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Chapter 2

Building the heart from human pluripotent stem cells: Towards advanced disease modeling, tissue engineering and regenerative medicine

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Submitted.
Abstract

Human pluripotent stem cell (hPSC) models of cardiac differentiation have proven valuable to study heart development and disease. In this concise review, we present a brief overview of lineage specification in the heart, current developments for generation of cardiac subtypes from hPSCs and implications for disease modeling, tissue engineering and regenerative medicine.
Introduction

Cardiovascular disease is a global epidemic responsible for 17.3 million deaths each year (Mozaffarian et al, 2015). Research aimed at understanding the development of the heart and etiology of the diseases affecting this complex organ is essential for devising new therapeutic strategies to combat cardiac disease.

The heart is a muscular organ made up of cardiomyocytes (CMs), endothelial cells, smooth muscle cells, fibroblasts, epicardial cells and endocardial cells. CMs make up approximately 1/3rd of the heart (Tirziu et al, 2012) and are the fundamental work unit responsible for conduction and contraction. The non-CM fraction of the heart contributes greatly to CM differentiation, proliferation and function (Tirziu et al, 2012). For the scope of this review, we focus on the subtypes that constitute the CM fraction of the heart.

Structurally, the mammalian heart is broadly divided into four chambers- two atria and two ventricles interspersed with the components of the conduction system (CS) that ensures activation of the cardiac muscle. Rhythmic contraction and relaxation of the heart ensures proper supply of blood and nutrients throughout the body. Each cycle of the cardiac muscle is initiated by a specialized group of CMs of the sinoatrial node (SAN) situated at the entrance of the right atrium. These cells set pace and rhythm of the heartbeat and are also aptly called ‘pacemaker cells’. An electrical impulse generated by the SAN cells spreads through the atria causing them to contract and forcing blood into the ventricles. The impulse then travels to the atrioventricular node (AVN), where it is delayed before being transmitted to the ventricles, in order to allow sufficient time for the atria to contract. Following this, the electrical activity spreads to the His-Purkinje network, which innervates the muscular ventricular walls. This results in ventricular contraction and subsequent expulsion of blood from the heart to the lungs and the body.

The electrical activity recorded as an action potential (AP) is markedly different between the SAN, atria, AVN, ventricles and the His-Purkinje network. Any disturbance to this precisely orchestrated conduction process in the heart may lead to fatal arrhythmias. In the last decade, research using animal models has given valuable insight into the genetics and physiology of the heart. In the recent years, this has been complemented by pluripotent stem cells (PSCs) including human embryonic stem cells (hESCs) and human induced PSCs (hiPSCs) (Takahashi et al, 2007) that now give us the opportunity to study cardiac differentiation and disease in vitro. As the field continues to progress towards recapitulating complex cardiac disorders and engineer three-dimensional (3D) models of the heart in a dish, we review our current understanding of cardiac lineage specification in vivo, an overview of current protocols for generating cardiac subtypes in vitro, its implications for disease modeling, present limitations and future perspectives with respect to tissue engineering and regeneration.

Developmental aspects of lineage specification in the heart

The heart is the first functional organ in the embryo and is morphologically complete by 8 weeks of gestation in humans (Wloch et al, 2007). The concerted action of a myriad of signaling pathways and transcriptional networks guide the specification of cardiac lineages. The heart is patterned from a part of the Mesp1 expressing mesodermal germ layer (Saga et
Two progenitor fields termed the first heart field (FHF) and the second heart field (SHF) contribute to the developing heart (Kelly et al., 2001; Meilhac et al., 2004). It is now understood that these cardiac progenitors are segregated as early as the gastrulation stage of embryogenesis and are programmed to contribute to distinct lineages (Lescroart et al., 2014; Devine et al., 2014). Wingless-related integration site (Wnt) signaling is essential for the induction of Mesp1 expressing cardiac mesoderm. In contrast, repression of Wnt signaling and increased bone morphogenetic protein (BMP) activity guides early cardiogenesis (Marvin et al., 2001; Schultheiss et al., 1997).

Bilateral cardiogenic pools comprising the FHF fuse to form a linear heart tube. Transcription factors such as Nkx2.5 and Tbx5, associated with early cardiogenesis are expressed in the FHF, which is also marked by Hcn4 (Liang et al., 2013; Später et al., 2013). The linear heart tube, which is mainly of left-ventricular identity, serves as a scaffold for addition of cells from the SHF.

Although not exclusive to the SHF, Isl1 is widely regarded as a SHF marker (Cai et al., 2003). The SHF is further compartmentalized into anterior and posterior domains, a process in which Retinoic acid (RA) signaling (Ryczekbusch et al., 2008) plays a crucial role. Furthermore, canonical Wnt signaling has been implicated in maintaining the proliferation of SHF to allow expansion and growth of the heart (Kwon et al., 2007; Lin et al., 2007). Isl1 and Fgf8/Fgf10 expressing anterior SHF is deployed at the arterial pole of the heart tube and addition of cells from these precursors form the right ventricle and outflow tract (Kelly et al., 2001; Zaffran et al., 2004; Ilagan et al., 2006). On the other hand, Isl1 expressing posterior SHF adds cells to the venous pole of the linear heart to form majority of the atria (Cai et al., 2003; Galli et al., 2008). An excess or deficiency in RA results in morphological defects of the outflow and inflow tract (Niederreither et al., 2001; Vermot et al., 2003) suggesting an important role for this signaling cascade in development of these cardiac structures.

The precise origin of the CS components however is incompletely understood. It has been suggested that the avian pacemaker is derived from a region of the mesoderm caudal to the SHF that is positive for Wnt8c. Due to distinct location of these precursors, it has been proposed that this region constitutes a third heart field (Bressan et al., 2013), which is directed by canonical Wnt signaling towards a pacemaker lineage. Studies in mouse have demonstrated that cells of the SAN are mainly derived from Isl1+ progenitors, indicating a possible SHF origin (Liang et al., 2013). Additional cell lineage tracing experiments are necessary to determine the origin of SAN cells especially in the light of the finding that Isl1 is also expressed in the cardiac neural crest and therefore does not mark SHF derivatives alone (Engleka et al., 2012).

The embryonic atrioventricular canal (AVC), important for septation of chambers and formation of valves is also thought to contain precursors of the AVN based on morphological cues (Virágh & Challice, 1982) and molecular analysis (Hoogaars et al., 2004; Horsthuis et al., 2009; Aanhaanen et al., 2009). Fate mapping studies in the mouse have revealed contributions of both the FHF and the SHF to the AVC (Dominguez et al., 2012; Später et al., 2013). BMP signaling is indispensable for the proper patterning of the AVC (Stroud et al., 2007) and during later developmental stages, notch signaling is required for maturation of myocardial cells in this region (Rentschler et al., 2011).
The atrioventricular bundle (AVB), bundle branches and the Purkinje fibers comprise the ventricular conduction system (VCS). The AVB is believed to originate from the apex of the interventricular septum (Lamers et al, 1991; Chan-Thomas et al, 1993). Retrospective clonal analysis performed in mouse using nlacZ/nlacZ reporter system supports this hypothesis and it was proposed that differentiation of the VCS follows a biphasic development model (Miquerol et al, 2010). Based on the clonal relationship, myocardium of the ventricles and peripheral CS share a common origin followed by limited proliferative outgrowth (Miquerol et al, 2010). Interestingly, VCS innervating the right and left ventricles have different embryological origins (Miquerol et al, 2013) evident by the expression of Hcn4 or Isl1 in their progenitors, thus representing FHF and SHF respectively. Moreover, differentiation to Purkinje cells is augmented by neuregulin and endothelin signaling from the endothelium (Gourdie et al, 1998; Rentschler et al, 2002).

**Figure 1.** Cardiac lineage diversification from early progenitors. (A) Mesp1+ cardiac mesoderm is organized into HCN4+ first heart field (FHF) and Isl1+ second heart fields (SHF); BMP signaling has a positive effect while Wnt signaling is inhibitory at this stage. SHF is further delineated as anterior SHF (aSHF) and posterior SHF (pSHF), a process controlled by RA signaling. FHF gives rise to left atrium (LA), right atrium (RA), left ventricle (LV) and the atrioventricular canal (AVC). aSHF, positive for Fgf8 and Fgf10 forms the right ventricle (RV) and outflow tract (OFT). pSHF provides cells to developing LA, RA, sinus venosus (SV) and the AVC. (B) Brief list of genes preferentially expressed in various cardiac compartments.
During the course of cardiac specification and differentiation, selective transcriptional activation or repression in distinct cardiac compartments maintains their identity. In fetal and adult heart, orphan nuclear transcription factors, Coup-tfI and Coup-tfII are expressed selectively in the atrial myocardium (Devalla et al., 2015). In mice, Coup-tfII was identified as regulating atrial identity by directly repressing a number of ventricular genes (Wu et al., 2013).

Also, Slcn, a gene involved in calcium cycling in the cell is preferentially expressed in the atrial myocardium. The mRNA expression of Slcn increases progressively in the atria of mice during fetal development and persists in the adult atria (Minamisawa et al., 2003) (Vangheluwe et al., 2005). While the aforementioned genes are expressed in both the atrial chambers, expression of homeodomain transcription factor, Pitx2 is restricted to the left atrium where it suppresses the SAN transcriptional program to ensure that pacemaking activity is confined to the right atrium(Franco et al., 2000; Wang et al., 2010). Members of the hairy-related transcription factor family, Hey1 and Hey2 are also expressed differentially in the cardiac chambers and regulate atrioventricular boundary formation in the heart (Kokubo et al., 2007). Hey1 is expressed in the developing atria and is downregulated at E15.5 in mice. Hey2 on the other hand, is expressed in fetal as well as adult ventricles (Nakagawa et al., 1999). Another transcription factor that is exclusive to the ventricles, both in fetal and adult stages is the Iroquois homeobox gene, Irx4 where it controls the expression of chamber-specific myosin heavy chain genes (Bao et al., 1999). In addition, Helix-loop-helix transcription factors, Hand1 and Hand2 are both expressed in the cardiac crescent, but become progressively restricted to left and right ventricles respectively as heart development progresses (Srivastava, 1999).

Similarly, the molecular signature of the CS myocardium distinguishes it from working myocardium and differences also exist within the components of the CS. Several transcription factors such as Shox2, Isl1, Tbx3 and Tbx18 have been shown to be essential for development and function of the SAN (Blaschke et al., 2007; Hoogaars et al., 2007; Wiese et al., 2009; Christoffels, 2006; Liang et al., 2015). Whilst T box transcription transcription factor, Tbx3 is expressed in all of the CS (Hoogaars et al., 2004), expression of Isl1, Shox2 and Tbx18 is localized to the SAN region. In addition, Tbx3 expression persists in the mature CS (Hoogaars et al., 2004). As discussed before, Isl1 is expressed in SHF progenitors (Cai et al., 2003) but as development proceeds, its expression is confined to the SAN region and declines gradually in the postnatal heart (Liang et al., 2015). The homeodomain transcription factor Shox2 is expressed in sinus venosus and SAN region of the developing heart (Blaschke et al., 2007). Lastly, Tbx18 is first present in the sinus horns precursors, which has been shown to contribute to the head region of the SAN myocardium(Wiese et al., 2009). In the developing AVC, Bmp2 is specifically expressed early on and is required for the activation of Tbx2 and Tbx3 in this region (Stroud et al., 2007; Yamada et al., 2000).

Along with transcription factors and structural markers, a number of ion channels are differentially expressed in CM subtypes. Hcn4, encoding the inward current, If is initially expressed in the FHF progenitors but as development progresses, its expression is predominant in the components of the CS, particularly SAN and AVN (Später et al., 2013). Hcn4 expression has also been reported in fetal and adult SAN of the human heart (Chandler et al., 2009; Sizarov et al., 2011). In the atria, potassium channel genes Kcnaj5, Kcnj3 and
Kcnj5 are preferentially expressed. Kcna5 encodes the Kv1.5 channel while Kcnj3 and Kcnj5 encode K\textsubscript{ir}3.1 and K\textsubscript{ir}3.4 channels respectively. Kv1.5 channels conduct the ultrarapid delayed rectifier potassium current, I\textsubscript{Kur} and K\textsubscript{ir}3.1/3.4 channels form heteromultimeric complexes to conduct acetylcholine-activated current I\textsubscript{\textsubscript{K,ACH}} (Wang et al, 1993; Krapivinsky et al, 1995).

In summary, differential developmental origins together with localized activation of signaling pathways and transcriptional networks confer distinct molecular and functional profiles to atrial, ventricular and conduction cells essential for their diverse physiological roles in the heart.

**Differentiation of pluripotent stem cells to cardiomyocyte subtypes**

Human pluripotent stem cells (hPSCs) differentiating to CMs represent a particularly useful model for studying early lineage specification of CMs during human development. Reproducing the molecular and functional profile of specialized subtypes of the heart in vitro is essential to not only for furthering our understanding of developmental paradigms involved in lineage specification but also for establishing heart models that faithfully recapitulate the in vivo cell types, valuable for applications in disease modeling, drug/toxicity testing and regenerative medicine.

Since the introduction of the first protocols to steer PSCs to CMs (Kehat et al, 2001; Mummery et al, 2002), many advances have been made to enhance the efficiency of in vitro cardiac differentiation approaches. Recent techniques have introduced more refined, small molecule-based strategies, to generate high percentages of CMs (Burridge et al, 2014; Lian et al, 2012; 2015). However, majority of these protocols result in heterogeneous mix of various cardiac subtypes, with ventricular-like CMs being the most prominent cell type. The use of fluorescent reporter lines for purifying CMs in in vitro cultures is discussed in detail elsewhere (Hartogh & Passier, 2015).

Recently, we and others have shown that RA drives PSCs to atrial-like CMs (Gassanov et al, 2008; Zhang et al, 2010; Devalla et al, 2015). We extensively characterized RA-driven atrial-like CMs and reported that they respond to atrial-specific ion channel blockers, thus making these cells a valuable tool for preclinical pharmacological testing (Devalla et al, 2015). In another study, BMP receptor antagonist, Gremlin2 also enhanced differentiation of mouse embryonic stemcells (mESCs) to CMs with an atrial-like phenotype (Tanwar et al, 2014). Directed differentiation protocols have also been developed for generation of ventricular-like CMs from hPSCs. Inhibition of RA signaling (Zhang et al, 2010) or canonical Wnt signaling (Karakikes et al, 2014) during cardiac differentiation has been shown to result in a relatively homogeneous population of ventricular-like CMs.

For the generation of pacemaker-like cells, several approaches including overexpression of some key transcription factors such as Tbx3 or Shox2 have yielded promising results (Jung et al, 2014; Ionta et al, 2015). Overexpression of human SHOX2 during differentiation of mESCs favored a SAN gene program (Ionta et al, 2015), exhibited increased automaticity in vitro and were able to pace mouse hearts subjected to complete heart block. On the other hand, differentiation of human TBX3 expressing mESC clones resulted in a higher percentage of pacemaker-like cells in embryoid bodies (EBs) (Jung et al, 2014). Seeding these induced...
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pacemaker-like cells on cultivated ventricular slices of murine hearts ex vivo demonstrated increased beating rates in slices compared with those co-cultured with control EBs. Recently, we have shown that inducing MYC expression in differentiating hPSCs and subsequent addition of a cocktail of mitogens and small molecules such as SB431542 (inhibitor of the TGFβ superfamily type I activin receptor-like kinase (ALK), ALK4, 5, 7), IGF1 and SAG (inhibitor of hedgehog signaling) maintained them in a proliferative progenitor state (Birket et al, 2015). Moreover, modulation of fibroblast growth factor (FGF) and BMP signaling in these cardiac progenitor spheres (CPCs) resulted in distinct precursor populations, which could be further differentiated towards SAN-like or ventricular-like CMs.

Although these studies exemplify potential strategies for the derivation of pacemaker cells in vitro, they involve genetic manipulation. Therefore, growth factor or small-molecule based approaches that can be applied over a wide range of cell lines would be superior. In this regard, it has been shown that manipulating Neuregulin/ErbB signaling during differentiation alternates the ratio of nodal versus working myocardium-like cells obtained in culture (Zhu et al, 2010). Since the chicken GATA6-GFP promoter-enhancer reporter construct used in this study labels the AVN and AVB in the adult heart (Davis et al, 2001), nodal-like cells generated in this study may resemble one of those cell types. Additional characterization of these nodal-like cells is necessary to define their precise molecular and functional profile.

Cardiac Purkinje cells from in vitro mESC differentiations were efficiently purified using a Contactin2-egfp reporter line (Maass et al, 2015). A small molecule screen on differentiating mESCs marked by CCS-lacZ and further validated with the Contactin2-egfp cell line identified that sodium nitroprusside efficiently directed differentiation towards Purkinje cells by activating cyclic AMP signaling (Tsai et al, 2015).

<table>
<thead>
<tr>
<th>Cardiac subtype</th>
<th>Source cell type</th>
<th>Approach</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrial-like</td>
<td>mESCs/hESCs</td>
<td>Exogenous addition of retinoic acid</td>
<td>Gassanov et al 2008, Zhang et al 2011, Devalla et al 2015</td>
</tr>
<tr>
<td>Ventricular-like</td>
<td>hESCs/iPSCs</td>
<td>Inhibition of Wnt signaling; Inhibition of retinoic acid signaling</td>
<td>Karakikes et al 2013, Zhang et al 2011</td>
</tr>
<tr>
<td>SAN-like</td>
<td>mESCs/hESCs</td>
<td>Overexpression of Shox2; Overexpression of Tbx3; Modulation of FGF and BMP signaling</td>
<td>Jung et al 2014, Ionta et al 2015, Birket et al 2015</td>
</tr>
<tr>
<td>Nodal-like</td>
<td>hESCs</td>
<td>Inhibition of neuregulin/ErbB signaling</td>
<td>Zhu et al 2010</td>
</tr>
<tr>
<td>Purkinje-like</td>
<td>mESCs</td>
<td>Activation of cAMP signaling</td>
<td>Tsai et al 2015</td>
</tr>
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Table 1. Overview of current approaches described for the generation of cardiac subtypes from PSCs

Pathologies affecting specific cardiac subtypes and disease modeling with hPSCs

A number of cardiac diseases originate or are localized to specific cell types of the heart. In this section, we briefly summarize some of those disorders and also discuss the current developments regarding in vitro disease modeling with hPSCs.

Diseases of the SAN, collectively termed as sinus node dysfunction (SND) are the major
reason for pacemaker implantations in North America and Western Europe (Gillis et al, 2012). SAN diseases characterized by abnormal impulse initiation or propagation by the SAN and include conditions such as sinus bradycardia, sinus arrest, chronotropic incompetence, sinoatrial block and bradycardia-tachycardia syndrome. The term ‘sick sinus syndrome’ is commonly used to refer to the variety of symptoms associated with SND. Although aging remains a major risk factor for the malfunction of the sinus node due to decline in mechanical sporadic cases of congenital SND have been reported, mainly due to mutations in ion channel genes such as SCN5A or HCN4 (Anderson & Benson, 2010). In vitro hPSC-based disease models of SND have not been developed so far due to lack of robust protocols for generation of pacemaker-like cells.

Atrial fibrillation (AF) is marked by increased pace and quivering of the atrial chambers in an irregular pattern. It is the most common arrhythmia encountered by physicians in the clinic and an estimated 33 million people worldwide suffer from this disease (Chugh et al, 2014). Untreated AF can be life threatening, often leading to stroke, heart failure or even sudden death. Risk for AF increases with age. However, mutations in ion channel genes, particularly those encoding myocardial potassium channel genes such as KCNA5, KCNE2, KCNQ1 or the sodium channel gene SCN5A have been implicated in familial AF (Olesen et al, 2013). Additionally, GWAS studies have identified a number of single nucleotide polymorphisms (SNPs) associated with AF, particularly the 4q25 locus that is in close proximity to the PITX2 transcript (Olesen et al, 2013). Since protocols have now been devised for steering differentiation of hPSCs to atrial-like CMs (Zhang et al, 2010; Devalla et al, 2015), modeling AF in vitro may now be possible. Rapid atrial pacing may be simulated by an optogenetic approach (Bruegmann et al, 2010) followed by assessing electrophysiological parameters of diseased CMs.

Pathological conditions affecting the AVN include AV conduction block or AV nodal reentrant tachycardia (AVNRT) (Mozaffarian et al, 2015). AVN is responsible for delaying the electrical impulse from the atria before it passes to the ventricles. It also acts as an accessory pacemaker in the event of SAN failure. AV block results in obstruction of electrical impulse propagation from atria to the ventricles. AVNRT on the other hand is a result of slow and fast electrophysiological pathways present in the AVN that lead to a reentry circuit. The electrical impulse thus self-perpetuates in the AVN becoming the focal point of heart rate and rhythm causing the atria and ventricles to trigger rapidly. Modeling diseases of the AVN in vitro is rather complex due to lack of knowledge in directing differentiation towards AVN-like cells. Moreover, a model employing the use of multiple cell types such as SAN-like, atrial-like and AVN-like cells to study impulse propagation between these cell types would better address the mechanisms leading to AV block and reentry.

Numerous disorders affect the physiology and functioning of the cardiac ventricles. Ventricular tachyarrhythmias including ventricular fibrillation (VF) and ventricular tachycardia (VT) are the most frequently reported cardiac rhythms leading to sudden cardiac death. Multiple mechanisms such as reentry, abnormal automaticity or triggered activity in the ventricular myocardium may underlie ventricular tachyarrhythmias. Cardiomyopathies, classified as dilated or hypertrophic significantly affect ventricular contraction and function. Systolic dysfunction and enlarged ventricular chamber characterize dilated cardiomyopathy (DCM). In hypertrophic cardiomyopathy (HCM), systolic and diastolic dysfunction is evident which
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is accompanied by thickening of the ventricular wall. Patients with DCM or HCM are at increased risk for ventricular arrhythmias, heart failure or sudden death. Salient features of a few disorders, where a phenotype is evident in ventricular-like CMs have been successfully recapitulated in vitro using hPSC-CMs. It has been particularly advantageous to study ion channel mutations using hPSC models due to the availability of an electrophysiological readout to assess diseased CMs. To date, hiPSCs have been generated to study long QT syndrome (LQTS) (Moretti et al, 2010; Davis et al, 2012), catecholaminergic polymorphic ventricular tachycardia (CPVT) (Itzhaki et al, 2012; Jung et al, 2012), arrhythmogenic right ventricular dysplasia (ARVD) (Kim et al, 2013), LEOPARD syndrome (Carvajal-Vergara et al, 2010), DCM (Sun et al, 2012), HCM and hypoplastic left heart syndrome (HLHS) (Jiang et al, 2014).

Although proof of principle that hiPSC-CMs reproduce disease phenotypes in vitro has now been demonstrated time and again, complex phenotypes that involve pathophysiological conditions of the heart involving multiple cardiac cell types have not been modeled by hPSCs. Knowledge of guiding differentiation of hPSCs to enriched populations of atrial-like, ventricular-like or nodal-like cells and advances in cardiac tissue engineering will enable the modeling of cardiac disorders specific to certain subtypes.

Assembling a heart in vitro- current limitations and future directions

Although protocols are now emerging for directed differentiation of hPSCs to cardiac subtypes in vitro, several challenges lie ahead of us for realizing the full potential of these cells especially for drug discovery/screenings and regenerative medicine.

Identifying cell-surface markers

One shortcoming of the current in vitro protocols for producing and using hPSC-CMs is the lack of specific cell surface markers that distinguish cardiac subtypes in culture. Only recently, a combination of VCAM1 and SIRPA were reported to facilitate efficient purification of hPSC-CMs from other cell types. For isolating presumptive pacemaker-like cells, CD166 has been proposed as a marker, as it is expressed on mESC-derived progenitor cells that progress to become functional SAN-like cells (Scavone et al, 2013). However, an earlier study reported the expression of CD166 in hESC-derived CMs (Rust et al, 2009). Post sorting, these cells gradually lost NKX2.5 expression and displayed an increased rate of proliferation, a finding that prompted the authors to conclude that CD166 is useful for sorting immature CMs. Additional studies are required to assess the possibility of using this marker for identifying pacemaker cells in hPSC differentiations. To date, no surface markers have been identified for the purification of hPSC-derived atrial-like and ventricular-like CMs. Since most directed differentiation protocols merely enrich for a certain subtype rather than resulting in homogenous populations, antibodies directed against surface antigens that are selective for atrial, ventricular or nodal cells would be valuable for sorting these cells for further applications.

Importance of generating right/left CM subtypes

Protocols addressing the derivation of right and left cardiomyocyte subtypes would further advance in vitro cardiac modeling. One of the major sites of AF initiation lies around the
myocardium of the pulmonary sleeves in the left atrium. In a canine model of rapid atrial pacing, it has been observed that left atrial activation is more arrhythmogenic than right atrial activation (Niwano et al, 2007). Although it has been demonstrated that the myocardial connection between the left atrium and pulmonary sleeve serve as predilection sites for AF (Haïssaguerre et al, 1998), it is yet to be fully elucidated whether intrinsic transcriptional and functional differences between right and left atrial CMs alter their susceptibility to arrhythmias. Incidentally, genetic variants in close proximity to Pitx2 on the 4q25 locus have been linked to AF. Pitx2 expression is reduced in patients with chronic AF (Chinchilla et al, 2011) and mice heterozygous for Pitx2 have an increased susceptibility to arrhythmias (Kirchhof et al, 2011). Similarly, the right and left ventricles also exhibit physiological differences and as mentioned before have distinct embryological origins. In the postnatal heart, right and left ventricles operate under different mechanical load. Whilst left ventricular dysfunction has been well studied in the context of myocardial ischemia or cardiomyopathies, little is known about molecular and cellular mechanisms that contribute to right ventricular dysfunction. In mice, α,–adrenergic receptor stimulation evoked contrasting inotropic responses from right and left ventricular trabeculae (Wang, 2006). Also, exposure to chronic high altitude hypoxia resulted in differential gene expression changes in right and left ventricles (Strniskova et al, 2006). These studies point to innate differences between the ventricular chambers and relevant in vitro models will shed light on some of these aspects.

**Improving maturity of hPSC-CMs**

*Figure 2. Methods to improve maturity of hPSC-CMs.* Structural, functional and metabolic characteristics of hPSC-CMs resemble human fetal CMs. Several recent studies have reported methods to improve their maturity by addition of components such as T3 hormone to cell culture media, co-culture with endothelial cells or even altering their biophysical parameters through electrical stimulation or cyclic stretch.
One of the limitations to using hPSC-derived CMs for translational medicine is their immature phenotype. Structurally, hPSC-CMs lack t-tubules and have an underdeveloped calcium cycling apparatus (Gherghiceanu et al., 2011). At the electrophysiological level, they present depolarized resting membrane potential and a decreased upstroke velocity (Mummery et al., 2012). The force of contraction generated by hPSC-CMs is weaker than human fetal CMs from second trimester (Ribeiro et al., 2015). Moreover, as in embryonic CMs, hPSC-CMs prefer glycolysis for energy production in contrast to adult CMs, which rely on oxidative phosphorylation (Lopaschuk et al., 2010; Rana et al., 2012). A number of studies have presented methods ranging from altering culture media to biophysical parameters to improve maturation of hPSC-CMs (Ribeiro et al., 2015; Yang et al., 2014; Nunes et al., 2013) and a few of these are summarized in Figure 2. Despite these advances, maturity of hPSC-CMs is not at par with their adult counterparts and additional strategies are required to address this issue.

The promise of tissue engineering

Efficient derivation of specific cardiac subtypes in vitro will give us the opportunity to mimic microenvironment of the heart with the help of tissue engineering technologies. Moreover, a number of pharmaceutical compounds are precluded from clinical use due to unwanted side effects and potential risk of proarrhythmia on the non-target CM subtype. Therefore, assembling various CM subtypes in vitro much like the cardiac tissue in vivo would be beneficial to assess drug efficacy/safety testing on various subtypes as well as modeling patient-specific diseases.

In the last decade, a variety of biomaterial scaffolds and substrates have been employed to pattern engineered heart tissue (EHT) from a variety of cell types. Most widely studied constructs for seeding CMs have used a cell-hydrogel system with collagen/matrigel (Zimmermann et al., 2002; Zimmermann et al., 2006) or fabricated scaffolds using biodegradable elastomers such as poly glycerol sebacate (Radisic et al., 2006). The advantage of this system is the possibility to miniaturize and automate the EHT construction process making it an attractive tool for applications in in vitro screening assays. A number of studies have also reported the design of vascularized EHT constructs from hPSC-CMs (Caspi et al., 2007b; Stevens et al., 2009; Tulloch et al., 2011). Moreover, a recent report suggests that hPSC-CMs in an EHT display improved sarcomeric organization (Schaaf et al., 2011) and chronic electrical stimulation further improved their maturity and function (Hirt et al., 2014).

Another strategy to create EHT in vitro used decellularized whole hearts while leaving the framework for vasculature intact to enable perfusion (Ott et al., 2008; Lu et al., 2013). Proof of principle that this method can be successful for making bioengineered hearts was demonstrated in decellularized rat hearts and recellularizing it with neonatal rat CMs (Ott et al., 2008). More recently, multipotent KDR<sup>low</sup>/C-kit<sup>neg</sup> cardiovascular progenitors from hiPSCs were injected into decellularized mouse hearts (Lu et al., 2013). These hearts exhibited spontaneous contractions 20 days after perfusion and an electrocardiogram could be recorded, albeit an irregular wave pattern indicating the lack of synchronous conduction. Interestingly, this study noted an effect of extracellular matrix on CM commitment. The CMs populating the atria expressed higher levels of MLC2A, SLN and lower levels of IRX4 compared to CMs that populated the ventricles (Lu et al., 2013). Although this approach would be favorable for the implantation of appropriate cardiac subtypes due to preserved chamber geometry, infusing
decellularized heart matrix with hPSC-derived atrial or ventricular CMs at the desired location and maintaining their viability maybe challenging. Also, this method would be less suitable for application in automated preclinical screenings. Nonetheless, it maybe well suited for modeling the pressure-volume relationship of the heart, studying multicellular phenotypes in cardiac disease and overall physiological function in an \textit{in vitro} setting.

![Figure 3. Strategies to build a heart in vitro.](image)

Furthermore, generating 3D organoids from hPSCs is now an exciting new area of research and protocols for derivation of cerebral, gastric, intestinal, kidney and liver organoids have been reported (Willyard 2015). Generation of organoids from hPSCs was accomplished in a 3D culture system through a stage-specific differentiation protocol recapitulating endogenous developmental processes. The resulting 3D tissue has been reported to contain self-organizing clusters of multiple cell types similar to \textit{in vivo} organs. Designing methods to generate organoids encompassing various cardiac subtypes along with smooth muscle and endothelial cells would enable understanding of multicellular interactions. Since hPSCs cultured as EBs with appropriate growth factors generate a mixture of atrial, ventricular or nodal cells, it seems feasible to generate heart organoids in 3D aggregates. However, a key step to achieve this goal would be that of producing subtype specific progenitors and their spatial organization in 3D format to effectively yield organoids with ratios of atrial, ventricular and nodal cells in a manner similar to native cardiac tissue.

Current approaches to engineer heart tissue are summarized in Figure 3 and the idea of fabricating mini organs in a dish is extremely valuable for studying cardiac physiology in its totality.

Towards regenerative medicine

The mammalian heart has limited ability to replenish damaged or necrotic cells following injury and transplantation of CMs generated \textit{in vitro} seems like an attractive option for cardiac repair. Although posed with many challenges, transplantation of hPSC-CMs into damaged hearts of small animal models has demonstrated that these cells graft with the host
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myocardium and may improve function, even if temporarily (Caspi et al., 2007a; van Laake et al., 2007; Laflamme et al., 2007). Human EHT constructs described above have also been transplanted into infarcted rodent myocardium, which showed long-term survival and grafting but strikingly did not lead to any functional improvement (Riegler et al., 2015). Another study performed in monkeys with induced ischemia-reperfusion injury injected 1 billion hESC-CMs to the infarcted myocardium and found that hESC-CMs remuscularized significant areas of damaged myocardium (Chong et al., 2014). Despite this encouraging finding, this study also reported the occurrence of arrhythmia post injection of hESC-CMs even though it was not life threatening. These studies reiterate the necessity for improved protocols to enable application of hPSC-CMs for regenerative therapies. Efficient cardiac subtype production and purification along with maturity as mentioned before is especially important for realizing the application of hPSC-CMs for heart regeneration. Ventricular CMs are needed for delivery into an infarcted myocardium and presence of any pacemaker cells in this preparation may trigger arrhythmias. Similarly, purified patches of SAN-like CMs would be relevant for developing a biological pacemaker for the treatment of rhythm disorders.

In other recent developments, the prospect of using hPSC-derived cardiovascular progenitors (CVPs) for cardiac repair is being tested. One study has demonstrated a favorable effect of transplanting hESC-derived CVPs (Menasche et al., 2015a) into a patient with severe heart failure (Menasche et al., 2015b). SSEA1+ hESC-CVPs also expressing ISL1 (Menasche et al., 2015a) were embedded in a fibrin scaffold and delivered surgically to the infarcted area of the patient. An improvement in left ventricular ejection fraction was noted at 3 months and the beneficial effect was sustained at 6 months post transplantation. Interestingly, there were no arrhythmias, tumor formation or immune rejection in the patient. Despite this encouraging finding, randomized controlled trials/long-term follow up and mechanisms leading to improved function need to be evaluated before concluding the effectiveness of this therapy. In a similar approach, multipotent hESC-CVPs co-expressing KDR and PDGFRα were transplanted into infarcted rodent myocardium (Fernandes et al., 2015). Subsequent analysis of the graft suggested that KDR+/PDGFRα+ CVPs differentiated primarily to CMs in vivo and an improvement in systolic function was observed comparable to infarcted hearts transplanted with hESC-CMs. Although the differentiation potential of KDR+ mesodermal CVPs in vitro to CM as well as EC lineages has been demonstrated earlier (Yang et al., 2008), transplantation of these CVPs did not result in improved vascularization of the graft compared to hESC-CMs. Results from this study suggest that transplantation of hESC-CVPs is not particularly advantageous compared with hESC-CMs.

It is clear that many hurdles need to be overcome before large-scale clinical trials can be initiated to evaluate the use of hPSC-CMs for cardiac repair. Nonetheless, these cells hold great promise due to their unlimited supply and resemblance to human CMs. However, efficient cardiac subtype production and purification along with maturity as mentioned before is especially important for realizing the application of hPSC-CMs for heart regeneration. Ventricular CMs are needed for delivery into an infarcted myocardium and presence of any pacemaker cells in this preparation may trigger arrhythmias. Similarly, purified patches of SAN-like CMs would be relevant for developing a biological pacemaker for the treatment of rhythm disorders. Moreover, engineered grafts containing hPSC-derived atrial or ventricular-like CMs could be tested for treatment of congenital heart conditions such as septal defects.
Concluding remarks

Recent advances in stem cell biology have enhanced our understanding of cardiac differentiation and disease. But now, it is time to put effort into translating these models for preclinical and possible clinical applications. One of the parameters to attain this goal is improving current culture conditions and protocols to yield mature cardiac subtype populations. This, along with current state-of the art knowhow such as bio and nano material technologies, microfluidics and 3D bioprinting will enable the development of organ-on-a-chip model to mimic physiology and organization of human tissues in vitro. Such a multidisciplinary approach integrating biology with technology to build the human heart in a dish will pave way for advance disease modeling and drug screening ultimately leading to personalized medicine.

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