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Optical recording of action potentials in human induced pluripotent stem cell-derived cardiac single cells and monolayers generated from long QT syndrome type 1 patients

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Abstract

Induced pluripotent stem cells (iPSCs) from type 1 long QT (LQT1) patients can differentiate into cardiomyocytes (CMs) including ventricular cells to recapitulate the disease phenotype. Although optical recordings using membrane potential dyes to monitor action potentials (APs) were reported, no study has investigated the disease phenotypes of cardiac channelopathy in association with the cardiac subtype at the single cell level. We induced iPSC-CMs from three control and three LQT1 patients. Single cell analysis using a fast-responding dye confirmed ventricular cells were the dominant subtype (control-iPSC-CMs: 98%, 88%, 91%; LQT1-iPSC-CMs: 95%, 79%, 92%). In addition, LQT1-iPSC-ventricular cells displayed an increased frequency of early afterdepolarizations (p value = 0.031). Cardiomyocyte monolayers constituted mostly of ventricular cells derived from LQT1-iPSCs showed prolonged AP duration (APD) (p value = 0.000096). High throughput assays using cardiomyocyte monolayers in 96 well-plates demonstrated I_{Kr} inhibitors prolonged APDs in both control- and LQT1-iPSC-CM monolayers. We confirmed the optical recordings of APs in single cells and monolayers derived from control- and LQT1-iPSC-CMs can be used to assess arrhythmogenicity, supporting the feasibility of membrane potential dye-

based high-throughput screening to study ventricular arrhythmias caused by genetic channelopathy or cardiotoxic drugs.

Introduction

Type 1 long QT syndromes (LQT1), a frequent type of congenital LQT syndrome (cLQTS) [1], is caused by a reduction of slow delayed rectifier K⁺ current (I_{Ks}) and is associated with a loss-of-function mutation in the *KCNQ1* gene [2]. *KCNQ1* A341V is known as one of the most frequent and severe *KCNQ1* mutations [3]. Its coexpression with wild type *KCNE1*, a beta subunit of the I_{Ks} channel, causes a pathological reduction of I_{Ks} [4], suggesting the importance of building physiological conditions to recapitulate the pathology in vitro. Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) were shown to remodel ion channels that regulate the electrophysiological activity of human heart and to successfully recapitulate the LQT1 phenotype [5-8].

Previous papers related to cLQTS patient-specific hiPSC-CMs verified the disease phenotype by using either the manual patch clamp method alone [5, 7] or in combination with the microelectrode array (MEA) system [6, 8]. However, the field potential duration (FPD), which reflects QT on electrocardiograms (ECG), of ventricular hiPSC-CM monolayers is longer than that of atrial iPSC-CMs when using the MEA system [9], suggesting a high proportion of ventricular

hiPSC-CMs is required to analyze ventricular arrhythmic disease. Patch clamp analysis is standard for classifying the cardiac subtype, but it requires much time and expertise [10]. Automated patch-clamp techniques may resolve these drawbacks, but they cause cells to produce spontaneous action potentials (APs) that are erratic due to enzymatic treatment immediately before the analysis [11].

FluoVolt (FV) is a new membrane potential VF2.4.Cl dye [12, 13]. It modulates photo-induced electron transfer (PeT) from an electron donor to a fluorophore through a synthetic molecular wire [12, 14] and responds on a microsecond timescale, which makes it faster than Förster resonance energy transfer (FRET)-based voltage sensitive dyes (VSDs) [15, 16]. Moreover, it has a higher fluorescence ratio (about 20% $\Delta F/F$ per 100 mV greater) and lower phototoxicity than electrochromic dyes such as di-4-ANEPPS [12, 17], enabling classification of the subtype of single cardiomyocytes [18].

FV and VF2.1.Cl have been used to analyze APs from iCell® cardiomyocyte monolayers or type 3 LQTS-iPSC-CM monolayers and also drug effects on multiwell plate readers [19, 20]. However, the proportion of ventricular type cells in those monolayers was not examined. Since arrhythmogenic change occurs in ventricular cells in LQTS, it is crucial to investigate the

electrophysiological properties of this specific subtype.

In the present study, we propose a new method that incorporates FV and combines optical measurements of the membrane potential of single cardiomyocytes and in cardiac monolayers to identify the subtype and associate the subtype with the cardiomyocyte properties. We show the effectiveness of this method for modeling type 1 long QT (LQT1) using patient hiPSC-CMs.

Materials and Methods

Generation of human iPS cells from three type1 LQTS patients

This study was approved by the Ethics Committees of the Graduate School of Medicine Kyoto University and the Kyoto University Hospital. Written informed consent was obtained from the patients in accordance with the Declaration of Helsinki. LQT1-iPSCs were generated from three patients using episomal vectors as described previously [21]. Three iPSC lines derived from healthy donors (201B7 [22], 409B2 [21] and 692D2 [23]) were used as control iPSCs. Human iPSCs were maintained on STO feeder layers cultured with primate ES cell medium (ReproCell, Yokohama, Kanagawa, Japan), as previously described [22].

Cardiac differentiation and fluorescence-activated cell sorting

Human iPSCs were differentiated by forming embryoid bodies (EBs), as previously described [24, 25] (see the Supplementary Material for further details). On day 29, EBs were dissociated and dispersed onto a fibronectin-coated 6-cm dish. On the following day, seeded cells were collected by Accumax for 10 minutes and subjected to fluorescence-activated cell sorting (FACS) (Aria II, BD Biosciences). To purify cardiomyocytes, SIRPa-positive and lineage (CD31, CD49a, CD140b, CD90, or TRA-1-60)-negative cells were sorted [26] and cryopreserved with STEM-CELLBANKER (Nippon Zenyaku Kogyo, Kooriyama, Japan) at -80°C. A few weeks later, the cryotubes were transferred to a liquid nitrogen storage tank.

Quantitative polymerase chain reaction (qPCR)

Total RNA was extracted after FACS sorting using QIAzol Lysis Reagent (QIAGEN, Hilden, Germany). qRT-PCR was performed with TaqMan gene expression assays (Thermo Fisher Scientific, Waltham, MA, USA). For further details, see the Supplementary Material.

Thawing frozen iPSC-CMs

For perforated patch clamp recording, the cryotubes were thawed in a 37°C water-bath, centrifuged at 300 g for 5 min, and seeded on fibronectin-coated No.2 cover glass (Matsunami, Osaka, Japan) filled with StemPro 34 SFM (Thermo Fisher Scientific) containing buffer and 10 ng/ml VEGF. Five to ten days after seeding, the cells were subjected to patch clamp experiments.

Current clamp recording

Perforated whole-cell patch was performed with current clamp mode using amphotericin B (Sigma-Aldrich, St Louis, MO, USA), an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) and pCLAMP software (Molecular Devices). Maximum diastolic potential (MDP), AP amplitude (APA), and action potential duration (APD) at 90% repolarization (APD₉₀) were calculated from the average AP of 10 consecutive and stable waves in 1 Hz pacing mode with pCLAMP software. For detailed protocols, see the Supplementary Material.

Voltage clamp recording

I_{Ks} currents were recorded from single cardiomyocytes with the ruptured whole cell patch clamp technique. I_{Ks} was calculated by subtracting the currents after perfusion with extracellular solution containing 30 μ M (-)-[3R,4S]-chromanol 293B (Tocris Bioscience, Bristol, UK) from the currents before perfusion. I_{Ks} currents were elicited by depolarizing steps from a holding potential of -40 mV to -20, 0, 20, and 40 mV for 4 s. This was followed by a 2 s repolarization phase. For detailed protocols, see the Supplementary Material.

Seeding high density iPSC-CM monolayer

2 μ l of 50 μ g/ml fibronectin was placed on a 35-mm-diameter glass bottom dish (Matsunami), or 5 μ l was placed on a 96-well plate with clear flat bottom wells (Corning, Corning, NY, USA). One hour later, fibronectin was aspirated, and a 2 μ l bead of thawed 4×10^4 iPSC-CM suspension was placed on the 35-mm dish or a 5 μ l bead of thawed 5×10^4 iPSC-CM suspension was placed on the 96-well plate. After another one-hour incubation, the appropriate volume of media was added gently. The dish or plate was incubated at 37°C, 5% CO₂. The composition of the media

was the same on days 7 to 29. Medium was exchanged every 2 to 3 days.

Loading of FV

When loading FluoVolt (FV; Thermo Fisher Scientific), the medium was exchanged with modified Tyrode's solution and FV (0.1% volume). Pluronic surfactants were not used in this study.

Twenty minutes after loading, the medium was refreshed with modified Tyrode's solution and used in each assay.

Optical recording of APs from single cells or cardiomyocyte monolayers

Cells were imaged in a 35-mm-diameter glass bottom dish at 37°C and modified Tyrode's solution identical to that used for patch clamp recordings. Subarray images were recorded every 8 ms in single cells or every 4 ms in monolayer using AquaCosmos software (Hamamatsu Photonics).

The regions of interest (ROIs) in the monolayers were defined as whole pixels of 512x32 at 1 Hz pacing. Graphing and calculation of APD₉₀ were performed with OriginPro 2016 (OriginLab, Northampton, MA, USA). For further details, see the Supplementary Material.

Optical recording of APs from cardiomyocyte monolayers on a high-throughput plate reader

The FDSS/ μ Cell imaging platform (Hamamatsu Photonics) was used. The LED current was set to 300 mA. An output excitation wavelength was used with standard settings of 492 BP 20 nm and emission filter 540AF40 (Omega optical, Brattleboro, VT, USA). Binning was set to 4x4, and the measurement interval was every 4 ms. Cardiomyocyte monolayers 7-10 days after seeding on each well were stimulated at 1 Hz with 1 ms depolarizing pulses at 10 V. Analysis of APD₉₀ was performed with FDSS Waveform Analysis software for cardiomyocytes (U8524-12; Hamamatsu Photonics).

Movie

Blinking monolayers were recorded with AquaCosmos software at 30 fps.

Statistical analysis

All statistical analyses were verified with an unpaired t test using EXCEL2016 (Microsoft,

Redmond, WA, USA). Values considered statistically significant are denoted as * $p < 0.05$ and ** $p < 0.005$ in the figures.

Results

Establishment of three LQT1 patient-derived-iPS cell lines

We selected three LQT1 patients as donors for the iPSC derivation. One of the donors was a 50-year-old woman (II-2 in Figure 1a) who experienced presyncope several times when she was in junior high school and underwent recurrent syncope in her thirties. She showed prominent QT prolongation in resting and exercise ECG (Figure 1b). The other donors were her two daughters whose QT intervals were prolonged according to school medical examinations. Genetic testing diagnosed the mother and two daughters as type 1 long QT syndrome with *KCNQ1* A341V mutation (c.1022C>T) (Figure 1c). The mutation is located at the transmembrane region in segment 6 near the pore of the I_{Ks} channel (Figure 1d) and is reported as one of the severest types of LQT1 [3]. Medical therapy (beta-blockade) and lifestyle measures were sufficient to prevent recurrent events in the three patients. Five of the six family members positive for *KCNQ1* mutation experienced

syncope, and the sixth (III-1 in Figure 1a) did not. All carriers showed QTc prolongation on ECG.

iPSCs were generated from the peripheral blood of the three patients using a nonviral method [23] and were differentiated into cardiomyocytes via EB formation [24, 25] (Figure 1e).

There was no significant difference in the expression of genes that affect APD between control- and LQT1-iPSC-CMs (Supplementary Figure S1).

Patch clamp analysis

Ventricular-type cardiomyocytes derived from the three control- and three LQT1-iPSC lines were subject to current clamp recordings (Figure 2a). Although there was no significant difference in MDP or APA, there was a significant difference in APD₉₀ during pacing at 1 Hz between control- and LQT1-iPSC-CMs (Figure 2b) (p value, 0.0026). In addition, voltage clamp recordings revealed much smaller chromanol 293B-sensitive I_{Ks} currents from LQT1-iPSC-CMs than controls (Figure 2c,d).

Action potentials recorded by FV dye in single hiPSC-CMs

We classified the cardiomyocytes into subtypes based on the APs of single cells obtained by FV (Figure 3a,b and Supplementary Figure S2). Ventricular, atrial, and nodal cardiomyocytes were defined as $APD_{90}/APD_{50} < 1.4$, $1.7 < APD_{90}/APD_{50}$, and $1.4 < APD_{90}/APD_{50} < 1.7$, respectively, as previously reported (Supplementary Figure S2) [27, 28]. We labeled ventricular cells whose APD was more than 1 second as “ventricular cells with long APD”. These cells were more frequently observed among LQT-iPSC-CMs (Figure 3b,c) than control-iPSC-CMs (Figure 3a,c). The ventricular-like cardiomyocytes in control and LQT1-iPSC-CMs constituted the major population (201B7 (control), 98%; 409B2 (control), 88%; 692D2 (control), 90%; LQT1A1 (mother), 95%; LQT1B1 (elder sister), 79%; and LQT1C1 (younger sister), 93%) (Figure 3c). The frequencies of ventricular cells with long APD in control-iPSC-CMs (201B7, 16%; 409B2, 17%; 692D2, 19%) were lower than those in LQT1-iPSC-CMs (LQT1A1, 50%; LQT1B1, 31%; and LQT1C1, 32%) (Figure 3c). Intriguingly, we observed more early afterdepolarizations (EADs) in LQT1-iPSC-CM populations than in control-iPSC-CM populations (Figure 3d,e) (p value, 0.031).

Action potentials recorded by FV in hiPSC-CM monolayers

We next measured the APDs during pacing in high-density cardiomyocyte culture (Figure 4a). Ten consecutive waves after dye loading were averaged (Figure 4b and Supplementary Movie). APD₉₀ from LQT1-iPSC-CMs were significantly longer than those from control-iPSC-CMs (Figure 4c) (p value, 0.000096).

APD measured by FV on high-throughput plate reader

We next measured hiPSC-CM monolayers by FV in a higher throughput system consisting of a 96-well plate. The APs on monolayers were stable before and after 1 Hz pacing (Figure 5a). The APD₉₀ averaged from 10 consecutive waves of LQT1B1-iPSC-CMs was longer than that from control-iPSC-CMs, and cisapride prolonged the APD₉₀ of cardiomyocytes derived from both iPSC groups (Figure 5b). In addition, we administered a beta stimulant to mimic the conditions under exertional or emotional stress, in which the disease phenotypes are more prominent [5, 6]. In the presence of 100 nM isoproterenol (ISP), we assessed the effect of several agents on drug-induced long QT syndrome (diLQTS). Erythromycin and cisapride prolonged APD₉₀ in both iPSC-CM groups under ISP, but the APD₉₀ of LQT-iPSC-CMs was consistently higher at all concentrations

(Figure 5c,d). These findings support the applicability of the membrane dye system to high-throughput-based drug discovery and toxicology testing.

Discussion

The proportion of a cardiac subtype in iPSC-CMs or cardiac monolayers and the AP parameters were reported to vary depending on the culture duration [29], suggesting the importance of a high proportion of the ventricular subtype when modeling the disease phenotypes of ventricular arrhythmias. In this study, we have proposed a simple method that uses FV to measure both single cardiomyocytes and cardiac monolayers derived from normal and LQT1-iPSCs. Optical recordings of single cardiomyocytes identified the cardiac subtypes of each cells and confirmed their variable electrophysiological properties (Figure 3a-c), which was consistent with previous reports of a genetically encoded membrane potential sensor [15, 16]. High electrophysiological variability of single iPSC-CMs warrants assessment of a large population of ventricular cells for modeling ventricular arrhythmia. Our results demonstrated the feasibility of optical recording to identify cardiac subtypes and assess the electrophysiological properties of large number of iPSC-CMs

simultaneously.

The monolayered cells successfully reduced the variations of APD when synchronized with electrical stimulation at a constant frequency. As previously reported, FPD, an electrophysiological parameter highly correlated with APD [30], of ventricular hiPSC-CM monolayers was longer than that of atrial iPSC-CM monolayers [9], suggesting that the high proportion of the ventricular subtype would be appropriate for analyzing the APs of LQT-iPSC-CM monolayers and diLQTS models. Combined with single cell-based identification of cardiac subtypes, our optical recordings confirmed that iPSC-CM monolayers composed mainly of ventricular cells recapitulated the prolonged AP duration of cLQTS and diLQT. In addition, this system was applicable to high throughput analysis to investigate the response to drugs.

Although conventional styryl voltage-sensitive dyes, such as di-4-ANEPPS and di-8-ANEPPS, have the ability to respond quickly to voltage changes, they have low sensitivity and non-negligible phototoxicity [17, 31]. FV and di-4-ANBDQBS, a near-infrared fluorescent voltage-sensitive dye, can precisely track the transmembrane voltage with lower photodynamic damage than di-4-ANEPPS [12, 32, 33]. Furthermore, FV has been used for a high-throughput screening [19].

ArcLight is a genetically encoded sensor of membrane potential that has brighter fluorescence and negligible phototoxicity, and has been used to study cardiomyocytes derived from human embryonic stem cells [15] and LQT2 iPSC-CMs [16]. However, the recorded APs do not agree with those from patch clamp techniques due to the slow temporal response of ArcLight [34]. VSFP-CR [35], a derivative of VSFP2.3 [36], was previously combined with subtype-specific marker genes (*MLC2v*, *SLN*, and *SHOX2*) to analyze the APs of three iPSC-CM subtypes [37]. Although genetically-encoded voltage indicators enabled promotor specific voltage response, it takes much time to establish the genetically-modified cell lines stably. On the other hand, FV can be rapidly used with many cell lines, as it needs only 20 min loading.

DiLQTS, which is an acquired LQTS, is mainly caused by blockage of the I_{Kr} channel and is more frequent than cLQTS. It is well known that the mutation of genes responsible for cLQTS could contribute to an increased risk of diLQTS [38, 39]. DiLQTS has a similar mutation rate as cLQTS for the three major genes responsible for cLQTS [40], leading to the hypothesis that type 1 cLQTS patient-derived iPSC-CMs are appropriate for studying drugs that cause QT prolongation by blocking I_{Kr} current. In the present study, we used FV to identify drugs that prolonged QT in

LQT1-iPSC-CM and control-iPSC-CM monolayers in a high-throughput manner (Figure 5b-d).

Despite our encouraging results, it should be noted that FV has some limitations because the measured APs are based on a relative scale. Nor do the APs provide MDP or APA values, unlike patch clamp analysis. Furthermore, hiPSC-CMs are heterogeneous in electrophysiological properties, including their APDs and automaticities, making it difficult to measure the APs of paced cardiomyocytes simultaneously. Thus, in FV experiments, APDs in single cells were obtained from unpaced cells with different beating frequencies. Heterogeneity of the induced cardiomyocytes may also cause variations in the recordings. Studies have investigated ways to mature hiPSC-CMs [41, 42], which could reduce the heterogeneity and thus contribute to more robust and precise high-throughput screening.

Although many reports have used the AP morphology-based classification of iPSC-CM subtypes [27, 28, 43, 44], it has recently been argued that AP morphology is not a reliable indicator [45, 46]. Future methods that can distinguish cardiomyocyte subtypes induced from pluripotent stem cells would contribute to measuring long APD in LQT-iPSC-CMs more precisely.

Conclusions

In summary, using an FV-based optical measurement system, we successfully identified ventricular-type cells as the major population in cardiac subtypes and found that they have a higher frequency of APD prolongation and EAD when derived from LQT1 patient iPSCs. Further, monolayers with this major population were suitable for analyzing two-dimensional AP in LQT-iPSC-CMs and diLQTS models in a high-throughput manner.

Conflicts of Interest

Dr. Yoshida owns stocks in iPS Portal, Inc. Although Takeda Pharmaceutical Company Ltd. was not a sponsor of this work, some data collection was conducted by using equipment leased by the company. Also, Takeda Pharmaceutical Company Ltd. is paying the salary of Dr. Takaki independently of this work.

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Supplementary Materials

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Figure legends

Figure 1: Type1 long QT syndrome family background and cardiac differentiation from human iPSCs. (a) Family pedigree. The squares indicate males and the circles indicate females. Closed symbols mark patients confirmed by their DNA sequences. Hexagrams mark members who have a syncope history. The QTc values of three patients before taking a beta-blocker are stated. (b) ECG of II-2 in Figure 1a before the patient started taking a beta-blocker. (c) Sanger sequencing of the three patients and one control. (d) Schematic figure of KCNQ1 protein. The black circle indicates the mutation site within the transmembrane region. The lower side locates intracellular. (e) Outline of the cardiac differentiation. Lower, representative shapes of beating EBs. Scale bar, 200 μm .

Figure 2: Patch clamp analysis of cardiomyocytes from control- and LQT-iPSC lines. (a) Representative APs of 1-Hz paced a control-iPSC-CM and an LQT-iPSC-CM from II-2 in Figure 1a. Ten consecutive waves are shown. (b) MDP, APA, and APD₉₀ from cardiomyocytes derived from the six lines. 201B7 (n=6), 409B2 (n=5), 692D2 (n=6), LQT1A1 (n=5), LQT1B1 (n=5), and LQT1C1 (n=7). Data are represented as means $\pm$ SEM. ** $p < 0.005$. (c) Representative current traces

from control- and LQT1-iPSC-CMs. Upper, the protocol in current clamp recording. Middle, representative traces before and after perfusion with 3R4S-chromanil 293B (30 $\mu\text{mol/l}$). Lower, 3R4S-chromanil 293B-subtraction. (d) I-V plots of I_{Ks} at the end of the depolarizing step. 692D2 (control) (n=3), LQT1B1 (n=3). * $p < 0.05$.

Figure 3: AP measurements of single cells using a membrane potential dye. (a) Examples of AP waves from control (409B2) measured by the dye. ROIs (1)-(5) are ventricular type. ROI (5) (green wave) shows long APD. (b) Examples of AP waves from LQT1B1. ROIs (1)-(4) are ventricular type with long APD. ROI (4) (purple wave) shows EADs. ROI (5) (green wave) is nodal type. (c) Proportion of subtypes in 3 control- and 3 LQT1-iPSC-CMs. 201B7 (n=128), 409B2 (n=41), 692D2 (n=92), LQT1A1 (n=192), LQT1B1 (n=101), and LQT1C1 (n=202). Ventricular-type cells predominate in all six lines. (d) Representative EADs in a ventricular type cell from LQT1B1. The EADs were defined as over 20% of the AP amplitude. Arrows show EADs. (e) Occurrence ratio of EADs in ventricular cardiomyocytes from the six lines. The number of ventricular cells was used as the denominator. * $p < 0.05$.

Figure 4: AP measurements in a cardiomyocyte monolayer. Data are represented as means $\pm$ SEM.

(a) An example phase contrast image of the monolayer culture. Scale bar, 500 μ m. (b)

Representative AP of a paced control-iPSC-CM monolayer. APD₉₀ was calculated from the average

of 10 consecutive waves. (c) APD₉₀ from 3 control- and 3 LQT-iPSC-CM monolayers. 201B7 (n=3),

409B2 (n=3), 692D2 (n=3), LQT1A1 (n=3), LQT1B1 (n=3), and LQT1C1 (n=3). LQT-iPSC-CMs

showed longer APD than controls. ** $p < 0.005$.

Figure 5: AP measurements in cardiomyocyte monolayers using a high-throughput plate reader. (a)

Representative AP waves on a 96-well plate. Pacing at 1 Hz starts 10 s after recording. (b) Cisapride

prolonged APD₉₀ of both control- and LQT1-iPSC-CMs. 692D2 (control) (n=3), LQT1B1 (n=5).

(c) Cisapride prolonged APD₉₀ of both control- and LQT1-iPSC-CMs with 100 nM isoproterenol.

692D2 (n=5), LQT1B1 (n=3). (d) Erythromycin prolonged APD₉₀ of both control- and LQT1-iPSC-

CMs with 100 nM isoproterenol. 692D2 (n=4), LQT1B1 (n=3). LQT-iPSC-CM monolayers in 300

nM and 1000 nM cisapride were not synchronized at 1 Hz pacing. Data are represented as means $\pm$

SEM. ** $p < 0.005$, * $p < 0.05$.

Figure 1

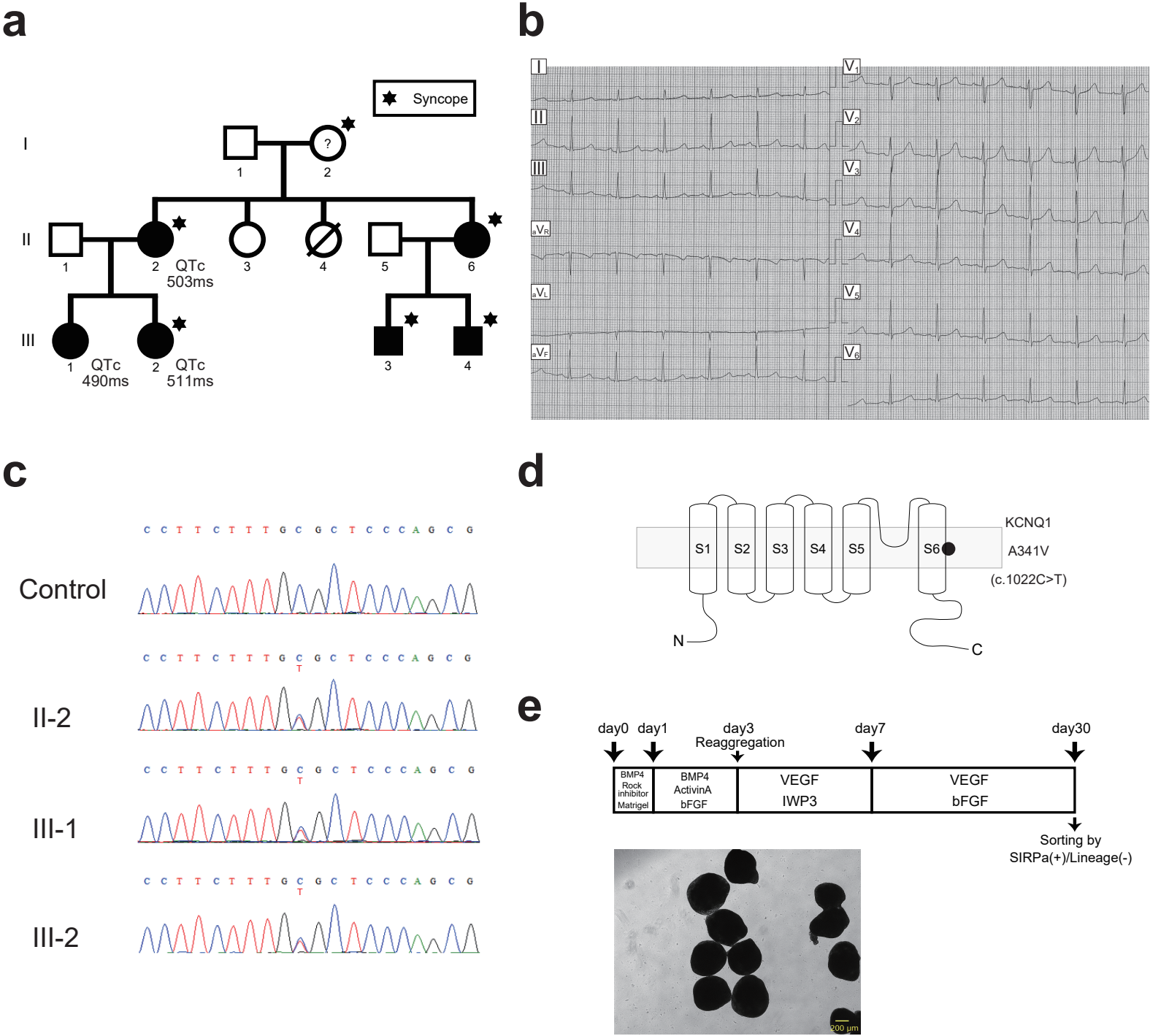
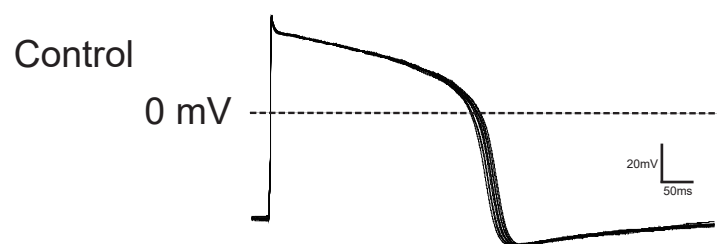
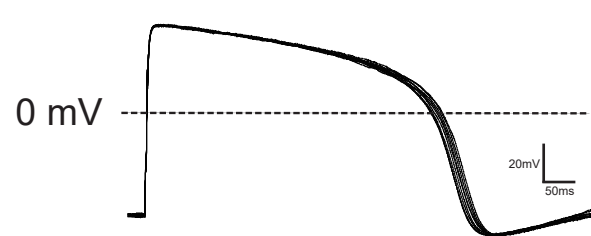


Figure 2

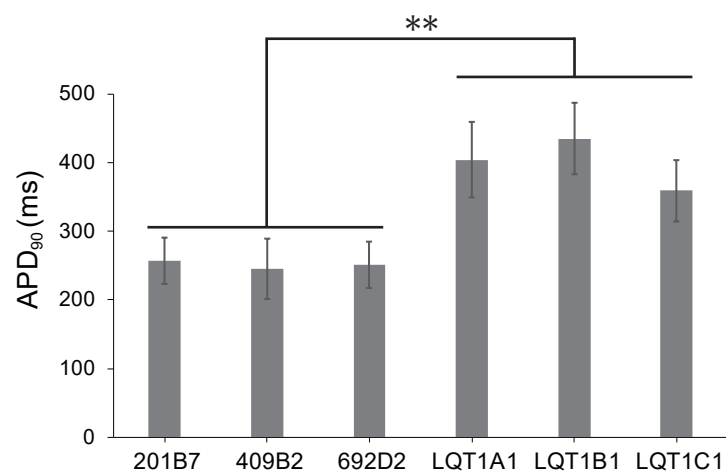
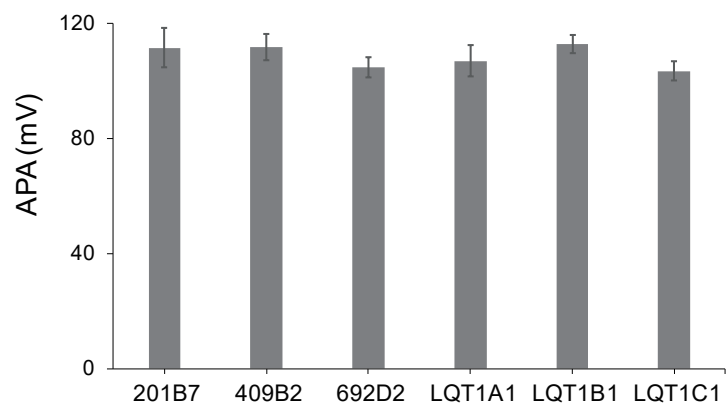
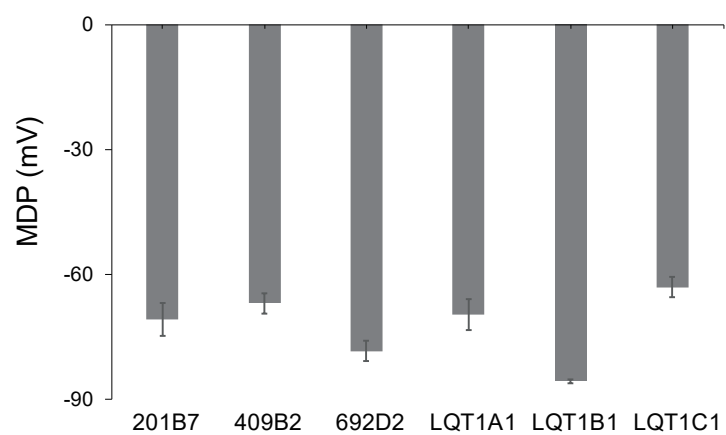
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LQT1



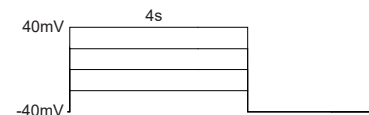
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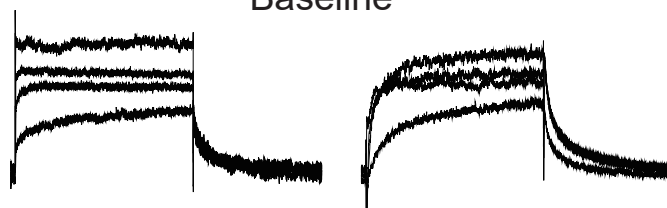
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Control

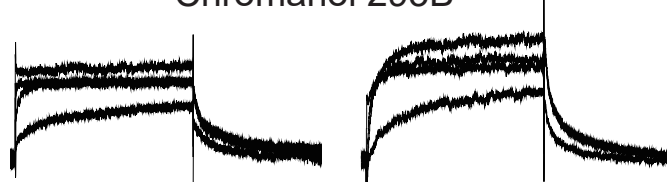
LQT1



Baseline



Chromanol 293B



Subtraction



d

I_{Ks}

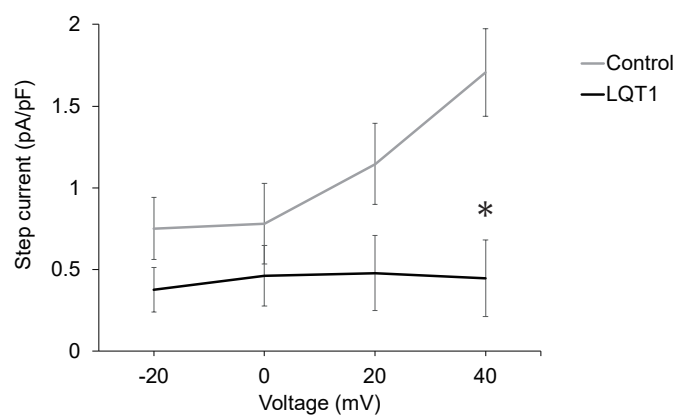
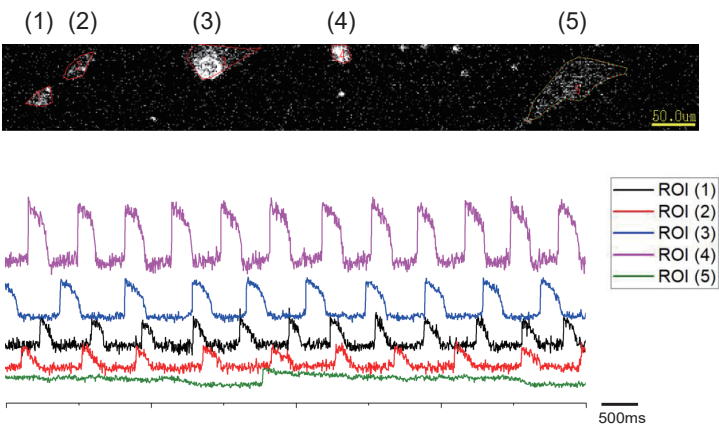
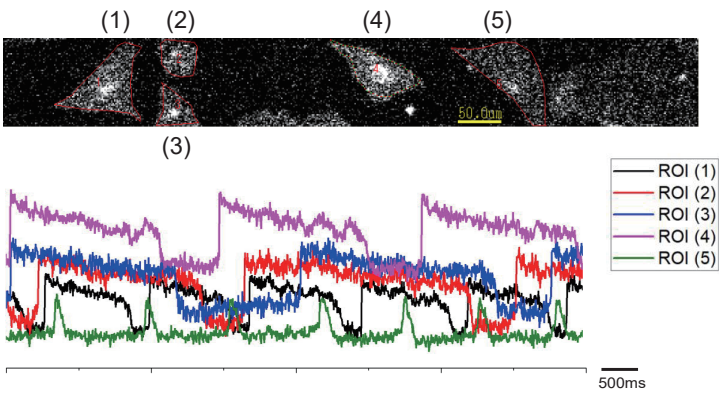


Figure 3

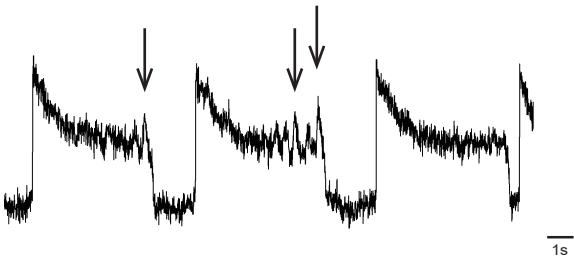
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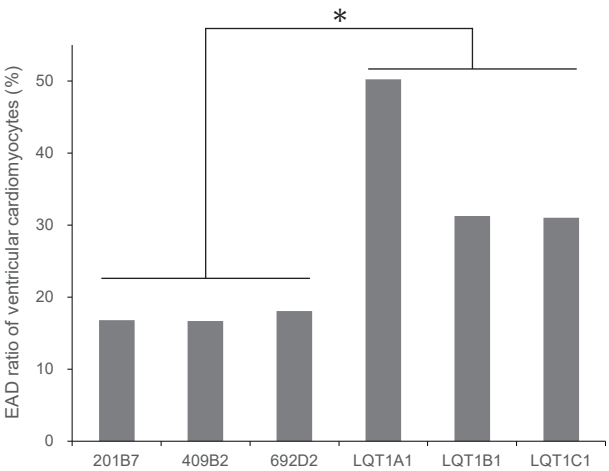
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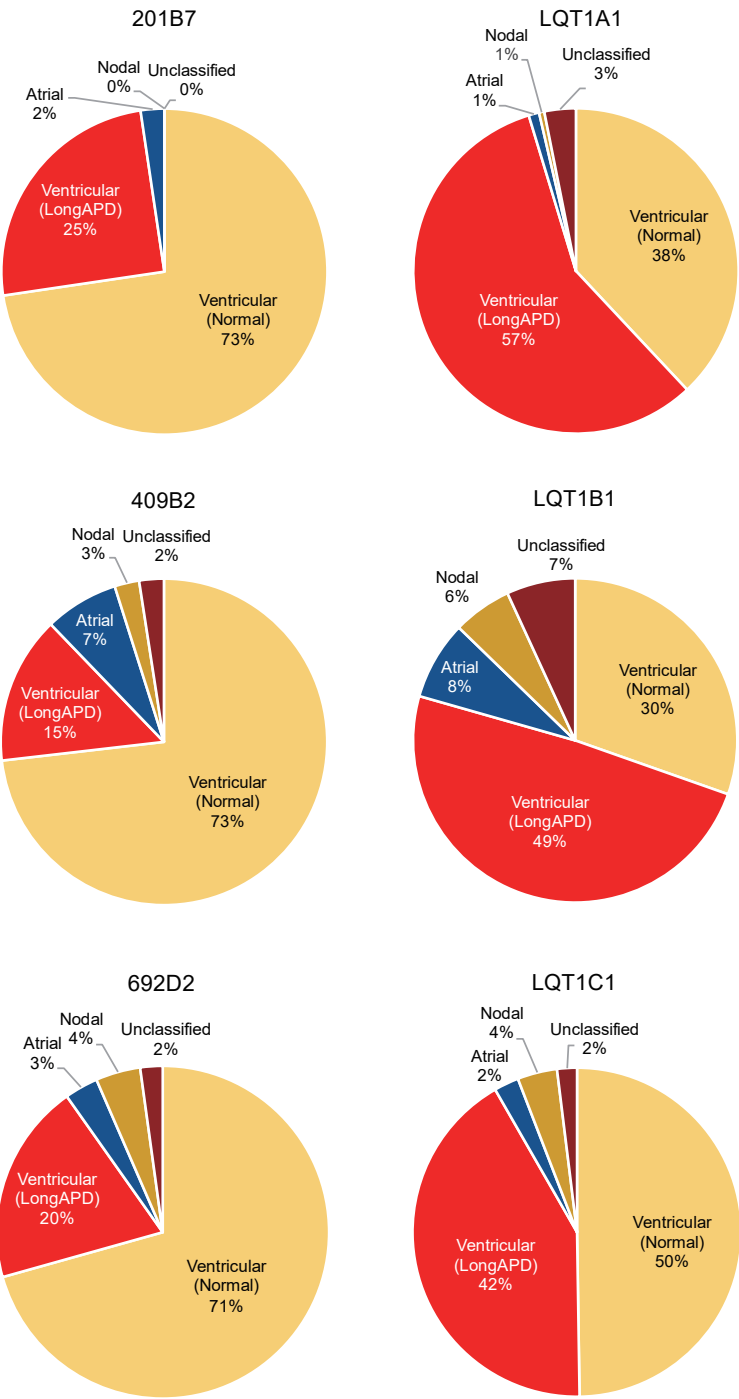


Figure 4

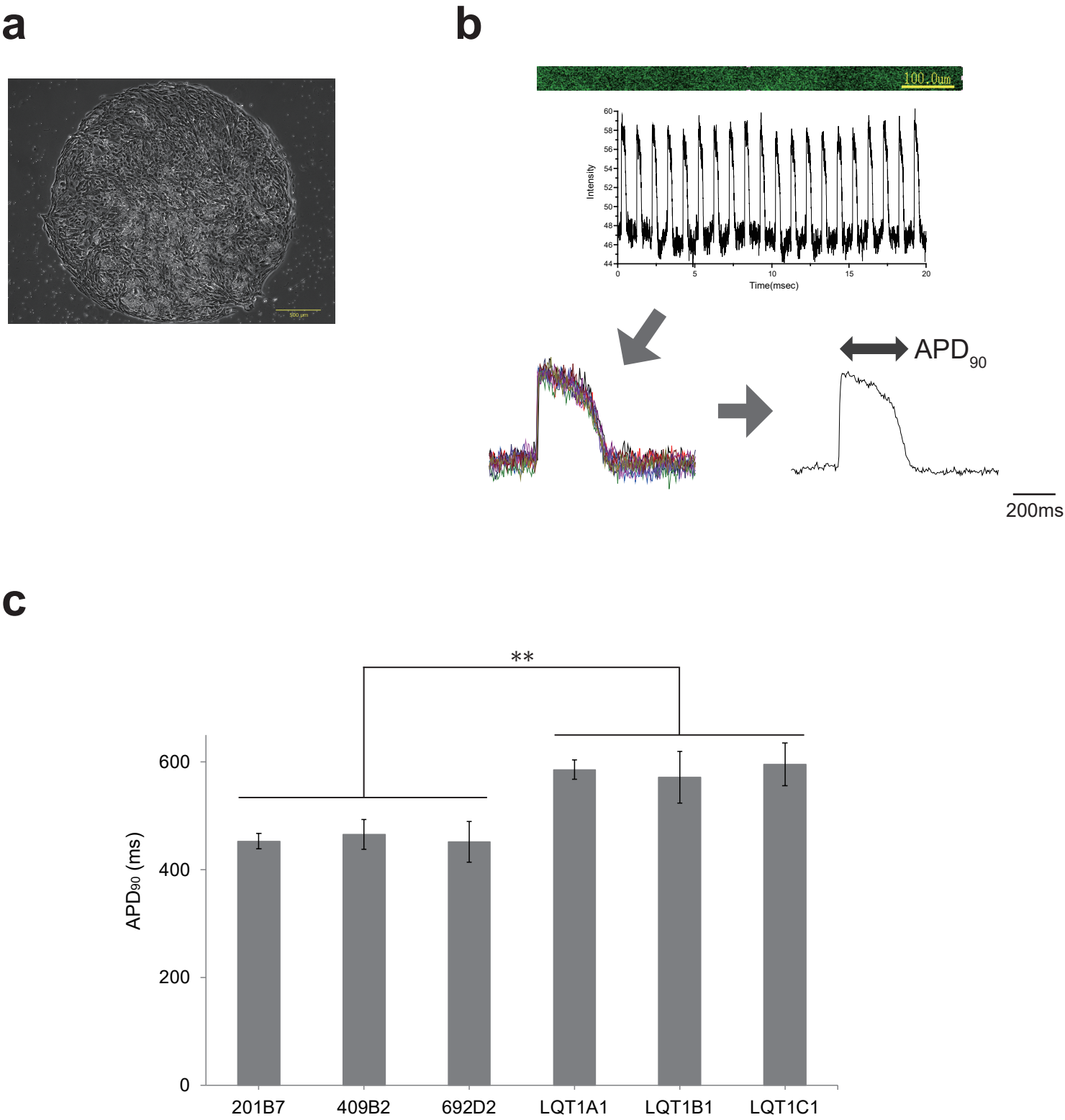
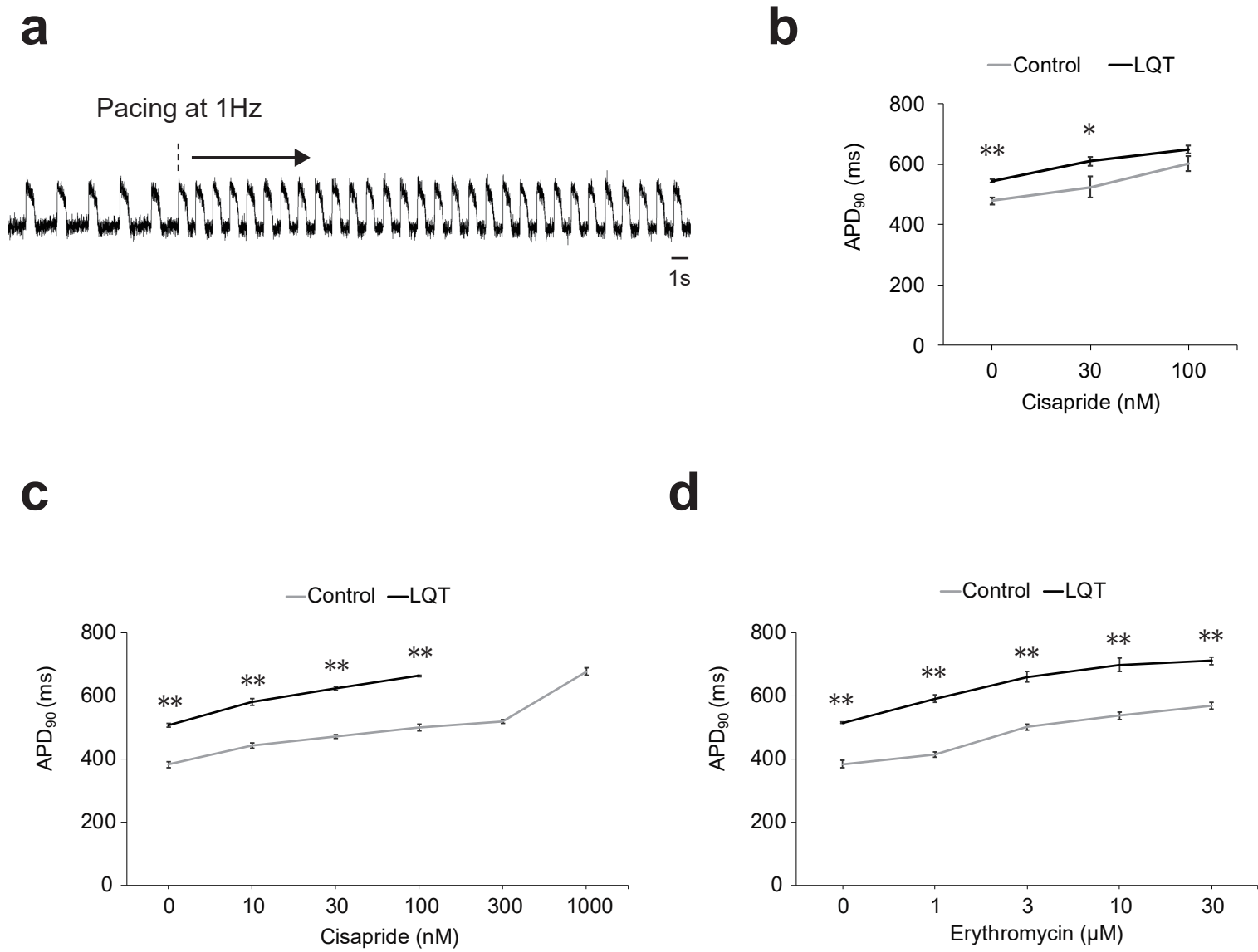


Figure 5



Supplementary Methods

In vitro cardiac differentiation

Human iPSCs were differentiated by forming EBs [1, 2]. On day 0, undifferentiated iPSCs were dissociated with Accumax (Innovative Cell Technologies, San Diego, CA, USA), and 5,000 cells were aggregated using a 96-well plate coated with Ultra-Low Attachment Surface, round bottom (Corning, Corning, NY, USA) in StemPro34 SFM (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with buffer (2 mM L-glutamine (Thermo Fisher Scientific), 4×10^{-4} M monothioglycerol (MTG) (Sigma-Aldrich, St Louis, MO, USA), 50 μ g/ml ascorbic acid (Sigma-Aldrich), 150 μ g/ml transferrin (Roche Diagnostics, Basel, Swiss), 50 U/ml penicillin, and 50 μ g/ml streptomycin), 10 μ M Y-27632 (WAKO, Osaka, Japan), 2 ng/ml BMP4 (R&D Systems, Minneapolis, MN, USA), and 0.5% BD Matrigel Matrix Growth Factor Reduced (BD Biosciences, San Jose, NJ, USA). On day 1, medium, including human recombinant activin A (12 ng/ml) (R&D Systems), BMP4 (18 ng/ml), and bFGF (10 ng/ml) (R&D Systems), were added into the wells to reach a final concentration of 6 ng/ml activin A, 10 ng/ml BMP4, and 5 ng/ml bFGF. On day 3, EBs were collected, dissociated with Accumax, and suspended in differentiation medium supplemented with buffer plus 10 ng/ml VEGF (R&D Systems) and 1 ng/ml IWP-3 (Stemgent, Lexington, KY, USA). 10,000 cells were aggregated in a new 96-well plate coated with Ultra-Low Attachment Surface, round bottom. On day 7, EBs were picked up into a 6-well or 10-cm plate coated with Ultra-Low Attachment Surface, and the medium was changed to StemPro 34 SFM containing buffer plus 10 ng/ml VEGF and 5 ng/ml bFGF. For the maintenance of iPSC-CMs, the culture medium was renewed every 2-3 days. Day 0-12 EBs were incubated at 37°C, 5% O₂, 5% CO₂, and 90% N₂. From day 12, the condition was changed to 37°C, 5% CO₂, and 95% air.

Quantitative polymerase chain reaction (qPCR)

Total RNA was extracted and purified from cells after FACS sorting using QIAzol Lysis Reagent (QIAGEN, Hilden, Germany) with the miRNeasy Mini Kit (QIAGEN) according to the manufacturer's protocol. The RNA concentrations were determined using NanoDrop 2000 (Thermo Fisher Scientific). Reverse transcription reaction from purified RNA was performed using ReverTra Ace (Toyobo, Osaka, Japan) with the oligo dT20 primer. qRT-PCR was performed with TaqMan gene expression assays (Thermo Fisher Scientific)

using the StepOnePlus Real-Time PCR system (Thermo Fisher Scientific). Expression levels were normalized against the expression level of an internal housekeeping gene (*ACTB*). The qPCR Taqman probes used in this study were as follows.

CACNA1C: Hs00167681_m1, *RYR2*: Hs00181461_m1, *SLC8A1*: Hs01062258_m1, *KCNQ1*: Hs00923522_m1, *KCNE1*: Hs00897540_s1, *KCNH2*: Hs04234270_g1, *KCNE2*: Hs00270822_s1, *SCN5A*: Hs00165693_m1, and *ACTB*: Hs99999903_m1.

Current clamp recording

Perforated whole-cell patch was performed with current clamp mode using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) and pCLAMP software (v9.2 or 10.4; Molecular Devices) with the IX73 inverted microscope (Olympus, Tokyo, Japan) and phase contrast objectives UPLFLN10XPH and UPLFLN20XPH (Olympus). Patch electrodes were made of borosilicate capillaries (1B150F-4; WPI, Sarasota, FL, USA) using a micropipette puller (P-97/IVF or P-1000IVE, Sutter Instruments, Novato, CA, USA). The level of stretching and heating was adjusted to obtain a resistance of 2–4 M Ω . The patch electrodes were filled with 300-500 μ g/mL amphotericin B (Sigma-Aldrich) plus intracellular solution containing 130 mM KOH, 130 mM L-Aspartic acid, 20 mM KCl, 5 mM NaCl, 10 mM HEPES, 5 mM Mg-ATP, 10 mM EGTA, and 1 mM MgCl₂. The pH was adjusted to 7.2 with KOH. APs were recorded from iPSC-CMs superfused with modified Tyrode's solution, which consisted of 120 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 0.84 mM Na₂HPO₄, 0.28 mM MgSO₄, 0.22 mM KH₂PO₄, 27 mM NaHCO₃, and 5.5 mM glucose, pH7.4, at 35-37°C. After the Axopatch amplifier was set to current clamp mode, spontaneous APs were recorded from spontaneously contracting hiPSC-CMs 5-10 days after seeding using the gap-free protocol, and paced APs were recorded from spontaneously contracting or quiescent cells using a pacing protocol of 4 ms duration, 0.2-1 nA amplitude, and 1 Hz frequency. All signals were filtered at 5 kHz, digitized at 10 kHz using Digidata 1322A or 1550A (Molecular Devices), and analyzed with pCLAMP 9.2 or 10.4 software (Molecular Devices). MDP, APA, and APD₉₀ were calculated from the average AP of 10 consecutive and stable waves in pacing mode.

Voltage clamp recording

I_{Ks} currents were recorded from single cardiomyocytes 5-10 days after seeding with the ruptured whole cell patch clamp method [3]. I_{Ks} currents were calculated by subtracting

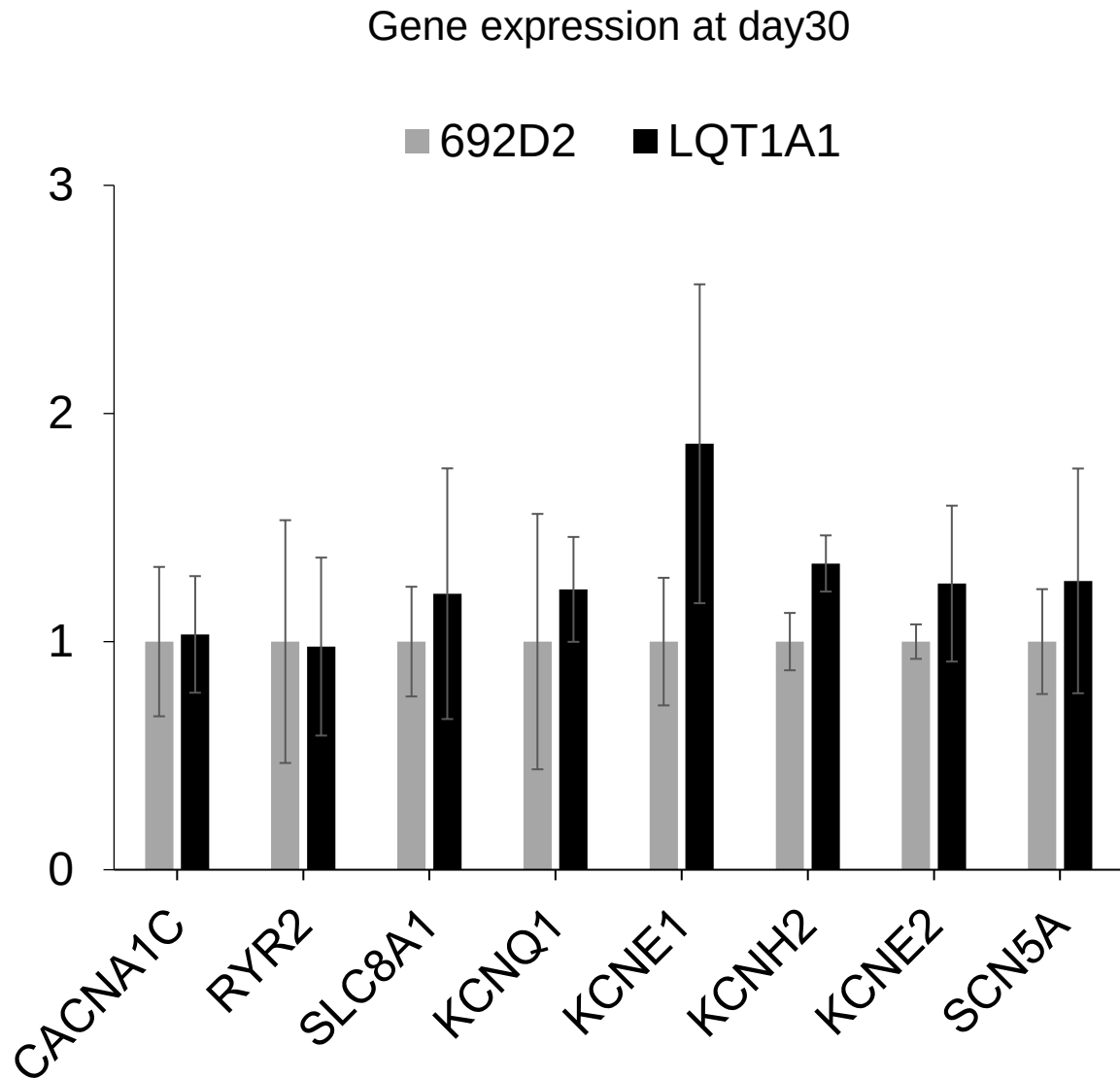
the currents after perfusion with extracellular solution containing 30 μ M (-)-[3R,4S]-chromanol 293B (Tocris Bioscience, Bristol, UK) from the currents before perfusion. The extracellular solution consisted of 150 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 1 mM Na-pyruvate, 15 mM HEPES, and 1 μ M nifedipine, pH7.4. The extracellular solution was perfused at 35-37°C using a peristaltic pump, MINIPULS 3, type MP-2 (Gilson, Middleton, WI, USA). The patch electrode was filled with the internal pipette solution containing 125 mM K-Aspartate, 20 mM KCl, 10 mM EGTA, 5 mM MgATP, 5 mM HEPES, 1 mM MgCl₂, 2 mM Na₂-phosphocreatinine, and 2 mM Na₂-GTP, pH7.2. Patch electrodes were made of borosilicate capillaries (1B150F-4) using a micropipette puller (P-97/IVF) whose level of stretching and heating was adjusted to obtain a resistance of 4-6 M Ω .

Data were acquired at 10 kHz with Digidata 1322A, low-pass filtered at 2 kHz with Axopatch 200B amplifier, and filtered at 1 kHz with Clampfit software (Molecular Devices) after recording. I_{Ks} currents were elicited by depolarizing steps from a holding potential of -40 mV to -20, 0, 20, and 40 mV for 4 s. This was followed by a 2-s repolarization phase.

Optical recording of APs from single cells or cardiomyocyte monolayers

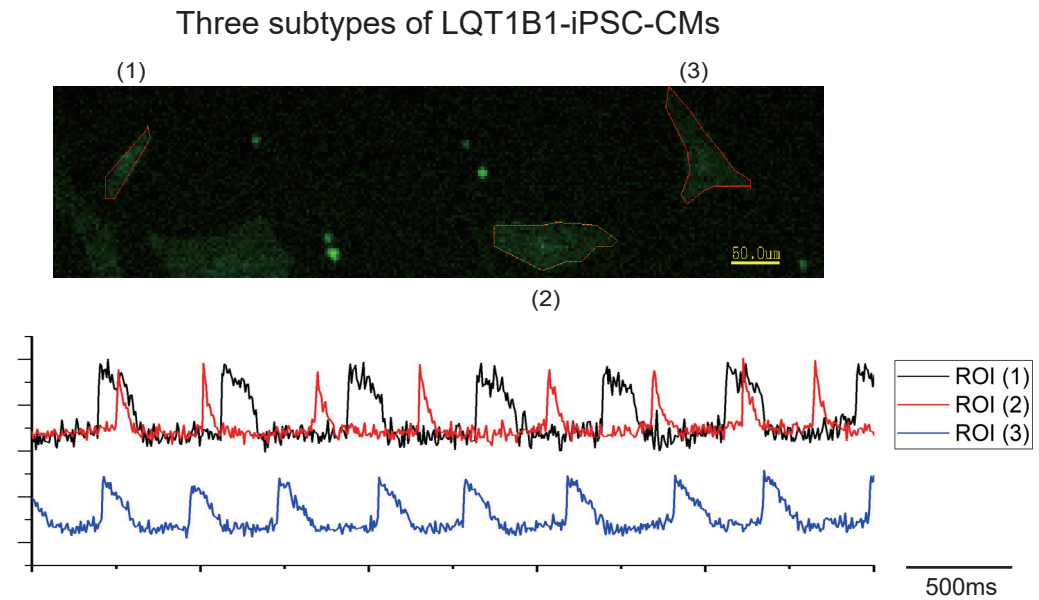
Cells were imaged in a 35-mm-diameter glass bottom dish at 37°C and modified Tyrode's solution identical to that used for patch clamp recordings fed with 5% CO₂, 20% O₂, and 75% N₂ perfused through water at 37°C using Humidification Bottle (Carl Zeiss, Jena, Germany) and Heating Device Humidity S1 (Carl Zeiss). One hour after setting the cells, fluorescence measurements were taken with the Axio Observer inverted fluorescence microscope (Carl Zeiss), Objective Fluar 10x/0.50 (Carl Zeiss), Xe lamp C7773 (Hamamatsu Photonics), EM-CCD camera ImagEM (Hamamatsu Photonics), filter set zeiss 16 (Excitation: BP 485/20, Beam Splitter: FT510), emission filter 535DF55 (Omega optical, Brattleboro, VT, USA), and AquaCosmos2.6 software (Hamamatsu Photonics) set at Ex 490 BP 10 nm. The Xe lamp was set at 100% output. Subarray images were recorded every 8 ms in single cells 5-10 days after seeding or every 4 ms in monolayer 10-15 days after seeding. Binning was set to 1x1. The regions of interest (ROIs) in the monolayers were defined as whole pixels of 512x32. The monolayers were stimulated at 1 Hz with 1 ms depolarizing pulses at 10 V using a pulse stimulator, Master-9 (A.M.P.I., Jerusalem, Israel), with an interelectrode distance of 12 mm (Intermedical, Osaka, Japan) [4]. Graphing, superimposed waveform traces, and calculation of APD₉₀ were performed with OriginPro 2016 (OriginLab, Northampton, MA, USA).

Supplementary Figures
Supplementary Figure S1.



qPCR analysis after FACS sorting of SIRPa(+)/lineage(-) at day 30 from 692D2 (n=3) and LQT1A1 (n=3). The relative gene expression was set to 1 for genes that affect ion currents in control line 692D2. There was no significant difference in the expression of any genes between 692D2- and LQT1A1-hiPSC-CMs. Data are represented as means $\pm$ SEM. Expression levels were normalized against the expression level of an internal housekeeping gene (*ACTB*).

Supplementary Figure S2.



Action potentials of three major cardiomyocyte subtypes from LQT1B1-iPSCs recorded by FluoVolt dye. ROI (1), (2), and (3) show ventricular-, atrial-, and nodal-type cardiomyocytes, respectively.

Supplementary Reference

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