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Cardiomyocyte maturation; It takes a village to raise the kid...

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Cardiovascular disease continues to be the leading cause of morbidity and mortality in the United States and the costs associated with the management of these diseases exceeds 300 billion dollars [1]. The most common form of heart disease, coronary heart disease, results from a narrowing of the blood vessels due to the buildup of plaque in the arteries. Over time, coronary heart disease weakens the heart muscle and can lead to heart failure; a condition where the heart muscle can no longer generate enough force to efficiently deliver blood to the body. Despite recent advances in medical and device therapies, there are no effective treatment strategies for heart failure. Although the death rates of heart disease have declined in the last decade, the disease burden remains high [1]. The risk of cardiovascular disease also increases with age due to diminished cardiac function associated with age-related morphological and structural changes and loss of cardiomyocytes [2]. Treatment modalities have been hampered by the fact that the maintenance and repair mechanisms of the heart are limited. Thus, novel approaches and techniques to improve or restore cardiac function must be developed, including the development of cardiomyocyte (CM) models for in vitro disease modeling, drug screening, cell-based therapies, and toxicity studies. CM toxicity models are critically important because cardiotoxicity is a primary reason drugs fail pre-clinical studies or clinical trials and accounts for the majority of drug recalls [3].

Cardiac muscle is composed of interconnected cardiomyocytes (CM) layered in an end-to-end pattern forming bundles, termed myofibers, which are adjacent to interstitial fibroblasts, blood vessels and extracellular matrix components [4]. CM are a highly specialized, force-generating, rod-shaped cell type with characteristic striations. CM striations are the result of bundles of myofibrils divided into contractile units, or sarcomeres, comprised of several contractile proteins including actin (thin myofilament) and myosin (thick myofilament) [4]. Sarcomeres are flanked by Z disks, which form a transverse sarcomeric scaffold to efficiently propagate force transmission [5]. CM are constantly subjected to physical stretching as a result of the contractile activity of the heart and contain a large number of mitochondria due to high levels of physiological activity [6]. The majority of CM are

terminally differentiated due to irreversible cell cycle arrest [7]. In humans, postnatal CM growth is primarily hyperplastic for the first few months after birth as the heart continues to develop. After this period, CM exit the cell cycle, transition to hypertrophic growth, and terminally differentiate, all of which coincides with CM binucleation [8].

CM organization is a critical factor for the coordination of cardiac contractile function. CM attach to one another through specialized portions of the membrane termed intercalated discs [5]. Intercalated discs contain a large number of gap junctions that facilitate the passage of ions between cardiomyocytes, which is essential for propagation of the action potential [9]. Excitation-Contraction Coupling (ECC) refers to the mechanism through which the cardiac action potential initiates contraction of the cardiomyocyte [10]. Membrane depolarization induces a large influx of calcium ions, resulting in an intracellular spike of calcium that initiates the coordinated shortening of the actin-myosin fibrils to produce the CM contraction [9]. The rapid depolarization of the membrane potential is transduced to the interior of the cell through T-tubules, which release calcium ions stored within the sarcoplasmic reticulum [11]. T-tubule networks result from invaginations of the sarcolemma that occur at the Z line at the end of each sarcomere and serve to ensure that the entire cell depolarizes rapidly and homogeneously [11]. CM modulate their contractile output in response to a wide variety of stimuli by altering ion currents, calcium handling and myofilament properties [9].

The limited regenerative and repair capacity of adult cardiomyocytes (CMs) in vivo is a major obstacle in the utilization of primary adult CM for a variety of clinical applications. Although adult cardiac stem cells as a replenishing pool to generate adult CM has been described, the true nature of these cells is very controversial and has been put in question recently. However, pluripotent stem cells and their potential to produce CM have been studied for decades and the field has developed a reliable scientific foundation based on developmental principles. It all began with the first generation of human pluripotent stem cells (hPSCs) and human embryonic stem cells (hESCs) by a group led by James Thomson in 1998 [12, 13]. At that point in time, murine embryonic stem cell culture and differentiation protocols had been extensively studied. Thus, it took only three years until the first human CM were generated through a protocol that was similar to protocols developed in mice [14]. The main principle of this approach is the generation of an embryoid body (EB), a complex mixture of different cell types that also contains CM when treated appropriately. However, research on these cells related to function and maturation was hampered by the inability to directly observe and study CM in the complex three-dimensional structure of EBs. Further development in protocols and cell-culture engineering led to the development of monolayer differentiation protocols for hPSC-derived CM [15]. About the same time, Yamanaka's and Thomson's groups reported the generation of hPSC from somatic cells [16, 17]. These landmark discoveries bypassed the major ethical obstacle related to the use of hESCs and ushered in the era of 'personalized' medicine, allowing for the generation of patient-specific hPSC-derived cell products for drug / toxicology screens, in vitro disease modeling and cell therapies.

The generation of hPSCs-derived CM closely resembles the developmental program with, of course, certain limitations, including the lack of complex embryonic structure formation and

associated interactions. The timing and incubation conditions of cytokines/growth factors in hPSCs cell culture used to initiate cellular fate decision are constantly evolving. In vivo cardiomyogenesis is initiated through Nodal-dependent formation of the mesoderm [18]. Subsequent BMP/FGF signaling maintains mesoderm and activates Wnt signaling which induces the mesoendodermal transcription factors Brachyury and Eomes. Both induce the master regulator for early cardiac progenitor cells, MESP1 [19]. The activation of MESP1 shuts down Wnt signaling and activates a complex signaling cascade that directs the multilineage cardiac progenitor cells, which contributes to cardiac endothelial and vascular smooth muscle cells, to lineage restricted immature CMs.

These developmental principles have been utilized to develop differentiation protocols for hPSC-derived CMs. Over the past 10 years, stage-specific FGF/BMP/Wnt -dependent activation/inhibition of developmental signaling pathways resulted in reliable generation immature CMs. The generation of human cardiac progenitor cells was reported by Keller's group that used a combination of EB, monolayer cultures, and staged activation with Activin A/BMP/FGF and subsequent inhibition of Wnt through DKK1 [20]. The precise movement through the different stages and cell-fate checkpoints are monitored through cell surface markers as KDR and PDGFR- α that allows for the monitoring and optimization for the desired outcome[21]. These adapted protocols were further modified to generate functional CMs under complete defined and growth factor free conditions. These modifications allowed for large, industrial quality production of CMs that are critically required for all the different applications related to patient care [22]. All we need are hPSC-derived CM that resemble the ones found in an adult human heart, but are we there yet?

hPSC-CM phenotypically resemble fetal CM in structure, proliferative capacity, metabolism and electrophysiology [23]. hPSC-CM vary in maturity and phenotypically change over time in culture. The patterning of myofibrillar formation differs in adult CM compared to hPSC-CM [24]. Adult CM are not readily obtained and do not survive very long in vitro whereas hPSC-CM can be cultured for long-periods of time while maintaining viability and contractile properties [25]. The differences in survival time make it difficult to compare the effects of damaging agents on adult CM and hPSC-CM [26]. In general, hPSC-CM display characteristic early-phase phenotypic markers, including a small, rounded morphology, fetal CM electrophysiological output, and ability to proliferate until a month after cellular contraction begins [23]. Thereafter, hPSC-CM can acquire more mature, late-phase phenotypic markers, including development of an oblong morphology, adult CM electrophysiological output and loss of proliferative ability [23]. Although the hPSC-CM differentiate into a more mature phenotype, they are not identical to adult CM. Gene expression profiling of hPSC-CM and adult CM showed differences contractile and cytoskeletal genes [23]. The majority of adult CM are bi- or multinucleated, whereas hPSC-CM contain a single nucleus, resembling embryonic CM [27]. Mature adult CM rely on oxidative metabolism (fatty acid β -oxidation) for ATP synthesis whereas hPSC-CM rely on glycolysis [28]. hPSC-CM and adult CM have opposing force-frequency relationships in response to increased stimulation frequency that is dependent on the availability of calcium and electrical coordination across the cell. Adult CM display a positive force-frequency relationship which is associated with an increase in contraction amplitude and intracellular calcium transients [24]. hPSC-CM show a negative force-frequency relationship in response

to increased stimulation frequency, presumably due to the diminished calcium handling properties of these cells [24]. It is important to consider that cardiomyocyte subtypes (e.g., ventricular-, atrial-, and nodal type) exist within the heart that differentially affects cellular electrophysiology [29]. Thus, future studies characterizing the cardiac lineage specification, differentiation, and maturation of hPSC-CM need to be conducted to determine the most suitable cells for in vivo transplantation.

In a landmark study, Chong et al [30] found that human stem cells have the capacity to regenerate a monkey heart. Prior to this study, it was not known whether transplantation of human embryonic stem cell-derived CM (hESC-CM) would engraft, functionally integrate, and repair the host myocardium after ischemic injury in a clinically relevant large-animal model [30]. The team, led by Dr. Charles Murry, a pioneer in the field of regenerative cardiovascular stem cell therapy, provided 'proof of concept' data demonstrating that hESC-CM injected hearts remuscularized the infarcted areas and, importantly, electrically coupled to the primate heart [30]. However, non-fatal ventricular arrhythmias were observed in hESC-CM-engrafted primates, which the authors argued could be attributed to the electrically immature hESC-CM.

Emerging data suggest that the immature state of hPSC-CM is a primary factor limiting their clinical application. It is still unclear if hPSC-CM have the capacity to adopt a fully mature, adult-like CM phenotype, such as the structural and contractile properties. Factors that have been shown to influence hPSC-CM maturation include prolonged cell culture, substrate stiffness, patterned substrates, and electrical stimulation [23]. It is well established that the thyroid hormones, including triiodothyronine (T3), have pleiotropic effects on cardiac development and adult cardiac function, including effects on cardiac contractility, energy metabolism and mitochondrial remodeling [31]. T3 has also been shown to induce the maturation of in vivo fetal sheep cardiomyocytes [32], cultured neonatal mouse and rat CM [33], and mouse ES-derived CM [34]. T3 suppressed fetal gene expression in neonatal CM and contributed to normal cardiac maturation [35].

Previous studies on CM maturation have relied primarily on electrophysiological output and less on the structural and functional capability of the cells. A recent study Dr. Murry's team [36] examined the effect of the T3 on the maturation of hPSC-CM using an extensive array of morphological, molecular, and functional parameters. Treatment of hPSC-CM with T3 for one week increased hPSC-CM cell size, anisotropy (geometrically aligned), and sarcomere length with no concomitant increase in cell number. Gene expression profiling of control and T3 treated cells showed that T3 increased α -MHC and sarcoendoplasmic reticulum ATPase (SERCA2a), decreased β -MHC, and did not alter the ratio of N2BA/N2B titin isoform expression. To determine the contractile properties of the CM, elastomeric micropost arrays data, which decouples substrate rigidity from adhesive and surface properties [37], showed that T3 decreased the proliferative activity of hPSC-CM by 50%. In addition, the decreased proliferative activity corresponded with an increase in the mRNA and protein expression levels of the cell cycle inhibitor p21. There was no difference in the percent of binucleated cells, a mature phenotype, between control and T3 treated hPSC-CM. T3 treated hPSC-CM improved contractile force generation, exhibited faster calcium transients, and increased maximal mitochondrial respiration capacity compared to untreated

controls. Although these data showed that T3 treatment induced maturation of hPSC-CM, the cells do not completely recapitulate the adult CM phenotype. The authors acknowledge the limitations of using a single agent to induce complex characteristics and suggest that a combinatorial approach of factors known to induce maturation, including increased culture time, may be more effective.

More detailed studies on the maturation of hPSC-CM should include measurements of the electrophysiological, contractile, and calcium-transient outputs to assess structural and functional performance over time [23]. The remaining challenges of hPSC-CM models is how to induce these cells to phenotypically and functionally mimic adult CM and extensive analysis of their molecular and functional properties must be performed to determine their compatibility with the host myocardium, especially with regard to intracellular calcium handling. Translational applications of the mature hPSC-CM would ideally match the electrical and mechanical properties of the myocardium, minimizing the risk of arrhythmia while increasing functional performance [36]. The field of regenerative medicine is rapidly evolving and the findings by Dr. Murry's group underscore the potential of cell-based therapies for drug and toxicity screens, human disease modeling, and therapeutic treatments for cardiovascular disease. Perhaps the promise of cell-based therapies is not so far off after all.

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