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Developmental paradigm

A precisely orchestrated interplay of genetic and epigenetic factors guides cardiogenesis. The heart, which starts out as a simple peristaltic tube, is remodeled to a complex four-chambered organ during embryonic development. In chapter 2, we presented a brief overview of cardiac lineage specification.

Retinoic acid (RA) signaling regulates the formation of posterior heart structures including the atria and sinus venosus (Niederreither et al., 2001; Hochgreb et al., 2003). In chapter 3, we showed that, in differentiating hESCs, addition of RA after mesoderm specification results in CMs with an atrial-like identity. We also noted an increase in expression of COUP-TF transcription factors following treatment with RA. COUP-TFII is expressed in the atria but not ventricles of the heart (Pereira et al., 1999; Wu et al., 2013; Devalla et al., 2015) and our data implies an upstream role for RA in initiating its expression in differentiating atrial cells. Furthermore, we also showed that COUP-TFI is induced during atrial differentiation and that it is also expressed exclusively in the atrial chambers.

Contractile function of the adult heart is an energy demanding process and it has recently been revealed that this highly oxidative organ mediates global metabolism (Grueter et al., 2012). However, little is known about heart-specific metabolic genes. In chapter 4, we described the preferential expression of Tecrl in the developing mouse heart. TECRL is an enzyme with predicted role in lipid metabolism. Strikingly, a number of genes with enzymatic function in the cell have essential roles during embryonic development (Pederson et al., 2004; Lopez-Sanchez et al., 2010). The expression of Tecrl at E8.5 in the inflow tract of mouse heart coincides with the formation of single contracting heart tube. As looping begins at E9.5, Tecrl begins to be expressed in both atria and ventricles. Its expression persists throughout development as well in the adult heart.

Sequential contraction of atria and ventricles is responsible for blood flow through the heart. The cardiac conduction system (CCS) propagates rhythmic electrical impulses to ensure organized contraction of the cardiac muscle. As shown in chapter 6, CCS in developing human fetal embryos expressed molecular markers such as TBX3 and HCN4, also previously identified in mouse (Hoogaars et al., 2007; Stieber et al., 2003). Transcriptional profiling of additional genes such as ISL1 and TBX18 indicated comparable findings between mouse and human CCS. This suggested evolutionary conservation of molecular mechanisms involved in formation of the CCS in humans. This work also serves as a reference for gene expression in the human CCS.

Translational impact

In chapter 3, we established that hESC-atrial CMs are a robust model for atrial-selective pharmacology, which has major implications for drug discovery and development to combat atrial fibrillation (AF). AF is the most common sustained arrhythmia and it imposes a huge socioeconomic burden worldwide. Existing antiarrhythmic drugs for the treatment of AF carry the risk of causing ventricular proarrhythmia or negative inotropy. This highlights the need for developing atrial-selective drugs that promise safety and efficacy. However, current preclinical screening assays to identify atrial-specific drugs use non-cardiac cell lines or animal models,
both of which have limitations in predicting the drug responses on the human heart. Our research uncovered a role for COUP-TFI and COUP-TFII in hESC-atrial CMs, which warrants further investigation into the role of these transcription factors in cardiac development and disease. We tested the effect of Vernakalant, a recently approved drug for the treatment of AF in Europe and noted that effects in hESC-atrial CMs were comparable to those observed in native human CMs in sinus rhythm. We also showed that hESC-atrial CMs predict atrial-selectivity of novel ion channel blockers, XEN-D0101 and XEN-R0703. Through this study, we addressed the impending need for a preclinical screening model resembling the physiology of a human atrial CM.

In chapter 4, we demonstrated that a recessive splice-site mutation in TECRL is associated with catecholaminergic polymorphic ventricular tachycardia (CPVT). This disease is characterized by adrenergically induced arrhythmias. Strikingly, a substantial proportion of individuals with CPVT, do not have an identified mutation. Exome sequencing of affected children in a consanguineous family allowed us to identify TECRL as the candidate gene responsible for the disease phenotype. Genetic testing of TECRL in families clinically diagnosed with CPVT and where RYR2 or CASQ2 screening is negative could reveal the prevalence of this mutation. Using patient-specific hiPSC-CMs, we showed altered calcium-handling properties in TECRL mutant CMs that were partly rescued by treatment with Flecainide. Flecainide has been shown to prevent catecholamine-induced arrhythmias in CPVT patients with RYR2 or CASQ2 mutations. Based on the response observed in hiPSC-CMs, Flecainide may also be effective in patients with TECRL mutations. However, since Flecainide did not entirely stop delayed after depolarizations (DADs) in mutant CMs, additional therapeutic strategies maybe needed to restrain arrhythmias in patients with TECRL mutations.

In chapter 5, we presented evidence for altered mitochondrial function in TECRL mutant CMs. Dysfunctional mitochondria have been linked to ventricular arrhythmias and sudden cardiac death in a guinea pig model of ischemia/reperfusion (Brown et al, 2010). Interestingly, similar mechanisms have been observed in various neurological disorders like amyotrophic lateral sclerosis (ALS), Alzheimer’s and Parkinson’s diseases. For example, In ALS with mutations in SOD1, decreased mitochondrial function, activation of unfolded protein response (UPR) and membrane hyperexcitability was observed in hiPSC-derived motor neurons (Kiskinis et al, 2014; Wainger et al, 2014). Further studies are needed to investigate how altered metabolism impacts calcium handling in TECRL mutant CMs. Rescuing mitochondrial dysfunction may preclude arrhythmias in CPVT associated with TECRL mutations. Most importantly, the work in chapters 4 and 5 demonstrates that hiPSC technology is valuable for modeling rare variants of unknown significance in order to evaluate the molecular and functional features of the disease phenotype in vitro.
Future directions

**Genome editing**

The introduction of CRISPR (clustered regularly interspaced short palindromic repeat)-Cas9 (CRISPR-associated nuclease 9) technology (Sander & Joung, 2014) has made it possible to perform targeted genome editing in hPSCs with unprecedented efficiency (Musunuru, 2013). Introduction/correction of mutations or gene knockouts can be done efficiently and with high specificity to the intended locus (Merkle et al., 2015). Although there are still some caveats with Crispr/Cas9 technology due to off-target effects, this technology has nevertheless dramatically altered the way we study gene function and model complex genetic disorders in stem cells. Using CRISPR/Cas9 system, sequence variants identified in genome-wide association studies (GWAS) can be introduced to test their implications for AF. *In vitro* models of familial AF can also be established by generating hiPSCs from patients harboring mutations in ion channel genes such as *KCNA5* (Olson et al., 2006). As described in chapter 3, atrial-like CMs can be generated from genetically modified or patient-specific hiPSC lines to study their phenotype. In addition, CRISPR/Cas9 will be valuable for genetic correction of TECRLc.331+1G>A mutation described in chapters 4 and 5. Using this approach, pathways relevant to TECRL function can be further defined and may lead to identification of druggable targets.

**Target/Drug discovery**

hPSC-derived CMs can be a powerful tool for drug discovery as they represent a physiologically-relevant model of a human CM. Numerous studies have shown the value of hPSC-derived CMs for safety pharmacology (Braam et al., 2010; Braam et al., 2013; Navarrete et al., 2013). This is particularly relevant as United States food and drug administration (US FDA) and European medicines agency (EMA) support a comprehensive in vitro proarrhythmia assay (CiPA) initiative from 2015, which proposes that cardiotoxicity profiling should include testing pharmaceutical compounds on human PSC-derived CMs (Cavero & Holzgrefe, 2014). Furthermore, hiPSC lines recapitulating long QT syndrome, hypertrophic cardiomyopathy, and dilated cardiomyopathy were used for high throughput drug screenings which revealed disease-specific patterns of cardio-toxicity (Liang et al., 2013). In chapter 3, we showed that hESC-atrial CMs respond to drugs targeting atrial-selective ion channels. Generating specific cardiac subtypes would be crucial for integrating hPSC-CMs at earlier stages of drug discovery with an aim to reduce drug attrition in late stages of development. Moreover, pharmacological testing of patient-specific hiPSC-CMs can be useful to reveal effectiveness of currently used drugs in newly identified genetic variants causing previously known disease phenotypes. In chapter 4, we studied members of a family who presented clinical symptoms of CPVT. Through DNA sequencing, a homozygous TECRLc.331+1G>A mutation was identified in affected family members. Single CMs derived from hiPSCs of symptomatic patient recapitulated abnormal electrophysiological properties observed in CPVT and application of flecainide decreased DADs. Technical advances in generating virus-free, non-integrating methods to reprogram hiPSCs in a short amount of time have now set the stage for developing therapies matching to an individual patient.
**Tissue engineering**

To reliably model complex diseases *in vitro*, it is also important to mimic cell-cell interactions. In AF, for example, fibroblast-CM interactions are believed to promote reentry and formation of ectopic impulses (Yue *et al.*, 2011). A prerequisite to achieve this is to efficiently generate various cardiac cell types and assemble them in a conformation/ratio in a manner similar to the human heart. Approaches integrating biophysical techniques now make it possible to engineer three-dimensional (3D) structures of living cells to facilitate construction of functional organs. One such technology that has revolutionized the field of medicine is 3D printing. It has already been successfully used for the production of medical devices such as implants and prostheses (Murphy & Atala, 2014). Recent advances have enabled the application of this technique for printing living tissue (termed 3D bioprinting). hPSC-CMs can be layered to reproduce 3D architecture of the heart and employing microfluidics can simulate blood flow in the tissue. The idea of fabricating mini organs in a dish is extremely valuable for studying cardiac physiology in its totality (Bhatia & Ingber, 2014).

**Cardiac regeneration**

Lastly, efforts are ongoing to test the beneficial effects of hPSC-CMs for regenerating damaged hearts. So far, only modest beneficial effects have been observed in small animal models (Caspi *et al.*, 2007; Laflamme *et al.*, 2007; van Laake *et al.*, 2007). To realize the use of hPSC-CMs for clinical use, a number of challenges have to be overcome. Foremost among those, is improving the maturity of hPSC-CMs. Structural, metabolic and electrophysiological properties of these CMs resemble human fetal CMs and generate weaker contractile forces (Ribeiro *et al.*, 2015, Birket *et al.*, 2015b). Moreover, spontaneously depolarizing immature CMs pose proarrhythmic risk when transplanted into native hearts (Chong *et al.*, 2014). Once an effective solution for improving maturity is identified, it will be crucial to produce homogeneous ventricular cells under current good manufacturing practice (cGMP) guidelines. Interestingly, a recent study has demonstrated a favorable effect of transplanting hESC-derived "committed cardiac progenitors" into a patient with severe heart failure (Menasche *et al.*, 2015b). ISL1+/SSEA+ hESC-derived cardiac progenitors (Menasche *et al.*, 2015a) were embedded in a fibrin scaffold and delivered surgically to the infarcted area of the patient. Although the study reported no arrhythmias, tumor formation or immune rejection in the patient, it is to be noted that the follow-up time in this case was only 3 months. There was also an improvement in left ventricular ejection fraction and despite the encouraging results from this clinical case report, additional criterion such as long-term follow-up and mechanisms leading to improved function need to evaluated before concluding the effectiveness of this therapy. It is certainly a long way before large-scale clinical trials can begin for testing the use of hPSC-CMs for cardiac repair but these cells hold great promise due to their unlimited supply and resemblance to a human CM.
References


