Ismail N, Bishop AE, Polak JM, Mantalaris A. The benefit of human embryonic stem cell encapsulation for prolonged feeder-free maintenance. *Biomaterials* 2008;**29**:3946–52
85. Kumashiro Y, Yamato M, Okano T. Cell attachment-detachment control on temperature-responsive thin surfaces for novel tissue engineering. *Ann Biomed Eng* 2010;**38**:1977–88
86. Shimizu T. Cell sheet-based tissue engineering for fabricating 3-dimensional heart tissues. *Circ J* 2014;**78**:2594–603
87. Stevens KR, Pabon L, Muskheli V, Murry CE. Scaffold-free human cardiac tissue patch created from embryonic stem cells. *Tissue Eng Part A* 2009;**15**:1211–22
88. Masumoto H, Ikuno T, Takeda M, Fukushima H, Marui A, Katayama S, Shimizu T, Ikeda T, Okano T, Sakata R, Yamashita JK. Human ips cell-engineered cardiac tissue sheets with cardiomyocytes and vascular cells for cardiac regeneration. *Sci Rep* 2014;**22**:6716
89. Stevens KR, Kreutziger KL, Dupras SK, Korte FS, Regnier M, Muskheli V, Nourse MB, Bendixen K, Reinecke H, Murry CE. Physiological function and transplantation of scaffold-free and vascularized human cardiac muscle tissue. *Proc Natl Acad Sci USA* 2009;**106**:16568–73
90. Shiba Y, Fernandes S, Zhu WZ, Filice D, Muskheli V, Kim J, Palpant NJ, Gantz J, Moyes KW, Reinecke H, Biber BV, Dardas T, Mignone JL, Izawa A, Hanna R, Viswanathan M, Gold JD, Kotlikoff MI, Sarvazyan N, Kay MW, Murry CE, Laflamme MA. Human ES-cell-derived cardiomyocytes electrically couple and suppress arrhythmias in injured hearts. *Nature* 2012;**489**:322–5
91. Nakamura Y, Matsuo J, Miyamoto N, Ojima A, Ando K, Kanda Y, Sawada K, Sugiyama A, Sekino Y. Assessment of testing methods for drug-induced repolarization delay and arrhythmias in an ips cell-derived cardiomyocyte sheet: multi-site validation study. *J Pharmacol Sci* 2014;**124**:494–501
92. Jacot JG, Martin JC, Hunt DL. Mechanobiology of cardiomyocyte development. *J Biomech* 2010;**43**:93–8
93. Kim DH, Lipke EA, Kim P, Cheong R, Thompson S, Delannoy M, Suh KY, Tung L, Levchenko A. Nanoscale cues regulate the structure and function of macroscopic cardiac tissue constructs. *Proc Natl Acad Sci USA* 2010;**107**:565–70
94. Kim C, Majdi M, Xia P, Wei KA, Talantova M, Spiering S, Nelson B, Mercola M, Chen HS. Non-cardiomyocytes influence the electrophysiological maturation of human embryonic stem cell-derived cardiomyocytes during differentiation. *Stem Cells Dev* 2010;**19**:783–95
95. Ieda M, Fu JD, Delgado-Olguin P, Vedantham V, Hayashi Y, Bruneau BG, Srivastava D. Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. *Cell* 2010;**142**:375–86
96. Qian L, Huang Y, Spencer CI, Foley A, Vedantham V, Liu L, Conway SJ, Fu JD, Srivastava D. In vivo reprogramming of murine cardiac fibroblasts into induced cardiomyocytes. *Nature* 2012;**485**:593–8
97. Ben-David U, Benvenisty N. The tumorigenicity of human embryonic and induced pluripotent stem cells. *Nat Rev Cancer* 2011;**11**:268–77
98. Shih C-C, Forman SJ, Chu P, Slovak M. Human embryonic stem cells are prone to generate primitive, undifferentiated tumors in engrafted human fetal tissues in severe combined immunodeficient mice. *Stem Cells Dev* 2007;**16**:893–902
99. Zhao T, Zhang ZN, Rong Z, Xu Y. Immunogenicity of induced pluripotent stem cells. *Nature* 2011;**474**:212–16
100. Guha P, Morgan JW, Mostoslavsky G, Rodrigues NP, Boyd AS. Lack of immune response to differentiated cells derived from syngeneic induced pluripotent stem cells. *Cell Stem Cell* 2013;**12**:407–12
101. Tachibana M, Amato P, Sparman M, Gutierrez NM, Tippner-Hedges R, Ma H, Kang E, Fulati A, Lee HS, Sritanaudomchai H, Masterson K, Larson J, Eaton D, Sadler-Fredd K, Battaglia D, Lee D, Wu D, Jensen J, Patton P, Gokhale S, Stouffer RL, Wolf D, Mitalipov S. Human embryonic stem cells derived by somatic cell nuclear transfer. *Cell* 2013;**153**:1228–38
102. Yamada M, Johannesson B, Sagi I, Burnett LC, Kort DH, Prosser RW, Paull D, Nestor MW, Freeby M, Greenberg E, Goland RS, Leibel RL, Solomon SL, Benvenisty N, Sauer MV, Egli D. Human oocytes reprogram adult somatic nuclei of a type 1 diabetic to diploid pluripotent stem cells. *Nature* 2014;**510**:533–6
103. Ma J, Guo L, Fiene SJ, Anson BD, Thomson JA, Kamp TJ, Kolaja KL, Swanson BJ, January CT. High purity human-induced pluripotent stem cell-derived cardiomyocytes: electrophysiological properties of action potentials and ionic currents. *Am J Physiol Heart Circ Physiol* 2011;**301**:H2006–17
104. Chong JJ, Yang X, Don CW, Minami E, Liu YW, Weyers JJ, Mahoney WM, Van Biber B, Cook SM, Palpant NJ, Gantz JA, Fugate JA, Muskheli V, Gough GM, Vogel KW, Astley CA, Hotchkiss CE, Baldessari A, Pabon L, Reinecke H, Gill EA, Nelson V, Kiem HP, Laflamme MA, Murry CE. Human embryonic-stem-cell-derived-cardiomyocytes regenerate non-human primate hearts. *Nature* 2014;**510**:273–7