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IK1-enhanced human-induced pluripotent stem cell-derived cardiomyocytes: an improved cardiomyocyte model to investigate inherited arrhythmia syndromes

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Vaidyanathan R, Markandeya YS, Kamp TJ, Makielski JC, January CT, Eckhardt LL. IK1-enhanced human-induced pluripotent stem cell-derived cardiomyocytes: an improved cardiomyocyte model to investigate inherited arrhythmia syndromes. Am J Physiol Heart Circ Physiol 310: H1611–H1621, 2016. First published April 8, 2016; doi:10.1152/ajpheart.00481.2015.—Currently available induced pluripotent stem cell-derived cardiomyocytes (iPS-CMs) do not ideally model cellular mechanisms of human arrhythmic disease due to a lack of a mature action potential (AP) phenotype. In this study, we create and characterize iPS-CMs with an electrically mature AP induced by potassium inward rectifier (IK1) enhancement. The advantages of IK1-enhanced iPS-CMs include the absence of spontaneous beating, stable resting membrane potentials at approximately –80 mV and capability for electrical pacing. Compared with unenhanced, IK1-enhanced iPS-CMs calcium transient amplitudes were larger (P < 0.05) with a typical staircase pattern. IK1-enhanced iPS-CMs demonstrated a twofold increase in cell size and membrane capacitance and increased DNA synthesis compared with control iPS-CMs (P < 0.05). Furthermore, IK1-enhanced iPS-CMs expressing the F97C-CAV3 long QT9 mutation compared with wild-type CAV3 demonstrated an increase in AP duration and late sodium current. IK1-enhanced iPS-CMs represent a more mature cardiomyocyte model to study arrhythmia mechanisms.

arhythmia; potassium ion channel; LQTS; iPS-CM

NEW & NOTEWORTHY

IK1-enhanced induced pluripotent stem cell-derived cardiomyocytes (iPS-CMs) more closely represent the cell size, structure, multinucleation, electrophysiology, and calcium handling properties of adult human cardiomyocytes. These IK1-enhanced iPS-CMs will permit advanced studies of arrhythmia mechanisms such as rotor and reentry generation in plated iPS-CMs or drug safety testing due to the mature cellular phenotype.

INDUCED PLURIPOTENT STEM (iPS) cells have opened new avenues to investigate many human diseases (33). Patient-specific iPS-derived cardiomyocytes (iPS-CMs) have been used to investigate inherited arrhythmia syndromes such as long QT syndrome (LQTS) and catecholaminergic polymorphic ventricular tachycardia (9, 21, 31, 34). These proof of concept studies have recapitulated some characteristic features of human disease (8, 23). However, despite advances in differentiation and culturing techniques, currently available iPS-CMs have several features of immature cardiomyocytes, which limit their application for modeling cellular arrhythmia mechanisms and inherited arrhythmic disease. The electrophysiological limitations occur predominately due to action potential (AP) diastolic properties including a relatively depolarized resting membrane potential and spontaneous automaticity due to a small inward rectifier (IK1) density and unopposed pacemaker current (Ii) (5, 18). In turn, this results in partial inactivation of ion channels such as the cardiac sodium channel to reduce current (INa) amplitude. Automaticity also interferes with electrical pacing and slow AP rates cannot be reproduced or studied. Additionally, iPS-CMs are small in size (small cell capacitance measurements) and lack a well-developed sarcomere pattern.

In the heart, IK1 contributes to phase 3 repolarization and maintains the resting membrane potential with the dominant IK1 component derived from Kir2.1 protein subunits encoded by the KCNJ2 gene. In addition to these electrophysiological functions, Kir2.1 is pivotal to fetal mouse cardiomyocyte maturation (6) and it is an important component for normal facial muscular/skeletal development and growth (32). Membrane hyperpolarization, induced by Kir2.1, is the key triggering event that initiates skeletal myotube differentiation and maturation (11). For these reasons, we developed IK1-enhanced iPS-CMs to overcome their electrophysiologic immaturity and to test the effect on growth and development of iPS-CMs.

We then used IK1-enhanced iPS-CMs as an expression model to study a non-ion channel mutation in CAV3, which encodes the scaffolding protein caveolin-3 (Cav3) and causes long QT syndrome type 9 (LQT9) (30). We previously reported using a heterologous expression model (HEK293 cells) that CAV3 LQT9 mutations increase late INa (INa-L) without affecting peak INa (INa,P) density and steady-state activation or inactivation (3, 30) and reduced IK1 density by decreasing surface membrane expression of Kir2.1 (28). Other groups have attempted to compensate for the small density of IK1 in iPS-CMs by electronic addition of IK1 or viral infection of Kir2.1 (2, 17), but, to date, augmentation of IK1 has not been systematically studied for its effects on AP characteristics, membrane currents, or cardiomyocyte growth. In the present study we 1) quantify the AP characteristics and effects of pacing; 2) define the percentage of IK1 enhancement; 3) demonstrate calcium transients more similar to adult myocytes; 4) quantify increased cell size, capacitance, and DNA synthesis; and 5) model LQT9 with AP prolongation and production of early afterdepolarizations (EADs).
METHODS

iPS-CMs. iPS-CMs (iCells) were obtained from Cellular Dynamics International (Madison, WI) and handled according to manufacturer specifications. iCells were chosen because they have previously been extensively characterized (18). The cells were cultured for 11–30 days. The iPS-CMs were split 24 h before cellular electrophysiology experiments and plated on 12-mm-precoated coverslips (BD Biosciences, San Jose, CA). Additionally, a second transgene and vector free human iPS-CM line (19-9-11) (33) was used to compare Kir2.1 protein levels.

Infection with adenovirus. iPS-CMs were infected with adenoviral constructs created using the ViraPower Adenoviral Expression System (Invitrogen, Grand Island, NY). Adenoviral constructs of wild-type (WT)-Kir2.1, WT-CAV3, and mutant F97C-CAV3 were created and grown as described previously (3). All adenoviral constructs expressed Internal ribosome entry site enhanced green fluorescent protein (IRES GFP) to identify infected iPS-CMs. GFP adenoviral infection efficiency was close to 100%. In all, five adenoviral infection schemes were studied: 1) iPS-CMs infected with GFP viral control, 2) iPS-CMs infected with Kir2.1 IRES GFP, 3) iPS-CMs infected with Kir2.1 IRES GFP and CAV3 IRES GFP, 4) iPS-CMs infected with Kir2.1 IRES GFP and F97C-CAV3 IRES GFP, and 5) iPS-CMs infected with CAV3 IRES GFP. In experiments, where Kir2.1 + WT-CAV3 or F97C-CAV3 were coexpressed, serial infections were done as follows: 1) iPS-CMs were infected with Kir2.1 IRES GFP adenovirus, and 2) 24 h later the same iPS-CMs were infected again with WT-CAV3 or F97C-CAV3 IRES GFP adenoviral constructs. Twenty-four hours after the second infection the iPS-CMs were split using trypsin (0.25%) onto 12-mm-precoated coverslips for cellular electrophysiology experiments. Experiments were recorded within a window of 2–4 days after splitting.

Cellular electrophysiology. Electrophysiology experiments were done using an Axopatch 200B amplifier and pCLAMP 10 (Molecular Devices, Sunnyvale, CA). APs were recorded under current clamp mode at 32°C. Voltage-clamp data were recorded at room temperature. Borosilicate glass pipettes (3–4 MΩ) were used (Model P-97; Sutter Instruments, Novato, CA). Whole cell capacitance was calculated by integrating the area under the capacitive transient and dividing this value by the step pulse (5 mV). Representative recordings of capacitive transients with protocol are shown in Fig. 1E.

Action potentials. The bath solution contained the following (mmol/l): 148 NaCl, 5.4 KCl, 1 MgCl2, 1.8 CaCl2, 0.4 NaH2PO4, 5.5

Fig. 1. Potassium inward rectifier (Ik1) from Ik1-enhanced induced pluripotent stem cell-derived cardiomyocytes (iPS-CMs). A: representative Ik1 recorded from iPS-CMs in baseline (dark grey), barium (500 μM, light grey), and barium-sensitive current (black). B: average current density from Ik1-enhanced iPS-CMs n = 7. Recordings were done using a ramp protocol shown in inset. C: sample Ik1 current recordings from iPS-CMs baseline (black), barium (dark grey), and barium-sensitive current (light grey). D: sample action potentials from 2 different iPS-CMs showing spontaneous activity and different maximum diastolic potentials. Results from the analysis of spontaneous activity are reported in Table 1. E: protocol used to record capacitance is shown at top and representative capacitance traces from iPS-CMs (black) and Ik1-enhanced iPS-CMs (grey) are shown at bottom.
glucose, and 15 HEPES (pH 7.4, NaOH). The pipette solution contained the following (mM/l): 150 KCl, 2 CaCl$_2$, 5 EGTA, 10 HEPES, and 5 MgATP (pH 7.2, KOH). Myocytes were paced at 0.5, 1, 2, and 3 Hz with a brief depolarizing pulse generated using a programmable digital stimulator (DS5000; WPI, Sarasota, FL). AP amplitude, action potential duration at 10% (APD$_{10}$), 50% (APD$_{50}$), and 90% (APD$_{90}$) of repolarization, and maximum upstroke velocity (dV/dt$_{max}$) were measured (pCLAMP 10; MatLab 6.0, Natick, MA). For each AP from each myocyte studied, pacing was done at each frequency for >100 beats before recording 5 consecutive APs for analysis (N = number of myocytes and n = number of AP).

**Inward rectifier potassium current.** $I_{K1}$ was recorded using bath solution containing the following (mM/l): 148 NaCl, 5.4 KCl, 1.0 MgCl$_2$, 1.8 CaCl$_2$, 0.4 NaH$_2$PO$_4$, 5.5 glucose, and 15 HEPES (pH 7.4, NaOH). Pipette filling solution contained the following (mM/l): 150 K-glucconate, 5 EGTA, 10 HEPES, and 5 MgATP (pH 7.2, KOH). Calcium currents and calcium-sensitive chloride currents were blocked with nifedipine (5 μmol/l) in the bath solution. $I_{K1}$ was recorded using ramp protocol with a velocity of 1.5 mV/s between −120 to +20 mV from a holding potential of −50 mV. BaCl$_2$ (500 μmol/l) was added to block $I_{K1}$ and currents were reported as barium subtracted.

**Sodium current.** Bath solution contained the following (mM/l): 20 NaCl, 1 MgCl$_2$, 1 CaCl$_2$, 0.1 CdCl$_2$, 10 HEPES, 11 glucose, and 132.5 CsCl 132.5 (pH 7.4, CsOH). Pipette filling solution contained the following (mM/l): 135 CsCl, 10 NaCl, 2 CaCl$_2$, 5 EGTA, 10 HEPES, and 5 MgATP (pH 7.2, CsOH). $I_{Na}$, $I_{Na-v}$, and $I_{Na-L}$ were recorded and analyzed as previously described (3, 26, 29). $I_{Na-L}$ is reported as the ratio of average current between 600 and 700 ms to the $I_{Na-v}$. To measure $I_{Na}$, steady-state inactivation, 1,000-ms long prepauses were applied before stepping from −110 and −20 mV in 10-mV increments, followed by 0 mV step for 25 ms. Capacitance compensation used a P/4 protocol.

**Calcium transients.** Intracellular calcium currents were measured in iPS-CMs infected with Kir2.1 IRES GFP or IRES GFP viral control and incubated with the cell permeant ratiometric calcium sensitive dye fura-2AM, 20 μmol/l (Molecular Probes, Eugene, OR). Cells were loaded with the fura-2-AM dye for 10 min at room temperature and then washed for 30 min at room temperature. Spontaneous calcium release from internal cellular stores was measured without electrical pacing. Next, electrical pacing was achieved by field stimulation (40 V, 37°C) at frequencies of 0.5 and 1 Hz. Calcium transients were continuously recorded using a photomultiplier tube with images sampled at 1 kHz and analyzed using commercially available software (IonOptix, Milton, MA).

**Immunocytochemistry.** Immunocytochemistry was performed as previously described (28). $I_{K1}$-enhanced iPS-CMs and viral control-infected iPS-CMs were plated on coverslips and fixed using 4% formaldehyde and permeabilized with 0.1% Triton X-100. Cells were incubated with anti-actinin and anti-MLC2 (myosin light chain 2, ventricular specific) antibody overnight at 4°C and then secondary antibodies were added after washing. Coverslips were mounted using Prolong gold and imaged under a Leica confocal microscope. Multiple fields of view were analyzed by two reviewers and blinded to the cell type.

For experiments to detect DNA synthesis, iPS-CMs infected with Kir2.1 were incubated in 10 μmol/l 5-bromo-2-deoxyuridine (BrDu; Invitrogen) in maintenance medium for 4 days. Cells were fixed using 4% paraformaldehyde and washed. DNA was denatured in 2 M HCl/0.1% Triton X-100 in PBS solution for 15 min at room temperature and washed with PBS solution. Primary antibodies for Kir2.1 (Santa Cruz Biotechnology) and BrDu (Invitrogen) were used to colabel the cells. Alexa Fluor 647 anti-mouse or Alexa Fluor 405 anti-rabbit (Invitrogen) was used to detect BrDu/Kir2.1, respectively. Nuclei were stained with DAPI. The BrDu-positive cells were counted relative to the total number of cells in iPS-CMs from five to six equal-sized fields of view for each group to comprised ~1,700 cells per group. From these same fields of view, we also quantified the number of mononucleated, binucleated, and trinucleated that were DAPI positive and/or BrDu positive in both groups (33). Samples were analyzed by two individuals blinded to the groups.

**Measurement of cell size.** In addition to measuring cell capacitance (see cellular electrophysiology above), cell size was also measured optically. iPS-CMs infected with IRES GFP Kir2.1 and IRES GFP viral control were split 24 h after infection and plated on 12-mm glass bottom dishes. Forty-eight hours after infection cells were imaged on a confocal microscope. Images were then transferred to ImageJ software (National Institutes of Health, Bethesda, MD) where they were converted into eight bit binary images for blinded measurements. The tracing tool (automated or manual) was used to highlight the edge of the cell following which the area inside the contour was calculated using standard function in ImageJ after calibration.

**Statistics.** All data are presented as mean ± SE. Statistical comparisons were carried out using Student’s unpaired (see Figs. 1, 3–7) t-test or ANOVA with Bonferroni post hoc test (see Figs. 2 and 5), using OriginLab (Northampton, MA) or Microsoft Excel software.

**RESULTS**

$I_{K1}$ from $I_{K1}$-enhanced iPS-CMs. After plating, iPS-CMs contracted spontaneously within 48 h at which time the plating media were changed to maintenance media (18). Endogenous $I_{K1}$ density from these and other iPS-CMs has been reported to be small relative to adult cardiomyocytes (5, 18). We found similarly small endogenous $I_{K1}$ (see Fig. 1C) and low or undetectable Kir2.1 without $I_{K1}$-enhancement demonstrated spontaneous activity and different maximum diastolic potentials (Fig. 1D and Table 1). Following Kir2.1 infection iPS-CMs became quiescent; they lacked phase 4 diastolic depolarization and had resting membrane potentials between −77.2 ± 2.5 to −79.8 ± 0.9 mV, which is similar to adult ventricular myocytes (see Table 1). $I_{K1}$ density measurements from the $I_{K1}$-enhanced iPS-CMs are shown in Fig. 1. Figure 1A shows example barium sensitive current (black) traces compared with baseline conditions (dark grey), using the voltage ramp protocol shown in Fig. 1B, inset. Summary current density data are shown in Fig. 1B (N = 7). The peak inward current density at −120 mV was −29.8 ± 6.9 pA/pF and peak outward current density at −50 mV was 4.7 ± 1.5 pA/pF. When compared with previous reports of endogenous $I_{K1}$ in iCell cardiomyocytes (18), $I_{K1}$-enhanced iPS-CMs density at depolarized voltages was increased approximately fivefold. On the other hand, the inward (−120 mV) and outward (−60 mV) current densities of $I_{K1}$-enhanced iPS-CMs are similar in magnitude to adult human $I_{K1}$ reported by Koumi et al. (12).

**AP characteristics of $I_{K1}$-enhanced iPS-CMs.**APs from $I_{K1}$-enhanced iPS-CMs were separated into atrial- and ventricular-like based on APD; iPS-CMs with a ratio of APD$_{30–40}$ to APD$_{70–80}$ at 0.5 Hz ≥1.5 were classified as atrial-like cardiomyocytes and those with a ratio >1.5 were classified as ventricular-like cardiomyocytes (18). This classification of the $I_{K1}$-enhanced iPS-CMs identified 17 of the 18 cells recorded (95%) as ventricular-like. Figure 2 (see also Table 1) shows the AP characteristics of ventricular-like iPS-CMs. Figure 2A shows example AP recordings paced at 0.5, 1, 2, and 3 Hz to test for rate adaptation typical of adult cardiomyocytes. The resting membrane potential and dV/dt$_{max}$ did not vary significantly with different pacing frequencies (Fig. 2, B and C).
Table 1. AP characteristics of uninfected iPS-CMs, I<sub>K1</sub>-enhanced ventricular-like iPS-CMs, and I<sub>K1</sub>-enhanced iPS-CMs expressing either WT-CAV3 or F97C-CAV3

<table>
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<tr>
<th>Cell Type</th>
<th>Frequency, Hz</th>
<th>MDP, mV</th>
<th>APD&lt;sub&gt;10&lt;/sub&gt;, ms</th>
<th>APD&lt;sub&gt;50&lt;/sub&gt;, ms</th>
<th>APD&lt;sub&gt;70&lt;/sub&gt;, ms</th>
<th>APD&lt;sub&gt;90&lt;/sub&gt;, ms</th>
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<tr>
<td>iPS-CMs</td>
<td>1.7±0.3</td>
<td>-49.2±2.8</td>
<td>53.4±14.3</td>
<td>181.9±39.8</td>
<td>232.2±41.2</td>
<td>292.4±44.6</td>
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<tr>
<td>I&lt;sub&gt;K1&lt;/sub&gt;-enhanced</td>
<td>0.5 Hz</td>
<td>-79.8±0.9</td>
<td>72±36.6</td>
<td>379±87.8</td>
<td>413.3±89.5</td>
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<td></td>
<td></td>
<td>1 Hz</td>
<td>-79.5±1.3</td>
<td>43.7±21</td>
<td>256±46.1*</td>
<td>277±45.9*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 Hz</td>
<td>-77.9±1.9</td>
<td>15.5±5.5*</td>
<td>141.8±20.7†</td>
<td>167±21.8†‡</td>
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<tr>
<td></td>
<td></td>
<td>3 Hz</td>
<td>-77.2±2.5</td>
<td>9.3±4.3*</td>
<td>101.4±18*‡</td>
<td>130±15.3†‡</td>
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<tr>
<td>Kir2.1 + WT-Cav3</td>
<td>0.5 Hz</td>
<td>-80.1±1.0</td>
<td>109.6±12.1</td>
<td>589.4±29.9</td>
<td>633.6±34</td>
<td>642.2±34</td>
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<td></td>
<td>1 Hz</td>
<td>-78.5±1.0</td>
<td>87.3±15.5</td>
<td>362.1±26</td>
<td>401.8±26.5</td>
<td>410.5±26.7</td>
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<td></td>
<td>2 Hz</td>
<td>-78.5±1.0</td>
<td>27.5±9</td>
<td>169.9±31.5</td>
<td>205±34.3</td>
<td>214.9±35.9</td>
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<tr>
<td>Kir2.1 + F97C-Cav3</td>
<td>0.5 Hz</td>
<td>-74.9±0.8</td>
<td>154.9±23.3</td>
<td>814.3±60‡</td>
<td>887.48±67‡</td>
<td>898.4±67.7‡</td>
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<td></td>
<td>1 Hz</td>
<td>-75.2±1.1</td>
<td>74.5±5.7</td>
<td>569.8±7.5‡</td>
<td>607.7±8.2‡</td>
<td>616.1±8.4‡</td>
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</table>

Values are means ± SE. APD, action potential duration; iPS-CMs, induced pluripotent stem cell-derived cardiomyocytes; WT, wild type; RMP, resting membrane potential; MDP, maximum diastolic potential. Uninfected iPS-CMs AP characteristics are based on their spontaneous beating rate, not paced frequency and MDP is significantly higher than I<sub>K1</sub>-enhanced iPS-CMs. Statistical significance was calculated by one-way ANOVA with Bonferroni correction. *Significant difference on comparison with respective APD at 0.5 Hz. †Significant difference on comparison with respective APD at 1 Hz. ‡Significant difference on comparison with respective APD and pacing frequency of Kir2.1 + WT-Cav3.

Table 1 and Fig. 2D show the results for ventricular-like iPS-CMs and rate dependence of APD at pacing frequencies of 0.5, 1, 2, and 3 Hz. Ventricular-like cardiomyocyte APD shortened progressively as rate increased.

**Calcium transients from I<sub>K1</sub>-enhanced iPS-CMs.** Intracellular calcium transients are an integral component of cardiac physiology coupling excitation to contraction. Calcium transients at baseline from the I<sub>K1</sub>-enhanced iPS-CMs were absent because cardiomyocytes are quiescent due to robust I<sub>K1</sub>, while the viral control (GFP)-infected iPS-CMs demonstrated spontaneous calcium transients (Fig. 3A, left). The basal calcium fura 340/380 ratios were not significantly different between the two groups (Fig. 3B, left) nor was the time constant of the calcium transient decline at both the 0.5- and 1-Hz pacing frequency (Fig. 3B, right). However, I<sub>K1</sub>-enhanced iPS-CMs demonstrated significantly greater calcium transient amplitudes compared with control (P = 0.036, 0.5 Hz and P = 0.040, 1 Hz). There was approximately a twofold increase in the peak calcium transient amplitude of I<sub>K1</sub>-enhanced iPS-CM compared with control (Fig. 3B, middle) at both the 0.5- and 1-Hz pacing frequency. Furthermore, in I<sub>K1</sub>-enhanced iPS-CMs we observed a typical positive staircase pattern of increased intracellular calcium upon increasing stimulation rate consistent with previously described rate-dependent intracellular calcium dynamics in adult cardiomyocytes (4, 13). This pattern was not found in the control GFP expressing iPS-CMs.
Kir2.1 infected iCells

Ips-Cms, Cstaining pattern (Fig. 4, normal growth of the heart is associated with increased cardiopositive, indicating ventricular myocytes.

Effect of Kir2.1 expression in adult cardiomyocytes (6). To assess the increased cell size; these characteristics have been linked to mental characteristic of adult cardiomyocytes maturation is 1,101/H11006

Immunolabeled iPS-CM area was compared between Kir2.1 and viral control-infected iPS-CMs, using ImageJ analysis. The average area of Kir2.1-infected iPS-CMs was 1,776/20, F97C-CAV3 (61.8/2.0 pF, n = 19), or uninfected iPS-CMs (44.3 ± 12.0, n = 4). Immunolabeled iPS-CM area was compared between Kir2.1 and viral control-infected iPS-CMs, using ImageJ analysis. The average area of Kir2.1-infected iPS-CMs was 1,776 ± 167 μm² compared with viral control-infected iPS-CMs was 1,101 ± 79 μm² (P < 0.05; Fig. 4B). Kir2.1-enhanced iPS-CMs and viral control-infected iPS-CMs were stained with MLC2V (ventricular myocyte marker), α-actinin (actin marker), and DAPI (nuclear marker). Compared with viral control-infected iPS-CMs, Kir2.1-enhanced iPS-CMs showed a denser MLC2V staining pattern (Fig. 4, C and D). Most cells were MLC2V positive, indicating ventricular myocytes.

Kir2.1 expression increases DNA synthesis in iPS-CMs. The normal growth of the heart is associated with increased cardiomyocyte DNA synthesis early in development due primarily to cellular proliferation. Later in development as myocyte hypertrophy contributes more to the growth of the heart, DNA synthesis is associated more with increased ploidy as well as multinucleation in a fraction of cardiomyocytes (7, 20). Because standard culture conditions following differentiation of iPS-CMs lead them to rapidly exit the cell cycle (33), we tested whether Kir2.1 expression changed this behavior in vitro producing cells that would proceed down the maturation pathway that is associated with increased DNA synthesis. We note a twofold increase in cell size and capacitance in Kir2.1-enhanced iPS-CMs and thus investigated the proliferative state of the nuclei in Kir2.1-enhanced iPS-CMs and viral control-infected iPS-CMs. Kir2.1-enhanced and viral control-infected iPS-CMs were incubated with BrdU, a thymidine analog that incorporates into newly synthesized DNA and can be identified with an anti-BrdU antibody(33). Figure 5A shows representative images of iPS-CMs infected with viral control (Fig. 5, Au-Ac) or Kir2.1 (Fig. 5, Ad-Af) stained with DAPI (blue) to identify nuclei and BrdU (red) to identify new DNA synthesis. Merged images of the regions are shown in Fig. 5, Ac and Af. For the Kir2.1-enhanced iPS-CMs, 31% (525 of 1,692 cells) of the nuclei were positive for BrdU compared with 5% (93 of 1,785 cells) of the viral control-infected iPS-CMs (Fig. 5B, top, P = 0.0001). The same samples were also analyzed for mononucleated, binucleated, or trinucleated cells. As shown in Fig. 5B, bottom, the percentage of bi- and trinucleated cells in Kir2.1-enhanced iPS-CMs was increased compared with viral control iPS-CMs: Kir2.1-enhanced iPS-CMs that were binucleated totaled 100/1,692 vs. 33/1,785

Kir2.1-enhanced iPS-CMs exhibit increased cell size. A fundamental characteristic of adult cardiomyocytes maturation is increased cell size; these characteristics have been linked to Kir2.1 expression in adult cardiomyocytes (6). To assess the effect of Kir2.1-enhancement on iPS-CM size, the whole cell membrane capacitance was determined from whole cell patchclamp electrophysiology measurements and the cell area was measured from imaging studies. We used multiple comparison controls because viral infection alone may affect cell size. For cell capacitance we compared Kir2.1-infected iPS-CMs with viral control-infected iPS-CMs. As shown in Fig. 4A, Kir2.1-infected iPS-CMs demonstrated a membrane capacitance of 177.9 ± 9.9 pF (n = 8), approximately three times that of iPS-CMs infected with WT-CAV3 (59.2 ± 1.4 pF, n = 20), F97C-CAV3 (61.8 ± 1.0 pF, n = 19), or uninfected iPS-CMs (44.3 ± 12.0, n = 4).

Innovative Methodology

iPS-CM MODEL FOR INVESTIGATING COMPLEX INHERITED ARRHYTHMIAS

H1615

Fig. 3. Characterization of the calcium transients from Kir1-enhanced iPS-CMs and viral control-infected iPS-CMs. A: representative calcium recordings from viral infected control iPS-CMs (black) and Kir1-enhanced iPS-CMs (grey). Spontaneous activity was recorded from the groups shown at left, the same cells paced at 0.5 and 1 Hz are reported at middle and right, respectively. B: average data of the basal calcium concentration (left), the peak amplitude (middle), and the time constant of calcium reuptake (right) for viral control-infected (black) and Kir1-enhanced (grey) iPS-CMs. The peak amplitude and time constant were calculated at both the 0.5- and 1-Hz pacing frequency. *P < 0.01.
in viral control cells (P = 0.004) and I_{K1}-enhanced iPS-CMs that were trinucleated totaled 9/1,692 vs. 6/1,785 in viral control (P = NS). The expression pattern of Kir2.1 (Fig. 5C) shows that cells with BrdU-positive nuclei also expressed Kir2.1 (Fig. 5, Ch and Ck, red), yet some BrdU-negative cells expressed Kir2.1.

**LQT9 CAV3 mutation expression in I_{K1}-enhanced iPS-CMs increases APD and I_{Na,L}**. Mutations in CAV3 cause LQT9 and the LQT9-associated F97C-CAV3 mutation increases I_{Na,L}, as shown in HEK293 cells (30) and in rat myocytes (3). Here, we compare I_{Na,P} and I_{Na,L} in I_{K1}-enhanced iPS-CMs infected with either WT-CAV3 or F97C-CAV3 and iPS-CMs with WT or F97C-CAV3, I_{Na,P} density increased in I_{K1}-enhanced iPS-CMs compared with iPS-CMs (Fig. 6, A and B). This is consistent with observations from other groups who have found that I_{Na,P} density increases in the presence of Kir2.1(19). F97C-CAV3 increased I_{Na,L} but did not affect I_{Na,P} in both I_{K1}-enhanced and iPS-CMs. Interestingly, I_{Na,L} was increased in I_{K1}-enhanced iPS-CMs compared with iPS-CMs infected with WT-CAV3 (Fig. 6, C and D). In Fig. 7, we demonstrate AP analysis of I_{K1}-enhanced iPS-CMs expressing F97C-CAV3 or WT-CAV3. Example APs from iPS-CMs expressing either WT-CAV3 or F97C-CAV3 and paced at 0.5, 1 and 2 Hz are shown in Fig. 7, A and B, D and E, and G and H. iPS-CMs expressing F97C-CAV3 could not be paced at 2 Hz due to a prolonged APD and developed (EADs; Fig. 7H). APD averaged data are shown for pacing at 0.5 Hz (Fig. 7C) and 1 Hz (Fig. 7E) and show that APD_{50}, APD_{70} and APD_{90} were prolonged for iPS-CMs expressing F97C-CAV3 compared with iPS-CMs expressing WT-CAV3 (see also Table 1). The resting membrane potential for I_{K1}-enhanced iPS-CMs expressing F97C-CAV3 was slightly depolarized (−74.9 ± 0.8 mV at 0.5 Hz and −75.2 ± 1.1 mV at 1 Hz) compared with I_{K1}-enhanced iPS-CMs expressing WT-CAV3 (−80.1 ± 0.9 mV at 0.5 Hz, −80.3 ± 0.9 mV at 1 Hz)
The increased cyte phenotype. APD shortens as pacing frequency increases (14, 24) and the prolongation of APD and generation of EADs.

DISCUSSION

The key findings are that our $I_{K1}$-enhanced iPS-CMs: 1) have a stable resting membrane potential with loss of spontaneous automaticity; 2) display $I_{K1}$ density analogous to adult cardiomyocytes; 3) show AP characteristics and calcium transients similar to adult cardiomyocytes; 4) exhibit cell size and membrane capacitance similar to adult cardiomyocytes; and 5) model a CAV3 mutation found in LQTS with demonstration of prolongation of APD and generation of EADs.

$I_{K1}$-enhancement creates iPS-CM with mature cardiomyocyte phenotype. The increased $I_{K1}$ density in the $I_{K1}$-enhanced iPS-CMs was similar in magnitude to that reported in human ventricular myocytes (12). In accordance with most mammalian animal models, including human ventricular myocytes, APD shortens as pacing frequency increases (14, 24) and the APD values we measured are similar to the previously reported range for human ventricular myocytes (15, 16, 22). Establishing a more negative resting membrane potential increases the availability of sodium and L-type calcium channels, and the $dV/dt$ values we obtained were in the range of adult cardiomyocytes. Membrane stabilization of $I_{K1}$-enhanced iPS-CMs is demonstrated by larger calcium transient amplitude, compared with controls without a change in the basal calcium levels or calcium transient rate of decay. Additionally, we note a typical stimulation rate-dependent increase in intracellular calcium, consistent with previous adult cardiomyocyte reports of rate-dependent increase in intracellular sodium and calcium (4, 13).

$I_{K1}$-enhancement increases cell size and DNA synthesis. In addition to establishing an electrically more mature phenotype, our data also suggest that $I_{K1}$-enhancement induces maturation of multiple properties of the iPS-CMs. It has been demonstrated in human skeletal muscle that inward rectifier potassium channels, specifically Kir2.1, induce membrane hyperpo-
K1 membrane hyperpolarization was shown by this same group to trigger the calcineurin pathway, which in turn activates myogenic transcription factors, myogenin and myocyte enhancer factor-2, and initiates essential steps involved in differentiation and maturation of skeletal muscle (10). Potassium channels may regulate cell size and volume by permeation-dependent mechanisms and cellular hyperpolarization and possibly via modulation of signaling cascades by protein-protein interactions as reviewed by Urrego et al. (27). Our I_K1-enhanced iPS-CMs showed a nearly twofold increase in cell capacitance values compared with previous reports for iPS-CMs (18). This effect was not due to the presence of adenovirus or GFP, as adenoviral infection alone did not generate this effect. I_K1-enhanced iPS-CMs demonstrated significantly more DNA synthesis identified by BrdU staining and increased percentage of binucleation compared with viral control-infected iPS-CMs. An interesting finding was the trend that nuclei in both groups positive for BrdU were also positive for Kir2.1, suggesting a role of Kir2.1 in increased DNA synthesis, which may include karyokinesis. The AP characteristics, cell size, pattern of staining of MLC2V and cTNT, and binucleation suggest a change in cell maturation state. Used in context of previous findings in skeletal myocytes, Kir2.1 may trigger cardiac myocyte differentiation and karyokinesis, and further investigation is needed.

Disease modeling. I_K1-enhanced iPS-CMs allow for more detailed disease modeling with LQTS pathognomonic findings of a prolonged APD that fails to shorten with pacing and EAD production. In current clamp, the establishment of a stable resting membrane potential increases sodium channel availability.
ability and may increase both peak and late current (1, 19). Compared with WT-CAV3, F97C-CAV3-infected iPSCMs produced an increase in $I_{Na,L}$ without affecting $I_{Na,P}$. Interestingly, in $I_{K1}$-enhanced iPSCMs, $I_{Na,P}$ increased twofold compared with iPSCMs and, in parallel, $I_{Na,L}$ was significantly increased in WT-CAV3 and F97C-CAV3 $I_{K1}$-enhanced iPSCMs. The increase in both $I_{Na,P}$ and $I_{Na,L}$ in $I_{K1}$-enhanced iPSCMs is an interesting finding. We speculate that in the absence of Kir2.1, Nav1.5 is more available to associate with Cav3. Association with WT-Cav3 (a known neuronal nitrite oxide synthase inhibitor) suppresses nitrosylation of the sodium channel, therefore keeping late sodium current low. In the presence of Kir2.1 expression, the expression of Nav1.5 increases for channels associated with WT-Cav3 and channels outside of caveolae. The Nav1.5 channels outside of caveolae are released from WT-Cav3-mediated neuronal nitrite oxide synthase inhibition and are more nitrosylated, increasing late sodium current. Therefore, we expected to see a relative increase in $I_{Na,P}$ and $I_{Na,L}$ in $I_{K1}$ enhancement. However, F97C-CAV3 plus $I_{K1}$ enhancement would not result in an increase in $I_{Na,P}$ but will increase $I_{Na,L}$ since this mutation has been shown to decrease Kir2.1 trafficking, thus Nav1.5 expression modulation would not occur (3, 28). $I_{K1}$ enhancement in patient-specific cell lines or iPSCMs modified by CRISPR technology is a logical extension of this work and may aid in this disease characterization as well as therapeutics testing.

**Technology applications.** Current and future application of iPSC-CM includes drug testing and cardiac regenerative therapy. For drug testing, iPSC-CM are one cell model used by the pharmaceutical industry and the research community for testing of drug safety and efficacy. Drugs that prolong the QT interval are a particular problem, largely related to the vulnerability of drug binding to the pore S6-region of Kv11.1 (25). Drug screening previously performed with noncardiac cells is now also being performed with iPSC-CMs. Our $I_{K1}$-enhanced iPSC-CMs may offer a more mature cardiac AP over a wide range of pacing rates for improved drug safety testing, so that pause-induced or bradycardic-dependent arrhythmia mechanisms (such as Torsade de Points, the pathognomonic arrhythmia of LQT) can now be studied. From a cellular therapeutics perspective, $I_{K1}$-enhanced iPSC-CMs, with their lack of spontaneous beating, may be more suitable for application to cardiac

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**Fig. 7. Expression of F97C-CAV3 in $I_{K1}$-enhanced ventricular iPSC-CMs prolongs APD and induces early afterdepolarizations (EADs).** A, D, and G: representative AP from $I_{K1}$-enhanced iPSC-CMs expressing WT-CAV3 paced at 0.5, 1, and 2 Hz, respectively. B and E: representative AP from $I_{K1}$-enhanced iPSC-CMs expressing F97C-CAV3 paced at 0.5 and 1 Hz, respectively. C: APD$10$, APD$50$, APD$70$, and APD$90$% repolarization from peak at a pacing frequencies of 0.5 calculated from ventricular-like $I_{K1}$-enhanced iPSC-CMs expressing WT-CAV3 (black) or F97C-CAV3 (grey). F: APD$10$, APD$50$, APD$70$, and APD$90$% repolarization from peak at a pacing frequencies of 1 Hz calculated from ventricular-like $I_{K1}$-enhanced iPSC-CMs expressing WT-CAV3 (black) or F97C-CAV3 (grey). H: EADs generation in $I_{K1}$-enhanced iPSC-CMs expressing F97C-CAV3 when paced at 0.33Hz. I: resting membrane potential reported at 3 pacing frequencies from iPSC-CMs coexpressing Kir2.1 and WT/F97C-CAV3. *Significant difference between the 2 groups at the respective APD. $N$ = number of cells, $n$ = number of AP (scale bars in G applicable to all but H).
regeneration. For these reasons, we anticipate that our \( I_{K1} \)-enhanced iPS-CMs will change the manner in which drug and disease therapeutics are tested and generated.

**Conclusion.** We conclude that \( I_{K1} \)-enhanced iPS-CMs more closely represent the cell size, electrophysiology, and calcium handling properties of adult human cardiomyocytes. The characterization provided in this Innovative Methodology report represents an essential step forward in the utilization of this cellular system to model human disease and toxicology.

**Limitations.** There are limitations to our experiments. While we have robust Kir2.1 expression, other isoforms (Kir2.2 and Kir2.3) normally present in human cardiomyocytes were not investigated. We also did not investigate the effects of F97C-CAV3 on Kir2.1 density given our previous report that LQT9 causing CAV3 mutations affects Kir2.1 density. This decision was made due to the 25–30% variability in Kir2.1 current density seen post viral infection. This variability did not affect the variability of resting membrane potential in Kir2.1-infected iPS-CMs but is the same variability by which F97C-CAV3 decreases Kir2.1 current density in heterologous cell model (28). We are currently pursuing new methods to address this degree of variability. The experiments studied only short-term adenoviral infection (3–5 days) of genes for \( I_{K1} \) and CAV3 (WT or F97C). Longer time periods were not studied; thus it is not known if the results we found would persist or change over time.

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