Turning Potential Into Action: Using Pluripotent Stem Cells to Understand Heart Development and Function in Health and Disease

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SUMMARY

Pluripotent stem cells hold enormous potential for regenerative therapies, however their ability to provide insight into early human development and the origins of disease could arguably provide an even greater outcome. This is primarily due to their contribution to the establishment of a powerful knowledge base of human development, something which all researchers and clinicians can potentially benefit from. Modeling human heart development and disease using pluripotent stem cells has already provided many important insights into cardiogenesis and cardiovascular disease mechanisms however, it is important to be aware of the complexities of this model system. Thorough contemplation of experimental models and specialized techniques is required to provide high-quality evidence of the intricacies of both normal early development, and when this process goes awry in disease states. STEM CELLS TRANSLATIONAL MEDICINE 2017;6:1452–1457

SIGNIFICANCE STATEMENT

This Perspective article provides a brief overview of the current and potential uses of pluripotent stem cells for investigating early development of the human heart in both health and in disease states. Additionally, it provides guidance and insight into the complexities of establishing pluripotent stem cell models to probe early cardiogenesis and cardiovascular disease.

“Heart and Brain are the two lords of life. In the metaphors of ordinary speech and in the stricter language of science, we use these terms to indicate two central powers, from which all motives radiate, to which all influences converge.”

— George Henry Lewes

INTRODUCTION

Understanding early human development is a complicated and technically difficult process. The formation of a complex biological organism such as a human is the result of a plethora of intricate cellular mechanisms and biochemical processes interacting in time and space to form a highly sophisticated entity with multiple systems, organs, and intermediate structures. Further, understanding the development of the heart is of great interest not only for developmental biologists, but also for those studying diseases resulting in structural or functional cardiovascular deficits, and those treating individuals with these deficits. According to the World Health Organization, cardiovascular disease is the leading cause of death globally [1], therefore it is imperative we increase our understanding of how the heart develops and functions, both under normal physiological conditions, as well as in diseased states.

When attempting to examine organogenesis in humans, it is particularly difficult to uncover the pathways involved in this complex process due to technical difficulties and ethical concerns associated with human-based research. Further, because of these limitations there is—justifiably—restricted availability of human tissue available for scientific purposes, even though access to this material would help to unlock the secrets of early development. In 1981, a discovery was made which changed the way we looked at mammalian development-pluripotent cells were “born.” First discovered as embryonic stem cells in mouse [2], this was then controversially mimicked in humans [3], and heralded a new era...
in developmental biology. However, as they say, necessity is the mother of invention, and pluripotent stem cells were then sensation-ally reinvented in 2007 as the universally ethically acceptable induced pluripotent stem cells (iPSC) [4]. The appeal of pluripo-tent stem cells (PSC) was not lost on any who understood their potential—cells which have the infinite capability to produce the plethora of cell types found in the human body. Since then, the concept of a “make-your-own cure” to degenerative diseases has been the twinkle in the eye of the public, clinicians and stem cell researchers alike. It is important however, to realize the even greater potential of these cells—uncovering the intricacies of human development to create a developmental knowledge base for all to benefit from. Therefore, human PSC (hPSC) have become a powerful tool in allowing researchers to examine early human development, circumventing the need for primary tissues [5–7].

In this Perspective article, we will briefly discuss what is known about normal heart development and the different factors involved in early cardiogenesis and how hPSC can help us to better understand this process. Current in vitro uses for hPSC-CMs are addressed along with the clinical applications of these cells. Finally, some practicalities of working with these cells are shared in order to give insight to the intricacies of their successful use.

**PLURIPOTENT STEM CELLS HELPING TO UNRAVEL HEART DEVELOPMENT**

The heart is a very complex organ. The cellular bulk of heart tissue mainly consists of cardiomyocytes and fibroblasts however these are not the only cell types which play an important role in cardiac development and function. Cells of the conduction system (nodal, bundle of His, bundle branch and Purkinje cells), inflammatory cells, blood vessels, endocardial and epicardial cells must also be considered. All these cell types play an important role in the correct functioning of the heart, and the body as a whole. The heart responds to sympathetic/parasympathetic stimulation—for example through psychological stress or increases/reductions in blood pressure and volume—as well as to pain, ischemia, oxygen concentration variation, changes in blood flow, and chemical/pharmacological insult.

The heart is the first functional organ created during mammalian embryonic development, derived from the mesodermal germ layer established in the early embryo. There are two distinct heart fields which contribute to the formation of the intricately complex four-chambered heart in a spatially and temporally defined manner. Upon gastrulation, the primitive streak is formed consisting of a layer of cells that establish the embryonic midline. In second week of human gestation, mesenchymal cells migrate through the streak and move out to create heart-forming regions. By week 3, this crescent shaped mesodermal region, often called the cardiac crescent, expands to form a primitive heart tube, made up of an exterior layer of myocardial cells and an interior layer of endocar-dial cells, separated by an extracellular matrix [8]. The heartbeat is initiated around this stage, followed by the progression of numerous cell divisions to form the common atrium and ventricles. The electrocardiogram also becomes clear at this stage, where it can be observed that cells of the primary heart tube possess low con-tractility and velocity, as opposed to cells of the chamber myocardium, which have high contractility and velocity [9], indicating early divergent identities of cardiomyocytes in regards to their electrophysiological properties in the early heart.

The “transcriptional machinery” of the heart is made up of an evolutionarily conserved group of transcription factors that reinforce one another’s expression and can either act singularly, or in conjunction, for the expression of genes required for proper cardiac development [10]. Signaling pathways associated with early cardiac development include the bone morphogenetic protein, Wnt and fibroblast growth factor signaling pathways. Also associated are sonic hedgehog (Shh) and Notch proteins.

The concept of inducing cardiogenesis in PSC is to attempt to mimic the normal development of the heart in a dish. In vitro dif-ferentiation of PSCs into cardiomyocytes can be broken down to three critical steps; (a) mesodendronal differentiation, (b) meso-derm generation, and (c) cardiac specification. These three pro-cesses can, and have been, achieved by combining different cytokines and small molecules in a tightly time- and dose-dependent manner [11]. Cardiac specification can also be influ-enced by the manipulation of the microenvironment, such as using matrices [12], carrier proteins [13] or coculturing with specifc cell types [14]. This is further described in Figure 1.

Although hPSCs can be differentiated to CMs, so far it has been shown that they are relatively immature and maintain fetal characteristics. Efforts toward maturing these cells in vitro will help to further understand how functional and developmental characteristics change over time.

**CURRENT IN VITRO USES FOR PSC-DERIVED CARDIAC CELLS**

The various uses of hPSC to date have included: modeling disease with known mutations [15]; creating cells for drug testing pur-poses [16, 17]; understanding the concept of cell identity and potential [18]; creating cell-specific populations for possible use in regenerative models of disease [19, 20], and of course, examining both normal early human development [7] and in diseased states [21]. Some more creative uses of these cells have also been sug-gested, one being the use of hPSC-derived cardiomyocytes (hPSC-CMs) for making microbimachines [22]. Here, we will focus on the disease modeling applications of these cells.

The use of hPSC-CMs for modeling disease has shown to be fruitful not only in monogenic diseases, but also for complex dis-eases such as trisomies and syndromes [21], where the problem of penetrance or individual disease variations could have theoreti-cally stymied their successful use. An extremely important factor in effectively modeling complex disease is ensuring all controls used are appropriate (e.g., related individuals, generated in the same laboratory in the same manner, identical culture conditions, etc.) [23]. This is even more crucial when the target disease is highly heterogeneous, or when only subtle differences between patients and controls are observed. The confounding factors which may affect the experimental outcomes include; the origin of primary reprogrammed cell, reprogramming method, genetic background, age, sex, comorbidities, and so forth. Therefore, a superior experimental design would be one which, beside the disease phenotype, has the most similarity between patients and controls. Additionally, having multiple clones from the same indi-vidual helps to eliminate the effects of reprogramming itself on the cells and any heterogeneity ensuing from the experimental processes performed.

Further bolstering models of heterogenous disease is to make a cohort of numerous patients, which ensures a more robust out-come and confirms that specific observed phenotypes are not merely due to genetic characteristics of individual patients. In the
The first hPSC-CMs were generated using embryoid body (EB) formation [57]. Using this method, cells are simply detached and transferred into low attachment dishes in serum containing medium, without growth factors. Cells from all three germ layers are generated, including cardiomyocytes at low yield [58, 59].

Embryoid bodies

Mummery et al. were the first to report of hPSC-CMs generation by co-culturing with the visceral endoderm cell line (END2) [14]. In this method, PSCs are in direct contact with endothelial cells and autocrine and paracrine factors secreted from endothelial cells induce cardiac differentiation.

Coculture

Growth factors

First reported in 2007. Activin A and BMP4 were the first growth factors used to induce cardiac differentiation [60]. Since then, BMP4 has been widely used in cardiac differentiation protocols either alone or in combination with other growth factors such as FGF and VEGF [12, 61, 62]. The Wnt signalling pathway, also important in early heart development, has a bi-phasic role in cardiac genesis. Wnt is required in early stages of cardiogenesis for the formation of mesoderm, however prolonged presence of Wnt inhibits further differentiation [63]. Multistep cardiac differentiation protocols utilize this knowledge to efficiently produce large numbers of hPSC-CMs [61].

Small molecules

Protocols using small molecules target specific signalling pathways which drive cardiogenesis. The advantages of these over growth factors are their longer half-life, and are significantly less expensive, which additionally makes them good candidates for use in scale-up applications [64]. In a search for improved differentiation protocols, small molecules have been used for differentiating hPSC to cardiomyocytes either on their own [37, 39] or in combination with growth factors [65].

Subtype-specific differentiation

Most of the commonly used differentiation protocols generate a mix of atrial, ventricular and nodal cells in different ratios. Subtype-specific cardiomyocyte generation is highly desirable for both drug discovery purposes and future clinical use, however limited success has been reported to date. Some methods include inhibition of NRG1β/Erbb pathway for nodal cell [70] and modification of retinoic acid signalling pathway cells to elicit an atrial or ventricular subtype [71].

GENETIC MANIPULATION

A number of genetically engineered hPSC lines have been generated to date using, for example, cardiac transcription factors (NKH2.5 [66], FLI1 [67]) and sarcomeric proteins (αMHC [68], MLCK2 [69]). These methods can either use fluorescent proteins or antibiotic selection to purify and sort cardiac populations.

THE CLINICAL LANDSCAPE OF PSC-DERIVED CARDIAC CELLS

Heart failure places an enormous burden on health systems worldwide. Patients suffering from heart failure have a poor 5-year mortality rate of approximately 50% [28] with limited treatment options. Currently, the best treatment for heart failure is transplantation, which for the majority of patients, is not a likely reality. The main cause of heart failure is ischemic heart disease [29], however congenital diseases, such as hypoplastic left heart syndrome and transposition of the great arteries, should also be considered in this burden, even though the number of affected individuals is comparatively small.

Preclinical trials using hPSC-CMs in non-human primates and porcine models have shown some promise for the treatment of heart failure [29–31], and although hPSC-CMs therapies have yet to be rigorously tested in humans, there has been evidence that other stem cell types may provide some benefit. Therapeutic trials using cardiac stem cells, mesenchymal stem cells, and bone marrow-derived cells have variably been shown to improve certain endpoints, however their true outcomes remain controversial [32, 33]. Improvements in these models are suspected to be a consequence of paracrine functions of the transplanted cells and not due to differentiation into cardiomyocytes, however this has not yet been definitively ascertained [28]. The first trial of hPSC-derived cardiac progenitor cells involving a single post-myocardial infarction patient was performed in 2015 [34], providing evidence of the feasibility of using hPSC for treatment of heart failure. Much work is still required to discover if this will be a promising option for treatments in the future.

For stem cell therapies to work, it is postulated that transplanted cells should be able to fully integrate and mature into cells similar to the native tissue [29], whereas others propose an earlier or progenitor type cell be transplanted to allow the cells opportunity to adapt to the native tissue environment [34]. It will only be through rigorous high-quality studies that this will eventually be ascertained.

An important step for the clinical success of hPSC for regenerative medicine is being able to reproducibly generate large numbers of highly-purified cells under clinical good manufacturing practice conditions [35, 36]. Although many articles have reported on ways to produce highly pure cardiomyocyte populations, the success of these methods has been variable from line to line. The reality is that every individual stem cell line is different, and thus responds differently to differentiation protocols, even in experienced hands and when well defined [11]. To date, it has been extremely difficult to reproducibly create functional cells with highly-purified populations from numerous genetically unique lines [37–39]. The largest study done to date using a small molecule-based cardiac differentiation protocol reported high reproducibility on a record 23 genetically individual pluripotent stem cell lines (51 total lines, including clones) [40].
clinical applications of hPSC-CMs will be hindered by this huge roadblock in the regenerative medicine field if personalized therapies are the way forward to treatment.

**PRACTICALITIES OF MAKING HPSC-CMS**

The laboratory environment is one that is highly contrived and obviously not precisely mimicking normal development. Although some excellent methods have been published on how to efficiently differentiate hPSC into cardiomyocytes, fibroblasts and extracellular matrix which can be formed within ring shaped molds, embedded with rigid posts, or elongated into rigid mesh. These can be used to measure the force of contraction of PSC-CM, and has also been shown to induce sarcomere assembly and maturation. Patch clamping is the gold standard method for the electrophysiological characterization of cardiomyocytes. Using this method, functionality of the cells, maturity level and even subtype of the cells can be revealed. This method, however, is extremely time consuming and low-throughput. Multielectrode array (MEA) is a non-invasive method for detection of field potential. This method can be used for high-throughput safety screening of drugs. Excitation-contraction coupling can be investigated using dye transfer techniques. **Stress responses:** Cellular responses to external stress, such as culturing cells in low oxygen level and stretch, can also be used to probe the functionality of hPSC-CMs characteristics. (see References [41, 52, 54–57, 73–75])

If making one or one million cardiomyocytes, it is important to remember that this cell is merely one building block of a larger, more complex organ. One must consider the outcome of what any experiment is set up to achieve (Fig. 2). The heart is not only resulted to an intermediate state of maturation. The best result achieved so far is to neonatal stage. This area is currently the focus of a large number of studies which have the aim to produce more mature type cells. One possible approach would be to combine two or more of the maturation methods in order to enhance the final outcome.

When considering hPSC-CMs and their role in modeling both development and cardiac disease, one must not only consider the role of the cell functionally, but also the role of cell-to-cell interaction (e.g., intercalated discs, gap junctions, desmosomes), and the environment in which the cells reside, (e.g., extracellular matrix, three-dimensional [3D] spatially, shear forces). Given the complexity of mechanical, structural and hemodynamic interactions in normal development, it is challenging to isolate specific signals which may affect development and remodeling responses in vivo, particularly since stretch and strain often vary in vitro [51]. Therefore, existing experimental techniques still remain inadequate in uncovering differences between diseased versus nondiseased, and in vitro and in vivo produced cells. This however, should not preclude the use of these cells in complex models of cardiac disease and development, as many undiscovered or surprising outcomes have still been revealed using current techniques [21].

**Figure 2.** Experimental procedures to consider when performing experiments with PSC-CMs. **Expression:** hPSC-CMs can be analyzed for the expression of proteins, RNA, methylation markings. **Cellular characteristics:** These methods used to examine the physical characteristics of cells will give insight into many types of diseases, particularly those with functional consequences. **Function:** The most challenging aspect of using hPSC-CM is assessing their function. Methods included are specialized and require expert advice. For example, engineered heart muscle is a 3D tissue construct made of mixture of cardiomyocytes, fibroblasts and extracellular matrix which can be formed within ring shaped molds, embedded with rigid posts, or elongated into rigid mesh. These can be used to measure the force of contraction of PSC-CM, and has also been shown to induce sarcomere assembly and maturation. Patch clamping is the gold standard method for the electrophysiological characterization of cardiomyocytes. Using this method, functionality of the cells, maturity level and even subtype of the cells can be revealed. This method, however, is extremely time consuming and low-throughput. Multielectrode array (MEA) is a non-invasive method for detection of field potential. This method can be used for high-throughput safety screening of drugs. Excitation-contraction coupling can be investigated using dye transfer techniques. **Stress responses:** Cellular responses to external stress, such as culturing cells in low oxygen level and stretch, can also be used to probe the functionality of hPSC-CMs characteristics. (see References [41, 52, 54–57, 73–75])

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**Expression**
- Protein (e.g., western blot, immunostaining)
- mRNA (e.g., qPCR, transcriptome sequencing)
- Methylation (e.g., whole genome methylation)

**Cellular characteristics**
- Cell types present (e.g., examine MiC2v/a isoforms)
- Cell proliferation rates (e.g., BRDU assay)
- Mitochondrial content (e.g., Mito tracker)
- Cellular coupling (e.g., gap junctions)

**Stress responses**
- Low oxygen culture
- Stretch
- Pharmacological agents
- Turbulent flow

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merely an electrically stimulated muscular pump for the blood, but a neurally integrated, hemodynamically active organ which responds to changes in the body as a whole, compensating homeostatically to changes in blood pressure, stress, and access to nutrients and oxygen. The importance of the 3D structure of the heart and each cell’s contribution to the organ must be also acknowledged, and finally the importance of cell coupling—both electrically and physically—must also be considered. The development of engineered tissues [52] or organoids [53] now gives us the opportunity to examine interactions between different cell types and now have an even better understanding of what may be occurring physiologically in the heart. Engineered heart muscle is a 3D tissue construct made of mixture of cardiomyocytes, fibroblasts and extracellular matrix which can be formed within ring shaped molds [52], embedded with rigid posts [54, 55], or elongated into rigid mesh [41, 56]. These can be used to measure the force of contraction of hPSC-CMs, and has been also shown to induce sarcomere maturation and maturation [57]. To make these tissues requires more than a million cells, which limits greater applications of these structures. A current aim in this sphere is to miniaturize this system in order to facilitate their mass production, and thus, enhance their application in disease modeling and drug screening platforms [57]. With the plethora of uses these versatile cells provide, it is exciting to consider what might be the next step in this already electrified field of research.

**Author Contributions**

H.F. and A.B.: manuscript writing, final approval of the manuscript.

**Disclosure of Potential Conflicts of Interest**

The authors indicated no potential conflicts of interest.

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**References**

1. World Health Organization. Cardiovascular Diseases Factsheet. 2016; Available at [http://www.who.int/mediacentre/factsheets/fs317/en/](http://www.who.int/mediacentre/factsheets/fs317/en/). Accessed January 2017.

2. Evans MJ, Kaufman, MH. Establishment in culture of pluripotent cells from mouse embryos. Nature 1981;292:154–156.

3. Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. Science 1998;282:1145–1147.

4. Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007;131:861–872.

5. Patterson M, Chan DN, Ha I, et al. Defining the nature of human pluripotent stem cell progeny. Cell Res 2012;22:178–193.

6. Pankratz MT, Li XJ, Lavaute TM, et al. Directed neural differentiation of human embryonic stem cells via an obligate primordial germ line [41,56]. The second uses to map the genome, particularly to design strategies. Dev Dyn 2016;245:1130–1144.

7. Srivastava D. Making or breaking the heart: From lineage determination to morphogenesis. Cell 2006;126:1037–1048.

8. Dunwoodie SL. Combinatorial signaling in the heart orchestrates cardiac induction, lineage specification and chamber formation. Semin Cell Dev Biol 2007;18:54–66.

9. Bondue A, Lapouge G, Paulissen C, et al. Stage-specific optimization of activin/nodal and BMP signaling promotes cardiomyocyte differentiation of mouse and human pluripotent stem cell lines. Cell Stem Cell 2011;8:228–240.

10. Zhang J, Kloc M, Wilson QF, et al. Extracellular matrix promotes highly efficient cardiogenesis of human pluripotent stem cells: The matrix sandwich method. Circ Res 2012;111:1125–1136.

11. Fonoudi H, Veynaneh M, Fatthali F, et al. IS11 protein transduction promotes cardiomyocyte differentiation from human embryonic stem cells. PLoS One 2013;8:e55577.

12. Mummery C, Ward-van Oostwaard D, Doevendans P, et al. CFPR/Cas9 differentiation of human embryonic stem cells to cardiomyocytes: Role of cocculture with visceral endoderm-like cells. Circulation 2003;107:2733–2740.

13. Bellin M, Casini S, Davis RP, et al. Isogenic human pluripotent stem cell pairs reveal the role of a KCNH2 mutation in long-QT syndrome. EMBO J 2013;32:3161–3175.

14. Liang P, Lan F, Lee AS, et al. Drug screening using a library of human induced pluripotent stem cell-derived cardiomyocytes reveals disease-specific patterns of cardiotoxicity. Circulation 2013;127:1677–1691.

15. Merchola M, Colas A, Willems E. Induced pluripotent stem cells in cardiovascular drug discovery. Circ Res 2013;112:534–548.

16. Bosman A, Sartiani L, Spinelli V, et al. Molecular and functional evidence of HCN4 and caveolin-3 interaction during cardiogenesis and cardiomyocyte differentiation from human embryonic stem cells. Stem Cells Dev 2013;22:1717–1727.

17. Neofytou E, O’Brien CG, Couture LA, et al. Hurdles to clinical translation of human induced pluripotent stem cells. J Clin Invest 2015;125:2551–2557.

18. Lait PA, Hei DJ, Raval AN, et al. Induced pluripotent stem cells for post-myocardial infarction repair: Remarkable opportunities and challenges. Circ Res 2014;114:1328–1345.

19. Bosman A, Letourneau A, Sartiani L, et al. Perturbations of heart development and function in cardiomyocytes from human embryonic stem cells with trisomy 21. Stem Cells 2015;33:1439–1446.

20. Park SJ, Gazzola M, Park KS, et al. Phototactic guidance of a tissue-engineered soft-tissue construct made of mixture of cardiomyocytes, fibroblasts and extracellular matrix which can be formed within ring shaped molds [52], embedded with rigid posts [54, 55], or elongated into rigid mesh [41, 56]. These can be used to measure the force of contraction of hPSC-CMs, and has been also shown to induce sarcomere maturation and maturation [57]. To make these tissues requires more than a million cells, which limits greater applications of these structures. A current aim in this sphere is to miniaturize this system in order to facilitate their mass production, and thus, enhance their application in disease modeling and drug screening platforms [57]. With the plethora of uses these versatile cells provide, it is exciting to consider what might be the next step in this already electrified field of research.

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1. World Health Organization. Cardiovascular Diseases Factsheet. 2016; Available at [http://www.who.int/mediacentre/factsheets/fs317/en/](http://www.who.int/mediacentre/factsheets/fs317/en/). Accessed January 2017.

2. Evans MJ, Kaufman, MH. Establishment in culture of pluripotent cells from mouse embryos. Nature 1981;292:154–156.

3. Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. Science 1998;282:1145–1147.

4. Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007;131:861–872.

5. Patterson M, Chan DN, Ha I, et al. Defining the nature of human pluripotent stem cell progeny. Cell Res 2012;22:178–193.

6. Pankratz MT, Li XJ, Lavaute TM, et al. Directed neural differentiation of human embryonic stem cells via an obligate primordial germ line [41,56]. The second uses to map the genome, particularly to design strategies. Dev Dyn 2016;245:1130–1144.

7. Srivastava D. Making or breaking the heart: From lineage determination to morphogenesis. Cell 2006;126:1037–1048.

8. Dunwoodie SL. Combinatorial signaling in the heart orchestrates cardiac induction, lineage specification and chamber formation. Semin Cell Dev Biol 2007;18:54–66.

9. Bondue A, Lapouge G, Paulissen C, et al. Mesp1 acts as a master regulator of multipotent cardiovascular progenitor specification. Cell Stem Cell 2008;3:69–84.

10. Kato S, Witty AD, Gagliardi M, et al. Stage-specific optimization of activin/nodal and BMP signaling promotes cardiomyocyte differentiation of mouse and human pluripotent stem cell lines. Cell Stem Cell 2011;8:228–240.

11. Zhang J, Kloc M, Wilson QF, et al. Extracellular matrix promotes highly efficient cardiogenesis of human pluripotent stem cells: The matrix sandwich method. Circ Res 2012;111:1125–1136.
37 Zhu WZ, Van Biber B, Laflamme MA. Methods for the derivation and use of cardiomyocytes from human pluripotent stem cells. Methods Mol Biol 2011;767:419–431.
38 Lian X, Hsiao C, Wilson G, et al. Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical Wnt signaling. Proc Natl Acad Sci USA 2012;109:E1884–E1887.
39 Burridge PW, Matsa E, Shukla P, et al. Chemically defined generation of human cardiomyocytes. Nat Methods 2014;11:855–860.
40 Fonoudi H, Ansari H, Abbasalizadeh S, et al. Large-scale production of cardiomyocytes from human pluripotent stem cells using a highly reproducible small molecule-based differentiation protocol. J Vis Exp 2016.
41 Bian W, Badie N, Himel HDT, et al. Robust T-tubulation and maturation of cardiomyocytes using tissue-engineered epicardial mimetics. Biomaterials 2014;35:3819–3828.
42 Spach MS, Heidlig FJ, Barr RC, et al. Cell size and communication: Role in structural and electrical development and remodeling of the heart. Heart Rhythm 2004;1:500–515.
43 Lundy SD, Zhu WZ, Regnier M, et al. Structural and functional maturation of cardiomyocytes derived from human pluripotent stem cells. Stem Cells Dev 2013;22:1991–2002.
44 Jacot JS, Kita-Matsu H, Wei KA, et al. Cardiac myocyte force development during differentiation and maturation. Ann N Y Acad Sci 2010;1188:121–127.
45 Wang PY, Yu J, Lin JH, et al. Modulation of alignment, elongation and contraction of cardiomyocytes through a combination of nanotopography and rigidity of substrates. Acta Biomater 2011;7:3285–3293.
46 Heidi Au HT, Cui B, Chu ZE, et al. Cell culture chips for simultaneous application of topographical and electrical cues enhance phenotype of cardiomyocytes. Lab Chip 2009;9:564–575.
47 Martherus RS, Vanherle SJ, Timmer ED, et al. Electrical signals affect the cardiomyocyte transcriptome independently of contraction. Physiol Genomics 2010;42A:283–289.
48 Deng XF, Rokosh DG, Simpson PC. Autonomous and growth factor-induced hypertrophy in cultured neonatal mouse cardiac myocytes. Comparison with rat. Circ Res 2000;87:781–788.
49 Foldes G, Mioulane M, Wright JS, et al. Modulation of human embryonic stem cell-derived cardiomyocyte growth: A testbed for studying human cardiac hypertrophy? J Mol Cell Cardiol 2011;50:367–376.
50 Lee YK, Ng KM, Chan YC, et al. Triiodothyronine promotes cardiac differentiation and maturation of embryonic stem cells via the classical genomic pathway. Mol Endocrinol 2010;24:1728–1736.
51 Zhu R, Blazaek A, Poon E, et al. Physical developmental cues for the maturation of human pluripotent stem cell-derived cardio-myocytes. Stem Cell Res Ther 2014;5:117.
52 Zimmermann WH, Schneiderberger K, Schubert P, et al. Tissue engineering of a differentiated cardiac muscle construct. Circ Res 2002;90:223–230.
53 McCracken KW, Cata EM, Crawford CM, et al. Modelling human development and disease in pluripotent stem-cell-derived gastric organoids. Nature 2014;516:400–404.
54 Hansen A, Eder A, Bonstrup M, et al. Development of a drug screening platform based on engineered heart tissue. Circ Res 2010;107:35–44.
55 Steoer A, Neuber C, Baldauf C, et al. Automated analysis of contractile force and Ca2+ transients in engineered heart tissue. Am J Physiol Heart Circ Physiol 2014;306:H1353–H1363.
56 Tulloch NL, Muskelhi V, Razumova MV, et al. Growth of engineered human myocardium with mechanical loading and vascular coculture. Circ Res 2011;109:47–59.
57 Huebsch N, Lskill P, Deveshwar N, et al. Miniaturized iPSC-cell-derived cardiac muscles for physiologically relevant drug response analyses. Sci Rep 2016;6:24726.
58 Kehat I, Kenyagin-Karsenti D, Snir M, et al. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. J Clin Invest 2001;108:407–414.
59 He JQ, Ma Y, Lee Y, et al. Human embryonic stem cell-derived cardiac muscle cells to develop into multiple types of cardiac myocytes: Action potential characterization. Circ Res 2003;93:32–39.
60 Xu C, Police S, Rao N, et al. Characterization and enrichment of cardiomyocytes derived from human embryonic stem cells. Circ Res 2002;91:501–508.
61 Laflamme MA, Chen KY, Naumova AV, et al. Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. Nat Biotechnol 2007;25:1015–1024.
62 Yang L, Soonpaa MH, Adler ED, et al. Human cardiovascular progenitor cells develop from a KDR+ embryonic stem-cell-derived population. Nature 2008;453:524–528.
63 Burridge PW, Thompson S, Milrod MA, et al. A universal system for highly efficient cardiac differentiation of human induced pluripotent stem cells that eliminates interline variability. PLoS One 2011;6:e18293.
64 Ueno S, Weidinger G, Osugi T, et al. Biphasic role for Wnt/beta-catenin signaling in cardiac specification in zebrafish and embryonic stem cells. Proc Natl Acad Sci USA 2007;104:9685–9690.
65 Fonoudi H, Ansari H, Abbasalizadeh S, et al. A universal and robust integrated platform for the scalable production of human cardiomyocytes from pluripotent stem cells. Stem Cells Transl Med 2015;4:1482–1494.
66 Minami I, Yamada K, Otsuji TG, et al. A small molecule that promotes cardiac differentiation of human pluripotent stem cells under defined, cytokine- and xeno-free conditions. Cell Rep 2012;2:1448–1450.
67 Elliott DA, Braam SH, Koutsis K, et al. NKF2-S(EGFP/W) hESCs for isolation of human cardiac progenitors and cardiomyocytes. Nat Methods 2011;8:1037–1040.
68 Bu L, Jiang X, Martin-Puig S, et al. Human iSL1 heart progenitors generate diverse multipotent cardiovascular cell lineages. Nature 2009;460:113–117.
69 Anderson D, Self T, Mellor IR, et al. Transgenic enrichment of cardiomyocytes from human embryonic stem cells. Mol Ther 2007;15:2027–2036.
70 Huber I, Itzhaki I, Caspi O, et al. Identification and selection of cardiomyocytes during human embryonic stem cell differentiation. FASEB J 2007;21:2551–2563.
71 Zhu WZ, Xie Y, Moyes KW, et al. Neuregulin/ErbB signaling regulates cardiac subtype specification in differentiating human embryonic stem cells. Circ Res 2010;107:776–786.
72 Zhang Q, Jiang J, Han P, et al. Direct differentiation of atrial and ventricular myocytes from human embryonic stem cells by alternating retinoid signals. Cell Res 2011;21:579–587.
73 Egashira T, Yuasa S, Tohyama S, et al. Patient-specific induced pluripotent stem cell models: Characterization of iPSC Cell-derived cardiomyocytes. Methods Mol Biol 2016;1353:343–353.
74 Kanda Y, Yamazaki D, Kurokawa J, et al. Points to consider for a validation study of iPSC cell-derived cardiomyocytes using a multi-electrode array system. J Pharmacol Toxicol Methods 2016;81:196–200.
75 Marcus IC, Illaste A, Heuking P, et al. Functional characterization and comparison of intercellular communication in stem cell-derived cardiomyocytes. Stem Cells 2015;33:2208–2218.