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Monitoring Human-Induced Pluripotent Stem Cell-Derived Cardiomyocytes with Genetically Encoded Calcium and Voltage Fluorescent Reporters

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SUMMARY

The advent of the human-induced pluripotent stem cell (hiPSC) technology has transformed biomedical research, providing new tools for human disease modeling, drug development, and regenerative medicine. To fulfill its unique potential in the cardiovascular field, efficient methods should be developed for high-resolution, large-scale, long-term, and serial functional cellular phenotyping of hiPSC-derived cardiomyocytes (hiPSC-CMs). To achieve this goal, we combined the hiPSC technology with genetically encoded voltage (ArcLight) and calcium (GCaMP5G) fluorescent indicators. Expression of ArcLight and GCaMP5G in hiPSC-CMs permitted to reliably follow changes in transmembrane potential and intracellular calcium levels, respectively. This allowed monitoring short- and long-term changes in action-potential and calcium-handling properties and the development of arrhythmias in response to several pharmaceutical agents and in hiPSC-CMs derived from patients with different inherited arrhythmogenic syndromes. Combining genetically encoded fluorescent reporters with hiPSC-CMs may bring a unique value to the study of inherited disorders, developmental biology, and drug development and testing.

INTRODUCTION

The ability to reprogram adult somatic cells into pluripotent stem cells by a set of transcription factors has revolutionized biomedical research (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). The generated human-induced pluripotent stem cells (hiPSCs) can be coaxed to differentiate into a variety of cell lineages (including cardiomyocytes [Zhang et al., 2009; Zwi et al., 2009]) that can then be utilized for the development of autologous cell-replacement therapies, disease modeling, and drug discovery (Robinton and Daley, 2012).

In the cardiac field, hiPSC lines were established from healthy individuals (Zhang et al., 2009; Zwi et al., 2009) and from patients inflicted with acquired (heart failure) (Zwi-Dantsis et al., 2013) and inherited cardiac disorders. Among the latter, patient-specific hiPSC-derived cardiomyocytes (hiPSC-CMs) models of different inherited arrhythmogenic syndromes (Bellin et al., 2013; Caspi et al., 2013; Itzhaki et al., 2011a, 2012; Jung et al., 2012; Moretti et al., 2010) and diverse cardiomyopathies (Lan et al., 2013; Sun et al., 2012) were established. The patient/disease-specific hiPSC-CMs were shown to recapitulate the disease phenotypes in culture, to provide mechanistic insights into disease processes, and to evaluate existing and novel therapies. Similarly, hiPSC-CMs were also proposed as a valuable tool for drug development (Mercola et al., 2013), demonstrating, for example, their value for safety pharmacology by screening the proarrhythmic effects of certain compounds (Braam et al., 2013; Liang et al., 2013; Zwi et al., 2009).

One of the key prerequisites for achieving the goals of these applications is to develop efficient tools to study the functional properties of the hiPSC-CMs and specifically of their electrophysiological and excitation-contraction-coupling properties. To this end, different electrophysiological techniques (patch-clamp (Itzhaki et al., 2011a) and multielectrode extracellular potential recordings [Zwi et al., 2009]) and imaging modalities (using voltage- or calcium-sensitive fluorescent dyes) were utilized. While providing valuable information, these methodologies also display inherent limitations, such as relatively low-throughput (patch-clamp), limited electrophysiological information (extracellular recordings), phototoxicity (voltage and calcium sensitive dyes), and inability to obtain long-term repeated recordings (patch-clamp, fluorescent dyes). Consequentially, a method that allows long-term, serial, and cellular functional phenotyping of healthy and diseased hiPSC-CMs is direly needed, especially if it can be achieved in a non-invasive, high-resolution, and large-scale manner.

The developments in the field of genetically encoded fluorescent indicators may provide a possible solution to the aforementioned challenges. Genetically encoded indicators are composed of a sensing element, which is usually fused to an autofluorescent protein (like circularly permuted enhanced GFP; cpEGFP) that alters its fluorescent intensity as a result of conformational changes in

the sensing element. While utilized in numerous neuroscience-related experimental models (Akemann et al., 2010; Cao et al., 2013; Grienberger and Konnerth, 2012; Looger and Griesbeck, 2012; Tian et al., 2009), the use of similar indicators in non-neuronal tissues, such as the heart, has been more limited (Addis et al., 2013; Chong et al., 2014; Kaestner et al., 2014; Leyton-Mange et al., 2014). Here, we aimed to transfer these emerging technologies to the cardiac field, specifically focusing on genetically encoded calcium indicators (GECIs) (Grienberger and Konnerth, 2012; Kaestner et al., 2014; Tian et al., 2009) and genetically encoded voltage indicators (GEVIs) (Jin et al., 2012; Kralj et al., 2012; Leyton-Mange et al., 2014), in an attempt to establish experimental platforms to monitor the functional activity of hiPSC-CMs. To this end, we aimed to express GCaMP5G (Addis et al., 2013; Tian et al., 2009), a GECI that displays improved dynamic range, improved sensitivity, and maintains relatively stable expression levels, and ArcLight A242 (Cao et al., 2013; Jin et al., 2012; Leyton-Mange et al., 2014), a new variant of the Ciona intestinalis voltage-sensitive (CiVS)-based fluorescent protein voltage sensor (Barnett et al., 2012; Murata et al., 2005) super-family, in both healthy and diseased hiPSC-CMs.

RESULTS

Expression of ArcLight in hiPSC-CMs

Dermal fibroblasts from a healthy individual were reprogrammed to generate hiPSCs by retroviral delivery of *SOX-2*, *KLF-4*, and *OCT4*. The generated hiPSC colonies displayed characteristic morphology, expressed the pluripotency markers NANOG, SSEA4, OCT-4, and TRA-1-60 (Figure S1A), and maintained a normal karyotype (Figure S1B). Pluripotency of the hiPSCs was verified by the presence of cell derivatives of all three germ layers in differentiating EBs (Figure S1C) and by formation of teratomas in SCID-beige mice (Figure S1D). Finally, hiPSCs showed silencing of the three retroviral transgenes (Figure S1E) and reactivation of endogenous pluripotency genes *NANOG*, *SOX2*, and *OCT4* (Figure S1F).

Cardiomyocyte differentiation of the hiPSCs was achieved using a modification of the monolayer-based directed differentiation system (Wang et al., 2014). This resulted in efficient differentiation with the percentage of cardiac troponin I (cTnI)-positive cells, ranging from 80% to 95% in the different hiPSC lines used. Gene expression analysis revealed the expression of cardiac-specific genes (*NKX2.5*, *MLC-2V*, *MYH-6*, and *MYH-7*) by the hiPSC-CMs and the downregulation of pluripotent genes (Figure S1G). Immunostaining studies showed positive staining for sarcomeric α-actinin and cTnI (Figure S1H).

The hiPSC-CMs were dispersed into single cells by enzymatic dissociation and plated on Matrigel-coated coverslips. Lentiviral transduction was used to deliver the ArcLight transgene to the plated cells, resulting in robust expression of the fluorescent reporter (Figure 1A). Importantly, as shown in Movie S1, the fluorescence levels of the ArcLight-expressing hiPSC-CMs changed during the cardiac cycle, with a reduction in fluorescence intensity concomitant with membrane depolarization (during the action potential), followed by a rise in fluorescence during membrane repolarization and at the resting state.

To quantify the fluorescence changes, we utilized the line-scan mode of the confocal microscope (Figures 1A–1C). The optical signals derived were highly stable, allowing continuous recordings of several optically derived action potentials (APs) from the same cardiomyocyte (Figure 1B) and detailed characterization of each sampled AP (Figure 1C). Interestingly, similar to previous reports using intracellular recordings (Zhang et al., 2009), three types of hiPSC-CMs' optical AP morphologies were noted (ventricular-, atrial-, and nodal-like; Figure 1D), suggesting the presence of different cardiomyocyte subtypes.

Monitoring the Effects of Ion-Channel Modulators

Next, we evaluated the ability of ArcLight to detect changes in the AP morphology of the hiPSC-CMs, focusing on alterations in AP duration (APD) because of the importance of this parameter for modeling inherited arrhythmogenic syndromes (short and long QT syndromes) and for the field of cardiac safety pharmacology. To this end, we applied specific ion-channel modulators designed to either block the slow (I_{Ks}) or fast (I_{Kr}) components of the delayed rectifier potassium current or to augment the late-sodium current (I_{NaL}), simulating alterations in currents most relevant to the congenital long QT syndrome types 1, 2, and 3, respectively.

Application of the I_{Kr} blocker E4031 (500 nM) led to significant prolongation of the optical APD (Figure 1E, top left) in the hiPSC-CMs with APD_{90} (time to 90% repolarization), increasing from 329 ± 22 ms to 571 ± 66 ms ($p < 0.01$, $n = 27$; Figure 1E, right). Interestingly, E4031 was highly arrhythmogenic in many hiPSC-CMs (48%), leading to development of early afterdepolarizations (EADs) and triggered beats (Figure 1E, bottom). Similarly, blocking of I_{Ks} with chromanol-293B (30 μM) also prolonged APD_{90} in the hiPSC-CMs ($p < 0.01$, $n = 29$, Figure 1F). Consistent with the notion that I_{Ks} does not play as an important role in hiPSC-CMs repolarization as I_{Kr}, the arrhythmias induced by chromanol were significantly less frequent with only 3% of the cells displaying EADs. Finally, application of ATX-II (30 nM), known to augment I_{NaL}, also led to marked APD_{90} prolongation ($p < 0.01$, $n = 28$; Figure 1G), which was associated with arrhythmogenic activity in